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INVITED REVIEW

Flaxseed lignan: Metabolism, extraction and isolation techniques, potential health benefits and applications in dairy foods

Elizabeth Thomas [1], Narender Raju Panjagari [1](✉), Sangita Ganguly [1], Suman Kapila [2] and Ashish Kumar Singh [1]

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Abstract: Flaxseed lignan has been recognized as a potent phytoestrogen for the treatment of health problems such as cancer, diabetes, hyperlipidaemia, cardiovascular diseases, postmenopausal related diseases and so on. Existing literature suggests that higher dietary or supplementary intakes of lignans and other phytoestrogens have been linked to improved cognitive performance in middle-aged and older people. The lignan secoisolariciresinol diglucoside is found in abundance in flaxseed. Flaxseed lignan metabolites may offer health benefits due to their weak estrogenic or anti-estrogenic effects, antioxidant activity or other yet-to-be-identified mechanisms. The current review explores the metabolism of flaxseed lignan by gut microbiota, its bio accessibility, health benefits of flaxseed lignan in humans and the possible mechanisms, with data from animal and clinical studies in the last few years to back up that assertion, application in food and dairy industry and makes recommendations for future research.

Key words: *Linum usitatissimum* L.; Lignan; Secoisolariciresinol diglucoside; Enterodiol; Enterolactone; Menopause; Functional property; Food applications

Introduction

The health and therapeutic advantages of flaxseed (*Linum usitatissimum* L.) and its derivatives (ground flax, flax oil, defatted flax, flax fiber, and lignan extract) have been proven. Flaxseed includes polyunsaturated oil, soluble and insoluble (dietary) fiber, and the plant lignan secoisolariciresinol diglucoside (SDG), which may all help with disease prevention and health promotion (Raole and Raole, 2022). Some of the possible advantages of flaxseed include alpha-linolenic acid (ALA) as an antihypertensive agent (Verma et al. 2020), enterolignans generated from SDG, enterolactone (ENL) and enterodiol (END) as antioxidants and 17-β estradiol mimetics (Albuquerque et al. 2020), and dietary fiber for cholesterol reduction (Prasad et al. 2020). SDG is a phytoestrogen, or plant hormone (Zare et al. 2022) and flaxseed has been determined to be the greatest source of SDG among diverse plant foods, with almost 1000 times the amount of SDG found in sesame seed, pumpkin seed, wheat, lentils, soybeans, pears, prunes, garlic, asparagus and carrot (Ebrahimi et al. 2021). SDG levels in defatted flaxseed powder have been reported to vary between 6 to 29 g/kg (Kaur and Sharma, 2021) equivalent to 3.4 to 14.40 mg/100g secoisolariciresinol (SECO) (Hyvärinen et al. 2006a). Matairesinol (0.002 g/kg), pinoresinol (0.007 to 0.248 g/kg), lariciresinol (0.028 to 0.033 g/kg), and isolariciresinol (0.102 g/kg) are other lignans identified in flaxseed, although their concentrations are low in comparison to SECO (Edel et al. 2015). Through its mammalian metabolites, enterodiol and enterolactone, SDG is thought to have phytoestrogenic properties (Hu et al. 2007). SDG is initially converted to END in the intestines by microflora, which can subsequently be further metabolized to ENL. SDG is deglycosylated into mammalian lignans by the activity of β-glycosidases, which assures lignan bioavailability and peripheral circulation in humans (Braune and Blaut, 2016). It has also been claimed that flaxseed flour and defatted meal produced the greatest output of END and ENL *in vitro*, up to 800 times that of others (Akter et al. 2021). Other lignans in flaxseed can also be converted to END and/or ENL, although their overall impact on enterolignan concentration is considerably lower than SDG's. *In vivo*, these enterolignans undergo enterohepatic circulation as they get conjugated in the liver following intestinal absorption and are discharged in bile or urine, where they are reabsorbed and repackaged as β-glucuronide or sulphate conjugates (Edel et al. 2015).

Flaxseed contains lignans in the secondary wall of the sclerite cells of the seed's outer integument (Chhillar et al. 2021). It is stored as a hydroxymethyl glutaryl ester-linked complex (SDG-HMG) (Sainvitu et al. 2012). SDG has emerged as a possible dietary

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component for health promotion being a vital substance in plant foods with functional value (Rodríguez-García et al. 2019). *Linum* spp. lignans have been found to be of substantial functional value and are thus intensively investigated. *Linum album*, which is a rich source of the anticancer drug podophyllotoxin (PTOX), has emerged as a paradigm for lignan-based research in other *Linum* species. Arylnaphthalene lignans were found in many different *Linum* species, including *Linum altaicum* (purple flax) and *Linum glaucum* (Chhillar et al. 2021).

Earlier investigations have revealed that SDG is metabolized by bacteria in the intestinal tract of humans and animals to enterodiol (END) and enterolactone (ENL) (Taibi et al. 2021), which have beneficial effects against osteoporosis, cardiovascular disease, hyperlipemia, breast cancer, colon cancer, prostate cancer and menopausal syndromes (Kezimana et al. 2018).

Mechanism of conversion of SDG into END and ENL

Plant lignans may be degraded and transformed to enterolignans (Li et al. 2022) (entero- from Greek *enteron* meaning "intestine") by bacteria in the intestine (Borriello et al. 1985). Enterolactone (ENL) and enterodiol (END) are the two main enterolignans or mammalian lignans generated by mammalian gut bacteria. Following consumption of SDG or similarly glycated lignans, such as pinoresinol diglucoside or sesaminol triglucoside (STG), the sugar moieties are hydrolyzed by *O*-linked deglycosylation in the large intestine, resulting in SECO and the other aglycones (Li et al. 2022). Four reactions must occur while converting SDG to ENL (Senizza et al. 2020). SDG is first transformed to SECO by *O*-linked deglycosylation, and then SECO is converted to the intermediate dihydroxyenterodiol (DHEND) via *O*-linked demethylation (Seyed-Hameed et al. 2020). From here, DHEND may be transformed to END by dehydroxylation, and then to ENL via END dehydrogenation. Alternatively, DHEND can be dehydrogenated to create a lactone ring, resulting in the formation of a second intermediate dihydroxyenterolactone (DHENL), which can subsequently be dehydroxylated to generate ENL (Ruiz de la Bastida et al. 2021; Yoder et al. 2015). ENL, the primary mammalian enterolignan generated in the rumen, is transported into physiological fluids, possibly benefiting human health in terms of menopausal symptoms, hormone-dependent malignancies, cardiovascular disease, osteoporosis, and diabetes (Schogor et al. 2014; Seyed-Hameed et al. 2020). Kuijsten et al. (2006) evaluated the enterolignan pharmacokinetics in healthy men and women taking a single dose of purified SDG (1.31 µmol/kg body weight) and reported that enterolignans were detected in plasma 8 to 10 hours following intake of pure SDG. END and ENL had a maximum plasma concentration after 14.8 h and 19.7 h of SDG ingestion, respectively and the mean elimination half-life of END (4.4 h) was less than that of enterolactone (12.6 h). The pharmacokinetics of the SDG supplemented through different food systems are yet to be identified.

Microorganisms involved in the conversion of SDG to END and ENL

Several researchers have assessed the ability of different gut bacteria to carry out the processes required for the conversion of glycosylated lignans to enterolignans (Senizza et al. 2020; Taibi et al. 2021). *Bacteroides fragilis*, *Bacteroides ovatus*, *Clostridium cocleatum*, *Clostridium saccharogumia*, *Clostridium ramosum*, and *Bacteroides distasonis* have all been found to be capable of doing *O*-deglycosylation, with the first four able to completely deglycosylate SDG within 20 hours of the experiment (Yoder et al. 2015). Deglycosylated SECO then undergo demethylation to create its intermediary DHEND. *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, *Eubacterium limosum*, *Clostridiaceae bacterium* END-2 (commonly known as 'strain END-2') and *Blautia producta* were shown to be capable of catalysing this process (Clavel et al. 2006a; Jin and Hattori, 2010). Following demethylation, *Eggerthella lenta* and *Clostridium scindens* can act on the intermediate to produce END by removing a hydroxyl group from each aromatic ring (Clavel et al. 2006b). *Lactonifactor longoviformis* and the above mentioned 'strain END-2' finish ENL synthesis by forming the lactone ring (Jin and Hattori, 2010). Alternatively, following SECO demethylation, *L. longoviformis* may produce the lactone ring, in which case END does not develop but rather a second intermediate, DHENL, which may then go on to make ENL (Yoder et al. 2015).

Figure 1 depicts the potential metabolism for converting SDG to END and ENL, as well as the microorganisms engaged in these processes. Bacteria generate both END and ENL enantiomers, and human exposure to the enantiomers arises from the interplay between the initial type of substrate and the makeup of the bacterial consortia (de Silva and Alcorn, 2019). When individuals consume their normal diets, (-) ENL predominates in serum, but when supplemented with flaxseed, (+) ENL increases significantly while (-) ENL increases very little. Furthermore, the types of END and ENL extracted after incubating SDG with gut bacteria was (+) END and (+) ENL (Saarinen et al. 2010). McCann et al. (2021) explored the connections between gut microbiota and lignan metabolism. For six weeks, 252 healthy postmenopausal women were given ten grams of ground flaxseed every day. Microbial colonies were discovered in urine and stool samples. There were *Slackia*, *Senegalimassilia*, *Klebsiella* and *Lactobacillus* present, all of which were linked to ENL production. Bacteria previously associated to colorectal cancer and cardiovascular disease such as *Pyramidobacter*, *Odoribacter* and *Fusobacteria*, were shown to be significantly reduced in the FS intervention.

Extraction and Isolation of SDG from Flaxseed

SDG is further polymerized (or oligomerized) in flaxseed, where it exists as part of a bigger complex comprised of five SDG residues linked by ester linkages to four 3-hydroxy-3-methylglutaric acids (Dauwe et al. 2021). SDG (35%), cinnamic acid glycosides, and

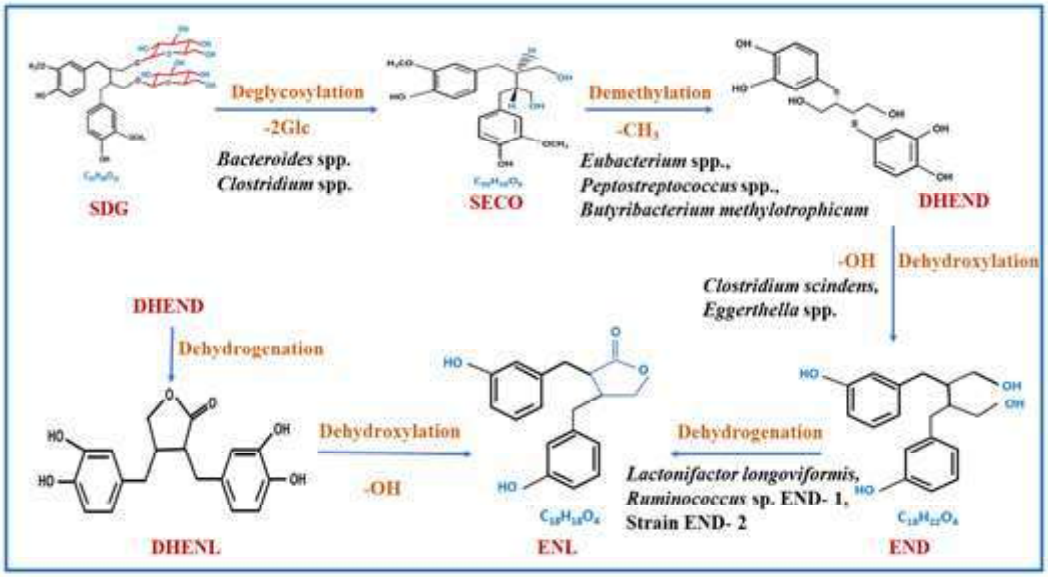

Fig. 1 Potential metabolism for converting SDG to END and ENL and the microorganisms engaged in these processes (Plotted according to the data obtained from Yoder et al. (2015)

hydroxymethyl glutaric acid (HMGA) are common components of this lignan complex (Hosseinian and Beta, 2009). The structure of SDG oligomer is illustrated in Figure 2.

As reported by Eliasson et al. (2003) these ester linkages may be readily and selectively broken by alkaline hydrolysis to produce SDG. To separate additional lignans from flaxseed, the glycosidic link of SDG must be broken.To separate this lignan complex from flaxseed, a simple method is usually required, while further procedures are required to isolate SDG from the complex. The lignans and gums (viscous soluble fibre) in flaxseed are mostly found in the hulls that surround the seeds, whereas the bulk of the proteins and lipids are found in the kernel/embryo. Carbohydrates (48.3 %), proteins (16.8 %), crude oil (26.5 %), moisture (5.0 %), and ash (3.5 %) are the chemical constituents in flaxseed hulls, while, on a dry basis the embryo fraction includes 22.0 % carbohydrates, 23.9 % proteins, 47.7 % crude oil, 3.6 % moisture, and 3.8 % ash (Hosseinian and Beta, 2009). The hull is divided into two distinct fractions, the mucilage fraction and the fibre fraction. The outer layers of hull comprises the mucilage portion and is rich in water soluble carbohydrates whereas the inner layers of hull forms the fibre fraction and is particularly rich in insoluble fibres and lignans. SDG is a component of mucilage fraction. The methods for the extraction of lignan complex make use of SDG's solubility in alcohol and water. The solvent-to-meal ratio during extraction ranges from 5:1 to 7:1 and from 12:1 to 16:1 (Hosseinian and Beta, 2009). For base hydrolysis in water or alcohol to release SDG, sodium hydroxide or calcium hydroxide are typically used (Zhuang et al. 2021). SDG must be released from its polymeric lignan precursor by breaking the ester-linkages in the complex. The SDG concentrates produced with calcium hydroxide easily separate from insoluble calcium salts, yielding a non-hygroscopic and relatively pure product. The base is generally used at a concentration of about 1 normal, and it is preferably used at a concentration of approximately 3-7 percent w/v (Hosseinian and Beta, 2009). The hydrolysis process is

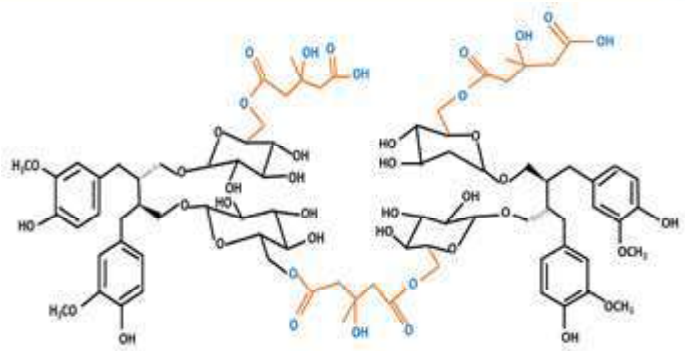

Fig. 2 Structure of SDG oligomer containing SDG and 3 hydroxy-3-methyl-glutaric acid (HMGA) units (shown in different colour)

Fig. 3 Conversion of secoisolariciresinol diglucoside (SDG) to anhydrosecoisolariciresinol (ASH)

typically carried out over a period of 4 to 24 hours (Zhuang et al. 2021). This procedure also destroys the cyanogenic glycosides, resulting in an extract free of cyanogenic glycosides or free cyanide. Apart from alkaline hydrolysis, acid and enzymatic hydrolysis are described in the literature for extraction of SDG (Renouard et al. 2010; Sicilia et al. 2003). The stability of acid hydrolysis products has lately been questioned, because under acidic environment SECO can be partly converted to its anhydrous form anhydrosecoisolariciresinol (AHS) which is also called as

shonanin (Lehraiki et al. 2010). The conversion of SDG to AHS is shown in Figure 3. The enzymatic technique is a gentle and selective procedure, but it does not provide full lignan hydrolysis since these metabolites concentrate in the seed's highly resistant layer (Lehraiki et al. 2010).

