Regd. No. 15665/68

Covered by Clarivate Analytics Services: Emerging Sources Citation Index

INDIAN JOURNAL OF DAIRY SCIENCE JANUARY-FEBRUARY VOL. 74, NO. 1, 2021

Contents

ISSN 0019-5146 (Print)
ISSN 2454-2172 (Online)

INVITED REVIEW

Astaxanthin – King of antioxidants as immune modulator and anti-inflammatory for enhancing productive performance and health of animals
SV Singh, Yallappa M Somagond and Aditya Deshpande 1

RESEARCH ARTICLES

DAIRY PROCESSING

Heat stability of high-protein ultrafiltration retentate: Effect of concentration factor and stabilizing salts
Ganga Sahay Meena, Ashish Kumar Singh, Vijay Kumar Gupta, Harleen Kaur Gujral and Rashmeet Kaur Malhotra 8

Microbiological and physicochemical changes during ripening of Camembert cheeses made from raw and pasteurized cow milk produced in Tizi-Ouzou (north of Algeria)
Hillal Sebbane, Dalila Almi, Sonia Hadouchi, Louiza Hedjel, Noria Smail-Saadoun and Abderrahmane Mati 18

Preparation of low-fat *paneer* using Glucono delta-lactone as an acidulant
Mamata Chaudhary, Suneeta Pinto, CN Dharaiya and Preeti Paul 30

Physico-chemical changes during processing and storage of UHT milk
Suvartan Ranvir, Rajan Sharma, Kamal Gandhi, Pranali Nikam and Bimlesh Mann 39

Quality assessment of market sample of *khoa* based *ramdana lai*
Pragya Bharti, Sanjeev Kumar and Suryamani Kumar 48

Optimization and characterization of functional chocolate with addition of butter fruit milkshake powder as a source of phenolic, flavonoid and carotenoid
Aparna Kumari, Shreya Pandey, Anil Kumar Chauhan and Meenakshi Singh 54

Genotypic identification of extended spectrum â-lactamase producing *Escherichia coli* in dairy supply chain
Amarjeet Kumar, Rashmi Hogarehalli Mallappa, Avinash Jaswal, Brijesh Kumar, Naresh Kumar and Raghu Hirikyathanahalli Vishweswaraiah 61

Factors affecting the milk yield, milk composition and physico-chemical parameters of ghee in lactating crossbred cows
N Veena, JS Hundal, M Wadhwa and AK Puniya 68

Shelf life enhancement of low calorie and fiber-enriched *Sandesh* by modified atmosphere packaging
Himanshu Kumar Rai, Dinesh Chandra Rai, Arvind and Shikha Pandhi 74

Formulation of pea milk fortified *dahi* incorporated with jamun seed extract
Shikha Pandhi and Amrita Poonia 80

ANIMAL PRODUCTION & REPRODUCTION

Efficiency of imputing missing genotypes by INDUSCHIP v2 in HF Crossbred cattle
Sujit Saha, Nilesh Nayee, Heena A Shah, Swapnil Gajjar, A Sudhakar, Sandeep K Donthula and Hardik V Poojara 89

Multiple linear regression analysis using monthly test day milk yield predicting the first lactation production performance for sire evaluation in Murrah buffaloes
Manvendra Singh, Ved Prakash, Shaktikant Das, Sonam Dixit and AK Gupta 96

Effect of area specific mineral mixture supplementation on milk production, biochemical and blood mineral status of Black Bengal goats
GM Kedare, Vipin, M Karunakaran, A Mandal, MK Ghosh and S Naskar 102

DAIRY ECONOMICS & EXTENSION

Influence of FMD vaccination stress on milk production in crossbred dairy cattle of Kerala
Pramod S, R ThirupathyVenkatachalapathy, Lasna Sahib and Bibin Becha B 107

Indian J Dairy Sci 74(1): 1-7
https://doi.org/10.33785/IJDS.2021.v74i01.001

INVITED REVIEW

Astaxanthin – King of antioxidants as immune modulator and anti-inflammatory for enhancing productive performance and health of animals

SV Singh, Yallappa M Somagond and Aditya Deshpande

Received: 10 December 2020 / Accepted: 28 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Astaxanthin is the potent antioxidant predominantly found in living organisms that are present in marine environment. It is known as “King of antioxidant” or “Super vitamin E” because of its potent antioxidant property. Antioxidant property of astaxanthin known to modulate different biological activity relating to antioxidant defence system, inflammation, immunity and ameliorating adverse effect of oxidative stress during summer season. Its use has been found in improving both male and female reproductive health and production performance of bovines. Astaxanthin helps in hastening of sexual maturity, improving sperm quality, increasing fertility and better development of embryos. It is considered as one of the essential feed additive in combating the adverse effect of climate change on production performance of animals. This review mainly focuses on source, structure and use of astaxanthin as an antioxidant, anti-inflammatory, immune stimulator and for improving productive performance of bovines.

Keywords: Astaxanthin, Bovines, Heat stress, Immunity, Inflammation

Introduction

Antioxidants are “free-radical scavengers” that can evade slow damage to cells caused by free radicals, which are produced in body as a reaction to environmental and other stresses. In the animals, carotenoids are considered as part of the antioxidant defence system. Carotenoids are pigment that plays an essential role in mitigating oxidative processes. They are potent antioxidants that can scavenge mono-molecular oxygen and peroxyl radicals. They influence cellular signalling and activate redox sensitive controlling pathways (Stahl and Sies, 2005). High concentration of carotenoids is commonly found in plants, algae and microorganisms. Animals and humans do not synthesis these carotenoids, therefore its necessary to provide them in diet (Sandmann, 1994). Carotenoids acts by inhibiting chain reaction by taking out free radicals, as these carotenoids contain polyene chain and long double bonds they perform as potent antioxidant.

Astaxanthin is considered one of the strongest carotenoids which is capable of protecting cells, lipids and membranes lipoproteins from oxidative damage (Naguib, 2000). This review mainly focuses on source, structure and use of astaxanthin as an antioxidant, anti-inflammatory, immune stimulator and reproductive health of bovines.

Sources of astaxanthin (Ast)

Astaxanthin is predominantly found in organisms that are present in marine environment i.e. in microalgae, plankton, krill and seafood and also present in yeast, fungi, complex plants and the feathers of some birds (flamingos and quail) (Hussein et al. 2006). Astaxanthin was previously extracted from krill (*Euphausia superba*) (Takaichi et al. 2003) and recently from green algae (*Haematococcus pluvialis*) or red yeast (*Phaffia rhodozyma*) by various cultivation methods which help to obtain pure astaxanthin (Ambati, 2014) Astaxanthin obtained from algae are two times more effective as compared to synthetic astaxanthin (Guerin, 2001). *Haematococcus pluvialis* is a green microalga that builds up high levels of astaxanthine in stress environment like high salinity, deficiency of nitrogen content, high temperature and light (Choi et al. 2002). It is regarded as the foremost source of natural astaxanthin. Holtin et al. (2009) reported that *Haematococcus pluvialis* comprises approximately 4% of free form, 2% of diester type and 94% of monoester type.

Structure of astaxanthin

Astaxanthin consists of special molecular structure on each ionone ring, i.e. presence of hydroxyl and keto molecules, which are accountable for the potent antioxidant properties and superior position in the membrane (Liu and Osawa, 2007). Astaxanthin comprises conjugated double bonds, hydroxyl and keto groups

Animal Physiology Division, ICAR-National Dairy Research Institute, Karnal-132 001, India

S V Singh (✉)
Animal Physiology Division, ICAR-National Dairy Research Institute, Karnal-132 001, India
Email:sohanvir2011@gmail.com)

(Figure 1). It has both lipophilic and hydrophilic properties (Higuera-Ciapara et al. 2006). Astaxanthin has two oxygenated groups on each ring structure that are responsible for its increased antioxidant characteristics (Guerin et al. 2003). In general lipid soluble antioxidants protects lipid soluble part of cells and water-soluble antioxidants protect the water-soluble portion of cells, but the unique structure of astaxanthin enables it to extend throughout cell membrane to play both roles and provides the cell membrane stability. Astaxanthin's polyene chain traps radicals in the cell membrane, while the astaxanthin terminal ring can scavenge radicals on the inner and outer parts of the cell membrane (Miki, 1991).

Naturally astaxanthin occurs in different forms (Table 1) esterified and free form and as isomers (Higuera-Ciapara et al. 2006). Synthetic astaxanthin is a molecule similar to that formed in living organisms and consists of a mixture of isomers 1:2:1 (3S, 3S), (3R, 3S), and (3R, 3R), respectively.

Mechanism of action of astaxanthin

Astaxanthin helps in neutralizing free radicals (nitrogen, sulphur, carbon and oxygen free radical) either by donating electrons to unpaired electrons or by quenching of unpaired electrons. Astaxanthin binds with free radical to form unreactive compound and help in quenching electron out of membrane (Figure 2) and resisting it from converting into pro-oxidant molecule.

Pharmacokinetics of Astaxanthin

Astaxanthin is carotenoid xanthophyll compound, is absorbed into body comparable to lipids and conveyed via lymphatic system (Figure 3). It get incorporated with lipoproteins and reaches tissues (Rao et al. 2013). Astaxanthin is a fat soluble compound, with enhanced absorption when supplemented along oils in diet. Absorption of astaxanthin also depends on associated dietary components. Bioavailability of astaxanthin in humans was improved when given with lipid-based diets, high levels of carotenes gets solubilized in the oil phase of the food matrix which can lead to better absorption (Olson, 2004; Rao et al. 2013). Lara et al. (2007) also stated that combination of astaxanthin and fish oil showed summative effect which contributed to improved immune response in wistar rats.

Biological Activity of Astaxanthin

Astaxanthin exhibited higher bioactivity than other antioxidants, since it could attach from inside to outside with cell membrane (Yuan et al. 2011). Astaxanthin is used as a nutrient in aquatic animals and as a pigmentation of broilers and egg yolk in the poultry industry (Cyanotech, 2000). Astaxanthin products are used as tablets, capsules, syrups, oils, soft gels, creams, biomass and granular powders for commercial applications in nutraceuticals. It acts as potent antioxidant, used as a dietary supplement to treat cardiovascular and neurodegenerative

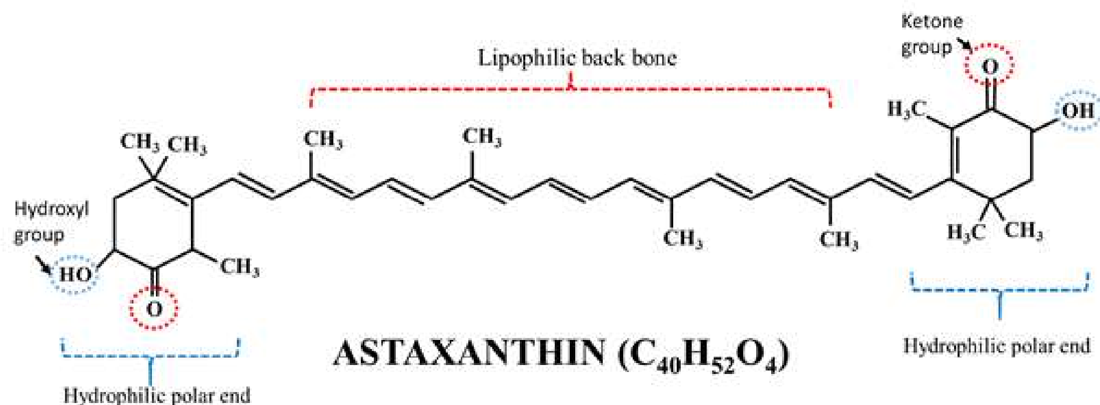


Fig. 1 Chemical structure of Astaxanthin, Molecular formula - $C_{40}H_{52}O_4$ and Molar mass - 596.84 g/mol (Higuera-Ciapara et al. 2006)

Role of astaxanthin in molecular system (Biswal, 2014)

Quenching of singlet oxygen	Reduces free radical concentration
Removing of radicals	Prevent chain reactions
Inhibiting lipid peroxidation	Membrane structure is maintained
Regulation of gene expression	Improvement of immune system
Acts as COX-2 inhibitor	Reduces inflammation
Inhibits enzyme activity of iNOS	Reduces nitric oxide level
Reduces C-reactive protein (CRP),	Decreases systemic inflammation
Suppresses inflammatory gene expression	Subduing of NF-kappa Beta activation and I(kappa)B
Suppresses inflammatory agents	Prostaglandin E2, IL-10, TNFalfa
Anti-androgen effects	Inhibition of enzyme 5-a-reductase

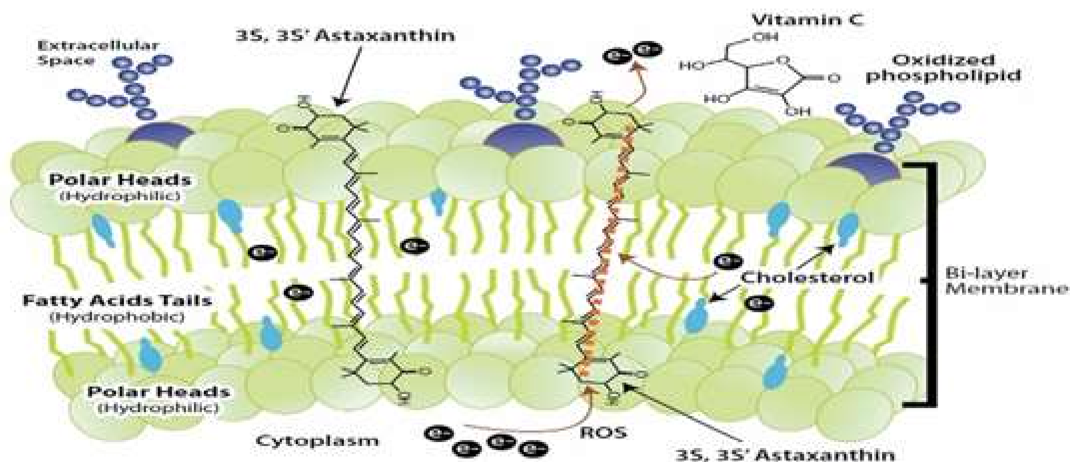


Fig. 2: Presence of astaxanthin in cell membrane (Pashkow et al. 2008)

Table 1 Different forms of astaxanthin

Source of astaxanthin	Different forms	Reference
Antarctic krill (*Euphausia superba*)	3R, 32 R - occurs as the esterified form	(Foss et al. 1987)
Wild Atlantic salmon	3S, 32 S occurs as the free form	
Haematococcus pluvialis	(3S, 32 S) - occurs as the stereoisomer form	[Hussein et al. 2006]
Xanthophyllomyces dendrorhous	(3R, 32 R) - occurs as the stereoisomer form	

disorders, acts as anticancer agent, prevents diabetes, and stimulates immune system. It is used as a feed additive in combating adverse effect of heat stress in bovines (Table 2).

Astaxanthin as a potent antioxidant

Astaxanthin is known as "king of carotenoids" or "supervitamin E" (Pan et al. 2003). It is having both direct and indirect antioxidant actions. Directly scavenges free oxygen radicals to stop chain reactions involved in oxidative damage and indirectly activates enzymes of antioxidant defence system (Buesen et al. 2015). Astaxanthin's ability to penetrate biological membranes with a defensive effect of lipid peroxidation within and outside of the cell membrane is one of its main features (Goto et al. 2001). Antioxidant strength of astaxanthin is 10 times more than zeaxanthin, lutein, canthaxanthin, β-carotene and 100 times greater than α-tocopherol and reported that many of carotenoid antioxidants like lycopene and zeaxanthin, or vitamins C and E under certain conditions become pro-oxidants but astaxnthin never turns into a pro-oxidant (Miki, 1991). Several in- vitro (Speranza et al. 2012) and in-vivo studies (Lee et al. 2011; Augusti et al. 2012) demonstrated antioxidant activity of astaxanthin.

Astaxanthin increases the activity of antioxidant enzymes (SOD, Catalase, GPx) considerably in astaxanthin treated LS-180 cell line (Hormozi et al. 2019) and in rats supplemented with astaxanthin orally (Rao et al. 2013). Yang (2011) reported that astaxanthin is capable of restoring the activity of non-enzymatic antioxidants such as GSH, vitamins C and E and other antioxidant enzymes. Dietary supplementation of astaxanthin can provide antioxidant protection for cells and used in preventing atherosclerotic cardiovascular disease (Pashkow et al. 2008). It is considered as one of the potent antioxidant, as it increases the activity antioxidant enzymes (Choi et al. 2002). Increased expression of caspase 3 and Bax, which showed its anti-proliferative effects in the LS-180 cell line (Tang et al. 2018; Hormozi et al. 2019). Astaxanthin enhances liver function, upturns the potential for defence against oxidative stress and influences on mechanisms of biodefense significantly (Amar et al. 2001).

Astaxanthin has been used as dietary supplement in ameliorating adverse effect of oxidative stress in bovines in hot humid and hot dry season. Beneficial impact of astaxanthin supplementing in murrah buffaloes (Priyadarshini, 2017) and in Tharparkar and Karan Fries heifers (Kumar, 2018) helped to preventing adverse effects of summer stress and improved antioxidant defence system during the summer season. Higher levels of TAC in astaxanthin supplemented heifers helped in reducing the generation of singlet radicals during oxidative stress conditions. Decreased levels of TBARS and lipid peroxidation in treatment group indicated decreased levels of stress in heifers and potent antioxidant activity of astaxnthin in heifers (Kumar and Singh, 2020). Astaxanthin is able to ameliorate adverse effects of oxidative stress in lactating buffaloes by decreasing cortisol and other stress markers in supplemented group (Somagond et al. 2019). Dietary supplementation of astaxanthin during harsh climatic conditions makes animals more heat tolerant and resilient.

Table 2 Use of astaxanthin in bovines

Animal	Dosage	Effects of astaxanthin	Reference
Karan Fries bulls	0.25mg/kg BW/day/animal	• Improved the semen quality during summer season	Soren et al. (2017) Soren and Singh (2018)
Pregnant dairy cows	1mg/kg body weight	• Elevated corpus luteal (CL) function.	Kamada et al. (2017)
Murrah buffaloes (pre and postpartum Murrah buffaloes)	0.25mg/kg BW/day/animal	• Inhibited the level of inflammatory cytokines by blocking NF-κB activation • Improved the milk production performance of animal	Priyadarshini and Aggarwal, (2018)
Karan Fries and Tharparkar heifers	0.25mg/kg BW/day/animal	• Higher weight gain and early attainment of puberty during summer season • Improved the antioxidant status, immunity, growth rate and decreased rate of cell death	Kumar and Singh (2020).
Lactating Murrah Buffaloes	0.25mg/kg BW/day/animal	• Increased milk yield by 5.39% by astaxanthin supplementation alone and 15.6% by combination of astaxanthin + prill fat • Reduced physiological responses and stress indicators levels during summer stress	Somagond et al. (2019) and Somagond et al. (2020)

Anti-inflammatory and immunomodulatory action of astaxanthin

Astaxanthin is an effective antioxidant for terminating inflammation in biological systems. Several studies reported that astaxanthin mainly of marine origin showed its potent antioxidant and anti-inflammatory action (Fassett and Coombes, 2011). There was significant decrease in infection and gastric inflammatory conditions in H. pylori-infected mice when they are treated with algal cell extracts containing astaxanthin (Liu and Lee, 2003). Anti-inflammatory effects of astaxanthin shown through through inhibiting the expression genes like inducible NO synthase (iNOS), cyclooxygenase-2, tumor necrosis factor-alpha (TNF-alpha), and interleukin-1beta (IL-1beta) involved in pro-inflammatory and blocking of NF-kappa B activation and reduced inflammatory mediators. Treatment with astaxanthin helps in reducing the secretion of pro-inflammatory cytokines from NF-κB transcription factor by ROS induced production in H_2O_2-stimulated mononuclear U937 cells (Speranza et al. 2012).

Astaxanthin is also involved in decreasing expression of Bcl2 genes and increases the expression of Caspase 3 and Bax genes, thereby, inhibiting the cells growth and proliferation and inducing apoptosis of cancer cells. So it was said that astaxanthin is also involved treating and prevention of cancers and strengthening the immune system in humans (Pashkow et al. 2008). Similarly Priyadarshini and Aggarwal (2018) reported dietary supplementation of astaxnthin in Murrah buffaloes prevented expression of genes involved in pro-inflammatory reactions, as a result of suppressing activation of NF kB and decreased level of inflammatory mediators. Kumar and Singh (2019) revealed that impact of heat stress was ameliorated in Karan Fries and Tharparkar heifers through NF-κB down regulation, which regulated the expression of genes involved in inflammatory reactions and quenching of free radicals. There were lower

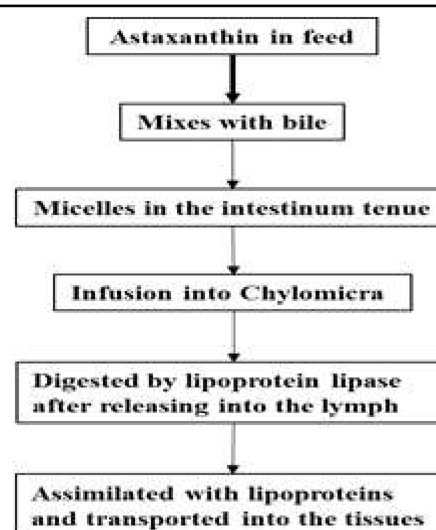


Fig. 3 Pharmacokinetics of astaxanthin (Olson, 2004)

cortisol levels in astaxanthin supplemented groups which showed heat stress alleviation effect during summer season.

In contrast to β-carotene and vitamin E, astaxanthin displayed greater immunomodulatory effects in mouse model (Jyonouchi, 1991). Studies in crustaceans, mainly on shrimp astaxanthin helped in improving immune response and increases tolerance to stress (Darachai et al. 1999). Dietary supplementation of astaxanthin increased antibody production and helped in restoring humoral immune response in older mice (Jyonouchi, 1991). Park et al. (2010) reported that astaxanthin acts as antiviral agent which is involved in enhancing natural killer cells activity, there by destroying virus infected cells. Dietary supplementation of astaxanthin in Murrah buffaloes increased the amount of IgG levels, which could neutralise reactive oxygen species and protected immune cells from lipid peroxidation (Priyadarshini and Aggarwal, 2018).

Astaxanthin in reproductive health of animals

Effect on female reproduction

Despite the fact that astaxanthin is solemnly used for pigmentation and as potent antioxidant, it has many other uses relating to reproduction in fish, i.e., hastening of sexual maturity, improvement in fertilization, survival of eggs and better development of embryos (Putman, 1992). Negative effects of heat stress on in vitro embryo production in summer season can be ameliorated by dietary supplementation of astaxanthin. It helps in restoring reproductive performance of dairy cows exposed to heat stress through regulation oxygen free radicals and antioxidant enzymes in oocytes and cumulus-oocyte complexes (Ispada et al. 2018). Young et al. (1995) described that by dietary supplementation of astaxanthin derived from *Haematococcus pluvialis* was sufficient to elicit its antioxidant effect by detoxification of accumulated peroxides and improving ovary functions. Dietary supplementation of astaxanthin showed increase in corpora lutea (CL), implant sites and foetuses, thereby, decreasing abortion in Minks (Hansen et al. 2001). The addition of astaxanthin to the culture medium help in development of early bovine embryos in culture medium by ameliorating adverse effect of heat stress. In addition, astaxanthin promotes embryonic development during heat stress condition by altering expression genes related to stress (SHC1 and SOD2) (Namekawa et al. 2010). Addition of astaxanthin to maturation medium (0.5 ppm) improved growth and maturation of porcine oocyte and also increased the number of oocytes reaching metaphase II and considerably reduced apoptosis of oocytes exposed to H_20_2 during in vitro maturation (IVM) (Do et al. 2015). Jang et al. (2010) revealed that addition of astaxanthin enhanced the growth of cultured embryos in bovine oviduct epithelial cells. Kuroki et al. (2013) also reported that astaxanthin supplementation during IVM enhanced mitochondrial membrane potential, which can help in rescuing oocyte from ROS generated due to heat induction.

Effect on male reproduction

In astaxanthin supplemented group of goldfish (*Carassius auratus*), higher sperm motility and fertilization rate was reported by Tizkar et al. (2015). Astaxanthin supplementation to human patient showed positive effect on sperm cells by decreasing the ROS and secretion of inhibin B, hence improving sperm quality and its function and increased conception rates or fertility (Comhaire, 2005). Concentrations of 2µM and 4µM of astaxanthin appeared to be successful dose to increase sperm potency during oxidative stress conditions (Salamon and Maxwell, 2000). Astaxanthin treatment in male mice greatly improved weight of testis and sperm morphological characteristics. Astaxanthin was able to restore sperm DNA damage because of generation of ROS in Cyclophosphamide (CP) induced toxicity. It also indicated germ cell protection of astaxanthin against chemo toxicity (Tripathi and Jena, 2008). Astaxanthin fed rats have shown a substantial reduction in ROS production in semen and improved male fertility (Mortazavi et al. 2014). Semen extender added with astaxanthin helped in improving the sperm quality and function of ram by reducing of malondialdehyde and ROS during storage (Fang et al. 2015) and bull (Farzan et al. 2014, Soren et al. 2017) semen at 5 °C for 72 hours. During production of miniature pig semen straw, adding of astaxanthin to frozen storage solutions, prevented damages during freeze-thawing process and maintained quality of sperms and improved fertility rate (Lee and Kim, 2018).

Kumar and Singh (2020) revealed that dietary supplementation of astaxanthin to Karan Fries and Sahiwal heifers helped in increasing level of growth hormone (GH), conservation of energy, increased growth performance and early attainment of puberty in supplemented group. Holstein bulls supplemented with astaxanthin enhanced the sperm progressive motility and reduced the lipid peroxidation rate in semen (Farzan et al. 2014). Addition of astaxanthin (0.25mg/kg body weight) revealed low level of antioxidant enzymes in semen, indicating positive effect on semen quality in Karan Fries bulls in heat stress conditions (Soren et al. 2017). Dietary Supplementation of astaxanthin in summer to crossbred (Karan Fries) bulls improved the quality of semen by improving antioxidant activity, which was indicated by decreased malondialdehyde concentration and higher expression of mitochondrial gene in spermatozoa like, mitochondrial transcriptions factor A (TFAM), citrate synthases (CS) and succinate dehydrogenase (SDH),. Supplementation with astaxanthin also enhanced semen quality parameters (volume, motility, concentration, and acrosome integrity) and reduced major abnormalities in semen over non supplemented bulls. Therefore, astaxanthin supplementation can be used as one of strategies in improving semen quality of crossbred bulls and amelioration of adverse effect of heat stress was suggested by Soren and Singh (2018).

Astaxanthin which acts as a potent antioxidant helps in amelioration of adverse effect of heat stress and improving productive performance of animals. Kumar and Singh (2020) reported astaxanthin supplementation in diets of heifers helped in improvement of growth, immunity, which subsequently leads to early attainment of puberty and increased productive life of cattle. Astaxanthin supplementation along with prill fat was able to maintain body condition and health status of buffaloes by mitigating adverse effect during summer season (Somagond et al. 2020). Priyadarshni (2017) revealed that astaxanthin supplementation enhanced immunity and lowered stress marker levels in Murrah buffaloes and improved reproductive health and milk yield by 7% during heat stress condition. There was 5.39% and 15.6% increase in milk yield in astaxanthin and astaxanthin in combination with prill fat fed groups of buffaloes over control group, respectively during summer season (Somagond et al. 2019).

Conclusion

Astaxanthin as a feed additive helps in amelioration of adverse effects of heat stress, acts as anti-inflammatory, improves immunity, reduces stress levels in bovines. Therefore, astaxanthin can be used as an important feed additive to combat the adverse effect of climate change/ stress and for improving production performance of animals during adverse climatic conditions.

References

Amar EC, Kiron V, Satoh S, Watanabe T (2001) Influence of various dietary synthetic carotenoids on bio defence mechanisms in rainbow trout, Oncorhynchus mykiss (Walbaum). Aquac Res 32: 162-173

Ambati RR, Phang SM, Ravi S, Aswathanarayana, RG (2014) Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. Mar Drugs 12: 128-152

Augusti PR, Quatrin A, Somacal S, Conterato GM, Sobieski R, Ruviaro AR, and Emanuelli T (2012) Astaxanthin prevents changes in the activities of thioredoxin reductase and paraoxonase in hypercholesterolemic rabbits. J Clin Biochem Nutr 51: 42-49

Biswal S (2014) Oxidative stress and astaxanthin: The novel supernutrient carotenoid. Int J Res Health Allied Sci 3: 147

Buesen R, Schulte S, Strauss V, Treumann S, Becker M, Groters S, Van Ravenzwaay B (2015) Safety assessment of [3S, 32 S]-astaxanthin– Subchronic toxicity study in rats. Food Chem Toxicol 81:129-136

Choi YE, Yun YS, Park JM (2002) Evaluation of factors promoting astaxanthin production by a unicellular green alga, Haematococcus pluvialis, with fractional factorial design. Biotechnol Prog 18: 1170-1175

Comhaire FH, Garem YE, Mahmoud A, Eertmans F, Schoonjans FRANK (2005) Combined conventional/antioxidant “Astaxanthin” treatment for male infertility: a double blind, randomized trial. Asian J Androl 7: 257-262

Cyanotech 2000 NatuRose®. Available at www.cyanotech.com/ html/asta/natu/naturose.html [accessed 12 December 2007]

Darachai J, Piyatiratitivorakul S, Menasveta P (1999) Effect of astaxanthin on growth and survival of Penaeus monodon larvae. In Proceedings of the 37th Kasetsart University Annual Conference 36-41

Do LTK, Luu VV, Morita Y, Taniguchi M, Nii M, Peter AT, Otoi T (2015) Astaxanthin present in the maturation medium reduces negative effects of heat shock on the developmental competence of porcine oocytes. Reprod Biol 15: 86-93

Fang YI, Zhong R, Chen L, Feng C, Sun H, Zhou D (2015) Effects of astaxanthin supplementation on the sperm quality and antioxidant capacity of ram semen during liquid storage. Small Rumin Res 130: 178-182

Farzan M, Chamani M, Varnaseri H (2014) The antioxidant effect of astaxanthin on quantitative and qualitative parameters of bull sperm. Indian Journal of Foundamental and Applied Life Sciences 4: 425-430

Fassett RG, Coombes JS (2011) Astaxanthin: a potential therapeutic agent in cardiovascular disease. Mar Drugs 9(3): 447-465

Foss P, Renstrøm B, Liaaen-Jensen S (1987) Natural occurrence of enantiomeric and Meso astaxanthin 7"-crustaceans including zooplankton. Comp Biochem Physiol B 86: 313-314

Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, Terada H (2001) Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochim Biophys Acta Biomembr 1512: 251-258

Guerin M (2001) Pigmentation of red seabream with natural astaxanthin derived from the alga Haematococcus pluvialis: comparison with synthetic astaxanthin. In Aquaculture 2001 conference, Lake Buena Vista, Florida, January 2001. World Aquaculture Society

Guerin M, Huntley ME, Olaizola M (2003) Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol 21: 210-216

Hansen KB, Tauson AH, Inborr J (2001) Effect of supplementation with the antioxidant astaxanthin on reproduction, pre-weaning growth performance of kits and daily milk intake in mink. J Reprod Fertil Suppl 57: 331-334

Higuera-Ciapara I, Felix-Valenzuela L, Goycoolea FM (2006) Astaxanthin: a review of its chemistry and applications. Crit Rev Food Sci Nutr 46: 185-196

Holtin K, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K (2009) Determination of astaxanthin and astaxanthin esters in the microalgae Haematococcus pluvialis by LC-(APCI) MS and characterization of predominant carotenoid isomers by NMR spectroscopy. Anal Bioanal Chem 395: 1613

Hormozi M, Ghoreishi S, Baharvand P (2019) Astaxanthin induces apoptosis and increases activity of antioxidant enzymes in LS-180 cells. Artif Cells Nanomed Biotechnol 47: 891-895

Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006) Astaxanthin, a carotenoid with potential in human health and nutrition. J. Nat. Prod 69: 443-449

Ispada J, Rodrigues TA, Risolia PHB, Lima RS, Gonçalves DR, Rettori D, Paula-Lopes FF (2018) Astaxanthin counteracts the effects of heat shock on the maturation of bovine oocytes. Reprod Fert Dev 30: 1169-1179

Jang HY, Ji S J Kim, YH Lee, HY Shin, JS, Cheong HT, Yang BK (2010) Antioxidative effects of astaxanthin against nitric oxide induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. Reprod Domest Anim 45: 967-974

Jyonouchi H, Hill RJ, Tomita Y, Good RA (1991) Studies of immunomodulating actions of carotenoids. I. Effects of β carotene and astaxanthin on murine lymphocyte functions and cell surface marker expression in in vitro culture system. Nutr Cancer 16: 93-105

Kamada H, Akagi S, Watanabe S (2017) Astaxanthin increases progesterone production in cultured bovine luteal cells. J Vet Med Sci 17-0044

Kumar S (2018) Influence of astaxanthin supplementation on growth performance, immunity and antioxidant status in heifers during

summer season (Doctoral dissertation, National Dairy Research Institute, Karnal)

Kumar S, Singh SV (2019) Inhibition of NF-κB signaling pathway by astaxanthin supplementation for prevention of heat stress–induced inflammatory changes and apoptosis in Karan Fries heifers. Trop Anim Health Pro 51: 1125-1134

Kumar S, Singh SV (2020) Influence of astaxanthin supplementation on attainment of puberty and lipid peroxidation in Sahiwal and Karan Fries (Holstein× Tharparkar) heifers during summer season. Biol Rhythm Res 51: 15-28

Kuroki T, Ikeda S, Okada T, Maoka T, Kitamura A, Sugimoto M, Kume S (2013) Astaxanthin ameliorates heat stress-induced impairment of blastocyst development *In Vitro*:–astaxanthin colocalization with and action on mitochondria. J Assist Reprod Gen 30: 623-631

Lara JJ, Economou M, Wallace AM, Rumley A, Lowe G, Slater C, Lean ME (2007) Benefits of salmon eating on traditional and novel vascular risk factors in young, non-obese healthy subjects. Atherosclerosis 193: 213-221

Lee DH, Kim CS, Lee YJ (2011) Astaxanthin protects against MPTP/ MPP+-induced mitochondrial dysfunction and ROS production in vivo and in vitro. "Food Chem. Toxicol 49: 271-280

Lee E, Kim D (2018) Effects of astaxanthin on miniature pig sperm cryopreservation. Biomed Res Int. doi.org/10.1111/and.13389.

Liu BH, Lee YK (2003). Effect of total secondary carotenoids extracts from Chlorococcum sp. on Helicobacter pylori-infected BALB/c mice. Int Immunopharmacol 3: 979-986.

Liu X, Osawa T (2007) Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity in vitro compared to the all-trans isomer. Biochem Biophys Res Commun *357*: 187-193

McNulty H, Jacob RF, Mason RP (2008) Biologic activity of carotenoids related to distinct membrane physicochemical interactions. Am J Cardiol 101: S20-S29

Miki W (1991) Biological functions and activities of animal carotenoids. Pure Appl Chem 63: 141-146

Mortazavi M, Salehi I, Alizadeh Z, Vahabian M and Roushandeh AM (2014) Protective effects of antioxidants on sperm parameters and seminiferous tubules epithelium in high fat-fed rats. J Reprod Infertil 15: 22

Naguib YM (2000) Antioxidant activities of astaxanthin and related carotenoids. J Agric Food Chem 48: 1150-1154

Namekawa T, Ikeda S,Sugimoto M, Kume S (2010) Effects of astaxanthin containing oil on development and stress related gene expression of bovine embryos exposed to heat stress. Reprod Domest Anim 45(6): e387-e391

Olson J A (2004) Carotenoids: absorption, transport, and metabolism of carotenoids in humans. Pure Appl Chem 66: 1011-1016

Pan CH, Chien YH, Hunter B (2003) The resistance to ammonia stress of *Penaeus monodon, Fabricius juvenile* fed diets supplemented with astaxanthin. J Exp Mar Biol Ecol 297: 107-118

Park JS, Chyun JH, Kim YK, Line LL, Chew BP (2010) Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. Nutr. Metab 7: 18

Pashkow FJ, Watumull DG, Campbell CL (2008) Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. Am J Cardiol 101: 58-S68

Priyadarshini L (2017) Immunity, antioxidant, energy status and production performance in pre and post-partum Murrah buffaloes during summer and winter seasons (Doctoral dissertation, National Dairy Research Institute; Karnal)

Priyadarshini L, Aggarwal A (2018) Astaxanthin inhibits cytokines production and inflammatory gene expression by suppressing IκB kinase-dependent nuclear factor κB activation in pre and postpartum Murrah buffaloes during different seasons. Vet World 11: 782

Putman M (1992) A review of the nature, function, variability, and supply of pigments in salmonid fish. EAS Special Publication

Rao AR, Sindhuja HN, Dharmesh SM, Sankar KU, Sarada R, Ravishankar GA (2013) Effective inhibition of skin cancer, tyrosinase, and antioxidative properties by astaxanthin and astaxanthin esters from the green alga *Haematococcus pluvialis*. ýJ Agric Food Chem 61: 3842-3851

Salamon S, Maxwell WMC (2000) Storage of ram semen. Anim. Reprod Sci 62: 77-111

Sandmann G (1994) Carotenoid biosynthesis in microorganisms and plants. In EJB Reviews. Springer, Berlin, Heidelberg 129-146

Somagond YM, Singh S, Deshpande A, Sheoran P, Chahal VP (2020) Physiological responses, energy metabolites and prolactin levels of buffaloes supplemented with dietary astaxanthin, prill fat and their combination during heat stress. Indian J Anim Sci 90: 55-60

Somagond YM, Singh SV, Deshpande A (2019) Effect of dietary supplementation of astaxanthin, prill fat and combination on stress indicators, milk yield and composition during heat stress in buffaloes. Biol Rhythm Res 1-11.doi.org/10.1080/09291016.2019.1658426

Soren S, Singh SV (2018) Semen quality, lipid peroxidation and expression of mitochondrial gene in ejaculated sperm of Karan Fries (Tharparkar x Holstein Friesian) bulls supplemented with astaxanthin. Indian J Anim Sci 88: 1346-1352

Soren S, Singh SV, Kumar S (2017) Effect of astaxanthin supplementation on semen (Karan Fries Bulls) storage at 5 C. Int J Curr Microbiol Appl Sci 6: 23-28

Speranza, L, Pesce M, Patruno A, Franceschelli S, Lutiis MAD, Grilli A, Felaco M (2012) Astaxanthin treatment reduced oxidative induced pro-inflammatory cytokines secretion in U937: SHP-1 as a novel biological target. Mar Drugs 10: 890-899

Stahl W, Sies H (2005) Bioactivity and protective effects of natural carotenoids. Biochim Biophys Acta Mol Basis Dis 1740: 101-107

Takaichi S, Matsui K, Nakamura M, Muramatsu M, Hanada S (2003) Fatty acids of astaxanthin esters in krill determined by mild mass spectrometry. Comp Biochem Physiol B Biochem Mol Biol 136: 317-322

Tang X, Liu B, Wang X, Yu Q, Fang R (2018) Epidermal growth factor, through alleviating oxidative stress, protect IPEC-J2 cells from lipopolysaccharides-induced apoptosis. Int J Mol Sci 19: 848

Tizkar B, Kazemi R, Alipour A, Seidavi A, Naseralavi G, Ponce-Palafox JT (2015) Effects of dietary supplementation with astaxanthin and β-carotene on the semen quality of goldfish (Carassius auratus). Theriogenology 84: 1111-1117

Tripathi DN, Jena GB (2008) Astaxanthin inhibits cytotoxic and genotoxic effects of cyclophosphamide in mice germ cells. Toxicology 248: 96-103

Yang Y, Seo JM, Nguyen A, Pham TX, Park HJ, Park Y, Lee J (2011) Astaxanthin-rich extract from the green alga Haematococcus pluvialis lowers plasma lipid concentrations and enhances antioxidant defense in apolipoprotein E knockout mice. J Nutr 141: 1611-1617

Young FM, Luderer WB, Rodgers RJ (1995) The antioxidant β-carotene prevents covalent cross-linking between cholesterol side-chain cleavage cytochrome P450 and its electron donor, adrenodoxin, in bovine luteal cells. Mol Cell Endocrinol 109: 113-118

Yuan JP, Peng J, Yin K, Wang JH (2011) Potential health promoting effects of astaxanthin: A high value carotenoid mostly from microalgae. Mol Nutr Food Res 55: 150-165

Indian J Dairy Sci 74(1): 8-17
https://doi.org/10.33785/IJDS.2021.v74i01.002

RESEARCH ARTICLE

Heat stability of high-protein ultrafiltration retentate: Effect of concentration factor and stabilizing salts

Ganga Sahay Meena[1], Ashish Kumar Singh[1], Vijay Kumar Gupta[1], Harleen Kaur Gujral[2] and Rashmeet Kaur Malhotra[2]

Received: 05 October 2020 / Accepted: 20 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Pasteurized cow skim milk (PCSM, 1×) was concentrated using ultrafiltration (UF) from 2 ×-7 × concentration factor (CF) to obtain retentates with different protein to total solids (TS) ratio. Alteration in chemical make-up led to poor thermal stability in UF retentates. Therefore, this study was aimed to investigate the changes in permeate flux, chemical composition, pH, viscosity and heat coagulation time (HCT) of UF/DF retentates as a function of CF. Impact of high-shear (20,000 rpm/ 5 min), diafiltration (DF) with 150mM NaCl solution and stabilizing salts on HCT of high-protein 7×UF retentates were also evaluated. With increase in CF; TS, protein, fat, ash, calcium contents and viscosity increased significantly ($p<0.05$), while permeate flux, lactose content, pH and HCT of retentates significantly decreased ($p<0.05$). Poor HCT (1 min) of 7×UF retentate was significantly increased ($p<0.05$) up to 60 min by addition of stabilizing salts (NaH_2PO_4, Na_2HPO_4 and $Na_3C_6H_5O_7$). Correlation was established between chemical constituents, pH, viscosity, HCT, permeate flux and concentration factor. Overall, this investigation established that identification of correct stabilizing salts via HCT-pH curve of PCSM and their appropriate addition converted thermally unstable (poor HCT) high-protein (7× UF) retentates to thermally stable product. Hence, they could be now explored in number of food applications.

Keywords: Diafiltration, Heat stability, Protein concentration, Stabilizing salts, Ultrafiltration, Viscosity

[1]Dairy Technology Division, ICAR- National Dairy Research Institute, Karnal, India-132001

[2]Amity Institute of Food Technology, Amity University, Noida, Uttar Pradesh, India-201303

Ganga Sahay Meena(✉)
Dairy Technology Division, ICAR- National Dairy Research Institute, Karnal, India-132001
Email:gsiitkgp@gmail.com
+91-184-2259485 (O); +91 9996129094 (M)

Introduction

Membrane processing had enabled the dairy sector to separate, fractionate, concentrate and purify milk proteins without applying harsh conditions related to temperature and pH. Ultrafiltration (UF) is a well-established, pressure-driven membrane process that finds maximum use in dairy industry for concentration and purification milk proteins. At a particular temperature, a part of water soluble milk constituents (such as lactose, vitamins and minerals) are selectively allowed to pass through UF membrane into permeate under the influence of applied trans-membrane pressure (TMP) and membrane molecular weight cut off (MWCO). Contrary to this, water insoluble/ colloidal (fat, calcium and protein) milk compounds are rejected by UF membrane based on their size, shape, charge and MWCO and further concentrated in UF retentate with increase in concentration factor. Concentration factor is the ratio of initial weight or volume of feed to final weight or volume of retentate (Meena et al. 2016). At similar TS level, the chemical composition of concentrates produced by either reverse osmosis (RO) or by multiple effect evaporation was different than that of UF retentate (Meena et al. 2016). Diafiltration (DF) is accomplished in the same UF plant to increase protein purity which is otherwise not possible with UF process alone. Thus, both UF and DF process causes alteration in chemical composition of UF/DF retentates and the same is responsible for different physico-chemical, rheological and functional characteristics of those retentates compared to that of concentrated milks.

Heat coagulation time (HCT) also known as thermal stability is very important functional property of liquid and concentrated milks. During high-heat processing of milks, it plays a prominent role. Singh (2004) defined HCT as the resistance (as time in minutes) shown by liquid and concentrated milks prior to onset of their heat induced coagulation at 140ºC and 120 or 130ºC, respectively. Chemical composition, more precisely protein and

calcium contents, pH and salt balance ratio have major influence on HCT of liquid and concentrated milks. HCT is mainly governed by stability of their milk proteins. As per Solanki and Gupta (2009), even slight change in delicate salt balance of milk, resulted from any process or additives, have major influence on their thermal stability.

HCT of liquid and concentrated milks (manufactured using multiple effect evaporators) has been mostly targeted in scientific investigations. Contrary to conventionally concentrated milks (maximum HCT between 6.4-6.6 pH), at similar protein or TS levels, UF and DF retentates exhibited entirely different HCT-pH profile. This was collectively governed by the extent of protein concentration, change in pH and alteration in delicate ionic equilibrium between serum and micellar proteins during skim milk concentration in UF/DF processes (Holt et al. 1981). Apart from intrinsic and extrinsic factor (Singh 1995), HCT of concentrated milks was also influenced by composition of salts (Sweetsur and Muir 1980) and beta lactoglobulin (β-Lg) as they showed inverse correlation with HCT (Muir and Sweetsur 1978). Singh and Creamer (1992) found liquid milks were more heat stable than concentrated milks on entire pH range.

Sweetsur and Muir (1980) evaluated the impact of pre-heating (90ºC/10 min) treatment and observed that it caused slight increase in HCT of UF retentate, while doubled the HCT of ordinary produced concentrated milk. Partial removal of calcium from liquid or concentrated milks tend to increase their thermal stability. Ozimek et al (1988) observed non-significant differences between HCT of fresh and a week old samples of UF retentate. It was demonstrated in our earlier investigation that stabilizing salts significantly ($P<0.01$) improved the HCT of homogenized 5× UF retentate from 1.45 min (pH 6.41) to 120 min (at pH 6.5, 6.6, 7.0) and 80 min (pH 6.6), respectively (Meena et al. 2016). However, such studies have not been so far conducted on highly concentrated (such as 7× UF/DF) retentates containing higher protein to TS ratio.

UF retentate acts as raw material or an ingredient during production of high-protein milks, *dahi/*curd (Meena et al. 2016), yoghurt (Yadav et al. 2018), dairy whiteners (Khatker et al. 2012) and milk protein concentrate (MPC) powders with different protein contents (Meena et al. 2017b). High-protein beverages which are preserved by conventional sterilization or ultra-high temperature treatments demands better thermal stability in high-protein liquid (retentates) and dried (MPC powders) ingredients (Meena et al. 2017a). Practically, proteins present in skim milk are concentrated using UF to achieve 0.60 protein to TS ratio in UF retentate. Its further purification with DF is necessary to obtain higher (>0.60) protein to TS ratio. Indeed, degree of protein concentration is directly proportional to increase in TS and calcium contents of UF/DF retentates which also exert detrimental effect on their HCT values.

Addition of stabilizing salts in concentrated milks to improve their heat stability is well- documented and commercially being explored in production of evaporated milks (Solanki and Gupta, 2009). These findings are not as such applicable on high-protein (6-7×) UF/DF retentates because of their higher protein and calcium contents at similar TS levels. Studies dedicated to improvement in HCT of such retentates are still scanty.

Therefore, present investigation has been aimed (i) to investigate the changes taking place in chemical composition, pH, viscosity and HCT of UF/DF retentates as well as in permeate flux as a function of concentration factor (1× to 7×) and (ii) to evaluate the impact of high-shear treatment (20,000 rpm/ 5 min) and stabilizing salts such as monosodium phosphate (MSP-NaH_2PO_4), disodium phosphate (DSP-Na_2HPO_4) and trisodium citrate (TSC-$Na_3C_6H_5O_7$) on HCT of high-protein 7× UF/DF retentates.

Materials and Methods

Materials

Fresh cow milk of morning shift was obtained from cattle yard at Experimental Dairy of ICAR-national Dairy Research Institute (ICAR-NDRI), Karnal; it was heated to 40±1ºC, centrifugally separated and, pasteurized at 73±1ºC/15s using a commercial scale milk pasteurizer (make-GEA Westfalia, capacity-10 KLPH). This pasteurized cow skim milk (PCSM) was collected in pre-sterilized stainless steel (SS) cans. All the chemicals used in present investigation were of analytical grade and procured from Sigma-Aldrich, (St. Louis, MO, US). Pre-serialized borosilicate glass bottles (make- BOROSIL®, capacity- 1000 mL) were used for the purpose of sample collection. For calcium estimation, only acid wash glassware were used.

Methods

Manufacturing of UF retentates (UFR) via concentration of PCSM

The SS cans containing PCSM were partially immersed in hot water, stored in a cheese vat at 80±1ºC. For better heat transfer, PCSM was slowly stirred using a pre-sterilized SS plunger. Thereafter, this pre-heated PCSM was filtered through a clean muslin cloth and poured in the balance tank of a pilot scale UF plant (make- Tech Sep, France), equipped with a tubular heat exchanger, retentate and permeate flow meters, thermometer and different pressure gauges. This plant was also equipped with two tubular membrane modules containing total 1.68m^2 area of zirconium oxide (mineral) membrane. The MWCO of this UF membrane was 50 kilo Dalton. Before each run, the UF plant was properly sanitized and completely drained. Total 300 kg PCSM was used in each run.

Maintaining constant TMP of 1 bar, PCSM (1×) was concentrated to 2×, 3×, 4×, 5×, 6× and 7× concentration factors (ratio of initial

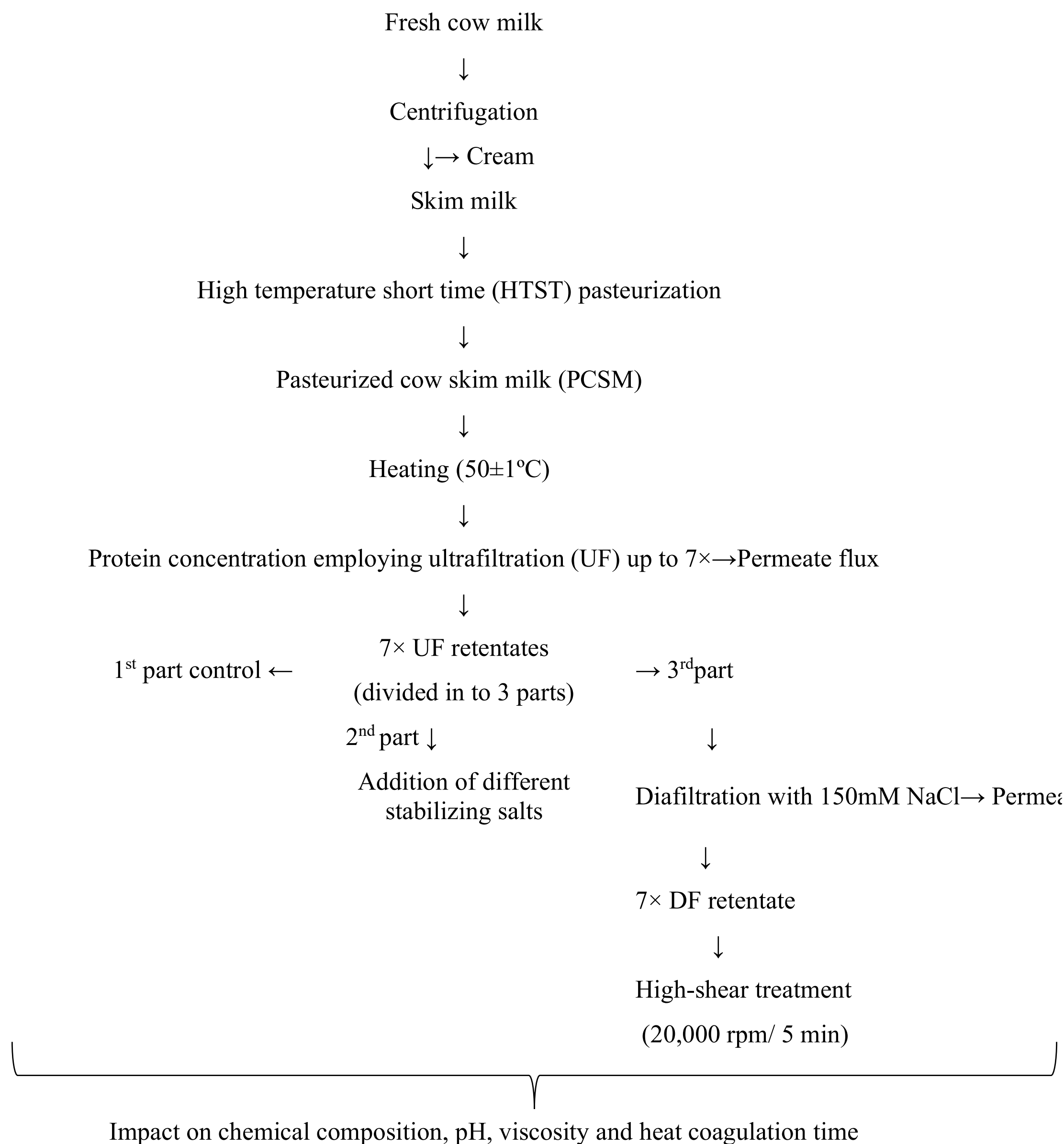


Fig. 1 Manufacturing of ultrafiltration (UF) - diafiltration (DF) retentates from pasteurized cow skim milk (PCSM).

weight of feed to final weight of retentate, usually denoted by ×) in UF plant at a constant temperature (50±1ºC). The weight of PCSM and permeate samples were determined using an electronic weighing balance. At any point of time, total weight of permeate subtracted from total weight of PCSM provided the actual retentate weight. At each concentration factor, the values of permeate flux in liter per hour per m^2 (LMH) were directly recorded from the permeate flow meter and used to calculate flux mean as per following equation (St-Gelais *et al.* 1991).

$$\text{Flux mean} = \text{Final flux} + 0.33 \times (\text{Initial flux} - \text{Final flux})$$

At each concentration factor, representative samples of 1×-7× UF retentates were collected in pre-sterilized glass bottles. The 7× UF retentate (7× UFR) sample was divided into 3 parts. First part was treated as control, second part was used to evaluate the effect of different stabilizing salts while third was further subjected to diafiltration treatment as mentioned below.

Diafiltration and high-shear treatment of 7× UF retentate

The 150mM NaCl solution was prepared in reverse osmosis (RO) water and used for the diafiltration of third part of 7×UF retentate in 1:1 ratio. Diafiltration was accomplished in same pilot scale UF plant via removal of exactly similar amount of permeate to the amount of added NaCl solution. The retentate thus produced

was referred as 7× DF retentate (7× DFR). A part of this was also subjected to high-shear treatment (20,000 rpm for 5 min) using a ULTRA-TURRAX® homogenizer (make: Ika, model: T18 digital). During high-shear treatment, sample temperature was maintained between 5-10ºC by keeping it in an ice bank. In order to keep a check on microbial growth, NaN_3 (0.03% w/w) was added in all samples prior to their refrigerated storage (4±1ºC), until their further analysis.

Chemical analysis and calcium determination

Standard gravimetric methods as reported in Indian standards (IS: 12333 1997 and IS: 1479 part III 1961) were used to determine TS and ash contents of PCSM and all retentate samples. Further, their crude protein contents were estimated adopting Macro Kjeldahl Method (IDF 1993) and multiplied with a constant factor (6.38) to get their actual protein contents. Gerber method (IS: 1224 part I 1977) was used to obtain fat contents of all these samples, while following equation was used to determine their lactose contents, respectively:

$$\text{Lactose content} = [\text{TS} - (\text{protein content} + \text{fat content} + \text{ash content})]$$

The standard AOAC (2005) method was used to determine calcium content of these samples using an atomic absorption spectrophotometer (make-Shimadzu; model- AA-7000).

pH measurement and its adjustment by stabilizing salts addition

To measure the pH of PCSM and different UF and DF retentates at 20±1ºC, a Eutech pH meter (make-Thermo Scientific, model-cyberscan 1100) was used. First of all, pH of PCSM and 7× UF retentate were adjusted in the range of 6.0 to 7.2 (with a constant increment of 0.1 unit) using standard 0.1N HCl and 0.1N NaOH solutions. These pH adjusted PCSM samples were used to determine its HCT-pH curve (Figure 3.a) that was further explored to find suitable stabilizing salt (s) for improving the HCT of 7×UFR. Thereafter, 1.0% (w/v) solutions of NaH_2PO_4 (MSP), Na_2HPO_4 (DSP) and $Na_3C_6H_5O_7$ (TSC) salts were prepared in distilled water and used to adjust the desired pH of 7×UFR as shown in Figure 3.b. The 0.2-0.5 % mixture of MSP and DSP (2:1 w/w) salts with and without 0.7% TSC were directly added in 7×UFR in order to evaluate their effect on HCT via total 11 treatments (T1-T11) as shown in Table 2. In order to nullify the effect of buffering action of milk proteins, initially adjusted pH of each sample was re-checked and re-corrected after constant duration of 1 h at 20±1ºC.

Determination of heat coagulation time (HCT)

Under standard test conditions, resistance shown by liquid and concentrated milk samples prior to onset their thermal coagulation is called HCT. In this investigation, HCT of PCSM was measured at 140ºC while that of UF/DF retentates was measured at 120ºC adopting the method reported by Khatkar et al. (2014).

Measurement of apparent viscosity

A Rheometer (MCR 52, Anton Paar, Germany) equipped with stainless steel cone plate CP75-1° probe was used to measure apparent viscosity (now onwards written as viscosity) of PCSM, UF and DF retentates at a fixed shear rate of 100 per second and temperature (20ºC). Total twenty measurements were recorded for each sample. The analysis was conducted in triplicate for each sample.

Statistical analysis

One-way analysis of variance (ANOVA) and Pearson correlation were employed for statistical analysis of the obtained data using SAS Enterprise guide (SAS 2008). Using Duncan's multiple range test, the mean were compared (Duncan 1955).

Results and Discussion

PCSM concentration by UF and DF: effect on chemical composition, pH and HCT of UF/DF retentates and permeate flux

Table 1 shows the change in chemical composition, pH, and HCT of UF/DF retentates and permeate flux at each concentration factor (also denoted by '×') during PCSM concentration by UF process. Compared to TS, protein and ash contents of PCSM (1×), these components of all UF (2×-7×) retentates and DF (7×DFR) retentates were significant different ($p<0.05$) with each other and showed a significant increase ($p<0.05$) with increase in concentration factor. Similar increasing trend was also observed in fat contents of these UF retentates. The fat contents of 7×UFR and 7×DFR were statistically at par ($p>0.05$) with each other, but significantly higher ($p<0.05$) than the fat contents of PCSM and remaining UF retentates. Over PCSM (1×) sample on dry matter (DM or TS) basis (Table 1), the protein, fat, ash and calcium contents of 2×-7× UF retentates increased in the range of 52.34-74.72, 1.73-2.48, 6.40-7.96 and, 2.16-2.61%, respectively. Contrary to marked increase in above mentioned constituents, significant decrease ($p<0.05$) in lactose content, pH, HCT and permeate flux values were observed with increase in concentration factors in all retentates. The lactose content (on DM basis), pH and HCT of PCSM (1×) were 55.37%, 6.67 and 60 min (Table 1) which decreased to 14.25%, 6.32, 1 min and 13.30%, 6.24 and 1 min , respectively in 7×UF retentate and 7×DF retentate samples.

Permeation of water soluble milk components into permeate through UF membrane along with subsequent concentration of colloidal milk constituents owing to their selective retention could easily explain such changes in concentration of milk components present in different UF/DF retentates. Similar changes in milk constituents were also reported by Meena et al (2016); Khatkar and Gupta (2014); Mistry (2002) during concentration of PCSM by ultrafiltration. Further, 2× to 7× concentration of PCSM (1×) markedly increased their calcium contents (on DM basis) in the

range of 2.16 to 2.61% compared to 1.42% calcium in PCSM. This is mainly attributed to concentration of milk proteins particularly casein. This is because only a part of soluble calcium present in PCSM passes into permeate through UF membrane, but at the same time its colloidal calcium, present in the form of colloidal calcium phosphate (CCP) got concentrated with concentration of caseins. Hence, the overall concentration of calcium enhances with increase in degree of protein concentration as shown in Table 1.

The increase in TS and ash contents seems to be responsible for significant ($p<0.05$) decrease in pH values of 2× to 7× UF retentates as a function of concentration factors (Table 1). Ferrer et al. (2011) reported that during ultrafiltration of skim milk, concentration of different minerals (such as K^+, Mg^+ and Ca^{2+}) also increases. Further, such rise in mineral contents induces changes in cationic profile of milk based on their association with milk proteins that ultimately reduces pH of UF retentates. Gradual decrease in pH of retentates during ultrafiltration was earlier also reported by Meena et al (2016); Ferrer et al. (2011). Apart from this, significant reduction ($p<0.01$) was observed in pH of 7×DFR than the pH of 7×UFR. This was attributed to applied diafiltration of 7× UFR with 150mM NaCl solution. Meena et al (2016) also observed decrease in pH of 5× DF retentate.

Thermal stability of concentrated milk is majorly influenced by the concentration of its constituents and its delicate salt equilibrium. It is clearly evident from Table 1 that with increase in concentration factor, chemical composition of different UF and DF retentates markedly changed. Furthermore, this also changed their pH and calcium contents which have prominent role in stabilization of milk proteins. Thus, alteration in chemical composition, decrease in pH and increase in calcium contents might have collectively disturbed the delicate salt equilibrium of UF and DF retentates and the same could be attributed to significant ($p<0.05$) decrease in their HCT values as shown in Table 1. Indeed, higher calcium content and lower pH are detrimental for thermal stability of concentrated milk samples and the same can easily explain relatively lower HCT values of UF retentates subjected to higher concentration factors. Ozimek et al (1998) also reported that with increase in volume concentration ratio, a decreasing trend was observed in HCT values of UF retentates.

As expected a gradual and significant ($p<0.05$) reduction was observed in permeate flux with the advancement of UF concentration (Table 1). Increase in TS and viscosity as well as decrease in pH of UF retentates acts as the prime factors promoting concentration polarization and fouling of UF membrane. Both concentration polarization and fouling are inversely proportional to permeate flux (Meena et al. 2016). In nutshell, reduction in stability of milk proteins have detrimental impact on permeate flux during ultrafiltration. The calculated mean flux values at each concentration factor are shown in Table 1 that clearly displayed a decreasing trend. Such reduction in permeate

Table 1 Chemical composition, pH, HCT of retentates and permeate flux at different concentration factors during ultrafiltration and diafiltration of PCSM

CF	TS	Protein	Fat	Lactose	Ash	Calcium	pH	HCT	Permeate
			Percent					(min)	flux LMH
1×	$8.47^h \pm 0.01$	$3.02^h \pm 0.09$	$0.1^g \pm 0.00$	$4.69^a \pm 0.00$	$0.66^h \pm 0.01$	$0.12^g \pm 0.00$	$6.67^a \pm 0.02$	$60.00^a \pm 0.88$	$99^a \pm 0.58$
		(35.66)	(1.18)	(55.37)	(7.79)	(1.42)			
2×	$11.56^g \pm 0.06$	$6.05^g \pm 0.07$	$0.2^f \pm 0.00$	$4.58^b \pm 0.02$	$0.74^g \pm 0.01$	$0.25^f \pm 0.00$	$6.57^b \pm 0.01$	$60.00^a \pm 0.58$	$68.33^b \pm 0.88$
		(52.34)	(1.73)	(39.61)	(6.40)	(2.16)			
3×	$15.07^f \pm 0.02$	$9.07^f \pm 0.04$	$0.3^e \pm 0.00$	$4.52^c \pm 0.01$	$1.17^f \pm 0.01$	$0.35^e \pm 0.00$	$6.57^b \pm 0.00$	$60.00^a \pm 0.58$	$59.33^c \pm 0.88$
		(60.19)	(1.99)	(29.99)	(7.76)	(2.32)			
4×	$18.23^e \pm 0.04$	$12.1^e \pm 0.06$	$0.4^d \pm 0.00$	$4.38^d \pm 0.01$	$1.34^e \pm 0.00$	$0.43^d \pm 0.00$	$6.56^{bc} \pm 0.00$	$25.00^b \pm 0.88$	$43.67^d \pm 1.45$
		(66.37)	(2.19)	(24.03)	(7.35)	(2.35)			
5×	$21.63^d \pm 0.08$	$15.14^d \pm 0.08$	$0.51^c \pm 0.01$	$4.22^e \pm 0.00$	$1.76^d \pm 0.01$	$0.53^c \pm 0.00$	$6.51^c \pm 0.00$	$6.00^c \pm 1.2$	$23^e \pm 0.58$
		(70.00)	(2.36)	(19.51)	(8.13)	(2.35)			
6×	$25.01^c \pm 0.05$	$18.21^c \pm 0.06$	$0.6^b \pm 0.00$	$4.12^f \pm 0.00$	$2.08^c \pm 0.01$	$0.66^b \pm 0.01$	$6.44^d \pm 0.00$	$2.00^d \pm 0.00$	$16.67^f \pm 0.88$
		(72.81)	(2.40)	(16.47)	(8.32)	(2.63)			
7×	$28.28^b \pm 0.01$	$21.31^b \pm 0.07$	$0.7^a \pm 0.00$	$4.03^g \pm 0.01$	$2.25^a \pm 0.01$	$0.74^a \pm 0.00$	$6.32^e \pm 0.01$	$1.00^e \pm 0.00$	$12^g \pm 0.58$
		(74.72)	(2.48)	(14.25)	(7.96)	(2.61)			
7×DF	$29.23^a \pm 0.06$	$22.45^a \pm 0.03$	$0.7^a \pm 0.00$	$3.89^h \pm 0.03$	$2.19^b \pm 0.00$	$0.75^a \pm 0.00$	$6.24^f \pm 0.02$	$1.00^e \pm 0.00$	$8.67^g \pm 0.33$
		(76.81)	(2.39)	(13.30)	(7.49)	(2.56)			

Mean ± S.E. (n=3), mean values with different superscripts ^{abcdefg} in a row are significantly different with each other ($p<0.01$). Values shown in parentheses are on dry matter basis (% of TS). HCT of PCSM (1×) was determined at 140 ^{0}C while that of other retentates were determined at 120 ^{0}C.