Eliasson et al. (2003) compared two different methods for the extraction of SDG. In the first method the SDG complex was extracted using a mixture of 1,4-dioxane - 95% aq. ethanol (1:1 v/v). The alcoholic extract was then subjected to alkaline hydrolysis using 0.3 *M* aqueous sodium hydroxide in order to isolate SDG from SDG-HMGA complex. In other method defatted flaxseed flour was subjected to direct alkaline hydrolysis using 2 *M* NaOH and distilled water. The authors reported that the direct alkaline hydrolysis resulted in higher yield as compared to the other method. Hosseinian and Beta (2009) attempted to extract SDG with just water followed by direct alkaline hydrolysis in an attempt to eliminate the usage of organic solvents described in prior techniques and to achieve a product free of solvent residues. This technique was claimed to be appropriate for extracting SDG from flaxseed hull in large amounts with high purity for nutritional supplements or nutraceutical applications. Base hydrolysis is generally performed at temperatures above room temperature (50 to 100 °C). A greater temperature is required to separate SDG from bigger molecular weight components like protein and starch residues, which are coagulated and precipitated by heat. The pH range is 10 to 13, with 11.8 to 12.5 being the ideal range. The pH of the solution must be acidified to a pH range between 3 and 8.5 after hydrolysis to prevent the ionisation of any functional groups in the aliphatic and aromatic parts of the SDG molecule. Apart from the traditionally applied methods for SDG extraction, later the efficiency of microwaves and ultrasound for SDG extraction was carried out (Beejmohun et al. 2007; Corbin et al. 2015; Nemes and Orsat, 2011; Zhang and Xu, 2007). Microwave assisted extraction (MAE) for 3 min with 1 *M* sodium hydroxide alkaline treatment was reported to produce the highest quantity of SDG (16.1 mg/g), p-coumaric acid glucoside (3.7 mg/g), and ferulic acid glucoside (4.1 mg/g), regardless of irradiation power proving MAE as an efficient method for SDG extraction in improving SDG yield as well as in saving time and energy (Beejmohun et al. 2007). Similarly Corbin et al. (2015) developed an efficient ultrasound assisted extraction (UAE) method for SDG. The UAE technique has been shown to be extremely effective for reducing mucilage entrapment of flaxseed phenolics because deep modification of the seedcoat ultrastructure and mucilage release occurs during ultrasonic treatment. According to Corbin et al. (2015) the conditions which were found to be optimal for UAE of SDG from flaxseeds include water as solvent supplemented with 0.2 N sodium hydroxide for alkaline hydrolysis of the SDG–HMGA complex, an extraction time of 60 minutes at a temperature of 25 °C, and an ultrasound frequency of 30 kHz. Under these conditions the yield of SDG was reported as 23.6 mg/g on dry basis. In their study, Thomas et al. (2023) assessed the efficacy of various extraction methods, including direct alkaline hydrolysis coupled with magnetic stirring, microwave, and ultrasound, in extracting SDG from defatted flaxseed. The extraction methods employed yielded SDG quantities ranging from 11.74 to 14.30 mg g^{-1} flaxseed on a dry matter basis. The optimal production of SDG was found to be achieved by the utilization of direct alkaline hydrolysis (using a 1 M aqueous NaOH solution) in conjunction with magnetic stirring (at a rate of 400 rpm for 1 hour at a temperature of 60 °C).

Supercritical carbon dioxide (SC-CO_2) is a non-toxic and cost-effective solvent. It can extract polar phenolic compounds from plant components in conjunction with polar modifiers. This extraction occurs at temperatures and pressures above the critical point of carbon dioxide, which is 31 °C and 7.4 MPa, respectively. Comin et al. (2011) conducted a study to evaluate the effect of the supercritical carbon dioxide method on flaxseed SDG extraction. The analysis revealed that the optimal conditions for extracting SDG were 7.8 mol% ethanol, 45 MPa pressure, and 60 °C temperature. Nevertheless, using SC-CO_2 extraction resulted in substantially less SDG than conventional extraction techniques. Supercritical antisolvent fractionation (SAF), which uses the non-polarity of CO_2 to precipitate certain chemicals from a solution, is another attractive but understudied method. According to Perretti et al. (2013), SAF produced maximum lignan content of 12.96 g L^{-1} when treated for 180 min at a pressure of 30 MPa and a flow rate of 15 kg/h of CO_2.

Pressurized low polarity water (PLPW) extraction, also known as subcritical water extraction, is a technology that modifies the properties of water to improve its extraction ability by heating the water to temperatures of up to 374 °C and maintaining the pressure at a level high enough to keep the water in a liquid state. When water is heated from 25 to 200 °C, its dielectric constant drops from 79 to 35, approaching values close to those for ethanol or methanol. At 25 °C, the polarity of pure water is about the same as that of water-methanol or water-acetonitrile combinations (Cacace and Mazza, 2006). The feasibility of extracting lignans from flaxseed meals using pressurized low-polarity water (PLPW) was evaluated in a fixed bed extraction cell. Maximum lignan extraction was obtained at a high temperature (180 °C), flow rate of 0.6–2 mL/min, high pH (9) and a co-packing ratio of 1:1.5 meal to glass beads (Ho et al. 2008). In another investigation, the maximum yield (12.94 mg/g) and extraction yield (72.57%) were obtained using 5 g of flaxseed meal sticks at 180 °C for 15 minutes, 1.500 pressure, and 40% fresh water (Ozkaynak-Kanmaz and Ova, 2013).

Following extraction, the hydrolysate is concentrated by a rotary evaporator before being subjected to either a liquid/liquid partition, such as an ethyl acetate/water system, or an anion exchange to enrich the lignans further. The resulting lignan-enriched solution is subjected to chromatographic separation to extract lignans with a purity higher than 90%. To separate SDG from other contaminants, the SDG-containing hydrolysate is passed through a glass column filled with Sephadex anion exchange resin or C-18 reverse-phase resin (Hosseinian and Beta,

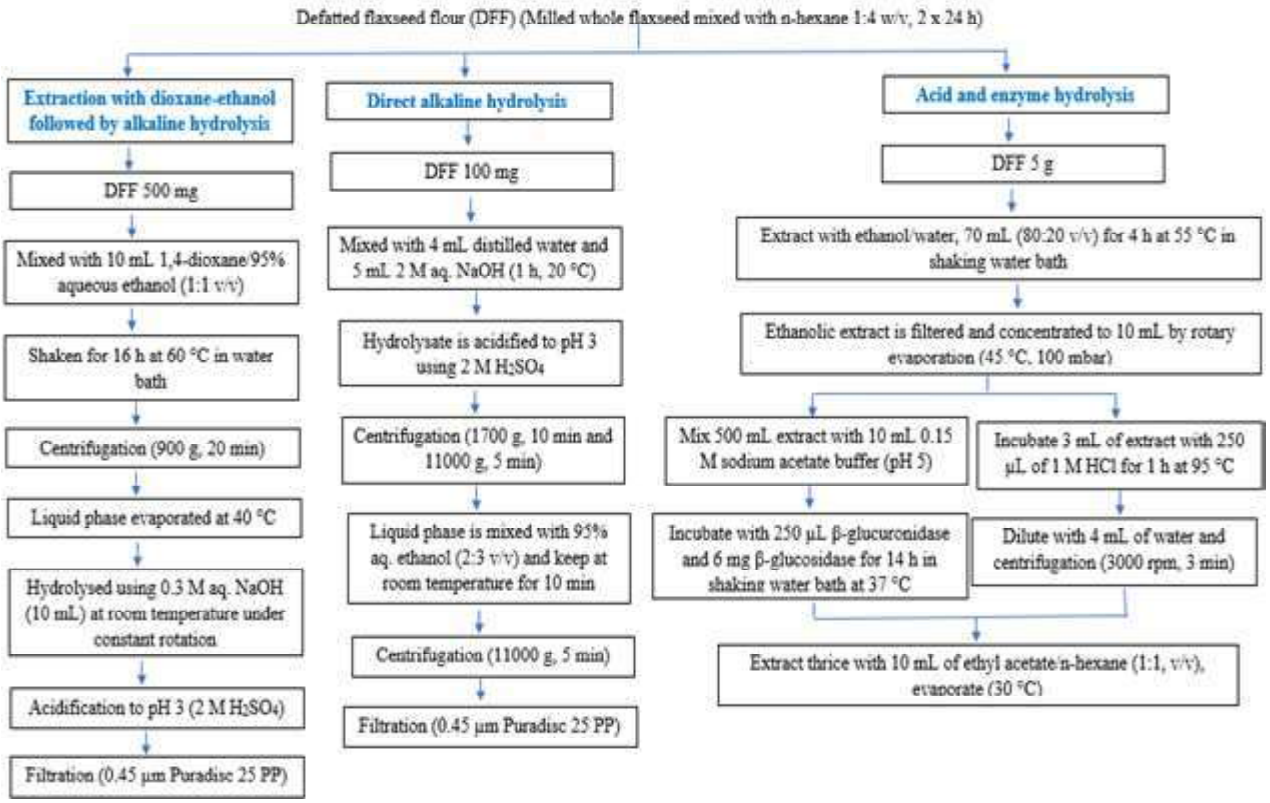

Fig. 4 Different methods for extraction of SDG (Eliasson et al. 2003; Johnsson et al. 2000; Sicilia et al. 2003)

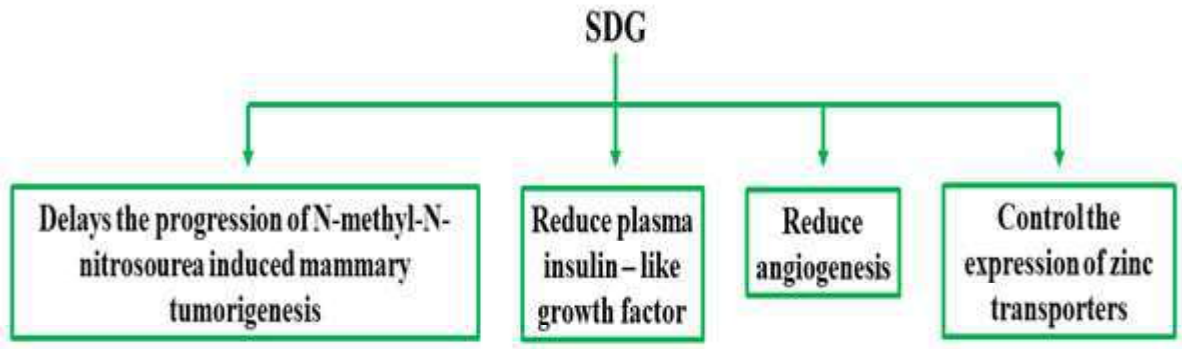

Fig. 5 Potential impact of SDG in breast cancer prevention

2009). The SDG is recovered by evaporation through freeze, spray, or vacuum drying. In a study, SDG was predicted to be recovered using lyophilization at a purity of 31% and yield of 3.2% by weight from defatted flaxseed with a 90% recovery rate (Dobbins and Wiley, 2004). Steps for extracting SDG from whole flaxseed by direct alkaline hydrolysis, acid and enzyme hydrolysis and extraction with dioxane ethanol followed by alkaline hydrolysis are illustrated in Figure 4.

Health benefits with consumption of the flax lignan secoisolariciresinol diglucoside

In human clinical trials and animal models, research on the health effects of whole flaxseed or flaxseed products has shown positive changes in blood lipid profiles, diabetes, and inflammation, as well as protection against certain types of cancer (Hu et al. 2022). At least three health-promoting components are present in flaxseed: soluble fibers or mucilage (approximately 6% of dry weight); high levels of polyunsaturated fatty acids (73% of the total fatty acid), especially α-linolenic acid, ω-3 PUFA (approximately 20% of dry weight); and the plant lignan secoisolariciresinol diglucoside. Many of the health benefits of flaxseed are primarily attributable to lignan, specifically SDG.

Cancer

Lignans are phytoestrogens found in nature and have health benefits for many diseases, including cancer. Several *in vivo* studies (Chikara et al. 2017; Ren et al. 2016; Scherbakov et al. 2021) have linked the anticancer effects of flaxseed to its main lignan, secoisolariciresinol diglucoside. *In vitro* investigations of breast, colon, and prostate cancers demonstrate that ENL inhibits tumor growth. Several studies suggest that ENL's antiproliferative capability results from its effects on the cell cycle and cell death induction (Tannous et al. 2020; Xiong et al. 2015). The cytotoxic effects of ENL appear to be specific to cancer cells. ENL (25–75 mM for 24 and 48 hours) inhibited the proliferation of human prostate cancer cells without affecting the vitality of healthy prostate epithelial cells (Chen et al. 2007).