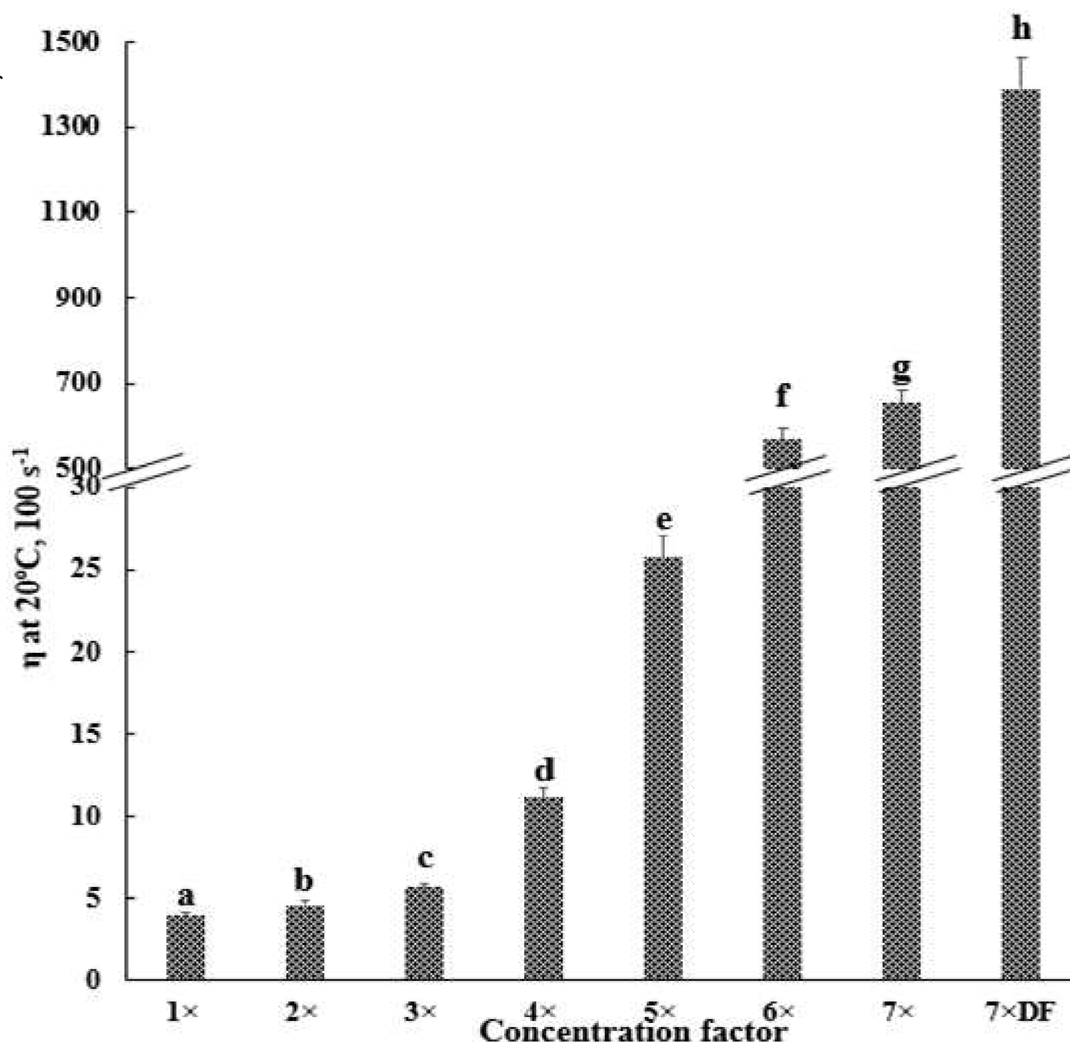


Fig. 2 Change in viscosity (mPa.s) of UF-DF retentates as a function of concentration factor. Mean ± S.E. (n=3), mean values with different superscripts [abcdefgh] are significantly different with each other at $p<0.05$.

flux with increase in UF concentration factors has been reported by Patil et al (2019); Uttamrao et al (2019); Patil et al (2018).

Effect on viscosity values of different samples

As a function of concentration factor, in comparison to PCSM, the changes in viscosity (at 100 per s shear rate and 20ºC temperature) of 2×- 7× UF retentates and 7× DF retentate are shown in Figure 2. The viscosity values of all these samples were significantly different ($p<0.05$) with each other. Further, a significant increase ($p<0.05$) in viscosity values of these samples were observed with consecutive increase in concentration factors. Indeed, such increase in viscosity values of UF/DF retentates were expected owing to marked increase in their TS, protein and ash contents along with marked decrease in their pH values (Table 1). All these factors collectively enhanced the viscosity values of UF/DF retentates. Meena et al. (2016) also reported such increase in viscosity during skim milk concentration up to 5×. It was observed that during 1×- 5× concentration of PCSM, the increase in viscosity was in the range of 3.90-25.70 mPa.s (Figure 2), however, it drastically increased in the range of 567.80- 1391.8 mPa.s for 6× and 7× UF retentates and 7×DF retentate. This could be easily explained by their relatively higher TS, protein and mineral (particularly calcium) contents than that of PCSM and 2× to 5× UF retentates. Viscosity of UF/DF retentates plays a crucial role during their spray drying and directly influences physical, reconstitution and functional properties of milk protein concentrate (MPC) powders. Shinde et al (2020) reported that more than 150 mPa.s (20 ºC and 50 per s) viscosity may create problems during atomization of UF/DF retentates. Hence, 6×, 7×UF retentates and 7×DF retentate might need additional treatment for their efficient atomization.

Selection of stabilizing salts via HCT-pH curve of PCSM

The UF and DF retentates obtained through the concentration of PCSM act as intermediate product. In liquid form these are used in different applications such as standardization of cheese milk, manufacturing of plain *dahi* (Meena et al. 2015), yoghurt (Yadav et al. 2018), high-protein milks, high-protein beverages,

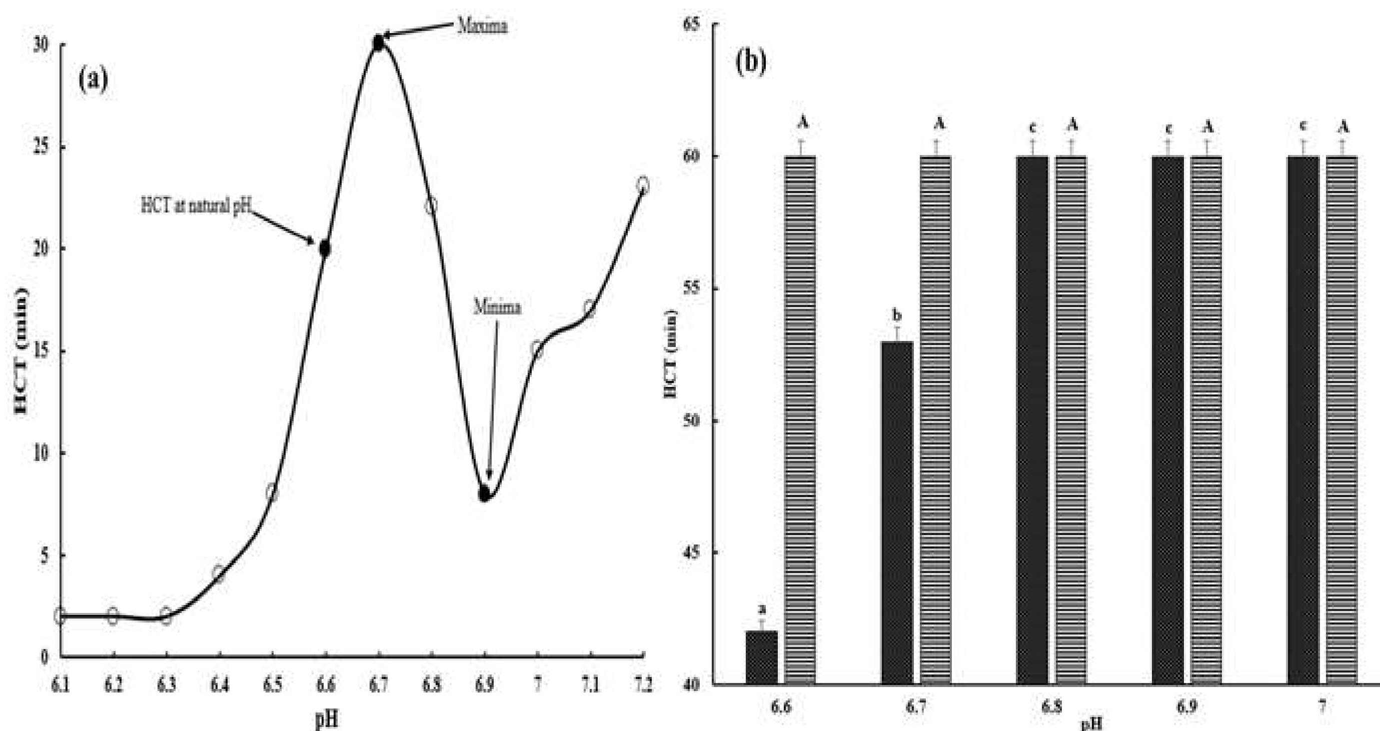


Fig. 3. The HCT-pH curve of PCSM at 140ºC (**a**); effect of NaH_2PO_4 & Na_2HPO_4; NaH_2PO_4 & $Na_3C_6H_5O_7$ on HCT of 7×UF retentate at 120ºC (**b**). Mean±S.E. (n=3) with similar superscripts [A & c] are at par (p>0.05) while those with different superscripts [abc] are significantly different with each other at p<0.05.

liquid dairy whiteners (Khatkar et al. 2014) and high-protein yoghurts. Further, through spray drying, they are converted to high-protein powders such as dairy whiteners (Khatkar et al. 2014), low-lactose powders (Solanki and Gupta 2014) and MPC powders containing protein in the range of 42-89% (Meena et al. 2017b) and milk protein isolates with >90% protein content. However, the major problem with high-protein UF/DF (6×-7×) retentates is their poor HCT (Table 1). Hence, tailoring and maintaining good heat stability in such retentates still remains as a key challenge and acts as a major obstacle in production of high-quality, high-protein products.

Thermal stability of UF retentates could be improved by the addition of stabilizing salts (Meena et al. 2016), however, the type of salts and their levels depends on the typical characteristics of a particular retentate. Selection of the type of stabilizing salts to be added is not a straight forward choice always and varies between addition of acidic (MSP-NaH_2PO_4) or basic phosphates (DSP-Na_2HPO_4) and citrates (such as TSC- $Na_3C_6H_5O_7$). Deeth and Hartanto (2009) recommended the use of MSP, if the natural pH of the milk is higher than the pH of maximum stability. Furthermore, addition of Na_2HPO_4 or $Na_3C_6H_5O_7$ was suggested if the natural pH of the milk is lower than the pH of maximal stability (Singh et al. 1995).

In current investigation, this was verified by adjusting the pH of PCSM (natural pH-6.6) from 6.0 to 7.2 and recording their HCT values at each pH level as shown in Figure 3.a. It is clear from HCT-pH curve (Figure 3.a) that PCSM showed maximum HCT at pH 6.7 and the same was markedly higher than its HCT at natural pH (6.6). These results are in good agreement with the earlier findings reported by Singh (2004). The HCT-pH curve (Figure 3.a) clearly demonstrated that the natural pH of the PCSM was lower than the pH of maximal stability, hence, Na_2HPO_4 and $Na_3C_6H_5O_7$ were selected to improve the heat stabilities of high-protein 7× UF/DF retentates.

Improvement in HCT of 7×UF retentate: Effect of stabilizing salts addition

As per Table 1, the 7×UF retentate had poor (1 min) HCT at its native pH (6.23). The HCT of 7×UF retentates whose pH was adjusted with 0.1N HCl and 0.1N NaOH solutions ranged from 2-5 min (for pH values 6.0-6.6) and 60 min for higher pH values (data not shown). This demonstrated a lead that the samples adjusted to pH values higher than natural pH of 7×UF retentate, markedly increased its HCT values. The pH of 7×UF retentate was also adjusted from 6.60 to 7.0 as shown in Figure 3.b using MSP (only to decrease pH, if required) and DSP as well as MSP and TSC solutions. As evident in Figure 3.b, the adjustment of pH with MSP and DSP solutions, significantly increased (p<0.05) the HCT of 7×UF retentate in the range of 42 - 60 min in the studied pH range. Thereafter, the test was discontinued. Further, the adjustment of pH with MSP and TSC solutions, significantly

increased ($p<0.05$) the HCT of 7×UF retentate to 60 min in similar pH range (Figure 3.b). Hence, Figure 3.b clearly indicate that in similar pH (6.60-7.0) range, the combination of MSP and TSC was more efficient in HCT improvement of 7×UF retentate compared to MSP and DSP salts. Observed marked improvement in HCT of 7×UF retentate could be attributed to stabilizing salts induced protection of milk proteins via increasing pH leading to decrease in calcium ion activity as both favor enhancement in HCT. In 6.1-7.0 pH range, changes in HCT values of homogenized 5× UFR samples were investigated by Meena et al. (2016) and it was concluded that its HCT (1 min at pH 6.41) was markedly enhanced in the range of 80-120 min in 6.5-7.0 pH range adjusted with DSP and TSC salts. Le Ray et al (1998) described that addition of sodium phosphate, sodium citrate and sodium chloride protected reconstituted casein micelles dispersions against heat coagulation by decreasing the amount of miceller minerals and enhancing stabilizing forces such as steric repulsions. Hence, similar effects could also explain increase in HCT values of 7×UF retentate at different pH values in present investigation. Indeed, observed variation in HCT values of 7×UF retentate emphasizes the importance of pH and salt balance and because of this reason, addition of the suitable stabilizing salt (s) in optimum levels is crucial as well as helpful in achieving most desired pH and salt balance ratio in studied 7×UF retentate. Khatkar et al. (2014) also reported similar findings during conversion of UF retentate into dairy whiteners.

In lack of scientific literature, HCT of 7×UF retentate containing stabilizing salts (as mentioned above) was not possible. Therefore, observed results have been compared with earlier reported studies on UF retentates concentrated to lower concentration factors. Meena et al (2016) evaluated the changes in heat stability of homogenized 5×UF retentate as a function of pH adjustment from 6.1 to 7.0 pH range using DSP and TSC salts. Both salts significantly ($P<0.01$) improved the HCT of this retentate from 1.45 min (native pH-6.41) to 80-120 min in studied range (6.5-

Table 2 Effect of high shearing and stabilizing salts addition on HCT of 7×DF retentates

Treatments	Code	HCT (min, at 120 ^{0}C)
7×UFR (produced by Ultrafiltration of PCSM)	T1	$1.00^d \pm 0.00$
7×DFR (produced by diafiltration of 7×UFR with 150mM NaCl solution)	T2	$1.00^d \pm 0.00$
T2+High-shear (20,000 rpm/ 5 min) treated 7×DFR	T3	$1.00^d \pm 0.00$
T2+ Addition of 0.2 % mixture of MSP and DSP (2:1 w/w)	T4	$1.00^d \pm 0.00$
T2+ Addition of 0.3 % mixture of MSP and DSP (2:1 w/w)	T5	$1.00^d \pm 0.00$
T2+ Addition of 0.4 % mixture of MSP and DSP (2:1 w/w)	T6	$1.00^d \pm 0.00$
T2+ Addition of 0.5 % mixture of MSP and DSP (2:1 w/w)	T7	$1.00^d \pm 0.00$
T4+ 0.7% TSC	T8	$28.00^a \pm 0.01$
T5+ 0.7% TSC	T9	$25.00^b \pm 0.06$
T6+ 0.7% TSC	T10	$20.00^c \pm 0.04$
T7+ 0.7% TSC	T11	$20.00^c \pm 0.04$

Mean ± S.E. (n=3), mean values with different superscripts [abcd] in a column are significantly different with each other ($p<0.05$).

Table 3 Correlation between concentration factor (CF) chemical constituents and physical properties (pH, HCT, viscosity) and permeate flux during ultrafiltration of pasteurized cow skim milk

	CF	TS	Protein	Fat	Lactose	Ash	Calcium	pH	HCT	Flux	Viscosity
CF	1.000										
TS	0.996**	1.000									
Protein	0.997**	1.000**	1.000								
Fat	0.991**	0.999**	0.998**	1.000							
Lactose	-0.997**	-0.991**	-0.993**	-0.987**	1.000						
Ash	0.977**	0.990**	0.988**	0.992**	-0.974**	1.000					
Calcium	0.991**	0.998**	0.997**	0.998**	-0.985**	0.989**	1.000				
pH	-0.955**	-0.936**	-0.940**	-0.923**	0.952**	-0.892**	-0.930**	1.000			
HCT	-0.899**	-0.919**	-0.915**	-0.928**	0.891**	-0.930**	-0.916**	0.734*	1.000		
Flux	-0.962**	-0.973**	-0.973**	-0.979**	0.959**	-0.965**	-0.979**	0.871**	0.942**	1.000	
Viscosity	0.843**	0.802**	0.808**	0.775*	-0.849**	0.755*	0.787*	-0.925**	-0.560	-0.688*	1.000

Pearson correlation denoted by ** and * are significant at the $p<0.01$ and $p<0.05$ levels, respectively

7.0) of pH. HCT of 10% and 20 % fat (% of TS) containing cow skim milk based UF retentates were only 14 min that enhanced to 69 and 68 min upon addition of 0.5% mixture of MSP and DSP (2:1w/w), respectively. Apart from this, addition of 0.4 % mixture of MSP and DSP (2:1 w/w) salts caused marked improvement in thermal stability of medium and full fat homogenized liquid dairy whiteners (Khatkar and Gupta 2012; Khatkar et al. 2014). Addition of 0.5% mixture of MSP and DSP (2:1 w/w) caused notable improvement in HCT of 5.48×UF retentate containing 21.28% TS. The maximum HCT (61 min) was observed at pH 6.72, however, addition of this salts mixture to higher levels gradually decrease its HCT.

Improvement in HCT of 7×DF retentate: Effect of stabilizing salts addition

The pH and HCT of 7×DF *retentate* were significantly lower ($p<0.05$) than that of 7×UF *retentate* as shown in Table 1. In order to bring the desired changes in techno-functional properties of milk proteins particularly in casein, UF retentates were intentionally subjected to either diafiltration with NaCl/KCl solutions or high-shear treatment. Such diafiltration decreases calcium content of DF retentate via exchanging sodium and calcium ions during this process, while high-shear treatment induces physical modifications in casein structure and markedly improve functional properties (particularly solubility) of resultant MPC powders. However, these approaches have detrimental effect on heat stability of retentates as shown in Table 2. Homogenization have been reported to decrease the heat stability values of liquid, evaporated and concentrated milks (Sweetsur and Muir 1980) and UF retentate (Meena et al. 2016).

Therefore, the effect of MSP and DSP (2:1 w/w) salts mixture on HCT of 7×DFR with and without TSC have been studied by different treatments as shown in Table 2. It was observed that HCT of T1, T2 and T3 samples were at par ($p>0.05$) with each other that further did not improve upon even 0.2-0.5% (T4-T7) addition of MSP and DSP (2:1 w/w) salts mixture. Contrary to this, further addition of 0.7% TSC in T4, T5, T6 and T7 led to significant improvement ($p<0.05$) in HCT values in treated samples (T8-T11). The HCT values of these samples were in the range of 20-28 min, respectively. Such variation in HCT values underlines the vital importance of salt balance and pH over the control on heat stability of 7× DF retentates as well. This is clear from Figure 3.b and Table 2 that the addition of MSP, DSP and TSC were capable in enhancing the heat stability of 7× UF retentate and 7× DF retentate samples. Although, the extent of rise in HCT values was higher in 7× UF retentates than 7× DF retentate.

Hence, findings of this investigation will be of prime importance to dairy sector in improving the thermal stability of high-protein containing UF/DF retentates. This will also widen the applications of liquid retentates in different food applications apart from helping in improving the physical, reconstitution and functional properties of dairy based high-protein powders manufactured from them.

Correlation between concentration factor, chemical composition, pH, HCT, viscosity of retentates and permeate flux during PCSM concentration by ultrafiltration

During concentration of PCSM by ultrafiltration, TS ($r = +0.996, p < 0.01$), protein ($r = +0.997, p < 0.01$), fat ($r = +0.991, p < 0.01$), ash ($r = +0.977, p < 0.01$), calcium ($r = +0.991, p < 0.01$) and viscosity ($r = +0.843, p < 0.01$) showed positive correlation, while lactose ($r = -0.997, p < 0.01$), pH ($r = -0.955, p < 0.01$), HCT ($r = -0.899, p < 0.01$) and permeate flux ($r = -0.962, p < 0.01$) showed negative correlation with concentration factor as shown in Table 3. Almost similar correlation was observed between TS and remaining parameters, while protein content showed positive correlation with fat ($r = +0.998, p < 0.01$), ash ($r = +0.988, p < 0.01$), calcium ($r = +0.997, p < 0.01$) and viscosity ($r = +0.808, p < 0.01$), however, it was negatively correlated with lactose ($r = -0.993, p < 0.01$), pH ($r = -0.940, p < 0.01$), HCT ($r = -0.915, p < 0.01$) and permeate flux ($r = -0.973, p < 0.01$), respectively. The ash content revealed positive correlation with calcium ($r = +0.989, p < 0.01$) and viscosity ($r = +0.755, p < 0.05$), but showed negative correlation with pH ($r = -0.892, p < 0.01$), HCT ($r = -0.930, p < 0.01$) and permeate flux ($r = -0.965, p < 0.01$). Apart from this, calcium content was negatively correlated with pH ($r = -0.930, p < 0.01$), HCT ($r = -0.916, p < 0.01$) and permeate flux ($r = -0.979, p < 0.01$) and positively correlated with viscosity ($r = +0.787, p < 0.05$). The pH was positively correlated with HCT ($r = +0.734, p < 0.05$) and permeate flux ($r = +0.871, p < 0.01$), however it was negatively correlated with viscosity ($r = -0.925, p < 0.01$), while HCT was positively correlated with permeate flux($r = +942, p < 0.01$). Furthermore, correlation of fat and lactose with other parameters has been shown in Table 3, respectively.

Conclusion

Concentration of PCSM by ultrafiltration caused noticeable increase in colloidal milk components and viscosity, however this also decreased the pH and permeate flux as a function of concentration factor. Such alteration in chemical composition disturbed the delicate salt equilibrium and led to gradual decrease in HCT up to 3× concentration, however, marked increase in TS, protein and calcium contents along with decrease in pH, drastically reduced the HCT of UF (5× - 7×) and 7× DF retentates. Even, applied diafiltration with 150mM NaCl solution and high-shear (20,000 rpm/ 5 min) treatment were found detrimental for HCT. Tailoring and maintaining good heat stability in high-protein retentates is acts a key challenge and major hurdle in production of high-quality products from such retentates. Combined use of stabilizing salts, identified from classical HCT-pH curve of PCSM at appropriate levels restored desired equilibrium between milk constituents and markedly improved thermal stability of high-protein UF/DF retentates. Establishment of correlation between

concentration factor, milk constituents, pH, viscosity, HCT and permeate flux provided better understanding about thermal stability of retentates. In nutshell, this investigation had established that poor heat stability of high-protein containing UF/DF retentates can be restored through the addition of suitable stabilizing salts in appropriate amounts. Further, such retentates with improved thermal stability could be explored in number of liquid products and they can also result in high-protein powders with improved physical, reconstitution and functional properties.

Acknowledgements

Authors thankfully acknowledge the Director, ICAR-NDRI, Karnal for providing the required facilities to accomplish this research work.

References

AOAC (2005) Official methods of analysis, 481, 18th edn. The association of official analytical chemists, Gaithersburg

Deeth HC, Hartanto J (2009) Chemistry of milk-role of constituents in evaporation and drying. In: Dairy powders and concentrated products, pp: 1-27

Ferrer MA, Alexander M, Corredig N (2011) Does ultrafiltration have a lasting effect on the physico-chemical properties of the casein micelles? Dairy Sci Technol 91:151-170

Holt C, Dalgleish DG, Jenness R (1981) Inorganics constituents of milk. 2. Calculation of the ion equilibrium in milk diffusate and comparison with experiment. Anal Biochem 113:154-163

IDF (1993) Lait: De´termination de la teneur en azote; IDF Standard 20 B. International Dairy Federation, Brussels

Indian Standard (1961) Methods of test for dairy industry-part II: chemical analysis of milk. Bureau of Indian Standards, IS-1479, Manak Bhavan, BIS, New Delhi

Indian Standard (1977) Determination of fat by the Gerber method-part I: milk. Bureau of Indian Standards, IS-1224, Manak Bhavan, BIS, New Delhi

Indian Standard (1997) Milk, cream and evaporated milk-determination of total solid content (Reference method). Bureau of Indian Standards, IS-12333, Manak Bhavan, BIS, New Delhi

Khatkar SK, Gupta VK (2012) Physicochemical and functional quality attributes of dairy whitener prepared from ultrafiltration process. J Food Process Preserv 38:1145-1154

Khatkar SK, Gupta VK, Khatker AB (2014) Studies on preparation of medium fat liquid dairy whitener from buffalo milk employing ultrafiltration process. J Food Sci Technol 51(9):1956-1964

Le Ray C, Maubois JL, Gaucheron F, Brulé G, Pronnier P, Garnier F (1998) Heat stability of reconstituted casein micelle dispersions: changes induced by salt addition. Le Lait 78(4):375-390

Meena GS, Singh AK, Arora S, Borad S, Sharma R, Gupta VK (2017a) Physico-chemical, functional and rheological properties of milk protein concentrate 60 as affected by disodium phosphate addition, diafiltration and homogenization. J Food Sci Technol 54:1678-1688

Meena GS, Singh AK, Panjagari NR, Arora S (2017b) Milk protein concentrates: opportunities and challenges-a review. J Food Sci Technol 54:3010-3024

Meena GS, Singh AK, Borad S, Raju PN (2016) Effect of concentration, homogenization and stabilizing salts on heat stability and rheological properties of cow skim milk ultrafiltered retentate. J Food Sci Technol 53:3960-3968

Meena PK, Gupta VK, Meena GS, Raju PN, Parmar PT (2015) Application of ultrafiltration technique for the quality improvement of dahi. J Food Sci Technol 52:7974-7983

Mistry VV (2002) Manufacture and application of high milk protein powder. Lait 82:515-522

Muir DD, Sweetsur AWM (1978) The effect of concentration on the heat stability of skim milk. J Dairy Res 45:37-45

Ozimek L, Sirlaorkul S, Dziuba J, Pikus W (1988) Heat stability of skim milk concentrated by ultrafiltration. J Dairy Sci 71(1):109

Patil AT, Meena GS, Upadhyay N, Khetra Y, Borad S, Singh AK (2018) Production and characterization of milk protein concentrates 60 (MPC60) from buffalo milk. LWT-Food Sci Technol 91:368-374

Patil AT, Meena GS, Upadhyay N, Khetra Y, Borad S, Singh AK (2019) Effect of change in pH, heat treatment and diafiltration on properties of medium protein buffalo milk protein concentrate. J Food Sci Technol 56:1462-1472

Shinde AP, Meena GS, Handge JU (2020) Effect of sodium triphosphate and sodium hexametaphosphate on properties of buffalo milk protein concentrate 60 (BMPC60) powder. J Food Sci Technol, 1-11. https://doi.org/10.1007/s13197-020-04712-y

Singh H (1995) Heat-induced changes in caseins including interactions with whey proteins, in heat-induced changes in milk. In: Fox PF (ed) Special Issue 9501, International Dairy Federation, Brussels, pp 86-99

Singh H (2004) Heat stability of milk. Int J Dairy Technol 57:111-119

Singh H, Creamer LK (1992) Heat stability of milk. In: Fox PF (ed) Advanced dairy chemistry-I proteins. Elsevier, London, pp 621-656

Solanki P, Gupta VK (2009) Effects of stabilizing salts on heat stability of buffalo skim milk ultrafiltered-diafiltered retentate. J Food Sci Technol 46:466-469

Solanki P, Gupta VK (2014) Manufacture of low lactose concentrated ultrafiltered-diafiltered retentate from buffalo milk and skim milk. J Food Sci Technol 51:396-400

St-Gelais D, Hache S, Gros-Louis M (1991) Combined effects of temperature, acidification, and diafiltration on composition of skim milk retentate and permeate. J Dairy Sci 75:1167-1172

Sweetsur AWM, Muir DD (1980) Effect of concentration by ultrafiltration on the heat stability of skim milk. J Dairy Res 47:327-335

Uttamrao HJ, Meena GS, Borad SG, Punjaram SA, Khetra Y, Upadhyay N, Singh AK (2019) Effect of disodium phosphate and homogenization on physico-chemical and rheological properties of buffalo skim milk based ultrafiltered retentate. J Food Sci Technol 56:2426-2435

Yadav V, Gupta VK, Meena GS (2018) Effect of culture levels, ultrafiltered retentate addition, total solid levels and heat treatments on quality improvement of buffalo milk plain set yoghurt. J Food Sci Technol 55:1648-55

Indian J Dairy Sci 74(1): 18-29
https://doi.org/10.33785/IJDS.2021.v74i01.003

RESEARCH ARTICLE

Microbiological and physicochemical changes during ripening of Camembert cheeses made from raw and pasteurized cow milk produced in Tizi-Ouzou (north of Algeria)

Hillal Sebbane[1], Dalila Almi[1], Sonia Hadouchi[2], Louiza Hedjel[2], Noria Smail-Saadoun[3] and Abderrahmane Mati[1]

Received: 01 August 2020 / Accepted: 10 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: This study was carried out on two types of Camembert produced in the region of Tizi-Ouzou (northern Algeria), one artisanal (AC) made from raw milk and the other industrial (IC) made from pasteurized milk. This work shows the effect of milk quality and cheese making processes on the progress of physicochemical and microbiological parameters throughout a maturation period of 12 days. The result shows that the repining microflora and undesirable microbial populations were significantly higher ($P < 0.05$) in the artisanal Camembert (AC) than in the industrial Camembert (IC). The physicochemical and microbiological parameters, during the ripening of the cheeses developed in a similar way with significant differences according to the type and the stage of maturation. During the cheese repining period, AC showed more extensive ($P < 0.05$) lipolysis and proteolysis than IC. SDS-PAGE of water-soluble proteins (WSP) and insoluble fractions showed more extensive degradation of αs-casein (αs-CN) than β-casein (β-CN). The WSP profile, analyzed by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC), was highest in AC than IC. The highest WSP profile was recorded at the 12th day of repining. The pathogenic flora decreased during the maturation process in AC. This development was confirmed by the results of the antibacterial effect of WSP, performed by the disc diffusion technique on *Escherichia coli* and *Staphylococcus aureus* strains. From this study, it can be concluded that AC has a better organoleptic quality, safe for the Algerian consumer and more profitable for the Algerian cheesemakers

Keywords: Algeria, Camembert, Cheese ripening, Lipolysis, Proteolysis, Raw and pasteurized milks

Introduction

Since the early 1990s, Algeria has implemented a dairy policy in order to enhance the value of local production from cattle breeding (Mamine et al. 2011). With the launch of the National Agricultural Development Plan (NADP) in 2000 and its extension to rural areas, total milk production increased from 1.9 billion liters in 2004 to 3.3 billion in 2018, covering about 60% of national milk needs. This production, even if it faces an insufficient collection circuit (Kali et al. 2011), has enabled some artisanal dairy farmers to process milk into raw milk cheese production. Nevertheless, this approach is quite innovative in Algeria and the microbiological and physicochemical characterization of raw milk cheeses is attracting the interest of public authorities and researchers (Aissaoui et al. 2011; Meribai et al. 2017). Indeed, hygiene requirements have become an important consideration in the production of artisanal cheeses, particularly those with a protected designation of origin (PDO), which are produced with raw milk, such as Camembert de Normandie (France), often produced in mountain areas (Gérard, 2015).

Traditional cheese making, especially for the Camembert, is distinguished from the industrial production by various aspects, and the better organoleptic qualities are obtained with the raw milk (Richard and Zadi 1983). Organoleptic properties are mainly influenced by the growth of lactic acid bacteria and the combined effect of proteolysis and lipolysis on the cheese matrix during the ripening process (Gebreyowhans et al. 2020). The release of bacteriocins and bioactive peptide fractions, endowed with antibacterial activities, inhibit bacterial pathogens (Mane and McSweeney, 2019), such as *Listeria ssp.* in the case of Camembert (Wan et al. 1997).This is how we set out to evaluate the quality of two soft cheeses, one made from raw milk and the other from pasteurized milk. We particularly explored the physicochemical

[1]Laboratory of Analytical Biochemistry and Biotechnology (LABAB), Mouloud Mammeri University, Tizi Ouzou, Algeria

[2] Faculty of Biological Sciences and Agronomic Sciences, Mouloud Mammeri University, Tizi Ouzou, Algeria

[3]Natural Resources Laboratory, Mouloud Mammeri University, Tizi Ouzou, Algeria

Hillal Sebbane (✉)
Laboratory of Analytical Biochemistry and Biotechnology (LABAB), Mouloud Mammeri University, Tizi Ouzou, Algeria
Email: hillalmicrobio@yahoo.com

and microbiological characteristics of these cheeses at different stages of the ripening process and demonstrate the safety of raw milk cheeses, especially Camembert, in Algeria. To the best of our knowledge, this is the first study to assess the antimicrobial effect of water-soluble peptide fractions extracted from Camembert.

Materials and Methods

Sampling procedure and cheesemaking

The samples of milk used for the production of Camembert, came from two farms in the region of Tizi-Ouzou. The farms are made up of cows of dairy cattle breeds (Holstein and Montbéliard), with an average age of 4 to 8 years and they are at the same stage of lactation. The maturing period is set at 12 days, with reference to Algerian industrial practices. Two types of Camembert, one artisanal, the other industrial, produced in the region of Tizi-Ouzou (northern Algeria), were selected within the framework of this study. The analyses were based on 108 samples from each Camembert type and on 27 samples from each kind of milk (raw and pasteurized). The sampling of the products were carried from March to June during the period 2014-2016, in order to avoid the seasonality effect on the physicochemical and microbiological composition of the milk. Three samples per month were collected from each milks (raw and pasteurized milk) and cheeses at four ripening stages: first, fourth, sixth and twelfth days (Table 1). Placed in a cooler, the samples were directly taken to the laboratory and the analyses were performed during the following 24 hours.

The moulding of the cheeses (AC and IC) was performed into polyurethane perforated moulds (cylinders 10 to 11 cm in diameter and 13 to 14 cm in height). Demoulding of cheeses (AC and IC) is carried out when the lactic acidity of the curd reaches values of 9.5 to 11% (w/w) and a pH of 4.5 to 4.9.

The artisanal Camembert (AC), made from raw milk, was produced in the locality of Ouacif (Tizi-Ouzou, Algeria) by an artisan cheesemaker breeding dairy cattle under extensive holding conditions and transforms milk to Camembert according to the guidelines of the French legislation related to cheeses and cheese specialties (Art. 9 and 14 of the decree n° 2013-1010 of the 12th of November 2013). Milk was heated up to 35 °C till it reaches an acidity of 22 to 25 °D (2.2 to 2.5 g/l of lactic acid) or at pH 6.30 to 6.35, before a liquid rennet (standard strength of 1:10000, Halal calf Rennet, Chr. Hansen Inc., Danemark) was added (15-20 ml/100 L of milk). After coagulation (1h10 min-1h30 min), the moulding of the cheese was performed directly with a ladle into polyurethane perforated moulds, without stirring the curd, at a rate of 5 ladles per mould, with a rest period of 15 to 20 minutes between each ladle, without stirring the coagulum. Draining was done on stainless drains (24 to 48 h, 25-28 °C) at a relative humidity (RH) of 95%, turning the cheese every five hours, before it was worked with sprinkling of fine salt. Then, the cheese (AC) was sprayed with a solution of *Penicillium camemberti* spores ($2x10^4$ spore/ml) and ripened during 12 days at a temperature of 10 to 12 °C and a RH of 90 to 95%, with a rotation every two to three days.

The industrial Camembert (IC), made from pasteurized milk, was produced in a cheese dairy located in Draa Ben Khedda (Tizi-Ouzou), which was supplied with milk from a close locality (Freha, Tizi-Ouzou). Without undergoing any standardization of fat and protein contents, milk is first pasteurized at 90 °C for 20 seconds and then cooled to 36 °C. After adding $CaCl_2$ (15-20 g/100 L of milk), milk was seeded with mixed lactic ferments (Mesophilous: DI-PROX M 229, BioProx, France/Thermophilous: DI-PROX TPM 2, BioProx, France) with 0.03% of fungal flora (Chr. Hansen Inc., Danemark) of *Penicillium camemberti* and *Geotricum candidum* in order to reach the final concentrations of 10^4 spores/ml and $2x10^4$ spores/ml, respectively. After the fermentation period of milk (20-25 minutes at 36°C), the pH reached a value of 6.35, commercial rennet fungal powder Marzyme™ (standard strength of: 150000, Danisco. France) was added (1.5 g/50 Kg of milk), then the coagulum is divided into small cubes (Ø = 30 mm). To accelerate draining, the coagulum was stirred twice during 15 minutes with an interval of five minutes. The whey was evacuated by pumping it out, then the moulding was carried out by

Table 1 Distribution of the samples according to the kind of the milk and the ripening stage

Sample	Ripening days	Sampling plan									Total number of samples
		1st year			2nd year			3rd year			
		1st month	2nd month	3rd month	1st month	2nd month	3rd month	1st month	2nd month	3rd month	
Artisanal or industrial camembert	1	3	3	3	3	3	3	3	3	3	27
	4	3	3	3	3	3	3	3	3	3	27
	6	3	3	3	3	3	3	3	3	3	27
	12	3	3	3	3	3	3	3	3	3	27
Total samples of Camembert at all stages of cheese repining											108
Raw or pasteurized milk		3	3	3	3	3	3	3	3	3	27

mechanically filling the moulds. The coagulum is drained in polyurethane molds placed on stainless drains during 15 to 20 hours at RH of 90 to 95% (27 °C-5 h and 20 °C-15 h), with rotations every five hours. After it was removed from the mold, the cheese was salted by brining (NaCl = 40%, pH = 4.5) during 30 to 60 minutes at 13 °C. The cheese surface was dried in a draining room during 15 hours (14 °C, RH = 85 %) with two rotations. Then, it was sprinkled with a suspension of *Penicillium camemberti* spores (2.10^9 Spore/ml) (Chr. Hansen Inc., Danemark) and ripened during 12 days (12-13 °C, RH = 90-95 %) with a rotation every two to three days.

Physicochemical analysis

Physicochemical parameters during cheese ripening were analysed after removing the cheese crust. Samples of milk were analyzed with Lacto-scan SP (Milkotronic LTD, Bulgaria) for density, fat and proteins). The pH was measured by immersing the pH-meter glass electrode (Hanna-instrument, Italy) directly in the products (cheeses and milks) (Bouton et al. 1994).The titratable acidity (TA) of the milk was expressed in Dornic degree (°D) (AFNOR, 1980) and as % of lactic acid for the cheese (Sulieman et al. 2012) The dry-matter (DM) was determined using an infrared desiccator IR35 (Denver instrument, Germany) by evaporation (105°C/20 min) of 3 g of milk (AFNOR, 1980) or 5 g of cheese (Randoin and Jourdan ,1952). The Fat content of cheese was determined by Van-Gulik butyrometer (Funkgerber Instrument, Germany) (JORADP, 2014).

Protein fractionation

Protein concentration was determined using the Lowry protein procedure at 750 nm with Folin-Ciocalteu's phenol reagent (Lowry et al. 1951). Bovine Serum Albumin was used as a standard. From slurry prepared from by mixing 20 g of cheese (crust removed and crushed) in 40 ml of sodium citrate buffer (0.5 M, pH 7), three protein fractions, which are the total protein content (TP), the acid soluble protein (ASP) and the non-protein nitrogen (NPN), were obtained by combining the methods reported by Gripon et al. (1975) and Guerra-Martinez et al. (2012).

Free amino acids

Total levels of free amino acids (FAA) in the pH4.6-Soluble fractions of the cheese were determined by the 2, 4, 6, trinitrobenzene-1-sulfonic acid (TNBS) method described by Polychroniadou (1988) modified by Bouton et al. (1993). Cheese extracts were prepared, as mentioned above. The use of a range of glycine standard (0.01-0.5 mM) allowed expressing the measurements in mEq glycine/g of protein.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Proteolysis during cheese ripening from the water-soluble proteins (WSP) and insoluble protein fractions at pH 4.6 was evaluated by SDS-PAGE (Barac et al. 2016). The fractions were prepared according to the techniques reported by Dupas et al. (2009).The PAGE-SDS was performed according the method described by Laemmli (1970). A marker kit was for bovine serum albumin (67 kDa), ovalbumin (45 kDa), β-Lactoglobulin (18 kDa) and α-Lactalbumin (14 kDa).

Reversed-phase high performance liquid chromatography (RP-HPLC) of the cheese solutions water-soluble fractions

The proteolysis during cheese repining was also assessed by RP-HPLC. Samples of WSP (3 mg/ml) were prepared in a Bis-Tris

Table 2 Milk and cheese microbial flora enumerated during ripening

Microflora	Culture medium	Incubation
Aerobic mesophilic flora (AMF)	PCA-Agar	30°C/24-72 h
Total coliforms (TC)	VRBL agar	30°C/24-48 h
Faecal coliforms (FC)		44°C/24-48 h
Faecal streptococci (FS)	BEA (bile esculineazide) agar followed by catalase and Gram reactions, growth at 37° C and at 6.5% NaCl in BHI	37° C/24-48 h
Mesophilic lactobacilli (MLAB)	MRS agar followed by a catalase test and a Gram stain	30°C/24-48 h
Mesophilic lactic *Streptococcus* (MLS)	M17 agar followed by a catalase test and a Gram stain	30°C/24-48 h
Yeast and molds (Y-L)	OGA agar	22°C/3-7 d
Staphylococcus aureus	Presumtive testing: Giolitti-Cantoni broth Confirmatory test: Baird Parker agar with the coagulase test	37°C/48 h
Salmonella spp. (samples:25 ml for milk or 25 g for cheese)	Enrichment on SFB broth and isolation on Hektoen agar	37 °C/48 h

buffer with a pH 8 and filtred through 0.45 µm nylon filters (Sartorius, Germany). Twenty µl of filtrate was injected in a Lichrosorb C8 column (5 µm, 125x4, 1mm). The mobile phase was constituted by a solvent A (10% acetonitrile, 0.1% trifluoroacetique acid: TFA) and a solvent B (60% acetonitrile, 0.02 % TFA). The elution was performed using a 0-50% binary gradient of the solvent B during 50 minutes with a flow rate of 0.2 ml/min at 35 °C. The detection was performed under UV at 214 nm.

Lipolysis

The lipolysis was assessed by measuring the amount of free fatty acids (FFA_s) according to the method described by Deeth et al. (1975). The FFAs were expressed in equivalent of oleic acid per 100 g of fat, referring to a standard curve of oleic acid (0.10-10 mg/ml).

Microbiological analysis

Milk and cheese microflora during ripening were determined by the conventional techniques for which the incubation conditions and the medium used are reported in Table 2. Cheese samples were collected at 1, 6 and 12 days of repining. The dilutions were prepared either directly from milk or from cheese prepared according the method described by Lenoir (1963) after some modifications. After removing the crust, 10 g samples of cheese were homogenized in a Stomacher 400 (Seward Medical, Londres, UK) with 90 ml of sterile sodium citrate (2%) at 40 °C. Decimal dilutions of milk and cheese solution were prepared with Ringer's solution. Germ counts were expressed according to the formula of Joffin and Joffin (1999).

Anti-microbial activity of water-soluble extract

The raw water-soluble extracts (WSE) were extracted at the 12th day of cheese ripening, as mentioned above (fractionation of WSP), without acidification. In order to eliminate the antagonistic effects of the pH and H_2O_2 , the WSE was neutralized to pH 6.8 with NaOH (2N) and after addition of few drops of catalase (1 mg/ml),it was incubated for one hour at 30 °C (Sahraoui et al. 2015). The raw WSE and the neutralized WSE were filtered through 0.22 µm filters .The agar-disc diffusion assay was performed according to protocol reported by Motta and Brandelli (2002).

The antibacterial activity was tested against *Escherichia coli* ATTCC 25922 and *Staphylococcus aureus* ATTCC 25923. All the strains were standardized by spectrometry (620 nm) at an optical density (OD) of 0.08-01, according to the Antibiogram Committee CASFM/EUCAST (2019).

Aliquots of 20 µl were applied on five discs (6 mm): two discs of the raw WSE, two discs of neutralized WSE and one disc with broth (as negative control). The Petri dishes were placed in a refrigerator at 4 °C for two hours. The antimicrobial effect of WSE (expressed in mm) was examined after incubation for 24 hours under appropriate culture conditions. Three replicates were performed on different dates.

Statistical analysis

The physicochemical composition and microbiological counts, between raw milk and pasteurized milk, were assessed by the Student's t-test (5% level of significance). An Analysis of variance (ANOVA), using the Tukey test for pair wise comparison of means, was performed (significant level of 5 %), to follow the changes in the physicochemical and microbiological parameters during cheese ripening. A principal component analysis (PCA) was run in order to analyze the effect of the ripening stage on the physicochemical and microbiological interactions during cheese maturation. The statistical analyses were performed using STATbox software ver. 6.4.

Results and Discussion

Physicochemical characteristics of milk

The milk physicochemical characteristics are reported in Table 3. Among the analyzed parameters, significant difference was only found between the DM and milk TP (P < 0.05). The sampling season can affect the physico-chemical and microbiological quality of milk (Spike and Freeman, 1967; Nalepa et al. 2018). The effect of seasonality was discarded in this study, as sampling occurred during the same periods of the year.

Changes in physicochemical parameters during ripening

The statistical analyses of the physicochemical parameters (Table 4) showed significant differences (P < 0.05) depending on the stage of ripening and the type of cheese.

Table 3 Physicochemical compositions of the milk

Composition	Raw milk	Pasteurized milk
pH	$6.69^a \pm 0.02$	$6.66^a \pm 0.06$
TA (°D)	$17^a \pm 0.93$	$15.66^a \pm 0.41$
Densité	$1.03^a \pm 0.01$	$1.027^a \pm 0.7$
DM (g/l)	$122.1^b \pm 0.3082$	$108.13^a \pm 2.31$
FAT (g/l)	$33.66^a \pm 2.16$	$36^a \pm 0.61$
TP (g/l)	$29.45^b \pm 0.09$	$26.66^a \pm 0.41$

Results in the same column for the same parameter with different superscripts are significantly different at P < 0.05.

The pH is negatively correlated with titratable acidity between the different cheese types and the ripening stages. From the first to the twelfth day of ripening, the pH values increased from 4.59 and 4.67 (IC) to 5.13 and 5.36, respectively. This is due to the deacidification of the cheese, which is better assessed through measuring the titratable acidity. Indeed, cheese acidity decreased between the first day (9.59% for the artisanal cheese and 9.11% for the industrial) and the last dayof ripening (5.83% artisanal cheese and 6.17% for the industrial**)**

In the case of Camembert the neutralization and deacidification of cheese is due to the assimilation of lactic acid and the releasing of NH_3 by the fungal flora, especially *Penicillium camemberti* and *Geotichum candidum,* which promoted the action of proteolytic enzymes and increased the proteolysis index (ASP%, NPN%, and FAA) (Leclercq-Perlat et al. 2004).

Dry Matter increased during ripening with significant differences between the stages and the cheese types. The values range from 39.40% (AC) and 40.89% (IC) at the first day, to 50.09% (AC) and 49.51% (IC) at the 12th day of ripening. These findings may be attributed to the dehydration during cheese repining. This phenomenon is caused by water loss and exchanges in volatile compounds (ammoniac, fat volatile fatty acids, etc.) between the cheese surface and the ripening room environment (Bertolino et al. 2011). The dehydration of cheese affects the contents of DM, fat and protein during ripening process. Indeed, at the last ripening day, the fat content reached at 25.99% for the AC and 28.75% for the IC and the protein contents attained 15.25% for the AC and 17.20% for the IC.

Progress of proteolysis and lipolysis during ripening

The proteolysis index, ASP/TP (ASP %) and NPN/TP (NPN %) ratios, have been used by many authors as cheese ripening indicators in order to estimate the degree of the proteolysis (Leclercq-Perlat, 2000). Statistical analysis showed significant differences between the types of cheeses and stages of ripening. ASP% increased from the first to the twelfth day of ripening, with the following values respectively: 5.22% to 22.24% for AC and from 4.69% to 17.44% for IC. The non-protein nitrogen values also increased from 0.310% to 0.44% for AC and from 0.22% to 0.28% for IC. The results of the free amino acids contents during cheese repining, revealed significant differences ($P<0.05$) between the cheeses. Free fatty acids increased from 0.12 (first day) to 0.25 (12th day) for AC and from 0.09 (first day) to 0.23(12th day) for IC. The combined effect of the microflora and indigenous enzymes of the raw milk explains the high degree of the proteolysis in AC. The action of intracellular enzymes, released during cell lysis, on the proteolysis of the cheese caseins has been reported by Saboya et al. (2001).The heat treatment of milk slows down cheese proteolysis by modifying the total flora and the proteolysis system during cheese ripening, such as the Camembert (Samelis et al. 2009). The proteolysis activity of the plasmin in ripened cheeses depends on the technology applied in milk processing. The effect of plasmin on caseins of cheese made from pasteurized

Table 4 Changes in physicochemical parameters during ripening of artisanal and industrial cheeses

Physico-chemical parameters	Cheeses	Ripening period (days)			
		1	4	6	12
pH	Artisanal ^{A}	$4.59^a \pm 0.38$	$4.60^a \pm 0.49$	$4.90^{ab} \pm 0.35$	$5.13^{ab} \pm 0.38$
	Industrial^B	$4.67^{ab} \pm 0.53$	$4.94^{ab} \pm 0.42$	$5.28^{ab} \pm 0.4$	$5.36^b \pm 0.46$
TA (g /100g of cheese)	Artisanal ^{B}	$9.59^f \pm 0.18$	$8.70^{de} \pm 0.43$	$8.06^{cd} \pm 0.42$	$5.83^a \pm 0.23$
	Industrial ^{A}	$9.11^e \pm 0.64$	$7.90^c \pm 0.39$	$7.04^b \pm 0.52$	$6.17^a \pm 0.22$
DM (g/100 g of cheese)	Artisanal ^{A}	$39.40^a \pm 2,49$	$41.84^a \pm 4.46$	$48.11^b \pm 4.48$	$50.09^c \pm 2.57$
	Industrial ^{A}	$40.89^b \pm 4.61$	$42.61^b \pm 3.41$	$47.11^{cd} \pm 2.59$	$49.51^d \pm 2.98$
FAT (g/100 g of cheese)	Artisanal ^{A}	$18.76^a \pm 0.82$	$20.15^a \pm 0.68$	$23.71^b \pm 0.77$	$25.99^c \pm 0.44$
	Industrial ^{B}	$22.95^b \pm 1.53$	$23.61^b \pm 1.92$	$27.11^{cd} \pm 1.59$	$28.75^d \pm 1.64$
TP (g/100g of cheese)	Artisanal ^{A}	$10.01^a \pm 1,34$	$12.07^b \pm 1.55$	$14.19^c \pm 0.63$	$15.25^c \pm 0.67$
	Industrial ^{B}	$12.17^b \pm 0.32$	$14.27^c \pm 0.77$	$15.29^c \pm 0.85$	$17.20^d \pm 0.78$
APS-PT (g/100g of TP)	Artisanal ^{B}	$5.22^a \pm 0.55$	$5.52^a \pm 0.53$	$12.49^b \pm 0.59$	$22.24^c \pm 2.87$
	Industrial ^{A}	$4.69^a \pm 0.27$	$4.63^a \pm 0.33$	$6.97^a \pm 0.42$	$17.44^c \pm 2.85$
NPN-PT (g/100 g of TP) %	Artisanal ^{B}	$0.31^b \pm 0.07$	$0.34^b \pm 0.06$	$0.42^c \pm 0.03$	$0.44^c \pm 0.04$
	Industrial ^{A}	$0.22^a \pm 0.03$	$0.22^a \pm 0.03$	$0.21^a \pm 0.03$	$0.28^{ab} \pm 0.05$
FAA(mEq glyc/g TP)	Artisanal ^{A}	$0.12^{ab} \pm 0.03$	$0.14^{bc} \pm 0,01$	$0.18^{cd} \pm 0.02$	$0.25^e \pm 0.02$
	Industrial ^{B}	$0.09^a \pm 0.004$	$0.13^{ab} \pm 0.003$	$0.21^{cd} \pm 0.03$	$0.23^d \pm 0.014$
FFA (Eq Oleic acid /100g of FAT)	Artisanal ^{B}	$0.90^a \pm 0.31$	$2.04^c \pm 0.006$	$2.61^d \pm 0.38$	$3.19^e \pm 0.18$
	Industrial ^{A}	$0.99^a \pm 0.11$	$1.22^{ab} \pm 0.14$	$1.28^{ab} \pm 0.007$	$1.40^b \pm 0.23$

Results in the same row or column for the same parameter with different superscripts (in upper case or lower case) are significantly different at $P < 0.05$

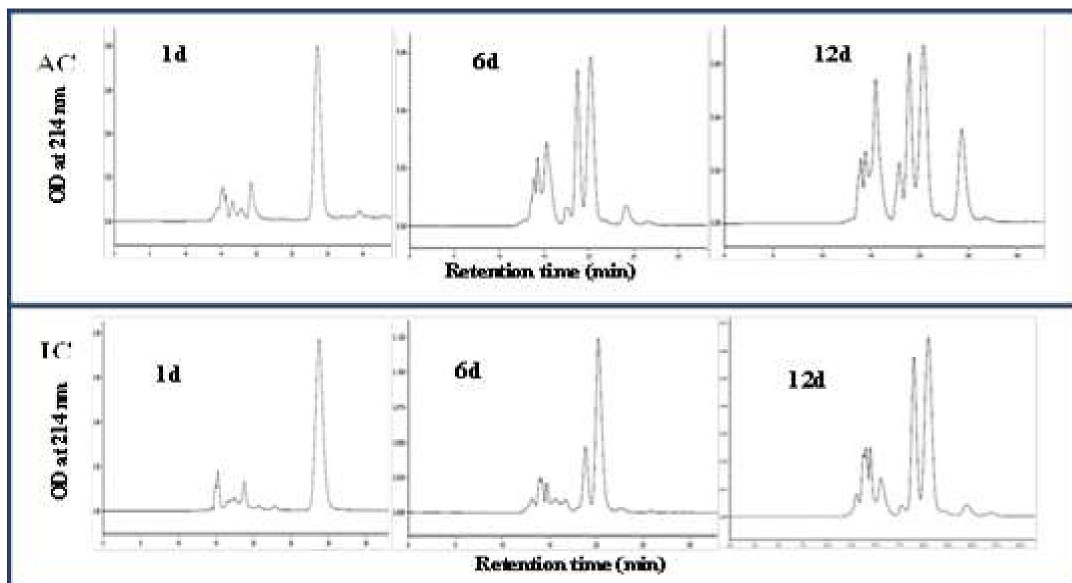


Fig. 1 RP-HPLC water soluble peptide (WSP) fractions from AC and IC at different periods of cheese repining (1, 6 d and 12 days)

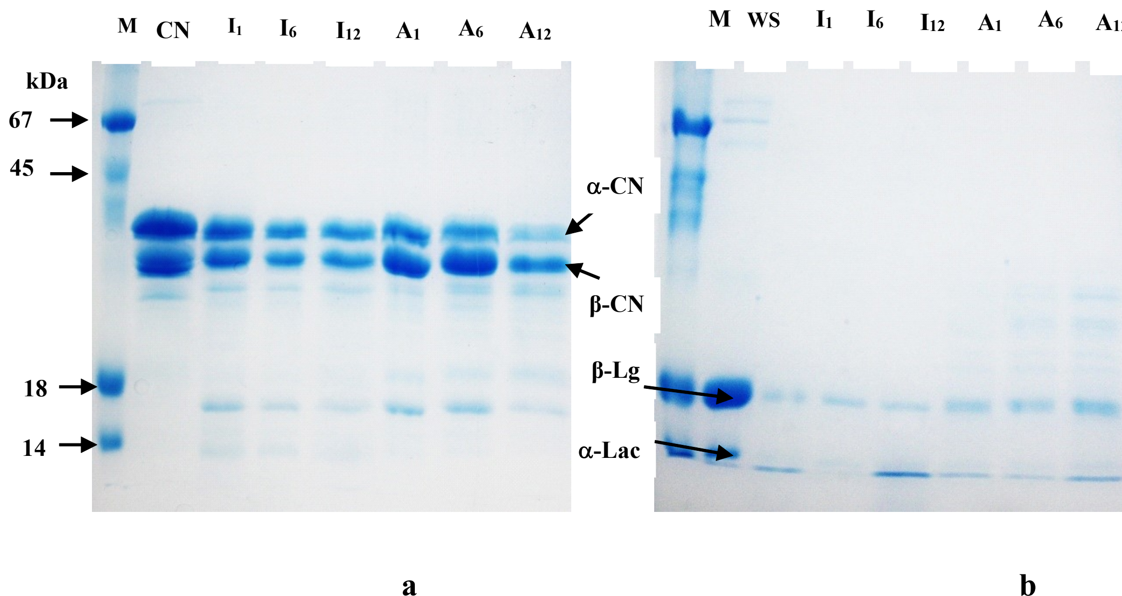


Fig. 2 SDS-PAGE electrophoretic profiles of the insoluble (a) and WSP (b) fractions at pH 4.6 of AC and IC at different days of repining (1, 6 and 12 days)

milk, decreases significantly with increasing temperatures (Benfeldt et al. 1997; Buffa et al. 2000). The action of the rennet, the plasmin, the proteases and the microbial peptidases on the caseins throughout ripening releases peptides and amino acids, which increases the ripening index: ASP%, NPN% and FAA % (Gonzalez et al. 2003; Upreti et al. 2006; Orlyukand Stepanishchev, 2014).

Free fatty acids during cheese ripening, increase as does the proteolysis and the fat content. Similar results have been reported by Batool et al. (2018). FFA presents significant differences between the stages and the types of cheeses. The high level of FFA in the AC can be explained by the micro floral composition of raw milk and the lipoprotein lipase activities. Indeed, the negative effect of pasteurization on LPL activities and the highest of lipolysis degrees in cheeses made from raw milk compared to those made from pasteurized milk have been reported by several authors (McSweeney et al. 1993; Franco et al. 2001).

RP-HPLC profiles

The RP-HPLC profiles of WSP of the two cheeses, showed an increase in the number of peaks during the ripening process (Figure 1). These peaks were more frequent in AC than in IC. This is due to the high level of proteolysis in AC, the proteolysis activity of the indigenous milk enzymes, the microbial enzymes

as well as the activity of the residual rennet. The heat treatment has a negative effect on the proteolysis process during cheese repining. Benfeldt and Sorensen (2001) showed an inverse relationship, during cheese ripening, between the temperature of heat treatment of milk and the proteolysis as followed by an RP-HPLC. According to Trujillo et al. (1997) and Van Hekken et al. (2007), most of the peptides which are released during ripening, are generated from the caseins hydrolysis by rennet and plasmin with a molecular weight ranging from 10 to 20 kDa.

Electrophoresis of WSP and insoluble proteins fractions

The figure 2_a, shows that the indigenous casein migrates as two main bands corresponding to αs-CN and β-CN. The band intensity decreased throughout ripening, leaving two news bands of low molecular weight (Figure 2_b).

This trend was most pronounced for AC at the 12th day of ripening. These profiles revealed that the caseins proteolysis changes over time and with the type of the cheese, with higher αs-CN proteolytic rates than with β-CN. Indeed, new bands with lower molecular weight and the increase in peaks of WSP obtained with RP-HPLC, explain the higher proteolysis index and the free amino acids rates observed in AC than in IC. Our findings are consistent with those reported by Gobbetti et al. (2002), who noted that chymosin hydrolyzes easily αs_1-CN than β-CN, releasing a peptide identified as α_{s1}-I-casein f(24-199). Chymosin is also able to hydrolyze β-CN (Addeo et al. 1980). The main cleavage site of chymosin in β-CN is Leu^{192}-Tyr^{193}. However, in pure solution, it can hydrolyze β-CN at seven sites, generating f1-192, f1-189, f1-163/4/5 et f1-139 peptides, which are called -β-I^{I}, -β-I^{II}, -β-II and -β-III respectively (Visser, 1993). Orlyuk and Stepanishvhev (2014) reported that between the first and the 21st days of the Camembert ripening, the content of αs-CN and β-CN decreased respectively, by 53% and 25%. Proteolysis during the Camembert ripening is mainly due to the action of five proteinases: rennet (chymosin and pepsin), plasmin, aspartyl and methaloprotease of *Penicillium caseicolum.* However, the rennet seems to be inactive on β-CN, but acts early on αs_1-CN with a similar action to that of aspartyl-proteinase on αs_1-CN. On the contrary, the action of the plasmine on β-CN is observed at the 21st day, while the proteolysis of β-CN by the aspartyl and by the methaloproteases is detectable after seven and ten days of ripening respectively (Mane and McSweeney, 2019).

Growth of microflora during ripening

The results of milk microorganism counts are reported in table 5 .The samples comply with the standards described in the Official Journal of the People's Democratic Republic of Algeria n° 39 of July 2017 (JORADP, 2017).The tests to detect pathogens in the milks and the cheeses, especially for *Staphylococcus aureus* and *Salmonella*, were negative. The number of microorganisms is significantly higher in the raw milk than in the pasteurized. This reflects the lethal effect of the pasteurization on milk microflora.

The results of the microflora evolution during ripening show an inverse relationship between two microbial groups (Figure 3). The group a, including faecal streptococci and faecal and total coliforms, decreased gradually during ripening. The group b, which consists of total aerobic mesophilic flora, streptococci, Lactobacillus, yeast and molds, increased during the ripening process. The trends were similar for both studied cheeses. The differences were rather concerned with variations in the number and in the distribution of the microflora. Indeed, the growth of the microflora during ripening of AC recorded significant differences between the first and the twelfth days for AMF (7.41±0.35 log cfu/g and 9.35±0.31 log cfu/g respectively), MLAB (4.41±0.23 log cfu/g and 5.98±0.49 log cfu/g respectively), FC (1.60 ±0.08log cfu/g and 0.33l±0.41og cfu/g respectively) and FS (1.33±0.2 log cfu/g and 0.58±0.15 log cfu/g respectively). However, IC recorded a slight contamination of TC from the beginning until the sixth day of ripening then it dropped drastically at the twelfth day. With regard to fecal coliform, it disappeared from the sixth day of ripening stage of IC.