Dietary components like SDG can increase mammary gland differentiation early and prevent breast cancer (Kezimana et al. 2018). SDG concepts on breast cancer prevention are shown in Figure 5. The advancement of N-methyl-N-nitrosourea-induced mammary tumorigenesis causes cancer. SDG slows the progression of N-methyl-N-nitrosourea by altering terminal end-bud differentiation (Rickard et al. 2009; Tan et al. 2004). SDG decreases plasma insulin-like growth factor I, decreasing breast cancer risk (Rickard et al. 2000). Zinc is abundant in breast cancer tissues. SDG can regulate the expression of Zn transporters (Zhang et al. 2008a). Finally, the vascular endothelial growth factor increases angiogenesis, which helps cancer development. END and ENL may prevent breast cancer by decreasing angiogenesis (Jungeström et al. 2007). Numerous *in vivo* and *in vitro* studies show that cancer cells, particularly breast cancer cells, express P-glycoprotein (P-gp) (Zhang et al. 2021). Morsy et al. (2020) mitigated P-gp-induced cancer using secoisolariciresinol (SECO). SECO, and secoisolariciresinol-4',4"-diacetate, derivatives of SDG suppressed breast cancer cell growth (Scherbakov et al. 2021). Also, SDG decreased mouse tumor volume by inhibiting nuclear factor-kappa B (Bowers et al. 2019). Chen et al. (2009) examined the effects of whole flaxseed (100 g/kg diet) and SDG (1 g/kg diet) on breast cancers in athymic postmenopausal mice. Whole flaxseed and SDG reduced palpable tumour size by enhancing apoptosis. Shah and Patel (2016) found that male Sprague-Dawley rats given 500 mg/kg SDG-rich *L. usitatissimum* extract for 18 weeks protected type 2 diabetes-related colon cancer.

Cell cycle regulatory genes in lung tissue are dramatically altered by lignan-rich flaxseed (Lim et al. 2021). In animal models, flaxseed protects the lungs against oxidative damage and inflammation, two significant factors in lung cancer growth and propagation. Chikara et al. (2017) found that ENL is a promising adjuvant therapy for lung cancer (Figure 6). ENL stops lung cancer cell proliferation in the G1 phase, the first of four cell cycle stages in eukaryotic cells (Tannous et al. 2020).

Lipid profile and cardiovascular health

Estrogen and phytoestrogens regulate cardiovascular disease during postmenopause (Rietjens et al. 2016). According to the third National Cholesterol Education Program report, 20–30 g of fibre and 5–10 g of soluble fraction daily lowers saturated fat and cholesterol absorption (Prim et al. 2012). Aqueous flaxseed extract was tested for anti-hepatotoxicity in Albino rats. One hundred grams of ground seeds were suspended in 500 mL of distilled water and filtered after 24 hours. The study used the concentrated extract after desiccating it in an oven at 30 °C for 24 hours. Supplementing 400 mg/kg of defatted flaxseed aqueous extract for 30 days enhanced liver function markers (Mushatet and Jawad, 2020). Okhti et al. (2016) studied the effects of dietary flaxseed-derived lignan on fatty liver disease in rabbits. 40 mg/kg/day of extracted pure flax lignan for 14 days reduced inflammatory cells in clogged blood vessels and sinusoids and moderate fibrosis in rabbit liver tissue's portal region around the bile ducts.

Inflammation

SDG helps to prevent oxidative stress and inflammation in preclinical models of diabetes and heart disease (Parikh et al. 2019). Pietrofesa et al. (2016) tested flaxseed lignan for asbestos-induced acute inflammation in mice. Asbestos-exposed mice fed a control diet had acute inflammation with increased peritoneal lavage fluid (PLF), WBCs, and pro-inflammatory cytokines, but those fed flaxseed lignans had a significant decrease. SDG reduced systemic inflammatory response-induced blood-brain barrier leakage and leukocyte adhesion and motility in aseptic encephalitis. SDG directly suppresses inflammatory cell-blood-brain barrier interactions and leukocyte inflammation (Rom et al. 2018).

Postmenopausal symptoms

As shown in Figure 7, flaxseed lignan inhibits postmenopausal symptoms by mimicking estrogen. However weakly, END and ENL bind estrogen receptors α and β due to their structural similarity to 17-β-estradiol. Mammalian lignans preferentially bind ERα (Sacco et al. 2011). ERα in osteoblasts, osteoclasts, and their progenitors regulates bone turnover genes (Sacco et al. 2011). ENL and 6-hydroxy-ENL prefer ERα over Erβ (Adlercreutz, 2007). Kim et al. (2002) investigated postmenopausal women's urine phytoestrogen excretion and bone mineral density. In 88 postmenopausal women, urine phytoestrogen metabolites were linked with bone mineral density (BMD). BMD favourably correlates with urine enterolactone, the metabolic end product of flaxseed lignan, which inhibits menopausal osteoporosis.

Sturgeon et al. (2011) administered postmenopausal women 15 g of powdered flaxseed daily for six weeks to test its influence on diabetes. Flaxseed supplementation affects diabetes markers IGF-1, IGF-BP3, and C-peptide. Lemay et al. (2002) found that flaxseed

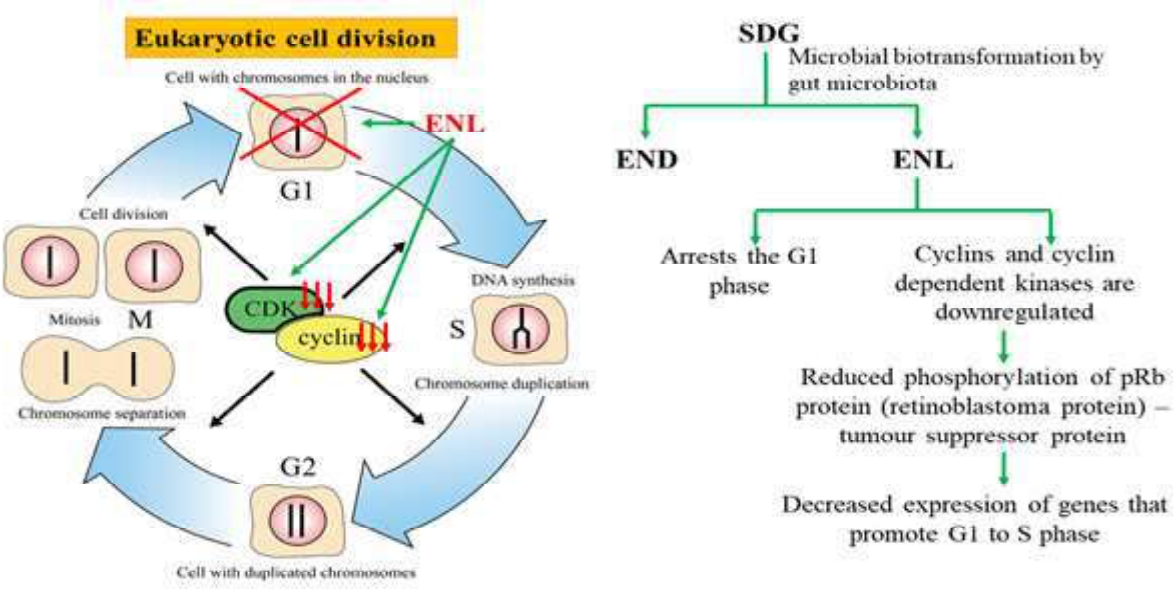

Fig. 6 Possible mechanisms for the anticarcinogenic property of ENL derived from SDG

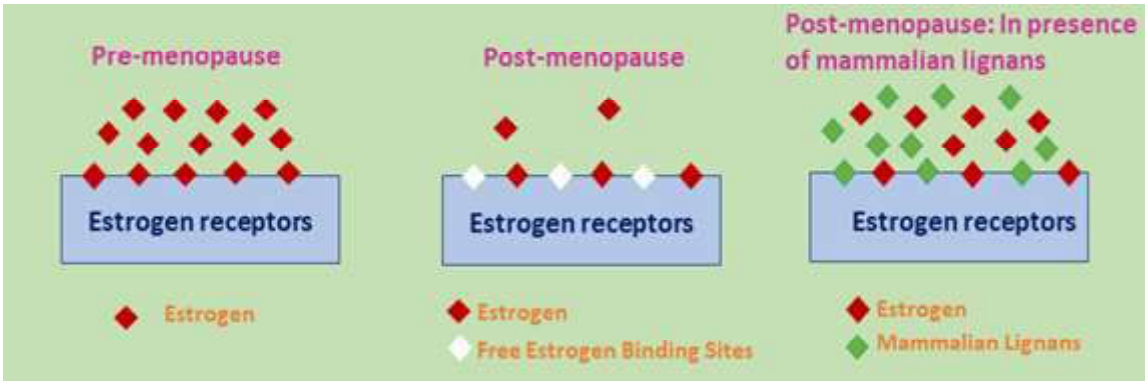

Fig. 7 Binding of mammalian liganans to estrogen receptors in absence of estrogen

reduces glucose and insulin levels and moderate menopausal symptoms. Thirty-eight women were fed bread with 25 grams of ground flaxseed and 46 milligrams of lignan for three months to compare menopausal symptoms. After three months, flaxseed significantly reduced hot flashes and KMI (Simbalista et al. 2010). Hallund et al. (2008) supplemented healthy postmenopausal women's diets with 500 mg of flaxseed lignan complex daily for six weeks to evaluate inflammatory markers. C-reactive protein, a liver-released inflammation marker, fell dramatically in the test individuals after the study. The effect of SDG in alleviating various diseases and disorders are summarized in Table 1.

Application of flaxseed lignan in dairy and bakery products

As whole seeds or flaxseed powder, flaxseeds have long been used in baking and other culinary products, but their use in milk and milk products is restricted due to their distinct flavor. Milk has demonstrated that it is an effective matrix for developing functional foods. Bioactive components, such as phytosterols, peptides, and omega-3 fatty acids, are commonly added to dairy products (Hyvärinen et al. 2006a). Given the health benefits of flaxseed lignans, developing functional dairy products based on flaxseed lignan supplementation will be an exciting strategy. In addition, milk is well known for promoting bone health. The casein-derived bioactive peptide VLPVPQK has been shown to stimulate osteoanabolism *in vitro* and in an ovariectomized rat model of osteoporosis (Mada et al. 2018). Thomas et al. (2023) evaluated the effect of SDG on the growth of probiotic bacteria *Lactiplantibacillus plantarum* for its possible use for the development of SDG enriched fermented milk. A fermentation dynamics investigation was conducted on buffalo milk samples with varying concentrations of lignan (0 to 400 mg of SDG per 100 mL of milk) and co-cultures comprising *Lactiplantibacillus plantarum*, *Streptococcus salivarius* ssp. *thermophilus*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The findings of the study indicate that the fermentation dynamics of *Lactiplantibacillus plantarum* and the starter cultures in the milk were not influenced by SDG. They also evaluated the effect of SDG enriched fermented milk in alleviating postmenopausal osteoporosis. A functional set *dahi* with a desirable probiotic (*Lactiplantibacillus plantarum* A5) count of 9.36 log CFU/mL and excellent techno-functional attributes (DPPH: 41.95% RSA, firmness: 485.49 g, sensory overall acceptability: 8.51) was developed to contain 260 mg of SDG in 20 g of *dahi*. The

Table 1: Functional relevance of SDG

Experiments	Targeted health benefit	Key findings	Reference
Defatted flaxseed aqueous extract 400 mg/kg body weight was administered orally to Albino rats for 30 days	Cholesterol	Administration of defatted flaxseed aqueous extract orally for 30 days increased liver function indicators including Alanine transaminase (ALT), Aspartate transaminase (AST), and Alkaline phosphatase (ALP)	(Mushatet and Jawad, 2020)
Rabbits were administered with 40 mg/kg/day of extracted pure flax lignan for 14 days	Fatty liver disease	The flaxseed lignan lowered inflammatory cells in clogged blood arteries and sinusoids, as well as in mild fibrosis in the portal region around the bile ducts of rabbit liver tissue	(Okhti et al. 2016)
SDG rich extract (500 mg/kg) was administered orally to rats for 18 weeks	Diabetes and colon cancer	SDG-rich extract of *L. usitatissimum* had a chemopreventive impact on colon cancer linked with type 2 diabetes mellitus, which might be mediated by CDK4 inhibition	(Shah and Patel, 2016)
Mice induced with ulcerative colitis were administered with SDG in doses of 100 and 200 mg/kg/day orally	Anti-inflammatory activity	SDG is effective against inflammatory bowel diseases such as ulcerative colitis	(Xu et al. 2016)
Patients aged between 45-75 years were supplemented with 100 mg and 200 mg lignan rich extract of flaxseed hulls (LinumLife EXTRA) for 8 weeks	Benign Prostatic Hyperplasia	Flaxseed hull extract supplementation offered better alleviation in obstructive symptoms of BPH, such as the sensation of incomplete bladder emptying, "stopping and beginning" when urinating, a weak urine stream, and "straining while urinating".	(Simons et al. 2015)
Estimating antidepressant-like effect of SDG (160 mg/kg) in ovariectomized mice subjected to unpredictable chronic stress	Antidepressant activity	• Chronic stress-induced increases in the serum corticosterone and adrenocorticotropic hormone were reversed by treatment with SDG • SDG's behavioural effects in ovariectomized mice may be connected to their regulating effects on the neuroendocrine-immune network and neurotrophin factor expression	(Ma et al. 2013)
Rats were fed with control diet (NC), control diet with 0.02% SDG lignan- enriched flaxseed powder (NCL), high-fructose and fat diet (HFD) and high-fructose and fat diet with 0.02% SDG lignan-enriched flaxseed powder (HFDL) for 12 weeks	Lipid profile, hypertension	• The total cholesterol in mg/dl for NC, NCL, HFD and HFDL were 69, 67, 70 and 63, respectively. The LDL cholesterol in mg/dl for NC, NCL, HFD and HFDL were 10, 11, 17 and 9, respectively and the HDL cholesterol in mg/dl for NC, NCL, HFD and HFDL were 39, 38, 27 and 35, respectively. • SDG-lignan supplementation could lower systolic blood pressure by 45% in the rats fed the HFDL diet compared to the rats on the HFD diet	(Park and Velasquez, 2012)
Feeding of basal diet and basal diet supplemented with 1 g SDG per kg diet for 8 weeks to mice	Breast tumor	• SDG metabolites, enterolactone and enterodiol are structurally similar to human estrogen (17β-estradiol), they have binding affinity to estrogen receptors (ER) and thus may modulate hormone-related diseases such as breast cancer. • SDG have been shown to attenuate tumorigenesis through reduction in cell proliferation and angiogenesis, as well as an increase in apoptosis via modulation of the estrogen receptor and growth factor-signaling pathways	(Truan et al. 2012)

Supplemented 15 g of ground flaxseed per day to postmenopausal women for 6 weeks	Diabetes	Flaxseed supplementation impacted circulating levels of IGF-1, IGF-BP3, or C- peptide which are markers of diabetes	(Sturgeon et al. 2011)
Evaluating the effects of SDG (100 mg/day) intake on hypercholesterolemia and liver disease risk factors in moderately hypercholesterolemic men	Hepatoprotective and anti-cholesterolemic activity	SDG reduces blood- cholesterol levels and also reduces the risk of liver disease	(Fukumitsu et al. 2010)
Antibacterial properties of SDG isolated from Indian flaxseed cultivars were evaluated	Anti-bacterial activity	Inhibition of bacterial diseases caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Bacillus cereus* and *Escherichia coli*	(Rajesha et al. 2010)
Basal diet of mice in postmenopausal condition was supplemented with SDG (1g/kg diet) for 8 weeks	Breast tumor	SDG significantly reduced the tumor growth primarily through reducing tumor cell proliferation rather than increasing apoptosis.	(Saggar et al. 2010)
38 postmenopausal women were fed with 25 g of ground flaxseed containing about 46 mg lignan for 3 months in the form of bread	Postmenopausal symptoms	Flaxseed significantly reduced hot flashes and Kupperman Menopausal Index (KMI)	(Simbalista et al. 2010)
Compared the effects of whole flaxseed (100 g/kg diet), SDG (1 g/kg diet), and flaxseed hull (18 g/kg diet) on breast tumour development in postmenopausal athymic mice.	Breast tumour	Whole flaxseed and SDG significantly reduced ER- and growth factor-related biomarkers, indicating that down regulation of the ER- and growth factor-mediated cell signalling pathway is the optimal mode of action for both whole flaxseed and SDG	(Chen et al. 2009)
Sixty-two hypercholestrolemic postmenopausal women were fed with 40 g/day of flaxseed powder in baked products	Cholesterol	Both total cholesterol and LDL cholesterol decreased significantly at 5th week by 8% and 11%, respectively	(Bloedon et al. 2008)
Low-fat muffins supplemented with 500 mg SDG were fed for 6 weeks to postmenopausal women	Inflammation	Significant decrease (0.88 to 0.80 mg/L) in C-reactive protein (CRP) was observed in test women	(Hallund et al. 2008)
Administered 30 g flaxseed in bread and muffins to postmenopausal women for 3 months	Cholesterol	Observed reduction in total cholesterol and LDL cholesterol by 7% and 10%, respectively and 22% reduction in lipoprotein (a) which is a predictor of atherosclerosis.	(Patade et al. 2008)
Patients were administered with 300 and 600 mg SDG in the form of a lignan-enriched flaxseed extract over a period of 4 months	Lower urinary tract symptoms	The International Prostate Symptom Score (IPSS) of treatment groups were significantly decreased and were reported to be as -3.67 (control), -7.33 (300 mg SDG) and -6.88 (600 mg SDG). The QOL score (quality of life, a subjective score evaluated as part of LUTSs) was also observed to be improved in treatment groups than control.	(Zhang et al. 2008b)
Determining the efficacy of SDG (20 mg/kg) in a hypercholesterolemic myocardial infarction rat model	Angiogenic and cardioprotective activity	Provides protection against cardiovascular diseases	(Penumathsa et al. 2007)
Investigating the effect SDG (360 mg lignan per day) on glycemic control, lipid profiles and insulin sensitivity in type 2 diabetic patients	Anti-diabetic activity	Daily supplementation of SDG improved glycemic control in type 2 diabetes individuals in a small but statistically significant way, without altering fasting glucose, lipid profiles, or insulin sensitivity	(Pan et al. 2007)

developed product was administered to ovariectomized (OVX) rats. According to the study ovariectomy decreased serum calcium, estrogen, and bone ash calcium levels by 32.27, 30.95, and 48.46 percent, respectively, compared to control group, while daily administration of SDG-enriched *dahi* (20 g) for eight weeks restored them. The proximal tibial metaphysis and distal femoral epiphysis micro-CT study showed that the ovariectomy lowered bone mineral density (BMD) by 11.06% and 9.18%, respectively, and lowered Trabecular thickness (Tb. Th) by 12.66% and 11.86%, respectively, while increasing Trabecular separation (Tb. Sp.) by 90.69% and 87.70%, respectively, compared to the sham control-group rats. SDG-enriched *dahi* improved BMD by 16.06 and 12.24% and Tb. Th by 35.32 and 19.62%, respectively, and decreased Tb. Sp by 47.04 and 47.22%, respectively, in OVX rats. The results suggest that the developed set *dahi* may help treat postmenopausal osteoporosis (Thomas et al. 2024).

Some studies have examined the effect of flaxseed lignan on the physicochemical and sensory properties of dairy products. In one such study, (Jeong et al. 2017) prepared bioactive *kefir* with added flaxseed extract at 1% to 3%. They reported that the pH and sensory scores were not substantially altered by the addition of flaxseed extract in comparison to control *kefir*. The effect of supplementation with 3.75 percent flax lignan on various properties of *misti dahi* made by substituting 10% honey for sugar was analyzed (Paul et al. 2016). The developed product had the same pH, moisture, acidity, total solids, and whey syneresis values as the control and 20-day shelf life. In addition, they discovered that 100 g/mL of lignan-enriched *misti dahi* inhibited -amylase by 48.41%, compared to 17.54% in the control group. This suggests that flax lignan-enriched *misti dahi* may be an anti-diabetic agent to enhance human health and treat diabetes. In another investigation, Hyvärinen et al. (2006a) investigated the stability of SDG when it was added to cold- and hot-dairy products such as milk, cheese, yoghurt, and whey drinks. SDG was not adversely affected by fermentation, pasteurisation, or chilling.

Manufacturers are increasingly incorporating polyunsaturated fatty acids (PUFA), rich in omega-3 fatty acids, into their formulations to improve the nutritional value of processed foods. Conversely, these components are susceptible to oxidation reactions, which can result in rancidity and potentially toxic substances. To mitigate this issue, Matumoto-Pintro et al. (2011) added flaxseed lignan extract (50–200 mg SDG/kg beverage) to dairy beverages enriched with PUFA, which were then tested for their resistance to light- and heat-induced oxidation. Initial concentrations of propanal and hexanal in dairy beverages containing SDG were reduced by 87% and 58%, respectively, indicating that flaxseed lignan offered protection against light- and heat-induced oxidation during the preparation of PUFA-enriched dairy beverages. According to the report, the optimal SDG concentration to prevent heat- and light-induced lipid oxidation in dairy beverages is less than 50 mg/L.

Popular in Chinese cuisine, the Chinese steamed bun, also known as *Mantou*, is a variety of steamed bun or bread-like items (Laohasongkram et al. 2011). It is made with wheat flour, water, and a leavening agent such as yeast or traditional sourdough. The aromatic compounds, such as aldehydes produced in the product as a result of enzymatic or spontaneous oxidation of the wheat flour fraction during fermentation, are desirable because they affect the overall aroma profile of bread. However, their excessive production during processing can result in off-flavors (Zhang et al. 2016). (Hao and Beta, 2012) analyzed the antioxidative potential of flaxseed hull extract in steamed Chinese bread. The addition of flaxseed hull extract (1 g extract/100 g flour) increased the total phenolic content of the product by 138.34% and the antioxidant activities in terms of DPPH activity and ORAC values by 90.69% and 67.43%, respectively; therefore, flaxseed hull extract can be developed as a functional food ingredient to improve the phytochemical content and antioxidative potential of refined flour products, such as steamed bread. The amount of lignan or flaxseed added to the bread, the type of flaxseed used, and the type of bacterial or yeast cultures used in the leavening process are all factors that influence the amount of SDG in bread. A significant amount of SDG (73-75%) was recovered from the samples, both in free and complex forms, demonstrating that SDG could tolerate heat during the milling, fermentation, and cooking procedures (Muir and Westcott, 2000). Similarly, bread containing whole flaxseed and defatted flaxseed flour preserved a significant level of lignan content following the dough, proofing, baking, and storing steps (Hyvarinen et al. 2006b).

Conclusion

Lignans forms a large group of phytoestrogens with chemical structures characterized by C_6 - C_3 units linked by a β-β' (8-8') carbon bond and multiple mechanisms of action. Lignans are ER ligands that act like estrogens in some tissues, such as mammary, ovary, prostrate, bone, cardiovascular tissue etc. Many estrogenic herbs have been used in Asian countries for thousands of years to treat various diseases and flaxseed lignan is an emerging nutraceutical ingredient capable for the prevention and treatment of diseases like cancer, diabetes, hyperlipidaemia, cardiovascular diseases, postmenopausal related diseases and so on. This review concludes that flaxseed lignan consumption can significantly reduce these diseases to a large extent. Further studies are necessary for exploring low-cost extraction and isolation techniques and also to evaluate the effect of incorporation of flaxseed lignan to different dairy and food products.

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Conflict of interests

The authors declare that they have no conflict of interests.

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RESEARCH ARTICLE

Comparison of acid casein-based Mozzarella cheese analogue with natural Mozzarella cheese during refrigerated storage

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Abstract: Mozzarella cheese analogue was prepared using acid casein and partially hydrogenated palm kernel oil as protein and fat source respectively and compared with natural Mozzarella cheese during refrigerated storage. Moisture and soluble nitrogen of analogue and natural cheese were statistically similar while analogue had higher pH and acid degree value. Moisture of the samples reduced during storage while pH, soluble nitrogen and acid degree value increased. Hardness and cohesiveness of both the samples were similar. Natural cheese had higher springiness and chewiness while analogue showed higher gumminess and adhesiveness. Hardness, gumminess and chewiness reduced during storage while other textural characteristics remained unaffected. Natural cheese had better functional characteristics such as shredability, meltability, fat leakage and stretchability. Meltability, fat leakage and stretchability improved during storage while shredability deteriorated. Natural cheese showed superior sensory characteristics, viz. appearance, flavour, melting, stringiness and chewiness throughout the storage.

Keywords: Mozzarella cheese; Mozzarella cheese analogue; physico-chemical characteristics; textural characteristics; functional characteristics; sensory characteristics

Introduction

Cheese analogue is generally defined as a product prepared by blending different constituents, including non-dairy fat and protein, either singly or in combination and producing a product similar to cheese. The production of cheese analogue is increasing globally because of continuous increase in consumer demand since the consumers want a product which possesses appreciable functional characteristics and acceptable sensory characteristics. The manufacturers are also interested in developing cheese analogues due to ease of production and cost-effectiveness (Dharaiya et al. 2019).

Refrigerated storage of Mozzarella cheese and its analogues results in some desirable changes such as mellowness, melt and stretch as well as undesirable changes such as sliminess on surface, poor shredability, excessive fat leakage etc. which are mainly result of proteolytic changes. The starter culture as well as rennet has been implicated in leading to such storage changes in natural Mozzarella cheese. Plasmin has been implicated for the changes observed in the functional properties of Mozzarella cheese analogue. Storage stability of cheese product is important from the perspective of the pizza makers, so that the desired functional properties may be maintained during its estimated usage period (Alinovi et al. 2020).

Therefore, the aim of current investigation is to observe the changes taking place in physico-chemical, textural, functional and sensory properties of Mozzarella cheese and its analogue

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during refrigerated storage and to recommend optimum period of storage to obtain suitable characteristics for pizza-making.

Materials and Methods

Acid casein (87.12% protein) was obtained from M/s. Mahaan Protein Ltd., Kosikalan, India. Specialty palm kernel oil (partially hydrogenated) based vegetable fat (Melting point – 32°C) was obtained from M/s. Kamani Oil Industries Pvt. Ltd., Mumbai, India. Calcium chloride, dihydrate; di-sodium hydrogen orthophosphate, dihydrate; tri-sodium citrate, dihydrate; anhydrous citric acid; lactic acid and lecithin were procured from M/s. Loba Chemie Pvt. Ltd., Mumbai, India. Pre-gelatinized starch (Pregenil XT) was obtained from M/s. Madhu Hydrocolloids Pvt. Ltd., Ahmedabad, India. Mozzarella cheese flavouring paste was supplied by M/s. Adare Food Ingredients Pvt. Ltd., Vitthal Udyognagar, India. Vacuum-evaporated salt (*Tata* brand) and fresh pre-baked pizza loaves were obtained from a local market. Rennet (*Maxiren* brand) was supplied by DSM Food Specialities Ltd., Mumbai, India.

Preparation of natural Mozzarella cheese: Mozzarella cheese was prepared using the method standardized by Chavhan et al. (2015).

Preparation of Mozzarella cheese analogue

Mozzarella cheese analogue (MCA) was prepared as per the method described by Dharaiya et al. (2021). The formulation of Mozzarella cheese analogue is depicted in Table 1.

Chemical analysis

Mozzarella cheese and analogue samples were analysed for moisture, soluble nitrogen (by semi-micro Kjeldahl method) and acid degree value using the method prescribed by FSSA (2015) as well as pH (Dharaiya et al. 2021).

Texture profile analysis

Texture of the experimental cheese samples was analysed using the method described by Dharaiya et al. (2021).

Functional characteristics

The experimental cheese samples were evaluated subjectively for shredability, considering ease of shredding, length and thickness of shreds and the behaviour of shreds after shredding. A stainless-steel shredder with pore size diameter of 3 mm was used for shredding. Meltability of experimental samples, stretchability and fat leakage was analysed using the methods described by Dharaiya et al. (2019).

Sensory evaluation of cheese as a pizza topping

The experimental cheese samples were examined for pizza making by conducting baking trials as per the method described by Dharaiya et al. (2021).

Statistical analysis

In each part of the study, data obtained from 4 independent trials were statistically analyzed using completely randomized design. Statistical analysis was carried out using one-way ANOVA through MS-Excel.

Results and Discussion

Chemical changes in analogue and natural Mozzarella cheese during storage

The changes in the physico-chemical properties during refrigerated storage of Mozzarella cheese and its analogue have been shown in Table 2.

Moisture

Moisture content of experimental cheese samples reduced significantly ($P<0.05$) during storage, though there was no significant difference between natural and analogue cheese samples but period of storage (P) and interaction between type of cheese and period of storage (C×P) significantly ($P<0.05$) decreased moisture content of cheese samples.