The decrease of contamination and the pathogen microflora, during the ripening process of cheese raw milk, is due to the increase in the ripening microflora, which modifies the physico-chemical parameters (a_w and pH) and releases peptides with antibacterial activity (Fontan et al. 2001; Arenas et al. 2004; Kirdar et al. 2018). However, some studies demonstrated that coliforms decrease during the first week and then increase till the end of the ripening process, with higher rates for the Camembert made

Table 5 Microbiological count in milk

Microflora (log_{10} CFU/mL)	Raw milk	Pasteurized milk
Aerobic mesophilic flora (AMF)	4.99^b ±0.306	3.71^a ±0.42
Total coliforms (TC)	1.66^b ±0.11	0.32^a ±0.24
Faecal coliforms (FC)	0.19^b ±0.17	0.0033^a ±0.004
Faecal streptococci (FS)	0	0
Mesophilic lactobacilli (MLAB)	3.63^b ±0.46	0.96^a ±0.178
Mesophilic lactic *streptococcus* (MLS)	2.49^b ±0.22	0.4^a ±0.14
Yeast and molds (Y and M)	1.57^b ±0.19	0.15^a ±0.19

Results in the same column for the same parameter with different letters are significantly different at $P < 0.05$

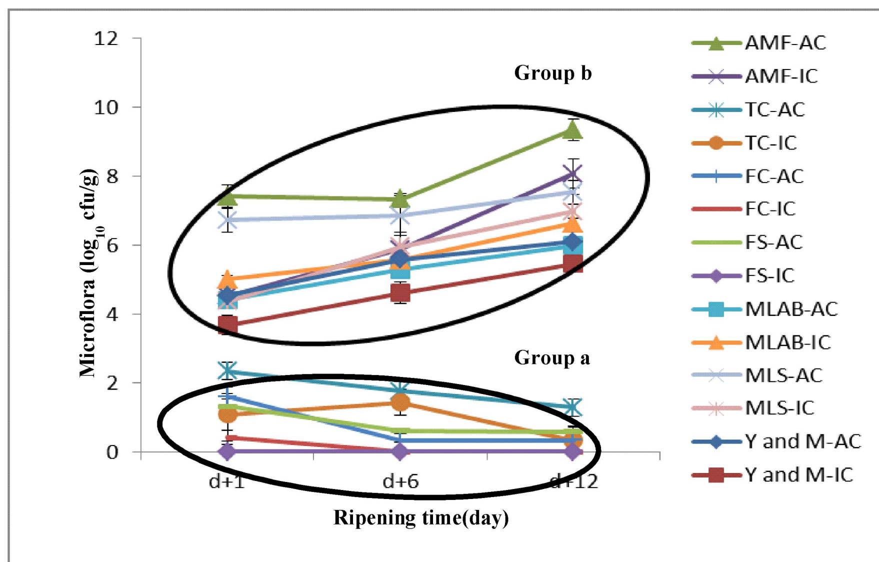


Fig. 3 Microflora evolution in the cheeses during ripening

Legend: Artisanal Camembert (AC), Industrial Camembert (IC). **Group a:** Contamining flora (Total coliforms (TC), Faecal coliforms (FC), Faecal streptococci (FS)). **Group b:** Ripening flora (Aerobic mesophilic flora (AMF), Mesophilic lactobacilli (MLAB), Mesophilic lactic streptococcus (MLS), Yeast and molds (Y and M)).

Table 6 Antibacterial activity statistics for the raw and neutralized supernatants

Strains	Supernatants	Cheeses type	Inhibition zone diameter (mm) Mean ± SD
Staphylococcus aureus ATCC 25923	Raw	IC	10.8 ± 0.97
		AC	10.5 ± 0.98
	Neutralized	IC	10 ± 1.94
		AC	8.8 ± 0.32
Escherichia coli ATCC 25922	Raw	IC	11.7 ± 0.21
		AC	11.3 ± 0.21
	Neutralized	IC	8.2 ± 0.64
		AC	7.9 ± 0.32

from raw milk than for those made from pasteurized milk (Mourgues et al. 1977; Rutzinski et al. 1979).

Antimicrobial activity analysis

The results of the analysis of the supernatant antimicrobial activity are reported in the Table 6. The presence of inhibition zones after neutralization of the supernatants suggests that the antibacterial effect could only be due to the presence of antibacterial substances, such as bacteriocins and bioactive peptides (Ortolani et al. 2010; Corrêa et al. 2011).

Cheese protection against pathogens proliferation can be performed by the indigenous antimicrobial agents of milk, such as lactoferrin (Farnaud and Evans, 2003), lactoperoxydase system (LPS) (Seifu et al. 2005) and lysozyme (Claeys et al. 2013). These agents are inactivated by the heat treatment (Conesa et al. 2010; Dumitraşcu et al. 2012; Claeys et al. 2013).These results explain in large part the decrease in the contaminating microflora during the ripening of AC and the role of the lactic microflora of the raw milk during the cheese ripening, thanks to their proteolytic activity and the production of bacteriocins.

Most of known varieties of cheeses contain a certain amount of kappacins (A and B) in form of caseinomacropeptide, with an antimicrobial activity, which are released as a result of the action of coagulant enzymes (rennet or other coagulant enzymes) on the κ-casein (Vajiheh, 2012). In the case of the Camembert, many studies demonstrated the effect of lactic bacteria and their products on the inhibition of *Listeria ssp* during cheese ripening (Wan et al. 1997). Lignitto et al. (2012) and Nguyen Thi et al. (2013) reported that inhibition of *Listeria monocytogenes* and *Listeria innocua* by water soluble extracts of cheese made from raw milk is attributed to peptides with MW < 1 kDa and to the

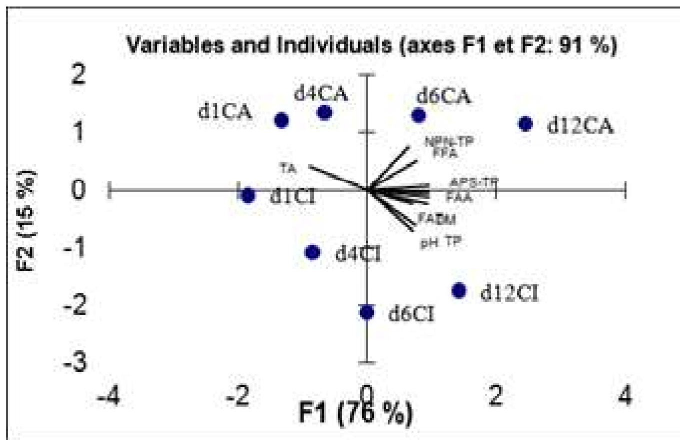


Fig. 4 Factorial plane 1-2 of the component analysis for the physicochemical changes during cheese repining

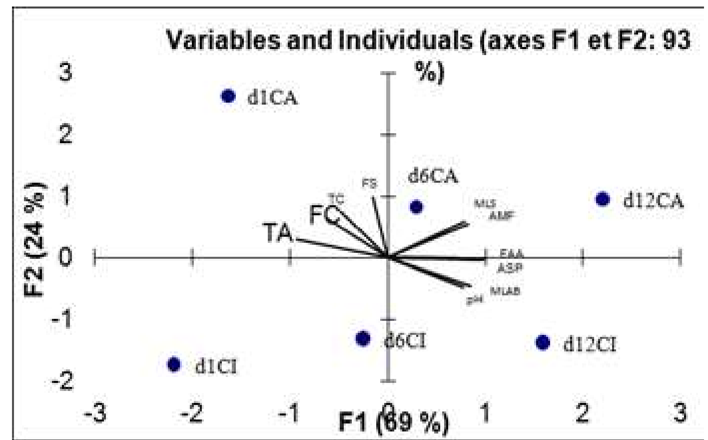


Fig. 5 Factorial plane 1-2 of the component analysis for the physicochemical and microbiological parameters interactions during ripening

antimicrobial peptides αs_1-CN f(1-23) et αs_2-CN f(183-207). The antimicrobial activity of the cheese water-soluble extracts could not only be attributed to the known intact bacteriocins with MW > 3 kDa, but also for the fraction with MW > 10 kDa (Drider et al. 2006; Pritchard et al. 2010). During the ripening process of the Camembert, 15 peaks-RPHPLC were identified as potentially bioactive, all stemming from β-CN with predominance of antibacterial fragments β-CN f(193-209) (Galli et al. 2019).

Interactions among the physicochemical and microbiological parameters and their progress during ripening

A PCA was run to analyze the interactions between the physicochemical and microbiological parameters and their evolution during ripening. The results related to physicochemical parameters during cheese repining are presented in Figure 4.

The first two axes explain 91% of the total variance. Axis F1 represents the maturity (temporal) gradient during the ripening process. F2 represents the type of the product. The projection of the means obtained from the three years of study from the same stage of ripening shows that F1 opposes the artisanal Camembert to the industrial. The small distance between d1AC and d4AC shows the high correlation (low changes) registered between the two first considered ripening stages of AC. In contrast, the temporal gradient revealed great changes (lower correlations) from the second stage of ripening, expressed here by an increase in the distance between successive stages (d4AC, d6AC, and d12AC). At the same stages, these differences were less pronounced during IC ripening, which registered an appreciable change only between the two last stages (J6IC and J12IC). The degree of maturation of AC is the result of the combined effects related to the production conditions and the physicochemical and microbiological proprieties of the raw milk. Consequently, IC is richer in DM, fat and proteins, but less matured than AC. Moreover, the factorial plane 1-2 highlights the concentration of the points representing fat, DM and proteins close to the last stage of ripening for IC, while the points representing the maturation index (ASP, NPN, FAA and FFA) are gathered towards this final ripening stage of AC.

The results of the component analysis for the physicochemical and microbiological parameters interactions during ripening are presented in Figure 5.

The axes F1 and F2 expressed 69 % and 24 % of the total variance respectively. The change in the physicochemical parameters is opposed to the contaminating microorganisms. F2 opposes the coliforms (faecal and total), the faecal streptococci to the mesophilic lactic bacteria (*Streptococci* and *Lactobacillus*), the MFAT, molds and yeasts. This axis also opposes contaminating germs to pH, FAA and ASP.

A scan of figures 4 and 5 explains the decrease of pathogenic organisms during ripening of the artisanal cheese. This could be due to the action of indigenous enzymes of the raw milk and increase in of lactic bacteria and the fungal flora, which lead to the release of bioactive peptides, with antibacterial effects by their proteolytic action, and production of bacteriocins by lactic bacteria. These results are confirmed by the correlation matrix obtained between the physicochemical and microbiological parameters, which revealed that molds and yeasts have an impact on the proteolysis, with significant ($P < 0,05$) positive correlations with the proteolysis products of ASP ($r = 0.88$) and FAA ($r = 0.90$). MLAB showed significant negative correlations with the pathogenic organisms TC ($r = -0.87$), FC ($r = -0.84$) and FS ($r = -0.86$). This explains the effect of MLAB on the decrease of these pathogenic germs during ripening of the cheeses made from raw milk and the role of lactic microflora in their bio-conservation.

Conclusions

The physicochemical parameters showed the same trends and recorded higher values in the Camembert made from raw milk. The inhibition of *Escherichia coli* and *Staphylococcus aureus* by the neutralized water-soluble fractions revealed the effect of the bioconservation of the camembert made from raw milk by an eventual presence, in the cheese, of bacteriocins and bioactive peptides with antibacterial effect. The current study opens up new perspectives within the framework of the dairy industry in Algeria, encouraging stockbreeders and farmers to transform the raw milk into healthy dairy products and provide steady and more active incomes than those generated from selling milk to collectors.

Acknowledgements

We would like to thank Dr Said Slimani, who read through the text, for his suggestions regarding the English and we also would like to thank Miss Lamia Bouadjela and Zobiri Assia for their technical assistance, from university Mouloud Mammeri Tizi-Ouzou (Algeria).

References

Addeo F, Pelissier JP, Chianes AL (1980) Specific action of milk-clotting enzymes on water buffalo caseins. I. Effect of chymosin on β-casein. J Dairy Res 47:421-426. doi:10.1017/S0022029900021324

AFNOR (Association Française de Normalisation) (1980) Lait. Détermination de la matière sèche. NF V04 207.In : AFNOR (Ed.), Recueil de normes françaises. Laits et produits laitiers. Méthodes d'analyse. Paris : Normalisation française, 33-34

Aissaoui ZO, Benatallah L, El Hannachi G, Zidoune MN (2011) Manufacture and characteristics of the traditional Algerian ripened bouhezza cheese. J Food Agric Enviro 9: 69-100

Arenas R, Gonzalez L, Bernardo A, Fresno JM, Tornadijo ME (2004) Microbiological and physico-chemical changes in Genestoso cheese, a Spanish acid curd variety, throughout ripening. Food Control 15: 271-279. doi:10.1016/S0956-7135(03)00067-7

Barac M, Smiljanic M, Žilic S, Pesic M, Stanojevic S, Vasic, M, Vucic T, Kostic A (2016). Protein profiles and total antioxidant capacity of water soluble and insoluble protein fractions of white cow cheese at different stage of ripening. Mljekarstvo 66: 187-197.doi: 10.15567/mljekarstvo.2016.0303

Batool M, Nadeem M, Imran M, Imran TT, Jalees AB, Ayaz M (2018) Lipolysis and antioxidant properties of cow and buffalo cheddar cheese in accelerated ripening. Lipids Health Dis 17: 1-10.doi:10.1186/s12944-018-0871

Benfeldt C, Sorensen J, Katrine H, Petersen E, Petersen T (1997) Heat treatment of cheese milk: Effect on plasmin activity and proteolysis during cheese ripening. Int Dairy J 7: 723-731. doi: 10.1016/S0958-6946(97)00083-6

Benfeldt C, Sorensen J (2001) Heat treatment of cheese milk: effect on proteolysis during cheese ripening. Int Dairy J 11: 567-574. doi :10.1016/S0958-6946(01)00078-4

Bertolino M, Dolci P, Giordano M, Rolle L, Zeppa G (2011) Evolution of chemico-physical characteristics during manufacture and ripening of Castelmagno PDO cheese in wintertime. Food Chem 129: 1001-1011. doi:10.1016/j.foodchem.2011.05.060

Bouton Y, Guyot P, Dasen A, Grappin R (1993) Activité protéolytique de souches de lactobacilles thermophiles isolées de levains et de Comté : I. Validation sur mini fromages des techniques de laboratoire. Le Lait 73: 265-279. doi: 10.1051/lait: 1993325

Bouton Y, Guyot P, Dasen A, Grappin R (1994) Activité protéolytique de souches de lactobacilles thermophiles isolées de levains et de Comté : I I .Application en sites industriels. Le Lait 74: 33-46. doi: 10.1051/lait: 199414

Buffa M, Trujillo AJ, Roy C, Guamis B (2000) Changes in chemical and microbiological characteristics of goat cheese made from raw, pasteurized or high-pressure-treated milk. High Pressure Res 19: 27-32. doi :10.1080/08957950008202531

Claeys WL, Cardoen S, Daube G, De Block J, Dewettinck K, Dierick K, De Zutter L, Huyghebaert A, Imberechts H, Thiange P, Vandenplas Y, Herman L (2013) Raw or heated cow milk consumption: review of risks and benefits. Food Control 31: 251-262. doi :10.1016/j.foodcont.2012.09.03

Conesa C, Rota C, Castillo E, Perez MD, Calvo M, Sanchez L (2010) Effect of heat treatment on the antibacterial activity of bovine lactoferrin against three food borne pathogens. Int J Dairy Technol 63: 209-215.doi: 10.1111/j.1471-0307.2010.00567.x

Corrêa APF, Daroit DJ, Coelho J, Meira SM, Lopes FC, Segalin J, Risso PH, Brandelli A (2011) Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. J Agr Food Chem 91: 2247-2254. doi:10.1002/jsfa.4446

Deeth H C,Fitzgerald C H, Wood A F (1975) A convenient method for determining the extent of lipolysis in milk. Aust J Dairy Technol 30:109-111

Drider D, Fimland G, Héchard Y, McMullen LM, Prévost H (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol 70:564-582. doi: 10.1128/MMBR.00016-05

Dumitraşcu L, Stănciuc N, Stanciu S, Râpeanu G (2012) Thermal inactivation of lactoperoxidase in goat, sheep and bovine milk - A comparative kinetic and thermodynamic study. J Food Eng 113: 47-52.doi.org/10.1016/j.jfoodeng.2012.05.028

DupasC, Adt I, Cottaz A, Boutrou R, Molle D, Jardin J, Jouvet T, Degraeve P (2009) A chromatographic procedure for semi-quantitative evaluation of caseinphosphopeptides in cheese. Dairy Sci Technol 89: 519-529.doi:10.1051/dst/2009027

Farnaud S, Evans RW (2003) Lactoferrin: a multifunctional protein with antimicrobial properties. Molecul Immunol 40: 395-405. doi:10.1016/S0161-5890(03)00152-4

Fontan MCG, Franco I, Prieto B, Tornadijo ME, Carballo J (2001) Microbiological changes in 'San Simoon' cheese throughout ripening and its relationship with physico-chemical parameters. Food Microbiol 18: 25-33.doi:10.1006/fmic.2000.0351

Franco I, Prieto B, Urdiales R, Fresno JM, Carballo J (2001) Study of the biochemical changes during ripening of Ahumado de Aliva cheese: a Spanish traditional variety. Food Chem 74: 463-469. doi: 10.1016/S0308-8146(01)00164-9

Galli BD, Baptista DP, Cavalheiro FG, Negrab F, Eberlin M N, Gigante ML (2019) Peptide profile of Camembert-type cheese: Effect of heat treatment and adjunct culture *Lactobacillus rhamnosus* GG. Food Res Int 234:71-75. doi:10.1016/j.ijfoodmicro.2016.06.025

Gebreyowhans S, Zhang S, Pang X, Yang B, Wang T, Wu Z, Lu J, Iaping J (2020) Changes in texture, composition and sensory characteristics of Camembert cheese made from a mixture of goat milk and cow milk during ripening. Int J Dairy Technol. doi: 10.1111/1471-0307.126990

Gérard Y (2015) Contractualisation et modes de coordination dans la filière laitière. Économie rurale 345: 87-100. doi: 10.4000/economierurale.4580

Gobbetti M, Morea M, Baruzzi F, Corbo M R, Matarante A, Considine T, DiCagno R, Guinee T, Foxd PF (2002) Microbiological, compositional, biochemical and textural characterisation of Caciocavallo Pugliese cheese during ripening. Int Dairy J 12: 511-523

Gonzalez J, Mas M, Tabla R, Moriche J, Roa I, Rebollo JE, Cáceres P (2003) Autochthonous starter effect on the microbiological, physicochemical and sensorial characteristics of Ibores goat's milk cheeses. Le Lait 83:193-202. doi : 10.1051/lait:2003009

Gripon J C, Desmazeaud M J, Lebars D, Bergere J L (1975) Etude du rôle des microorganismes et des enzymes au cours de la maturation des fromages : II. Influence de la présure commerciale. Le Lait 55:502-516. doi : 10.1051/lait:197554828

Guerra-Martínez J A, Montejano J G, Martín-del-Campo S T (2012) Evaluation of proteolytic and physicochemical changes during storage of fresh Panela cheese from Queretaro, Mexico and its impact in texture. CYTA-J Food 10: 296-305.doi: 10.1080/19476337.2011.653791.

JORADP (Journal officiel de République Algérienne Démocratique et Populaire) (2014) Arrêté 17 du 17-12-2013 relatif à la détermination de la matière grasse dans le fromage. JORADP n°67 du 12-11-2014

JORADP (Journal Officiel de la République Algérienne Démocratique et Populaire) (2017) n°39 du2 juillet 2017. Arrêté interministériel du 2 Moharram 1438 correspondant au 4 octobre 2016 fixant les critères microbiologiques des denrées alimentaires, pp 13-15

Joffin C, Joffin J N (1999) Microbiologie alimentaire. CRDP d'Aquitaine, 5th edn, Bordeaux, pp 213-215

Kali S, Benidir M, Ait Kaci K, Belkheir B, Benyoucef MT (2011) Situation de la filière lait en Algérie: Approche analytique d'amont en aval. Livest Res Rural Dev 23:1-12

Kirdar SV, Kursun-Yurdakul O, Kalit S, Kalit MT (2018) Microbiological changes throughout ripening of Keş cheese. J. Cent. Eur. Agric 19:61-71. doi: /10.5513/JCEA01/19.1.2024

Laemmli V K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Leclercq-Perlat M N, Oumer A, Buono F, Bergere J L, Spinnler H E, Corrieu G (2000) behavior of *Brevibacterium linens* and *Debaryomyces hansenii* as ripening flora in controlled production of soft Smear Cheese from reconstituted milk: Protein Degradation. J Dairy Sci 83:1674-1683

Leclercq-Perlat M N, Buono F, Lambert D, Latrille E, Spinnler H E, Corrieu G (2004) Controlled production of Camembert-type cheeses. Part I: Microbiological and physicochemical evolutions. J Dairy Res 71: 346-354. doi: 10.1017/S0022029904000196

Lenoir J (1963) La flore microbienne du Camembert et son évolution au cours de la maturation. Le Lait 43: 262-270. doi: 10.1051/lait:1963425-42611

Lignitto L, Segato S, Balzan, S, Cavatorta V, Oulahal N, Sforza S, Degraeve P, Galaverna, G, Novelli E (2012) Preliminary investigation on the presence of peptides inhibiting the growth of *Listeria innocua* and *Listeria monocytogenes* in Asiago d'Allevo cheese. Dairy Sci & Technol 92: 297-308. doi: 10.1007/s13594-012-0057

Lowry O H, Rosebrough N J, Farr A L, Randall R J (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Mamine F, Bourbouze, Arbouche F (2011) La production laitière locale dans les politiques de la filière lait en Algérie. Cas de la wilaya de Souk Ahras. Livest Res Rural Dev 23: 1-2

Mane A, McSweeney PLH (2019) Proteolysis in Irish farmhouse Camembert cheese during Ripening. J Food Biochem. doi.org/10.1111/jfbc.13101

McSweeney PLH, Fox PF, Lucey J, Jordan KN, Cogan TM (1993) Contribution of the indigenous microflora to the maturation of Cheddar cheese. Int Dairy J 3: 613-634. doi: 10.1016/0958-6946(93)90104-8

Meribai A, Jenidi R, Hammouche Y, Bensoltane A (2017) Physico-chemical characterization and microbiological quality evaluation of klila, an artisanal hard dried cheese from Algerian's arid areas: Preliminary study. J New Sci Agric and Biotech 40: 2169-2174

Motta A S, Brandelli A (2002) Characterization of an antibacterial peptide produced by *Brevibacterium linens*. J Appl Microbiol 92:63-70. doi:10.1046/j.1365-2672.2002.01490.x

Mourgues R, Vassal L, Auclair J, Mocquot G (1977) Origine et développement des bactéries coliformes dans les fromages à pâte molle. Le Lait 57: 131-149. doi: 10.1051/lait:1977563-5645

Nalepa B, Olszewskaa M A, Markiewiczb L M (2018) Seasonal variances in bacterial microbiota and volatile organic compounds in raw milk. Int J Food Microbiol 267:70-76. doi:10.1016/j.ijfoodmicro.2017.12.024

Nguyen Thi P, Dupas C, Adt I, Degraeve P, Ragon M, Missaoui MF, Novelli E, Segato S, Dong PT, Oulahal N (2013) Partial characterisation of peptides inhibiting *Listeria* growth in two Alpine cheeses. Dairy Sci Technol 94: 61-72. DOI: 10.1007/s13594-013-0141-6

Orlyuk YT, Stepanishchev MI (2014) Assessment of proteolysis and lipolysis intensity in pechersky cheese ripening in the presence of *Penicillium camemberti* and *Penicillium roquefortimolds*. Foods and Raw Mater 2: 36-39. doi: 10.12737/4128

Ortolani MBT, Moraes PM, Perin LM, Vicosa GN, Carvalho KG, Silva JA, Nero LA (2010) Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese. J Dairy Sci 93: 2880-2886. doi: 10.3168/jds.2009-3000

Polychroniadou A (1988) A simple procedure using trinitrobenzenesulphonic acid for monitoring proteolysis in cheese. J Dairy Res 55: 585-596. doi: 10.1017/S0022029900033379

Pritchard S, Phillips M, Kailasapathy K (2010) Identification of bioactive peptides in commercial Cheddar cheese. Food Res Int 43: 1545-1548. doi: 10.1016/j.foodres.2010.03.007

Randoin L, Jourdan C (1952) Détermination des teneurs en eau et matière sèche, en calcium et en phosphore de deux variétés de fromages à pâte demi-dure et à moisissures internes. Le Lait 32 : 481-485. doi: https://doi.org/10.1051/lait:195231818

Richard J, Zadi, H (1983) Inventaire de la Flore Bactérienne dominante des Camemberts fabriqués avec du lait cru. Le Lait 63: 25-42. doi: org/10.1051/lait:1983623-6243

Rutzinski JL, Marth EH, Olson NF (1979) Behavior of *Enterobscterserogenes* and *Hafnia* Species during the Manufacture and Ripening of Camembert Cheese. J Food Prot 42: 790-793. doi:10.4315/0362-028X-42.10.790

Saboya LV, Goudédranche H, Maubois JL, Lerayer ALS, Lortal S (2001) Impact of broken cells of lactococci or propionibacteria on the ripening of Saint-Paulin UF-cheeses: extent of proteolysis and GC-MS profiles. Le Lait 81: 699-713

Sahraoui Y, Fayolle K, Leriche F, Le Flèche-Matéos A, Sadoun D (2015) Antibacterial and technological properties of *Lactococcus lactis ssp. lactis* KJ660075 strain selected for its inhibitory power against *Staphylococcus aureus* for cheese quality improving. J Food Sci Technol 52: 7133-7142. doi: 10.1007/s13197-015-1845-9

Samelis J, Lianou A, Kakouri A, Delbes C, Rogelj I, Bojana BM, Montel MC (2009) Changes in the Microbial Composition of Raw Milk Induced by Thermization Treatments Applied Prior to Traditional Greek Hard Cheese Processing. Journal Food Prot 72: 783-790.

Seifu E, Buys EM, Donkin EF (2005) Significance of the lactoperoxidase system in the dairy industry and its potential applications. Trends Food Sci Technol 16: 137-154. doi:10.1016/j.tifs.2004.11.002

Spike PW, Freeman A E (1967) Environmental influences on monthly variation in milk constituents. J Dairy Sci 50: 1897-1904

Sulieman EAM, Mohamed Ali RA, Abdel Razig KA (2012) Production and Effect of Storage in the Chemical Composition of Mozzarella Cheese. Int J Food Sci Nutr Engin 3: 21-26.doi: 10.5923/j.food.20120203.02

Trujillo AJ, Guamis B, Carretero C (1997) Hydrolysis of Caprine β-Casein by Plasmin. J Dairy Sci 80: 2258-2263. doi:10.3168/jds.S0022-0302 (97)76174-5

Upreti P, Metzger LE, Hayest KD (2006) Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on cheddar cheese quality: Proteolysis duringripening. J Dairy Sci 89:444-453

Vajiheh F (2012) Milk Proteins-derived antibacterial peptides as novel functional food ingredients. Ann Biol Res 3: 2520-2526

Van Hekken DL, Tunick MH, Tomasula, PM, Francisco J, Corral M, Gardea A (2007) Mexican Queso Chihuahua: rheology of fresh cheese. Int J Dairy Technol 60: 5-12. doi: 10.1111/j.1471-0307.2007.00291.x

Visser S (1993) Proteolytic Enzymes and Their Relation to Cheese Ripening and Flavor. J Dairy Sci: 329-350. doi:10.3168/jds.s0022-0302 (93)77354-3

Wan K, Harmark BE, Davidson AJ, Hillier JB, Gordon A, Wilcock MW, Hickey MJ (1997) Coventry-Inhibition of *Listeria monocytogenes* by piscicolin 126 in milk and Camembert cheese manufactured with a thermophilic starter. J Appl Microbiol 82: 273-280

Indian J Dairy Sci 74(1): 30-38
https://doi.org/10.33785/IJDS.2021.v74i01.004

RESEARCH ARTICLE

Preparation of low-fat *paneer* using Glucono delta-lactone as an acidulant

Mamata Chaudhary, Suneeta Pinto, CN Dharaiya and Preeti Paul

Received: 17 September 2020 / Accepted: 04 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: The study was aimed to develop a process to manufacture low-fat *paneer* using glucono delta-lactone as an acidulant. Carboxymethyl cellulose was used as a fat replacer. Low-fat *paneer* was prepared from 1.0 % fat mixed milk (Cow:Buffalo::55:45). Optimization of the manufacturing parameters was carried out by using Response Surface Methodology with central composite rotatable design using three independent variables viz. coagulation temperature, level of carboxymethyl cellulose and strength of glucono delta-lactone solution. The suggested solution from RSM analysis for low-fat *paneer* was coagulation temperature: 80°C, level of carboxymethyl cellulose: 600 mg/kg milk and strength of glucono delta-lactone solution: 2%. The developed product had 64.10±0.82% moisture, 6.53±0.16% fat and 18.21±1.01% FDM. The product had 27% higher protein and about 4 times less fat as compared to regular *paneer*. Results of the study revealed that an acceptable quality low-fat *paneer* can be manufactured by incorporating hydrocolloid and glucono delta-lactone.

Keywords: Carboxymethyl cellulose, Glucono delta-lactone, Low-fat *paneer*, Response surface methodology

Dairy Technology Department, SMC College of Dairy Science, Anand Agricultural University, Anand, India

CN Dharaiya (✉)
Dairy Technology Department, SMC College of Dairy Science, Anand Agricultural University, Anand, India
E-mail ID: chetandharaiya@gmail.com
Mobile No.: +91 7573013697

Introduction

Paneer is a heat and acid coagulated traditional Indian dairy product. In India, *paneer* production has been largely confined to the small unorganized sectors. *Paneer* is a soft cheese like product which is rich in fat and protein. The average fat content of *paneer* varies between 23 to 26% while protein content varies between 17 to 21%. Scientific evidence links high-fat diets with increased risk of obesity, atherosclerosis, high blood pressure and tissue injury (Madadlou et al. 2005). This has necessitated a higher demand for low-fat foods. Low-fat products are formulated or designed so as to meet the dietary needs of obese people, people at risk of cardiovascular disease, diabetics and people on diets for weight management (Sandrou and Arvanitoyannis 2003). The high cost of *paneer* is mainly attributed to its high fat content (23-26%) due to which many low and middle class income people refrain from consuming it and cannot afford to include it in their regular diet.

The role of fat in the development of acceptable flavour, body and texture as well as appearance of *paneer* is unquestionable. A reduction in fat content of *paneer* results in lower acceptability. Usually, low-fat cheese varieties are usually characterized by rubbery texture, flat flavour, poor meltability and undesirable dull colour lacking glossiness compared to their full fat counterparts (Mistry 2001). In literature, it has been reported that fat reduction in dairy products can be achieved by replacing fat with starches, polysaccharides, gums or fibres from cereal, vegetables and fruits (Tufeanu and Tia 2016). Murtaza *et al.* (2017) prepared low-fat cheddar cheese from 2.0% fat buffalo milk by adding xanthan gum and guar gums at different rates varying from 0.15 to 0.45%. They reported that guar gum can effectively be used @ 0.45% to produce a low-fat Cheddar cheese with functional, textural and sensory characteristics which were comparable to full fat cheese.

Glucono-δ-lactone (GDL), an acidulant, is a non-agricultural additive which can be produced in a non-synthetic way. GDL hydrolyses in water to form gluconic acid. The rate of acid formation is affected by temperature, concentration and the pH of the solution. It has low acid release at room temperature, but the rate of conversion to gluconic acid increases as the temperature increases (Fetahagić et al. 2002). It has been reported

to be one of the best acids to reduce pH without adversely affecting the acidic flavour profile of food products (Lucey and Kelly, 1994). It is commonly used for manufacture of silken soft tofu (Guo and Ono 2005). Shin et al. (2014) developed a low-fat tofu using GDL as acidulant. They reported that the physicochemical and sensory properties were comparable to full-fat tofu.

It was hypothesized that use of hydrocolloids in combination with GDL would help in improving the body and texture characteristics of low -fat *paneer*. Hence a low-fat *paneer* was prepared using GDL as an acidulant in combination with hydrocolloids.

Materials and Methods

Whole milk (6.1% fat/9.1% MSNF), standardized milk (4.5% fat/ 8.5% MSNF) and skim milk (0.2% fat/9.1% MSNF) were collected from Vidya Dairy, Anand. Carboxymethyl cellulose (CMC) (Loba-chemicals private Ltd., Mumbai) and Glucono delta-lactone (GDL) (Roquette India Pvt. Ltd., Mumbai) were used as a hydrocolloid and an acidulant respectively in low-fat *paneer* (LFP). Citric acid (Loba-chemicals private Ltd., Mumbai) used as coagulant for manufacture of control sample of *paneer*.

Preparation of *paneer*

Low-fat *paneer* was prepared using different levels of CMC, coagulation temperatures and strengths of GDL solution as suggested by Response Surface Methodology (RSM) to choose the best combination to get the most acceptable LFP. The selected range on the basis of preliminary trials were: 75 to 85°C for coagulation temperature (A), 600 to 900 mg/kg of milk for level of CMC (B) and 2.0 to 4.0% strength of GDL solutions (C). At temperatures lower than 75°C and lower strength of GDL solutions the curd was too soft to handle and at temperatures higher than 85°C and higher than 4% strength of GDL the paneer lacked the desired firmness and became too hard. Higher levels of CMC i.e. higher than 900 mg/kg affected the body and texture of paneer adversely and it became too sticky. The solutions were optimized by adopting a three factor Central Composite Rotatable Design (CCRD) consisting of total 20 experiments Table 1.

Milk (standardized to 1.0% fat) was heated to 40-45°C and CMC (level mentioned in Table 1) was added in the form of a paste (prepared in lukewarm milk, about 100 times its weight). Milk was further heated to 90°C for 5 min, cooled to the coagulation temperature (as mentioned in Table 1) and coagulated with GDL solution (strength as mentioned in Table 1), pre-heated to coagulation temperature, which was added slowly to the milk with slow stirring until a coagulum and clear whey separated out. The coagulum was allowed to settle for 5 min for completion of the coagulation process. Care was taken to see that the temperature of whey was above 70°C at the time of draining. Draining of whey was completed in 5 min. The coagulum was transferred to a rectangular sterilized stainless steel *paneer* hoop lined with a clean and sterile muslin cloth. The curd was pressed for 20 min by applying a pressure of 2.0 to 2.5 kg/cm^2. *Paneer* blocks were removed from the hoop and immersed in pasteurized chilled water (about 4°C) for 2 hours. The drained *paneer* blocks (200 g each) were vacuum packed in pre-sterilized laminated pouches with UV light (12 μ polyester + 50 μ LD/LLDPE) and stored at 7±1°C. Control sample of *paneer* (CP) was prepared from standardized milk (4.5% fat/8.5% SNF milk) using the method described by Aneja et al. (2002).

Physico-chemical analysis

Moisture, fat, protein and ash content as well as titratable acidity and pH of *paneer* samples were determined as per the procedure described by FSSAI (2015) .

Textural analysis

Two-bite compression testing of *paneer* samples was done with Lloyd Instrument, Hampshire, UK (Model No. 01/2962) using 5 KN load-cells which moved at a speed of 20 mm/min. All the textural measurements were conducted in a room maintained at 23±1°C temperature and 65±1% RH. The *paneer* samples were taken for texture measurement after tempering the same at 20±1°C. Cubic samples of the experimental *paneer*, with edges of 2.00±0.06 cm, were placed in such a manner that fibers were oriented perpendicular to the cylindrical compression anvil. The cubic samples were compressed up to 70% of their initial size. Average of five cubic samples was reported.

Sensory evaluation

Each block of *paneer* was cut into approximately 25 g rectangular pieces and tempered to 20±2°C before judging. The sensory panel (n=9) was composed of faculty members of the institute. The products were evaluated using 100-point scale as described in IS: 15346 (2003) in a well-ventilated sensory evaluation laboratory. Lukewarm water was uses for rinsing mouth.

Statistical analysis

The data was analysed using Response Surface Methodology (RSM) Design Expert (Version 8.0.3) which was employed to carry out optimization of selected parameters in the final product formulation. Comparison of LFP with CP was analysed using Completely Randomized Design (CRD).

Results and Discussion

All the 20 batches of LFP were evaluated for their sensory and textural attributes as well as selected compositional parameters. The results of the study obtained for sensory scores, textural properties and selected compositional attributes of LFP are presented in Table 1. The regression analysis of suggested models

for sensory scores, textural properties and FDM (fat on dry matter basis) of low -fat *paneer* is presented in Table 2.

Effect of coagulation temperature, level of CMC and strength of GDL solution on sensory characteristics of low -fat *paneer*

As seen in Table 2 that the calculated F-values are more than the Table F-values at 5% level of significance which indicate the significance of the model terms. It can also be seen that, the coefficient of determination (R^2) which reflects the proportion of variability in data explained or accounted by the model for flavour, body and texture, colour and appearance and total score were 0.8950, 0.8507, 0.8094 and 0.8624 respectively. A larger R^2 values suggest a better fit of the quadratic model. The Adequate Precision Value (APV) measure the signal to noise ratio. As seen in Table 2, the APV for sensory characteristics were greater than 4, which support the suitability of the model to navigate the design.

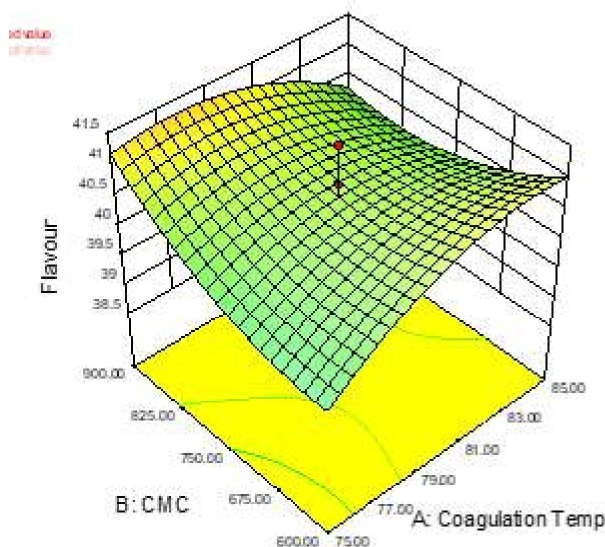
Flavour

The flavour score of LFP ranged from 37.7 to 42.2 out of 50 (Table 1). A significant (P<0.01) reduction in flavour score was observed with increase in strength of GDL solution (C). The interaction of coagulation temperature and level of CMC (AB) in milk showed significant (P<0.05) negative effect. At quadratic level, coagulation temperature and strength of GDL solution reduced flavour score significantly at 5% and 1% level respectively while level of CMC significantly (P<0.05) improved flavour score.

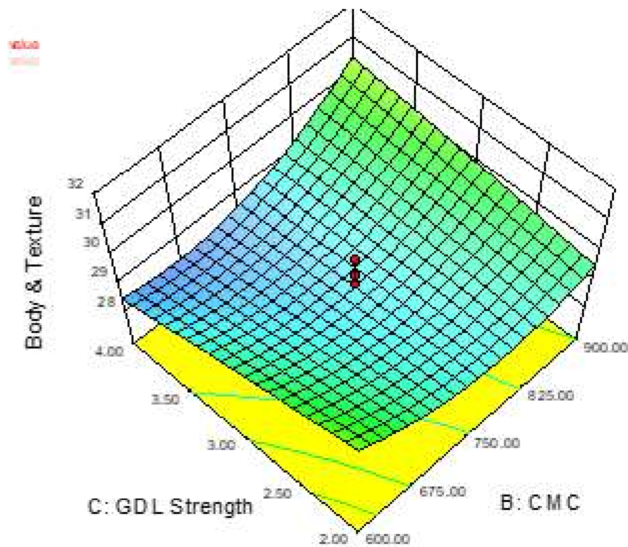
The results obtained in this study are in contrast to those reported by Ghosh et al. (2019), who observed reduction in the flavour score of LFP, prepared from 0.5% fat cow milk using 1% solution of citric acid as a coagulant, with increase in coagulation temperature from 65 to 80°C. Whereas, in present study, the highest flavour score was obtained at 80°C coagulation temperature. The difference observed could be due to the differences in type and level of coagulants used and type of milk used. Similar results were observed by Murtaza et al. (2017) who reported that with increase in level of guar gum (from 0.15 to 0.45%), an increase in flavour score of low-fat Cheddar cheese prepared from buffalo milk (2.0% fat) was observed. Salari et al. (2017) prepared cream cheese by addition of hydrocolloids such as xanthan gum and CMC and reported an improvement in taste of the product. The reduction in the flavour score could be

Table 1 Experimental design matrix and sensory characteristics, textural properties and FDM of low -fat *paneer*

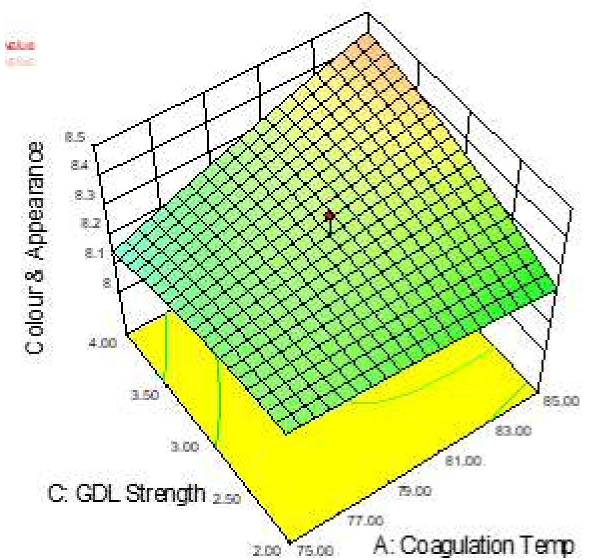
Run Order	Coagulation Temperature (°C) (A)	Level of CMC (mg/kg milk) (B)	Strength of GDL solution (%) (C)	Flavour Score (Out of 50)	B & T Score* (Out of 35)	C & A Score @ (Out of 10)	Total Score** (Out of 100)	Hardness (N)	Chewiness (Nmm)	Cohesiveness	FDM# (%)
1	80.00	750.00	4.68	37.70	29.00	8.25	79.95	20.58	64.95	0.492	18.88
2	75.00	600.00	2.00	40.50	31.50	8.50	85.50	19.48	61.44	0.520	18.67
3	85.00	900.00	4.00	39.00	30.25	8.50	82.75	25.14	83.11	0.513	17.32
4	88.41	750.00	3.00	39.20	29.00	8.35	81.55	23.34	75.67	0.500	17.49
5	80.00	497.73	3.00	41.00	31.25	8.25	85.50	23.77	74.40	0.501	21.23
6	80.00	750.00	3.00	40.45	28.89	8.37	82.72	19.54	60.91	0.500	17.74
7	85.00	900.00	2.00	40.40	29.40	8.25	83.05	13.68	39.97	0.465	19.78
8	80.00	750.00	3.00	41.40	29.78	8.37	84.55	19.45	76.15	0.492	18.31
9	80.00	750.00	3.00	40.20	28.05	8.37	81.62	19.68	60.56	0.495	17.40
10	85.00	600.00	4.00	39.30	29.08	8.37	81.75	28.23	85.21	0.478	19.64
11	75.00	900.00	2.00	39.40	31.00	8.00	83.40	15.35	43.77	0.462	21.26
12	71.59	750.00	3.00	39.20	31.20	8.20	83.60	17.16	48.34	0.505	17.29
13	80.00	750.00	3.00	40.60	28.90	8.25	82.75	19.45	60.50	0.492	18.29
14	80.00	1002.27	3.00	42.20	31.80	8.20	87.20	14.59	44.29	0.478	22.15
15	75.00	900.00	4.00	41.40	31.20	8.12	85.72	13.85	39.37	0.478	22.06
16	80.00	750.00	1.32	39.60	28.90	8.20	81.70	14.08	42.97	0.499	18.07
17	85.00	600.00	2.00	42.00	30.20	8.20	85.40	20.28	62.61	0.515	19.20
18	80.00	750.00	3.00	40.20	29.20	8.30	82.70	17.53	60.50	0.506	18.29
19	80.00	750.00	3.00	40.80	28.00	8.20	82.00	19.54	60.32	0.513	17.96
20	75.00	600.00	4.00	37.80	27.90	8.12	78.82	24.48	73.25	0.489	17.00



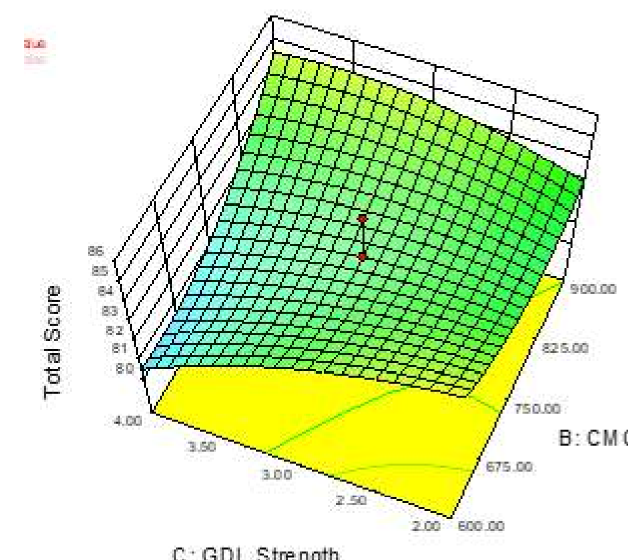
(a) Response surface of flavour as influenced by coagulation temperature (°C) and level of CMC (mg/kg milk)



(b) Response surface of body and texture score as influenced by level of CMC (mg/kg milk) and Strength of GDL solution (%)



(c) Response surface of colour and appearance as influenced by coagulation temperature (°C) and strength of GDL solution (%)



(d) Response surface of total score as influenced by level of CMC (mg/kg milk) and strength of GDL solution (%

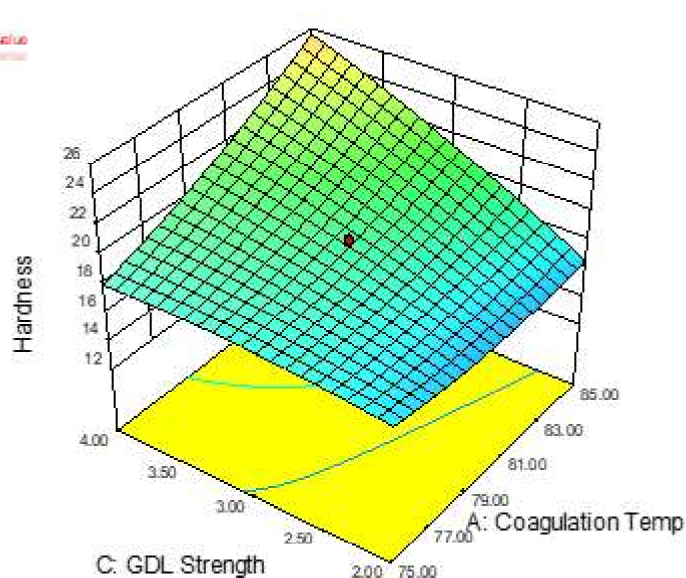
Fig. 1 Surface plot (3-D) for sensory characteristics of low -fat paneer

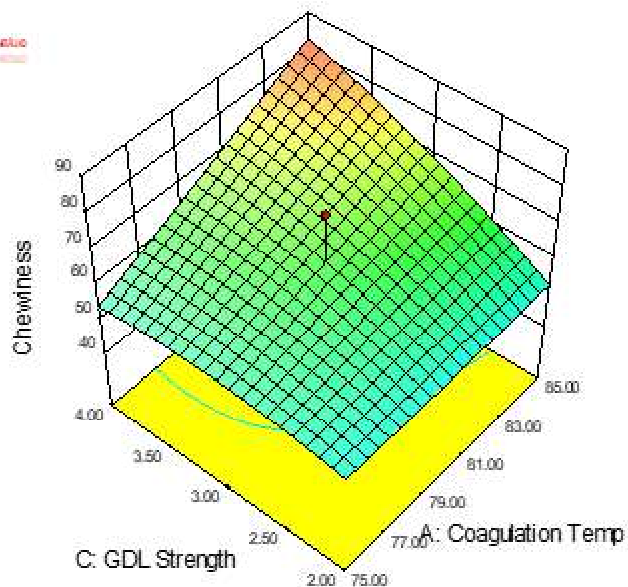
ascribed to lack of richness and flat flavour owing to lower fat content.

Body and Texture

The body and texture score of LFP as depicted in Table 1 varied from 27.9 to 31.8 (out of 35). The values from Table 2 suggested the coagulation temperature of milk showed significant ($P<0.05$) negative effect. At quadratic level, body and texture score significantly increased with increase in coagulation temperature ($P<0.05$) and level of CMC ($P<0.01$). Interaction of level of CMC and strength of GDL solution (BC) had significant ($P<0.05$) positive effect (Fig. 1(b)).

The results obtained in this study are in contrast to those reported by Ghosh et al. (2019) who reported that the body and texture score of low-fat *paneer* prepared from 0.5% fat cow milk increased when milk coagulation temperature increase from 50 to 60°C and





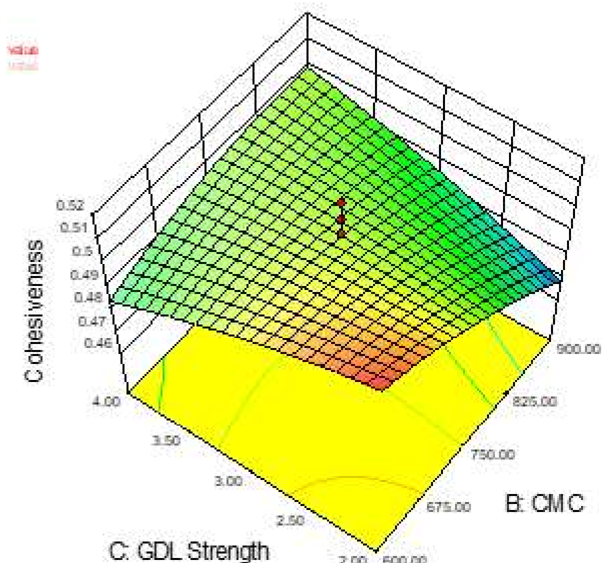
(a) Response surface of hardness as influenced by coagulation temperature (°C) and strength of GDL solution (%) (b) Response surface of chewiness as influenced by coagulation temperature (°C) and strength of GDL solution (%)

then decreased when coagulation temperature increase from 65 to 80°C. Whereas, in current study, it was found that the highest body and texture scores was obtained when the coagulation temperature was 80°C. The difference observed could be attributed to the differences in type and level of ingredients used and type of milk used. Low fat *paneer* is characterised by hard body, while addition of hydrocolloids like CMC helped in moisture retention by water-binding and thus increased moisture content which resulted in softer body and ultimately led to higher body and texture score.

Colour and Appearance

The colour and appearance scores of LFP depicted in Table 1 varied from 8.0 to 8.5 (out of 10). The observations from Table 2 suggested significant ($P<0.05$) increase in colour and appearance score with increase in coagulation temperature. Interaction of coagulation temperature with CMC (AB) or strength of GDL solution (AC) (Fig. 1(c)) and interaction of CMC and strength of GDL solution (BC) showed significant positive effect.

Ghosh et al. (2019) also reported that the positive correlation of milk coagulation temperature with colour and appearance of *paneer* of low-fat *paneer* prepared from 0.5% fat cow milk. Singh et al. (2015) also observed similar observation in low-fat fibre supplemented *paneer* (3.0% fat milk). Similar observations were made by Murtaza et al. (2017) who reported that with increases level of guar gum there was an increase in colour and appearance score of low-fat Cheddar cheese prepared from buffalo milk (2.0% fat). The higher score for colour and appearance could be attributed to higher glossiness due to higher moisture content because of addition of CMC.



(c) Response surface of cohesiveness as influencedby level of CMC (mg/kg milk) and strength ofGDL solution (%)

Fig. 2 Surface plot (3-D) for textural properties of low -fat paneer

Total score

The total score of LFP depicted in Table 1 ranged from 78.82 to 87.20 (out of 100). The observations from Table 2 suggested

Table 2 Regression analysis of suggested models for sensory characteristics, textural properties and FDM of low -fat *paneer*

Parameter		Sensory characteristics				Textural properties			Composition
		Flavour Score (Out of 50)	Body & Texture Score (Out of 35)	Colour & Appearance Score (Out of 10)	Total Score (Out of 100)	Hardness (N)	Chewiness (Nmm)	Cohesiveness	FDM (%)
Intercept		40.60	28.81	8.31	82.72	19.16	63.06	0.50	17.99
Linear Level	A	0.1172	-0.4668#	0.0613#	-0.2883	1.7986*	7.2515*	0.0009	-0.1987
	B	0.1917	0.3002	-0.0299	0.4620	-2.9208*	-9.2941*	-0.0089*	0.5460#
	C	-0.5855*	-0.2198	0.0182	-0.7870#	2.4778*	8.0630*	-0.0011	-0.1119
Interactive effect	AB	-0.5500#	-0.3031	0.0844#	-0.7688	0.6337	3.3512	0.0068	-1.1737*
	AC	-0.4250	0.3906	0.0856*	0.0512	1.9887*	7.2912#	0.0031	-0.1438
	BC	0.7500*	0.7219#	0.0719#	1.5438*	-0.3737	0.5412	0.0165*	-0.0538
Quadratic level	A^2	-0.4544#	0.4250#	-0.0089	-0.0383	0.6162	0.2134	-0.0003	-0.1920
	B^2	0.3942#	0.9288*	-0.0266	1.2964*	0.2379	-0.7270	-0.0047	1.3283*
	C^2	-0.6488*	-0.0346	-0.0266	-0.7100#	-0.4161	-2.6309	-0.0026	0.1916
R2		0.8950	0.8507	0.8094	0.8624	0.9253	0.8880	0.8446	0.8619
Model F-value		9.47	6.33	4.72	6.97	13.76	8.81	6.04	6.94
APV		11.22	7.63	8.80	10.34	13.59	10.61	10.46	9.45
Suggested Model		Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic

*Significant at 1% level ($P<0.01$); #Significant at 5% level ($P<0.05$);R^2 coefficient of determination; APV Adequate Precision Value; FDM Fat on Dry Matter; A, B and C refer to the coagulation temperature, level of CMC and strength of GDL solution respectively;

Table 3 Comparison of predicted v/s actual values of responses selected

Response	P Value	PredictedValue *	ActualValue @	Cal.t-Value #	Significance
Flavour[1]	0.1060	41.42	42.09	1.9013	NS
Body & Texture[1]	0.7561	30.38	30.15	0.3251	NS
Colour & Appearance[1]	0.0623	8.35	8.57	2.2854	NS
Total Score[1]	0.4921	85.15	85.81	0.7315	NS
Chewiness	0.3772	61.69	61.26	0.9534	NS
Cohesiveness	0.6728	0.5202	0.5137	0.4437	NS
Hardness	0.5196	19.11	19.35	0.6838	NS
FDM	0.1512	18.82	18.21	1.6444	NS

[1]Sensory score on 100 point score card; [*] Predicted values of Design Expert 8.0.3 package; [@] Actual values are average of seven trials of optimized product; [#] t-values found non-significant at 5% level of significance; NS = Non-significant; Tabulated t-value = 2.447 (Calculated t-value less than tabulated value)

Table 4 Comparison of proximate composition, sensory and textural properties of low -fat *paneer* with control *paneer*

Components	CP (Citric acid)	LFP (GDL+CMC)	CD (0.05)
Moisture (%)	61.80±0.82	64.10±0.64	0.89
Fat (%)	8.92±0.48	6.53±0.16	0.42
FDM	18.94±1.01	18.21±0.33	NS
Protein (%)	23.35±0.77	22.07±0.37	0.72
Ash (%)	2.62±0.12	2.45±0.15	NS
Carbohydrate (%)	3.26±0.29	4.84±0.27	0.34
Acidity (%LA)	0.45±0.015	0.47±0.019	NS
pH	5.96±0.025	5.94±0.029	NS
% Fat recovery	87.37±1.43	87.87±2.38	NS
% Total solids recovery	47.67±0.96	46.61±0.66	NS
% Yield	12.29±0.65	13.42±0.35	0.62
Flavour Score (Out of 50)	45.05±1.94	42.09±2.27	2.46
Body & Texture Score (Out of 35)	31.57±1.62	30.16±1.78	NS
Colour and Appearance Score (Out of 10)	8.21±0.23	8.57±0.31	0.32
Total Score (Out of 100)	89.84±2.56	85.81±3.42	3.52
Hardness (N)	22.93±1.75	19.34±1.22	1.80
Cohesiveness	0.46±0.02	0.52±0.03	0.04
Chewiness (Nmm)	64.17±2.79	61.26±3.29	NS
Springiness (mm)	6.08±0.29	6.38±0.23	NS
Gumminess (N)	10.55±0.51	9.17±0.49	0.61

strength of GDL solution had significant ($P<0.05$) negative effect on total score at linear level, it indicates increase in strength of GDL solution there was a decrease in total score of LFP. It was found that higher level of CMC in milk (B^2) had significant ($P<0.01$) positive effect on total score, while higher level of strength of GDL solution (C^2) had significant ($P<0.05$) negative effect on total score at quadratic level. The interaction of level of CMC and strength of GDL solution (BC) had significant ($P<0.01$) positive effect on total score of LFP (Fig. 1(d)).

Similar observation reported by Murtaza et al. (2017) who reported that with increases level of guar gum the overall acceptability score increased in low fat Cheddar cheese prepared from buffalo milk (2.0% fat). Thus the results are in agreement with those reported in literature. However, the results obtained in this study are in contrast to those reported by Ghosh et al. (2019). They reported that the total score of low-fat *paneer* prepared from 0.5% fat cow milk increased when milk coagulation temperature increase from 50 to 60°C and then decreased when coagulation temperature increase from 65 to 80°C. Whereas, in our study, it was found that the highest total score was obtained when the coagulation temperature was 80°C. The difference observed could be due to the differences in type and level of ingredients used and type of milk used. The flavour score of LFP was reduced while body and texture score as well as colour and appearance score was increased, resulting in rise in total score.

Effect of coagulation temperature, level of CMC and strength of GDL solution on textural properties and FDM of low-fat *paneer*

Textural properties selected as responses for LFP were Chewiness, Cohesiveness and Hardness and from composition FDM was selected. The values of these responses along with their factors as per run order are given in Table 1. R^2 for hardness, chewiness, cohesiveness and FDM were 0.9253, 0.8880, 0.8446 and 0.8619 respectively (Table 2).

Hardness

The values of hardness of LFP shown in Table 1 ranged from 13.68 to 28.23 N. The values presented in Table 2 revealed with increase in coagulation temperature of milk (A) and strength of GDL solution (C) there was a significant ($P<0.01$) increase in hardness of LFP. However, level of CMC showed significant ($P<0.01$) negative effect on hardness of LFP at linear level. The interaction of coagulation temperature of milk and strength of GDL solution (AC) had significant ($P<0.01$) positive effect on hardness of LFP (Fig. 2(a)). Increasing coagulation temperature and strength of GDL solution resulted in reduction in moisture content which led to increased hardness while increasing the level of CMC increased water-binding and thus moisture content of final product resulting in reduced hardness. Hardness of low-fat fibre supplemented paneer prepared from toned milk (3.0% fat/8.5% SNF) increased significantly ($P<0.05$) with increase in amount of citric acid solution and coagulation temperature (Singh et al. 2015) Murtaza et al. (2017) reported that increase in concentration of guar gum and xanthan gum (independently) from 0.15 to 0.45% there was a decrease in hardness 1399.54g to 1321.78g and 1440.23g to 1389.00g respectively of low-fat Cheddar cheese (2.0% fat milk).

Chewiness

The values of chewiness (Nmm) of LFP shown in Table 1 ranged from 39.37 to 85.21 Nmm. The observations from Table 2 suggested coagulation temperature and strength of GDL solution had positive correlation with chewiness of LFP. However, level of CMC had negative correlation with chewiness of LFP. Coagulation temperature of milk and strength of GDL solution (AC) had significant (P<0.05) positive interaction effect on chewiness (Fig. 2(b)). Increasing coagulation temperature and strength of GDL resulted in reduced moisture and thus increased total solids as well as protein content while increasing the level of CMC resulted in higher moisture and lower total solids and protein in final product. Chewiness has direct relationship with protein content (Mistry, 2001). Similar results observed by Singh et al. (2015) who reported that coagulation temperature (P<0.05) and amount of citric acid solution (P<0.01) had significant positive effect on chewiness at linear level of low-fat fibre supplemented *paneer* made from 3.0% fat and 8.5% SNF. Similarly Murtaza et al. (2017) also reported that with increase in level of hydrocolloids xanthan gum and guar gum (independently) from 0.15 to 0.45% showed decrease in chewiness of low fat Cheddar cheese prepared from buffalo milk from (2.0% fat).

Cohesiveness

The values of cohesiveness of LFP shown in Table 1 ranged from 0.46216 to 0.52023. The values from Table 2 suggested with increase in level of CMC there was a significant (P<0.01) decrease in cohesiveness of LFP. The interaction effect of level of CMC and strength of GDL solution (BC) had significant (P<0.01) positive effect on cohesiveness of LFP (Fig. 2(c)). Reduction in cohesiveness with higher level of CMC could be ascribed to higher moisture content associated with higher CMC. Similarly to our results, Singh et al. (2015) also reported that the interaction of coagulation temperature and amount of citric acid solution had non-significant effect on cohesiveness of low-fat fibre supplemented *paneer* (3.0% fat milk). Similar results also observed by Murtaza et al. (2017) also reported that with increase in level of hydrocolloids *viz.* xanthan gum and guar gum (independently) from 0.15 to 0.45% resulted into decrease in the cohesiveness of low fat Cheddar cheese (2.0% fat milk) from 0.866 to 0.824 and 0.876 to 0.812, respectively.

Fat on Dry Matter (FDM)

The values of FDM shown in Table 1 ranged from 17.00 to 22.15%. The observations from Table 2 suggested with increase in level of CMC there was significant (P<0.05) increase in FDM of LFP. The interaction of coagulation temperature of milk and level of CMC (AB) showed significant (P<0.01) negative effect on FDM of LFP. Increasing the level of hydrocolloids such as CMC helps in retention of fat (Salari et al. 2017). Similar results observed by Murtaza et al. (2017) who reported that with increase in level of hydrocolloids (xanthan gum and guar gum independently) there was an increase in FDM content of low-fat Cheddar cheese prepared from buffalo milk (2.0% fat).

Optimization of low -fat *paneer* (LFP)

Optimization of process for the development of LFP was carried out with the objective of determining the best possible combination(s) of different levels of factors *viz*. coagulation temperature, level of CMC and strength of GDL solution that would lead to the most acceptable product in terms of sensory characteristics.

The goals for all sensory responses were set to be maximized. The predicted formulation for optimized LFP from RSM analysis consisted of 79.67°C (~80°C) coagulation temperature, 600 mg CMC per kg milk (w/w of milk) and 2.0% GDL solution. The predicted sensory scores, textural properties and FDM of LFP are depicted in Table 3. The final product was manufactured employing the suggested formulation and the actual results obtained were compared with these predicted values of the criteria/responses selected for process optimization. The calculated t-values of for all the parameters are reported in Table 3. The calculated t-values being less than the table values, it is inferred that there was non-significant (P<0.05) difference between the predicted and actual values of responses.

LFP prepared using the optimized solution was manufactured using 80°C coagulation temperature, @ 600 mg/kg milk CMC (w/w of milk) and 2.0% GDL solution. The compositional, physico-chemical, sensory characteristics and textural properties of the low-fat *paneer* (LFP) was compared with a *paneer* (CP) made by using citric acid (1% solution w/v) as a coagulant. The average values of the chemical composition of the developed reduced-fat *paneer* i.e. LFP and CP are showed in Table 4. Moisture content of LFP is significantly (P<0.05) higher than that of CP due to addition of hydrocolloid such as CMC which translated into significantly (P<0.05) lower fat, protein and carbohydrate content as well as higher yield. Flavour score of LFP is significantly (P<0.05) lower due to flat flavor owing to higher moisture while colour and appearance score is significantly (P<0.05) higher due to more whiteness in LFP but total score of LFP remained significantly (P<0.05) lower. Hardness of LFP is significantly (P<0.05) lower while cohesiveness is significantly (P<0.05) higher due to higher moisture content. Yellamanda *et al.* (2006) reported acidity values of low-fat *paneer* prepared form milk containing 2.0% fat and 9.0% MSNF to be 0.42-0.43%. According to FSSR (2011) standards for low fat *paneer*, a product can be qualified as low fat if it meets the following requirements: Moisture (max. % m/m) 70% and milk fat (% m/m on dry basis): 20% maximum. Based on the results obtained in this study, LFP can be classified as "Low-Fat *Paneer*" under the FSSR (2011) regulations, since it met the requirements with respect to moisture and fat content.