Moisture loss during storage of cheese due to evaporation is a common phenomenon. Such reduction in the moisture content of Mozzarella cheese during refrigerated storage has also been reported by Ehsannia and Sanjabi (2016) and Jana and Tagalpallewar (2017).

pH

The type of cheese (C) and the interaction between type of cheese and period of storage (C×P) had a significant (P d" 0.05) influence on the pH values of the product. The pH of analogue and natural cheeses increased progressively with increase in the

Table 1: Formulation of Mozzarella cheese analogue

Ingredients	Rate of addition (%)
Vegetable fat	15.00
Acid casein	21.30
Tri-sodium citrate	0.90
Disodium hydrogen phosphate	1.60
Lecithin	0.15
Citric acid	0.18
Calcium chloride	0.30
Pre-gelatinized starch	2.00
Cheese flavouring	3.00
Common salt	1.00
Water	54.57

storage period. The rise in pH of analogue was greater up to 21st day, thereafter it tended to stabilize while the pH was of natural cheese increased linearly through-out the storage period. However, such change in the pH was non-significant. Cheese analogue had higher initial pH values than that of natural cheese due to presence of emulsifying salts; such higher pH values associated with analogue was maintained throughout the storage period. (Goncalves and Cardarelli, 2021)

Soluble nitrogen (SN)

The type of cheese (C) failed to exert significant impact on soluble nitrogen while period of storage (P) and interaction between them (C×P) had significant (P<0.05) impact. The fresh cheeses (0 day) were statistically identical in their SN content. SN increased progressively during storage. The increase in SN content at 35th day over the initial content was greater in case of natural cheese (i.e. 1.26%) than in that of analogue (i.e. 1.07%). The lower magnitude of rise in SN content in analogue compared to natural cheese could be due to absence of residual rennet activity and the varying pH conditions in cheese. A similar trend has been reported by several researchers in analogue and natural Mozzarella cheese (Alinovi et al, 2020); processed Mozzarella cheese (Khetra et al. 2015) and processed cheese analogue (Ehsannia and Sanjabi, 2016).

Acid degree value (ADV)

There was a significant (P≤0.05) influence of type of cheese (C), storage period (P) as well as their interaction (C x P) on the ADV of cheeses. ADV increased significantly (P≤0.05) especially from 28th day of refrigerated storage. Fresh natural cheese showed lower ADV. An increase in ADV and Free Fatty Acids (FFA) content of natural cheese during refrigerated storage has also been reported by Jeewanthi and Paik (2018).

Textural changes in analogue and natural Mozzarella cheeses during refrigerated storage

The changes in textural characteristics during refrigerated storage of natural cheese and analogue is presented in Table 3.

Hardness

The type of cheese (C) failed to influence the hardness of cheeses, while the period of storage (P) and the interaction between type of cheese and period of storage (C×P) had a significant (Pd"0.05) influence on the hardness of cheeses. The hardness of analogue gradually increased till 21st day of storage, followed by a gradual decrease till the end (35th day) of storage while natural cheese showed a gradual decrease in its hardness throughout the storage period. In spite of lower moisture content

Table 2: Changes in physico-chemical properties of analogue and natural Mozzarella cheese during refrigerated storage

Cheese samples	0 day	7 days	14 days	21 days	28 days	35 days	Average
			Moisture content (%)				
ACMCA	53.42±0.35^{a}	53.32±0.29^{a}	53.24±0.31^{a}	53.11±0.39^{a}	52.92±0.25^{b}	52.75±0.31^{b}	53.13
NMC	53.65±0.30^{a}	53.41±0.33^{a}	53.27±0.34^{a}	53.14±0.28^{a}	53.04±0.22^{a}	52.89±0.24^{b}	53.23
Average	53.54^{p}	53.37^{p}	53.26^{p}	53.13^{p}	52.98^{q}	52.82^{q}	
CD (0.05)	Cheese type (C)=NS; Period of storage (P)=0.45; C x P=0.67						
			pH				
ACMCA	5.89±0.07^{a}	5.91±0.05^{a}	5.94±0.06^{a}	5.98±0.08^{a}	6.01±0.07^{a}	6.04±0.09^{a}	5.962^{x}
NMC	5.41±0.05^{b}	5.41±0.06^{b}	5.42±0.04^{b}	5.42±0.08^{b}	5.43±0.07^{b}	5.44±0.09^{b}	5.422^{y}
Average	5.65	5.66	5.68	5.70	5.72	5.74	
CD (0.05)	Cheese type (C)=0.12; Period of storage (P)=NS; C x P=0.29						
			Soluble nitrogen (% of total nitrogen)				
ACMCA	1.24±0.14^{a}	1.44±0.18^{a}	1.66±0.21^{a}	1.81±0.15^{a}	2.06±0.14^{a}	2.31±0.15^{b}	1.753
NMC	1.28±0.23^{a}	1.53±0.18^{a}	1.71±0.21^{a}	1.98±0.15^{a}	2.35±0.19^{b}	2.54±0.17^{b}	1.898
Average	1.260^{p}	1.485^{p}	1.685^{p}	1.895^{q}	2.205^{q}	2.425^{q}	
CD (0.05)	Cheese type (C)=NS; Period of storage (P)=0.48; C x P=0.88						
			Acid degree value				
ACMCA	0.58±0.14^{a}	0.63±0.11^{a}	0.72±0.10^{b}	0.79±0.12^{b}	0.85±0.15^{b}	0.96±0.13^{b}	0.755^{x}
NMC	0.19±0.13^{a}	0.22±0.14^{a}	0.25±0.09^{a}	0.29±0.11^{a}	0.34±0.13^{a}	0.39±0.11^{a}	0.280^{y}
Average	0.385^{p}	0.425^{p}	0.485^{p}	0.540^{p}	0.595^{p}	0.675^{q}	
CD (0.05)	Cheese type (C)=0.19; Period of storage (P)=0.23; C x P=0.46						

ACMCA-Acid casein based Mozzarella cheese analogue; NMC – Natural Mozzarella cheese; x & y shows significant difference in type of cheese; p & q shows significant difference during storage; a & b shows significant difference for interaction between type of cheese and period of storage

in analogue, it possessed lower hardness which could be ascribed to the differences in the protein structure and hydration of protein. The increase in the hardness of cheeses during storage could be attributed to a concomitant decrease in the moisture content (Table 2) and possible increase in the water holding capacity (WHC) of proteins, whereas decline in the hardness at a later stage of storage could be ascribed to the proteolytic changes (Table 2) which shadowed the effect of decrease in moisture

Table 3: Changes in textural properties of analogue and natural Mozzarella cheese during refrigerated storage

Cheese samples	0 day	7 days	14 days	21 days	28 days	35 days	Average
			Hardness (N)				
ACMCA	14.48±1.21^{a}	16.50±1.54^{b}	18.44±1.42^{b}	19.09±1.16^{b}	15.93±1.35^{b}	12.57±1.61^{a}	16.17
NMC	18.22±1.53^{b}	16.72±1.42^{b}	15.21±1.62^{a}	14.80±1.16^{a}	14.51±1.23^{a}	12.79±1.09^{a}	15.38
Average	16.35^{p}	16.61^{p}	16.83^{p}	16.95^{p}	15.22^{p}	12.68^{q}	
CD (0.05)	Cheese type (C)=NS; Period of storage (P)=1.85; C x P=3.71						
			Cohesiveness				
ACMCA	0.354±0.07	0.361±0.09	0.380±0.11	0.387±0.08	0.390±0.09	0.396±0.07	0.378
NMC	0.398±0.06	0.387±0.09	0.368±0.10	0.343±0.08	0.321±0.05	0.310±0.09	0.354
Average	0.376	0.375	0.374	0.365	0.356	0.353	
CD (0.05)	Cheese type (C)=NS; Period of storage (P)=NS; C x P=NS						
			Springiness (mm)				
ACMCA	3.66±0.19^{a}	3.77±0.24^{a}	3.81±0.30^{a}	3.89±0.22^{a}	3.72±0.27^{a}	3.51±0.31^{a}	3.73^{x}
NMC	5.12±0.25^{b}	5.28±0.27^{b}	5.35±0.32^{b}	5.36±0.24^{b}	5.20±0.21^{b}	5.03±0.19^{b}	5.22^{y}
Average	4.390	4.525	4.580	4.625	4.460	4.270	
CD (0.05)	Cheese type (C)=0.31; Period of storage (P)=NS; C x P=0.76						
			Gumminess (N)				
ACMCA	511.10±9.73^{a}	594.16±7.53^{b}	703.05±6.69^{b}	740.48±8.41^{b}	621.87±7.52^{b}	497.74±8.95^{a}	611.40^{x}
NMC	725.02±8.12^{b}	648.10±9.04^{b}	453.46±7.68^{a}	389.94±8.22^{a}	389.22±7.65^{a}	367.79±7.69^{a}	495.58^{y}
Average	618.06^{p}	621.13^{p}	578.26^{p}	565.21^{p}	505.55^{q}	432.76^{q}	
CD (0.05)	Cheese type (C)=68.05; Period of storage (P)=83.02; C x P=166.04						
			Chewiness (N-mm)				
ACMCA	18.63±1.31^{a}	22.53±1.63^{b}	26.35±1.52^{b}	28.34±1.95^{b}	23.14±1.72^{b}	17.50±2.31^{a}	22.75^{x}
NMC	37.04±2.21^{c}	34.22±1.95^{c}	24.25±2.04^{b}	20.92±1.62^{a}	20.28±1.88^{a}	15.46±1.84^{a}	25.36^{y}
Average	27.84^{p}	28.38^{p}	25.30^{q}	24.63^{q}	21.71^{q}	16.48^{r}	
CD (0.05)	Cheese type (C)=2.52; Period of storage (P)=3.89; C x P=6.76						
			Adhesiveness (N-mm)				
ACMCA	0.276±0.07	0.290±0.08	0.295±0.10	0.285±0.06	0.282±0.11	0.258±0.09	0.281^{x}
NMC	0.206±0.06	0.213±0.07	0.228±0.05	0.227±0.09	0.223±0.07	0.218±0.10	0.219^{y}
Average	0.241	0.252	0.262	0.256	0.252	0.238	
CD (0.05)	Cheese type (C)=0.15; Period of storage (P)=NS; C x P=NS						

ACMCA-Acid casein based Mozzarella cheese analogue; NMC – Natural Mozzarella cheese; x & y shows significant difference in type of cheese; p, q & r shows significant difference during storage; a, b & c shows significant difference for interaction between type of cheese and period of storage

content of cheeses. Jeewanthi et al. (2016) reported reduction in the expressible serum in Mozzarella cheeses during their refrigerated (4°C) storage; such phenomena are indicative of increase in the water holding capacity of proteins. Similar findings were reported by Jeewanthi and Paik (2018).

Cohesiveness

None of the parameters studied i.e. type of cheese (C), period of storage (P) and their interaction (C×P) had any significant influence on the cohesiveness of the cheeses. Similar results were obtained by Jeewanthi and Paik (2018).

Springiness

The springiness of cheeses was significantly (Pd"0.05) affected the type of cheese (C) and the interaction between type of cheese and period of storage (C×P); the springiness of cheeses remained unaffected by the period of storage (P). Natural cheese had significantly (P<0.05) higher springiness than analogue which could be ascribed to the reduced fat particle size in case of natural cheese resulting in more extensive protein-protein and protein-fat interaction yielding product having desired springiness. The varying rate of hydration of casein in analogue and natural cheese also might have led to the observed difference in the springiness. The dehydrated casein is rehydrated during preparation of cheese analogue while it is naturally hydrated in milk (Badem and Ucar, 2016).

Gumminess

The gumminess values were significantly (P<0.05) affected by the type of cheese (C), the period of storage (P) and their interaction (C x P). The gumminess of analogue was significantly (P<0.05) higher than that of natural cheese. Since gumminess value is derived as a product of hardness and cohesiveness, the trend shown by hardness and cohesiveness is also reflected in the values of gumminess. Higher gumminess is not a desirable characteristic. During storage of Mozzarella cheese, the gumminess was reported to increase initially, followed by a decrease during subsequent storage (Rizwan-ur-Rehman et al. 2017).

Chewiness

The chewiness of experimental cheeses was significantly (P<0.05) influenced by the type of cheese (C), the period of storage (P) as well as their interaction (C×P). Since chewiness is product of springiness and gumminess, the trend exhibited by gumminess and springiness is reflected in the values of chewiness. The mellowing in the structure of Mozzarella cheese during ageing, as a result of proteolytic changes and possibly increased water holding capacity of protein, has a favourable influence on the perceived chewiness of cheese (Jana and Tagalpallewar, 2017).

Adhesiveness

The type of cheese (C) had a significant (P≤0.05) effect on the adhesiveness of product, while the period of study (P) and their interaction (C×P) failed to influence the adhesiveness of cheese appreciably. The difference in the type and proportion of emulsifying salts and the difference in the micro-structure of cheese matrix must have led to the observed differences in the adhesiveness of cheese samples (Jana and Tagalpallewar, 2017).

Changes in the functional characteristics of analogue and natural Mozzarella cheeses during refrigerated storage

Since the analogue and natural Mozzarella cheeses have application mainly as a pizza topping, both the type of cheeses was evaluated for their pizza related baking qualities at an interval of 7 days till 35th day of refrigerated storage (7±1°C). The results related to the changes in the baking qualities of cheeses are presented in Table 4.

Shredability

The shredding property of analogue was found superior to that of natural cheese in the beginning of the storage but with progress of the storage period, the shredability of analogue deteriorated while that of natural cheese improved initially and deteriorated after 21 days of storage. The lower moisture content and presence of emulsifying salts in analogue resulted in better shredability. The improvement in the shredability of natural cheese initially could be attributed to absorption of free moisture into the block of cheese (Banville et al. 2013). The deterioration of shredability towards the end of storage could be due to proteolysis. Similar trend has been observed by Dharaiya et al. (2019).

Meltability and melting time

The meltability of cheeses was significantly (P≤0.05) affected by the cheese type (C), period of storage (P) and their interaction (C×P). The meltability of both types of cheeses increased progressively with the advent of storage. The meltability of Mozzarella cheese increases during storage due to hydrolysis of β-casein and it is correlated with the soluble nitrogen content of cheese (Liu et al. 2024). Meltability of cheese is associated with its moisture in non-fat substances (MNFS) and cheese pH; lowering in pH improves cheese meltability. The progressive increase in proteolysis during ageing of cheese also promotes its meltability, while in case of analogue raising the levels of emulsifying salts (Kamath et al. 2022) and adjusting higher pH value tended to improve the meltability of product. An increase in the meltability of natural cheese and analogue during refrigerated storage has been reported by most researchers (Alinovi et al. 2020).

Owing to the improvement in melting property of cheese, the time required for the product to melt in the baking oven (at 230°C)

tended to decrease progressively with the advancement in the storage period. An inverse relation has been noted between meltability and melting time in the oven.

Fat leakage

The storage period (P) failed to exert any significant influence on the fat leakage of cheeses, while the type of cheese (C) and interaction (C×P) had a significant (P<0.05) influence on it. A gradual decrease in the fat leakage of analogue was noticed during the refrigerated storage. Contrary to this, natural cheese showed gradual increase in the fat leakage throughout the storage period of 35 days. Emulsifying salts used in cheese analogue preparation are known to modify the proteins to emulsify the fat in the cheese matrix (Arief and Manab, 2024). The pH of cheese analogue was higher than that of natural cheese resulting in reduced fat leakage. The increase in the fat leakage in natural cheese could be associated to the difference in the emulsification of milk fat in cheese matrix and the lower pH. The plasticizing treatment given to cheese curd in case of natural cheese might be making the cheese matrix porous and reducing the emulsified state of milk fat contributing to the greater fat leakage. The increase in the fat leakage in natural cheese during refrigerated storage has also been reported by several workers (Alinovi et al. 2020).