Conclusion

Based on the results obtained in this study it can be concluded that the optimum parameters for manufacture of low-fat *paneer* using GDL as coagulant are: using a coagulation temperature of 80°C, addition of 600 mg/kg CMC (w/w) of milk and using GDL in the form a 2.0% solution. The quantity of GDL required to coagulate one kg milk was 3.8 to 4.0 g. The yield of *paneer* obtained was 13.4 kg/100 kg milk. The product had 27% higher protein as compared to regular *paneer*. The fat content in the developed product was about 4 times lower than that of regular *paneer*. The developed product has a good potential of being marketed as a healthy alternative to *paneer*.

References

Aneja, RP, Mathur, BN, Chandan, RC, Banerjee, AK (2002) Heat-acid coagulated products. In: Gupta PR (ed) Technology of Indian Milk Products, Dairy India Yearbook, New Delhi, pp 133-142

Fetahagić S, Maćej OD, Denin-Đurđević JD, Jovanović ST (2002) The influence of GDL concentration on milk pH change during acid coagulation. J Agric Sci 47:75-85

FSSAI (2015) Manual of Methods for Analysis of Foods – Milk and Milk Products, Government of India, New Delhi, pp 54, 56, 135, 139, 143, 145

FSSR (2011) Food Safety and Standards (Food Products Standards and Food Additives) Regulations, Ministry of family and health affair, Government of India, New Delhi, India

Ghosh D, Debnath A, Chetri A (2019) Effect of processing parameters on quality attributes of cow skim milk *Paneer*. Pharm Innovation J 8:429-431

Guo S, Ono T (2005) The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. J Food Sci 70:258–262

IS: 15346 (2003) Method for Sensory Evaluation of *Paneer*/Chhana. Bureau of Indian Standards, Manak Bhavan, New Delhi

Lucey J, Kelly J (1994) Cheese yield. Int J Dairy Technol 47:1-14

Madadlou A, Khosrowshahi A, Mosavi ME (2005) Rheology, micro-structure and functionality of low-fat Iranian White cheese made with different concentration of rennet. J Dairy Sci 88:3052–3062

Mistry VU (2001) Low fat cheese technology. Int Dairy J 11:413–422

Murtaza MS, Sameen A, Huma N, Hussain F (2017) Influence of hydrocolloid gums on textural, functional and sensory properties of low fat Cheddar Cheese from buffalo milk. Pak J Zool 49:27-34.

Salari S, Zanganeh M, Fadavi A, Ahmadi Z (2017) Effect of xanthan gum and carboxymethyl cellulose on chemical and sensory properties of cream cheese. Int J Adv Manuf Technol 8: 2-6

Sandrou DK, Arvanitoyannis IS (2003) Low-fat/calorie foods: current state and perspectives. Crit Rev Food Sci Nutr 40:427-447

Shin W, Kim W, Kim Y (2014) Physicochemical and sensory characteristics of a low-fat tofu produced using supercritical co_2 extracted soy flour. Food Sci Biotechnol 23:43-48

Singh G, Kumar A, Kumbhar BK, Dar BN (2015) Optimization of processing parameters and ingredients for development of low-fat fibre-supplemented *paneer*. J Food Sci Technol 52:709-719

Tufeanu R, Tiţa O (2016) Possibilities to develop low-fat products: a review. Acta Univ. Cibiniensis, Ser. E: Food Technol 20:3-19

Yellamanda S, Reddy K, Devi LN (2006) A Study on pickling of low fat *paneer*. Indian J Dairy Sci 59:125-127

Indian J Dairy Sci 74(1): 39-47
https://doi.org/10.33785/IJDS.2021.v74i01.005

RESEARCH ARTICLE

Physico-chemical changes during processing and storage of UHT milk

Suvartan Ranvir[1], Rajan Sharma[2], Kamal Gandhi[2], Pranali Nikam[2] and Bimlesh Mann[2]

Received: 20 June 2020 / Accepted: 19 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: In this study, physico-chemical and heat induced changes during the preparation and storage of UHT milk were evaluated. Parameters such as pH, acidity, viscosity, sedimentation, colloidal calcium (Ca), colloidal magnesium (Mg), hydroxyl methyl furfural (HMF), lactulose and color value were evaluated. During conversion of raw milk to UHT milk, there was slight increase in viscosity, colloidal Ca, colloidal Mg, HMF, lactulose and colour value, while decrease in pH value was observed. During storage of UHT processed milk there was significant ($p<0.001$) increase in viscosity, *a* value, *b* value, HMF and lactulose content, whereas a significant ($p<0.001$) decrease in pH, colloidal Ca, colloidal Mg and *L value* was observed. Increase in acidity, sedimentation content and formation of Maillard browning products adversely affected the quality of UHT milk. These changes were noticed more in UHT processed milk stored at 30°C vis-à-vis 5°C.

Keywords: UHT milk, Calcium, Magnesium, HMF, Lactulose, Sedimentation

Introduction

Ultra-high temperature (UHT) processing of milk is one of the promising heat processing technique for elongating the shelf life of milk. UHT processing of milk is carried out at high temperature (135-150 °C) for a short time (1-10 seconds), resulting in production of sterile milk which when packaged aseptically is stable for about 6 months at room temperature (Al-Saadi and Deeth, 2008; Ranvir et al. 2020a). During processing and storage of UHT milk, various physio-chemical and biochemical reactions take place, including Maillard reactions, degradation of lactose, hydrolysis of lipid, aggregation and denaturation of whey proteins, formation of β-lactoglobulin and k-casein complexes and disturbance of salt balance (Corredig and Dalgleish, 1996; Richards et al. 2014; Sakkas et al. 2014). Apart from these changes, heating process causes development of undesired colors and flavour (especially cooked) which significantly affects the nutritional and sensory quality of UHT milk (Elliott et al. 2003; Elliott et al. 2005; Al-Saadi and Deeth, 2008; Sakkas et al. 2014). These changes drastically affect the quality of UHT milk, which limits its shelf life and thus the consumer acceptance (Rauh et al. 2014; Gaur et al. 2018; Sunds et al. 2018). The heat sensitive components, which are already present or develop in milk during heat processing, allow a direct and quantitative assessment of the processing impact without the detailed knowledge of the actual heat treatment history of the product are called as heat load indicators or thermal time integrator's (TTIs) (Claeys et al. 2002). TTIs can be categorized into two groups; first one is type- I and another one is type- II indicators. Type I-indicators are heat sensitive components such as whey proteins, vitamins, enzymes etc. During processing and storage, these components undergo inactivation, degradation and denaturation. Type II-indicators are those which are not originally present or present at a low level and are developed during processing and storage of heated milk and milk products e.g. HMF and lactulose (Elliott et al. 2003; Mayer et al. 2010; Sakkas et al. 2014). Type I-indicators are more suitable parameters for the evaluation of low intensity heat processing conditions e.g. pasteurization, while type II-indicators are better for assessment of severe heat treatments, such as UHT processing (De Block et al. 1996; Sakkas et al. 2014; Meshram et al. 2018).

Mostly two types of problems occurs in UHT milk during its storage, the first one is age gelation and the second one is development of changes like off flavour, colour and nutritional loss. The rate of these changes is largely dependent on the storage time and temperature. They cause adverse impact on the quality of UHT milk, thus limiting consumer acceptance (Corredig and Dalgleish, 1996; Richards et al. 2014; Sakkas et al. 2014; Deeth and Lewis, 2017; Ranvir et al. 2020b). Some of the researchers

[1]Dairy Chemistry Division, Warner College of Dairy Technology, SHUATS, Prayagraj, UP, India

[2]Dairy Chemistry Division, National Dairy Research Institute Karnal-132 001, Haryana, India

Rajan Sharma (✉)
Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal, 132 001 India
Email: rajansharma21@gmail.com ; Mob. 9416120181

reported that during storage of UHT milk, there occurs reduction in sweet taste and development of sugary aromatic flavor (Clare et al. 2005; Jensen et al. 2015). The present study has been planned to assess the physico-chemical changes during conversion of raw milk to UHT milk and impact of storage time and temperature on the quality of UHT milk.

Materials and Methods

Reagents

Trifluoroacetic acid (TCA) was procured from Sisco Research Laboratories Pvt. Ltd, India. Sodium acetate was procured from Loba chemie, India. Other chemicals required in the study were procured from Sigma-Aldrich, Germany. All sample preparations and measurements were carried out using double distilled water (Cascada water purifier, UK) having conductance of 0.055 µS/cm^2.

Collection of milk samples

Raw milk and subsequently pasteurized and UHT processed (by indirect method) toned milk samples were collected from Verka dairy plant, Chandigarh, India. Raw and pasteurized milk were analyzed for their physico-chemical parameters and heat induced components. UHT milk was produced by heating milk at 140°C for 4 s by using an indirect tubular heat exchanger (STORK, Netherlands). Nine UHT milk (fat 3.0% and 8.5% SNF) samples were brought to the laboratory on the day of manufacture and divided into two groups; first group was stored at 5ºC and second group was stored at 30ºC. Samples were examined at a regular interval of one month till 4 months of storage.

pH

The pH was determined in UHT milk by using Lab India pH analyzer (M-420, Cyberscan pH Tutor, EUTECH Instruments, Thermo Fisher Scientific, Mumbai, India) with combined electrode at 20±2ºC. Prior to use, the pH meter was calibrated with standard buffer solution of pH 4, 7 and 11.

Acidity

Titratable acidity of milk samples was determined as per the procedure described in BIS (IS: 1479 (part-1) 2016).

Viscosity

The viscosity was measured at 20ºC under constant conditions using Ostwald viscometer in athermostatically controlled water bath.

Sedimentation

Sedimentation test was carried out as per the method suggested by Hassan et al. (2009). The UHT milk sample was drain out from the cartons by leaving the bottom 4 cm. The cartons were then inverted for about 10 minutes, up righted and kept in the exhaust hood to dry. The cartons were allowed to dry for 48 hours after opening the bottom flaps or wings of cartons to facilitate the drying of any sediment entrapped there. The cartons were weighed and then washed thoroughly to remove any sediment or residue adhering to the container. The washed cartons were again dried and weighed.

Colloidal Ca and colloidal magnesium content

UHT milk samples were defatted by centrifugation (Centrifuge 5810 R, Eppendorf, Sigma-Aldrich, Germany) at 4000x*g* for 10 min at 4°C and skim milk was ultrafiltered with Amicon® Ultra-15, 10KDa Centrifugal Filter Devices (Merck, Germany) using centrifuge (@ 5000x*g*, 40 min, 25°C). The permeate was discarded and retentate was analyzed for colloidal Ca and Mg content by using atomic absorption spectrometry (AA-7000; Shimadzu, Japan) as described in International Organization for Standardization (ISO, 8070 2007).

HMF

Total HMF content in UHT milk was determined using the method suggested by Cais-Sokolinska (2005). Ten millilitre UHT milk sample was acidified with 5 ml 0.3 N oxalic acid followed by heating at 100°C for 60 min. Content was then cooled to room temperature and mixed with 5 ml of 40% trichloroacetic acid solution. The mixture was thoroughly mixed and filtered thorough Whatman paper No. 42 filter paper. One millilitre of aqueous solution of TBA (0.05 M) was then added to 4 ml of the filtrate. The solution was mixed thoroughly and incubated in water bath maintained at 40°C for 40 min. It was then cooled to room temperature and absorbance was measured at 443 nm.

Lactulose

The lactulose content was measured by enzymatic method using kit (Cat No: K-LACTUL) procured from Megazyme, Bray, Wicklow.

Color Value

Tristimulus spectrophotometer Hunter Lab model Color Flex® [Hunter Associates Laboratory Inc., VA, U.S.A with software (version 4.10)] was used for measuring the color of UHT milk and the results were expressed in terms of the CIE-LAB system according to the method of Popov-Raljic et al. (2008). The instrument was standardized in day light at reflectance angle of 10° (illuminant D65/10° standard observer). The instrument was calibrated with standard black and white tiles as specified by the manufacturer. The light source was dual beam xenon flash lamp. Measurements were made on the milk sample taken in a glass sample cup (10 cm height and 6 cm diameter) supplied with the instrument by filling it to a fixed level (up to 3 cm) for each sample.

Statistical analysis

The results of physico-chemical changes during preparation and storage of UHT milk were compared using two-way ANOVA and that obtained between raw, pasteurized and freshly processed UHT milk through one-way ANOVA employing Bonferroni Post-Tests to compare results of different months using Prism Graph Pad (Prism version 7.01) software.

Results and Discussion

Assessment of the changes in physico-chemical parameters and production of heat induced components during pasteurization and UHT treatment is required to understand their impact on quality of milk. The changes in physico-chemical and heat induced parameters of raw milk, subsequently pasteurized and UHT processed milk are given in Table 1. During heating of milk, different chemical, physical and biochemical reactions take place. Comparing raw, pasteurized and UHT milk, there was a non-significant ($p>0.05$) change in acidity and viscosity, while the *a* value, HMF and lactulose content showed a significant ($p<0.001$) increase. HMF, colloidal Ca and colloidal Mg content was increased significantly ($p<0.05$) in pasteurized and UHT milk as compared to that of raw milk (Table 1). Thus, pasteurization and UHT treatment cause several physico-chemical and heat induced changes in milk. Similar results were also obtained by Jeurnink and De Kruif (1993), Elliot et al. (2003), Pestana et al. (2003) Gaucher et al. (2008) and Oh and Deeth (2017). Ritota et al. (2017) reported increase in pH, viscosity, HMF, lactulose, colloidal Ca, colloidal Mg and colour value and decrease in acidity during heating of milk. It is worth mentioning here that a small amount of lactulose (7.09 ± 0.071 mg/L) was also observed in raw milk. Previous researchers (Elliott et al. 2003; Lan et al. 2010) have also reported this important indicator of heat treatment in raw milk. Increase in viscosity during heating may be due to the denaturation of whey proteins (Jeurnink and De Kruif, 1993) and increase in pH may be due to lower whey protein associating with the micelles (Pestana et al. 2003). Burton (1984) reported that increase in colloidal Ca because of ionic Ca combined with the phosphates or the denatured proteins turns into the colloidal Ca form while the Ca moves to the inside of casein micelles, reduced the content of the ultrafiltrable Ca accordingly. There was also increase in colloidal Mg due to their migration into the calcium phosphate microgranules as evidenced by the increase in the size of these particles (Oh and Deeth, 2017). Maillard reaction formed HMF, Lactulose during heating of UHT milk (Sakkas et al. 2014) and also formed brown-coloured pigments (pyralysins and melanoidin) which caused change in colour value (Popov-Raljic et al. 2008).

Table 1 Assessment of the physio-chemical parameters in raw, pasteurized and UHT milk

Milk Type	pH	Acidity (%LA)	Viscosity (cp)	ColloidalCa (mg/100mL)	ColloidalMg (mg/100mL)	HMF (µM)	Lactulose (mg/L)	Colour		
								L	*a*	*b*
Raw	6.61±0.026^{a}	0.135±0.016^{a}	1.61±0.022^{a}	74.1±0.424^{a}	3.56±0.122^{a}	1.30±0.078^{a}	7.09±0.071^{a}	85.72±0.092^{a}	-1.57±0.007^{a}	9.26±0.056^{a}
Pasteurized	6.65±0.028^{a}	0.124±0.018^{a}	1.63±0.044^{a}	75.2±0.197^{b}	4.02±0.117^{b}	2.92±0.064^{b}	21.4±1.414^{b}	85.40±0.056^{ab}	-2.24±0.042^{b}	9.28±0.046^{a}
UHT	6.67±0.019^{a}	0.124±0.022^{a}	1.64±0.029^{a}	77.2±0.282^{c}	4.24±0.186^{c}	8.03±0.035^{c}	251±3.531^{c}	87.62±0.091^{c}	-1.80±0.042^{c}	9.33±0.042^{a}

Data are presented as mean ± SEM (n = 3).

$^{a-c}$Means within column with different lower case superscript are significantly different ($p<0.05$) from each other.

pH

The pH of UHT milk samples stored at 5 and 30°C was determined by pH meter and the results are presented in Fig. 1(a). The pH value of freshly prepared UHT milk sample was observed to be 6.67±0.019 which decreased significantly (*p*<0.001) to 6.54±0.019 and 6.45±0.032 after storage for 4 months at 5 and 30°C, respectively. It was observed that samples showed non-significant (p>0.05) decrease in pH value at 5°C, while samples stored at 30°C showed a significant decrease (p<0.01) after one month of storage period. Decrease in pH during storage of UHT milk may be due to the dephosphorylation of casein micelles, breakdown of lactose, precipitation of calcium phosphate, and proteolysis (Al-Saadi and Deeth, 2008). Our results are agreeing with the corresponding results obtained by McMahon (1996) and Hassan et al. (2009). Aldubhany et al. (2014) also reported that pH of UHT milk decreased during storage. Decrease in pH value might be a major factor for causing gelation during storage of UHT milk. As previously reported by Hassan et al. (2009) at neutral pH (6.7), the casein micelles are stable, while the lowering of the pH leads to aggregation of casein micelles and formation of a gel.

Acidity

The acidity in UHT milk samples was determined by titration method and it was expressed in terms of percent lactic acid. The results of acidity in UHT milk sample stored at 5 and 30°C are presented in Fig. 1 (b). The acidity value of freshly prepared UHT milk sample was observed to be 0.124±0.025 % lactic acid which increased significantly (p<0.001) to 0.144±0.017 and 0.164±0.0006 % after storage for 4 months at 5 and 30°C, respectively. Moreover, the statistical analysis indicated that the storage period had a significant effect on titratable acidity. The acidity percentage increased significantly (p<0.001) during storage at both 5 and 30°C temperature. The sample stored at 30°C showed higher rate of increase as compared to that stored at 5°C. The Maillard reaction takes place during processing and storage, which degrade the lactose into acids. Formic acid is responsible for increasing titratable acidity of milk during storage. Our results are in agreement with earlier study carried out by Aldubhany et al. (2014), who also reported that a progressive relation exists between the acidity of UHT milk samples and storage time and the rate of increase in acidity was slightly higher in UHT samples stored at room temperature as compared to sample stored under refrigeration.

Viscosity

Viscosity is an important property of any liquid food because of its direct impact on its appearance and consumer acceptance (DePeters and Cant, 1992). Casein micelles play an important role in determining the viscosity of skim milk (Clare et al. 2005); however factors like addition of NaCl, pressure, reaction with sugars, ionic strength, pH, and heat also affects the aggregation of the micelles which influences resistance to flow (Broyard and Gaucheron, 2015). Changes in the viscosity during storage of UHT milk are depicted in Fig. 2(a). Viscosity of freshly prepared UHT milk sample was found to be 1.64±0.029 cP which increased significantly (*p*<0.001) to 1.87±0.005 and 2.01±0.009 cP after storage for 4 months at 5 and 30°C, respectively. It was observed that samples showed non-significant (p>0.05) increase in the viscosity at 5°C, while that stored at 30°C showed a significant increase (p<0.001) after one month of storage period. It was also noted that UHT milk samples stored at 30°C showed higher viscosity than that of the sample stored at 5°C. Similar trends to our results of increase in viscosity was reported by Aldubhany et al. (2014), who also observed that higher increase in viscosity in UHT milk sample stored at 37°C than the sample stored at 4°C. Increase in viscosity is directly related to the proteolysis and proteolysis results in gelation during storage of UHT milk which leads to increase in viscosity value (Datta and Deeth, 2001; Rauh et al. 2014)

Sedimentation

The quality of sediment depends on the raw milk and on the type and severity of the heat treatments. For any one type of process, the amount of sediment increases with the severity of the heat treatment (Sweetsur et al. 1975; Vankatachalm and MacMahon, 1991). The amount of sediment decreases with homogenization pressure (Robinson, 1994). Sedimentation results of UHT milk samples during storage at 5 and 30°C are illustrated in Fig 2 (b). Sedimentation content in freshly processed UHT milk was observed to be 0.102±0.006 g which was significantly (p<0.001) increased to 1.937±0.018 and 3.417±0.026 g after storage for a period of 4 months at 5 and 30°C, respectively (Fig. 2b). The higher increase in sedimentation value was observed after 2 months of storage at 30°C. It was noted that sample stored at 30°C showed higher rate of increase of sedimentation than the sample stored at 5°C. The increase in sedimentation value during storage of sample may be due to the aggregation of proteins or protein particles of various sizes (Malmgren et al. 2017; Gaur et al. 2018). Similar to our findings, Ramsey and Swartzel (1984), Malmgren et al. (2017) and Gaur et al. (2018) also reported that sediment formation during storage of UHT milk was increased with storage period.

Colloidal Ca and colloidal Mg content

The equilibrium of Ca and Mg between soluble and colloidal phase in milk is important for their bioavailability and stability (Aldubhany et al. 2014). Colloidal Ca and colloidal Mg content in milk are around 66.5 and 33%, respectively (Tewari and Juneja 2007; Huppertz et al. 2017; Singh et al. 2019). The changes in colloidal fraction of Ca and Mg content in UHT milk samples stored at 5 and 30°C are presented in Fig.3 (a) and (b). Colloidal Ca was decreased significantly (*p*<0.001) during storage from

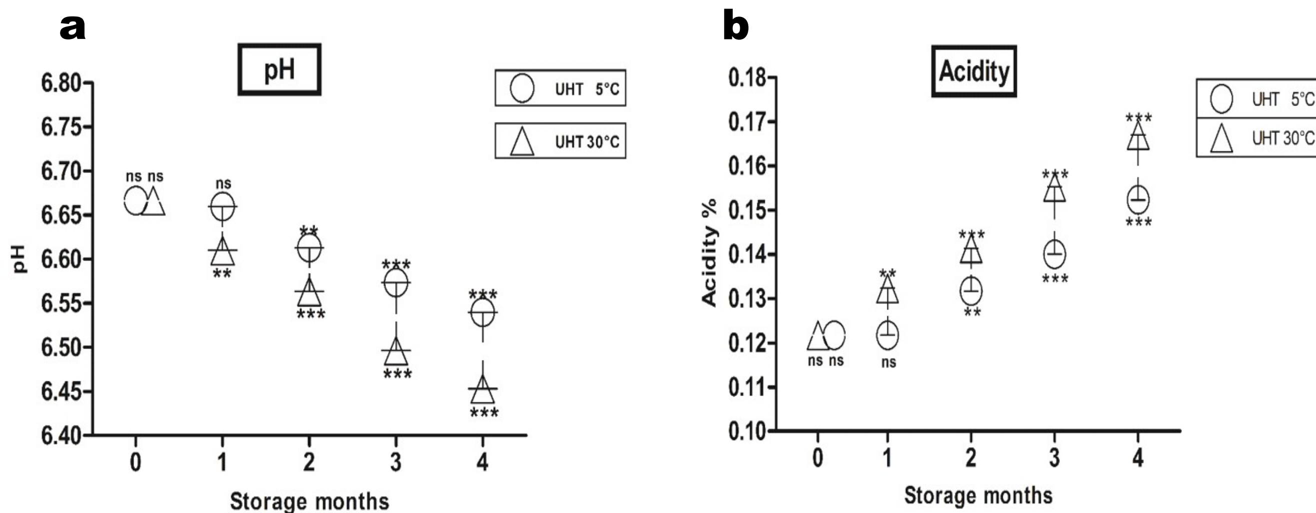


Fig. 1 (a). Changes in pH (b) Changes in acidity content during storage of UHT milk sample at 5°(O) and 30°C (△) after 0th, 1st, 2nd, 3rd, and 4th months.

Centre line shows the standard error; the circle (O) shows the milk sample stored at 5°C and the triangle (△) shows the sample stored at 30°C. All the readings were taken in triplicates. $^{ns}p > 0.05$, *p<0.05, **p<0.01 ***p<0.001.

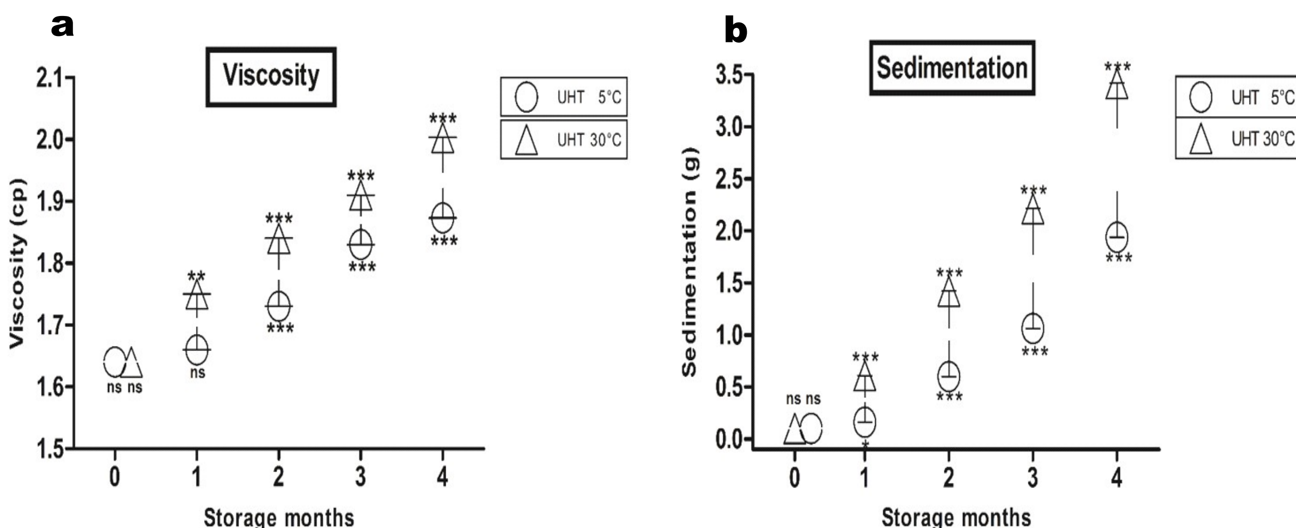


Fig 2 (a) Changes in viscosity (b) changes in sedimentation content of UHT milk sample at 5°(O) and 30°C (△) after 0th, 1st, 2nd, 3rd, and 4th months.

Centre line shows the standard error; the circle (O) shows the milk sample stored at 5°C and the triangle (△) shows the sample stored at 30°C. All the readings were taken in triplicates. $^{ns}p > 0.05$, *p<0.05, **p<0.01 ***p<0.001.

77.2±0.282 to 73.4±0.351 and 71.3±0.312 mg/100 mL at 5°C and 30°C, respectively after 4 month of storage (Fig 3a). Colloidal Mg also significantly (p<0.001) decreased during storage from 4.22±0.186 to 3.41±0.326 and 2.93±0.214 mg/100mL, at 5 and 30°C, respectively after 4 month of storage (Fig. 3b). Sample stored at 30°C showed greater conversion of colloidal Ca and Mg phase to soluble Ca and Mg phase than that stored at 4°C. The changes in colloidal Ca contents may be due to decrease in pH values during storage, especially when the samples were stored at 30°C. In concomitant to our results, Aldubhany et al. (2014) also reported that transfer of Ca and Mg from colloidal to soluble form occurs

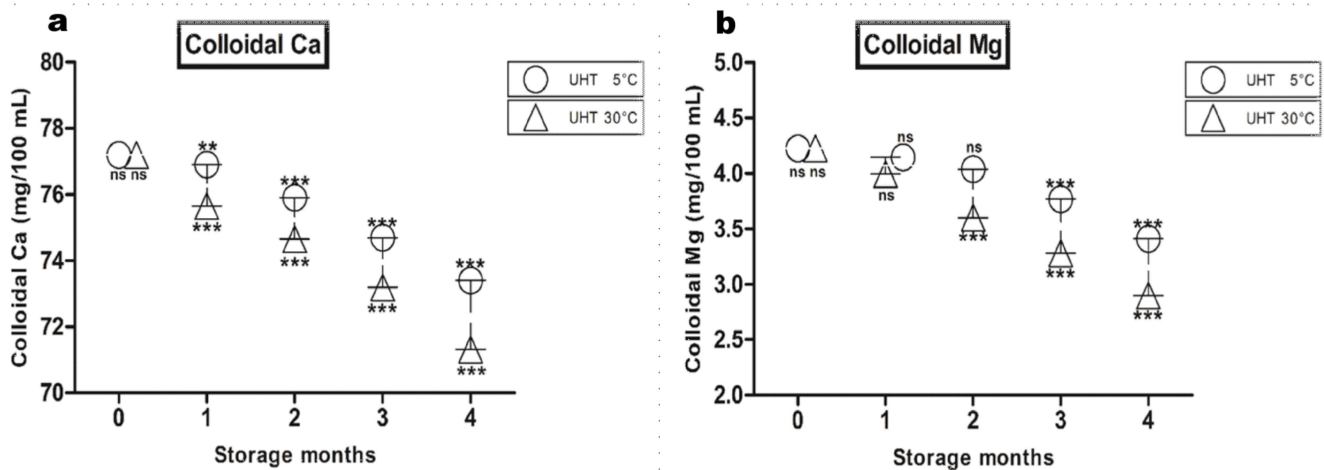


Fig. 3 (a) Changes in colloidal calcium (b) changes in colloidal magnesium content during storage of UHT milk sample at 5°(O) and 30°C (△) after 0th, 1st, 2nd, 3rd, and 4th months.

Centre line shows the standard error; the circle (O) shows the milk sample stored at 5°C and the triangle (△) shows the sample stored at 30°C. All the readings were taken in triplicates. $^{ns}p > 0.05$, $*p<0.05$, $**p<0.01$ $***p<0.001$.

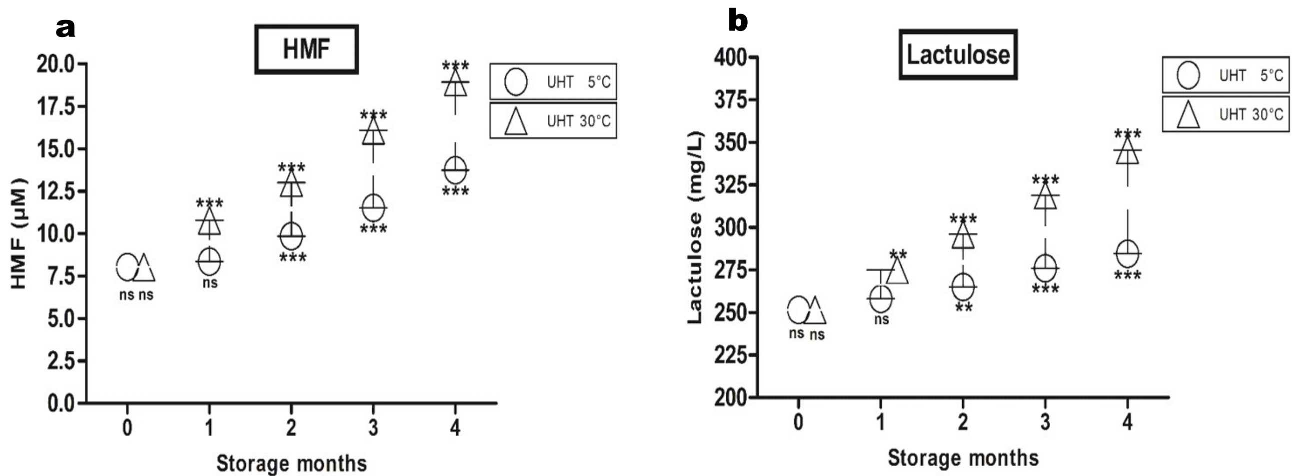


Fig. 4 (a) Changes in HMF content (b) changes in lactulose content during storage of UHT milk sample at 5°(O) and 30°C (△) after 0th, 1st, 2nd, 3rd, and 4th months.

Centre line shows the standard error; the circle (O) shows the milk sample stored at 5°C and the triangle (△) shows the sample stored at 30°C. All the readings were taken in triplicates. $^{ns}p > 0.05$, $*p<0.05$, $**p<0.01$ $***p<0.001$.

with storage and that the changes were more pronounced in UHT milk sample stored at 37°C than that stored at 4 and 22°C.

HMF

HMF is an important component used for assessing the intensity of heat treatment. It is also used as an indicator for degree of progress of the Maillard reaction. It is not present in raw milk or present only at trace level and formed during heating and storage of high heat treated milk and milk products (Albalá-Hurtado et al. 1997; Morales and Jiménez-Pérez, 1999; Morales et al. 2000; Murata et al. 2007; Ritota et al. 2017). In earlier stage of Maillard reaction, there occurs condensation of carbonyl group of lactose and [-amino group of lysine residue, followed by development of intermediate product HMF (Morales et al. 2000; Cais-Sokolinska, 2005). Amadori rearrangement product (1-amino-l-deoxy-2-ketoses) formed during severe heating is rapidly converted into HMF under acid conditions (Nursten, 2005). In this study, extent of HMF content was determined by using the method suggested by Cais-Sokolińska, (2005) and results are illustrated in Fig. 4 (a). The HMF content was significantly

Table 2 Color (*L, a, b)* value of UHT milk sample stored at 5°C and 30°C for 4 months

Storage period (months)	Storage Temperature	***L***	***a***	***b***
0	—	87.62±0.091^{ns}	-1.80± 0.042^{ns}	9.33±0.042^{ns}
1	5°C	87.27±0.155*	-1.04±0.021***	9.41±0.018^{ns}
	30°C	86.52±0.127***	-0.81± 0.077***	9.80±0.049***
2	5°C	86.22± 0.12***	-0.95±0.027***	9.55±0.023**
	30°C	84.91±0.092***	-0.65± 0.056***	9.90±0.029***
3	5°C	85.81±0.144***	-0.71±0.028***	9.69±0.042***
	30°C	80.22±0.106***	0.030± 0.023***	10.15±0.022***
4	5°C	84.57±0.106***	-0.55±0.027***	9.81±0.017***
	30°C	79.84±0.098***	0.18± 0.018***	10.42±0.070***

Superscript are significantly different, ^{ns}p >0.05, *p<0.05, **p<0.01 ***p<0.001 from each other.

(p<0.001) increased during storage from 8.03±0.035 to 13.74 ±0.091 and 18.94±0.106 µmol/L at 5 and 30°C, respectively after 4 month of storage. It was observed that there was about 1.7 times increase in the HMF content of the sample stored at 5°C and around 2.3 times increase in the sample stored at 30°C. Higher content of HMF in sample stored at 30°C may be due to the higher rate of Maillard reaction at higher temperature. Our results are in agreement with the Cais-Sokolińska, (2005) who reported that HMF value almost doubled in the UHT milk stored at 20°C after 24 weeks. The author also revealed that increase in the HMF content was higher in sample stored at 20°C than that stored at 4 and 8°C. Morales et al. (2000) reported that in freshly processed UHT milk sample, it is around 5.6-8.7 µmol/L. HMF is a product which originates from the Maillard reaction, and hence it can be assumed that, while the Maillard reaction progresses during UHT milk storage, the HMF content will proportionally increases (Morales and Jimenez-Perez, 1999).

Lactulose

Lactulose is a semi-synthetic disaccharide consisting of galactose and fructose molecule. During heating of milk and milk product lactose gets isomerized with the formation of lactulose (4-O-ß-D-galactopyranosyl-D-fructofuranose) by Lobry de Bruyn–Alberda van Ekenstein (LA) transformation (Hashemi and Ashtiani, 2010). Lactulose is considered to be a suitable indicator for differentiating UHT milk and pasteurized milk (Montilla et al. 2005). Fig. 4 (b) shows that the increase in lactulose concentration is greatly dependent on the storage time and temperature. Samples stored at 30°C showed increase in lactulose content than samples stored at 5°C. Lactulose content in freshly processed UHT milk was observed to be 251±3.531 mg/L which was significantly (p<0.001) increased to 284±3.535 mg/L and 345±2.121 mg/L after storage for a period of 4 months at 5 and 30°C, respectively. Comparing lactulose content of freshly processed UHT milk with samples stored over a period of 1 month, it was observed that samples stored at 5°C showed non-significant changes (p>0.05), while sample stored at 30°C showed a significant increase (p<0.001) in lactulose content. Our results are in agreement with the previous study carried out by (Birlouez-Aragon et al. 1998; Morales et al. 2000; Elliott et al. 2003; Elliott et al. 2005; Sakkas et al. 2014) who found that the lactulose content in UHT milk is in the range of 50–850 mg/L. Lactulose content up to 15 mg/L in pasteurized milk and 80 mg/L in high temperature pasteurized milk has been reported (Marconi et al. 2004; Feinberg et al. 2006). Simlar trend of considerable increase in the lactulose content during storage was also reported (Morales et al. 2000; Elliott et al. 2005; Feinberg et al. 2006). Morales and Jimenez-Perez (1999) observed that the maximum increase in lactulose content was at high temperatures and the highest value was recorded when milk was stored at 40 and 50°C for 90 days.

Color value

Heating process, storage condition and heat induced reaction like Maillard's reaction, causes physio chemical changes in milk, which impacts the color of milk (Popov-Raljic et al. 2008). The color determination denoted in *L, a, b* value. *L* represent for the index of lightness, *b(+)* represent for yellow color while *b(-)* represents for blue color. *A* (+) represent values for red color whereas *a* (-) represents for green color (Manzi et al. 2013). Changes in the *L, a and b* values of UHT milk samples during storage at 5 and 30°C are presented in Table 2. *L, a* and *b,* values of freshly processed UHT milk was observed to be 87.62±0.091, -1.80±0.042 and 9.33±0.042. It was observed that *L* value decreased significantly (p<0.001) to 84.57±0.106 and 79.84±0.098 at 5 and 30°C, respectively after 4 months of storage. The *a* value was increased significantly (p<0.001) to -0.55±0.027 and 0.18±0.018 at 5 and 30°C, respectively, while the b value significantly (p<0.001) increased to 9.81±0.017 and 10.42±0.070 at 5 and 30°C, respectively after 4 months of storage. It was observed that the sample showed significant (<0.05) decrease in *L value* and non-significant (p>0.05) change in the *b* value at 5°C, while that stored at 30°C showed a significant (p<0.001) change in both *L* and *b* value after one month of storage period. Sample stored at 30°C showed more changes in *L, a and b* value as compared to that stored at 5°C sample. Gaucher et al. (2008) also observed visual browning in milk samples during storage at

40°C. Popov-Raljic et al. (2008) reported that the average psychometric chroma *b** value was significantly higher ($p<0.01$) during storage of UHT milk with 3.2% milk fat.

Conclusion

In agreement with other studies, pasteurized milk showed less heat damage than the UHT processed milk sample. There was increase in acidity, viscosity, sedimentation value, while decrease in colloidal Ca and Mg during storage of UHT milk. There was continuous rise of heat induced components such as lactulose, HMF and colour value during storage of UHT milk. All these changes affected the quality of UHT milk during storage. Minimal changes were observed in that milk stored at 5°C rather than that stored at 30°C. So, the study indicated that it is better to store UHT milk at refrigeration temperature to preserve its quality.

Acknowledgement

Authors thank the Department of Dairy Chemistry, ICAR-NDRI, Karnal for providing funds.

References

Al-Saadi J, Deeth HC (2008) Cross-linking of proteins and other changes in UHT milk during storage at different temperatures. Aust J Dairy Technol 63:93–99

Albalá-Hurtado S, Veciana-Nogués MT, Izquierdo-Pulido M, Vidal-Carou MC (1997) Determination of free and total furfural compounds in infant milk formulas by high-performance liquid chromatography. J Agric Food Chem 45: 2128–2133

Aldubhany TAW, Gouda E, Khattab A, Dabour N (2014) Effects of storage on some physico-chemical characteristics of UHT milk stored at different temperature. Alexandria Science Exchange 3:107-114

Birlouez-Aragon I, Nicolas M, Metais A, Marchond N, Grenier J, Calvo D (1998) A rapid fluorimetric method to estimate the heat treatment of liquid milk. Int Dairy J 8(9):771–777

Broyard C, Gaucheron F (2015) Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Sci Technol 95:831–862

Burton, H (1984). Reviews of the progress of dairy science: the bacteriological, chemical, biochemical and physical changes that occur in milk at temperatures of 100–150° C. J Dairy Res 51:341-363

Cais-sokolinska D, Pikul J, Dankow R, Wojtowski J (2005). Changes in some selected physico-chemical and sensory parameters of UHT milk during storage at different temperatures. Milchwissenschaft 60: 37-40

Claeys WL, Van Loey AM, Hendrickx ME (2002) Kinetics of alkaline phosphatase and lactoperoxidase inactivation, and of beta-lactoglobulin denaturation in milk with different fat content. J Dairy Res 69: 541–553

Clare DA, Bang WS, Cartwright G, Drake MA, Coronel P, Simunovic J (2005) Comparison of sensory, microbiological, and biochemical parameters of microwave versus indirect UHT fluid skim milk during storage. J Dairy Sci 88: 4172–4182

Corredig M, Dalgleish DG (1996) Effect of different heat treatments on the strong binding interactions between whey proteins and milk fat globules in whole milk. J Dairy Res 63: 441-449

Deeth HC, Lewis MJ (2017) Changes during storage of UHT milk. High temperature processing of milk and milk products. John Wiley and Sons. pp 261-320

Datta N, Deeth HC (2001) Age gelation of UHT milk—a review. Food and Bioproducts processing 79:197-210

De Block J, Merchiers M, Van Renterghem R, Moermans R (1996) Evaluation of two methods for the determination of lactulose in milk. Int Dairy J 6: 217-222

DePeters EJ, Cant JP (1992) Nutritional factors influencing the nitrogen composition of bovine milk: A Review. J Dairy Sci 75:2043–2070

Elliott AJ, Dhakal A, Datta N, Deeth HC (2003) Heat-induced changes in UHT milks - Part 1. Aust J Dairy Technol 58: 3–10

Elliott AJ, Amenu B, Deeth HC (2005) Heat-induced and other chemical changes in commercial UHT milks. J Dairy Res 72: 442–446

Feinberg M, Dupont D, Efstathiou T, Louâpre V, Guyonnet JP (2006) Evaluation of tracers for the authentication of thermal treatments of milks. Food Chem 98: 188–194

Gaucher I, Mollé D, Gagnaire V, Gaucheron F (2008) Effects of storage temperature on physico-chemical characteristics of semi-skimmed UHT milk. Food Hydrocoll 22: 130–143

Gaur V, Schalk J, Anema SG (2018) Sedimentation in UHT milk. Int Dairy J 78: 92–102

Hashemi SA, Ashtiani FZ (2010) The isomerization kinetics of lactose to lactulose in the presence of sodium hydroxide at constant and variable pH. Food and Bioproducts Processing 88: 181–187

Hassan A, Amjad I, Mahmood S (2009) Microbiological and physicochemical analysis of different UHT milks available in market. Afr J Food Sci 3: 100–106

Huppertz T, Fox PF, Kelly AL (2017) The caseins: Structure, stability, and functionality. Proteins in Food Processing: Second Edition. pp 49–92

IS 1479 (Part-1) (1977) (Reaffirmed 2016) Determination of titratable acidity in milk. Handbook of food analysis-dairy products. Bureau of Indian Standards. Delhi India: Simco printing press

ISO 8070 (2007) Milk and milk products determination of calcium, sodium, potassium and magnesium content -Atomic absorption spectrometric method. International organization for standardization

Jensen S, Jansson T, Eggers N, Clausen MR, Larsen LB, Jensen HB, Bertram HC (2015) Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. Eur Food Res Technol 240: 1247-1257

Jeurnink TJ, De Kruif KG (1993) Changes in milk on heating: viscosity measurements. J Dairy Res 60: 139-150

Lan XY, Wang JQ, Bu DP, Shen JS, Zheng N, Sun P (2010). Effects of heating temperatures and addition of reconstituted milk on the heat indicators in milk. J Food Sci 75: C653-8

Manzi P, Di Costanzo M, Mattera M (2013) Updating nutritional data and evaluation of technological parameters of Italian milk. Foods 2: 254–273

Malmgren B, Ardö Y, Langton M, Altskär A, Bremer MG, Dejmek P,Paulsson M (2017) Changes in proteins, physical stability and structure in directly heated UHT milk during storage at different temperatures. Int Dairy J 71:60-75

Marconi E, Messia MC, Amine A, Moscone D, Vernazza F, Stocchi F, Palleschi G (2004) Heat-treated milk differentiation by a sensitive lactulose assay. Food Chemi 84: 447–450

Mayer HK, Raba B, Meier J, Schmid A (2010) RP-HPLC analysis of furosine and acid-soluble β-lactoglobulin to assess the heat load of extended shelf life milk samples in Austria. Dairy Sci Technol 90: 413–428

McMahon DJ (1996) Age-gelation of UHT milk: changes that occur during storage, their effect on shelf life and the mechanism by which age-gelation occurs. Int Dairy Fed. pp 315–325

Meshram BD, Asgar S, Adil S, Ranvir S (2018) Chemical markers for monitoring heat damage of processed milk. Int J Chem Stud 6: 2073-2081

Montilla A, Moreno FJ, Olano A (2005) A reliable gas capillary chromatographic determination of lactulose in dairy samples. Chromatographia 62: 311–314

Morales FJ and Jiménez-Pérez S (1999) HMF formation during heat-treatment of milk-type products as related to milk fat content. J Food Sci 64: 855–859

Morales FJ, Romero C, Jiménez-Pérez S (2000) Characterization of industrial processed milk by analysis of heat-induced changes. Int J Food Sci Technol 35:193–200

Murata M, Totsuka H and Ono H (2007) Browning of furfural and amino acids, and a novel yellow compound, furpipate, formed from lysine and furfural. Biosci. Biotechnol. Biochem71: 1717–1723

Nursten H (2005) The Maillard reaction chemistry, biochemistry and implications. The Royal Society of Chemistry, Cambridge, UK

Oh HE, Deeth HC (2017) Magnesium in milk. Int Dairy J 71: 89-97

Pestana JM, Gennari A, Monteiro BW, Lehn DN, Souza CFVD, Anema SG, Elwood P (2003) UK food standards agency workshop report: The effects of the dietary n-6: n-3 fatty acid ratio on cardiovascular health. Am J Food Tech 10(6):1640-1646

Popov-Raljic JV, Lakic NS, Lalicic-Petronijevic JG, Barac MB, Sikimic VM (2008) Color changes of UHT milk during storage. Sensors 8: 5961–5974

Ramsey JA, Swartzel KR (1984) Effect of ultra high temperature processing and storage conditions on rates of sedimentation and fat separation of aseptically packaged milk. J Food Sci 49: 257-262

Ranvir S, Sharma R, Gandhi K, Upadhyay N, Mann, B (2020a) Assessment of proteolysis in ultra-high temperature milk using attenuated total reflectance–Fourier transform infrared spectroscopy. Int J Dairy Technol 73: 366-375

Ranvir S, Sharma R., Gandhi K, Mann, B (2020b) Assessment of physico-chemical changes in UHT milk during storage at different temperatures. J of Dairy Res 87: 243-247

Rauh VM, Sundgren A, Bakman M, Ipsen R, Paulsson M, Larsen LB and Hammershøj M (2014) Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion. Int Dairy J 38: 199–207

Richards M, De Kock HL, Buys EM (2014) Multivariate accelerated shelf-life test of low fat UHT milk. Int Dairy J 36: 38–45

Ritota M, Di Costanzo MG, Mattera M, Manzi P (2017) New trends for the evaluation of heat treatments of milk. J Anal Methods Chem 1864832

Robinson RK (1994) Modern Dairy Technology: Volume 1 Advances in Dairy Processing Vol 1 McGraw Hill Book Co Inc, New York, USA.

Sakkas L, Moutafi A, Moschopoulou E, Moatsou G (2014) Assessment of heat treatment of various types of milk. Food Chem 159: 293–301

Singh M, Sharma R, Ranvir S, Gandhi K, Mann B (2019). Profiling and distribution of minerals content in cow, buffalo and goat milk. Indian J Dairy Sci 72: 480-488

Sunds AV, Rauh VM, Sørensen J, Larsen LB (2018) Maillard reaction progress in UHT milk during storage at different temperature levels and cycles. Int Dairy J 77: 56–64

Sweetsur, AWM, White JCD (1975) Studies on the heat stability of milk proteins III. Effect of heat induced acidity in milk. J Dairy Sci 42: 73-88

Tewari G and Juneja VK (2007) Advances in Thermal and Non-Thermal Food Preservation. John Wiley and Sons

Vankatachalm, N. and MacMahon, D.J. (1991) Effect of lactose concentration on age-gelation of UHT sterilized skim milk concentrate. J Dairy Sci 74:101-107

Indian J Dairy Sci 74(1): 48-53
https://doi.org/10.33785/IJDS.2021.v74i01.006

RESEARCH ARTICLE

Quality assessment of market sample of *khoa* based *ramdana lai*

Pragya Bharti[1], Sanjeev Kumar[2] and Suryamani Kumar[3]

Received: 27 November 2020 / Accepted: 11 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: *Khoa based Ramdana Lai* (*KBRL*) is a popular traditional *khoa* based sweets of Bihar. It is prepared by *khoa* blending with *ramdana*, sugar and other additives. The present investigation was planned to assess the quality of *KBRL* samples from different location of Bihar. A total of 36 *KBRL* samples were collected from various locations of Bihar and were analyzed for their sensory, physico-chemical, microbial, textural and colour characteristics. The sensory scores of *KBRL* in terms of flavour, body and texture, colour and appearance and overall acceptability; the colour characteristics in terms of *L*, a*, b** values; the textural characteristics in terms of hardness, springiness, cohesiveness, chewiness and gumminess and microbial quality measured in terms of TPC, yeast and molds and coliform count were found significantly (p≤"0.05) different among samples**.** The moisture, fat, protein, total carbohydrate and ash content of samples varied from 16.83 to 20.83%, 9.30 to 12.0%, 10.63 to 16.22%, 51.62 to 61.3% and 0.7 to 0.75%, respectively.

Keywords: Khoa, Physico-chemical, Ramdana, Ramdana Lai, Sensory, Textural characteristics

Department of Dairy Technology, S G Institute of Dairy Technology (BASU, Patna) Patna-14

Sanjeev Kumar (✉)
S G Institute of Dairy Technology (BASU, Patna), Patna-14
Email: suryamanikr@gmail.com)

Introduction

Milk based ethnic sweets have lot of market potential as their demand is increasing not only Indian market, but also in overseas market. Apart from *khoa* based confectionary like *burfi, peda, kalakand, gulabjamun* are quite popular in India and abroad. Among them one of the *khoa* based confectionary *Khoa based Ramdana Lai* (*KBRL*) is one of the most popular product of Bihar. It is mostly prepared by halwais generally at Barh, Dhanarua, Biharsharif, and Patna location of Bihar. It is articulasly prepared by blending of *khoa* with *ramdana* and certain proportion of sugar to make homogenous consistency with round shape and square shape.

In absence of technical know-how, the large scale production of these region specific milk products in organized sector is a challenging task (Singh and Kumar, 2006), which requires scientific investigations in terms of their characterization, standardization of technological parameters and strategic interventions for ensuring the safety and enhancing the shelf-life (Chawla et al. 2014). Due to the differences in the manufacture procedure adopted by the manufactures, the organoleptic and physico-chemical quality of *KBRL* vary greatly. Keeping this in view, the present study was undertaken to evaluate the quality of market sample of *KBRL* collected from various location of Bihar.

Material and Methods

Sample collection

The market samples of *KBRL* were procured from four different places of Bihar like Barh, Dhanarua, Biharsharif, and Patna. Nine sweet shops were selected in each city thus a total of 36 samples were procured for this survey work.

Sensory evaluation

The sensory analysis was carried out by serving market samples of *KBRL* among a panel of seven judges of faculty members of university. The samples were examined for colour and appearance, body and texture, flavour, sweetness and overall acceptability

on 9-point hedonic scale. The scores given by panel of judges were then statistically analysed.

Physico-chemical analysis

The fat content of *KBRL* market samples were determined by mojonnier fat extraction apparatus as specified in BIS (1961). The protein content was determined by micro kjeldahl method (Menefee and Overman, 1940). The moisture content by gravimetric method and ash content of samples were determined as method suggested in BIS (1981). The total carbohydrate content of *KBRL* samples was calculated by difference to achieve 100% of total contents. The method prescribed by Deeth et al. (1975) was used to estimate the FFA content of market samples of *KBRL*.

Colour measurement

Colour measurements were carried out using *Color Flex* (Hunter Associates Laboratory, Inc., Reston VA, USA) colour measurement system equipped with dual beam xenon flash lamp and universal software. The results were represented by the L^*, a^*, b^* notation. It is a 3D colour presentation method in which L^* is the lightness index of colour and equals 0 for black and 100 for white, a^* value is redness and greenness index the amount of red (0 to 60) or green (0 to -60) and b^* value is the yellowness (0 to 60) or blueness (0 to -60) index.

Textural characteristics

Various textural characteristics such as hardness (N), cohesiveness, springiness (mm), gumminess (N) and chewiness (N mm) were measured of *KBRL*, using *Stable Micro Systems Texture Analyzer* (Model TA.XT2i Stable Micro systems, double cycle compression, fitted with 25 kg load cell, combined with Texture Expert Exceed Software). A cylindrical shape of market samples with 1.0 cm diameter and 1.0 cm height was used for determining the textural characteristics. A data acquisition rate 200 pps with probe P75 was used and samples were compressed twice to 80 % of its height. The probe speed of 2.5 mm/sec during test and 2.5 mm /sec for pre-test and 5.0 mm/sec post-test were used throughout the study. All measurements were carried out at 25°C.

Microbiological analysis

All the market samples of *KBRL* were analyzed for total plate count (TPC), yeast and mould count and coliform count by standard method cited in BIS (1980). The results were expressed as cfu/g of sample.

Statistical analysis

The data were recorded and analyzed as per the standard procedures (Snedecor and Cochran, 1994) using SPSS version 22.0 statistical software employing one-way and two-ways ANOVA test. The data are presented as mean ± standard error (SE) of three replicates.

Results and Discussion

Physico-chemical properties

Moisture content

It has been observed (Table 1) that the moisture content of market samples were found in the range of 16.83±0.99 to 20.83±0.38. However, the results of present findings are in accordance with Keerthi et al. (2018) and Dastur and Lakhani (1971). Similar observations were also reported by Sharma et al. (2012), who observed that the moisture content of milk cake was slightly higher in control samples than market samples. The variation in the moisture content might be mainly due to the difference in method of manufacture, extent of desiccation, amount of sugar added, difference in chemical composition of base material used. The present findings are also in accordance with Banjare et al. (2015). Further, our study reported similar finding with Chawla et al. (2014) and Patel et al. (2006), who observed significant differences in moisture content in *dooda burfi*.

Fat

The fat content of market *KBRL* samples were significant ($p \leq 0.05$) as presented in Table 1. The difference in the fat content of *KBRL* samples might be attributed to the variation in the type of milk used (buffalo/cow) and their fat content, amount of sugar added and duration of desiccation. The present study was in alignment with Banjare et al. (2015). Keerthi et al. (2018) and Chawla et al. (2014) also reported that fat content of market sample *doda burfi* was in the range of 4.70 to 41.05%. Further, Puri et al. (2015) reported that the fat content of market sample of *cham-cham* was in the range of 2.39 to 9.4%.

Protein

The protein content was also found significant ($p \leq 0.05$) also even among the samples (Table 1). Our finding correlates with Keerthi et al. (2018) and Chawla et al. (2014) who reported the protein content variation of market samples of *doda burfi*. Further, Ghodeker et al. (1974) also reported that the protein content of market samples *burfi* and *peda* varied between 12.1 to 20.3 and 6.3 to 11.8%, respectively

Ash content

The ash content of market samples of *KBRL* were also found significant ($p \leq 0.05$) among samples (Table 1). Our findings correlate with the reports of Rajorhia and Srinivasan (1979), who studied that the ash content in *khoa* samples also showed fairly wide variations due to the type of milk used, extent of desiccation and addition of adulterants.

Total carbohydrate

The total carbohydrates of market samples of *KBRL* were found significant (p≤0.05) difference among the samples (Table 1). Sharma et al. (2012) was observed similar observation for milk cake. Further, Ghodeker et al. (1974) reported that variation in sugar content of market samples of *burfi* and *peda*.

Free fatty acids (FFA)

The FFA values of *KBRL* samples were in ranged from 0.11±0.03 to 0.21±0.05% oleic acid (Table 1). There was significant (p≤0.05) difference found among samples even from same market. Further, Ray et al. (2002) found that *peda* samples from Kolkata market having FFA in the range of 0.056 to 0.65% oleic acid. Similarly, Hemavathy and Prabhakar (1973) also reported that *burfi* comprised of FFA of 3.41 g oleic acid/kg fat, whereas Suresh and Jha (1994) reported FFA ranging from 0.2 to 0.6% oleic acid in *kalakand* samples.

Microbial analysis

It is evident (Table 2) that there was significant (p≤0.05) difference between market samples even in same market. The difference in the TPC of *KBRL* samples might be mainly due to unhygienic conditions prevailing during manufacture and quality of raw material used during preparation. Spoilage of dairy products by molds is a frequent occurrence in India mainly due to the prevailing tropical climate and high humidity. Since the mold spores are transmitted through air, they are ubiquitous in nature. Similar observation also reported by Karthikeyan and Pandiyan (2013). The difference in the coliform count of *KBRL* samples could be due to uncleaned hands of workers, poor quality of milk, unhygienic conditions of manufacturing unit, inferior quality of material used, water supplied for washing the utensils and post processing contamination. The results obtained in present study are in agreement with the results reported by Keerthi et al. (2018), Kumar and Sinha (1989) and Grewal and Tiwari (1990) for *peda*, Indigenous milk product and *rasmalai*, respectively.

Sensory characteristics

Flavour

It is evident (Table 3) that the flavour scores of various market samples of *KBRL* were found in the range from 7.05±0.39 to 8.33±0.25. All the values obtained from the samples collected from different region showed significant (p≤0.05) difference among them. The wide range in values of flavour score of samples collected from each region clearly revealed shop to shop variation. The samples of Barh and Dhanarua were found significantly higher (p≤0.05) than the samples from Biharsharif and Patna. The highest flavor score were obtained for Barh sample which might be due to the higher fat content and special skill of preparation of this product. These finding are supported by Reddy and Rajorhia (1990), who reported that an increase in flavour score of plain *peda* with the increase in fat content.

Colour and Appearance

All the values of the samples collected from different cities showed significant (p≤0.05) difference among them (Table 3). The difference in the colour and appearance score might be due to wide variation in raw material, amount of sugar added (during

Table 1 Proximate composition* of market samples of *KBRL*

Component (%)	Cities			
	Barh	Biharsharif	Dhanarua	Patna
Moisture	20.41±1.11[a]	17.93±0.01[cd]	18.50±1.41[b]	17.97±1.50[c]
Fat	11.83±0.38[a]	9.30±0.57[d]	11.37±0.37[b]	10.35±0.92[c]
Protein	15.97±0.37[a]	10.8±0.54[d]	12.79±0.87[b]	11.23±0.50[c]
Ash	0.72±0.04[a]	0.70±0.01[d]	0.72±0.03[a]	0.71±0.03[b]
Total Carbohydrate	51.05±1.37[d]	61.26±0.01[a]	56.55±2.27[c]	59.73±1.43[b]
FFA	0.11±0.01[d]	0.21±0.05[a]	0.12±0.01[c]	0.16±0.03[b]

Values in a row with different superscripts are significantly different markets (cities) at p≤0.05, *mean values±SE, n=3

Table 2 Microbial count* of market *KBRL* samples

Cities	Microbial count (cfu/g)		
	Total plate counts	Yeast and mould count	Coliform count
Barh	3.860±2.51[d]	3.777±1.23[d]	1.930±0.72[d]
Biharsharif	4.187±3.67[a]	4.070±2.07[b]	2.020±0.51[bc]
Dhanarua	4.163±3.31[ab]	4.127±1.61[a]	2.053±0.47[b]
Patna	4.107±3.21[c]	4.030±1.18[bc]	2.110±0.32[a]

Values in a column with different superscripts are significantly different markets (cities) at p≤0.05, *mean values±SE, n=3

heat treatment reaction between sugar and amino acid produce maillard browning) and method (direct or indirect). Sharma et al. (2001), recorded that increase in fat percentage in *khoa* resulted in improvement in its colour.

Body and Texture

The results obtained from different cities showed significant (p≤0.05) difference (Table 3). It is also observed that there was a wide range in values of body and texture score of samples collected from each region. It might be due to wide variation in chemical composition particularly moisture, fat and sugar levels. Our findings is in agreed with the result of Londhe and Pal (2007) who reported that significant effect on the body and texture score of brown *peda* with increase in the level of fat in milk and sugar, but to a certain extent and Banjare et al. (2015), who also concluded that there was significant difference among all the samples of *peda*

Overall acceptability

The highest mean score as 8.33±0.25 while the lowest mean score as 7.11±0.22 were obtained for the samples from Barh and Biharsharif, respectively which were significantly (p≤0.05) different from Patna and Dhanarua (Table 3). Among samples from different shops from same cities, all the values obtained from different shops showed significant (p≤0.05) variation. Difference in the overall acceptability might be due to wide variation in colour and appearance, body and texture, flavour, and also variation in chemical composition of market these samples. Our results are in accordance with Chawla et al. (2014) who characterized *Doda burfi*.

Rheological characteristics

Hardness

It is evident (Table 4) that wide ranges of variation in hardness were observed in all samples of *KBRL*. The samples of Barh had least hardness with mean value of 4.64±0.56 N with range between 3.44 to 5.35 N. It could be observed that hardness of *ramdana Lai* significantly (p≤0.05) differed between cities and shops. Similar findings were reported by Londhe et al. (2012) in *peda* samples. The increase in hardness of all brown *peda* samples during storage could be attributed to the difference in moisture content. Our observation is also in conformity with the findings of Banjare et al. (2015).

Springiness

The springiness was found significantly (p≤0.05) differed between samples of *KBRL* from different market areas and shops (Table 4). The samples of Barh, Dhanarua and Biharsharif had significantly (p≤0.05) lower springiness than samples of Patna. Similar findings were reported by Banjare et al. (2015) in case of *peda*. Further, our findings was in close agreement with Londhe et al.(2012) who observed that the springiness value of brown *peda* sample showed rapid increase from initial value of 0.16 to 0.23 mm.

Cohesiveness

KBRL samples from region Patna and Biharsharif had significantly (p≤0.05) lower cohesiveness than samples from Barh and Dhanarua region. After first bite of TPA, *KBRL* lost its original shape and texture and became little bit floury, which shows

Table 3 Sensory score* of market samples of *KBRL*

Cities	Sensory Characteristics			
	Flavour	Body & Texture	Colour & Appearance	Overall Acceptability
Barh	8.33±0.25[a]	7.94±0.03[a]	8.27±0.350[a]	8.33±0.25[a]
Biharsharif	7.05±0.39[cd]	7.05±0.16[d]	7.11±0.48[cd]	7.11±0.22[d]
Dhanarua	8.05±0.30[b]	7.77±0.26[b]	7.94±0.16[b]	8.00±0.25[b]
Patna	7.33±0.35[c]	7.27±0.26[c]	7.27±0.36[c]	7.33±0.25[c]

Values in a column with different superscripts are significantly different markets (cities) at p≤0.05, *mean values±SE, n=3

Table 4 Textural attributes* of market samples of *KBRL*

Textural property	Cities			
	Barh	Biharsharif	Dhanarua	Patna
Hardness (N)	4.64 ± 0.56[d]	14.63 ±3.49[a]	9.17±1.25[c]	10.40 ±1.84[b]
Cohesiveness (N)	0.132±0.02[b]	0.077± 0.08[c]	0.140±0.02[a]	0.071±0.04[d]
Springiness (mm)	0.203±0.02[b]	0.197±0.03[d]	0.200±0.01[c]	0.225±0.05[a]
Gumminess (N)	0.610±0.09[d]	1.127±0.31[a]	1.274±0.18[ab]	0.720±0.15[c]
Chewiness (N mm)	0.123±0.02[cd]	0.215±0.04[b]	0.256±0.04[a]	0.161±0.02[c]

Values in a row with different superscripts are significantly different markets (cities) at p≤0.05, *mean values±SE, n=3

Table 5 Colour attributes* of market samples of *KBRL*

Cities	Colour attributes		
	L^*	a^*	b^*
Barh	53.65±1.19[c]	8.45±0.37[b]	26.29±0.06[c]
Biharsharif	54.50±0.14[b]	7.65±0.92[c]	27.75±1.58[a]
Dhanarua	56.34±0.44[a]	5.78±0.15[d]	22.93±0.26[d]
Patna	52.72±0.21[d]	9.03±0.08[a]	27.06±0.86[b]

Values in a column with different superscripts are significantly different markets (cities) at p≤0.05, *mean values±SE, n=3

brittleness of product and lower cohesiveness were found in the samples from Patna and Biharsharif samples. Similar findings were recorded by Londhe et al. (2012) in *peda* sample and Palit and Pal (2005) in *burfi* sample during storage. Further similar findings were observed by Banjare et al. (2015) in case of *peda*.

Chewiness

The chewiness of *KBRL* differed significantly among market areas and shops (p≤0.05). Similar findings were observed by Banjare et al. (2015) in case of *peda*.