Stretchability

The type of cheese (C), the period of storage (P) and their interaction (C×P) were significantly (Pd"0.05) affected the stretchability of stored cheese samples. There was an increase in the stretchability of analogue up to 21 days followed by decline. In case of natural cheese, the stretchability gradually increased throughout the storage period up to 35 days. The fresh analogue cheese stretched to a greater extent than did fresh natural cheese. The initial improvement in the stretchability of Mozzarella cheese with ageing was ascribed in part to the age-related reduction in the concentration of intact para-casein as a result of proteolytic changes taking place during aging (Table 2). Such changes led to product having improved water binding capacity, getting progressively mellowed in body which thus must have led to permitting longer strands of cheese when stretched, post baking (Zedan et al. 2014; Goncalves and Cardarelli, 2021; Guo et al. 2023).

Table 4: Changes in functional properties of analogue and natural Mozzarella cheese during refrigerated storage

Cheese samples	0 day	7 days	14 days	21 days	28 days	35 days	Average
			Shredability*				
ACMCA	Very good	Very good	Good	Good	Fair	Poor	---
NMC	Good	Very good	Very good	Good	Fair	Fair	---
			Meltability#				
ACMCA	2.28±0.24^{a}	2.88±0.29^{a}	3.51±0.26^{a}	4.19±0.31^{a}	4.75±0.27^{a}	5.13±0.30^{b}	3.79^{x}
NMC	3.47±0.32^{a}	4.05±0.30^{a}	4.78±0.28^{a}	5.40±0.24^{b}	5.78±0.29^{b}	6.12±0.27^{b}	4.93^{y}
Average	2.875^{p}	3.465^{p}	4.145^{p}	4.975^{q}	5.265^{q}	5.625^{q}	
CD (0.05)			Cheese type (C)=0.98; Period of storage (P)=1.36; C x P=2.62				
			Melting time (sec)				
ACMCA	470.0±7.32^{a}	462.5±8.14^{a}	432.5±7.91^{a}	390.0±6.45^{a}	365.0±7.29^{b}	322.5±7.85^{b}	407.08^{x}
NMC	440.0±7.83^{a}	430.0±7.54^{a}	392.5±8.19^{a}	352.5±7.12^{b}	332.5±7.80^{b}	300.0±7.22^{b}	374.58^{y}
Average	455.00^{p}	446.25^{p}	412.50^{p}	371.25^{q}	348.75^{q}	311.25^{q}	
CD (0.05)			Cheese type (C)=22.29; Period of storage (P)=34.51; C x P=79.02				
			Fat leakage (cm^{2})				
ACMCA	3.86±0.18^{a}	3.40±0.22^{a}	3.14±0.29^{a}	2.90±0.25^{a}	2.59±0.26^{a}	2.36±0.19^{a}	3.042^{x}
NMC	4.19±0.21^{a}	4.91±0.25^{b}	5.51±0.23^{b}	6.05±0.28^{b}	6.75±0.29^{b}	7.54±0.21^{b}	5.825^{y}
Average	4.025	4.155	4.325	4.475	4.670	4.950	
CD (0.05)			Cheese type (C)=0.61; Period of storage (P)=NS; C x P=2.29				
			Stretchability (cm)				
ACMCA	14.5±1.29^{a}	17.5±1.35^{a}	19.5±1.94^{b}	22.5±1.75^{b}	20.0±1.23^{b}	14.0±1.64^{a}	18.0
NMC	12.5±1.52^{a}	15.0±1.98^{a}	17.5±2.05^{a}	20.75±1.63^{b}	22.75±1.41^{b}	24.75±1.56^{c}	18.875
Average	13.5^{p}	16.25^{p}	18.5^{q}	21.625^{r}	21.375^{r}	19.375^{q}	
CD (0.05)			Cheese type (C)=NS; Period of storage (P)=2.84; C x P=5.08				

ACMCA-Acid casein based Mozzarella cheese analogue; NMC – Natural Mozzarella cheese; x & y shows significant difference in type of cheese; p, q & r shows significant difference during storage; a, b & c shows significant difference for interaction between type of cheese and period of storage; *Subjective analysis; # Arbitrary value

Changes in the sensory quality of stored analogue and natural Mozzarella cheeses as pizza topping

The type of cheese (C) as well as the interaction between type of cheese and period of storage (C×P) significantly (P<0.05) affected all sensory parameters while period of storage (P) failed to significantly influence when judged as a pizza topping (Table 5).

Appearance

The appearance of Mozzarella cheese on pizza pie takes into consideration the melted cheese appearance, fat leakage and browning aspects. The appearance score for natural Mozzarella cheese was significantly (P<0.05) higher than that of analogue. The appearance score for both the samples were increased during first 21 days followed by a decrease. Natural cheese was whiter in colour and glossier. The analogue had a transparent appearance after melting which led to visibility of the pizza base beneath. Dharaiya et al (2021) also reported superior appearance score for natural cheese over analogue.

Flavour

Natural cheese possessed characteristic Mozzarella flavour with slight salty and acidic taste while analogue had characteristics sour taste even though pH was adjusted to higher level. The flavour score of analogue increased upto 21 days while that of natural cheese increased for initial 14 days followed by a decrease. Several researchers reported superior flavour of natural cheese over analogue (Dharaiya et al. 2021; Short et al. 2021).

Melting

Cheese is a network of interconnecting molecules of casein, hence, the hydrolysis of casein molecules influenced melting behaviour of Mozzarella cheese. The analogue sample had

Table 5: Changes in sensory characteristics of analogue and natural Mozzarella cheese during refrigerated storage

Cheese samples	7 days	14 days	21 days	28 days	35 days	Average
			Appearance score			
ACMCA	7.14±0.25^{a}	7.16±0.29^{a}	7.21±0.26^{a}	6.93±0.36^{a}	6.75±0.19^{a}	7.038^{x}
NMC	7.39±0.31^{a}	7.74±0.26^{b}	7.95±0.21^{b}	7.72±0.18^{b}	7.25±0.24^{a}	7.610^{y}
Average	7.27	7.45	7.58	7.33	7.00	
CD (0.05)			Cheese type (C)=0.33; Period of storage (P)=NS; C x P=0.69			
			Flavour score			
ACMCA	6.58±0.19^{a}	6.64±0.16^{a}	6.85±0.24^{a}	6.47±0.21^{a}	5.93±0.18^{a}	6.494^{x}
NMC	7.95±0.21^{b}	8.10±0.20^{b}	7.90±0.29^{b}	7.70±0.22^{b}	7.50±0.24^{b}	7.830^{y}
Average	7.26	7.37	7.38	7.09	6.71	
CD (0.05)			Cheese type (C)=0.65; Period of storage (P)=NS; C x P=1.45			
			Melting score			
ACMCA	7.21±0.29^{a}	7.41±0.25^{a}	7.32±0.30^{a}	7.01±0.26^{a}	6.52±0.24^{a}	7.09^{x}
NMC	7.08±0.21^{a}	7.59±0.26^{a}	7.89±0.28^{b}	7.63±0.19^{a}	7.47±0.23^{a}	7.53^{y}
Average	7.15	7.50	7.61	7.32	6.99	
CD (0.05)			Cheese type (C)=0.38; Period of storage (P)=NS; C x P=1.26			
			Stringiness score			
ACMCA	7.17±0.32^{a}	7.31±0.36^{a}	7.01±0.31^{a}	6.84±0.39^{a}	6.58±0.27^{a}	6.98^{x}
NMC	7.43±0.28^{b}	7.62±0.31^{b}	7.95±0.24^{b}	7.77±0.35^{b}	7.54±0.34^{b}	7.66^{y}
Average	7.30	7.46	7.48	7.31	7.06	
CD (0.05)			Cheese type (C)=0.36; Period of storage (P)=NS; C x P=0.80			
			Chewiness score			
ACMCA	7.27±0.41^{a}	7.37±0.35^{a}	7.06±0.21^{a}	6.93±0.29^{a}	6.73±0.31^{a}	7.07
NMC	7.14±0.37^{a}	7.41±0.31^{a}	7.68±0.26^{b}	7.27±0.24^{a}	7.02±0.32^{a}	7.30
Average	7.21	7.39	7.37	7.10	6.87	
CD (0.05)			Cheese type (C)=NS; Period of storage (P)=NS; C x P=0.72			
			Overall acceptability			
ACMCA	7.07±0.34^{b}	7.18±0.41^{b}	7.09±0.36^{b}	6.82±0.44^{a}	6.49±0.39^{a}	6.93^{x}
NMC	7.40±0.28^{b}	7.64±0.35^{c}	7.84±0.36^{c}	7.62±0.33^{c}	7.29±0.29^{b}	7.56^{y}
Average	7.24	7.41	7.47	7.22	6.89	
CD (0.05)			Cheese type (C)=0.36; Period of storage (P)=NS; C x P=0.48			

ACMCA-Acid casein based Mozzarella cheese analogue; NMC – Natural Mozzarella cheese; x & y shows significant difference in type of cheese; a & b shows significant difference for interaction between type of cheese and period of storage

suboptimal melting with non-uniform matting of the cheese shreds on pizza pie while natural cheese had uniform melting and fusion of cheese shreds. The melting behaviour of analogue improved during initial 14 day of storage while that of natural cheese increased during initial 21 days followed by deterioration. The improvement in the meltability of NMC during refrigerated storage has already been established (Sheikh et al. 2023).

Stringiness

Natural cheese reported superior stringiness than analogue. Natural cheese had thinner and non-fibrous strands while analogue had thicker and fibrous strands. Superior stringiness of natural cheese could be attributed to use of rennet in the preparation while acid casein based analogue is devoid of rennet. The stringiness of analogue improved up to 14th day of refrigerated storage while that of natural cheese improved up to 21st day of the storage followed by deterioration. Similar findings were reported by Short et al. (2021).

Chewiness

The type of cheese (C) and the period of storage (P) could not influence chewiness while their interaction (C x P) had significant ($P<0.05$) influence. In case of natural cheese, chewiness score increased during initial 21 days followed by deterioration while in case of analogue, it increased for initial 14 days and then deteriorated. The judges liked moderate chewiness in case of Mozzarella cheese. The cheese samples were very chewy at the end of storage. Sheikh et al. (2023) also had similar observations.

Overall acceptability

The period of storage (P) could not influence overall acceptability score while type of cheese (C) and the interaction (C x P) had significant ($P<0.05$) influence on it. Analogue had statistically ($P<0.05$) similar score during initial 21 days and then decreased while in case of natural cheese, overall acceptability score improved on 14th day and was statistically ($P<0.05$) similar up to 28th day and then decreased. The maximum score was observed for natural cheese on 21st day of refrigerated storage.

Conclusion

Mozzarella cheese analogue was associated with higher pH and acid degree value than that of natural cheese during refrigerated storage while moisture and soluble nitrogen were almost similar. Moisture of cheese samples reduced with advancement of storage while soluble nitrogen and acid degree value increased. Analogue had lower springiness, chewiness and adhesiveness while higher gumminess compared to its natural counterpart. Hardness, gumminess and chewiness of experimental samples reduced with progression of storage. Shredability of analogue deteriorated as the storage period advanced while that of natural cheese increased up to 14 days and then deteriorated. Analogue had comparatively lower meltability and fat leakage than that of natural cheese. Meltability and stretchability of cheese samples increased with storage. Sensory characteristics of analogue improved up to 14 days followed by deterioration while those of natural cheese improved up to 21 days of storage and then deteriorated.

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RESEARCH ARTICLE

Estimation of β-sitosterol as a tool to detect ghee adulteration with palm oil

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Abstract: Ghee is a popular dairy product in India and most expensive fat in India so it is prone to adulteration with highly manipulated inexpensive oils/fats, especially during the lean season. Dry fractionation is the most cost-effective method of modifying the physical properties of milk fat. β-sitosterol is a principal sterol in edible oil (palm oil). The use of chromatography to separate sterols from ghee fractions could be a novel way to distinguish between β-sitosterol and cholesterol. In the current study, ghee was added with palm oil at levels of 0, 5, 10 and 20%. β-sitosterol was estimated qualitatively using the reverse phase-thin layer chromatography method (RP-TLC) and found better spot in liquid fraction than control ghee even at a 5 % level. This result was quantified using RP-HPLC and shows L_{20} with palm oil (5%) 0.1755 ppm concentration, S_{20} with palm oil (5%) 0.0755 ppm concentration.

Keywords: Reversed-phase thin layer chromatography, Reversed-phase high performance liquid chromatography, Ghee, Palm oil, β-sitosterol.

Introduction

Humans require edible oils and fats as part of their daily diet (Pitts et al. 2007). Adulteration is defined as the process of lowering the quality or nature of a given substance by adding a foreign or inferior substance and removing vital elements (Gonzálvez et al. 2010). The adulteration is a very serious problem. However, because of their higher demand in the domestic and international markets, adulteration of expensive oil with inexpensive oil is a major issue (Yadav, 2018). Adulteration is not visible because of its small scale and low impact, but it has existed in society for a long time. The main cause of adulteration is deception, which increases their income by increasing the volume of suspected products. Adulteration is also practised by some greedy businesses in order to increase their profit margins (Ayza and Yilma, 2014).