Gumminess

KBRL samples of Barh and Patna had significantly (p≤0.05) lower gumminess values than the samples of Dhanarua and Biharsharif. Similar findings were reported by Banjare et al. (2015) in case of *peda*. Further, similar findings were also observed by Londhe et al. (2012) who reported that that the initial gumminess of laboratory made brown *peda* was 1343.8g but during storage in 20 days at 30ºC *peda* packed in cardboard box lined with butter paper was increased to 5335.71g

Colour attributes

L* value

The quantitative measurement of L^* value can be considered to indicate the severity of heat treatment (Rufian–henares et al. 2005). Statistical analysis revealed that there was significant (p≤0.05) differences in L^* value among all four groups studied. The lower L^* value of *ramdana Lai* could be due to intense heat treatment given, heat interactions between sugar (both milk sugar and added cane sugar) and milk constituents, leading to well-known Maillard reaction. Similar finding was reported by Singh et al. (2018) for *pinni* where L^* value of market samples was varied from 30.10 to 59.63.

a* value

It is evident (Table 5) that highest a^* (9.03±0.08) value was observed with the Patna's samples and lowest (5.78±0.15) value was for samples of Dhanarua. Statistical analysis revealed that there was significant (p≤0.05) differences in a^* value of *KBRL* among all four groups studied. It was well established that the colour of milk and milk products undergoes maillard browning which is characterized by two primary hues viz., brown and yellow. Kumar et al. (2006) estimated the colour value of *gulabjamun* and found that brownness of *gulabjamun* crust was comprised of brownness and yellowness. Similar finding was reported by Singh et al. (2018) for pinni in which reported that a^* value varied from 6.03 and 18.56.

b* value

It is observed (Table 5) that the market samples from Biharsharif (27.75±1.58) was yellower than Barh, Patna, Dhanarua as their b^* value were 26.29±0.06, 27.06±0.86, and 22.93±0.26, respectively. Statistical analysis revealed that there was significant difference (pd"0.05) in b^* value of *ramdana lai* among four group. Our finding was contradictory with the report by Kumar et al. (2006) who reported b^* value as 57.34 for G*ulabjamun* samples. This variation is mainly due to quite difference in the method of manufacture of *KBRL* and *Gulabjamun*. However, Singh et al. (2018) reported variation of b^* value from 19.75 to 39.92 in market samples of *pinni*

Conclusions

KBRL is an indigenous milk product of Bihar origin with considerable nutritional importance. The market samples procured from different shops from various cities of Bihar showed wide variation in their proximate composition, sensory attributes, textural profile, colour attributes and microbial qualities among the various cities and even within the different samples collected from same shop. The reason for variability could be attributed to the use of variable amount of *khoa*, type of *khoa* and addition of *khoa* and *ramdana* at intermittent stages of preparation. Further, the microbial quality of market samples also indicated a wide variation among the samples of various places of Bihar even also in same sweet maker shop. This might be because of poor attention followed for maintaining hygiene and sanitary condition of manufacturing, post contamination through packaging materials or other handling practices.

References

Banjare K, Kumar M, Goel BK (2015) Effect of storage on chemical, textural and sensory attributes of traditional indigenous sweet peda. Asian J Dairy Food Res 34: 104-108

BIS (1961) IS:1479, Methods of test for dairy industry, part-II: chemical analysis of milk. Indian Standards Institution, New Delhi

BIS (1980) SP: 18 Hand book of food analysis Part I: Genera methods. Bureau of Indian Standards, New Delhi

BIS (1981) IS: (SP: 18) "ISI Handbook of food analysis, Part XI: Dairy products," Indian Standards Institution, New Delhi

Chawla R, Patil GR, Singh AK (2014) Sensory characterization of doda burfi (Indian milk cake) using principal component analysis. J Food Sci Technol 51: 558-564

Dastur NN, Lakhani AG (1971) Chemical composition of khoa. Indian J Dairy Sci 24: 223.

Deeth HC, Fitz-Gerald CH (1975) A convenient method for determining the extent of lipolysis in milk. Australian J Dairy Technol 30: 109-111

Ghodeker DR, Dudani AT, Ranganathan B (1974) Microbiological quality of Indian milk products. J Milk Food Technol 37: 119-122

Grewal JS, Tiwari RP (1990) Microbiological quality of rasamalai. J Food Sci Technol 27: 178-179.

Hemavathy J, Prabhakar JV (1973) Changes in the carbonyl composition of a milk based sweetmeat-burfi during preparation and storage. J Food Sci Technol 10: 156-160.

Karthikeyan N, Pandiyan C (2013) Microbial quality of khoa and khoa based milk sweets from different sources. Intl Food Res J 20: 1443-1447

Keerthi S, Manthani V, Swarnalatha G (2018) Assessment of quality of Peda samples sold in Kamareddy district - A case study. Intl J Current Microbiol Applied Sci 7: 1427-1434

Kumar AJ, Singh RRB, Patel AA, Patil GR (2006) Kinetics of colour and texture changes in gulabjamun balls during deep-fat frying. LWT-Food Sci Technol 39: 827-833

Kumar V, Sinha RN (1989) Incidence of coliforms in indigenous milk products. Indian J Dairy Sci 42: 579-580

Londhe G, Pal D (2007) Developments in shelf life extension of khoa and khoa based sweets-an overview. Indian J Dairy Biosci 18: 1-9

Londhe G, Pal D, Raju PN (2012) Effect of packaging techniques on shelf life of brown peda, a milk-based confection. LWT-Food Sci Technol 47: 117-125

Menefee SG, Overman OR (1940) A semimicro-kjeldahl method for the determination of total nitrogen in milk. J Dairy Sci 23: 1177-1185.

Palit C, Pal D (2005) Studies on mechanized production and shelf life extension of burfi. Indian J Dairy Sci 58:12–16

Patel HA, Salunke P, Thakar PN (2006) Chemical, microbiological, Theological and sensory characteristics of peda made by traditional and mechanized methods. J Food Sci Technol 43: 196-199

Puri R, Khamrui K, Khetra Y, Malhotra R, Devraja HC (2016) Quantitative descriptive analysis and principal component analysis for sensory characterization of Indian milk product cham-cham. J Food Sci Technol 53: 1238- 1246

Rajorhia GS, Srinivasan MR (1974) Physico-chemical changes during manufacturing of khoa, XIX Inter, Dairy Congress, IE, 216

Ray PR, Bandyopadhyay AK, Ghatak PK (2002) Comparative studies on quality of market available and laboratory made peda. Indian J Dairy Sci 55: 83-85

Reddy CR, Rajorhia GS (1990) Equilibrium relative humidity of khoa based sweets (Peda & Burfi). Asian J Dairy Res 9: 200-204

Rufian-Henares JA, Guerra-Hernandez E, Garcia-Villanova B (2006) Colour measurement as indicator for controlling the manufacture and storage of enteral formulas. Food Contl 17: 489-493

Sharma GD, Yadav MC, Singh KP, Singh BP (2012) Physico chemical quality of milk cake. Indian Res J Ext Edu 1: 338-339

Sharma HK, Singhal RS, Kulkarni PR (2001) Effect of packaging under vacuum or under nitrogen on the keeping quality of danedar khoa. Intl J Dairy Technol 54: 107-110

Singh AK, Kumar A (2006) Technology of selected region specific milk products. In: 21st short course on Developments in traditional dairy products, Dec. 10-30, NDRI, Karnal: 225-234

Singh RK, Khamrui K, Jaglan V (2018) Physicochemical and colour attributes of market samples of north Indian sweet pinni. J Pharmacognosy Phytochem 7: 3370-3374

Snedecor GW, Cochran WG (1994) Statistical methods. 6th ed. Iowa state university press, Ames

Suresh I, Jha YK (1994) Sensory, biochemical and microbiological qualities of kalakand. J Food Sci Technol 31: 330-332

Indian J Dairy Sci 74(1): 54-60
https://doi.org/10.33785/IJDS.2021.v74i01.007

RESEARCH ARTICLE

Optimization and characterization of functional chocolate with addition of butter fruit milkshake powder as a source of phenolic, flavonoid and carotenoid

Aparna Kumari[1], Shreya Pandey[2], Anil Kumar Chauhan[1] and Meenakshi Singh[3]

Received: 21 October 2020 / Accepted: 20 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Butter fruit milkshake powder (BFMS) is a healthful mixture of avocado pulp and dairy components with high nutritional and antioxidant activity. In this study, an attempt has been made to enhance the nutraceutical and functional properties of the chocolate with BFMS powder at the 15-60% level based on the physical, nutritional, sensory attributes, and storage of study. Sensory studies of chocolate indicated that maximum sensory score was found at 30% incorporation of BFMS without adverse effects. BFMS powder's addition increased the contents of fat, dietary fibers, proteins, phenolic, flavonoid, and beta carotenoid content of chocolate. Mineral element (Ca, Mg, Fe, Zn, and Mn) were also increased with the addition of BFMS in chocolate. Storage studies of BFMS powder fortification in chocolate revealed that butter fruit milkshake chocolate (BFMSC) significantly more stable than control chocolate and accepted by consumers up to 60 days of storage. This study showed that BFMS powder fortified chocolate was nutritionally improved and economical with acceptable sensory properties. This may increase the market's revenue and enable chocolate companies to develop a functional new chocolate type.

Keywords: Avocado, Bioactive compound, Butter fruit milkshake powder, Chocolate; Shelf Life

[1]Department of Dairy Science and Food Technology, Institute of Agricultural Science , Banaras Hindu University, Varanasi, India

[2]Centre of Food Technology, Institute of Professional Studies, University of Allahabad, Allahabad, India

[3]Technology Management Directorate(Socio-economic Ministry Interface),Council of Scientific Research, New Delhi, India

Anil Kumar Chauhan (✉)
Department of Dairy Science and Food Technology, Institute of Agricultural Science, Banaras Hindu University, Varanasi-221005, India
E-mail: anilchauhancfst@gmail.com
Phone: 09450658188

Introduction

Chocolate is one of the most popular and consumed delicacies in worldwide. It is a complex multiphase system of particulate (cocoa, milk components, sugar) and continuous phases (milk fat, cocoa butter, and emulsifiers) and color, gloss, shape, surface texture, roughness, shininess, and translucency is its visual and appearance attributes (Briones et al. 2005). Chocolate represent a functional food product due to its high level of flavonoid content, which positively impacts human health (Wollgast and Anklam 2000). Latif (2013) also reviewed the impact of chocolate on the human health and illustrated that chocolate/cocoa is a rich source of antioxidants with radical scavenging activity due to large concentrations of flavonoids, epicatechin, catechin and, procyanidins. Cocoa mass also contains macro and micro minerals such as potassium (K), phosphorus (P), copper (Cu), iron (Fe), zinc (Zn), and magnesium (Mg).

In the last years, a marked change in food consumption patterns, consumers are getting more demanding in the food market for nutritious, palatable, affordable, and easily consumed food products (Mussatto and Mancilha 2007). Consumers would also like to have more alternative to choose food products than ever before. In view of the above, food technologist and researcher launch organic, prebiotic, probiotic, and high-cocoa polyphenol-rich chocolate that can be effectively found in the market (Erdem et al. 2014). Presently in COVID-19 pandemic situation, functional foods acquire a prominent place in the food market due to their capability to provide positive health effects beyond their conventional nutritional benefits. So, new food product development (NPD) has been increased using functional food ingredients. Also, government and companies invest in the food marketing field to develop new products.

Avocado (*Persea americana*) is a subtropical fruit, highly appreciated by consumers due to its nutritional values. Avocado (Butter fruit) a rich source of monounsaturated fatty acids that increase the level of HDL that is good for the heart; polyhydroxylated fatty alcohols (PFA) that suppress inflammatory response and protect against UV-induced damage in skin cells; lipophilic acetogenins exhibited the highest antioxidant capacity (Rosenblat et al. 2011; Souza et al. 2011;

Bhuyan et al. 2019). Additionally avocado is rich in vitamins such as tocopherol, ascorbic acid, pyridoxine, beta-carotene, and minerals like K and Mg (Fulgoni et al. 2013). According to Ortiz-Avila et al. (2015), avocado consumption gives protection from hypersensitivity, coronary heart disease, oxidative damage, anti-mycobacterial activity, and improves immune systems.

However, an avocado grows only in a particular region, and its shelf life is also very short, so there is a need for researchers and food technologists to discover a method to preserve the avocado with its nutritional properties. So, developed butter fruit milkshake powder (BFMS) revealed that these products provide a healthful mixture of dairy and fruit components with high nutritional and longer shelf-life. With these contexts, the objective of present study was to optimize and characterize the functional chocolate using butter fruit milkshake powder as a source of phenolic, flavonoid and carotenoid as well as cell viability of chocolate and the effect of storage (two months) on the sensory attributes was also evaluated.

Materials and Methods

Raw materials (cocoa powder, milk powder, butter, sugar) for the preparation of chocolate were purchased from the local market. All the required chemicals used were of analytical grade.

Preparation of Butter fruit milkshake powder (BFMS)

Lyophilized butter fruit milkshake powder (BFMS) was prepared by the method described by Pandey et al. (2020) in which avocado pulp, pasteurized toned milk (Amul), sugar, and maltodextrin was mixed at 84, 28, 10, and 6 % respectively at -20ÚC under 0.035 mbar pressure.

Preparation of chocolate

According to the methodology reported by Mayank and Kumar (2012), the chocolate was prepared with minor modifications. Ingredients used were BFMS, cocoa powder, sugar, butter, whole milk powder (fat 26%). The quantity used to prepare control chocolate as follows: 100 g cocoa powder, 30 g milk powder, 62 g butter, 150 g sugar, and water 117 mL. Four blends were prepared with the replacement of cocoa powder by BFMS that is 100:0(control); 85:15, 70:30; 55:45; and 40:60 as shown in Table1. Sugar syrup (one string) was made with a mixture of sugar and water in the oven for 4-5 min at 60ÚC. Then butter was mixed in sugar syrup well till melted and afterwards cocoa powder and milk powder mixed above mixture gradually till glossy. The mixer was then poured in a greased pan and refrigerated for 10-15 min until it became solid. Prepared control chocolate (CC) and BFMS chocolate (BFMSC) were packed and stored for further analysis.

Sensory admissibility of chocolate

Optimization of BFMS in chocolate was decided by organoleptic evaluation by 15 semi-trained panellists. The sensory evaluation was planned in quiet with properly illuminated lights, humid free & ventilated room by maintaining hygienic condition. The sensory evaluation was done according to method explained by Żyżelewicz et al. (2018), where sensory attributes, i.e., colour and appearance, consistency (hardness, smoothness) and mouthfeel, flavour, and overall acceptability. The sensory analysis was done using 5-point scale, 5 meant extremely desired quality, 4 was desirable quality, 3 was tolerable quality, 2 represented dislike, and one was for a defective product.

Determination of nutritional composition and trace elements

A moisture analyzer was used to analyze moisture, which works on halogen heating technology. The protein content was estimated with Kjeldahl nitrogen analyzer (Kel Plus –DISIYL EMS), fat content with SOCS-PLUS model SCS8, Fibertec™ 1023 - semi-automatic crude fibre analyzer is used to determine crude fibre. All proximate composition of chocolate sample was analyzed according to AOAC (2000) method. The energy value was determined by bomb calorimeter (Sundy Science and Technology Co., Ltd).

Macro and microelements like calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and manganese (Mn) were detected by using AAS (Atomic Absorption Spectrophotometer) (Perkin Elmer) according to the method explained by Aparna et al. (2018).

Texture and color of chocolate

Customer adequacy is affected by the texture and color of chocolate. Color Flex EZ determined color value in term of L*, a*, and b*, where L* (lightness/ darkness), a* (redness/ greenness) and b* (blueness/ yellowness).

The texture of the chocolate was determined by using a TA.XT plus texture analyzer with a sharp cutting blade probe. The hardness and breaking strength of chocolate were recorded by force required to break, and the average value was recorded.

Analysis of Nutraceuticals for FGSF, CC, and FGSC

Estimation of polyphenolics and flavonoids contents

BFMS, CC, and BFMSC sample extracts were prepared for the estimation of polyphenolics and flavonoids compounds by the procedure described Tyagi et al. (2020) in which formic acid / water /methanol(0.3/29.7/70 v/v/v) used as extracted agent.

Folin-Ciocalteu phenol reagent is used to evaluate the total polyphenol content (TPC) of the sample by the method described by Stoilova et al. (2007). Standard curve was prepared by gallic acid (5-60µg /mL; y = 0.00137x+0.3059; r2 = 0.989; x is the concentration of solution; y is the absorbance). The results were expressed as GAEg-1 of dw.

Flavonoid content in sample extracts was determined by using the $AlCl_3$colorimetric method explained by Tyagi et al. (2020). Rutoside trihydrate was used to plot standard curve (5-120µg/mL; y = 0.0054x+0.0821; r2 = 0.9974; x is the concentration of solution; y is the absorbance).The results were expressed in RTEg-1 of dw.

Carotenoid content

β-carotene was estimated by the method as suggested by Srivastava and Kumar (2003). 5g of a sample of butter fruit milkshake powder (BFMS) was grinded with few crystals of Na_2SO_4 and homogenized with 10 ml $(CH_3)_2CO$. It was decanted, and then the supernatant was collected and transferred supernatant to a separatory flask. 10 ml of petroleum ether was added in separating funnel and mixed. Two layers were found, the lower layer discarded and the upper layer collected and, OD was recorded at 452 nm. The result was expressed in µg/ 100mg.

Storage studies

Water activity

Water activity (a_w) of the chocolate samples was determined by water activity meter (Aqua-Lab, model 3TE, Decagon Devices Inc., Pulluman, WA, USA) with 25°C temperature.

Peroxide value (PV)

Peroxide value was estimated by the American Oil Chemist's Society (AOCS) (1990) method. 3–5g of the sample was taken in an iodine flask then 20 mL of CHClƒ followed by 30ml of CHƒ COOH were added. 1 mL of KI solution was added to the sample and left to stand in the dark for 15 min at room temperature. After 15 min, 75mL distilled water was added to the sample, and the content was titrated against $Na_2S_20_3$ (0.01 N) using starch as an indicator of a colorless endpoint. The PV was expressed as meq O_2/kg of fat.

Thiobarbituric Acid (TBA) Value

The TBA value of the sample was estimated by the procedure given by Tarladgis et al. (1960). 5g of chocolate samples were placed in a flask, and 25 mL of C_6H_6 followed by 20 ml aqueous trichloroacetic acid solution (0.67 % w/v in water) was added. The obtained sample was shaken in a mechanical shaker for 2 h. The aqueous layer was separated using a separating funnel and heated over a water bath for 20 min. The absorbance was measured at 540 nm using a spectrophotometer. TBA reactive substances were calculated from a standard curve of malonaldehyde, a breakdown product of tetraethoxypropane .

Cost analysis

Cost calculation of butter fruit milkshake powder fortified chocolate was calculated using the standard methods by taking to account of the yield of butter fruit milkshake powder and all cost of raw material and processing(labor and electric) charges at the laboratory level.

Results and Discussion

Sensory acceptability of BFMS in chocolate

The data related to sensory evaluation was presented in Table 2. The sensory rating for color and appearance, flavor, consistency, and overall acceptability declined with the increase in BFMS powder concentration from 0-60%. The color scores significantly decreased as increased the level of butter fruit milkshake powder (15-60%) and ranged from 4.56 to 3.94, and the control chocolate

Table 1 Ingredients used for preparation of control chocolate (CC) and butter fruit milkshake chocolate (BFMSC)

Ingredient	CC (0%)	BFMS (15%)	BFMS (30%)	BFMS (45%)	BFMS (60%)
Cocoa powder (g)	100	85	70	55	40
Milk powder (g)	30	30	30	30	30
Butter (g)	62	62	62	62	62
BFMS (g)	-	15	30	45	60
Sugar (g)	150	150	150	150	150
Water (mL)	117	117	117	117	117

Table 2 Sensory score of chocolate formulation with varying of BFMS level Sensory acceptability of control chocolate (CC) and *Tinospora cordifolia* chocolate (TCC)

Formulation Cocoa powder: BFMS	Color and appearance	Flavour	Consistency	OAA
Control chocolate	4.87±0.27	4.53±0.24	4.20±0.12	4.66±0.23
85%:15%	4.56±0.25	4.32±0.22	4.14±0.25	4.49±0.22
70% : 30%	4.40±0.25	4.13 ±0.2	4.09±0.28	4.33±0.14
55% : 45%	4.09±0.13	3.87 ±0.24	3.53±0.19	3.67±0.17
40% : 60%	3.94±0.27	3.07 ±0.11	3.17±0.15	3.23±0.21

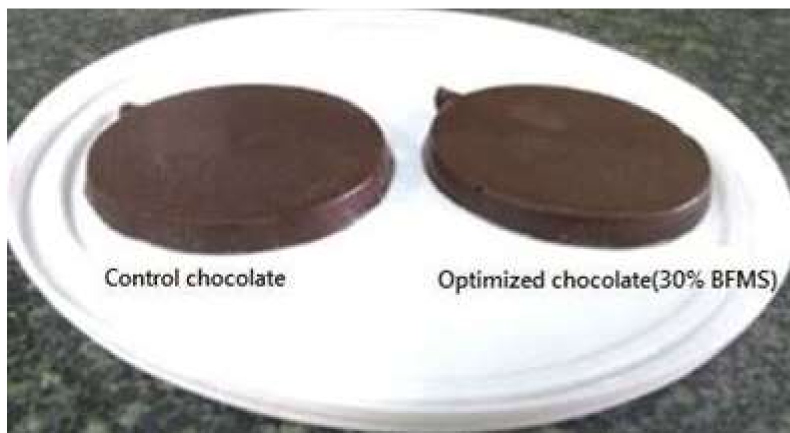


Fig.1 Optimized BFMS chocolate and control chocolate

Table 3 Nutritional composition of BFMS, CC, and BFMSC

Parameter	BFMS	CC	BFMSC
Moisture %	2.9±0.37	1.55 ±0.04	1.83±0.03
Protein %	17.2±0.14	5.92±0.07	11.98±0.01
Fat %	42.2±0.22	27.52±0.13	40.64±0.16
Ash%	2.78±0.09	1.54±0.04	2.14±0.02
Carbohydrate %	33.16	73.34	70.06
Crude Fiber %	0.84	0.47	0.822
Enegry Value(kcal/100g)	277.11±0.29	522±0.29	531.05±0.11
Ca(mg/100g)	13.45±0.26	1432±5.03	1456±6.33
Mg(mg/100g)	29±0.38	497±3.13	504±2.83
Fe(mg/100g)	0.63±0.07	24.6±2.03	26.5±1.89
Zn(mg/100g)	0.67±0.04	11.4±1.81	11.6±1.57
Mn(mg/100g)	0.15±0.02	2.97±.51	3.18±.37
Colour	L*(65.83±0.69)	L*(29.03±0.77)	L*(31.45±0.21)
	a*(-4.5±0.57)	a*(8.01±0.18)	a*(8.23±0.41)
	b (22.83±0.17)	b*(6.48±0.35)	b*(6.59±0.61)
Texture (Hardness) in kg	_	8.13 kg±0.948	9.48±0.88

Values are Mean ± Standard deviation (SD) of triplicates

had a significantly higher score (4.87). The hardness of BFMSC was slightly hard and feeling gritty within the mouth due to the high fiber content of BFMS. The overall acceptability score of control chocolate was 4.66, 15 % BFMSC (4.49), 30% BFMSC (4.33), 45 %(3.67) and 60 % BFMSC (3.23). These results showed that the addition of BFMSC above 30 % adversely affected the sensory parameter of chocolate. Thus, it could be concluded that chocolate can be prepared by using 30 % butter fruit milkshake powder with acceptable sensory quality (Fig. 1). This result conforms with Al-Marazeeq (2018), who reported that sensory attributes decreased with increasing the addition of wheat germ (6-15%) and found that 10% supplementation of the wheat germ was more acceptable than other concentration.

Nutrient composition and trace elements of BFMS, CC, BFMSC

The nutritional analysis results of BFMS, CC, and BFMSC are depicted in Table 3. Butter fruit milk powder fortified chocolate possessed a good quantity of protein 11.98%, fiber 0.822%, lipid 40.64, and the gross energy value for BFMSC was 531.05 kcal/100g. The obtained result was higher than control chocolate because butter fruit milkshake powder processed a good quantity of protein, fat, fiber, and ash, as shown in the previous study. Fat content (40.64%) of optimized chocolate was partly in the form of monounsaturated fatty acid, which is good for the heart. Al-Marazeeq (2018) found similar results of improved mineral, and protein content when incorporating wheat germ in chocolate.

Minerals are essential for the maintenance of human health. Trace elements were determined by the atomic absorption spectrophotometer shown in Table 3 revealed that the calcium (1456mg/100gm), magnesium (504mg/100gm), iron (26.5mg/100gm), zinc (11.6mg/100gm), and Mn (3.18 mg/100g) of optimized chocolate was higher than the control chocolate. These result obtained because the avocado pulp has a good quality of macro and microelement (Fulgoni et al. 2013)

Color and texture analysis

Table 3 shows, color and texture value of control chocolate and optimized chocolate. The color values of control chocolate were L* (29.03±0.77), a*(8.01±0.18), and b*(6.48±0.35). Addition of BFMS powder to the chocolate, brightness values (L*) was significantly increased, and the redness (a*) and the yellowness (b*) slightly increased when compared to the control sample. The addition of BFMS in chocolate affected textural. It was found the hardness proportionally increased with the addition of butter fruit milkshake powder in chocolate. The hardness of control chocolate is 8.13 kg, which increased with an increased level of BFMS. It happens because butter fruit milkshake powder has high moisture and fiber and makes a strong bond with cocoa protein. A previous study (Heo et al. 2019) also reported that muffin's hardness increased with the addition of enriched with dietary fiber from kimchi by product.

Phytochemical characteristics of CC, FGSF, and FGSC

Total phenolic and flavonoid content

Phenols are essential plant constituents. Various researches have confirmed that phenolic and flavonoid compounds are the most prominent antioxidative constituents in vegetables, fruits, and cereals (Choi et al. 2007). These constituents have a proportional relationship with total antioxidant activity. Table 4 shows the total phenolic and flavonoid content of BFMS, CC, and BFMSC. The total phenolic content of butter fruit milkshake powder fortified chocolate was 932.14 mg/100 g of dry weight, which was high in comparison to control chocolate (758.14mg/100g). TFC of the optimized chocolate extract against rutin was found to be 43.2mg/100g of dry weight, respectively, which was more than the control chocolate(43.2 mg/100g dw). Butter fruit milkshake powder has higher phenolic and flavonoid content because of avocado rich in phenolic and flavonoid compounds (Vinha et al. 2013), which proportionally increased the TPC and TFC content in BFMSC.

Beta carotene

BFMS is rich in carotenoid; hence the chocolate was prepared with BFMS Powder was rich in carotene content (71.13 μg/100g) shown in Table 4. Similar result was also reported by Hamdan et al. (2020) that carotenoid content of chocolate increases as incorporated with spirulina.

Shelf life study of chocolate

Effect on water activity

Several factors such as the raw materials used, the surface area of the materials, and the temperature and humidity of refining and conching may influence water activity of chocolate (Biquet and Labuza 1988). Water activity was increased from 0.391 to 0.532 in control chocolate (CC) and from 0.404to 0.408 in butter fruit milk shake chocolate (BFMSC) depicts in Table 5, during the storage of 0 to 60 days. The results depict that water activity of control chocolate and optimized chocolate increased during storage the 2 months of shelf life. Similar findings of increase in water activity in during storage were noticed in previous studies by Rossini et al. (2011) in white chocolate.

Effect on peroxide value

Quality loss and deteriorative problems in a food product are related to lipid degradation (Haak et al. 2006). In this regard, peroxide values increased for all chocolate samples during shelf life study for two months. The values presented in Table 5 depicts that the peroxide values of optimized chocolate 2.20±0.21 to 3.88±0.40and control chocolate 2.24±0.19to 4.11±0.13 increases with an increase in storage period from 0 to 60 days respectively. Moreover, the peroxide value for optimized chocolate was lower than CC during storage. This outcome is well supported by the observation of Rossini et al. (2011), who noticed that peroxide

Table 4 Total phenolic contents, total flavonoid contents and antioxidants potential of BFMS, CC, and BFMSC

Parameter	BFMS	CC	BFMSC
TPC(mg/100gdw)	416.2 ± 5.04	758.14± 7.15	932.14±5.19
TFC(mg/100g dw)	24 ± 1.54	36.02± 1.50	43.2±1.92
Beta carotenoid(μg/100g)	502± 7.09	0.45± 0.001	71.13± 7.86

Values are Mean ± Standard deviation (SD) of triplicates

Table 5 Water activity, TBARS, and Peroxide value of butter fruit milkshake powder chocolate samples stored at 20ÚC for up to 2month

	Water activity			Peroxide value (mEq O2/kg)			TBARS (μg/g)		
Days	0	30	60	0	30	60	0	30	60
Control chocolate(20ÚC)	0.391±0.005	0.412±0.010	0.532±0.016	2.24±0.19	2.65±0.33	4.11±0.13	5.84±0.34	6.51±0.52	8.1±0.58
BFMSC(20ÚC)	0.404±0.004	0.408±0.007	0.530±0.005	2.20±0.21	2.36±0.18	3.88±0.40	5.60±0.13	6.22±0.42	7.55±0.54

Values are Mean ± Standard deviation (SD) of triplicates

Table 6 Cost analysis calculation for cost of butter fruit milk shake powder

Particulars	Amount
Total weight of pulp obtained in 1 kg of raw avocado	655 g
% yield of powder in raw avocado=(weight of powder/weight of raw avocado)*100	655/1000*100=65.5%
Total cost of 655 gram of avocado milk shake powder(Cost of raw avocado+ Processing charges+ miscellaneous)	Rs.278/-
Cost of raw avocado	Rs.178/kg
Processing charges(electricity used in drying +labor charges+ miscellaneous)	Rs.100/kg
Cost for 1 kg of Butter fruit milk shake powder	(278/655)1000= Rs. 424.43/Kg
Butter fruit milk shake powder cost	424.43/Kg or 42.44/100g

Table 7 Cost estimation of chocolate

Ingredient	Quantity	Rate(Rs/100g)	Total cost of control chocolate(Rs/-)	Total cost of BFMSC(Rs/-)
Cocoa powder	100g for CC 70g for BFMSC	120	120	84
Milk powder	30g	50	15	15
Butter	62g	48	29.76	29.76
BFMS	0 for CC 30 for BFMSC	42.44	0	12.73
Sugar	150g	5	7.5	7.5
Water	117ml	2	2.34	2.34
Sub total			174.6	151.33

values of white chocolate prepared with antioxidants were lower than that of control.

Effect on TBA value

From the data represented in Table 5, it was noticed that increasing the storage period elevates the TBA value of both chocolate. The TBA value for optimized chocolate (BFMSC) were 5.60±0.13, 6.22±0.42, and 7.55±0.54, and for control chocolate (CC) were5.84±0.34, 6.51±0.52and 8.1±0.58 at 0, 30th, and 60th days of storage, respectively. There was no significant change observed in BFMSC and CC's TBA value from 0 to 60 days of storage. A similar finding was studied by Rossini et al. (2011) for white chocolate. The lower value of TBA delayed the onset of rancidity in chocolate.

Cost estimation

Cost analysis of BFMS powder

The cost analysis calculation of BFMS powder is shown in Table 6. The amount of total BFMS powder obtained from 1Kg of raw avocado after being processed (removal of moisture and wastage) was estimated 655 grams, and the yield of BFMS powder was found to be 65.5%, and the total cost for 1 Kg of BFMS powder was calculated as 424.43/Kg.

Cost estimation of chocolate

Cost of ingredient used and prepared chocolate shown in Table 7. The cost of optimized chocolate (Rs. 151.33/100g) in the laboratory was estimated to lower than control chocolate (Rs. 174.6/100g). The cost of cocoa powder used in control chocolate was higher than BFMS powder. Hence there was not much variation in the cost of control and optimized chocolate. So the use of butter fruit milkshake powder in preparation of optimized chocolate was found to be economical.

Conclusions

Butter fruit milkshake powder comprises protein, fiber, antioxidants, and phenolic compounds that are advantageous for health. Due to nutritional qualities and palatability of the butter fruit milkshake powder, the present study was carried out to make chocolate with BFMS that has acceptable sensory attributes and enhanced nutritional properties. The study findings based on sensory analysis suggested that 30% of butter fruit milkshake powder addition in chocolate in the place of cocoa powder is acceptable by consumers. BFMS powder's addition increased micro and macro minerals (Ca, Mg, Fe, Zn and Mn), proteins, dietary fibers, phenolic, flavonoids, and beta carotenoid contents of the chocolate. Shelf life investigation showed butter fruit milkshake powder fortified chocolate significantly more

stable than control chocolate and accepted by consumers up to 60 days of storage and presence of bioactive compounds in BFMSC can use to treat different irresistible illnesses. This kind of value-added chocolate may expand the food industry and the market with nutraceutical potential. Further, the histopathology and other compulsory examination are required before getting an endorsement from the administrative bodies. Different food items such as cookies, snack bars, nutritional bars, and candies can also be developed by supplementing the butter fruit milkshake powder.

References

Al-Marazeeq KM (2018) Evaluation of Proximate Composition and Sensory Attributes of Dark Chocolate Fortified with Wheat Germ. Adv J Food Sci Technol 14: 103-107

AOAC (2000) Official Methods of Analysis of the Association of Official Analytical Chemists, seventeenth ed. Association of Official Analytical Chemists, Gaithersburg, MD

AOCS (1990) Official Methods and Recommended Practices of American Oil Chemical Society,4th Edition

Aparna, Chauhan AK, Singh PK (2018) Estimation of toxic, trace and essential metals (Pb, cd, Fe, Zn, Mn, Cu, Mg, K) in fruit and vegetable product (jam, ketchup, pickles) by atomic absorption spectrophotometer. The Pharm. Innov 7: 313-317

Bhuyan DJ, Alsherbiny MA, Perera S, Low M, Basu A, Devi O A, Barooah MS, Li CG, Papoutsis K (2019) The odyssey of bioactive compounds in avocado (*Persea americana*) and their health benefits. Antioxidants 8: 426

Biquet IB, Labuza TP (1988) Evaluation of the moisture permeability characteristics of chocolate films as an edible moisture barrier. J Food Sci 53: 989–998

Briones V, Aguilera JM, Brown C (2005) Effect of surface topography on color and gloss of chocolate samples. J Food Eng 77: 776–783

Choi Y, Jeong HS, Lee J (2007) Antioxidant activity of methanolic extracts from some grains consumed in Korea. Food Chem 103: 130-138

Erdem Ö, Gültekin-Özgüven M, Berktas I, Ersan S, Tuna HE, Karadagˇ A, Özçelik B, Güneş G, Cutting SM (2014) Development of a novel synbiotic dark chocolate enriched with Bacillus indicus HU36, maltodextrin and lemon fiber: Optimization by response surface methodology. LWT- Food Sci Technol 56: 187–193

Fulgoni VL, Dreher M, Davenport AJ (2013) Avocado consumption is associated with better diet quality and nutrient intake, and lower metabolic syndrome risk in US adults: results from the National Health and Nutrition Examination Survey (NHANES) 2001–2008. Nutr Jo 12: 1

Godočiková L, Ivanišová E, Kačániová M (2017) The influence of fortification of dark chocolate with sea buckthorn and mulberry on the content of biologically active substances. Adv Res Life Sci 1: 26-31

Haak L, Raes K, Smet K, Claeys E, Paelinck H, De Smet S (2006) Effect of dietary antioxidant and fatty acid supply on the oxidative stability of fresh and cooked pork. Meat Sci 74: 476–486

Hamdan AB, Riaty C, Fitriya W, Ekantari N (2020) Effects of Nanoencapsulated Carotenoid of Spirulina platensis on the Sensory Profiles of Dark and Milk Chocolate. In *E3S Web of Conferences* (Vol. 147, p. 03022) EDP Sciences

Heo Y, Kim MJ, Lee JW, Moon B (2019) Muffins enriched with dietary fiber from kimchi by product: Baking properties, physical–chemical properties, and consumer acceptance. Food Sci Nutr 7: 1778-1785

Latif R (2013) Chocolate/cocoa and human health: a review. Neth J Med 71: 63-8

Mayank S, Kumar JD (2012) Chocolate formulation as drug delivery system for pediatrics. Indonesian J Pharm 23: 216-224

Mussatto SI, Mancilha IM (2007) Non-digestible oligosaccharides: a review. Carbohydr Polym 68:587-597

Ortiz-Avila O, Esquivel-Martínez M, Olmos-Orizaba BE, Saavedra-Molina A, Rodriguez-Orozco AR, Cortés-Rojo C (2015) Avocado oil improves mitochondrial function and decreases oxidative stress in brain of diabetic rats. J Diabetes Res :9

Pandey S, Aparna, Varghese S, Chauhan AK, Singh M (2020) Development of phytonutrient enriched avocado milkshake powder and its quality evaluation. Indian J Dairy Sci 73: 556-565

Rosenblat G, Meretski S, Segal J, Tarshis M, Schroeder A, Zanin-Zhorov A, Lion G, Ingber A, Hochberg M (2011) Polyhydroxylated fatty alcohols derived from avocado suppress inflammatory response and provide non-sunscreen protection against UV-induced damage in skin cells. Arch Dermatol Res 303:239-246

Rossini K, Noreña CP, Brandelli A (2011) Changes in the color of white chocolate during storage: potential roles of lipid oxidation and non-enzymatic browning reactions. J Food Sci Technol 48: 305-311

Souza DS, Pimentel JDR, Prado MM, Marques LG, Narain N (2011) Rehydration characteristics of freeze-dried avocado (Persea americana). In International Congress on Engineering and Food-ICEF11 (pp. 1-6). Athens, Greece: Food Process Engineering in a Changing World

Srivastava RP and Kumar S (2003) Fruits and vegetable preservation–principles and practices. International Book Distributing Co., India. Pages 360

Stoilova I, Krastanov A, Stoyanova A, Denev P, Gargova S (2007) Antioxidant activity of a ginger extract (Zingiber officinale). Food Chem 102: 764-770

Tarladgis BG, Watts BM, Younathan MT, Dugan Jr L (1960) A distillation method for the quantitative determination of malonaldehyde in rancid foods. J Am Oil Chem Soc 37: 44-48

Tyagi P, Chauhan AK & Aparna (2020) Optimization and characterization of functional cookies with addition of *Tinospora cordifolia* as a source of bioactive phenolic antioxidants. LWT-Food Sci Technol: 109639

Vinha AF, Moreira J, Barreira SV (2013) Physicochemical parameters, phytochemical composition and antioxidant activity of the algarvian avocado (Persea americana Mill.). J Agric Sci 5: 100

Wollgast J, Anklam E (2000) Polyphenols in chocolate: Is there a contribution to human health? Food Res Int 33: 449-459

Żyżelewicz D, Budryn G, Oracz J, Antolak H, Kręgiel D, Kaczmarska M (2018) The effect on bioactive components and characteristics of chocolate by functionalization with raw cocoa beans. Food Res Int 113: 234-244

Indian J Dairy Sci 74(1): 61-67
https://doi.org/10.33785/IJDS.2021.v74i01.008

RESEARCH ARTICLE

Genotypic identification of extended spectrum β-lactamase producing *Escherichia coli* in dairy supply chain

Amarjeet Kumar[1], Rashmi Hogarehalli Mallappa[2], Avinash Jaswal[1], Brijesh Kumar[1], Naresh Kumar[1] and Raghu Hirikyathanahalli Vishweswaraiah[1]

Received: 02 November 2020 / Accepted: 06 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Foodborne illnesses due to antibiotic-resistant bacteria represents a major public health problem in both developed and developing countries. Among 190 samples, 139 *Escherichia coli* positive isolates from raw milk, pasteurized milk, and human handlers were identified by phenotypic methods and genotypic methods. All *E. coli* isolates were found to be resistance to penicillin, oxacillin, erythromycin and clindamycin. The dominant type of resistance to cefotaxime and amoxiclave identically detected in 18.7% isolates followed by ampicillin in 17.98%, trimethoprim 15.82%, tetracycline 10.79%, nalidixic acid 7.91%, and piperacillin 7.79%. Four isolates have shown resistance (2.87%) to Ceftriaxone and ceftazodime, Cefotaxime and one isolate has shown resistance to Cefepime. Further, all four isolates were confirmed as extended spectrum β-lactamase (ESBL) producer by double disc diffusion test and ESBL chromogenic medium. Later, all four isolates were evaluated by PCR and they are observed as carrier of blaCTX M gene which is responsible for ESBL antibiotic resistance in *E.coli* but blaTEM and blaSHV genes were absent in all four ESBL isolates. Based on the above findings, it is concluded that ESBL antibiotic resistance in *E. coli* were more prevalent in milk and this may due to spread and acquirement of antibiotics resistance gene by plasmid and mobile genetic elements

Keyword: Antibiotic resistance, *E. coli*, Milk, ESBL

[1]National Referral centre, Dairy Microbiology Division, ICAR-NDRI, Karnal-132001. Haryana, India
[2]Molecular Biology Unit, Dairy Microbiology Division, ICAR-NDRI, Karnal-132001

Raghu Hirikyathanahalli Vishweswaraiah (✉)
National Referral Centre, Dairy Microbiology Division,
ICAR-National Dairy Research Institute, Karnal-132001. Haryana, India.
Email: raghu.nrcndri@yahoo.com ;4rvsy.dmndri@gmail.com
Tel: +91-184-2259534, Mobile: +91-9466963599

Introduction

The enzyme responsible for degradation of oxyimino-β-lactam antibiotics is Extended-spectrum β-lactamases (ESBL) and these antibiotics are used in the treatment of various serous humans and animal infections (Palmeira and Ferreira, 2020). ESBL were first identified in the year 1983 in the family Enterobacteriaceae and they are responsible serious risk to human health may be due to treatment failure in various severe cases of infections in 2013 (Adeolu et al. 2016). These enzymes are encoded by plasmids that confer resistance to the penicillins; to first-, second-, third-, and fourth-generation cephalosporin's; and to aztreonam but not to carbapenems (EFSA, 2011). Human-to-human spread of these type of enzyme producing bacterial pathogens have been demonstrated in public hospital settings, representing that human colonization is a pool for dissemination (Liebana et al. 2013). Furthermore, various reports are available for the isolation and identification of ESBL-producing bacteria from foods and food animals, suggesting the likely role of the food production chain as a pool for this group of pathogenic bacteria (EFSA, 2019, Odenthal et al. 2016). There are various data's are available regarding robust correlation between the incidence of ESBL-producing bacteria in foods (Algeria et al. 2020) and the prevalence of infections in humans may be anticipated that food obtained from animals may be infected with ESBL producers which contributing to the transmission within the population (EFSA, 2011, Carrottoli, 2008). Furthermore, ESBL producers also can the resistant bacteria can fetch additional genes having some virulence property; it is notable that strains of *Escherichia coli* (STEC) are known to be food-borne pathogens, have been confirmed as ESBL-producers, representing that the transference of the extracellular DNA from commensals to foodborne pathogenic strains is possible (Torpdahl et al. 2013).

The occurrence of ESBL-producing *Escherichia coli* is extensively growing throughout India. These pathogens pose a major threat to the treatment of infection and may cause tricky in the management of infections. This may create unnecessary problem with the widespread use of second- or third-generation antibiotics for the monitoring and treatment of bacterial infections (Van Hoek et al. 2015). ESBL *E. coli* is typically unaffected to routinely used antibiotics instigating a surge in the use of almost

all antimicrobials (i.e., carbapenems) in treatment. The *E. coli* strains harboring those resistance genes can easily spread to other pathogens leading to the spread of resistance (Odenthal et al. 2016). Hence, the presence of ESBL-producing *E. coli* in the dairy supply chain maybe arriving from healthy animals is the fact which has to be suitably premeditated. Therefore, in our present study, we are evaluating the occurrence of ESBL-producing *E. coli* in dairy supply chain.

Materials and Methods

Procurement and Maintenance of culture

The standard culture used in our study was *Escherichia coli* ATCC 25922 was purchased from American Type Culture collection. The culture was activated in nutrient broth followed by streaking on Violet red bile (VRBA) agar followed by incubation for overnight at 37°C. A single pure colony from VRBA after microscopic examination was picked up and maintained on nutrient agar slant by routine sub-culturing after every fortnight. All the experiments were conducted using overnight grown cultures. All media chemicals including Muller Hinton Agar and antimicrobial agents including discs are procured from Himedia lab (Mumbai, India).

Detection of *E. coli* using PCR

Species specific primers were used in Colony polymerase chain reaction(PCR) includes Forward:GGTAACGTTTCTACCGCAGAGTTG/ Reversed:CAGGGTTGGTACACTGTCATTACG, target gene of *E. coli* as phoA with a size of 468 bp (Shome et al. 2011) using a protocol given by Godambe et al. (2017).

Antimicrobial susceptibility tests (AST)

Antimicrobial susceptibility tests was carried out on Mueller-Hinton Agar (Hi-media, Mumbai, India) using the Kirby-Bauer disk diffusion method (Bauer et al. 1996). The data were evaluated and inferred according to National Committee for Clinical Laboratory Standards (NCCLS 1993). Reference strain of *E. coli* ATCC 25922 was used as a quality control strain for studying AST (CLSI 2007). The resistance strains were evaluated for minimum inhibitory concentration (MIC) using micro-dilution methods followed data inferred as per CLSI guidelines (CLSI 2007).

Extended Spectrum β-lactamase (ESBL) confirmatory test

The double disc synergy test (DDST) was performed for ESBL using amoxicillin/clavulanate, ceftazidime, ceftriaxone, aztreonam, and cefotaxime (Jarlier et al. 1988; Drieux et al. 2008). Further, phenotypic confirmation of ESBL positive isolates was carried out using CLSI (2012) guidelines. The test is considered as positive when a decreased susceptibility to cefotaxime is combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as 'champagne-cork' or 'keyhole (Drieux et al. 2008). A ratio of ceftazidime or cefotaxime MIC to ceftazidime or cefotaxime-clavulanic acid MIC equal to or greater than eight indicated the presence of ESBL (Drieux et al. 2008).

Phenotypic identification of ESBL in *E. coli*

An overnight grown culture of ESBL +ve isolate whose turbidity was adjusted to 0.5 McFarland solutions (Himedia Lab, Mumbai, India) was streaked on the Hicrome ESBL agar plates followed by incubation at 37°C for 24 h. The development of pink or purple colored colonies on the Hicrome ESBL agar plates considered as positive for ESBL.

Identification of ESBL by PCR

The following ESBLs resistance determinants were investigated by PCR for bla-CTXM, bla-TEM and bla-SHV gene (Karczmarczyk et al. 2011). Colony PCR were performed as per protocol given by Godambe et al. (2017) by targeting ESBL encoding genes [Primer Sequence (5'-3') such as CTX-M universal- F-CGATGTGCAGTACCAGTAA; CTX-M universal-R–TTAGTGACCAGAATCAGCGG, blaSHV-F-TTATCTCCCTGTTAGCCACC; blaSHV-R-GATTTGCTGATTTCGCTCGG, blaTEM-F-GCGGAACCCCTATTTG and blaTEM-R-ACCAATGCTTAATCAGTGAG] (Olesen et al. 2008) with a genome size of 585 bp for CTX-M (Batchelor et al. 2005) and 795 bp for blaSHV (Weill et al. 2004).

Results and Discussion

Detection of *E. coli in dairy supply chain*

In our study, the prevalence of *E. coli* in raw milk samples were found to be 57.27%, 20% in pasteurized milk samples, and 25% in swab of human handler working in dairy farms and plants by biochemical identification test such as Indole, Methyl red, Vogues Prausker test and citrate utilization test. From the above, 139 isolates were selected and confirmed as *E. coli* using specific enzyme substrate interaction using two stage enzyme assay and Hichrome ECD agar wherein all isolates have shown characteristic enzyme reaction and characteristic blue colored colonies on Hichrome ECD agar (Fig. 1). Later, these isolates were shown characteristic 468 bp amplicon bands on gelelectroporesis based on the use of species specific primer. The amplification was seen in the reference strain *E. coli* ATCC 25922 which was used as positive control. Almost similar prevalence rat

e of *E. coli* were reported by Soomro et al. (2014) wherein they found 57 and 52% prevalence of *E. coli* in raw milk respectively. Tadesse *et al.* (2018) and Badri et al. (2017) have shown lower

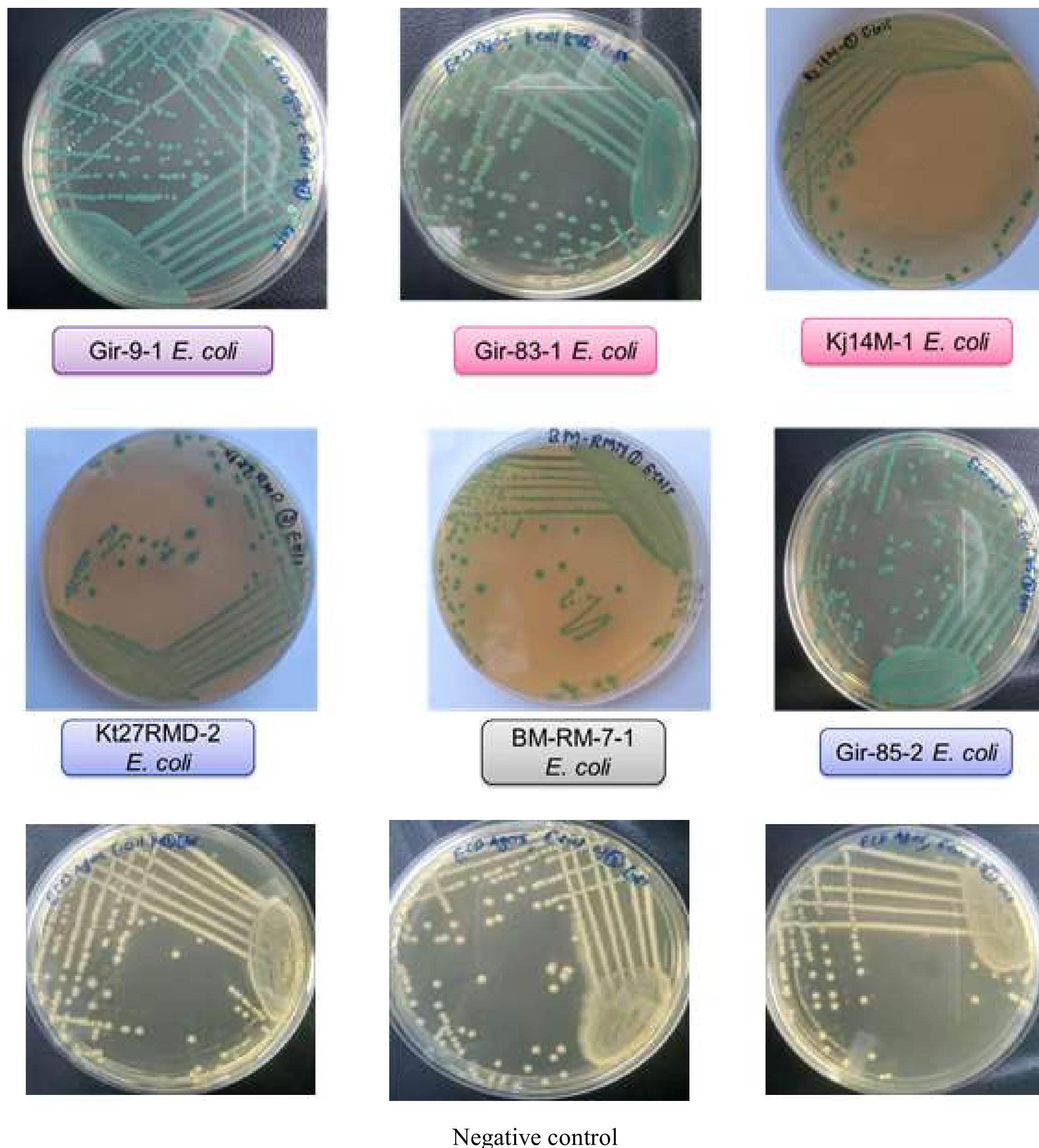


Fig. 1. Rapid detection of *E. coli* isolates on Hichrome ECD/MUG agar

prevalence rate (33%, 38%) of *E. coli* in raw milk samples respectively. The variation in prevalence rate of *E. coli* may be attributed to lapses in clean milk production, different geographic location and season, fecal contamination of milk, and due to poor hygiene and sanitary practices followed while milking and further handling (Thaker et al. 2012). The prevalence of *E.coli* in pasteurized milk may be due to post processing contamination and poor hygienic management subsequently the milk is pasteurized (Ali and Abdelgadir, 2011)

Antibiotic susceptibility test

Among 139 *E. coli* isolates, all isolates have shown resistance towards penicillin (P), oxacillin (OX) and erythromycin (E), respectively based on inhibition zone diameter by AST followed by 26 isolates have shown resistance towards amoxicillin (MC) and Cefotaxime (CTX), 25 isolates to ampicillin (AMP), 22 isolates to trimethoprim (TR), 15 isolates to tetracycline (TET), 11 isolates to nalidixic acid (NA), 10 isolates to piperacin (PI), 5 isolates to gentamycin (GEN) and chloramphenicol (C), 4 isolates ceftriaxone (CTR) and cefepime (CPM), 3 isolates to nitrofurantoin (NIT) and amikacin (AK), 1 isolate has shown resistance towards ceftazidime (CAZ) and ofloxacin (OF), and none of the isolates have shown resistance towards ampicillin-sulbactam (A/S), meropenem (MRP), ertapenem (ERP) and netilmicin (NET),

Table 1 Prevalence of antibiotics resistant pattern of *E. coli*

Name of antibiotics	No of Resistance isolates	No of Intermediate isolates	No of Susceptible isolates
P	139 (100%)	0	0
AMP	25 (17.98)	1 (0.71%)	113 (81.29%)
OX	139 (100%)	0	0
PI	10 (7.19%)	16 (11.51%)	113 (81.29%)
AMC	26 (18.7%)	40 (28.77%)	73 (52.51%)
A/S	0	8 (5.75%)	131 (94.24%)
CTX	4 (2.87%)	35 (25.17%)	100 (71.94%)
CPM	4 (2.87%)	35 (25.17%)	100 (71.94%)
CTR	4 (2.87%)	0	135 (97.12%)
CAZ	1 (0.71%)	8 (5.75%)	130 (93.52%)
NA	11 (7.91%)	24 (17.26%)	104 (74.82%)
CIP	2 (1.43%)	4 (2.87%)	133 (95.68%)
OF	1 (0.71%)	0	138
TR	22 (15.82%)	1 (0.71%)	116 (83.45%)
C	5 (3.59%)	2 (1.43%)	132 (94.96%)
NIT	3 (2.15%)	0	136 (97.84%)
CD	139 (100%)	0	0
TE	15 (10.79%)	3 (2.15%)	121 (87.05%)
E	139 (100%)	0	0
AK	3 (2.15%)	24 (17.26%)	112 (80.57%)
GEN	5 (3.59%)	6 (4.31%)	128 (92.08%)
MRP	0	0	139 (100%)
ETP	0	0	139 (100%)
NET	0	0	139 (100%)

P: Penicillin-G, AMP: Ampicillin, OX:Oxacillin, PI: Piperacin, AMC: Amoxicillin, A/S: Ampicillin-sulbalactam, CTX: Cefotaxime, CPM: Cefepime, CTR Ceftriaxone, CAZ: Ceftazidime, NA: Nalidixic Acid, CIP: Ciprofloxacin, OF: Oflaxacillin, TR: Trimethoprim, C: Chloramphenicol, NIT: Nitrofurantoin, CD: Clindamycin, TE: Tetracycline, E: Erythromycin, AK: Amikacin, GEN: Gentamycin, MRP: Meropenam, ETP: Erthaapenem, NET: Netilimycin

respectively (Table 1). Based on the AST, it was clear that four isolates are showing resistance towards ESBL and none were showing resistance to carbapenem group of antibiotics. The dominant types of resistance was observed in our results are in close association with the result of Rasheed et al. (2014) wherein he was reported 14.7% of the isolate from raw milk were showing resistance towards ESBL. This may be due acquiring of mobile genetic element such as plasmids, transposons, and Class 2 integrons (Singh et al. 2005).

ESBL *E. coli*

Based on diameter of zone of inhibition by AST methods four isolates of *E. coli* have found positive for ESBL. Further, these 4 positive isolates were confirmed phenotypically as ESBL using double disk diffusion test (DDDT) and Hi-Chrome ESBL agar base (Fig. 2). All four ESBL positive isolates have shown an inhibition zone diameter of ≤ 27 mm for CTX and CTR, followed by three isolates have shown a inhibition zone diameter of ≤ 22 mm for CAZ and CPM (Table 2). Overall prevalence of ESBL positive *E. coli* in raw milk samples was 3.27% No ESBL positive isolates were obtained from pasteurized milk and human handlers swab samples. All the ESBL positive isolates by DDDT and Chromogenic ESBL medium have shown identical resistance towards (P, AMP, PI, CTX, CTR, CAZ, and CPM) and (P, PI, CTR, CPM, TE) by 4 and 2 *E. coli* isolates, respectively. Duan et al. (2006) reported a 3.1% prevalence of ESBL producers among *E. coli* isolates from dairy cattle. In a Turkish study reported by Kucukbasmaci et al. (2008) reported 2.1% prevalence of ESBL producing Enterobacteriaceae isolated from dairy cattle. Gundogan and Avci (2013) reported 10% (2/20) prevalence of ESBL positive *E. coli* in milk which is slightly higher than current study. The prevalence rate of ESBL producing *E. coli* was 29.3% (17/22) in raw milk which is much higher than the current study (Badri et al. 2017).

PCR identification of ESBL in *E. coli*

All four ESBL positive isolates were further confirmed as ESBL *E.coli* by genotypic methods using colony PCR wherein all 4

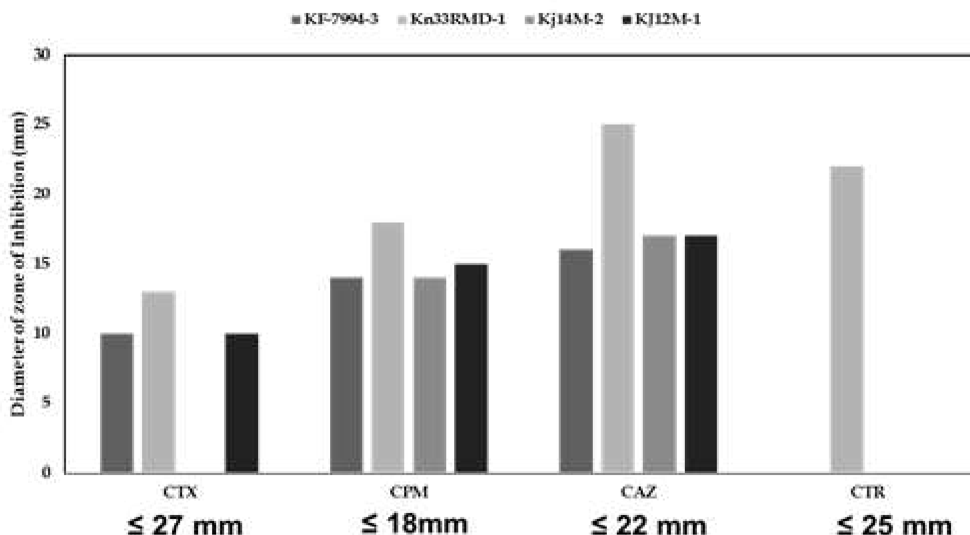


Fig. 2 Confirmation of ESBL +ve *E.coli* isolates by DDDT and ESBL chromogenic agar. a. double disc diffusion test b. ESBL +ve *E. coli* on Hicrhome ESBL agar c. Diameter of zone of inhibition in ESBL +ve silates by DDDT.

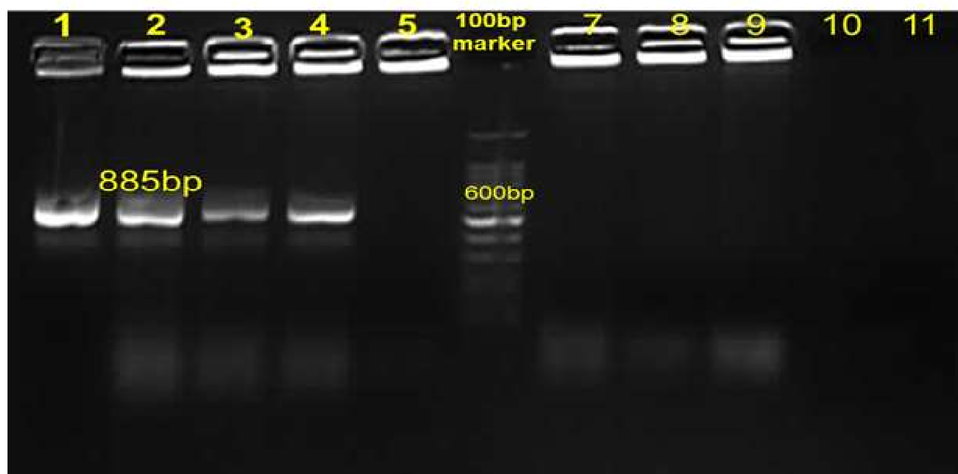


Fig. 3 ESBL producing isolates with primers specific for bla-CTXM and bla-SHV gene. Lane 1 to 4: ESBL producing isolates with bla-CTXM primer; Lane 5 to 9: ESBL producing isolates with bla-SHV primer; Lane 10: Negative control for bla-CTXM primer; Lane 11: Negative control for bla-SHV primer

isolates have shown bands for bla CTXM gene yielded 885 bp amplicon on agarose gel (Fig. 3). However, no amplified products were obtained with bla-SHV and bla-TEM primer. This indicates that all four ESBL producing isolates were harboring bla-CTX-M gene which encodes ESBL in *E. coli* isolates. Similar findings were reported by Batabyal et al. (2018) regarding the prevalence of ^{bla}CTX-M gene in 12 ESBL *E.coli* among 22 isolates obtained from West Bengal. Ghatak et al. (2013) have reported a one isolate was harboring New Delhi metallo β lactamase gene (bla_{NDM}) and another isolate was carrying ESBL gene – $bla_{CTX M}$. Further, Dhara

and Tripathi (2014) has reported ESBL *E.coli* were found positive for bla CTX M-3 gene (18 nos), bla CTX M-9 gene (6 nos), bla SHV gene: (5 nos) and bla TEM gene: (5 nos) and may cause health risk to consumers due to contamination by ESBL producing *E. coli*, their pathogenicity and treatment failure as a result of antibiotic resistant.

Conclusions

Based on the above findings it is concluded that four *E. coli isolates have shown* resistance to ESBL antibiotics like CTX, CTR, CAZ and CPM may indicate presence of multiple drug resistance gene on same mobile genetic elements. Further, all four ESBL positive *E. coli* isolates were harboring CTX-M gene which is linked with dairy animal. It also concluded that the prevalence of ESBL *E. coli* in raw milk may due to transmission and acquisition of antibiotics resistance gene by plasmid and mobile genetic elements.

Acknowledgment

AK is the recipient of Junior Research Fellow scholarship from Indian Council of Agriculture Research, DARE, and Government of India. Director, ICAR-NDRI, Deemed University for furnishing the required research facility for conducting the study on AMR in *E. coli*. Indian Network Fishery and Animals antimicrobial resistance (INFAAR), ICAR and FAO are highly acknowledged for their help in undergoing a training program on antimicrobial resistance in bacterial pathogens. The project was initially supported by Science and Engineering Research Board, DST, GOI, New Delhi under the Grant number: SERB/ECR/2017/002333.

References

Adeolu M, Alnajar S, Naushad S, Gupta RS (2016) Genome-based phylogeny and taxonomy of the ‘Enterobacteriales’: proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. Int J Syst Evol Microbiol 66: 5575-5599

Alegría Á, Arias-Temprano M, Fernández-Natal I, Rodríguez-Calleja JM, García-López ML, Santos Jan (2020) Molecular diversity of ESBL-producing *E. coli* from foods of animal origin and human patients. Int J Environ Res Public Health 17:1312.

Ali AA, Abdelgadir WS (2011) Incidence of *Escherichia coli* in raw cow’s milk in Khartoum state, British. J Dairy Sci 2:23–26

Badri AM, Ibrahim IT, Mohamed SG, Garbi MI, Kabbashi AS (2017) Prevalence of Extended Spectrum β-Lactamase (ESBL) Producing *E. coli* and *Klebsiella pneumonia* Isolated from Raw Milk Samples in Al Jazirah State, Sudan. Mol Biol *7*: 2

Batabyal K, Banerjee A, Pal S, Dey S, Joardar SN, Samanta I, Isore DP, Singh AD (2018) Detection, characterization, and antibiogram of extended-spectrum β-lactamase *E. coli* isolated from bovine milk samples in West Bengal, India. Vet World 10:1423

Batchelor M, Hopkins K, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, Liebana E (2005) blaCTX-M genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrob Agents Chemother 49: 1319-1322

Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45: 493-496

CLSI (2007 Performance standards. In: Institute CaLS, editor. M100-S17. vol. ISBN 1-56238-625-5, 1 edn. 940 West Valley Road, Suite 1400, Wayne 19087-1898 USA; 2007.