As the world's population continues to grow at an alarming rate, food is frequently tainted in order to meet the needs of this expanding population and feed the large-scale population. Most developed countries have a higher rate of food adulteration. Among them, it has become a very serious problem over the last two decades, posing serious health risks to almost all populations (Majed et al. 2016). β-sitosterol (Beta-sitosterol) is a phytosterol (plant sterol), a white waxy powder with a distinct odour that is one of the food additive components. FSSR, (2021) also recommended β-sitosterol estimation in ghee to check the purity is mandatory and it should be absent. Adulteration of fats and oils is currently a major problem all over the world, and rapid detection methods must be developed (Kou et al. 2018). It is critical to characterize milk fat for purity in order to maintain a consistent, well-defined quality. Because of the varied composition of the triglycerides contained in milk fat, detecting adulterants has always been difficult. The measurement of physico-chemical characteristics, elements of unsaponifiable matter, and evaluation of water-soluble and/or insoluble volatile fatty acids have all been used to detect foreign fats in milk fat. To detect adulteration in milk and milk products with foreign fats, TLC of unsaponifiable matter of milk fat, gas chromatography (GC) analysis of triacylglycerol (TAG) or fatty acid profile, and HPLC analysis of TAG and marker sterols of milk fat in combination with multivariate statistical data processing have been used. The majority of the above-mentioned characteristics, on the other hand, are only effective when large amounts of adulterants are employed, and they are not capable of detecting the type and level of added adulterants (Sharma et al. 2020). The resolution of cholesterol and ß-sitosterol, an index sterol in plant oils/fats, is limited in normal phase thin layer chromatography (Arun et al. 2005; Patel et al. 2011). RP-TLC methods based on silica gel-G plates impregnated with undecane (IDF 1966; Mathew and

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Kamath 1978). Thin layers of $CaCO_3$ and soluble starch (10 g + 4 g) impregnated with liquid paraffin (Ramamurthy et al. 1967), on the other hand, are time consuming and have reproducibility and sensitivity issues. β-sitosterol among the phytosterols is predominant in vegetable oil (palm oil), therefore the presence of β-sitosterol in ghee formed the basis for checking the adulteration of ghee with palm oil. Sofia (2005) detects ghee adulterated with palm oil only at a 20% level using various physicochemical characterization, especially Reichert-Meissl value. Upadhyay (2014) also detected palm oil in ghee at a 5% level using RP-TLC detects, but the spots were not clear. Therefore, in the present investigation the ghee has been fractionated in to liquid and solid fractions based on melting point. Further, the fractionated ghee used for the detection of β-sitosterol using RP-TLC at 5% level of adulteration with palm oil.

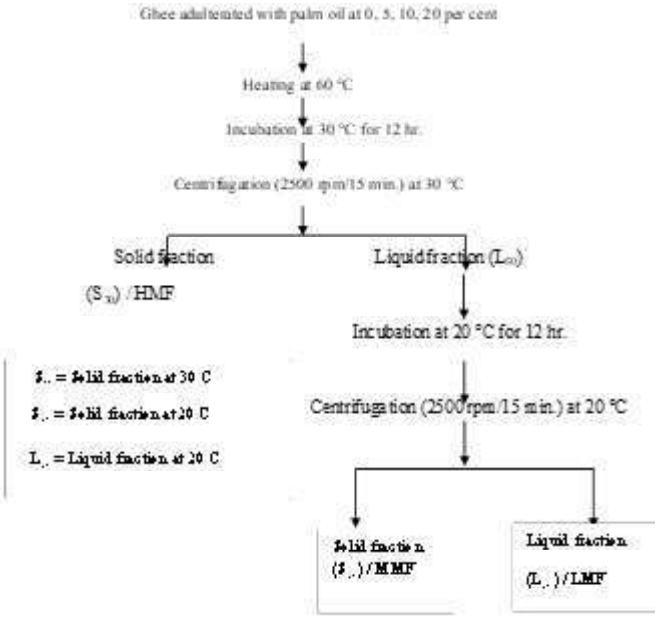
Materials and Methods

Butter of National brand was purchased from retail outlet near Hebbal, Bengaluru. Palm oil were purchased from the local Bengaluru market and used for the blending with ghee to their impact on physico-chemical characterization of ghee. β-sitosterol (Sigma Aldrich, USA), Cholesterol (Sigma Aldrich, USA) RP-TLC (reversed-phase thin layer chromatography) plates TLC silica gel 60 RP-18 F 254S (Merck Specialities Private Ltd., Mumbai, India).

Preparation of samples

Butter was then heated on direct flame in a stainless steel vessel and clarified into ghee with continous strring at a temperature of 115-117°C. Ghee was then filtered through muslin cloths, cooled, filled in airtight glass bottles for futher analysis.

Dry fractionation technique



The method of Kankare (1974) was followed fractionate ghee. The crystal memory was removed by heating ghee to 60°C. It was then progressively cooled to 30°C in an incubator for 12 hr. to crystallize. After centrifugation at 2500 rpm for 15 min. in a temperature-controlled centrifuge kept at 30°C, the liquid was separated from the crystals by decantation. At 30°C, solid fraction obtained (S_{30}) was considered a high melting fraction. The liquid fraction collected at 30°C was then incubated for a further 12 hr. at 20°C. After centrifugation at 2500 rpm for 15 min. in a temperature-controlled centrifuge kept at 20°C. The produced crystals were separated. The solid portion obtained at 20°C (S_{20}) was considered a medium melting fraction, whereas the amount that remained liquid at 20°C was referred to as the low melting fraction (L_{20}).

Extraction of Unsaponifiable Matter (USM) from the Ghee for RP-TLC

Unsaponifiable matter from fat samples was isolated essentially as per the method standardized by Sharma et al. (2009). To extract 0.2 g molten fat sample was taken in a 15 ml capacity screw capped tube followed by the addition of 5 ml of 5 % methanolic KOH. The tube was incubated in a water bath maintained at 90 °C with intermittent shaking after every 5 min., for about 20 min. After 20 min. of incubation, the tube was cooled to room temperature under tap water. One ml water and 5 ml hexane were added in the tube and tube was vortexes for 1–2 min. followed by centrifugation at 2,000 rpm for about 2 min. The upper hexane layer was pipetted out and in a small beaker of about 10 ml capacity and hexane was evaporated to get dried unsaponifiable matter. The dried unsaponifiable matter was redissolved in chloroform and volume was made to 500 µl in an eppendorf tube.

Reversed-phase thin layer chromatography (RP-TLC) of unsaponifiable matter (USM)

Method of sterols separation on C18 stationary phase as described by Jarusiewicz et al. (2005) was adopted in the study. Developing solvent consisting of Petroleum ether: Acetonitrile: Methanol (20:40:40 v/v) was added to a TLC glass chamber lined with filter paper on the three sides. Chamber was saturated for about 15 min. 6 µl of the unsaponifiable matter solution (500 µl solution in chloroform) was spotted on TLC silica gel 60 RP-18 F 254S plate at a distance of about 1 cm from the bottom along with solutions of standard cholesterol, β-sitosterol and mixture (β-sitosterol and cholesterol) as different spots and allowed to air dry. TLC plate was then developed in the developing chamber saturated with developing solvent till the solvent front had travelled about three-quarters of the length of the plate. The plate was then removed, dried, and sprayed with phosphomolybdic acid solution (20 % solution in ethanol) and kept at 90-95°C/3 min and spot of distinct blueish bands was compared with reference standard. The Rf value was calculated

by taking the ratio of distance moved by the solute (in cm) to the distance moved by the solvent (in cm).

Extraction of Unsaponifiable Matter (USM) from the Ghee for RP-HPLC

One gram of the fat sample was weighed for the extraction of USM in a screw-capped test tube and 25 mL of 5% methanolic KOH was added to it. The tube was kept in a water bath maintained at 90°C for about 50 min with vigorous shaking at regular intervals. 5 mL of water and 15 mL hexane were then added and the contents were vortexes for 1 min followed by centrifugation at 3000 rpm for about 5 min. The upper hexane layer was pipetted out and dried to obtain USM. The dried USM obtained was then dissolved in 300 µL of chloroform and the volume was made up to 500 µL with methanol. This sample was then filtered through 0.22 µm Millipore filter paper and subjected to RP-HPLC analysis. The reference standards of β-sitosterol of 1 mg/mL concentration were also run-on RP-HPLC and peak detection was made at 205 nm.

Analytical Conditions for HPLC

RP-HPLC was used to profile the samples of sterols. HPLC conditions as described by Oh et al. (2011) were adopted for the profiling and separation of sterols from the mixture of standard sterols vegetable oils, specific adulterant oil, and adulterated ghee samples. 20 µL of sample was injected into the HPLC column (Reversed phase C-18, 4.6 × 250 mm ID, 5µ 120 Å particle size, Dionex) held at 30°C (in a temperature-controlled column oven) for separation of sterols. Chromatography was initiated at a linear solvent (Acetronitrile:Isopropanol; 9:1, v/v) flow rate of 1.5 mL per min over a period of 30 min with a UV detector probe fixed at 205 nm for the detection of sterols.

Peaks Identification and their Confirmation:

USM of pure ghee and adulterated ghee samples (20 µL) were injected to examine for the presence of cholesterol and phytosterols. The identification of the peaks in the samples was done by comparing the retention time with that of reference standards. The appearance of the peak for β-sitosterol (phytosterol) in the adulterated samples was used as an indicator to confirm the presence of vegetable oils in adulterated ghee sample**s.**

Results and Discussion

Reversed-phase thin-layer chromatography

Reversed-phase thin-layer chromatography (RP-TLC) to check the purity of milk fat at low levels of adulteration with vegetable oil (palm oil). RP-TLC protocol to resolve the β-sitosterol and cholesterol (Jarusiewics et al. 2005) has been developed, where in new generation readymade RP-18 Silica gel-G F254S TLC were used. These plates are easy to use and have good reproducibility.

Separation of standard sterols and their mixture

It is evident from chromatogram (Fig. 1) that standard cholesterol and β-sitosterol showed difference in their mobility on RP-18 TLC silica gel G F254 S plate and even the mixture of cholesterol and ß-sitosterol was also resolved into two different bands corresponding to cholesterol and ß-sitosterol. The Rf value of cholesterol standard was calculated as 0.21, whereas that of β-sitosterol as 0.17. This indicated that the standardised conditions had the potential to be used for resolving sterols, especially cholesterol and ß-sitosterol in ghee samples adulterated with vegetable oils (palm oil).

RP-TLC Profile of Sterols in USM of Ghee Samples

It can be seen from the chromatogram that in case of control ghee samples (cow and buffalo) the position of prominent band was corresponding to the Rf value of cholesterol and there was no band corresponding to the Rf value of β-sitosterol (Fig. 1). However, in case of ghee with palm oil (5%), L_{20} with palm oil (5%) and S_{20} with Palm Oil (5%) a prominent band corresponding to the Rf value of β -sitosterol appeared as evident in Fig. 1. These observations clearly indicated that β-sitosterol was the prominent sterol in palm oils selected for the study, thereby the appearance of any β-sitosterol band in ghee sample could be considered as an indicator of adulteration of ghee with vegetable oils (palm oil).

Validity and specificity of RP-TLC method

The unsaponifiable matter from these samples was subjected to RP-TLC analysis to obtain the profile of sterols. It is evident from the RP-TLC chromatogram (Fig. 1) that control ghee samples not showed any band corresponding to the band of β-sitosterol. This clearly indicated that standardized protocol was very specific in detecting the added vegetable oils (palm oil) in ghee and method could be used to detect the adulteration of ghee with vegetable oils without showing any false positive results.

Detection of adulteration of ghee with palm oil

The unsaponifiable matter of the ghee samples adulterated with palm oils at different levels was subjected to the above standardized RP-TLC method. RP-TLC chromatograms (Fig. 1), clearly showed that the adulteration of ghee with palm oil could easily be detected even at the level of ghee with palm oil (5%), L_{20} with palm oil (5%) and S_{20} with Palm oil (5%) due to the presence of spot corresponding to the β-sitosterol which were not there in case of the control of ghee. Finding from the present work clearly demonstrated that the concentration of palm oil in the samples of milk fat increased the intensity of spots (both cholesterol and β-sitosterol) were prominent in fractionated ghee (especially liquid

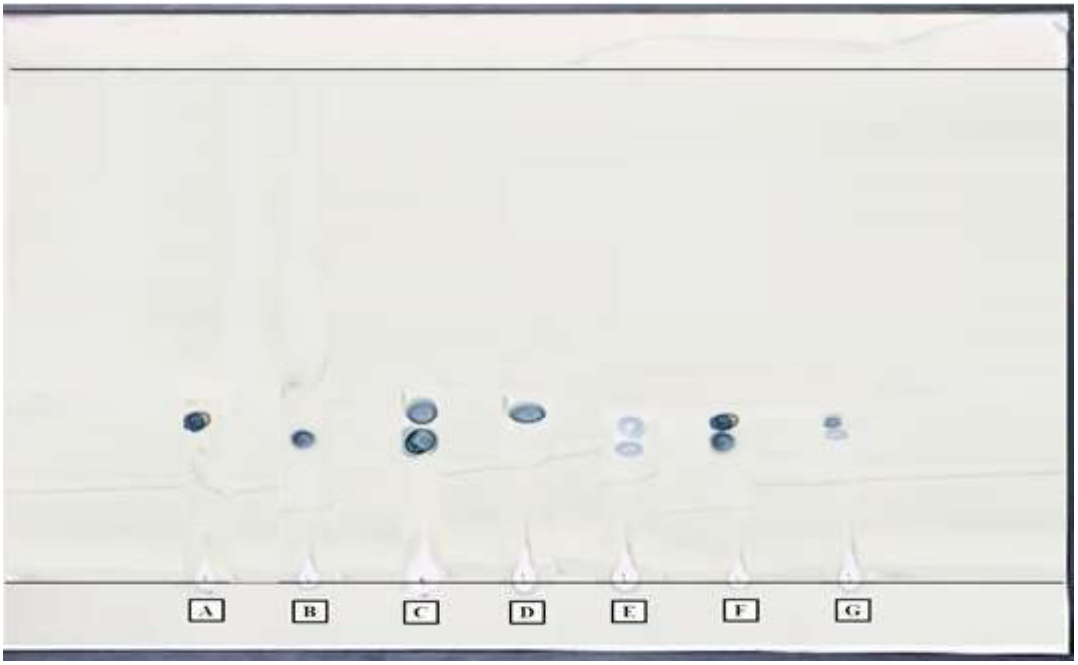


Fig. 1 Reverse phase thin layer chromatography (A) cholesterol (B) β-sitosterol (C) Mixture (β-sitosterol and cholesterol) (D) Control ghee (E) Ghee with palm oil (5%) (F) L_{20} with palm oil (5%) (G) S_{20} with palm oil (5%)

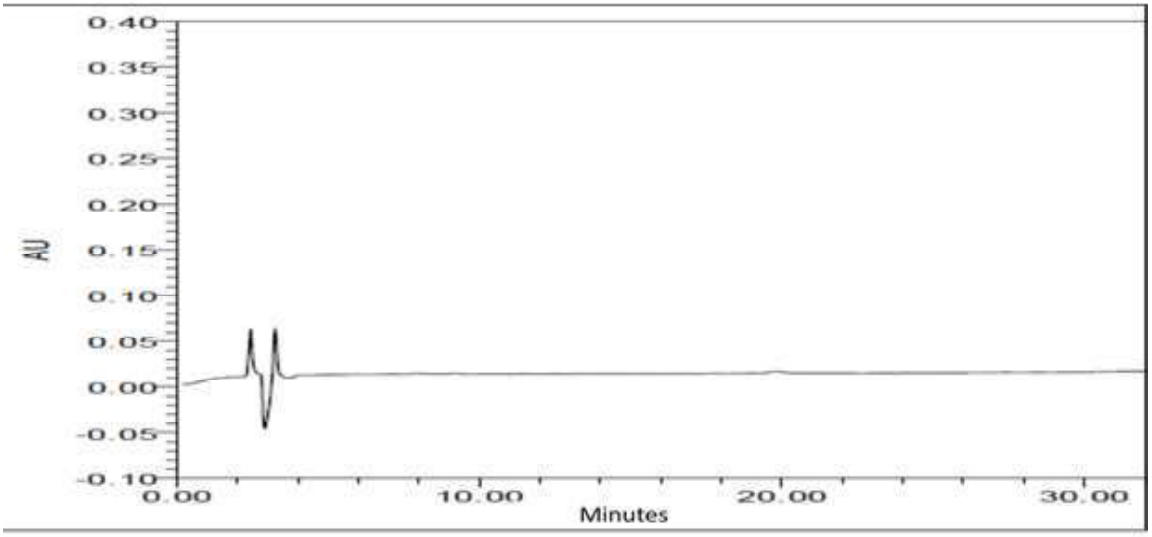


Fig. 2 Chromatography graph for control ghee

fraction) compared with control ghee. The result obtained are comparable with those of Rani et al. (2015) reported that RP-TLC chromatograms clearly showed that coconut oil added to ghee at 5 % level a very faint band were visible in the chromatogram, but at 7.5 % level, the visibility of band corresponding to β-sitosterol band were more. Similar results were also found by Upadhyay, (2014) reported that the concentration of vegetable oil in the samples of milk fat increased (from 5 to 15 % groundnut oil) the intensity of spot increased indicating the increase in the amount of β-sitosterol in the samples and thus enabling the detection of milk fat adulteration. Physicochemical characterization (Reichert-Meissl value) detects palm oil in ghee only at 20%. (Sofia, 2005) In the current investigation, the spots clear by using dry fractionation to detect the ghee adulterated with palm oil at a 5% level in less than 2 hours.