CLSI (2012) Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute (M100eS22). 2012 (s22nd Informational Supplement).

Dhara L, Tripathi A (2014) Genetic and structural insights into plasmid-mediated extended-spectrum β-lactamase activity of CTX-M and SHV variants among pathogenic Enterobacteriaceae infecting Indian patients. Int J Antimicrob Agents 43: 518-26.

Drieux L, Brossier F, Sougakoff W, Jarlier V (2008) Phenotypic detection of extended spectrum β lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect 14: 90-103.

Duan RS, Sit TH, Wong SS, Wong RC, Chow KH, Mak GC, Ho PL (2006) *Escherichia coli* producing CTX-M β-lactamases in food animals in Hong Kong. Microb Drug Resist 12: 145-148

EFSA (2011) BIOHAZ Panel Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. EFSA J 9: 2322

EFSA (2019) ECDC. The European union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. EFSA J 17: 5598.

Ghatak S, Singha A, Sen A, Guha C, Ahuja A, Bhattacharjee U, Das S, Pradhan NR, Puro K, Jana C, Dey TK (2013) Detection of New Delhi Metallo β Lactamase and Extended Spectrum β Lactamase genes in *E. coli* isolated from mastitic milk amples. Transbound Emerg Dis 60: 385-389

Godambe LP, Bandekar J, Shashidhar R (2017) Species specific PCR based detection of Escherichia coli from Indian foods. 3 Biotech 7: 1–5

Gundogan N, Avci E (2013) Prevalence and antibiotic resistance of extended-spectrum β-lactamase (ESBL) producing *E. coli* and *Klebsiella* species isolated from foods of animal origin in Turkey. Afr J Microbiol Res 7: 4059-4064

Jarlier V, Nicolas MH, Fournier G, Philippon A (1988) Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Clin Infect Dis 10: 867-878

Karczmarczyk M, Abbott Y, Walsh C, Leonard N, Fanning S (2011) Characterization of multidrug-resistant *Escherichia coli* isolates from animals presenting at a university veterinary hospital. Appl Environ Microbiol 77: 7104-7112

Kucukbasmaci O, Ciftcioglu G, Midilli K, Issa G (2008) Detection of extended spectrum β-lactamase producing Enterobacteriaceae from food animals In Turkey. Revue Méd Vét 159: 586-592

Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos AP, Mevius D, Peixe L, Poirel L, Schuepbach-Regula G, Torneke K (2013) Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food producing animals: An EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin Infect Dis 56: 1030-1037

NCCLS (National Committee for Clinical Laboratory Standards) (1993) Tentative Guidelines, M26-TNCCLS. Villanova, PA: 1993. Methods for determining bactericidal activity of antimicrobial agents

Odenthal S, Akineden Ö, Usleber E (2016) Extended-spectrum β-lactamase producing Enterobacteriaceae in bulk tank milk from German dairy farms. Int J Food Microbiol 238: 72–78

Olesen I, Hasman H, Møller Aarestrup F (2004) Prevalence of β-lactamases among ampicillin-resistant *E. coli* and Salmonella isolated from food animals in Denmark. Microb Drug Resist 10: 334-340

Rasheed MU, Thajuddin N, Ahamed P, Teklemariam Z, Jamil K (2014) Antimicrobial drug resistance in strains of *E.coli* isolated from food sources. Rev Inst Med Trop Sao Paulo 56: 341-346

Shome BR, Das Mitra S, Bhuvana M, Krithiga N, Velu D, Shome R, Isloor S, Barbuddhe SB, Rahman H (2011) Multiplex PCR assay for species identification of bovine mastitis pathogens. J Appl Microbiol 111: 1349-1356

Singh R, Schroeder CM, Meng J, White DG, McDermott PF, Wagner DD, Yang H, Simjee S, DebRoy C, Walker RD, Zhao S (2005) Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *E. coli* recovered from humans and food animals. J Antimicrob Chem 56:216-219

Soomro AH, Arain MA, Khaskheli M, Bhutto B (2002) Isolation of *E. coli* from raw milk and milk products in relation to public health sold under market conditions at Tandojam. Pak J Nutr 1: 151-152

Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, McDermott PF (2012) Antimicrobial drug resistance in *E. coli* from humans and food animals, United States, 1950–2002. Emerg Infect Dis 18: 741

Thaker HC, Brahmbhatt MN, Nayak JB, Thaker HC (2013) Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat. Vet World 6: 10-13

Torpdahl M, Nielsen EM, Scheutz F, Olesen B, Hansen DS, Hasman H (2013) Detection of a Shiga toxin- and extended-spectrum-β-lactamase-producing *E. coli* O157:H7 human clinical isolate. J Antimicrob Chemother 68: 1203–1204

Van Hoek AHAM, Veenman C, van Overbeek WM, Lynch G, de Roda Husman AM, Blaak H (2015) Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables. Int J Food Microbiol 204: 1–8

Weill FX, Demartin M, Tandé D, Espié E, Rakotoarivony I, Grimont PA (2004) SHV-12-like extended-spectrum-β-lactamase-producing strains of *Salmonella enterica* serotypes *Babelsberg* and *Enteritidis* isolated in France among infants adopted from Mali. J clin microbiol 42: 2432-2437

Indian J Dairy Sci 74(1): 68-73
https://doi.org/10.33785/IJDS.2021.v74i01.009

RESEARCH ARTICLE

Factors affecting the milk yield, milk composition and physico-chemical parameters of ghee in lactating crossbred cows

N Veena[1], JS Hundal[2], M Wadhwa[2] and AK Puniya[3]

Received: 08 April 2020 / Accepted: 07 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: The aim of this study was to investigate the effect of season, production status, stage of lactation, and parity on milk yield, milk composition and physico-chemical parameters of ghee in forty crossbred cows (CBCs). Milk samples were collected from individual animal during morning in both summer (May-August) and winter (November-February) season. The results revealed that milk yield was significantly influenced by the production status and lactation stage. The fat percentage was significantly affected by the stage of lactation and parity. However, protein percentage was affected by the season and stage of lactation. An effect of season, stage of lactation and parity on butyro-refractometer (BR) reading of ghee was significant. The Reichert-meissl (RM) value of CBCs ghee was significantly influenced by the parity whereas Polenske value by the production status and parity. Results also indicated that the RM value of CBCs ghee was found to be below the legal standard of minimum 28 in Punjab state.

Keywords: Crossbred cows, Milk yield, Milk composition, Physico-chemical parameters of ghee

Introduction

Development of breeding programs for changing the composition of milk requires knowledge of the relative influence of genetic (stage of lactation, pregnancy, breed, parity etc) and non-genetic (nutrition, season of calving, temperature etc) factors affecting milk constituents (Sudhakar et al. 2013). Augmenting lactation milk yield has been emphasized for increasing the productivity of dairy animals; however, milk constituents such as fat, protein and lactose percentages have so far received little attention in breeding programs.

The physico-chemical quality of ghee (clarified butterfat) is usually assessed by analyzing certain characteristics such as Reichert-Meissl (RM) value, Polenske value (PV), Butyro-refractometer (BR) reading, Iodine value and Saponification value. These analytical characteristics are mostly the reflections of the fatty acid composition of the milk lipid (Veena et al. 2020) and vary with region to region and season to season, depending on the type of breed and the feed. For instance, RM value is substantially a measure of the lower chain volatile water soluble fatty acids i.e butyric acid ($C_{4:0}$) and caproic acid ($C_{6:0}$) whereas Polenske value is a measure of lower chain volatile water insoluble fatty acids i.e caprylic acid ($C_{8:0}$) and capric acid ($C_{10:0}$). Butyro-refractometer (BR) reading, which measures the index of refraction between air and the liquid fat and varies with the nature of the fat, is usually determined at 40°C (Veena et al. 2018; Veena et al. 2020). However, the composition of milk as well as fatty acid composition is largely affected by various factors i.e., lactation stage, lactation number, breed, season and environmental factors. The available information on various factors influencing the composition of milk and physicochemical properties of milk fat/ ghee is scanty in crossbred cows. Moreover Punjab state is endowed with highly productive population of crossbred cattle, closer to the Holstein Friesian both in production traits and in appearance. Thus the present investigation was undertaken with the objective to study the effect of various factors affecting the milk yield, milk composition and chemical parameters of ghee, prepared from milk of crossbred cows.

Materials and Methods

Sample collection and analysis

Forty crossbred cows from the Livestock farm of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU,

[1]Department of Dairy Chemistry, College of Dairy Science and Technology; Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, 141 004, Punjab, India

[2]Department of Animal Nutrition, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, 141 004, Punjab, India

[3]Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal, 132 001, Haryana, India

N Veena[1*](✉)
Department of Dairy Chemistry, College of Dairy Science and Technology; Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, 141 004, Punjab, India
Email: veena.ndri@gmail.com

Ludhiana, Punjab, India) were kept under farm management. Diet of crossbred cows was met through green fodder (*ad lib.)* and concentrate according to milk production. All the experimental animals were offered identical ration to meet production/ maintenance requirement to negate the feeding effect on milk production. The animals had free access to water throughout the day. Feed samples offered in two different seasons (May – August and November - February) were collected and ground to pass through 1 mm sieve and then analyzed for fat, protein, acid detergent fibre (ADF) and neutral detergent fibre (NDF) content (Robertson and Van Soest, 1981).

The crossbred cows were grouped according to production status (high yielders, >15 kg/day; medium yielders, 8-15 kg/day; low yielders, <8 kg/day), stage of lactation (early - up to 100 days of postpartum; mid – 101 to 200 days; late – more than 201 days of postpartum) and parity (1st to 4th lactation). Milk samples were collected from individual animal during morning in both summer (May - August) and winter (November - February) season. Milk yield was recorded after complete milking. Contents of fat, solid-not-fat (SNF) and protein in milk samples were analyzed by a MilkoScreen (Indifoss Analytical Pvt Ltd, Ahmedabad, India). Cream was separated from each of the individual milk samples by centrifugal method in the cream separator. Cream samples were then converted to ghee by direct cream method as described by De (2005). Ghee samples were stored in refrigerator (4°C) till for further analysis. Ghee samples were analyzed for RM and PV as per the standard procedure described in IS: 3508 (ISI, 1966). Butyro refractometer (BR) reading of ghee was measured using digital butyro refractometer (Atago Co Ltd, Tokyo, Japan).

Statistical analysis

Milk samples were collected twice in each season from 40 animals. Mean values and pooled standard error of duplicate determinations were reported. Statistical significance was set at P<0.05. The data was analyzed in a factorial design (Snedecor and Cochran, 1994) by using the software package SPSS version 16 (SPSS 1996) and differences in mean were assessed by using Tukey's b test.

Table 1. Composition of the feed offered to CBCs in different seasons

Parameter	Season	
	Summer	Winter
Dry matter, kg /day	16.3	14.9
Roughage : Concentrate ratio	55:45	49:51
Crude Protein, %	15.5	20.1
Fat, %	2.97	3.59
NDF, %	52.42	37.8
ADF, %	32.39	22.7

Results are expressed as mean values, n=5

Results and Discussion

The chemical composition of feed offered to crossbred cows in different season is shown in the Table 1.The animals were offered green fodder and concentrate in the ratio of 55 to 45 and 49 to 51, respectively in the summer and winter season. The total dry matter intake was 16.3 and 14.9 kg/d, respectively in the summer and winter season. The crude protein and fat content were higher in the winter fodder, whereas NDF and ADF content were higher in the summer fodder.

Effect of season on milk yield, milk composition and physico-chemical parameters of ghee

The seasonal differences can attributed to the differences in quality and quantity of available fodder and climatological variation in different seasons which have direct impact on production traits. The data in Table 2 revealed that milk yield and fat content of lactating CBCs was not affected by the season. Many workers (Nehra 2011, Radhika et al. 2012) reported no significant effect of season and period of calving on the first lactation milk yield. Radhika et al. (2012) reported that season of calving had no significant effect on milk fat percentage. However, the protein and SNF content were significantly lower (P<0.05) in winter than summer season. According to Sarkar et al. (2006) milk composition traits were highest in hot humid season but lowest in milk yield as compared to other seasons. The daily yield of fat and protein was observed to be higher during summer (P<0.05) than winter season.

The RM and PV of ghee were found to be slightly higher in summer than that found in the winter, however, significant difference were not observed (Table 2). The RM value of ghee was found to be 25.04 in winter and 25.79 in summer, while PV was 1.08 and 1.12, respectively. The previous findings were expected because the summer milk fat contained higher levels of C_4 and C_6 acids than the one found in the winter milk fat (Laurelle et al. 1976). However, this discrepancy between both fats and in their fatty acid levels was due to the effect of the season of the year and consequently the plane of nutrition (McDowall and McGillivary, 1963; Hall, 1970; Gray, 1973). The BR reading was found to be lower (P<0.05) in summer compared to winter season.

Effect of production status on milk yield, milk composition and physico-chemical parameters of ghee

Effect of production status on milk yield, milk composition and physico-chemical parameters of ghee is represented in the Table 3. The milk yield varied from 5.58 kg/d (low yielders) to 19.71 kg/ d (high yielders) and significantly affected (P<0.05) by the production status. The fat content varied (p>0.05) from 3.63 (medium yielders) to 4.21 per cent (high yielders). The production status of animals showed no significant effect on fat, protein and SNF content. The daily fat and protein yield was affected

(p<0.05) by the production status of the animals and was observed to be higher in high yielding dairy animals as compared to low yielding animals and this is due to differences in daily milk yield.

The data on physiochemical parameters revealed that RM value was not affected by production status of the CBCs. The production status of animals influenced (p<0.05) the PV and it was observed to be low in low yielders (0.94) and high in high yielders (1.2). The BR value was not affected by the production status of animals and was within the normal range of 41-43.

Effect of stage of lactation on milk yield, milk composition and physiochemical parameters CBCs

The daily milk yield was observed to vary from 11.4 (mid lactation) to 14.5 kg (early lactation) (Table 4). The fat content of CBCs increased linearly with the advancement of lactation and values were higher (p<0.05) during late than mid and early lactation. The increase in total lipid contents may be due to the activity of fatty acid synthesizing enzymes particularly acetyl CoA carboxylase which is a regulatory enzyme in the fatty acid synthesis might have slightly increased in late lactation than early and mid lactation (Sharma et al. 2000). The daily fat yield was observed to be higher (p<0.05) from animals in late lactation in comparison to animals in early stage of lactation and correlates well with milk yield and fat content during different stages of lactation. Similarly protein content was also increased with progress of lactation stage. No influence of stage of lactation was observed on SNF content and protein yields. Bhoite and Padekar (2002) reported a non significant effect of stage of lactation for fat in Holstein crosses but a significant effect in crosses involving Jersey. Sarkar et al., (2006) reported that lactation stage had no influence on fat content but a significant effect on protein, SNF and lactose content.

The data on physicochemical properties of milk fat (Table 4) revealed that RM and PV were slightly higher in early and late lactation period compared to mid lactation, however, significant differences were not observed. Studies reported that proportions

Table 2. Effect of season on milk yield, milk composition and physico-chemical parameters of ghee

Parameter	Season[#]		
	Summer	Winter	PSE
Milk yield kg/day	12.17	13.20	0.50
Fat, %	4.00	3.78	0.19
Protein, %	4.03[b]	3.45[a]	0.070
SNF, %	9.85[b]	9.22[a]	0.15
Fat, kg/day	0.56[b]	0.36[a]	0.036
Protein, kg/day	0.52[b]	0.35[a]	0.032
RM value	25.79	25.04	0.30
Polenske value	1.12	1.08	0.05
BR reading	41.59[a]	42.88[b]	0.12

Mean values with different superscripts in a row differ significantly (P<0.05).
[#]Irrespective of lactation number, stage of lactation and production status. PSE-Poolesd standard error

Table 3. Effect of production status on milk yield, milk composition and physico-chemical parameters of ghee

Parameter	Production status[#]			
	High	Medium	Low	PSE
Milk yield , kg/day	19.71[c]	12.76[b]	5.58[a]	0.61
Fat, %	3.83	3.63	4.21	0.22
Protein, %	3.70	3.69	3.84	0.08
SNF, %	9.46	9.53	9.62	0.18
Fat, kg/day	0.62[b]	0.44[b]	0.32[a]	0.044
Protein, kg/day	0.57[b]	0.44[b]	0.29[a]	0.039
RM value	25.44	25.76	25.04	0.36
Polenske value	1.20[b]	1.18[b]	0.94[a]	0.058
BR reading	42.23	42.09	42.38	0.11

Mean values with different superscripts in a row differ significantly (P<0.05). [#]Irrespective of lactation number, stage of lactation and season. PSE-Poolesd standard error

of short- and medium-chain fatty acids ($C_{6:0}$ to $C_{14:0}$) increased during the first 3 months of lactation and decreased after that, whereas long chain fatty acids followed opposite trend (Mele et al. 2009; Stoop et al. 2009). Butyric acid showed a decreasing trend as lactation progressed for all parities while the proportion of $C_{6:0}$ to $C_{14:0}$ fatty acids were lower at the beginning of the lactation, increased first 100 days of lactation and remained steady for the rest of the lactation period (Bilal et al. 2014). The BR reading varied (p<0.05) in a narrow range of 41.9 (late) to 42.5 (mid) and increased up to mid stage of lactation and decreased thereafter.

Effect of parity on milk yield, milk composition and physio-chemical parameters of ghee

Effect of parity on milk yield, milk composition and physico-chemical parameters of ghee in CBCs is represented in the Table 5. With increase in parity the milk yield increased linearly but the differences were not significant. The milk fat was observed to be highest (p<0.01) in primiparous cows in comparison to multiparous cows (Table 5). A decrease in milk fat percentage of 0.2% over five lactations has been reported by Rogers and Stewart (1982). However, the protein and SNF content were not affected by the parity. Contrary to the present findings Suman (2009a) and Suman (2009b) observed significant effect of parity on SNF and protein content, respectively. Radhika et al., (2012) and Sarkar et al (2006) reported milk yield and milk composition traits were not differed significantly in different parities. The fat and protein yield was observed to be highest (p<0.01) in multiparous animals (4th lactation). Wathes et al. (2007) suggested that there are differences between primiparous and multiparous cows in the control of tissue mobilization that may promote nutrient partitioning into growth, as well as milk during the first lactation.

Both RM and PV were significantly influenced (P<0.05) by the parity but no systematic trend could be observed. The RM value varied from 24.7 (2nd lactation) to 26.6 (4th lactation). The PV was observed to be highest of ghee obtained from animals in 4th parity and lowest in 3rd parity. The BR reading varied in a very narrow range of 42.04 (1st) to 42.59 (2nd lactation). The BR reading was observed to be lower in the first parity and differ significantly from other lactation numbers.

Table 4. Effect of stage of lactationon milk yield, milk composition and physico-chemical parameters of ghee

Parameter	Stage of lactation#			
	Early lactation	Mid lactation	Late lactation	PSE
Milk yield, kg/day	14.45[b]	11.42[a]	12.17[a]	0.58
Fat, %	3.42[a]	4.04[b]	4.22[c]	0.21
Protein, %	3.58[a]	3.72[a]	3.92[b]	0.079
SNF, %	9.30	9.42	9.89	0.17
Fat, kg/day	0.38[a]	0.43[ab]	0.56[c]	0.041
Protein, kg/day	0.42	0.37	0.44	0.037
RM value	25.88	24.79	25.57	0.35
Polenske value	1.08	1.06	1.17	0.055
BR reading	42.23[b]	42.51[b]	41.97[a]	0.11

Mean values with different superscripts in a row differ significantly (P<0.05). #Irrespective of lactation number, production status and season. PSE-Poolesd standard error

Table 5. Effect of parity on milk yield, milk composition and physico-chemical parameters of ghee

Parameter	Parity#				
	1st	2nd	3rd	4th	PSE
Milk yield kg/day	12.0	12.32	12.47	13.95	0.65
Fat, %	4.50[b]	3.54[a]	3.99[a]	3.52[a]	0.24
Protein, %	3.44	3.81	3.76	3.95	0.09
SNF, %	9.0	9.69	9.61	9.85	0.20
Fat, kg/day	0.37[b]	0.34[a]	0.54[b]	0.60[b]	0.046
Protein, kg/day	0.28[a]	0.36[a]	0.46[a]	0.63[b]	0.041
RM value	25.15[ab]	24.70[a]	25.20[a]	26.61[b]	0.40
Polenske value	1.13[b]	1.04[ab]	0.99[a]	1.25[b]	0.062
BR reading	42.04[a]	42.59[b]	42.22[b]	42.09[b]	0.16

Mean values with different superscripts in a row differ significantly (P<0.05). #Irrespective of season, stage of lactation and production status. PSE-Poolesd standard error

Incidence of low RM value

The RM value of ghee prepared from crossbred cow's milk was found to be below the FSSAI standard of minimum 28 in Punjab state (FSSA, 2006), irrespective of season, stage of lactation, production status and parity. This may be attributed to the fact that crossbred cows of Punjab has more of Holstein Friesian inheritance and hence produce milk of low fat percentage with low RM value compared to indigenous breeds of cow. There are numerous references in the literature about the effect of various rations on the RM value of butterfat (Arup, 1929; Geisler, 1926, Hawley, 1933; Overman and Garrett, 1932; Sutton et al., 1932). In some instances extremely low RM numbers have been obtained (Arup, 1929; Hawley, 1933; Sutton et al., 1932). These were invariably caused by extreme or unusual feeding conditions. Stout and Wilster (1939) reported that the lowest RM value was produced in a region where alfalfa hay formed the major portion of the ration. It is recognized that feed influences butterfat composition and inherent glyceride composition. Kehar et al. (1956) who studied the effect of breed, season and locality on some of chemical constants of ghee prepared from milk collected from 12 farms in different parts of India. They observed that RM and Polenske values for ghee samples lay between 16 and 34.76 (average 24.29) and 1.1 and 2.8 (average 1.77), respectively. More studies needs to be conducted before a definite conclusion is drawn about the incidence of low RM value in the milk fat of CBCs in Punjab state.

Conclusions

From study, it was observed that effect of season, lactation stage, production status and parity were visible in milk yield, milk composition and chemical parameters of CBCs ghee. Average milk yield was significantly influenced by stage of lactation and production status. An effect of season, stage of lactation and parity on butyro-refractometer (BR) reading of ghee was significant. The fat percentage was significantly affected by the stage of lactation and parity. However, protein percentage was affected by the season and stage of lactation. The RM value of CBCs ghee was significantly influenced by the parity whereas PV by the production status and parity. Results of this study also revealed that RM value of CBCs ghee was appears to be below the FSSAI standard of minimum 28 in Punjab state. It is, therefore, necessary that much more detailed work should be undertaken on the effect of climate, feed, breed/species and method of management on the chemical parameters of ghee to ascertain the proper ranges for Indian standards.

Acknowledgement

Authors are thankful to the Punjab State Cooperative Milk Producer's Federation Limited, Chandigarh for providing financial assistance for carrying out this research work.

References

Arup PS (1929) The Composition of Irish winter butter. Analyst 54: 634-645

Bhoite UY, Padekar RN (2002) Factors affecting milk yield and composition. Indian J Animal Res 36: 67-69

Bilal G, Cue RI, Mustafa AF, Hayes JF (2014) Effects of parity, age at calving and stage of lactation on fatty acid composition of milk in Canadian Holsteins. Can J Anim Sci 94: 401-410

De S (2005) Outlines of Dairy Technology. Oxford Publishing Company, New Delhi

FSSA (2006) The Food Safety and Standards Act. Universal's, New Delhi, India

Geisler JG (1926) The Effect of Cottonseed Feeding on Butterfat. J Oil Fat Inds 3: 115-118

Gray IK (1973) Seasonal variations in the composition and thermal properties of New Zealand milk fat, I. Fatty acid composition. J Dairy Res 40: 207-214

Hall AJ (1970) Seasonal and regional variations in the fatty acid composition of milkfat. Dairy Industries 35: 20-24

Hawley H (1933) The Phytosterol acetate test as a routine method in the examination of butterfats with borderline Reichert-Meissl values. Analyst 58: 529-531

ISI (1966) Methods of Sampling and Test for Ghee (Butter Fat) (3508: 1966). Bureau of Indian Standards, Manak Bhavan, New Delhi

Kehar ND, Ray SN, Joshi BC, Raisarkar BC (1956) Chemical constants of pure ghee. In: Studies on Fats, Oils and Vanaspatis. Manager of Publications, Delhi, p. 5-12

Laurelle L, Van DM, Daenens P (1976) Fatty acid composition of Reichert-meissl and Polenske fractions in butterfat. J Dairy Res 43: 137-140

McDowall FH, McGillivary WA (1963) Studies on the properties of New Zealand butterfat, VI. Comparision of the properties and vitamin A potencies of butterfats produced by clover-fed and ryegrass-fed dairy cows. J Dairy Res 30: 47-57

Mele M, Dal Zotto R, Cassandro M, Conte G, Serra A, Buccioni A, Bittante G, Secchiari P (2009) Genetic parameters for conjugated linoleic acid, selected milk fatty acids, and milk fatty acid unsaturation of Italian Holstein-Friesian cows. J Dairy Sci 92: 392-400.

Nehra M (2011) Genetic analysis of performance trends in Karan- Fries cattle. M.Sc. Thesis, ICAR-National Dairy Research Institute (Deemed University), Karnal, India

Overman OR, Garrett OF. (1932) The Influence of certain balanced rations on the chemical and physical properties of milk fat. J Agric Res 45: 51-58

Radhika G, Ajithkumar S, Rani A, Sathian CT, Anilkumar K, Usha AP, Dinesh CN (2012) Milk yield and composition of crossbred cows in the hilly Wayanad district of Kerala, India. Indian J Animal Sci 82: 1251-1254

Robertson JA, Van Soest PJ (1981) The detergent system of analysis and its application on human food. In: James W.P.T. and O. Theander (eds), The Analysis of Dietary Fibre in Food, Marcel Dekkar Inc., New York, pp 123-158

Rogers GL, Stewart JA (1982) The effects of some nutritional and nonnutritional factors on milk protein concentration and yield. Aust J Dairy Technol 37: 26-32

Sarkar U, Gupta AK, Sarkar V, Mohanty TK, Raina VS, Prasad S (2006) Factors affecting test day milk yield and milk composition in dairy animals. J Dairying Foods HS 25: 129-132

Sharma KC, Sachdeva VK, Singh S (2000) A comparative gross and lipid composition of Murrah breed of buffalo and cross-bred cow's milk during different lactation stages. Arch Tierz Dummerstorf 43: 123-130

Snedecor GW, Cochram WG (1994) Statistical Methods. Oxford and IBH Publications, New Delhi

SPSS (1996) Statistical packages for social sciences. Version 12.0, SPSS Inc., Linois, USA

Stoop WM, Bovenhuis H, Heck JML, van Arendonk JAM (2009) Effect of lactation stage and energy status on milk fat composition of Holstein-Friesian cows. J Dairy Sci 92: 1469-1478.

Stout RE, Wilster GH. (1939) Some observations on the Iodine, Reichert-Meissl and Polenske values of milk fat produced during the winter in three sections of Oregon. J Dairy Sci 22: 424-425

Sudhakar K, Panneerselvam S, Thiruvenkadan A K, Abraham J, Vinodkumar G (2013) Factors effecting milk composition of crossbred dairy cattle in Southern India. Int J Food Agric Vet Sci 3: 229-233

Suman CL (2009a) Lactation trend of milk solid-not-fat in two-breed crosses of cattle at an organized farm. Indian J Animal Res 43**:** 12-16

Suman CL (2009b) Algebric functions describing lactation trend of milk Protein in two-breed crosses of cattle. Indian J Animal Res 43**:** 62-66

Sutton TS, Blcown JB, Johnston EW (1932) The Effect of corn oil on milk and butterfat production and on the composition of butterfat in the dairy cow. J Dairy Sci 15: 209-211

Veena N, Wadhwa M, Hundal JS, Puniya AK (2020) Factors affecting milk yield, milk composition and physic-chemical parameters of ghee in Murrah buffaloes of Punjab region. Indian J Dairy Sci 73: 40-45

Veena N, Wadhwa M, Mehta H, Barui AK, Puniya AK, Hundal JS, Grewal RS (2018) Effect of supplementing bypass fat on milk yield, milk composition and chemical parameters of ghee in crossbred Cows. Int J Curr Microbiol App Sci 7: 2604-2609

Wathes DC, Cheng Z, Bourne N, Taylor VJ, Coffey MP, Brotherstone S (2007) Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domest Anim Endocrinol 33: 203-225

Indian J Dairy Sci 74(1): 74-79
https://doi.org/10.33785/IJDS.2021.v74i01.010

RESEARCH ARTICLE

Shelf life enhancement of low calorie and fiber-enriched *Sandesh* by modified atmosphere packaging

Himanshu Kumar Rai, Dinesh Chandra Rai, Arvind and Shikha Pandhi

Received: 27 June 2020 / Accepted: 01 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: *Sandesh* is the most popular *chhana*-based sweet delicacy of the eastern part of India, especially West Bengal, India. *Sandesh* is a heat-acid coagulated product that is rich in high quality animal protein, fat, minerals, and vitamins. The present research was conducted to study the effect of three different combinations of gases i.e. 98% CO_2, 98% N_2 and 50% N_2: 50% CO_2 on the shelf life of low calorie and fiber-enriched *sandesh* samples. The samples packed under air were kept as control. The samples were stored in a BOD incubator at 25 and 37°C and analysed for microbial, textural and sensorial changes at an interval of 7 days up to 28 days. The samples packed with air showed significantly higher textural and sensorial changes and microbial spoilage as compared to the other three combinations. The results showed that samples packed with 50% N_2: 50% CO_2 combination had better shelf stability as compared to the samples packed under air, 98% N_2 and 98% CO_2.

Keywords: *Sandesh,* Sensory attributes, Shelf life, Textural properties

Introduction

Milk and milk-based products have been an important source of nourishment in our daily life (Kumar and Singh, 2017).India is the largest producer of milk in the world with an annual production of around 155.5 million tonnes (NDDB, 2017). About 50-55% of the product is converted to traditional Indian dairy products and forms an important part of the cultural heritage of India (Bandopadhyay and Khamrui, 2007). Advancement of technology and incorporation of novel ingredients to restructure traditional dairy products has been the current trend in the market which is majorly leaning towards the development of low calorie and more shelf-stable products (Gawande et al. 2012). *Sandesh* is a popular traditional dairy product prepared by acid or heat coagulation of milk and mainly admired in the eastern part of India, especially in West Bengal (Saha et al. 2018). It is famous for its palatability, aroma and nutritional value attributed by the presence of good quality proteins, fat, vitamins, minerals etc. It is prepared by the constant mixing of *channa* with sugar over medium heat (Bandyopadhyay et al. 2008). Cow milk is preferably used for the preparation of *channa* as it gives smooth texture with uniform grains which are mainly desired for preparation of *sandesh* (Husain and David, 2018). Limited shelf-life of these products due to their susceptibility to undergo deterioration during storage makes them unfit for consumption (Londhe et al. 2012). Innovations in packaging systems such as vacuum packaging, modified atmosphere packaging have provided an excellent solution by retaining the freshness of perishable as well as non-perishable foods for a comparatively longer time (Chowdhury et al. 2017).

Modified atmosphere packaging (MAP) has come up as an efficient approach to enhance the shelf-life of perishable products without the addition of any chemical additive (Floros and Matsos, 2005). It involves altering the composition of the product surroundings within the food package to protect the product from various microbial and oxidative changes during storage that is triggered by the presence of oxygen in the product surrounding (Jha et al. 2015). Therefore, the presence of a low concentration of residual oxygen in the product surrounding within the packaging system is the key approach utilized in the modified atmosphere packaging of foods for shelf life extension (Sandhya, 2010). Several types of research have been conducted to study the effect of modified atmosphere packaging on the preservation of *lal peda* (Londe et al. 2012), *paneer* (Thippeswamy et al. 2011) and *brown peda* (Jha et al. 2015) which demonstrated modified atmosphere packaging as a promising technique to extend the

Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221 005, India

Himanshu Kumar Rai (✉)
Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221 005, India
Email: raihimanshuias@gmail.com; Mobile: +919598232301

shelf-life of traditional dairy products. In view of above-mentioned prospect, the present work was undertaken to study the effect of modified atmosphere packaging on the shelf life of low calorie and fiber-enriched *sandesh* based on sensorial, microbial and textural parameters.

Materials and Methods

The present work was carried out in the laboratory of the Department of Dairy Science and Food Technology, Banaras Hindu University, Varanasi, India. Low calorie and fiber-enriched *sandesh* was manufactured using milk standardized to 3% fat and 8.5% SNF. The milk was procured from the Dairy Farm, Banaras Hindu University, Varanasi, India. Stevia and sorbitol used for the preparation of low calorie and fiber-enriched *sandesh* were procured from the local market of Varanasi, India and online sources. The level of sorbitol, oat flour, and stevia optimized using response surface methodology based on sensory scores were 4%, 15% and 0.25% respectively. The detailed manufacturing process of low calorie and fiber-enriched *sandesh* was represented with the help of a flow diagram (Figure 1).

Packaging and storage of low calorie and fiber-enriched *sandesh*

Three different combinations of gases i.e. 98% CO_2, 98% N_2 and 50% N_2:50% CO_2 were used to pack the *sandesh* samples. The samples packed under air were kept as control. The samples were stored in a BOD incubator at 25 and 37°C at 60% relative humidity and analyzed for microbial and textural changes at an interval of 7 days up to 28 days. Tray of dimension 18 x 12.5 cm^2 (Containing 6 pieces of low calorie and fiber enriched *sandesh*) manufactured by Elixir Technologies was used for packaging.

Microbial analysis

All the samples were subjected to total plate count (TPC), yeast and mold count (YMC) and coliform count. One gram of sample was properly mixed with distilled water. 1ml of resultant homogenate was added to 9ml of sterile saline water in a test tube and diluted serially to obtain a series of dilutions up to 10{ w . 1ml or 0.5ml of the appropriate dilutions from each tube was aseptically pipette out and plated on to different selective and differential media using pour plate technique. The TPC was determined on plate count agar (PCA) and incubated at 37°C for 24–48 hours. For mold and yeast detection, sample was spread on potato dextrose agar (PDA) and incubation was done at 25°C for 24–48 hours. Coliforms in the samples were estimated by plating on violet red bile agar (VRBA) before being incubated at 37°C for 24–48 hours (Kumbhar et al. 2009). The number of microbial counts was calculated using the following formula:

$$\text{Colony-forming units (CFU)/ml} = \frac{\text{No. of colonies x dilution factor}}{\text{Weight of aliquot taken (ml)}}$$

Texture profile analysis (TPA)

TPA on samples was performed by using the Texture Analyser (TA.XT plus texture profile analyzer, Stable Micro Systems, UK) to characterize the hardness, adhesiveness, springiness, cohesiveness and gumminess of the optimized *sandesh*. During the textural analysis, samples of optimized *sandesh* were cut into 1.5 cm^3 size pieces and their temperature was maintained at 25°C.

Sensory analysis

The sensory quality of low calorie and fiber-enriched '*sandesh*' samples were judged by a panel of 10 semi-trained judges from the Department of Dairy Science and Food Technology. The samples were evaluated for sensory attributes viz., color and appearance, flavor, sweetness, body and texture and overall acceptability based on a 9-point hedonic scale (Amerine et al. 1965).

Statistical Analysis

All experiments were performed in triplicate. Data is expressed as mean value. The means were compared using Duncan's multiple range test (DMRT) at $P < 0.05$. One-way analysis of variance (ANOVA) was performed as described by Snedecor and Cochran (1989), to test the significance of data in each trial and parameter.

Results and Discussion

Microbial changes of low calorie and fiber-enriched *sandesh* samples were analyzed at an interval of 7 days of storage. Total plate count and yeast and mold count on day zero was 4.87 and 2.85 log_{10} CFU/g, respectively. Coliforms were not detected in the samples during the entire study period. Microbial loads in different gas combinations are shown in Table 1. In the samples stored under air at 25 and 37°C, microbial activity reached the critical limits after 14 days of storage. Hence, the samples were discontinued after 14 days of storage. In other samples stored with 98% N_2, 98% CO_2 and 50% N_2:50% CO_2 at 25 and 37°C, there was an increase in the TPC and YMC, within 28 days and no coliforms were detected. Critical limits were reached at 28 days of storage. All the samples stored under MAP displayed a continuous increase in the total plate count and yeast and mold populations during 28 days of storage period. However, if compared with the samples containing 98% N_2 and 98% CO_2, the growth of the microbial populations was lower in the case of 50% N_2: 50% CO_2 atmosphere package during the 28 day storage period. The samples containing 98% nitrogen displayed delayed microbial growth as nitrogen is an inert gas that does not support microbial growth. However, as observed, samples with 98% CO_2 displayed increased inhibition of microbial growth when compared to samples containing 98% N_2, probably due to the bacteriostatic effect of CO_2, as mentioned in several earlier reports (Daniels et al. 1985; Banks and Annis, 1990; Davis, 1998; Devlieghere and Debevere, 2000). Smith et al. (1986) reported

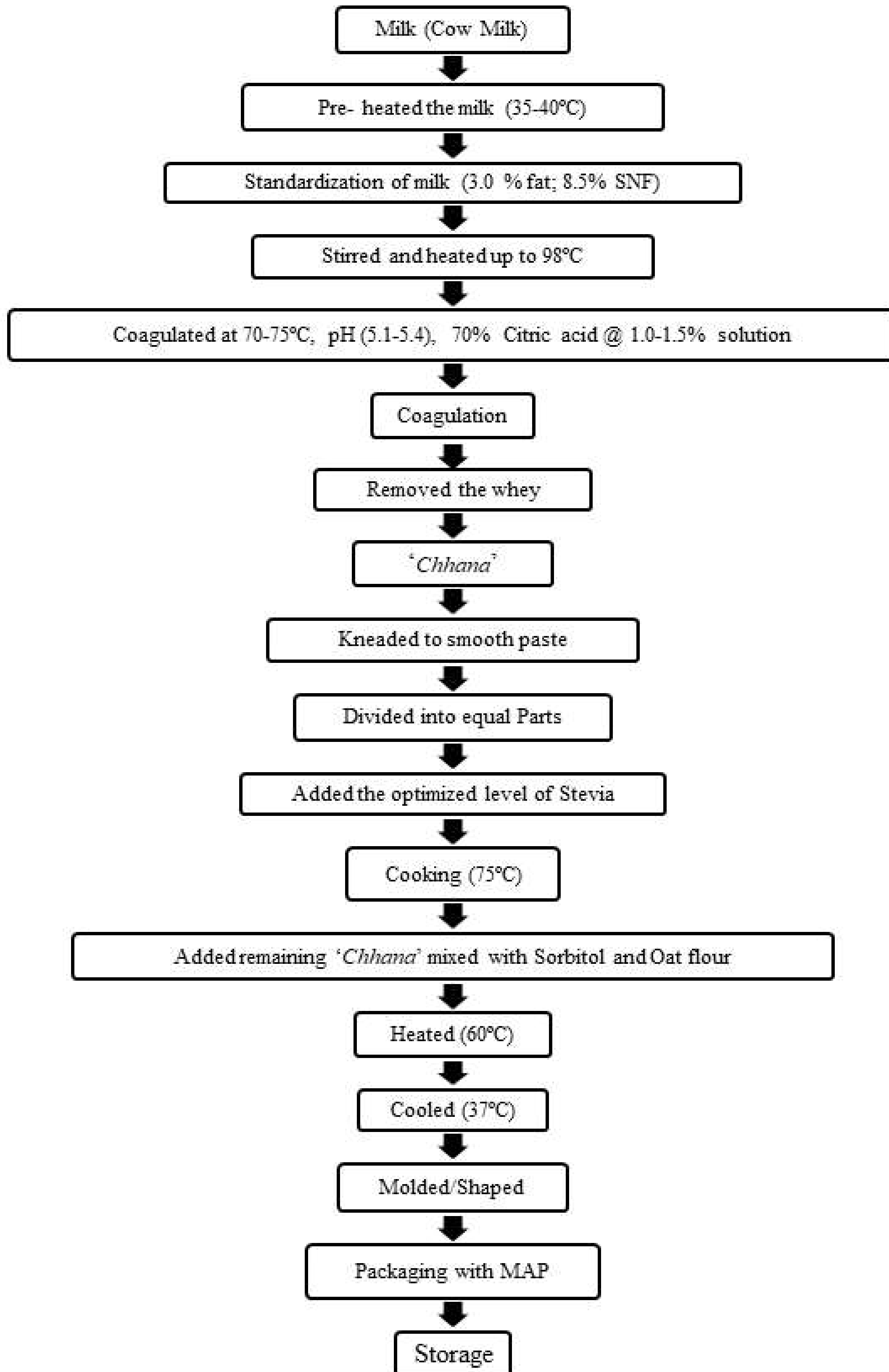


Fig. 1 Flow diagram of low calorie and fiber enriched *sandesh* preparation

that the gas packaged (40% N_2:60%CO_2) crusty rolls with the headspace O_2 concentration never increased beyond 0.05% and the rolls remained mold-free even after 60 days.

Changes in Textural properties

The textural properties of any product are important criteria to determine the acceptability of the product. Table 2 depicts the textural changes in *sandesh* during storage. There was a continuous increase in the hardness of the *sandesh* samples with an increase in the storage period for the control samples and the MAP samples. The hardness varied significantly with variation in the gas composition and the storage temperature. Table 2 shows that the hardness of the *sandesh* samples increased significantly (P<0.05) with an increase in the temperature, the hardness of the samples packed under air increased to a greater extent than those packed under the MAP conditions. The hardness of any product is directly related to its moisture content. It can be observed from Table 2, that the samples packed under 50% N_2: 50% CO_2 showed the minimum changes in hardness, which could be attributed to its better moisture retention under the MAP conditions.

The cohesiveness of a product is the ratio of the area under the second bite curve before reversal compression to under the first bite curve. It is the measure of the extent to which the structure of the product was disrupted during the first compression. The average cohesiveness of the samples remained constant throughout the storage period with no significant variation. Also, change in temperature did not show any significant effect on the cohesiveness during 14 days of storage but varied significantly after 14 days with temperature variation. The cohesiveness increased initially during the storage period, but later it decreased marginally. These observations are similar to those reported by Londhe et al. (2012) in brown *peda* and Jha et al. (2015) in lal *peda*. Loss in moisture content could be the reason for the loss of cohesiveness with the progression of the storage period. Gupta et al. (1990) also reported similar findings in *khoa* with an increase in total solids.

The springiness of a food refers to its ability to return to its original form after compression. It is the height that the product recovers between the first and the second compression. The springiness for *sandesh* samples did not vary significantly (P>0.05) for up to 07 days of storage at all the temperatures in the control and the MAP samples. However, the springiness increased with further increase in storage period and varied significantly (P<0.05) with variation in the storage temperature and gas composition. Jha et al. (2015) reported similar observations in lal *peda* samples stored under MAP conditions.

The adhesiveness of a product is related to its sensory stickiness. Table 2 shows that the average adhesiveness of the *sandesh* samples decreased gradually, with increasing the storage period. The average adhesiveness of the samples had a significant change with an increase in temperature from 25 to 37°C. This decline in the adhesiveness could be due to the decrease in free moisture during the storage. A similar trend in adhesiveness was observed in the lal *peda* samples by Jha et al. (2015).

Gumminess is the product of hardness and cohesiveness. It can be seen from Table 2, that the average gumminess of *sandesh* was significantly (P<0.05) affected by variation in the temperature.

Table 1 Microbial changes in low calorie and fiber enriched *sandesh* during storage packaged under air and under MAP

Days	Atmosphere	Total plate count (log_{10} cfu/g)		Yeast and mold count(log_{10} cfu/g)	
		25 ^{0}C	37 ^{0}C	25 ^{0}C	37^{0}C
0		4.87±0.004	4.87 ±0.04	2.85 ±0.02	2.85 ±0.02
07	Control	5.43 ±0.04^{aA}	5.58 ±0.03^{Aa}	2.94 ±0.03^{aA}	3.12±0.02^{aA}
	98 % CO_2	4.94 ±0.02^{aA}	4.99 ±0.02^{Ab}	2.87 ±0.01^{aA}	3.03±0.02^{aA}
	98 % N_2	4.97±0.03^{aA}	5.08 ±0.04^{Ab}	2.88±0.03^{aA}	3.08 ±0.03^{aA}
	50:50::CO_2:N_2	4.90. ±0.01^{aA}	4.95 ±0.05^{Ab}	2.86±0.02^{aA}	2.93±0.01^{aA}
14	Control	6.69 ±0.02^{aA}	6.82±0.01^{Aa}	3.42±0.02^{aA}	3.55 ±0.03^{aA}
	98 % CO_2	5.01± 0.03^{bA}	5.13 ±0.04^{Ab}	2.95 ±0.02^{bA}	3.19 ±0.02^{bA}
	98 % N_2	5.13 ±0.01^{bA}	5.48 ± 0.03^{Ab}	3.01 ±0.02^{bA}	3.26 ±0.04^{bA}
	50:50::CO_2:N_2	4.96 ±0.02^{bA}	5.16 ±0.03^{Ab}	2.89 ±0.01^{bA}	3.01±0.02^{bA}
21	98 % CO_2	5.38± 0.01^{aA}	5.68 ±0.03^{Ba}	3.09 ±0.03^{aA}	3.19 ±0.02^{aA}
	98 % N_2	5.43 ± 0.03^{aA}	5.81±0.03^{Ba}	3.15±0.01^{aA}	3.26 ±0.04^{aA}
	50:50::CO_2:N_2	5.10 ±0.03^{aA}	5.39 ±0.02^{Bb}	2.94 ±0.03^{aA}	3.01 ±0.02^{aA}
28	98 % CO_2	5.89 ±0.03^{aA}	6.19±0.02^{Aa}	3.18 ±0.03^{aA}	3.28±0.03^{aA}
	98 % N_2	6.01 ±0.04^{aA}	6.21±0.03^{Aa}	3.32 ±0.02^{aA}	3.45 ±0.02^{bA}
	50:50::CO_2:N_2	5.28 ±0.02^{bA}	5.78±0.02^{Ba}	3.12 ±0.03^{aA}	3.19 ±0.02^{aA}

Values are mean ± standard deviation, n=3

* Values with different small superscripts in columns are significantly different (p<0.05) within each interval

* Values with different capital superscripts in rows are significantly different (p<0.05) within each interval

Table 2 Textural changes in low calorie and fiber enriched *sandesh* during storage packaged under air and under MAP

Days	Atmosphere	Hardness		Cohesiveness		Springiness		Adhesiveness		Gumminess	
		25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C
0		3408±0.56	3408±0.56	0.052±0.05	0.052±0.05	0.25±0.02	0.25±0.02	15.60±0.03	15.60±0.03	177.21± 0.84	177.21± 0.84
7	Control	6220±0.36^{aA}	6570±0.43^{aB}	0.059±0.03^{aA}	0.063±0.03^{aA}	0.32±0.03^{aA}	0.35±0.01^{aA}	13.10±0.02^{aA}	12.50±0.04^{aA}	366.60± 0.55^{aA}	410.86±0.61^{aB}
	98% CO_2	4210±0.24^{bA}	4780±0.63^{bB}	0.056±0.03^{aA}	0.059±0.02^{aA}	0.27±0.03^{aA}	0.29±0.03^{aA}	13.70±0.01^{aA}	12.90±0.03^{aA}	235.76±0.46^{bA}	257.39±0.43^{bB}
	98% N_2	4420±0.44^{cA}	5101±0.24^{cB}	0.058±0.01^{aA}	0.061±0.01^{aA}	0.30±0.02^{aA}	0.32±0.05^{aA}	13.60±0.03^{aA}	12.78±0.01^{aA}	256.36±0.84^{cA}	280.62±0.37^{bB}
	50:50::CO_2:N_2	4050±0.65^{dA}	4308±0.58^{dB}	0.053±0.02^{aA}	0.055±0.03^{aA}	0.26±0.01^{aA}	0.28±0.06^{aA}	13.90±0.02^{aA}	13.10±0.02^{aA}	14.65±0.63^{bA}	232.75±0.91^{cB}
14	Control	9500±0.83^{aA}	9814±0.78^{aB}	0.066±0.04^{aA}	0.071±0.03^{bA}	0.38±0.05^{aA}	0.40±0.02^{aA}	12.60±0.03^{aA}	12.30±0.02^{aA}	527.11±0.23^{aA}	582.21±0.47^{aB}
	98% CO_2	5510±0.83^{bA}	5901±0.56^{bB}	0.059±0.02^{aA}	0.061±0.02^{cA}	0.33±0.06^{aA}	0.36±0.03^{aA}	13.60±0.04^{aA}	12.19±0.02^{aB}	336.17±0.47^{bA}	396.01±0.34^{bB}
	98% N_2	5620±0.45^{bA}	6080±0.14^{bB}	0.064±0.03^{aA}	0.065±0.03^{cA}	0.35±0.04^{aA}	0.38±0.02^{aA}	13.50±0.01^{aA}	12.08±0.03^{aB}	377.25±0.34^{cA}	430.31±0.63^{cB}
	50:50::CO_2:N_2	5380±0.64^{cA}	5840±0.86^{cB}	0.054±0.02^{aA}	0.059±0.04^{cA}	0.31±0.02^{aA}	0.32±0.04^{aA}	13.75±0.04^{aA}	12.25±0.04^{aB}	295.03±0.96^{bA}	09.29±0.24^{dA}
21	Control	12100±0.58^{aA}	13200±0.34^{aB}	0.061±0.01^{aA}	0.064±0.05^{aA}	0.54±0.03^{aA}	0.57±0.03^{aA}	9.60±0.04^{aA}	8.90±0.03^{aA}	670.01±0.83^{aA}	702.07±0.64^{aB}
	98% CO_2	6820±0.63^{bA}	7408±0.43^{bB}	0.058±0.05^{aA}	0.060±0.02^{aA}	0.38±0.05^{bA}	0.41±0.02^{bA}	13.20±0.03^{bA}	12.09±0.05^{bB}	415.11±0.39^{bA}	447.22±0.85^{bB}
	98% N_2	6970±0.22^{bA}	7620±0.23^{cB}	0.062±0.06^{aA}	0.063±0.04^{aA}	0.40±0.03^{bA}	0.43±0.02^{bA}	12.70±0.03^{bA}	11.60±0.06^{bB}	435.15±0.46^{bA}	491.09±0.48^{cB}
	50:50::CO_2:N_2	6602±0.36^{cA}	7301±0.42^{dB}	0.053±0.04^{aA}	0.058±0.03^{aA}	0.37±0.02^{bA}	0.40±0.01^{bA}	13.45±0.04^{bA}	12.80±0.04^{bA}	355.02±0.57^{cA}	381.05±0.73^{dB}
28	98% CO_2	9508±0.58^{aA}	9807±0.37^{aB}	0.057±0.02^{aA}	0.059±0.03^{aA}	0.49±0.03^{aA}	0.51±0.04^{aA}	12.80±0.02^{aA}	11.69±0.03^{aA}	580.11±0.64^{aA}	610.13±0.62^{aB}
	98% N_2	10008±0.82^{aA}	12118±0.29^{bB}	0.060±0.03^{aA}	0.062±0.01^{aA}	0.51±0.03^{aA}	0.53±0.02^{aA}	12.05±0.03^{aA}	11.09±0.03^{aB}	597.09±0.53^{aA}	641.11±0.34^{aB}
	50:50::CO_2:N_2	8108±0.57^{bA}	8438±0.61^{cB}	0.052±0.02^{aA}	0.057±0.01^{aA}	0.45±0.02^{aA}	0.48±0.02^{aA}	12.98±0.02^{aA}	12.10±0.05^{aA}	522.21±0.48^{bA}	545.21±0.24^{bB}

Values are mean ± standard deviation, n=3

* Values with different small superscripts in columns are significantly different ($p<0.05$) within each interval
* Values with different capital superscripts in rows are significantly different ($p<0.05$) within each interval

Table 3 Sensory changes in low calorie and fiber enriched *sandesh* during storage packaged under air and under MAP

Days	Atmosphere	Body and	texture	Flavour		Colour and	appearance	Sweetness		Overall acceptability	
		25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C	25 ^{0}C	37 ^{0}C
0		8.40±0.04	8.40±0.04	8.30±0.04	8.30±0.04	8.50±0.04	8.50±0.04	8.40±0.04	8.40±0.04	8.50±0.04	8.50±0.04
7	Control	6.50±0.03^{aA}	4.95±0.03^{aB}	6.00±0.03^{aA}	5.80±0.03^{aA}	6.70±0.03^{aA}	6.50±0.03^{aA}	7.00±0.03^{aA}	4.91±0.04^{aB}	6.70±0.03^{aA}	5.10±0.04^{aB}
	98% CO_2	7.90±0.02^{bA}	7.65±0.02^{bA}	7.60±0.02^{bA}	7.35±0.02^{bA}	8.10±0.02^{bA}	7.90±0.02^{bA}	8.05±0.02^{bA}	7.93±0.02^{bA}	7.86±0.02^{bA}	7.60±0.02^{bB}
	98% N_2	7.80±0.02^{bA}	7.60±0.02^{bA}	7.45±0.02^{bA}	7.20±0.02^{bA}	7.93±0.02^{bA}	7.68±0.02^{bB}	7.96±0.02^{bA}	7.80±0.03^{bA}	7.70±0.02^{bA}	.50±0.03^{bA}
	50:50::CO_2:N_2	8.00±0.03^{bA}	7.80±0.03^{cA}	7.70±0.03^{bA}	7.45±0.03^{bA}	8.21±0.03^{bA}	8.01±0.03^{bA}	8.30±0.03^{cA}	8.20±0.02^{cA}	7.95±0.03^{bA}	.75±0.02^{bA}
14	Control	5.30±0.04^{aA}	5.10±0.04^{aA}	5.93±0.04^{aA}	5.73±0.04^{aA}	6.03±0.04^{aA}	5.83±0.04^{aA}	6.23±0.04^{aA}	5.90±0.03^{aB}	5.90±0.04^{aA}	.85±0.03^{aA}
	98% CO_2	7.50±0.02^{bA}	7.30±0.02^{bA}	7.60±0.02^{bA}	7.24±0.02^{bA}	7.90±0.02^{bA}	7.70±0.02^{bA}	8.04±0.02^{bA}	7.45±0.02^{bB}	7.60±0.02^{bA}	.40±0.02^{bA}
	98% N_2	7.30±0.03^{bA}	7.10±0.03^{bA}	7.20±0.03^{cA}	7.00±0.03^{bA}	7.71±0.03^{bA}	7.56±0.03^{bA}	7.40±0.03^{cA}	7.30±0.04^{bA}	7.40±0.03^{bA}	.20±0.04^{bA}
	50:50::CO_2:N_2	7.70±0.01^{bA}	7.50±0.01^{cA}	7.80±0.01^{bA}	7.65±0.01^{cA}	8.00±0.01^{bA}	7.95±0.01^{bA}	7.60±0.01^{cA}	7.95±0.02^{cB}	7.90±0.01^{bA}	.75±0.02^{cA}
21	98% CO_2	7.21±0.03^{aA}	6.95±0.03^{aB}	7.21±0.03^{aA}	6.91±0.03^{aB}	7.41±0.03^{aA}	7.11±0.03^{aB}	7.40±0.03^{aA}	7.20±0.02^{aA}	7.40±0.03^{aA}	7.00±0.02^{aB}
	98% N_2	7.01±0.01^{bA}	6.80±0.01^{aA}	6.81±0.01^{bA}	6.70±0.01^{bA}	7.21±0.01^{aA}	6.95±0.01^{aB}	7.11±0.01^{aA}	6.90±0.02^{aA}	7.21±0.01^{aA}	6.72±0.02^{aB}
	50:50::CO_2:N_2	7.41±0.02^{cA}	7.17±0.02^{aA}	7.50±0.02^{cA}	7.20±0.02^{aA}	7.60±0.02^{aA}	7.36±0.02^{bB}	4.96±0.02^{bA}	4.81±0.03^{bA}	7.65±0.02^{bA}	7.20±0.03^{aB}
28	98% CO_2	6.80±0.01^{aA}	6.68±0.01^{aA}	6.80±0.01^{aA}	6.70±0.01^{aA}	6.90±0.01^{aA}	6.80±0.01^{aA}	6.50±0.01^{aA}	6.22±0.04^{aB}	6.38±0.01^{aA}	.20±0.04^{aA}
	98% N_2	6.53±0.03^{bA}	6.43±0.03^{bA}	6.63±0.03^{aA}	6.43±0.03^{bA}	6.73±0.03^{aA}	6.53±0.03^{bA}	5.33±0.03^{bA}	5.00±0.03^{bB}	6.23±0.03^{aA}	5.95±0.03^{bB}
	50:50::CO_2:N_2	7.10±0.03^{cA}	6.90±0.03^{cA}	7.00±0.03^{bA}	6.90±0.03^{aA}	7.20±0.03^{aA}	7.00±0.03^{aA}	6.80±0.03^{aA}	6.60±0.03^{aA}	6.70±0.03^{bA}	6.40±0.03^{aB}

Values are mean ± standard deviation, n=10

* Values with different small superscripts in columns are significantly different ($p<0.05$) within each interval
* Values with different capital superscripts in rows are significantly different ($p<0.05$) within each interval

It was lower in samples stored at 25°C, which gradually increased with an increase in the storage temperature to 37°C. The variation in gas composition had a significant (P<0.05) effect on the gumminess of *sandesh* sample during the storage period.

Changes in sensory attributes

The average score for all sensory attributes decreased significantly (P<0.05) with the storage period. The control samples were found to be unacceptable after 14 days, hence discarded from the further sensory evaluation. The samples packed under 50% N_2:50% CO_2, scored the maximum among all the samples for all the sensory properties at their respective storage temperatures and storage periods. The sensory scores for body and texture, flavor, color and appearance, sweetness and overall acceptability varied significantly (P<0.05) among MAP samples during storage at every temperature (Table 3). An increase in the storage temperature had a negative effect on the sensory score of the product and this can be deduced from the fact that the samples stored at 37°C showed lower sensory score as compared to samples stored at 25°C. The samples stored at 25°C packed under 50% N_2:50% CO_2 showed the highest sensory characteristics of the samples.

Conclusions

An attempt was made to evaluate the shelf life of *sandesh* stored under air and MAP with variation in gas composition viz. 98% N_2, 98% CO_2 and 50% N_2:50% CO_2 during two different storage temperatures (25 and 37°C). The samples stored under air had a short shelf life as compared to samples stored under MAP. Therefore, the MAP could be considered as a better option for storage of *sandesh*. The samples packed under 50% N_2:50% CO_2 was optimally effective in preserving the microbial and textural properties of *sandesh*. During the storage period, the textural property of optimized products also varies significantly. The hardness of the product increases significantly as the storage time increases and was more prominent at 37°C due to greater loss of moisture as compared to the storage temperature of 25°C. Gumminess and springiness also increase over the storage period and the adhesiveness of the experimental product decreases during the storage at both storage temperature. This study could prove to be helpful in the preservation of other traditional dairy products using MAP.

References

Amerine MA, Pangborn RM, Roessler EB (1965) Principles of sensory evaluation of foods. Academic Press, London

Bandyopadhyay M, Chakraborty R, Raychaudhuri U (2008) Antioxidant activity of natural plant sources in dairy dessert (Sandesh) under thermal treatment. LWT-Food Sci Technol 41: 816- 825

Bandyopadhyay P, Khamrui K (2007) Technological Advancement on Traditional Indian Desiccated and Heat Acid Coagulated Dairy Products. Bulletin-International Dairy Federation 415:1-7

Banks HJ, Annis PC (1990) Comparative advantages of high CO_2 and low O_2 types of controlled atmospheres for grain storage. In: Calderon M, Barkai-Golan R (eds) Food preservation by modified atmospheres. CRC Press Inc, Boca Raton, pp 93-122

Chowdhury T, Chattopadhyay SK, Saha NC (2017) Modified atmosphere packaging (MAP) and the effect of chemical preservative to enhance shelf life of khoa (heat desiccated milk product). J Packag Technol Res 1: 25-31

Daniels JA, Krishnamurthi R, Rizvi SSH (1985) A review of effect of carbon dioxide on microbial growth and food quality. J Food Prot 48:532-537

Davis HK (1998) Fish and shellfish. In: Blakistone BA (ed) Principles and applications of modified atmosphere packaging of foods, 2ndedn. Blackie Academic & Professional, London, pp 194-239

Devlieghere F, Debevere J (2000) Influence of dissolved carbon dioxide on the growth of spoilage bacteria. LWT-Food Sci Technol 33:531–537

Floros JD, Matsos KI (2005) Introduction to modified atmosphere packaging. In: Han JH (ed) Innovations in food packaging. Elsevier Ltd, London, pp159–172

Gawande H, Shendurse A, Dhotre A (2012) Low calorie traditional milk sweets in India: A review. Indian Food Ind 31: 43-51

Gupta SK, Patil GR, Patel AA, Garg FC, Rajorhia GS (1990) Instron texture profile parameters of khoa as influenced by composition. J Food Sci Technol 27:209–213

Husain SA, David J (2018) Studies on sensory attributes of herbal sandesh by incorporation of ashwagandha (*Withaniasomnifera*) and tulsi (*Ocimum sanctum*) at room temperature. J Pharmacogn Phytochem7: 2567-2571

Jha A, Kumar A, Jain P, Gautam AK, Rasane P (2015) Effect of modified atmosphere packaging on the shelf life of lal *Peda*. J Food Sci Technol 52: 1068-1074

Kumar A, Singh SS (2017) Preparation and quality assessment of *Chhanapodo* prepared by using buffalo, coconut and soy milk. Pharm Innovation J 6: 809- 814

Kumbhar SB, Ghosh JS, Samudre SP (2009) Microbiological analysis of pathogenic organisms in indigenous fermented milk products. Advance J Food Sci Technol 1:35-38

Londhe GK, Pal D, Raju PN (2012) Effect of packaging techniques on shelf life of brown peda, a milk-based confection. LWT- Food Sci Technol 47:117–125

NDDB (2017) National Dairy Development Board, Gujrat, India. http,// www.nddb.org/information/stats/milkprodindia

Saha N, Bhattacharjee S, Bhattacharyya S (2018) Preparation and pharmacognostic evaluation of sandesh, an Indian sweet dairy product, using natural colorant from *Clitoria ternatea* (Aparajita) flower. Int J Food Sci Nutr 3: 19-24

Sandhya (2010) Modified atmosphere packaging of fresh produce: current status and future needs. LWT- Food Sci Technol 43:381–392

Smith JP, Ooraikul B, Koersen WJ, Jackson ED, Lawrence RA (1986) Novel approach to oxygen control in modified atmosphere packaging of bakery products. Food Microbiol 3: 315–320

Snedecor GW, Cochran WG (1989) Statistical methods, 8th edn. IowaState University Press, Ames

Thippeswamy L, Venkateshaiah BV, Patil SB (2011) Effect of modified atmospheric packaging on the shelf stability of paneer prepared by adapting hurdle technology. J Food Sci Technol 48: 230–235

Indian J Dairy Sci 74(1): 80-88
https://doi.org/10.33785/IJDS.2021.v74i01.011

RESEARCH ARTICLE

Formulation of pea milk fortified *dahi* incorporated with jamun seed extract

Shikha Pandhi and Amrita Poonia

Received: 05 July 2020 / Accepted: 08 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Changing market trend have paved the way for development of value-added products fortified with plant-based constituents for supplementing their therapeutic potential. In sight of this, fortified dairy products have gained immense attention in recent times. The present study was undertaken with an aim to fortify animal-based nutrients with plant-based nutrients by developing a pea milk fortified *dahi* incorporated with jamun seed extract. A ratio of 5:1 (5 parts of water and 1 part of pea) for pea milk formulation and 3:2 (3 parts of pea milk and 2 parts of skim milk) for fortification of milk was used for *dahi* preparation. The developed product exhibit good per cent antioxidant activity of 80.1±8.84 and total phenolic content of 98.33±15.56 mg of gallic acid equivalent (GAE)/ml of *dahi* sample with an overall acceptability of 8.6 on 0 day followed by an overall acceptability score of 6.2 on 12th day of storage which is the predicted shelf life of the product with successive decline in per cent antioxidant activity and total phenolic content beyond this period. Other parameters such as water holding capacity, viscosity and syneresis were found to be 60.35 ±1.34 %, 38±0.2cP and 8.2±0.4 ml/100g, respectively. Physicochemical parameters and textural analysis was also performed. The final cost of product was estimated to be Rs.13 per cup (100g) which is quite comparable to the market *dahi*.

Keywords: *Dahi*, Fortified, Jamun seed extract, Pea milk

Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221 005, Uttar Pradesh, India

Amrita Poonia (✉)
Department of Dairy Science and Food Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India
Phone:9644305305
Email: dramritapoonia@gmail.com

Introduction

The conception of novel idea of functional food has paved the demand for development of food products that not only provides basic nutrition, but also affirms goof health and longevity (Fazila et al. 2018). *Dahi* is a fermented product obtained after fermentation of milk by addition of starter culture to offer easily digestible nutrients with potential therapeutic effects (Sandhya et al. 2018). This has made *dahi* a preferred product to be explored from a functional food aspect. In view of this, the researchers are continuously investigating different functional ingredients for their potential beneficial effects such as probiotics, prebiotics, plant extracts etc (Fazila et al. 2018). Plant consists of an array of bioactive components known for their potential therapeutic and health-promoting effects (Naczk and Shahidi, 2006). The incorporation of these plant constituents known as "phytochemicals" in to various food products is a leaning trend in the current market. They have known to exhibit high antioxidant, anticarcinogenic, antimicrobial, anti-atherogenic, anti-inflammatory (Zimmer et al. 2012), antidiabetic and antihypertensive activities (Yadav et al. 2018). Incorporation of these phytochemicals into dairy products has received considerable attention in the food industry to improve both nutritional and functional value of the product.

Syzygium cumini (commonly known as "jamun") is a very large evergreen tropical tree belonging to the family *Myrtaceae* (Pandhi and Poonia, 2019). The presence of various phyto-constituents contributes to the anti-diabetic, antioxidant and/or antimicrobial activity. Jamun seed powder has been used for centuries as a natural form for balancing the healthy blood sugar level and is the most studied plant part. They are reported to contain jamboline, gallic acid, ellagic acid, corilagin, 3,6-hexahydroxy diphenoylglucose, 4,6-hexahydroxydiphenoylglucose,1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol (Baliga et al. 2011). The glycoside, named Jamboline is mainly responsible for maintenance of glucose levels as in the normal limits (Desai et al. 2019). Further, the emerging demand for alternative protein sources apart from animal milk have necessitates the exploration of plant based protein sources. Soybean milk has been effectively explored for development of various commercial products due to

its distinct characteristics such as ability to bind calcium. In sight of this, the other important crop that is emerging is- yellow peas. Pea is among the oldest food legume crops that are recognized as an inexpensive and readily available source of protein, complex carbohydrates (especially starch), vitamins and minerals. The higher nutrient density of dry peas makes them a valuable food commodity that is capable of fulfilling the dietary requirements of millions of undernourished individuals worldwide (FAOSTAT, 2011). Dry peas possess many functional and nutritional properties and are generally consumed as dhal, stews, snacks, vegetables and flour. Various nutrients present in dry peas may help in lowering the risk of heart diseases, stroke, and various cancers, while enhancing quality of life by helping manage weight. The soluble fiber in dry peas and low glycemic index may help stabilize blood sugar levels, which is especially important for people with diabetes (Parihar et al. 2016). Pea's essential proteins can be extracted as pea milk using different method and can be used to fortify animal milk in different ratios as a "new raw material" (Denkova et al. 2013).

In view of the increasing consumer interest towards improving their overall health and reducing the risk of specific disorders provide great opportunities for expanding dairy based products to provide benefits beyond their traditional nutritional value. Present study aims at developing a pea milk fortified *dahi* incorporated with jamun seed extract for combining the benefits of peas with milk.

Materials and Methods

Fresh skim milk, dried jamun seeds (*Syzygium cumini*) and splitted yellow peas were procured from local market of Varanasi. Household *dahi* inoculums were used as a culture. Dried jamun seeds were grounded into a coarse powder. The packaging material used for packaging of the *dahi* was polypropylene cups that were covered on top with cellophane.

Preparation of jamun seed extract

25 grams of jamun seed powder was mixed with 100ml of ethanol and ultrasonicated for 40 sec at 20 KHz using a probe sonicator. It was then subjected to shaking for 3 hours and then filtered using Whatman paper no.1. The filtered extract was then concentrated using a vacuum rotary evaporator at 50 C and stored at 4-5 C for further use (Pandhi and Poonia, 2019).

Determination of total phenolic content

0.5ml of diluted extract was added to 2.5ml of 0.2N Folin-Ciocalteau reagent and placed for 5 minutes. 2ml of 75g/L of Na_2CO_3 was then added. The above solution was then kept for incubation at room temperature for 2 hours. Absorbance was measured at 760nm using 1cm cuvette UV-1800 spectrometer (Shimadzu, Japan). Gallic acid (0-800mg/L) was used to produce a standard calibration curve. The total phenolic content was expressed in mg of Gallic acid equivalent (GAE)/ml of extract after applying the dilution factor (Stankovic, 2011).

Radical scavenging activity (% DPPH inhibition)

0.5 ml of ethanol extract of jamun seed was taken and to it 2.5mL of DPPH solution (8mg/100mL ethanol) was added. A control was set up with 0.5ml distilled water as blank and left at room temperature for 30 min. The sample was centrifuged at 3000 rpm for 15 min. In cuvette, 0.5 ml of centrifuged solution was taken and to it 1mL of ethanol was added. Absorbance was taken at 517 nm separately for blank and sample using ethanol as a reference (Nishino et al. 2000).

% DPPH inhibition = $(A_B - A_S/A_B) \times 100$

Where,

A_B = OD for blank A_S = OD for sample

Formulation of pea milk based *dahi* fortified with jamun seed extract

Preparation of pea milk

Pea milk was prepared by adopting single stage extraction technique with slight modification (Upadhyay et al. 2011). Pea milk having water to pea ratio of 5:1 was prepared by first adding 1 kg pre-processed/treated pea with 2 L water in the grinder for grinding pea for at least 10 min followed by addition of remaining 3 L of water. The obtained mixture was allowed to filter through a muslin cloth and the filtrate was allowed to stand for 2 hours for settling of starch. Collect the upper water fraction as pea milk for further use. The settled starch was separated, dried and collected as a by-product.

Preparation of control dahi

Skim milk was taken, heated to 85°C and then cooled to 40°C. Milk was inoculated with *dahi* inoculum @ 2 % and then 100 ml of it was transferred to each cup. The samples were incubated at 40°C in incubator till the proper setting of *dahi (*Rathi et al. 2013).

Preparation of pea milk fortified dahi incorporated with jamun seed extract

Pea milk fortified *dahi* was prepared according to the method given by Rathi et al. (2013) with slight modification (Figure 1). Pea milk of ratio 5:1 was combined with skim milk in the ratio 3:2 (3 parts of pea milk and 2 parts of skim milk) and total solids of pea milk mixture was adjusted to 16 % by using (4%) skim milk powder, heated to 85ÚC and then cooled to 40°C. 1.5% of 10 times diluted extract was added and then pea extract combined milk was inoculated with household *dahi* @ 2 % and then 100 ml of it was transferred to polystyrene cups. The samples were

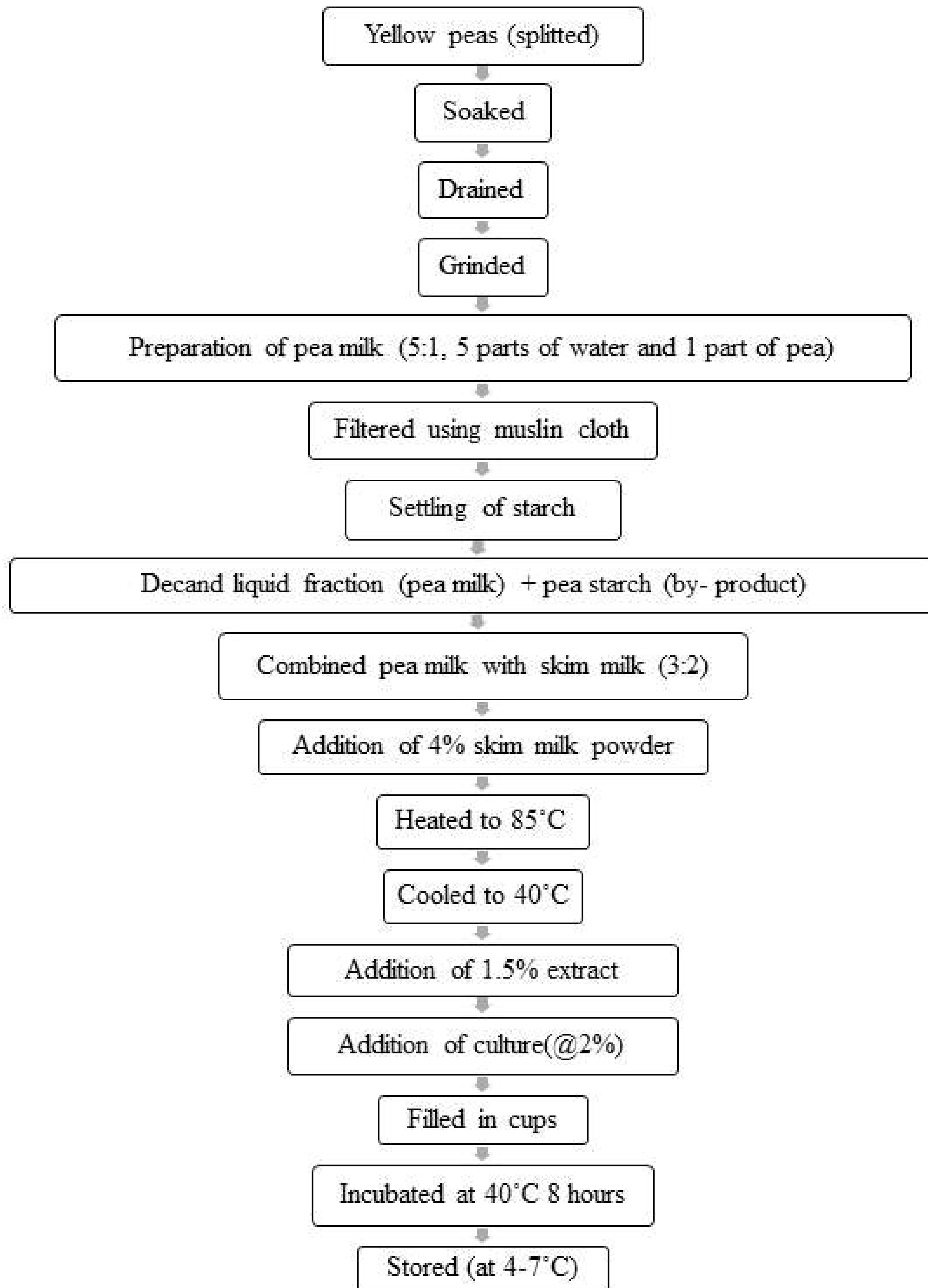


Fig. 1 Flow diagram for preparation of pea milk fortified *dahi* incorporated with jamun seed extract (JSE)

incubated at 40°C in an incubator till the setting of *dahi* (Figure 2).

Physicochemical analysis

Different physicochemical parameters such as % fat, % Titratable acidity, % Protein, % ash content, viscosity and pH were determined as per the methods described by Rangana (2001).

Fig. 2 Pea milk fortified *dahi* incorporated with jamun seed extract

Fig. 3 Showing syneresis in pea milk fortified *dahi* incorporated with jamun seed extract

Color measurement

L-a-b values for *dahi* were determined using a Hunter color system, ColorFlex EZ model which was first calibrated using a white tile and black tile. It measures color using a three-dimensional scales, such as CIE L*a*b*, have been developed to objectively quantify color values. This scale defines color as follows: L* (lightness) axis: black to white (0 to 100) a* (red green) axis: positive values are red; negative values are green; 0 is neutral b* (yellow – blue) axis: positive values are yellow; negative values are blue; 0 is neutral. All visible colors can be quantified within this 3-D rectangular space.

Determination of flavonoid content

10 grams of *dahi* sample was repeatedly extracted with 100 ml of 80% aqueous methanol at room temperature. The mixture was then filtered through a filter paper into a pre-weighed 250 ml beaker (Adham, 2015). The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The percentage flavonoid was calculated as:

$$\%\,Total\ Flavonoids = \frac{Weight\ of\ residue(g)}{Weight\ of\ sample\ taken(g)} \times 100$$

Water holding capacity (WHC) and syneresis

20 grams of *dahi* sample was taken in a 50 mL centrifuge tube and centrifuged at 2000 rpm for 10 minutes. The weight of clear whey separated was measured and the water holding capacity (WHC) was calculated using the formula (Remeuf et al. 2003):

$$\%\,WHC = \frac{ND - WD}{ND} \times 100$$

Where, ND=weight *dahi* WE = weight of expelled whey.

For syneresis, *dahi* in the container (100 g) was cleanly cut crosswise with a spatula and around the edge with a thin spatula. A stainless steel wire grid (8 mash/cm^2) slightly smaller than the inside diameter of the container, was placed gently on the surface of *dahi*. At 30 min interval, the volume of whey obtained was poured off into a 10 ml measuring cylinder, keeping wire grid in place with the help of a spatula, to prevent the *dahi* from falling out and volume of whey was noted (Marshall, 1982) (Figure 3).

Texture analysis

Textural parameters were analyzed using Texture Analyzer (TA.XT*plus* texture profile analyzer, Stable Micro System Ltd, Model TA-XT plus, UK) was calibrated using a 50 kg weight and the outlined in data acquisition software (Exponent Lite XT PLUS, Ver.4.0.13.0 lite). Back Extrusion Cell (A/BE) with 35 mm disc and extension bar using 5 kg load cell was used. Tests were carried out in standard size back extrusion container (50 mm diameter) with a test speed of 1.00 mm/sec and return speed of 5.00 mm/sec. Trigger force was taken to be 5g with 80mm return distance and 30mm distance. Tests were carried out either in a standard size back extrusion container (50 mm diameter) approx. 75% full

or in the original container immediately after the removal from storage. The extrusion disc should be positioned over the sample container. For comparison of cohesiveness and "work of cohesion", the probe must return to the same position above the samples after each test. To do this, it is necessary to calibrate the probe to a distance that is a starting distance, e.g. 30mm above the top of the container of the sample surface. When the probe is returning to the start (i.e. pulling out of the sample), it is recommended that the container is held to prevent it from lifting. Four factors that were analyzed are cohesiveness (g), consistency (g sec), Index of viscosity (g sec), firmness (g).

Sensory analysis

The sensory analysis was carried out using a 9- point hedonic scale by serving experimental sample and control sample to a panel of 11-judges from the Department of Dairy Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. The sensory parameters that were studied include color, body and texture, flavor, acidity and overall acceptability. The 9-point hedonic rating scale was arranged such that: Like extremely-9, Like very much-8, Like moderately-7, Like slightly-6, Neither like nor dislike-5, Dislike slightly-4, Dislike moderately-3, Dislike very much-2, Dislike extremely-1 (Amerine et al. 1965). The scores rated by the panel of judges were then statistically analyzed. The samples were code numbered to avoid identification and bias.

Statistical analysis

The data obtained for various physicochemical, textural and sensory parameters in the present study were analyzed for the significant difference in the treatments by subjecting to the analysis of variance (ANOVA) technique (one way) using Minitab 17 software.

Storage study

The *dahi* samples were stored at refrigeration temperature (4 – 7°C) and were drawn at 3days interval up to 21 days. The samples were analyzed for change in pH, % TA, sensory evaluation scores, Total phenolic content and % DPPH during storage using the same method indicated above with 0.5 ml of *dahi* sample. Also, the estimation of the shelf life of the product was determined until it retains all its sensory, nutritional properties. Stability of jamun seed extract in the product during storage can be determined by analyzing the total phenolic content, DPPH inhibition at different time intervals during storage. The microbiological analysis of the developed value-added *dahi* product was carried out as per the standard method for coliforms, yeast and molds using the violet red bile agar (VRBA) or potato dextrose agar (PDA), respectively (Kumbhar et al. 2009). 1g of the sample was diluted in 9ml of buffer blanks and subsequent dilutions were prepared up to 10 dilutions.

The number of microbial counts was calculated using the following formula:

$$\text{No. of Microorganisms/g} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Weight of aliquot taken(ml)}}$$

Cost estimation

The cost economics of the developed *dahi* was estimated by taking into account the cost of raw materials, processing cost and labor cost, i.e, fixed and variable cost.

Table 1 Moisture content and total solids content of pea extract

	% Total solids	% Moisture content
Pea extract	5.14±0.5	94.86±0.5
Pea extract + skim milk	9.42±1.4	90.58±1.4
Pea extract + skim milk + 4% SMP	15.94±2.0	84.06±2.0

Values are mean ± standard deviation, n=3

Table 2 Physicochemical parameters for pea milk fortified *dahi* incorporated with jamun seed extract

Parameters	Control *dahi*	Pea *dahi*
% Total solids	15.55±2.4	15.9±3.2
% Fat	0.1±0.00	0.1±0.00
% Protein	5.4±0.11	5.8±0.18
% Ash	1.09±0.02	0.76±0.08
pH	4.41±0.04	4.50±0.8
Viscosity(cP)	42±0.20	38±0.20
% Titratable acidity	0.80±0.10	0.82±0.02

Values are mean ± standard deviation, n=3

Results and discussion

Total phenolic content & antioxidant activity (% DPPH inhibition) of extract

Using the standard curve, the total phenolic content of the extract was determined and was found to be 425.90±15.2 mg of gallic acid equivalent/mL of extract. The results indicate the presence of higher total phenols with high yield of 12.76±1.45%. UAE (Ultrasonicated assisted extraction) was considered to be more effective than other conventional extraction because of its high efficiency and efficacy in the extraction of various bioactive constituents from the inactive solid matrix without any detrimental effects (Cho et al. 2006). The total antioxidant activity of the ethanolic extract was found to be 95.83±0.50%. The ultrasonication process involves collapse of bubbles that cause physical, chemical or mechanical disruption of the biological membrane of the extractable compound and hence, provides better efficiency and efficacy (Cares et al. 2009; Metherel et al. 2009). Higher phenolic content usually conveys higher antioxidant activity (Margaret et al. 2015).

Formulation of pea milk

Pea milk formulated with water to pea ratio of 5:1 (optimized ratio) was subjected to determination of moisture content and total solid content and results are indicated in Table 1. Skim milk powder @ 4 % was added to pea milk fortified milk to adjust total solid content to 16% as this percent of total solid is required for desirable body and texture of *dahi* (Chowdhury and Bhattacharyya, 2014).

Physicochemical analysis

Various physicochemical parameters of formulated *dahi* were determined and the results are tabulated in Table 2.

Texture analysis (TA)

The average values for the four texture parameters such as consistency, cohesiveness**,** index of viscosity, firmness were found to be 5419.2± 45.1, 86.16 ± 3.56, 176.56 ± 2.99, 219.055 ± 4.34 respectively. The values of consistency and firmness of *dahi* sample were found to be lower than that of control with consistency and firmness of 6252.1± 42.6 and 325.025 ± 3.31 respectively. This is in accordance with the study conducted by Sivakumar and Dhanalakshmi (2015) who also observed reduction in firmness and consistency of *dahi* on addition of plant extract. They suggested that plant extract might have showed inhibitory action on starter culture, thus delaying the curd formation process and affecting the overall texture of the final product.

Table 3 Sensory evaluation of developed product

Parameter	Sample	Control
Color	7.9±0.9	8.7±0.7
Flavor	7.6±1.2	8.9±0.6
Body and texture	8.3±0.7	8.4±0.3
Consistency	8.6±0.7	8.5±0.6
Acidity	7.1±0.6	7.8±0.2

Values are mean ± standard deviation, n=11

Water holding capacity (WHC) and syneresis

Water holding capacity of *dahi* relates to the ability of its constituents to hold water within *dahi* matrix. The % water holding capacity of the formulated *dahi* was found to be 60.35 ±1.34. There was a significant difference ($p<0.05$) between the water holding capacity of a control sample of *dahi* which was 70.23±1.56, the reason may be higher ability of milk protein to hold more water as compared to pea protein in combination with milk proteins (Table 3). Syneresis is the separation of whey from the curd and expressed as ml as depending on the method followed. The extent of syneresis is indicative of the quality of *dahi*. Consumers prefer *dahi* with no visible whey separation. Presence of separated whey indicates excessive fermentation and stored *dahi*, lacking consumer acceptance in the market. Syneresis for formulated *dahi* was found to be 8.2±0.4 ml/100g whereas, for control sample, it was found to be 7.8±0.4ml/100g which was lower than the formulated product, this could be due to the comparatively lower water holding capacity of the formulated product due to variation in the composition. As per the findings, the addition of plant extract containing polyphenols appears to encourage the contraction of protein matrix and aids the dismissal of whey, decreasing the amount of entrapped water in protein matrix (Masmoudi et al. 2020). The difference in the acidity could also be a major contributor of disruption of protein network (Ranadheera et al. 2012),

Color estimation

The L-a-b values for the formulated product was found to be L= 83.85±0.42, a =0.37±0.01, b =14.21±0.02. The L- value was more on the higher side indicating the lightness of the product. Low + a value indicate a tinch of red color in the product, + b indicates the color of the products to be towards yellow. The overall color of the product can be expressed as light yellow with a very light tint of red color. Addition of plant extract slightly lowers down the lightness of the product and results in slight shift toward darker side (Shokery et al. 2017).

Sensory analysis

The data obtained by sensory evaluation in the present study reveals that there was no significant difference between the consistency and body and texture of developed *dahi* and the control *dahi* the reason for this could be the similar level of % total solids in both which was the major contributor towards the body and texture. Color, flavour and acidity of the *dahi* were found to be significantly affected by the incorporation of extract

Table 4 Effect of storage period on the shelf life of *dahi* for 21days

Parameters	0 Day	3 Day	6 Day	9 Day	12 Day	15 Day	18 Day	21 Day
pH	4.50±0.8	4.40±0.4	4.31±0.5	4.11±0.8	4.05±0.7	3.90±0.4	3.82±0.2	3.2±0.4
% Titratable acidity	0.82±0.02	0.86±0.02	0.91±0.05	0.98±0.08	1.02±0.03	1.20±0.06	1.32±0.08	1.34±0.06
% DPPH inhibition	80.1±8.84	78.5±11.45	74.23±14.67	1.23±5.67	62.8±14.65	57.4±12.56	54.6±10.32	54.3±11.23
Total phenolic content (mg of gallic acid equivalent /mL of sample)	98.33±15.56	92.45±18.45	90.45±8.84	88.56±13.41	84.34±9.55	80.34±10.23	77.45±12.32	64.02±12.13
Yeast and mold count per gram	0	0	6	10	11	16	20	28
Coliform count per gram	0	0	0	0	0	0	0	0
Overall acceptability	8.6±0.4	8.2±0.2	7±0.3	6.8±0.2	6.2±0.4	5.5±0.3	5±0.4	4±0.2

Values are mean ± standard deviation, n=3

Table 5 Cost estimation of the developed product

Ingredient	Amount/ 100g cup	Cost (Rs.)
Yellow peas	12g	0.6
Jamun seed extract	1.5 ml	1.5
Skim milk	40 ml	2.8
Skim milk powder	4g	1.33
Packaging cost	1 cup+ aluminium foil	1.0
Inoculum	1.5g	0.165
	Total	7.39
Processing cost	Per cup	1.0
	Total	8.39
Marketing and distribution expenses @25% of product	Per cup	2.097
	Total	10.48
Profit margin @ 30% of cost of product	Per cup	2.517
	Total cost (per 100g cup)	13 Rs.

and fortification of pea milk in the *dahi.* The sensory score for flavour of developed *dahi* was lower as compared to the control, the reason for this could be the beany flavour that might have affected the score. The incorporation of extract and pea milk has contributed towards a yellowish color of the developed product that may have resulted in the lower sensory score for color parameter in the developed product. Further, the difference in the score for acidity might also be attributed by the incorporation of extract and pea milk in the product (Table 3). Addition of any herbal extract into a product or modification of its composition could be the sole reason for the lowered sensory scores by panellists (Bajalanlou and Pakbin, 2016)

Storage study

Storage study of formulated *dahi* was conducted with *s*amples which were seal packed in polyethylene bags and were store at 4-5°C during the whole storage period. The parameters that were studied during the storage study were pH, % titratable acidity (%TA), % DPPH inhibition, total phenolic content and overall acceptability. During storage of *dahi* sample pH had shown a decreasing trend in value whereas an increasing trend was observed in % TA. The justification for the observed trend was increased lactic acid concentration that simultaneously decreases the pH of *dahi*. The functional constituents such as phenols had shown a gradual decrease in concentration relating to loss of

phenols during storage. Loss of phenols during storage affect the antioxidant property hence a decreasing pattern was observed for % DPPH inhibition. From microbiological analysis, it was found that the coliform count of *dahi* was nil throughout the storage period. Yeast and mold count was also found to be less than the standard value of 100/g (Table 4). Considering all these parameters, the extent to which these parameters ensure safety and quality of product was chosen to be the estimated shelf life. A shelf life of 12 days was estimated for the product, taking into account the maximum possible concentration of phenols with almost all other quality and safety aspects were maintained. Yadav et al. (2007) evaluated low-fat probiotic *dahi* prepared with *Lactococci* starters and 2 adjunct probiotic cultures of *Lactobacillus acidophilus* and *Lactobacillus casei* under refrigerated storage at 7°C and found that *dahi* stored for 8 days may be acceptable to consumers. Parseeda and Nanu (2007) investigated changes in quality of curd stored under refrigeration (4–11°C) for a period of 21 days with respect to acidity, pH, and sensory parameters. The results indicated that the product had a shelf life of 9 days under refrigerated storage (Table 4).

Cost estimation

The estimated cost of the developed product was found to be Rs. 13 per 100g cup which is quite comparable to the commercial *dahi* product available in the market which generally cost Rs 20 or 22 per 200g of cup (Table 5). Hence, from the economy point of view the product was quite feasible.

Conclusion

Pea milk fortified *dahi* incorporated with jamun seed extract was successfully developed with an estimated shelf life of 12 days under refrigerated storage. The formulated product was developed in view of the increasing demand for products supplemented with plant based nutrients and phytonutrients. The developed product might be suitable for diabetic people as it contains phytonutrients extracted from jamun seeds and had shown higher antioxidant activity and total phenolic content. The developed product was found to be good in terms of body and texture but has a slightly lower liking for sensory parameters such as flavor and color. The estimated cost of the product was also comparable to the market *dahi.* Further, the pea starch collected as a by-product of pea milk can be dried and can be put forward for further useful applications such as a food thickener or as a base material for development of starch based edible film for food packaging.

References

Adham AN (2015) Comparative extraction methods, phytochemical constituents, fluorescence analysis and HPLC validation of rosmarinic acid content in *Mentha piperita*, *Mentha longifolia* and *Osimum basilicum*. J Pharmacogn Phytochem 3: 130-139

Amerine MA, Pangborn RM, Roessler EB (1965) Principles of sensory evaluation of foods. Academic Press, London.

Bajalanlou F, Pakbin B (2016) Chemical properties and sensory evaluation of probiotic yoghurt manufactured with aqueous extract of aloevera. J Biol Today's World 5: 197-202

Baliga MS, Bhat HP, Baliga BRV, Wilson R, Palatty PL (2011) Phytochemistry, traditional uses and pharmacology of *Eugenia jambolana* Lam.(black plum): a review. Food Res Int 44: 1776-1789

Cares MG, Vargas Y, Gaete L, Sainz J, Alarcon J (2009) Ultrasonically assisted extraction of bioactive principles from *Quillaja Saponaria Molina*. Physics Procedia 3: 169-178

Cho YJ, Hong JY, Chun HS, Lee SK, Min HY (2006) Ultrasonication assisted extraction of resveratrol from grapes. J Food Eng 77: 725-730

Chowdhury SR, Bhattacharyya AK (2014) Production, characterization and value addition of dahi made from raw, pasteurized and double pasteurized milk. Int J Res Eng Technol 3: 602-607

Denkova Z, Yanakieva V, Denkova R, Dobrev I, Kozludzhova S (2013) Examining the possibilities for application of pea milk in obtaining fermented probiotic foods. . ÍÀÓ×ÍÈ ÒÐÓÄÎÂÅ 53: 31-35

Desai GB, Kshirsagar RB, Sawate AR (2019) Effect of jamun (*Syzygium cumini*) seed powder extract on physico-chemical and organoleptic properties of beverage. The Pharma Innovation J 8: 1003-1010

FAOSTAT (2011) Food and Agriculture Organization Statistics. Food security data and definitions 2005–2007.

Fazilah NF, Ariff AB, Khayat ME, Rios-Solis L, Halim M (2018) Influence of probiotics, prebiotics, synbiotics and bioactive phytochemicals on the formulation of functional yogurt. J Functional Foods 48: 387-399

Kumbhar SB, Ghosh JS, Samudre SP (2009) Microbiological analysis of pathogenic organisms in indigenous fermented milk products. Adv J Food Sci Technol 1: 35-38

Margaret E, Shailaja AM, Venugopal Rao (2015) Evaluation of antioxidant activity in different parts of *Syzygium cumini (Linn*.). Int J Curr Microbiol Appl Sci 4: 372-379

Marshall RJ (1982) An improved method for measurement of syneresis of curd formed by rennet action on milk. J Dairy Res 49: 329-336

Masmoudi M, Ammar I, Ghribi H, Attia H (2020) Physicochemical, radical scavenging activity and sensory properties of a soft cheese fortified with *Arbutus unedo L.* extract. Food Biosci 35: 1-8

Metherel AH, Taha AY, Izadi H, Stark KD (2009) The application of ultrasound energy to increase lipid extraction throughput of solid matrix samples [flaxseed]. Prostaglandins, Leukotrienes and Essential Fatty Acids 81: 417-23

Naczk M, Shahidi F (2006) Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. J Pharm Biomed Anal 41:1523-1542

Nishino T, Shibahara-Sone H, Kikuchi-Hayakawa H, Lshikawa F (2000) Transit of radical scavenging activity of milk products prepared by Millard reaction and *Lactobacillus caseii* strain *shirota* fermentation through the hamster intestine. J Dairy Sci 83: 915-922

Pandhi S, Poonia A (2019) Phytochemical screening of jamun seeds using different extraction methods. Pharma Innovation J 8: 226-231

Parihar AK, Bohra A, Dixit GP (2016) Nutritional benefits of winter pulses with special emphasis on peas and rajmash. Biofortification Food Crops 8: 22-34

Parseeda R, Nanu E (2007) Keeping quality of curd under refrigeration. J Food Sci Technol 44: 400–403

Ranadheera CS, Evans C, Adams M, Baines S (2012) Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk. Food Chem 135: 1411-1418

Ranganna S (2001) Handbook of analysis of fruits and vegetable products. 2nd ed., Tata Mc-Graw-Hill Pub. Com. Ltd., New Delhi.

Rathi M, Upadhyay N, Dabur RS, G Ankit (2013) Formulation and physico-chemical analysis of whey-soymilk *dahi*. J Food Sci Technol 52: 968-975

Remeuf F, Mohammed S, Sodini, Tissier JP (2003) Preliminary observation on the effects of milk fortification and heating on microstructure and physical properties of stirred yoghurt. Int Dairy J 13: 773-782

Sandhya S, Khamrui K, Prasad W, Kumar MCT (2018) Preparation of pomegranate peel extract powder and evaluation of its effect on functional properties and shelf life of curd. LWT- Food Sci Technol 92: 416-421

Shokery ES, El-Ziney MG, Yossef AH, Mashaly RI (2017) Effect of green tea and Moringa leave extracts fortification on the physicochemical, rheological, sensory and antioxidant properties of set-type yoghurt. J Adv Dairy Res 5: 2

Sivakumar GM, Dhanalakshmi B (2015) Physico-chemical and organoleptic evaluation of *dahi* prepared with betel leaf extract. Shanlax Int J Arts, Sci Humanities 3: 134-137

Stankovic MS (2011) Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. Kragujevac J Sci 33: 63-72

Upadhyay N, Dabur RS, Rathi M (2011). Development and shelf life study of flavoured whey-soya milk beverage. Indian J Dairy Sci 64: 92-101

Yadav H, Jain S, Sinha RP (2007) Evaluation of changes during storage of probiotic *dahi* at 7 °C. Int J Dairy Technol 60: 205-210

Yadav K, Bajaj RK, Mandal S, Saha P, Mann B (2018) Evaluation of total phenol content and antioxidant properties of encapsulated grape seed extract in yoghurt. Int J Dairy Technol 71: 96-104

Zimmer AR, Leonardi B, Miron D, Schapoval E, Oliveira JR, Gosmann G (2012) Antioxidant and anti-inflammatory properties of *Capsicum baccatum*: from traditional suse to scientific approach. J Ethnopharmacol 139: 233

Indian J Dairy Sci 74(1): 89-95
https://doi.org/10.33785/IJDS.2021.v74i01.012

RESEARCH ARTICLE

Efficiency of imputing missing genotypes by INDUSCHIP v2 in HF Crossbred cattle

Sujit Saha[1], Nilesh Nayee[1], Heena A Shah[2], Swapnil Gajjar[1], A Sudhakar[1], Sandeep K Donthula[1] and Hardik V Poojara[1]

Received: 13 September 2020 / Accepted: 24 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: INDUSCHIP- an Illumina platform based custom made genotyping chip was designed with 45K polymorphic markers for Indian cattle breeds and 8K base SNPs of Illumina BovineLD chip to genotype indigenous and crossbred cattle in India. Current study was undertaken to assess the genotype imputation efficiency of INDUSCHIP v2 microarray in HF crossbred cattle and compare its efficiency of imputation with that of GGP-35K microarray. HD genotyping data of total 869 cattle from 14 indicine breeds, 2 crossbred (HF and Jersey crossbred) and 2 exotic breeds (HF, Jersey) were used for this study. Post quality control, only 846 animals and 449955 SNPs remained for imputation study. Only 23.65% of 35339 SNPs in GGP-35K chip are found to be common with INDUSCHIP v2 SNP panel. Imputation was carried out with the help of Beagle 5.0 software using subset of both INDUSCHIP v2 and GGP-35K SNP panels. The study revealed higher average concordance rate (CR) and squared correlation (DR^2) for INDUSCHIP v2 as compared to GGP-35K in crossbred HF population.

Keywords: Genotype Imputation, HF Crossbred cattle, INDUSCHIP, Single Nucleotide Polymorphism

[1]National Dairy Development Board (NDDB), Anand, Gujarat-388 001, India

[2]Centre for DNA Fingerprinting and Diagnostics (CDFD), Uppal, Secunderabad, Telangana-500 039, India

Sujit Saha (✉)
NDDB, Anand, Gujarat-388001, India
Email: ssaha@nddb.coop; sujitssahaabc@gmail.com

Introduction

Identification of polymorphic variants (SNPs) across the genome, development of high throughput genotyping and sequencing techniques has led to the generation of massive amount of genomic information on large number of individuals. In Livestock, these genomic information is mainly used for breeding purpose, known as Genomic selection (GS), where, superior individuals are selected for breeding at the very young age on the basis of Genomic enhanced Breeding values (GEBV), computed as a linear function of evenly spaced DNA markers (SNP)spread across the genome and their associated genotypes (Meuwissen et al. 2016). Genomic information from dense SNP chips provides an opportunity to increase rate of genetic progress in the breeding programs if a sufficient number of markers and animals with phenotypes are genotyped. More number of markers means greater linkage disequilibrium between SNPs and more chances of capturing genomic variation. However, several studies indicated that increase in SNP density, after a certain threshold, does not seem to improve the quality of realized genomic relationship in any significant way (Su et al. 2012, Chang et al. 2019).

Since, genotyping with HD SNP panels are expensive, it limits the number of animals to be genotyped. Hence, in practice, people preferred cost effective alternative called genotype imputation, which allows inference of the missing marker genotypes from individuals genotyped with low or medium density (LD) panels by using information from reference population genotyped with high-density panels (Carvalheiro et al. 2014).This not only makes it possible to increase the genomic information and predict missing genotypes (Marchini and Howie,2010) but to reduce genotyping costs and intensify genomic selection (Ventura et al. 2014) by genotyping more number of animals and combine data from different breeds (Larmer et al. 2014).

To implement genomic selection in India for indicus breeds and their taurine crosses, a medium-density customized chip i.e., INDUSCHIP v1 consisting of 45700 SNPs sampled from HD genotype of the mostly four indicus breeds (Gir, Sahiwal, Kankrej and Red Sindhi) and their taurine crosses (HF cross & Jersey cross) have been developed (Mrode et al. 2019). The genotyping

chip contained around 41000 SNPs from HD data having high MAF (0.25), uniformly distributed across the genome for all the breeds under study with an average distance between two consecutive SNPs around 65 kbps. In addition to the above, 2000 ancestry informative SNPs for above mentioned six breeds, ISAG recommended parentage SNPs and some known open-source genetic markers were also included (Nayee et al. 2017). Subsequently, INDUSCHIP v1 was upgraded to INDUSCHIP v2 (52363 SNPs) incorporating additional 6663 highly polymorphic SNPs (Saha et al. 2020).

Current study was undertaken to assess the genotype imputation efficiency of INDUSCHIP v2 micro array to HD level in Holstein Friesian crossbred (HFCB) population and compare its performance with other commercially available medium density chip i.e., GGP indicus-35K microarray developed by Neogen Geneseek operation on Illumina platform specially designed for indicine cattle.

Materials and Methods

Source of data

Total 869 number of Cattle belong to 14 different Indicine breeds (Amritmahal, Deoni, Gir, Hariana, Hallikar, Kankrej, Khillar, Kangayam, Ongole, Red Sindhi, Rathi, Sahiwal, Siri and Tharparkar) and 2 crossbred (HF crossbred-HFCB and Jersey crossbred-JCB) breeds were genotyped with 777K Bovine HD BeadChip (Illumina, Inc.,San Diego, CA). The genotype data for 2 taurine breeds, Holstein Friesian (HF) and Jersey, were obtained from Aarhus University, Denmark. The genotype candidates were selected mainly from frozen semen stations in India and certain state run livestock farms maintaining purebred animals of those breeds.

Data editing

Quality control checks were applied to raw data. SNPs located on autosomes, with call rate >95% and genotyping rate>90% were kept. Further, SNPs with a minor allele frequency (MAF) less than 0.01 and Hardy Weinberg equilibrium having p value less than 10^{-4} were excluded.

After quality control, out of a total of 869 animals of 14 different breeds (multi-breed) and 777962 SNPs, only 846 animals and 449955 SNPs remained for imputation study.

Retrieval of INDUSCHIP and GGP indicus-35K SNP panels:

50K SNP panel data (52363 SNP) of INDUSCHIP was retrieved from customized INDUSCHIP v2 manifest file (NDDB_ Induschip2_ 15061153X355693_B1.bpm). Around 2949 SNPs, which were present in INDUSCHIP v2 manifest file but was not found to be matching with HD SNPs, thus were excluded from this study. After quality control, finally 49399 SNPs remained, whose HD genotyping data was extracted as a subset to study the imputation efficiency of INDUSCHIP. Similarly, The SNP panel list of GGP indicus-35K medium density chip was obtained from NAGRP community data repository.

Creation of Test, Reference and Validation data sets:

From this data, randomly 11 HFCB animals were selected at a time to form test groups animals. While remaining animals 835 animals of multiple breed were taken as reference group with HD data obtained after quality control. Five such test groups were created. Subsequently, genotyping information for the INDUSCHIP and GGP indicus 35K SNP panel were retrieved as a subset from HD data for all the five test groups of animals.

Further, in order to study the concordance of imputation for missing genotypes, five validation data sets with HD genotype data for each group of test animals were also created.

A schematic diagram of the experimental design of this imputation study is presented in Figure 1.

Imputation using INDUSCHIP and GGP indicus-35K SNP panels

Imputation was carried out for 5 test groups of animals using genotyping information of INDUSCHIP v2 SNP panel and GGP indicus-35K SNP panel, respectively. During the study, instead of taking all the 29 autosomes, imputation was carried out for 5 selected autosomes (i.e. Chromosome no.1, 5, 15, 20 and 25) to compare the imputation efficiency.

PLINK (Purcell et al. 2007) software was used for quality control of the data, creation of test, reference and validation data sets as well as for preparing inputs file for Beagle. Imputation was carried out using Beagle 5.0 software (Browning et al. 2018), a population-based imputation program (does not rely on pedigree information)that adopts a stochastic procedure based on a Hidden Markov Monte-Carlo process to infer the probabilities of each haplotype/genotype (Carvalheiro et al. 2014).Imputation accuracy was assessed in terms of concordance rate i.e. the proportion of alleles or genotypes that are correctly imputed (Weigel et al. 2010) and squared correlation between the estimated allele dose and the true allele dose i.e. dosage r^2 (DR^2).The animal wise concordance rate between imputed and actual genotype was estimated using R statistical software and DR^2 values between markers are obtained from Beagle software output.

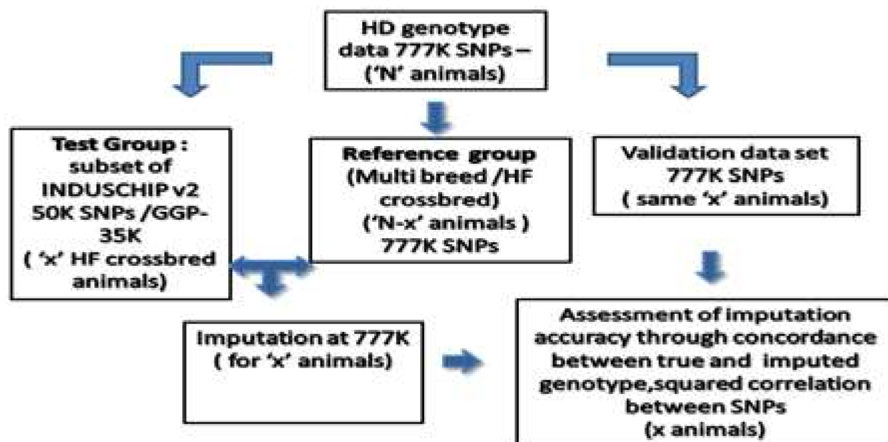
Results and Discussion

Characterization of INDUSCHIP v2 SNP chip

N*umber and Distribution of SNPs across autosomes:*

For an SNP array to be efficient in genotyping for a particular population, it is important to ensure that the selected SNPs are

Fig. 1 Schematic Diagram of the experimental design for imputation study



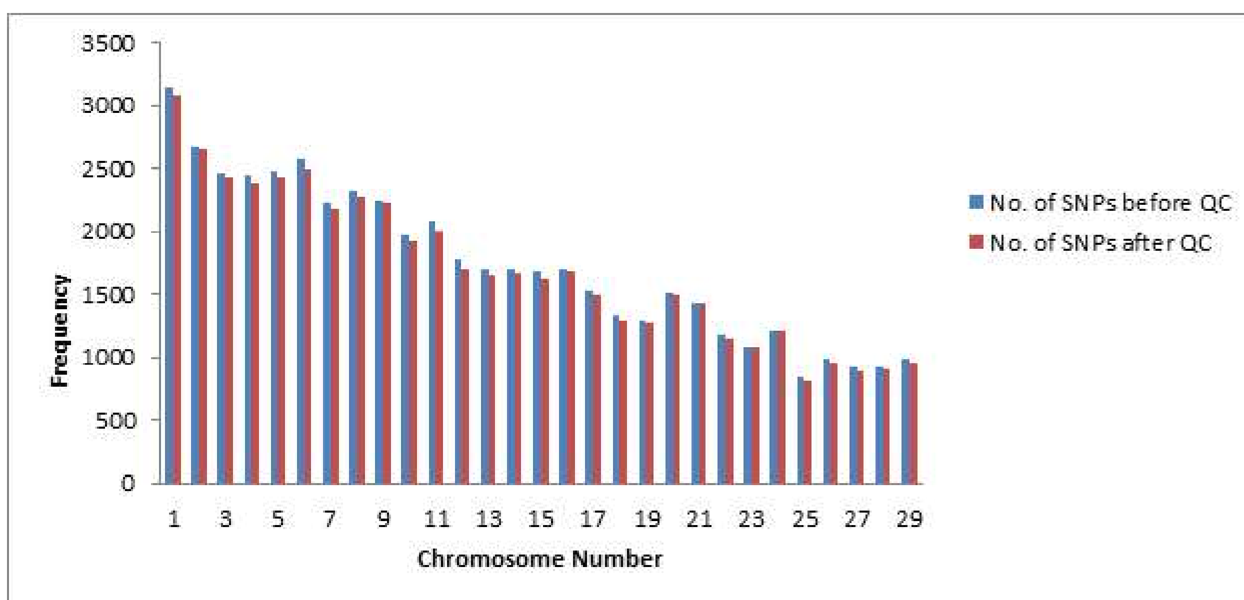


Fig. 2 Chromosome-wise distribution SNPs in INDUSCHIP v2 before and after quality control

distributed evenly covering the entire genome. INDUSCHIP was designed by selecting a subset of SNPs from Illumina BovineHD genotyping array. INDUSCHIP v2 manifest file revealed that there were altogether 52363 SNPs located in all chromosomes. Out of which only 50436 SNPs are located in 29 autosomes (96.3%). Distribution of SNPs across the autosomes in INDUSCHIP v2 vis-à-vis Illumina Bovine HD chip is presented in Table No.1. The data revealed that on an average 6.8% of the HD SNPs located per autosomes were selected in customized INDUSCHIP v2 microarray.

The average distance between the SNPs was found to be around 49.7 Kb across the autosomes. The maximum distance between SNPs was found in chromosome number 10 (52.52 Kb), while minimum distance (46.79 Kb) was observed in chromosome number 9.

Post quality control (QC), out of a total of 50436 SNPs located in autosomes, only 49399 SNPs remained for imputation study. The autosome wise distribution of SNPs before and after quality control (QC) is presented in Figure 2.

Minor allele Frequency

Autosome-wise distribution of minor allele frequencies (MAF) in HFCB population was estimated using PLINK and presented in Table no.2. MAF was classified into three different categories viz. Rare SNPs (MAF > 0 – <0.05), Intermediate SNPs (MAF >= 0.05 – 0.25), and Highly polymorphic SNPs (MAF > 0.25).The distribution of SNPs based on MAF in HFCB population for INDUSCHIP v2 SNP panel indicated that the majority of SNPs (around 73.27%) existing in INDUSCHIP v2 SNP panels are polymorphic having MAF >0.25 (Figure 3).

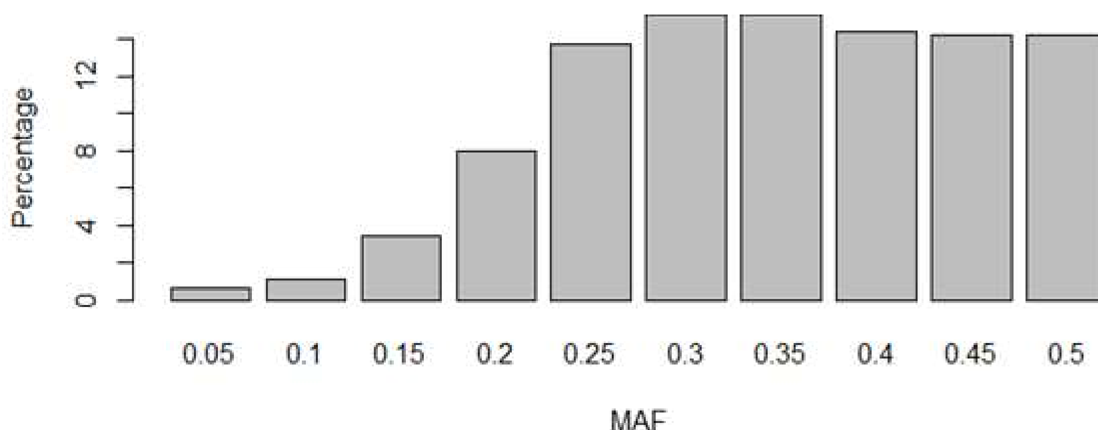


Fig. 3 MAF-wise distribution of SNPs (%) in INDUSCHIP v2

Table 1 Chromosome-wise distribution of SNPs in Illumina Bovine HD chip and INDUSCHIP v2 microarray

Chromosome No.	No. of SNPs in Bovine HD chip	No. of SNPs in INDUSCHIP v2	% SNP in INDUSCHIP v2 compared to Bovine HD chip	Average distance (in KB) between SNPs in INDUSCHIP v2
1	46495	3155	6.8	50.14
2	40056	2677	6.7	51.00
3	35579	2468	6.9	49.15
4	34980	2442	7.0	49.26
5	34842	2483	7.1	48.70
6	35519	2572	7.2	47.11
7	33168	2227	6.7	50.46
8	33529	2320	6.9	48.70
9	31060	2250	7.2	46.79
10	30449	1975	6.5	52.52
11	32015	2078	6.5	51.50
12	26127	1782	6.8	51.00
13	23594	1700	7.2	49.32
14	24780	1697	6.8	49.00
15	24755	1680	6.8	50.53
16	24178	1695	7.0	47.92
17	22266	1522	6.8	49.28
18	19386	1342	6.9	49.05
19	18908	1284	6.8	49.72
20	21490	1508	7.0	47.39
21	21175	1440	6.8	49.67
22	18034	1178	6.5	51.76
23	15215	1091	7.2	47.66
24	18620	1217	6.5	50.95
25	12931	838	6.5	50.92
26	15242	988	6.5	52.21
27	13152	922	7.0	49.18
28	13038	921	7.1	50.13
29	14710	984	6.7	51.31

Comparison of the efficiency of INDUSCHIP v2 and GGP indicus-35K microarray in imputing missing SNPs in HF crossbred cattle

Investigation on SNP markers available in GGP indicus-35K chip, respectively, revealed that out of total 35339 SNPs present in GGP indicus-35K chip, only 8361 SNPs are found (23.65 %)to be

Table 2 Chromosome-wise distribution of MAF in INDUSCHIP v2 microarray

Chromosome No.	Categories of Minor Allele Frequency (MAF)			Grand Total
	>0-0.05	>0.05-0.25	>-0.25	
1	14	814	2259	3087
2	34	768	1849	2651
3	21	588	1819	2428
4	14	586	1791	2391
5	21	651	1755	2427
6	13	660	1817	2490
7	14	586	1574	2174
8	20	856	1396	2272
9	5	603	1621	2229
10	5	453	1470	1928
11	5	468	1539	2012
12	10	455	1245	1710
13	9	595	1055	1659
14	18	499	1150	1667
15	9	401	1213	1623
16	8	471	1202	1681
17	12	351	1130	1493
18	6	302	989	1297
19	13	274	988	1275
20	13	375	1104	1492
21	13	394	1026	1433
22	7	236	910	1153
23	2	224	856	1082
24	3	326	877	1206
25	1	165	646	812
26	1	209	754	964
27	3	187	709	899
28	2	190	711	903
29	2	219	740	961
Total	298	12906	36195	49399
%	0.60	26.13	73.27	

common with INDUSCHIP v2 SNP panel. In GGP indicus-35K chip, around 81% of SNPs were found to be polymorphic with MAF > 0.25.

Imputation was carried out using genotype information at INDUSCHIP v2 SNP panel and GGP indicus-35K SNP panel for 5 chromosomes (i.e. Chromosome no. 1, 10, 15, 20 and 25, respectively) for all the five test group of animals.

The Concordance rate obtained from this study found to vary between 0.971 (Chromosome no.10) to 0.980 (Chromosome No.15) while imputing INDUSCHIP v2 SNP panel to HD level, while the same was varying from 0.961 (Chromosome no.10) to 0.974 (Chromosome No.15) for GGP indicus-35K (Table 3).

Caravalheiro et al. (2014), while imputing GGP20Ki and GGP75Ki panel to HD panel in Nellore animals, observed concordance rate of 97 and 99%, respectively.

The average DR^2 found to vary between 0.892-0.922 in INDUSCHIP v2, while it was 0.888-0.913 in GGP indicus-35K (Table 4).

The present study revealed that selected SNPs in customized INDUSCHIP v2, which was specifically designed for genotyping of indicine breeds and their crosses, were distributed uniformly covering the entire genome. Distribution of SNPs in INDUSCHIP v2 is found to be similar to the distribution of SNPs in other Bovine SNP chips like Illumina 50K and GeneSeek 75K (Mutukumalli et al. 2009).

The majority of the SNPs with high MAF (>0.25) across the autosomes, indicated existence of considerable heterozygosity in crossbred population and INDUSCHIP v2 appeared to be effective in capturing variability in the crossbred population. Malik et al. 2018 in his study using high throughput genotyping-by-sequencing (GBS) markers found that the MAF within the

Table 3 Average concordance rate of INDUSCHIP v2 and GGP indicus-35K in HFCB cattle

Group	INDUSCHIP v2					GGP indicus-35K				
	Chr1	Chr5	Chr10	Chr15	Chr25	Chr1	Chr5	Chr10	Chr15	Chr25
Test -1	0.986	0.984	0.970	0.987	0.978	0.983	0.977	0.958	0.984	0.969
Test -2	0.975	0.984	0.977	0.986	0.982	0.968	0.977	0.970	0.982	0.974
Test -3	0.982	0.980	0.968	0.975	0.980	0.977	0.972	0.958	0.966	0.976
Test -4	0.968	0.968	0.970	0.972	0.965	0.953	0.957	0.960	0.970	0.956
Test -5	0.971	0.968	0.971	0.975	0.969	0.963	0.956	0.960	0.968	0.956
Average	0.977	0.977	0.971	0.98	0.975	0.969	0.968	0.961	0.974	0.966

Table 4 Average DR^2 of INDUSCHIP v2 and GGP indicus-35K in HFCB cattle

Group	INDUSCHIP v2					GGP indicus-35K				
	Chr1	Chr5	Chr10	Chr15	Chr25	Chr1	Chr5	Chr10	Chr15	Chr25
Test -1	0.920	0.926	0.893	0.933	0.890	0.916	0.919	0.887	0.931	0.877
Test -2	0.900	0.923	0.918	0.930	0.908	0.897	0.916	0.915	0.922	0.898
Test -3	0.924	0.921	0.907	0.910	0.909	0.918	0.913	0.897	0.905	0.901
Test -4	0.892	0.906	0.902	0.918	0.877	0.883	0.886	0.897	0.895	0.896
Test -5	0.892	0.906	0.902	0.918	0.877	0.881	0.890	0.891	0.914	0.868
Average	0.906	0.916	0.904	0.922	0.892	0.899	0.905	0.897	0.913	0.888

Indian cattle varied from 0.103 (in Ongole cattle) to 0.177 (in Siri cattle), whereas the Holstein cattle had the lowest value of 0.089. Chagunda et al. 2018 reported average minor allele frequency of 0.29, 0.23, 0.18 and 0.13 for Holstein, Jersey, N'Dama and Gir cattle, respectively.

Comparing imputation efficiency between INDUSCHIP v2 and GGP indicus-35K expressed in terms of average concordance rate as well as squared correlation estimate (DR^2) between Imputed and actual genotypes revealed marginally better performance of INDUSCHIP v2 over GGP indicus-35K chip in Indian HF crossbred population. It may be attributed due to the fact that design of INDUSCHIP v2 chip was based on Indigenous breeds and its crosses (Nayee et al. 2017), while the SNP panels for GGP indicus-35K chip were selected from Australian Brahman, Droughtmaster, Guzerath, Gyr, Nellore, Santa Gertrudis, and tropical composite (Ferraz et al. 2018).

Conclusions

From the present study it can be concluded that the current version of customized INDUSCHIP micro array i.e. INDUSCHIP v2 was quite efficient in imputation at HD level, hence can be effectively used for genotyping and subsequent analysis. However, with the passage of time, as more and more number animals of different breeds spread across the country are genotyped and incorporated in reference population, it would be possible to improve its imputation efficiency further through expanding reference population and incorporating more informative SNPs for the Indian cattle population in future versions of INDUSCHIP micro array. Further, it may also lead to development of low density (LD) microarray with around 10000 informative SNPs and make genotyping facility available to the common dairy farmers at affordable cost.

Acknowledgements

Under Indo-Danish collaboration, technical guidance provided by the Scientists of Center for Quantitative Genetics and Genomics, Department of Molecular Biology and Genetics, Research Center Foulum, Aarhus University, Denmark, in selecting INDUSCHIP panel is duly acknowledged. The authors humbly thank NDDB management for funding and necessary support for this project. The cooperation extended by the Field staff and officials from various PT, PS projects, Livestock Farms for sample collection is duly acknowledged.

References

Browning BL, Zhou Y, Browning SR (2018) A one penny imputed genome from next generation reference panels. American J Hum Genet 103: 338-348. doi: 10.1016/j.ajhg.2018.07.015.

Carvalheiro R, Boison SA, Neves HHR, Sargolzaei M, Schenkel FS, Utsunomiya YT, O'Brien AMP, SolknerJ, McEwan JC, Van Tassell CP, Sonstegard TS, Garica, JF (2014) Accuracy of genotype imputation in Nelore Cattle. Genet Sel Evol.46: 69. doi:10.1186/s12711-014-0069-1.

Chang LY, Toghiani S, Aggrey SE and Rekaya R (2019) Increasing accuracy of genomic selection in presence of high density marker panels through prioritization of relevant polymorphisms. BMC Genet.20: 21. doi.org/10.1186/s12863-019-0720-5.

Chagunda MGG, Mujibi FDN, Dusingizimana T,Kamana O, Cheruiyot E, Mwai OA (2018) Use of high density single nucleotide polymorphism (SNP) arrays to assess genetic diversity and population structure of dairy cattle in smallholder dairy systems: The Case of Girinka programme in Rwanda. Front Genet.9: 438. doi:10.3389/fgene.2018.00438

Ferraz JBS, Wu X, Li H, Xu J, FerrettiR, Simpson B, Walker J, Silva LR, Garica JF, TaitRG Jr, Bauck S (2018) Design of a low density SNP chip for Bos indicus:GGP indicus technical characterization and imputation accuracy of higher density SNP genotypes. In: Proceedings of the 11th World Congress of Genetics Applied to Livestock Production. Auckland, New Zealand,6-11 February,2018.

Larmer SG, Sargolzaei M, Schenkel FS (2014) Extent of linkage disequilibrium, consistency of gametic phase, and imputation accuracy within and across Canadian dairy breeds. J Dairy Sci.97: 1-14. doi:10.3168/jds.2013-6826.

Marchini J, Howie B (2010) Genotype imputation for genome-wide association studies. Nat Rev Genet.11: 499-511. doi:10.1038/nrg 2796.

Matukumalli LK, Lawley CT, Schnabel RD, Taylor JF, Allan MF, Heaton MJ,O'Connell J, Moore SS, Smith TPL, Sonstegard TS, Van Tassell CP (2009) Development and Characterization of a High Density SNP Genotyping Assay for Cattle. PloS One 4: e5350-5063

Meuwissen T, Hayes BJ, Goddard ME (2016) Genomic Selection: A paradigm shift in animal breeding. Anim. Front. 6(1): 6-14. https://doi.org/10.2527/af.2016-0002

Mrode R, Ojango JMK, Okeyo AM, Mwacharo M (2019) Genomic Selection and use of Molecular tools in Breeding Programs for Indigenous and Crossbred Cattle in Developing Countries: Current Status and Future Prospects. Front Genet. 9: 694. doi:10.3389/fgene.2018.00694.

Nayee N, Saha S, Gajjar S, Sudhakar A, Trivedi KR (2017) Compendium of International Workshop on Genomic Selection for Genetic Improvement in Indian Dairy Animals. November 28-29, 2017, BAIF, Pune, India: 15-16

Purcell S, Neale B, Todd-Brown K, ThomasL, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC (2007) PLINK: a tool set for whole –genome association and population based linkage analysis. American J of Hum Genet.81: 559-75.doi: 10.1086/519795.

Saha S, Nayee N, Shah H, Gajjar S, Kishore G, Gupta RO, Trivedi KR (2020) Effect of composition and size of the reference population in genotype imputation efficiency of INDUSCHIP in HF Crossbred cattle. Indian J Dairy Sci.73: 250-255

Su G, Brondum BF, Ma P, Guldbrandtsen B, Aamanand GP, Lund MS (2012) Comparison of genomic predictions using medium-density(~54,000) and high-density (~777,000) single nucleotide polymorphism marker panels in Nordic Holstein and red dairy cattle populations. J Dairy Sci 95: 4657-4665

Ventura RV, Lu D, Schenkel FS, Wang Z, Li C, Miller SP (2014) Impact of reference population on accuracy of imputation from 6K to 50K single nucleotide polymorphism chips in purebred and crossbred beef cattle. J Anim Sci.92: 1433-1444. doi:10.2527/jas.2013-6638.

Weigel KA, Van Tassell CP, O'Connell JR, VanRaden PM, Wiggans GR (2010) Prediction of unobserved single nucleotide polymorphism genotypes of Jersey cattle using reference panels and population-based imputation algorithms. J Dairy Sci.93: 2229-2238. doi: 10.3168/jds.2009-2849

Indian J Dairy Sci 74(1): 96-101
https://doi.org/10.33785/IJDS.2021.v74i01.013

RESEARCH ARTICLE

Multiple linear regression analysis using monthly test day milk yield predicting the first lactation production performance for sire evaluation in Murrah buffaloes

Manvendra Singh[1], Ved Prakash[2], Shaktikant Das[3], Sonam Dixit[4] and AK Gupta[5]

Received: 25 September 2020 / Accepted: 01 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: A total of 9071 first lactation monthly test-day milk yield records of Murrah buffaloes were used to predict the first lactation 305-day milk yield (FL305DMY) by using stepwise backward regression analysis. For the prediction of FL305DMY best combination of monthly test-day milk yields were selected based on adjusted R^2 and RMSE values. The objective of the study was to compare various methods of sire evaluation *viz.,* least squares , simple regressed least squares , best linear unbiased prediction sire model and best linear unbiased prediction animal model in terms of accuracy and efficiency. The methods were compared on the basis of error variance, coefficient of determination, coefficient of variation and rank correlations among the methods. The accuracy of prediction of FL305DMY from monthly test-day milk yields were observed to be best for TD-2 (45th day), TD-4 (105th day) and TD-6 (165th day) combination with BLUP-AM as the most efficient method for sire evaluation. Individual, key monthly TD-6 (165th day) milk yield has high rank correlation with EBVs obtained from actual 305-day milk yield. It was concluded that the optimum combination of TD-2 (45th day), TD-4 (105th day) and TD-6 (165th day) or individual TD-6 (165th day) can be used for genetic evaluation of Murrah sires.

Keywords: FL305DMY, Multiple linear regression, Murrah buffalo, Test-day milk yield

[1]Krishi Vigyan Kendra, Banda, BUAT, Uttar Pradesh, India

[2]AGB Division, ICAR-NRC Camel, Bikaner, Rajasthan, India

[3]AGB Division, GADVASU, Ludhiana, India

[4]Animal Nutrition Division, ICAR-NDRI, Karnal, Haryana, India

[5]AGB Division, ICAR-NDRI, Karnal, Haryana, India

Manvendra Singh (✉)
Krishi Vigyan Kendra, Banda, BUAT Banda-210001, Uttar Pradesh, India
Email: manav31vet@gmail.com

Introduction

India is the home of the finest buffalo germplasm of the world. The country is bestowed with some of the most renowned buffalo breeds; Murrah, Nili-Ravi, Mehasana, Jaffrabadi. Presently the country is having 109.85 million heads of buffaloes (Livestock Census, 2020), constituting over 54.53 % of the total world buffalo population and holds first position in the world. Over the last few decades the contribution of buffaloes in the total milk production of the country is highest than any other livestock species. The contribution of buffaloes to the total milk production of India (187.75 million tonnes) is about 91.81 million tonnes, i.e. 49 % (BAHS, 2019).

Murrah is one of the best milch breed of buffaloes with superior genetic potential for milk production and is used for grading up the local non-descript buffaloes under the genetic improvement programmes across the country. The ultimate objective of any breed improvement programme is to bring fast and accurate genetic gain in the population. In the animal selection, female (dam) has limited scope due to inadequate number of replacement stock. As sires are considered as more than half of the herd, the key step is to identify and select the superior sires on the basis of their breeding values to bring desirable rate of genetic gain. An early and accurate judgment of sire's breeding value is crucial for long term genetic progress in the population.

In country like India, sires are generally evaluated on the basis of FL305DMY records of their daughters. This results in enhanced generation interval, lower genetic gain per unit of time, fewer numbers of daughters per sire due to smaller herd size, costly and time consuming of field recording of performance data etc. As a suitable alternative to these constraints, the test-day milk yield (TDMY) models have recently evoked considerable interest of the animal breeders for sire's selection in buffaloes (Chakraborty et al. 2010, Singh and Taylor, 2013, Chitra et al. 2016, Sahoo et al. 2019).

The different methods of sire evaluation *viz.,* least squares (LSM), simple regressed least squares (SRLS), best linear unbiased prediction (BLUP) and restricted maximum likelihood method (REML) have been used by different workers (Kumar et al. 2015,

Chitra et al. 2016, Sahoo et al. 2019). The main advantage of using TDMY models for sire evaluation is the possibility of selecting sires at younger age, resulting in reduced generation interval, increased intensity of selection attributed to the availability of more numbers of records on daughters. This will eventually lead to higher rates of genetic gain in milk yield. In addition, an animal can be included in the evaluation if it has at least one measurement and the larger amount of available data will increase sires evaluation accuracy and minimize possible bias due to excluding incomplete lactations (Ptak and Schaeffer, 1993, Schaeffer et al. 1977). Prediction of FL305DMY from TDMY records at various intervals has higher accuracy (> 80%) of prediction because of high genetic association between TDMY and complete milk production records (Chakraborty et al. 2010, Dongre et al. 2012, Sahoo et al. 2019).

Although test-day milk yield records offer greater advantage compared to 305-day milk yield in selection schemes but the investigation on fitting of prediction equations using monthly test-day milk yield records for sire evaluation is scanty in Murrah buffaloes. Hence, the present study was aimed to fit and compare the different prediction equations of first lactation 305-day milk yield using monthly test-day milk yields for sire evaluation in Murrah buffalo.

Materials and Methods

The present investigation was conducted on first lactation 9071 monthly test-day milk yield records of 965 Murrah buffaloes that calved during 1977 to 2012 sired by 98 bulls. The data on monthly test-day milk yields and first lactation 305 days or less milk yield were collected from history cum-pedigree sheets and daily milk yield recording registers maintained at Dairy Cattle Breeding Division, ICAR-National Dairy Research Institute, Karnal. Milk yield recorded on 15th day of calving was taken as first monthly test-day milk yield (TD1). Next 9 test-day milk yield records were taken at 30 days interval from first test-day (TD2 – TD10). The first lactation 305 days or less milk yield was also used in the present study. Only those records of Murrah buffaloes were considered that had produced milk for at least 100 days and minimum of 500 kg. To ensure the normal distribution, the outliers were removed and data within the range of mean ± 3SD were only considered for the present study.

Prediction of FL305DMY

Different monthly test-day milk yields were used for the prediction of FL305DMY by stepwise backward multiple linear regression analysis (SAS Enterprise Guide 4.3). Following model was used:

$$Y_i = a + b_i © x_i$$

Where, Y_i is the estimated first lactation 305-day milk yield of the ith animal; X_i is the test-day record of ith animal; a is intercept; b_i is the regression coefficient of first lactation 305- day milk yield on test- day records.

Sire evaluation

The breeding values of 98 Murrah sires with five or more daughters were estimated from the actual and predicted FL305DMY and key monthly test-day milk yields by regression analysis by applying four sire evaluation methods i.e., Least Squares Method (LSQ), Simple Regressed Least Squares (SRLS), Best Linear Unbiased Prediction Sire Model (BLUP-SM) and Best Linear Unbiased Prediction Animal Model (BLUP-AM).

Results and Discussion

Prediction of FL305DMY

The estimated intercept values, regression coefficients, adjusted R^2 and RMSE values for prediction of 305-day milk yield by best prediction equations using single, two and three test day combinations are presented in Table 1.

It was observed that the regression equation with 2 important variables TD-2 and TD-4 explained about 71% variability with RMSE value 218.37 kg of prediction in 305-day milk yield. Addition of one more variable TD-6 increased the predictability to about 83% i.e. an increase of 12% with RMSE value 163.18 kg. Hence regression equation with 3 variables *viz.* TD-2 (45thday), TD-4 (105thday) and TD-6 (165thday) was considered more appropriate (early test days with high accuracy) for prediction of FL305DMY with about 83% accuracy and 163.18 kg RMSE value. In a study on Murrah buffalo, Singh and Rana (2008) observed that the accuracy of prediction of 305-day milk yield using monthly test-day milk yields varied between 42% (TD-1) and 67% (TD-6). Sahoo et al. (2019) also observed that the regression equation with 2 important weekly test days (TD-7 i.e., 48th day and TD-22 i.e., 153rd day) explained about 86% variability with RMSE 162.41 kg and with 3 weekly test days (TD-7 i.e., 48th day, TD-22 i.e., 153rd

Table 1 Best prediction equations and their accuracy for estimation of FL305DMY

Test-Days	Best Prediction Equations	Adjusted R^2(%)	RMSE (kg)
1	Y_i = 226.31 + 213.27 *TD-4	65.95%	247.06
1	Y_i = 431.20 + 214.19 *TD-6	69.89%	242.10
2	Y_i = 73.08 + 76.51 *TD-2 + 155.16 *TD-4	70.96%	218.37
3	Y_i = -35.53+ 62.73 *TD-2 + 75.34 *TD-4 + 125.39 *TD-6	82.76%	163.18

day and TD-34 i.e., 237th day) explained about 88% variability with RMSE 147.14 kg, respectively in Murrah buffalo. However, Dongre et al. (2012) predicted the FL305DMY with R^2-value of 81.49 to 85.16% and RMSE 190.4 to 253.0 kg for different fortnightly test day milk yield combinations in Sahiwal cattle. Prediction of 305-day milk yield using individual key monthly test-day milk yields (TD-6 and TD-4) which have higher genetic and phenotypic correlations with 305-day milk yield gave prediction accuracy and RMSE value around 70%, 242.10 and 66%, 247.60 respectively.

Sire evaluation

The average estimated breeding values (EBVs) for Murrah sires based on the actual and predicted first lactation 305-day or less milk yield and key monthly test-day milk yields of their daughters for different sire evaluation methods are presented in Tables 2. It was observed that the BLUP-SM and BLUP-AM has the highest stability and least variability in terms of highest and lowest EBVs and found to be superior in comparison to other models on the basis of actual and predicted FL305DMY and key monthly test-day milk yields which were in agreement with the findings of Sahoo et al. (2019).

Effectiveness of sire evaluation methods

The sire evaluation methods were compared on the basis of results of rank correlations, error variance, coefficient of determination and coefficient of variation.

Spearman's Rank Correlations

It was observed that the highest rank correlation was found between BLUP-SM and BLUP-AM (0.94) while the lowest (0.62) was observed among SRLS and BLUP-AM for actual 305-day milk yield (Table 3). Others workers (Chaudhari et al. 2014, Chitra et al. 2016, Sahoo et al. 2019) have also observed that the BLUP method was one of the most efficient sire evaluation as it has lowest error variance than other methods in Murrah buffaloes. The rank correlations of predicted 305-day milk yield using TD-2 and TD-4 and TD-2, TD-4 and TD-6 with actual 305-day milk yield were found to be highest within a particular method e.g.

Table 2 Average expected breeding values (EBVs) of Murrah sires for actual and predicted first lactation 305-day milk yield and key monthly test-day milk yields by different sire evaluation methods

Sire evaluation methods	Average EBV (kg)	Number of sires above average EBV	Number of sires below average EBV	Maximum EBV (kg)	Minimum EBV (kg)
		Actual first lactation 305-day milk yield			
LSQ	1755.98	41 (41.8%)	57 (58.2%)	2428.02	1274.62
SRLS	1794.82	49 (50.0%)	49 (50.0%)	2328.40	1302.61
BLUP-SM	1845.10	42 (42.8%)	56 (57.2%)	1950.51	1733.94
BLUP-AM	1805.74	47 (47.9%)	51 (52.1%)	2087.38	1557.51
		Predicted first lactation 305-day milk yield (TD-2+TD-4)			
LSQ	1750.98	42 (42.8%)	56 (57.2%)	2336.66	1315.46
SRLS	1800.83	44 (44.8%)	54 (55.2%)	2352.45	1319.06
BLUP-SM	1826.65	43 (43.8%)	55 (56.2%)	1968.61	1732.94
BLUP-AM	1799.73	42 (42.8%)	56 (57.2%)	2165.08	1572.31
		Predicted first lactation 305-day milk yield (TD-2+TD-4+TD-6)			
LSQ	1752.00	40 (40.8%)	58 (59.2%)	2384.70	1323.11
SRLS	1805.17	49 (50.0%)	49 (50.0%)	2300.81	1381.10
BLUP-SM	1835.38	47 (47.9%)	51 (52.1%)	1924.66	1767.77
BLUP-AM	1804.74	44 (44.8%)	54 (55.2%)	2120.17	1565.59
		Key monthly test-day milk yield (TD-4)			
LSQ	7.20	41 (41.8%)	57 (58.2%)	9.70	5.01
SRLS	7.38	44 (44.8%)	54 (55.2%)	9.60	5.40
BLUP-SM	7.50	45 (45.9%)	53 (54.1%)	7.98	7.15
BLUP-AM	7.38	46 (46.9%)	52 (53.1%)	8.84	6.53
		Key monthly test-day milk yield (TD-6)			
LSQ	6.44	40 (40.8%)	58 (59.2%)	8.99	4.58
SRLS	6.56	48 (48.9%)	50 (51.1%)	4.87	2.29
BLUP-SM	6.54	50 (51.0%)	48 (49.0%)	6.99	6.21
BLUP-AM	6.55	49 (50.0%)	49 (50.0%)	7.92	5.39

Table 3 Rank correlations among EBVs of sires for actual 305-day milk yield by different sire evaluation methods

Methods	LSQ	BLUP-SM	SRLS	BLUP-AM
LSQ	1.00	0.78**	0.78**	0.76**
BLUP-SM		1.00	0.69**	0.94**
SRLS			1.00	0.62**
BLUP-AM				1.00

Table 4 Rank correlations among breeding values of sires for predicted 305-day milk yield and key monthly test-day milk yields with actual 305-day milk yield by different sire evaluation methods

Methods	BLUP-SM(305-DMY)	LSQ (305-DMY)	SRLS(305-DMY)	BLUP-AM (305-DMY)
	Predicted first lactation 305-day milk yield (TD-2+TD-4)			
BLUP-SM	0.77	0.61	0.48	0.70
LSQ	0.65	0.90	0.65	0.60
SRLS	0.55	0.65	0.91	0.47
BLUP-AM	0.75	0.60	0.45	0.76
	Predicted first lactation 305-day milk yield (TD-2+TD-4+TD-6)			
BLUP-SM	0.90	0.70	0.60	0.85
LSQ	0.70	0.94	0.71	0.66
SRLS	0.61	0.67	0.94	0.53
BLUP-AM	0.87	0.71	0.55	0.90
	Key monthly test-day milk yield (TD-4)			
BLUP-SM	0.75	0.59	0.50	0.70
LSQ	0.64	0.85	0.62	0.61
SRLS	0.55	0.63	0.88	0.48
BLUP-AM	0.72	0.55	0.44	0.74
	Key monthly test-day milk yield (TD-6)			
BLUP-SM	0.83	0.64	0.59	0.79
LSQ	0.68	0.86	0.69	0.65
SRLS	0.62	0.62	0.84	0.54
BLUP-AM	0.74	0.58	0.49	0.75

BLUP-SM of predicted 305-day milk yield with BLUP-SM of actual 305-day milk yield and so on for other methods (Table 4).

The comparison of rank correlations of EBVs obtained by using TD-2, TD-4 and TD-6 *vis-à-vis* TD-2 and TD-4 with EBVs of actual 305-day milk yield revealed that the rank correlations were higher for TD-2, TD-4 and TD-6 by either of the methods used for sire evaluation. In the case of key monthly test-day milk yields, the higher rank correlations were obtained for TD-6 with actual 305-day milk yield by any of the four methods of sire evaluation. The rank correlations were by and large higher for TD-6 followed by TD-4 among different sire evaluation methods.

All the different methods of sire evaluation were compared for their accuracy, efficiency and stability on the basis of the values of error variance, coefficient of determination and coefficient of variation, respectively (Table 5).

Error variance

The error variance is one of the criteria of judging the effectiveness of different methods of sire evaluation. An efficient method of sire evaluation must have minimum error variance. The results showed that the error variance of BLUP-AM was lowest as compared to the other methods. Thus, on the basis of error variance the BLUP-AM was considered more efficient followed by LSQ in actual and predicted 305-day milk yield and also for key monthly test-day milk yields. Similar observations were reported by Sahoo et al. (2019) in Murrah buffalo.

Coefficient of variation (CV)

The stability of different sire evaluation methods was judge by comparing the estimates of coefficient of variation. The nearer the CV (%) of the population for sire evaluation to the CV (%) of unadjusted data, the more stable is the method. The CV (%) of unadjusted data for 305-day milk yields was 29.21 per cent.

In case of actual 305-day milk yields, the coefficient of variation was most near to unadjusted data in SRLS method (26.03) and far apart in BLUP-AM method (23.59%). The CV of LSQ and BLUP-SM was almost same for actual 305-day milk yield. The coefficient of variation for predicted 305-day milk yield by using TD-2 and

Table 5 Comparison of relative efficiency and effectiveness of different sire evaluation methods for actual and predicted first lactation 305-day milk yield and key monthly test-day milk yields in Murrah buffalo

Methods	Error Variance (kg^2)	Relative Efficiency	Coefficient of determination (%)	Coefficient of variation (%)
	Actual first lactation 305-day milk yield			
LSQ	205116	88.50	20.90	25.79
SRLS	218346	83.13	21.46	26.03
BLUP-SM	207702	87.39	27.20	25.23
BLUP-AM	181531	100	44.97	23.59
	Predicted first lactation 305-day milk yield (TD-2+TD-4)			
LSQ	144584	84.57	22.35	21.71
SRLS	158507	77.14	29.07	22.10
BLUP-SM	145368	84.11	29.40	21.18
BLUP-AM	122278	100	50.37	19.42
	Predicted first lactation 305-day milk yield (TD-2+TD-4+TD-6)			
LSQ	174615	85.59	20.52	23.85
SRLS	187776	79.60	26.92	24.00
BLUP-SM	176148	84.85	25.6	23.25
BLUP-AM	149470	100	46.21	21.42
	Key monthly test-day milk yield (TD-4)			
LSQ	3.12	85.89	20.56	24.52
SRLS	3.35	80	26.80	24.78
BLUP-SM	3.13	85.62	25.60	23.98
BLUP-AM	2.68	100	45.90	22.16
	Key monthly test-day milk yield (TD-6)			
LSQ	2.83	80.91	19.18	26.11
SRLS	2.95	77.62	23.38	26.17
BLUP-SM	3.50	65.42	17.40	29.06
BLUP-AM	2.29	100	48.18	23.07

TD-4 and TD-2, TD-4 and TD-6 and for key test-day milk yields by LSQ, SRLS, BLUP-SM and BLUP-AM ranged between 19.42-22.10%, 21.42-24%, 22.16-24.78% and 23.07-29.06%; respectively indicating nearly equal stability of these methods. Sahoo et al. (2019) in Murrah buffalo also reported nearly equal stability of all the methods.

Conclusions

The prediction of 305-day milk yield from monthly test-day milk yields using TD-2 (45th day), TD-4 (105th day) and TD-6 (165th day) combination gave higher accuracy (82.76%) of prediction. Individual, key monthly TD-6 (165th day) milk yield has high rank correlation with EBVs obtained from actual 305-day milk yield. It was concluded that the optimum combination of TD-2 (45th day), TD-4 (105th day) and TD-6 (165th day) or individual TD-6 (165th day) can be used for genetic evaluation of Murrah sires due to their high rank correlations with EBVs of actual 305-day milk yield. BLUP-AM was found most efficient and accurate method followed by BLUP-SM for sire evaluation using actual and predicted 305-day milk yield and key monthly test-day milk yields in Murrah buffalo.

Acknowledgements

The authors are immensely grateful to Director, ICAR-NDRI, Karnal and Head DCB division for providing necessary facilities for conducting this research work.

References

BAHS (2019) Basic Animal Husbandry Statistics. Department of Animal Husbandry, Dairying & Fisheries. Ministry of Agriculture, Govt. of India

Chakraborty D, Dhaka SS, Pander BL, Yadav AS, Singh S, Malik PK (2010) Prediction of lactation milk yield from test day records in Murrah buffaloes. Indian J Anim Sci 80: 244-245

Chaudhari PN, Upadhay MR, Patel AC, Patel SB, Rank DN (2014) A comparison of different methods of sire evaluation for production and reproduction traits of Murrah graded buffaloes. Int J Res Hum Soci Sci 2: 16-24.

Chitra A, Jain A, Kumar M, Ratwan P, Gupta AK (2016) Genetic evaluation of sires for milk production and its composition traits in Murrah buffaloes. Indian J Dairy Sci 69: 721-724

Dongre VB, Gandhi RS, Singh A, Ruhil AP (2012) Comparative efficiency of artificial neural networks and multiple linear regression analysis for prediction of first lactation 305-day milk yield in Sahiwal cattle. Livest Sci 147: 192-197

Kumar V, Chakravarty AK, Patil CS, Shivahre PR, Gupta AK (2015) Comparison among different conventional methods of sire evaluation in Murrah buffalo. Indian J Anim Sci 85**:** 670-673

Livestock Census (2020) 20th All India Livestock Census. Department of Animal Husbandry, Dairying & Fisheries. Ministry of Agriculture, Govt. of India.

Ptak E, Schaeffer LR (1993) Use of test day yields for genetic evaluation of dairy sires and cows. Livest Prod Sci 34: 23–34

Sahoo SK, Singh A, Ambhore GS, Dash SK, Dubey PP (2019) Comparative efficiency of different multiple linear regression prediction equations of first lactation 305-day milk yield for sire evaluation in Murrah buffaloes. Indian J Anim Res 53: 1287-1291

Schaeffer LR, Minder CE, McMillan I, Burnside EB (1977) Non-linear techniques for predicting 305-day lactation production of Holsteins and Jerseys. J Dairy Sci 60: 1636-1644

Singh A, Rana JS (2008) Prediction of 305- day milk yield based on test-day values in Murrah buffaloes. Indian J Anim Sci 78: 1131-1133

Singh S Tailor SP (2013) Prediction of 305 days first lactation milk yield from fortnightly test and part yields. Indian J Anim Sci 83: 166-169

Indian J Dairy Sci 74(1): 102-106
https://doi.org/10.33785/IJDS.2021.v74i01.014

RESEARCH ARTICLE

Effect of area specific mineral mixture supplementation on milk production, biochemical and blood mineral status of Black Bengal goats

GM Kedare[1], Vipin[2], M Karunakaran[1], A Mandal[1], MK Ghosh[1] and S Naskar[3]

Received: 08 October 2020 / Accepted: 04 December 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: This study was conducted to find out the effect of area-specific mineral mixture (ASMM) supplementation on production performances, the biochemical and mineral profile of Black Bengal does during the peripartum period. For this purpose, 20 pregnant does were made into two groups according to the bodyweight viz., Control-T_0 (n=10) and Treatment-T_1 (n=10) and supplemented with 0 and 6 g of ASMM/animal/day, respectively starting from 3 months before the expected date of parturition to till the onset of the first post-partum estrus. Overall mean milk fat, total ash, total solids, solids not fat, milk crude protein and milk yield, blood glucose, non-esterified fatty acids, α-amino nitrogen, phosphorus and serum glutamic pyruvic transaminase concentration were not affected by ASMM supplementation. The overall mean of serum glutamic oxaloacetic transaminase, calcium, copper, and zinc concentration in control (T_0) and treatment (T_1) groups were 127.05±1.27 vs.132.73±1.26 IU/L, 7.83±0.08 vs. 8.19±0.08 mg/dl, 0.66±0.02 vs. 0.94±0.02 ppm, 0.69±0.01 vs. 0.97 ± 0.02 ppm, respectively and the values were higher in supplemented groups. More studies need to be conducted involving the use of ASMM supplementation to see its effect on the performance of goats.

Keywords: Area-specific mineral mixture, Biochemical, Goat, Minerals, Milk parameters

[1]ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani-741 235, West Bengal, India

[2]Center for Advanced Faculty Training in Animal Nutrition, ICAR-IVRI, Izatnagar, Bareilly-243 122, Uttar Pradesh, India

[3]ICAR-Indian Veterinary Research Institute, Eastern Regional Station, Kolkata-700037, West Bengal, India

Vipin (✉)
Center for Advanced Faculty Training in Animal Nutrition, ICAR-IVRI, Izatnagar, Bareilly-243 122, Uttar Pradesh, India
Email:vipinsingh729@gmail.com

Introduction

Goats (*Capra hircus*) are reared by farmers for purposes like meat, milk, hides, hair and dung. Black Bengal (*Capra hircus bengalensis*) breed of goat is mostly reared in the eastern and north-eastern region of India. Black Bengal goat is a smaller in body size but highly prolific and meat-type breed of goat and it has many desirable productive traits like early sexual maturity i.e. 6-8 months of age. Nutritional status affects the production potential and reproductive performance of farm animals. More often goats are generally malnourished, particularly with regards to micronutrients. There are twenty-two mineral elements which are very much essential for the health and production of animals (Sharma et al. 2009). Micro-minerals supplementation is required as most of the roughages, greens, concentrates are deficient in trace mineral elements. Ghosh et al. (2013) reported six minerals namely Ca, P, Zn, Cu Co, Mn are deficient in lower Gangetic part of West Bengal. But till now, the impacts of area specific mineral mixture (ASMM) on production performance of Black Bengal goat have not been studied. Concept of the area a specific mineral mixture is a new approach of low input and high output for the end-users. There is an abundant opportunity for exploiting the idea of ASMM for adjusting the deficiency of minerals to obtain the optimal production potential of animals to improve the economy of farmers.

Materials and Methods

Experimental animals

This study was approved by the Institutional Ethics Committee of ICAR-National Dairy Research Institute, India. Twenty numbers of Black Bengal does of 1st parity maintained at ERS-IVRI, Kalyani farm was selected and divided randomly into 2 groups of 10 animals each based on their body weight viz., control (T_0) and treatment (T_1). Control animals were managed under complete grazing with supplementation of concentrate @300 g/head/day starting from three months before parturition until the appearance of the first post-partum estrus. The animals in the treatment group were fed the same diets as in control group except supplemented with ASMM @ 6 g/head/day. This area

specific mineral mixture was prepared (Table 1) as per Ghosh et al. (2013).

Chemical analysis of feeds

Proximate composition of feeds and fodders was analysed as per AOAC (2005) and cell wall constituents were estimated as per Van Soest et al. (1991). One g representative samples of feeds and fodders fed to goats were taken and tri-acid digestion (HNO3: $HClO_4$: H2SO4 in ratio 3: 2: 1) was done for 1 to 2 h until the solution was clear. The samples were filtered with Whatman 42 filter paper. Then, a sufficient amount of deionized water was added to make the final volume up to 100 ml and samples were analysed by using AAS (Agilent-200 model). Plasma minerals were also analysed.

Analysis of blood parameters

Blood samples were collected at the weekly intervals from the jugular vein into heparinised tubes (20 IU heparin/ml of blood) and centrifuged at 3000 rpm for 30 min at 4ºC and plasma was separated and kept in the labelled storage vials of 5 ml capacity and stored at -20ºC till analysis. For determination of plasma NEFA, copper soap extraction methods modified by Shipe et al. (1980) was followed. Blood α-amino nitrogen concentration was estimated as per Goodwin (1970). Blood glucose was estimated using commercially available glucose test kit (GOD-POD Method, Span Cogent Diagnostics Ltd., India Product no# 93DP100-74). Alanine amino transaminase (ALT) and aspartate aminotransferase (AST) were estimated by 2, 4-DNPH (Reitman and Frankel Method) using a commercial kit (Span Diagnostics Ltd., India). Blood calcium was estimated using a commercially available calcium kit (Span Diagnostics Ltd., India; product no. 87L S100-60).

Estimation of milk composition

Milk samples were collected on day3 after parturition and then fortnightly till first postpartum heat. Complete milking was done, milk was collected aseptically in the clean and sterile sampling bottles. The milking was done in the morning and evening on the days of sample collection to assess the milk production. 10 ml of milk sample was preserved separately for total protein estimation while, the remaining portion was used for fat, solid-not fat, total solids and ash estimation.

Milk fat was estimated as per Gerber, (1892), the fat percentage was read directly by especially a calibrated butyrometer. For the estimation of milk total solids, 10 g of milk sample was taken in pre-weighted silica crucible placed at 80^0C in the hot air oven for overnight. Next day sample with crucible was weighed. Total solids and SNF contents were estimated by the difference. For estimation of milk total ash dry sample with silica, crucible was ashed at 550^0C in a muffle furnace for 3 h and total ash was estimated by the difference in weight after ashing of dried sample. The total nitrogen was estimated (AOAC 2005). Total nitrogen content was multiplied by 6.38 to get milk protein value.

Statistical analysis

Different statistical designs were considered to the analysis of data as per Snedecor and Cochran (1994) and analysis was carried out through the SPSS v16.

Results and Discussion

Mineral status of feeds

The average concentration of calcium (Ca), phosphorus (P) copper (Cu) and zinc (Zn) in green fodder were 0.25%,0.38%,7.98 ppm, and 44.46 ppm, respectively with corresponding values of 0.25%,0.24%,7.53 ppm, and 38.75 ppm, in concentrate mixture (Table 2). The fodders were deficient in P. The lower P level could be attributed to low pH of the soil (Singh et al. 2011) and the ionic acidity of soil might be induced the formation of a complex with iron and thereby lowered the availability of inorganic P. The critical concentration of Zn for feeds and fodder is 30 ppm (McDowell et al. 1983). Panda et al. (2016) reported content of Mn, Cu and Zn in different feed and fodder varied between 17.14-54.29, 4.55-38.90 and 17.51-47.78 ppm, respectively in the western undulating region of Orissa. Das et al. (2003) reported the feed and fodder available for animal feeds in hill zone region West Bengal were a good source of Cu (17.97-38.73 ppm) moderate source of Ca (0.26-89%) and Zn (31.91-57-35 ppm) and a poor source of P (0.14-17%). Samanta and Samanta (2002) studied in the coastal saline zone of West Bengal and noticed, the available feeds and fodder were sufficient in Ca, Cu and Zn but deficient in P concentration Similar findings have been reported earlier from the north-eastern part of India (Chander Datt and Aruna Chhabra 2005, Hegde et al. 2016, 2018).

Effect of the area-specific mineral mixture (ASMM) on milk yield and composition

The milk yield and composition were similar in two groups indicating that ASMM supplementation has no significant effect on these variables Table 3. Singh et al. (2016), Wu et al. (2000), Sharma et al. (2002), Rabiee et al. (2010) and Begum et al. (2010) reported non-significant (P>0.05) effect of feeding ASMM on milk components such as protein, fat and SNF of milk on. This

Table 1 Mineral composition of area specific mineral mixture

Minerals	Quantity (g)
Calcium	25.96
Phosphorus	20.08
Zinc	0.529
Copper	0.258
Cobalt	0.027
Manganese	0.011

Table 2 Chemical composition (% DM basis) and mineral status of concentrate and green fodder

Attributes	Concentrate mixture	Green fodder
Dry matter (%)	93.9±0.12	17.13±2.33
Organic matter (%)	93.72±0.07	86.81±0.63
Crude protein (%)	18.94±0.60	12.82±1.73
Ether extract (%)	4.51±0.10	2.66±0.27
Total ash (%)	6.28±0.07	12.92±0.62
Acid Insoluble ash (%)	1.77±0.06	3.36±0.28
Neutral detergent fiber (%)	32.54±1.67	57.6±5.65
Acid detergent fiber (%)	11.20±1.98	35.91±4.08
Calcium (%)	0.25	0.25
Phosphorus (%)	0.24	0.38
Copper (ppm)	7.53	7.98
Zinc (ppm)	38.75	44.46

Table 3 Effect of ASMM on milk yield and milk composition

Attributes	Control (T_0)	Treatment (T_1)	P
Milk fat (%)	5.74±0.03	5.86±0.04	>0.05
Total solids (%)	15.16±0.19	15.23±0.15	>0.05
Total ash (%)	0.78±0.01	0.80±0.01	>0.05
Solid not fat (%)	8.64±0.02	8.71±0.03	>0.05
Crude protein (%)	3.63±0.04	3.67±0.05	>0.05
Milk yield (g/day)	281.49±19.91	312.43±16.20	>0.05

finding also supported by Singh et al. (2016), Tiwari et al. (2013), Sahoo et al. (2017) and Nocek et al. (2006) who found the non-significant difference improvement in overall milk yields of control and area specific mineral mixture supplemented group. Singh et al. (2020) reported after introducing (50g) mineral mixture in the feed, average milk yield, fat and SNF content increased by 6.2, 5.3 and 1.8%, respectively, while milk yield increased by 0.49 L/day/animal.

Effect of ASMM on blood parameters

Non-esterified fatty acids (NEFA) and α–amino nitrogen (AAN) values differed non-significantly ($P<0.05$; Table 4) among groups at different time intervals. NEFA is produced due to the adipose tissue breakdown of fat in response to negative energy status. These circulating NEFAs are absorbed and metabolized for energy. The concentration of NEFA directly reflects the amount of adipose tissue breakdown. Clinical experience suggests serum NEFA concentrations are more sensitive to energy balance changes compared with body condition scoring in growing situations (Van Saun, 2000). Blood α-amino nitrogen indicates protein synthesis status of the animal, but here, the results indicate sufficient protein ration was provided in both groups. Hornick et al. (1996, 1998) reported plasma α-amino nitrogen increases during growth. Mondal and Prakash (2003) reported plasma α-amino nitrogen concentration increases, as the buffalo's age increases. Godara et al. (2015) reported no significant difference ($P>0.05$) in AAN levels between treatment and control group when supplemented with an area specific mineral mixture in black Bengal goats.

Blood glucose level was also similar in both the control and ASSM supplemented groups. Similar to our result, Ashry et al. (2012) and Behera et al. (2012) reported that feeding mineral mixture did not have any significant effect on blood glucose concentration. Contrary to our result, Godara et al. (2015) reported a significant difference ($P<0.05$) in glucose levels between treatment and control group when supplemented with area specific mineral mixture in black Bengal goats.

Effect of area-specific mineral mixture on serum enzymes

The plasma ALT level was similar in two treatment groups, however, plasma AST level was higher ($P<0.05$; Table 4) group T1 but its concentration was within the normal value. Similar to our results, Godara et al. (2015) reported a significant difference ($P<0.05$) in AST levels between treatment and control group when supplemented with area specific mineral mixture in black Bengal goats. Sharma et al. (2011) and Exton (1980) also observed that AST and ALT level was higher in mineral supplemented group as compared with the control group. Pandey et al. (2018) and Chaudhary and Patel (2019) observed no significant change in on serum AST concentration due to supplementation of either commercial or ASMM.

Effect of area-specific mineral mixture on plasma mineral profile

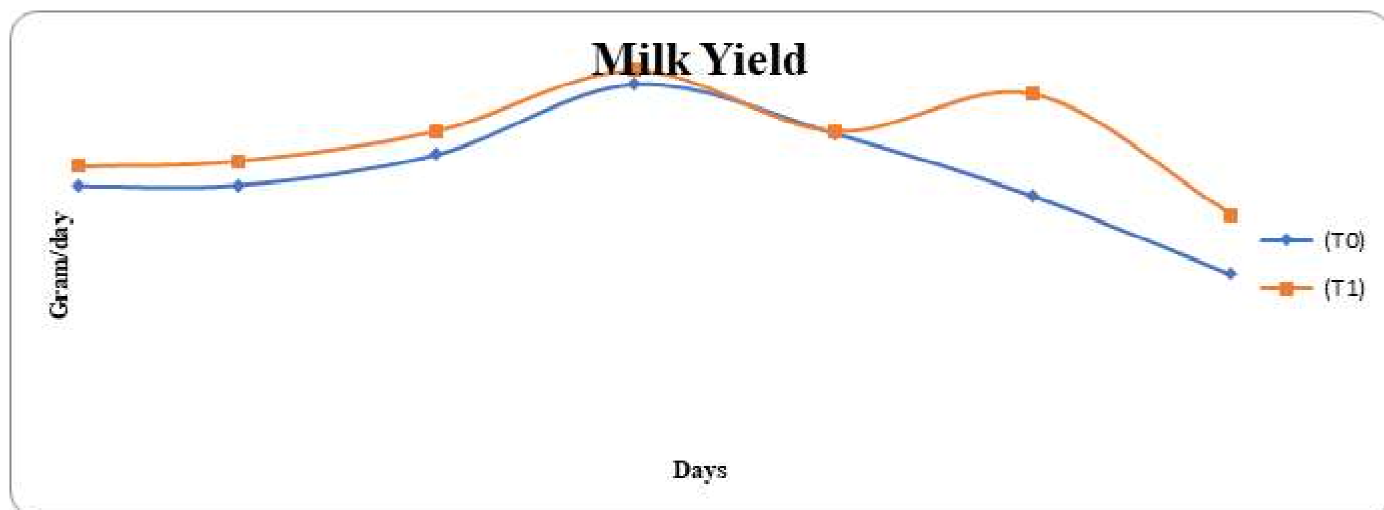


Fig. 1 Effect of ASSM on milk yield of Black Bengal does

Table 4 Effect of supplementation of ASMM on different blood parameters

Attributes	Control (T_0)	Treatment (T_1)	P
NEFA (µmol/L)	0. 28±0. 02	0. 26±0. 02	>0.05
α–amino nitrogen (mg/dl)	37. 74±0. 77	39.89±1.07	>0.05
Glucose (g/dl)	53.26±1.33	55.70±1.43	>0.05
ALT (IU/L)	29.73±0.74	30.92±0.55	>0.05
AST (IU/L)	127.05±1.27[b]	132.73±1.26[a]	<0.05
Calcium (mg/dL)	7.83±0.08[b]	8.19±0.08[a]	<0.05
Phosphorus (mg/dL)	4.72±0.17	4.96±0.21	>0.05
Copper (ppm)	0.66±0.02[b]	0.94±0.02[a]	<0.05
Zinc (ppm)	0.69±0.01[b]	0.97±0.02[a]	<0.05

a.b values bearing different superscripts in column differ significantly (P<0.05) from each other

The overall mean value Ca was higher (P<0.05) in the T1 group (Table 4). This might be because, vitamin D in the intestinal mucosa as 1, 25- dihydroxycholecalciferol acted by the opening of Ca channel and facilitates Ca uptake and transfer with the help of Ca binding protein (Hurwitz, 1990). Phosphorus concentration was found to be similar in both groups. The overall mean of copper (Cu) and zinc (Zn) differed significantly (P<0.05; Table 4) between the groups due to supplementation of ASMM which contains these minerals. Similar results were supported by other workers (Upadhyay, 2004; Chaudhary and Patel 2019; Niaz et al. 2017; Agrawalla et al. 2017 and Samanta et al. 2005).

Conclusions

It is concluded that supplementation of area-specific mineral mixture significantly improved the Ca, Zn and Cu concentration in plasma of Black Bengal does. Though supplementation of ASSM did not have significant improvement in milk production and its composition, more studies need to be carried out to see the effect of supplementation of the area-specific mineral mixture on the overall performance of goats.

Acknowledgements

The authors thank ICAR-NDRI (Deemed University), India for providing suitable experimental facilities.

References

AOAC (2005) Official Methods of Analysis. 18th Edition, Association of Official Analytical Chemists, Maryland, USA

Agrawalla J, Sethy K, Behera K, Swain RK, Mishra SK, Sahoo N, Mohapatra MR, Khadenga S (2017) Improved reproductive performance of crossbred cattle in Puri district of Odisha following supplementation of area specific mineral mixture. Indian J Anim Reprod 38: 43-45. 19

Begum I, Azim A, Akhter S, Anjum MI, Afzal M (2010) Mineral dynamics of blood and milk in dairy buffaloes fed on calcium and phosphorus supplementation. Pakistan Vet J 30: 105-109

Behera PC, Das M, Tripathy DP, Panigrahi B, Panda N (2012) Mineral supplementation and its relevance in improving conception rate in anestrus and repeat breeding heifers. Intas Polivet 13: 17-21

Chander Datt and Aruna Chhabra (2005) Mineral status of Indian feeds and fodders: A review. Indian J. Dairy Sci 58: 305-320

Chaudhary RK, Patel DC (2019) Augmenting blood profile and reproduction in buffaloes of tribal areas of Vadodara district (Gujarat)

through appropriate mineral mixture supplementation. The Indian J Vet Sci Biotechnol 14: 51-55

Das A, Biswas P, Rajendran D (2003) Micronutrient profile of feeds, fodders and animals in hill zone of West Bengal. Anim Nutr Feed Technol 3: 9-16

Devasena B, Reddy IJ, Ramana JV, Prasad PE, Prasad JR (2010) Effect of Supplementation of Area specific mineral mixture on reproductive performance of crossbred cattle a field study. Indian J Anim Nutr 27: 265-270

El Ashry GM, Hassan AAM, Soliman SM (2012) Effect of feeding a combination of zinc, manganese and copper methionine chelates of early lactation high producing dairy cow. Food Nutr Sci 3:

Exton JH (1980). Effect of diets low in calcium and phosphorus on development of growing lambs. Ameri J Physio 3: 235-238

Gerber N (1892) Die acid-butyrometrie, eine neue fettbestimmungs modification. Milch-Zeitung 53: 390-892

Ghosh MK (2013) Area-specific mineral formulation for feeding of yak and yak cattle hybrid. ERS, NDRI, Kalyani, West Bengal, India

Godara RS, Naskar S, Das BC, Godara AS, Ghosh MK, Mondal M, Bhat SA (2015) Effect of area specific mineral supplementation on biochemical profile in female Black Bengal goats. J Anim Res 5: 263-268

Goodwin JF (1970) Spectrophotometric quantitation of plasma and urinary amino nitrogen with flurodinitrobenzene. Stand Meth Clin Chem 6: 89-98

Hegde M, Jaisunder S, Kumaresan A, Datt C, Singh P, Singh A (2016). Micro mineral concentration in soil, plant and dairy cattle in Andaman Group of Islands. Indian J Anim Nutr 33: 394-398

Hegde M, Jaisunder S, Singh P, Datt C (2018) Macro mineral status in soil, plant and dairy cattle in Andaman group of islands. Indian J Anim Nutr 35: 415-420

Hornick JL, Eenaeme CV, Gauthier S, Baldwin P, Istasse L (1996) Glucose, alpha-amino nitrogen, and amino acid exchange across the hind limb in young double-muscled type bulls maintained at two growth rates. Canadian J Anim Sci 76: 193-202

Hornick JL, Van Eenaeme C, Diez M, Minet V, Istasse L (1998) Different periods of feed restriction before compensatory growth in Belgian Blue bulls: II. Plasma metabolites and hormones. J Anim Sci 76:260-271

Husain SS (1999) Sustainable genetic improvement of economic traits of Black Bengal goats through selective and crossbreeding. Bangladesh Agricu University Res Progr 10:72-80

McDowell LR, Conrad JH, Ellis GL, Loosli JK (1983) Minerals for grazing ruminants in tropical regions (p. 112). Gainesville: University of Florida.

Mondal M, Prakash BS (2003) Changes in plasma GH, LH and progesterone and blood metabolites following long-term exogenous somatoliberin administration ingrowing buffaloes (Bubalus bubalis). J Anim Vet Adv 2: 259-270

Niaz F, Sethy K, Swain RK, Behera K, Mishra SK, Karna DK, Mishra C (2017) Combined effect of concentrate and area specific mineral mixture supplementation on the performance of Ganjam goat in its native tract. Pharma Innovation 6: 320-323

Nocek JE, Socha MT, Tomlinson DJ (2006) The effect of trace mineral fortification level and source on performance of dairy cattle. J Dairy Sci 89:2679-2693.

Panda M, Panda N, Panigrahi B, Swain RK, Mishra A (2016) Mineral profile and metabolic status of cattle in western undulating zone of Odisha. Indian J Anim Nutr 33: 290-295

Pandey VK (2018) Effect of Area specific mineral mixture on serum biochemical parameters and milk yield in dairy cattle. Intl J Agric Sci 10: 4965-4968

Rabiee AR, Lean IJ, Stevenson MA, Socha MT (2010) Effects of feeding organic trace minerals on milk production and reproductive performance in lactating dairy cows: a meta-analysis. J Dairy Sci 93: 4239-4251

Sahoo B, Kumar R, Garg AK, Mohanta RK, Agarwal A, Sharma AK (2017) Effect of supplementing area specific mineral mixture on productive performance of crossbred cows. Indian J Anim Nutr 34: 414-419.

Samanta CS, Mondal MK, Biswas P (2005) Effect of feeding mineral supplement on the reproductive performance of anestrous cows, Indian J Anim Nutr 22: 177-184

Samanta A, Samanta G (2002) Mineral profile of different feed and fodders and their effect on plasma profile in ruminants of West Bengal. Indian J Anim Nutr 19: 278-281

Sharma MC, Joshi C, Das G (2009) Soil, fodder and serum mineral (cattle) and haematobiochemical profile in some districts of Central Uttar Pradesh. Indian J Anim Sci 79: 411-415

Sharma J, Kumar A, Tiwari DP, Mondal BC (2011) Effect of dietary supplementation of calcium, copper and manganese on nutrient utilization, growth, blood biochemical and mineral profile in crossbred heifers, Indian J Anim Sci 81:493-497

Sharma MC, Joshi C, Sarkar TK (2002) Therapeutic efficacy of minerals supplement in macro-minerals deficient buffaloes and its effect on haematobiochemical profile and production. Asian Australasian J Anim Sci 15: 1278-1287

Shipe WF, Senyk GF, Fountain KB (1980) Modified copper soap solvent extraction method for measuring free fatty acids in milk. J Dairy Sci 63:193-198

Singh DK, Sahu SP, Teufel N (2020) Mineral mixture feeding enhances dairy animals productivity: A study of Bihar, India. Indian J Anim Nutr 37: 26-30

Singh RK, Mishra SK, Swain RK, Dehuri PK, Sahoo GR (2011) Mineral profile of feeds, fodders and animals in mid-central table land zone of Orissa. Anim Nutr Feed Technol 11: 177-184

Singh S, Chhabra S, Randhawa SNS, Randhawa CS, Gupta DK (2016) Effect of area specific mineral mixture feeding on milk yield and composition of dairy animals of central zone of Punjab. Intl J Livest Res 6: 62-65

Snedecor GW, Cochran WG (1994) Statistical methods 8th edn. Oxford and IBH Publishing Corporation, Calcutta

Sultana S, Khan M, Hassan M, Khondoker M (2012) Effects of concentrate supplementation on growth, reproduction and milk yield of Black Bengal goats (Capra hircus). Bangladesh Vet 29: 7-16

SPSS (2010). Statistical packages for Social Sciences, Version 16, SPSS Inc., Illinois, USA

Tiwari MR, Shrestha BK, Mandal P, Panday LN (2013) Growth performance of Khari goats on supplementation of urea molasses mineral block (UMMB) in fodder based diets. Nepal J Sci Technol 13: 29-32

Tiwari R, Sharma MC, Singh BP (2013) Awareness and impact of area specific mineral mixture technology in field situation. Indian J Anim Sci 83: 435-437

Upadhyay AK (2004). Effect of mineral supplementation on conception of dairy animals. Indian J Vet Med 24: 34-38

Van Saun RJ (2000) Blood profiles as indicators of nutritional status. Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon

Van Soest PV, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74:3583-3597

Wu Z, Satter LD, Sojo R (2000) Milk production, reproductive performance, and fecal excretion of phosphorus by dairy cows fed three amounts of phosphorus. J Dairy Sci 83:1028-1041

Indian J Dairy Sci 74(1): 107-111
https://doi.org/10.33785/IJDS.2021.v74i01.015

RESEARCH ARTICLE

Influence of FMD vaccination stress on milk production in crossbred dairy cattle of Kerala

Pramod S, R ThirupathyVenkatachalapathy, Lasna Sahib and Bibin Becha B

Received: 18 June 2020 / Accepted: 24 November 2020 / Published online: 28 February 2021
© Indian Dairy Association (India) 2021

Abstract: Foot and mouth disease (FMD) is a contagious viral disease affecting cloven-footed animals which is endemic in India. The primary disease control strategy in India is mass vaccination using oil adjuvant killed FMD vaccine containing O, A and Asia 1 serotypes. Variations in milk yield following vaccination with oil adjuvant killed FMD vaccine was studied in 80 crossbred cattle, maintained at Livestock Research Station, Thiruvazhamkunnu for eight days. The study was conducted in August 2019. Rectal temperature, temperature-humidity index (THI) and milk quality were also monitored. Immunization resulted in transient febrile reaction(103.03±0.24 ^{o}F) and a significant reduction in milk yield ($p<0.01$). The average milk production (mean±SE) before vaccination was 7.68±0.28 kg, which dropped to 7.07±0.28 kg, on the day after vaccination. The highest losses were observed on the first day after vaccination, with a mean of 0.58±0.09 kg. Milk quality remained unaffected. Total milk yield of the herd rebounded to pre-vaccination levels by the sixth day after vaccination. The average cumulative production loss calculated over the period was 1.93 Kg per animal. Notable changes in feed or water consumption were not observed. The study observed that production loss due to vaccination is negligible in comparison to potential losses due to FMD in a herd.

Keywords: FMD vaccination, Milk production, Production loss, Vaccination stress

Kerala Veterinary and Animal Sciences University, Livestock Research Station, Thiruvazhamkunnu, Palakkad, Kerala, India

Thirupathy V R (✉)
Kerala Veterinary and Animal Sciences University, Livestock Research Station, Thiruvazhamkunnu, Palakkad, Kerala, India
Email: thirupathy@kvasu.ac.in

Introduction

Vaccination introduces killed or attenuated pathogens into the body of an animal, forcing the host to mount an immune response, as if it were a real infection. Injectible vaccines contain preservatives and materials like mineral oils which keep the antigens in suspension to prolong the immune reaction and to raise antibody titre to a protective level. Stress due to vaccination arises from the act of handling, injection and inflammatory reactions (Jo et al. 2014).

Foot and mouth disease (FMD) is a contagious viral disease affecting cloven-footed animals which is endemic in India. Office International des E´pizooties (OIE) recognized FMD as the most important constraint to international trade of animals and animal products. Losses of production, retarded growth, abortion, sub-fertility, etc. are noticed in recovered animals which erode the profitability of farming and drain the resources of the farmers (Pawar et al. 2010). Direct and indirect losses after the FMD outbreak are well studied, as indicated by publications by Laria et al. (2017); Baluka, (2016) and Sharma et al.(2016), among others. Anti FMD vaccination drives often stir discussions about production loss in the post-vaccination period among livestock farmers and technical personnel working alongside them. However, publications addressing this problem after vaccination in cattle were rare, which inspired the present study. The work was conducted at Livestock Research Station, Thiruvazhamkunnu (LRST) under Kerala Veterinary and Animal Sciences University to ascertain whether vaccination against FMD caused a significant reduction in milk yield or quality in the immediate post-vaccination period.

Materials and Methods

Crossbred lactating animals (n=80), maintained at Livestock Research Station,Thiruvazhamkunnu Tunder uniform management conditions were vaccinated using oil adjuvant FMD vaccine in August 2019. Milk production, milk quality parameters (*viz*. fat, solids not fat (SNF), protein, lactose content) and rectal temperatures were monitored after vaccination as detailed below. Milk production records (n=640) starting from one day before vaccination (day -1), the day of vaccination (day 0) and until six

days after vaccination (day 1 to day 6) were monitored. Animals were classified based on the number of days in milk as early stage (0-100 days), mid stage (101-200 days) and later stage(>200days) as suggested by Vijayakumar et al. (2017). There were 25 animals in the early stage, 27 animals in mid stage and 28 cows in the late stage of lactation. Rectal temperatures from a subsample of 12 animals were recorded using a digital thermometer (Omron) at daily intervals until day 4, starting from day 0. Milk quality parameters were quantified using Ekomilk Ultra pro milk analyzer (Milkana KAM98-2A) ultrasonic milk tester from another subset of six animals using samples collected from forenoon and afternoon milking. Temperature humidity index (THI) was calculated with data available from an automatic weather station (Campbell Scientific, CR 800 series data logger) maintained on the campus. The formula developed by Clemson University, USA, THI = T_{air}” (0.55"[0.55xRH/100])x(T_{air}-58.8)where, THI=Temperature Humidity Index, T_{air}= air temperature in Fahrenheit, RH =percent relative humidity(Kohli et al. 2014; Habeeb et al.2018) was used for calculating THI. Production data were analyzed using records of day 0 (day of vaccination) and day 1 (24 hrs after vaccination) with a paired sample t-test. Multiple paired t-tests with respect to day 0 were done to follow the variables throughout the period of study. Production losses were estimated based on the assumption that vaccination stress alone was the reason for the deviation, considering production on day 0, as the baseline production, whenever such differences appeared statistically significant.

Results and Discussion

Animals were maintained under uniform management conditions during the period of the study. Changes in feeding or drinking pattern were not observed. None of the animals were treated for systemic diseases or mastitis during the period of the study. Rectal temperature of animals, ambient temperature (average), ambient temperature (maximum), relative humidity (average) and temperature-humidity index observed/calculated during the first five days of study are presented in Table 1.

Weather parameters monitored during the period of the study indicated that the animals were more or less under uniform thermal stress. Cows were under mild thermal stress when THI remained between 72 and 79. The stress increased to a moderate level when THI was between 79 and 89(Kohli et al. 2014; Habeeb et al.

Table 1 Mean rectal temperature, weather parameters and THI

Days	Temperature			Relative humidity		THI	
	Rectal (°F) Mean±SE	Ambient (Max, °C)	Ambient (Avg, °C)	RH (Avg)	RH (Max)	Avg	Max
0	102.28±0.30	30.37	25.31	98.30	99.00	77.45	86.51
1	103.03±0.24	31.07	25.63	97.30	100.0	78.13	87.93
2	101.95±0.09	30.36	25.33	95.50	99.70	77.56	86.60
3	101.75±0.16	29.06	25.07	97.60	98.70	76.99	84.13
4	100.93±0.22	28.78	24.30	100.0	100.0	75.74	83.80

Table 2 Mean (±SE)milk composition (%)

Days	Forenoon (n=6)				Afternoon (n=6)			
	Fat	SNF	Lactose	Protein	Fat	SNF	Lactose	Protein
0	2.99±0.29	7.42±0.17	3.89±0.10	2.95±0.05	5.38±0.45	7.54±0.16	3.97±0.09	2.98±0.05
1	2.80±0.23	7.56±0.11	4.00±0.06	2.98±0.04	4.43±0.20	7.39±0.11	3.88±0.06	2.94±0.04
2	3.10±0.20	7.88±0.32	4.17±0.18	3.10±0.11	4.63±0.22	7.22±0.06	3.78±0.03	2.88±0.02
3	3.19±0.43	8.01±0.25	4.24±0.13	3.15±0.08	4.62±0.18	7.49±0.20	3.94±0.11	2.97±0.07

Table 3 Mean total milk production during pre/post-vaccination period

Pre/Post-vaccination period	Milk Production(Mean±SE,kg)					
	Forenoon (n=80)		Afternoon (n=80)		Total (n=80)	
	Total	Mean	Total	Mean	Total	Mean
Day -1	393.0	4.91±0.18	221.0	2.76±0.10	614.0	7.68±0.28
Day 0	393.5	4.92±0.17	218.5	2.73±0.11	612.0	7.65±0.28
Day 1	358.0	4.48±0.18**	207.5	2.59±0.12*	565.5	7.07±0.27**
Day 2	377.0	4.71±0.18**	204.0	2.55±0.11**	581.0	7.26±0.28**
Day 3	379.5	4.74±0.17	212.0	2.65±0.10	591.5	7.39±0.27*
Day 4	378.5	4.73±0.17*	210.0	2.63±0.09	588.5	7.36±0.26**
Day 5	366.0	4.58±0.18**	213.5	2.67±0.10	579.5	7.24±0.27**
Day 6	383.5	4.79±0.18	216.5	2.71±0.10	600.0	7.50±0.28

**P<0.01, *P<0.05, Superscripts indicate a significant difference between rows from day 0

2018). Animals remained under mild stress most of the time, which exacerbated to moderate stress when daily temperatures peaked. Therefore, it is reasonable to assume that production loss observed in the study on the first day after immunization was due to vaccination stress. Rectal temperatures of the sampled animals were elevated by about 1°F on the first day after vaccination. Though the increase in rectal temperature was not statistically significant, it was in the range of mild pyrexia, since the normal rectal temperature reported for a dairy cow was 100.4 to 102.8 °F (Susan et al. 2016).The average rectal temperature returned to baseline on the second day after vaccination and dropped consistently over the following days. A similar finding was reported in buffaloes, except that temperatures were recorded on day 3 after vaccination against FMD and fever was not noted (Sivajothi et al. 2018). Pyrexia could be due to the use of oil adjuvant which was known to induce reactions, like granuloma, abscesses or fever (Aucouturier et al. 2001).

Milk quality parameters remained unaffected by vaccination stress. The mean±SE values (%) of fat, SNF, lactose and protein observed in the present study are presented in Table 2. Observations from the present study coincide with the report by Rao et al. (2017) in this regard. Another interesting finding was that SNF in milk level remained below specifications (8.3%) maintained by food safety and standards regulations (2019). Fat content was also lower than specifications (3.2%) in the forenoon milk samples.

Milk production remained stable on the day of vaccination and the previous day. The average milk production (mean±SE) of the animals on the day before vaccination was 7.68±0.28 kg, which dropped to 7.07±0.28 kg, after vaccination. Consequently, milk production of the herd decreased by about 7.6 percent from 612 kg to 565.5 kg. The summary of post-vaccination milk production is presented in Table 3. A significant drop (P<0.01) in total milk production was noticed between day 0 and day 1 in animals across all stages of lactation. Both forenoon (P<0.01) and afternoon (P<0.05) milk productions differed significantly between day 0 and day 1. The difference in total milk production of herd with respect to day 0 remained significant until day 5 after vaccination.

The mean milk production for different stages of lactation is presented in Table 4. Total milk yield, *ie*. sum of forenoon and afternoon production; was reduced significantly in animals across all the three stages of lactation on day 1 after vaccination. Further, it remained significantly reduced on day 2 after vaccination in early lactation and mid-lactation animals. In early and mid-lactation groups, no significant difference was observed in forenoon milk production after vaccination. However, forenoon milk yield dropped significantly among animals in late lactation.

Significant reduction in milk yield was observed in afternoon milk production on day 2 (P<0.01), day 4 (P<0.05) and day 5 (P<0.05) after vaccination inthe early lactation group. In mid

Table 4 Lactation stage-wise mean milk production during pre/post-vaccination period

Pre/Post-vaccination period	Milk Production (Mean±SE,kg)								
	Early lactation (n=25)			Mid lactation (n=27)			Late lactation (n=28)		
	FN	AN	Total	FN	AN	Total	FN	AN	Total
Day -1	5.78±0.39	3.34±0.21	9.12±0.59	4.57±0.25	2.57±0.16	7.15±0.40	4.46±0.25	2.43±0.14	6.89±0.37
Day 0	5.66±0.37	3.44±0.21	9.10±0.57	4.59±0.27	2.57±0.15	7.17±0.40	4.57±0.24	2.25±0.15	6.82±0.39
Day 1	5.42±0.39	3.18±0.21	8.60±0.53**	4.39±0.24	2.41±0.17*	6.80±0.40**	3.71±0.22**	2.25±0.22	5.96±20.38**
Day 2	5.52±0.35	3.14±0.20**	8.66±0.54*	4.50±0.27	2.26±0.15**	6.76±0.41**	4.19±0.30*	2.30±0.18	6.50±0.41
Day 3	5.48±0.38	3.32±0.18	8.80±0.55	4.54±0.23	2.46±0.14	7.00±0.37	4.29±0.25*	2.32±0.13	6.52±0.38
Day 4	5.60±0.36	3.18±0.16*	8.78±0.50	4.54±0.25	2.39±0.13	6.93±0.37	4.14±0.24**	2.36±0.15	6.50±0.38*
Day 5	5.48±0.37	3.20±0.17*	8.68±0.53	4.26±0.24	2.57±0.16	6.83±0.40	4.07±0.24**	2.29±0.16	6.36±0.38**
Day 6	5.62±0.37	3.40±0.19	9.02±0.55	4.5±0.25	2.43±0.14	6.93±0.37	4.34±0.27	2.36±0.15	6.69±0.42

**P<0.01, *P<0.05, Superscripts indicate a significant difference between rows from day 0

Table 5 Parity wise mean milk production during pre/post-vaccination period

Pre/Post-vaccination period	Milk Production (Mean±SE,kg)								
	1st Parity (n=32)			2nd Parity (n=29)			Parity > 3 (n=19)		
	FN	AN	Total	FN	AN	Total	FN	AN	Total
Day -1	4.79± 0.33	2.62±0.19	7.42±0.52	5.31±0.24	2.98±0.14	8.29±0.37	4.50±0.36	2.65±0.22	7.15±0.55
Day 0	4.62±0.31	2.60±0.21	7.23±051	5.27±0.26	2.98±0.15	8.25±0.41	4.86±0.33	2.55±0.20	7.42±0.51
Day 1	4.43±0.33	2.45±0.19	6.89±0.52**	4.82±0.23*	2.87±0.21	7.70±0.37**	4.00±0.37**	2.39±0.24	6.39±0.50**
Day 2	4.53±0.33	2.46±0.19	7.00±0.51*	5.10±0.23	2.82±0.15	7.93±0.35*	4.42±0.35*	2.26±0.22*	6.68±0.57**
Day 3	4.51±0.32	2.57±0.18	7.09±0.50	5.24±0.23	2.84±0.14	8.08±0.36	4.36±0.31*	2.47±0.20	6.84±0.49*
Day 4	4.53±0.33	2.57±0.17	7.10±0.50	5.22±0.21	2.75±0.13	7.98±0.33*	4.31±0.30**	2.50±0.18	6.81±0.46**
Day 5	4.45±0.33	2.57±0.17	7.03±0.50	5.01±0.22	2.93±0.15	7.93±0.36	4.10±0.32*	2.42±0.19	6.52±0.50**
Day 6	4.57±0.32	2.60±0.18	7.18±0.50	5.22±0.26	2.60±0.19	8.18±0.37	4.50±0.38*	2.47±0.18	6.97±0.55

**P<0.01, *P<0.05, Superscripts indicate a significant difference between rows from day 0

Table 6 Production loss due to vaccination stress

Pre/Post-vaccination period	Production loss (kg)							
			Early lactation (n=25)		Mid lactation Stage 2 (n=27)		Late lactation Stage 3 (n=28)	
	Yield loss	Mean reduction±SE	Yield loss	Mean reduction±SE	Yield loss	Mean reduction±SE	Yield loss	Mean reduction±SE
Day 1	-42.5	0.58±0.09	-12.50	0.50±0.16	-10.00	0.37±0.12	-24.00	0.86±0.21
Day 2	-30.5	0.39±0.09	-11.00	0.44±0.18	-11.00	0.40±0.12	-10.50	0.38±0.15
Day 3	-20.5	0.26±0.10	-	-	-	-	-8.00	0.28±0.12
Day 4	-23.5	0.29±0.10	-	-	-	-	-12.00	0.42±0.12
Day 5	-32.5	0.41±0.10	-	-	-	-	-14.00	0.50±0.13
Total	-154		-23.50		-21.0		-68.5	

lactating animals, afternoon milk yield was significantly reduced on day 1 and day 2 after vaccination. However, no significant change was noticed in the afternoon milk yield of cattle in late lactation.

The parity wise mean milk production is presented in Table 5. A significant drop in milk production (P<0.01) was observed on day 1 after vaccination across all parity groups. Forenoon milk production was also reduced in second parity animals (P<0.05) and >3 parity group (P<0.01) on day 1 after vaccination. Average milk production in the forenoon remained significantly lower even on the 6th day after vaccination among animals of third and higher parity. Total milk production remained significantly reduced until 5 days after vaccination.

Cumulative losses with respect to total yield stood at 154.0 Kg, from day 1 to day 5 after vaccination, during which such differences were statistically significant. Production loss observed in the study due to vaccination stress is presented in Table 6.

The highest drop in milk yield was observed (7.6%) on the first day after vaccination, which averaged at 0.58 Kg per animal. Total milk yield returned to the baseline on the 6thday after vaccination. The average production loss calculated over the period was 1.93 Kg per animal .Among animals in late lactation, forenoon milk yield alone was affected, while afternoon yields remained stable. Nearly half of the animals in the third and higher parity group (n=9) were also in the late stage of lactation. Animals in the third stage of lactation were severely affected by vaccination stress so that they accounted for 44.5% of the total loss, besides taking 5 days to regain yield. Working in temperate regions, Canadian researchers (temperate region) reported that Holstein Frisian animals in early lactation suffered the highest production loss (Scott et al. 2001) after immunization with 9 way killed vaccines. This result is in contrast with the findings of the present study. The differences could be attributed to environmental and genetic factors.

Milk production loss after FMD outbreak among 59 cattle at Chazhoor panchayath in Thrissur district of Kerala was estimated at 80.68% and was valued at Rs. 2.53 Lakhs. The loss per animal in this regard was roughly 4,300 Rupees or USD 63 (Mathew and Menon, 2008). The loss observed in the present study due to stress following FMD vaccination was about Rupees 100 (USD 1.5) per animal which is negligible in comparison with probable post-outbreak losses.

Conclusions

Vaccination against FMD using oil adjuvant vaccines resulted in febrile reaction and production loss. Total milk yield regained to pre-vaccination levels on the sixth day after vaccination. Among early lactation and mid-lactation animals, total production returned to pre-vaccination level as early as the third day after vaccination. In the late lactation group, afternoon production remained stable even though forenoon yields returned to pre-vaccination state only after five days. The present study was conducted during the southwest monsoon when climate related stress remained at a mild to moderate level. The production loss could probably exceed 7.6% when other factors like heat stress in summer comes into play. This study throws some light on the need to formulate guidelines to reduce the stress associated with FMD vaccination and the season of vaccination. Loss in milk production due to vaccination stress is negligible when compared with potential loss due to the FMD outbreak.

Acknowledgments

The study was supported by the State plan project (2018-19) "Improvement of milk production in cattle and goats at LRST" funded by the Government of Kerala.

References

Aucouturier J, Dupuis L, Ganne V (2001) Adjuvants designed for veterinary and human vaccines. Vaccine 19: 2666-2672

Baluka SA (2016) Economic effects of foot and mouth disease outbreaks along the cattle marketing chain in Uganda. Vet World 9: 544-553

Habeeb AA, Gad AE, Atta, MA (2018) Temperature-humidity indices as indicators to heat stress of climatic conditions with relation to production and reproduction of farm animals. Int J Biotechnol. Recent Adv1: 35-50

Jo NC, Jung J, Kim JN, Lee J, Jeong SY, Kim W, Sung HG, Seo S (2014) Effect of vaccination against foot-and-mouth disease on growth performance of Korean native goat (*Capra hircuscoreanae*). J Anim Sci 92: 2578-2586

Kohli S, Atheya UK, Thapliyal A (2014) Assessment of optimum thermal humidity index for crossbred dairy cows in Dehradun district, Uttarakhand, India. Vet World 7: 916-921

Laria MA, Fani MM, Lyons NA, Azizi N (2017) Impact of FMD outbreak on milk production and heifers' growth on a dairy herd in southern Iran. Prev Vet Med 144: 117-122

Mathew L, Menon DG (2008) Economic impact of FMD in Chazhoor panchayath. Vet World 1: 5-6

Pawar SS, Tamilselvan RP, Saravanan S, Sanyal A, Mohapatra JK, Pattnaik B (2010) Foot and Mouth Disease (FMD): India and the world. Sci India 13: 36-42

Rao TKS, Kumar B, Sharma VK, Sriranga KR, Baishya A, Bhakat M, Mohanty TK (2017) Effect of vaccination on performance of dairy animals with special reference to bulls: a review. Theriogenol Insight 7: 185-197

Scott HM, Atkins G, Willows B, McGregor R (2001) Effects of 2 commercially-available 9-way killed vaccines on milk production and rectal temperature in Holstein-Friesian dairy cows. Can Vet J 42: 793-798

Sharma VB, Verma MR, Qureshi S, Bharti P (2016) Effects of diseases on milk production and body weight of cattle in Uttar Pradesh. IJAEB 9: 463-465

Sivajothi S, Reddy YVP, Reddy S, Vani S, Chandel S (2018) Evaluation of herbal anti-stressor product (Restobal) during the vaccination stress in buffaloes. Int J Curr Microbiol App Sci 7: 723-727

Susan EA, Michael AM, Dana GA (2016) The Merck Veterinary Manual. 11th Ed. Merck & Co., New Jersey

Vijayakumar M, Park JH, Ki, KS, Lim DH, Kim SB, Park SM, Jeong HY, Park BY, Kim TI (2017) The effect of lactation number, stage, length, and milking frequency on milk yield in Korean Holstein dairy cows using automatic milking system. Asian Australas J Anim 30: 1093-1098