Reverse phase high performance liquid chromatography (RP-HPLC)

An efficient, fast and reliable reversed phase high-performance liquid chromatography-based method was developed to detect specific adulterant in ghee (clarified butterfat) samples. The method is based on the detection of cholesterol and β-sitosterol as markers in the unsaponifiable matter of pure ghee and adulterated ghee samples, respectively. Validation of the method revealed that it was fast, economical, highly reliable, and comparable with the reversed-phase thin layer chromatography method with no false negatives.

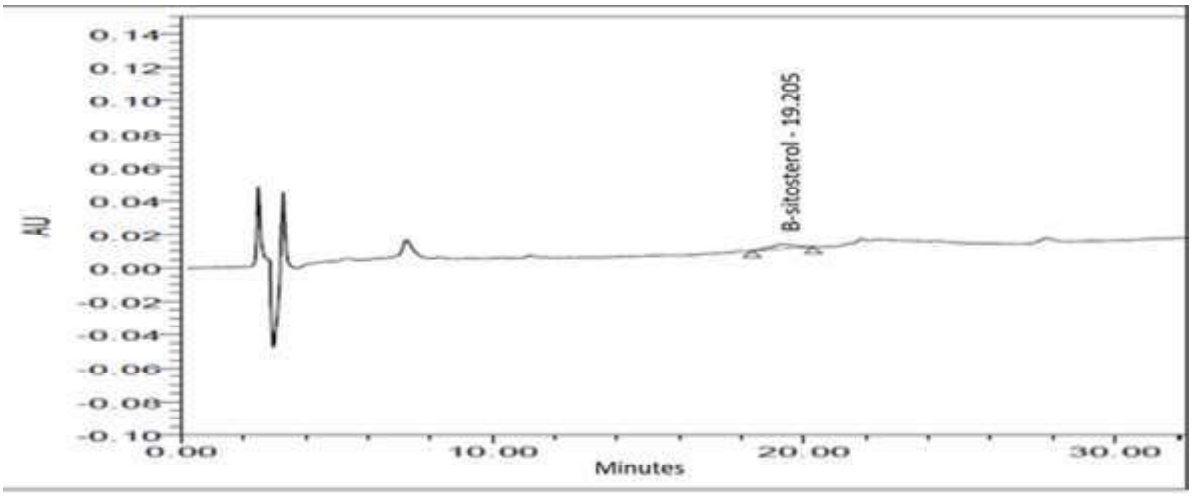


Fig.3 Chromatography graph for S_{20} with palm oil (5%)

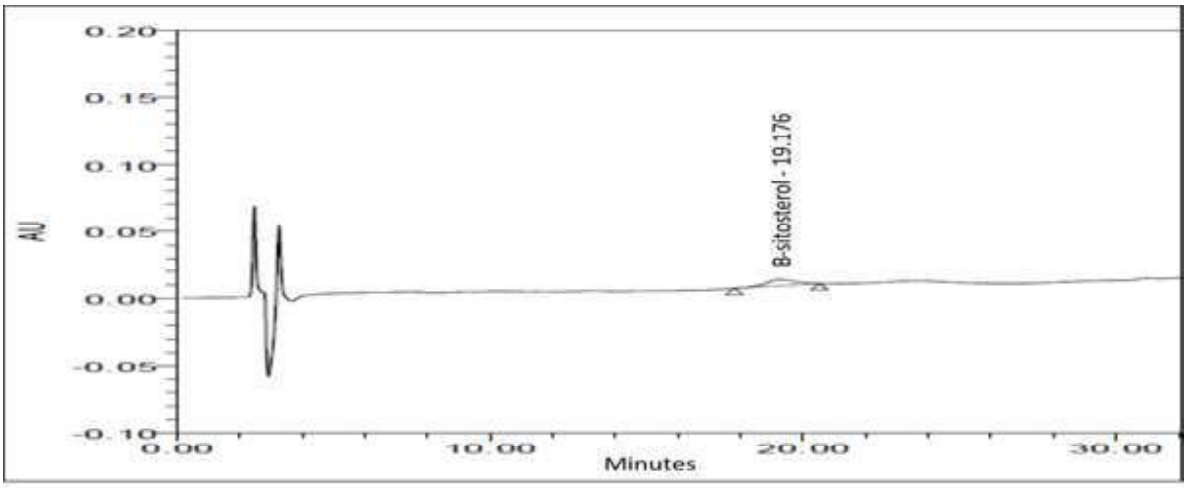


Fig.4 Chromatography graph for L_{20} with palm oil (5%)

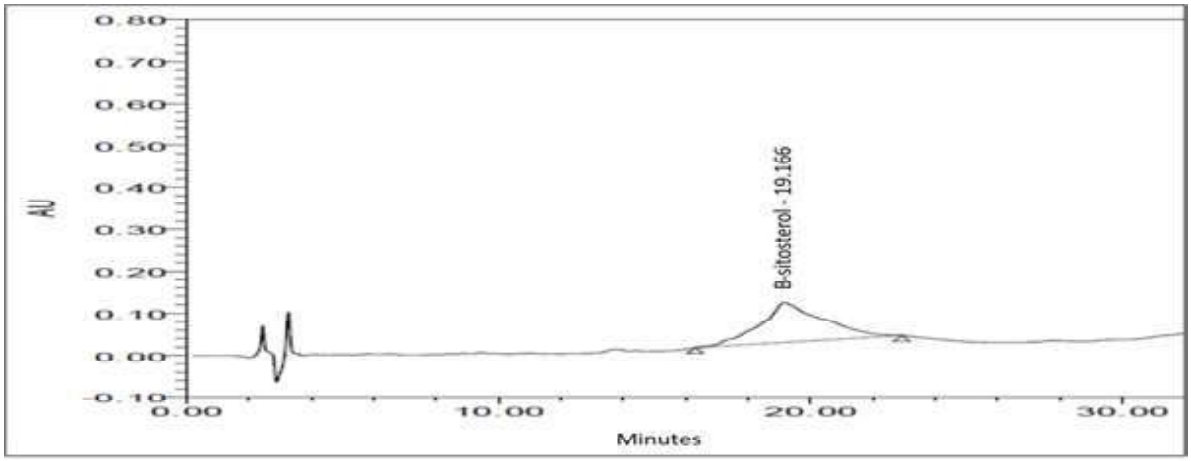


Fig.5 Chromatography graph for standard

Reversed-Phase High Performance Liquid Chromatographic (RP-HPLC) Conditions for the Profiling of USM

RP-HPLC conditions for the separation of standard sterols as described by (De, 2011) were adopted and standardized for the profiling of sterols in USM of ghee, adulterated samples. The retention time of individual standard sterols (β-sitosterol), was 19.166 min. (Figure 5). The results indicated that the β-sitosterol resolved properly as evident from their Rt values. Since β-sitosterol resolved properly using the standardized HPLC method and β-sitosterol served as principle markers sterol for vegetable

oils. Therefore, this standardized method was selected to compare the results with that of the RP-TLC method.

Validation of Standardized RP-HPLC Method

The USM from these samples were subjected to RP-HPLC analysis to obtain the profile of sterols which is depicted in Figure 5. A small variation in the retention time of β-sitosterol was observed in the samples used for the validation and was found to be 19.166 min. control ghee sample no peak corresponding to the Rt of β-sitosterol It is evident from the RP-HPLC chromatograms that L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples showed peak corresponding to the peak of β-sitosterol (Figure 3, 4). It was amply clear that in L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples peak corresponding to the Rt of β-sitosterol was 19.205, 19.176 min. observed, hence the standardized method was found to be very specific in detecting the adulterant oil in ghee, wherein major sterol, i.e., β-sitosterol, was the selected tracer component.

Detection of Adulterant Oils in Ghee

RP-HPLC chromatograms that L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples. Quantified using RP-HPLC shows L_{20} with palm oil (5%) 0.1755 ppm concentration, S_{20} with palm oil (5%) 0.0755 ppm concentration.

Conclusions

Reversed-phase thin layer chromatographic protocol has been standardized in the present study. The adulteration of ghee with adulterant oils such as palm oil could be detected up to 5 % level. Physicochemical characterization (Reichert-Meissl value) detects palm oil only at 20% for ghee adulterated with palm oil. Without fractionation, the intensity of the band on RP-TLC is not clear. As a result, fractionation using RP-TLC the intensity of band is clear in L_{20} with palm oil (20%) and RP-HPLC could be suggested in the current study even at a 5% level.

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RESEARCH ARTICLE

Physicochemical quality of cow milk collected from different sources in Adewa, Central zone of Tigray, Ethiopia

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Abstract: The study was conducted in Adewa, central zone of Tigray Ethiopia, aimed to asess the physicochemical quality of raw cow milk collected from smallholders, cafeterias and local vendors. A total of seventy samples of raw cow milk were collected and analysed. All samples were collected using proportional random sampling method. The mean values for pH, specific gravity, titratable acidity, freazing point, Total solid , fat, Solid not fat, protein, ash and lactose contents of milk samples collected from smallholders and cafeterias were 6.640±0.03, 1.032±0.04, 0.165±0.01, -0.530±0.07, 12.84±0.05, 3.98 ±0.04, 8.86 ±0.01, 3.69 ±0.03, 0.58 ±0.06, 4.58 ±0.0 and 6.40 ±0.00, 1.026 ±0.00, 0.19±0.004, -0.48±0.009, 14.81±0.003, 4.63 ±0.005, 10.2±00, 4.24±0.005, 0.73±0.0013 and 5.20 ±0.002 respectively. In the case of milk samples collected from local vendors PH, specific gravity, titratable acidity, freezing point, Total solid, Solid not fat, Protin ash and lactos content were 6.27±0.007, 1.022±0.00, 0.28±0.006, -0.49±0.001, 14.1±0.002, 4.12±0.008, 9.94±0.003, 4.41±0.003 and 0.74±0.002 respectively. The study found that the chemical quality of samples collected from cafeterias and local vendors was significantly higher (P<0.05) than samples from small scale milk producers. However, the physicochemical quality of samples from all three sources was within the standard level, except for pH and titratable acidity in the samples from cafeterias and local vendors. This indicates that a need for improved physicochemical quality standards, providing training and education for small-scale milk producers, cafeterias, and local vendors on proper milk handling and storage practices, to enhancing the overall quality of milk in the region

Keyword: Physical; Chemical; Milk; Cow; Quality

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Introduction

In developing countries like Ethiopia, dairy production plays a vital role in both rural and urban areas (Tegegne et al. 2013). Milk, a white liquid rich in protein, carbohydrates, fats, minerals, and vitamins, is a crucial component of the diet. It is obtained from various mammalian animals and consumed by humans (Pereira, 2014). Milk's nutritional richness and widespread availability make it an invaluable resource in improving health outcomes and promoting sustainable development.

The physiochemical composition of milk is a critical factor in determining its quality and suitability for producing processed milk products. Both the physical properties and chemical compositions of milk serve as indicators of its quality when it is hygienically normal (Kailasapathy, 2015). Freshly drawn milk exhibits variation in composition, structure, and properties. Even within the milk from a single milking of one cow, there can be differences (Alganesh et al. 2007). This variability underscores the importance of regular monitoring and assessment of milk quality, particularly in dairy production systems where factors such as breed, diet, milking practices, and animal health can influence milk composition.

Ensuring the production of quality milk is crucial to meet consumer demand and to facilitate the creation of high-quality dairy products. Quality milk is defined as milk that is free from pathogenic bacteria and harmful substances, has a good flavor, normal composition, adequate keeping quality, and low bacterial counts (Ahmedsham et al. 2018). It serves as the essential raw material for producing different dairy products(Saxena and Rai, 2013). However, In Ethiopia regions like Adwa district in Tigray, challenges exist in maintaining consistent milk quality due to variations in physicochemical composition caused by the addition of adulterants and the absence of quality measurement standards. In the study area Adwa district, where milk production primarily occurs through informal marketing systems, and there is no established quality control system between producers and consumers for ensuring milk quality. To address this gap, studies such as the one conducted in Adewa central zone of Tigray, Ethiopia, are essential. The objective of this studies were to compare the physical properties and chemical composition of

cow milk collected from different sources. By addressing the challenges related to milk quality in regions in Adwa district was essential for the sustainable growth of the dairy industry and the satisfaction of consumer demand for safe and nutritious dairy products.

Materials and Methods

Descriptions of the study area

The study was carried out in Adewa district which is located in central zone of Tigray, Ethiopia. Geographicaly location of Adwa district, lie between 380 53'55"E to 380 57' 30" E longitude and 140 08' 43" N to 140 11' 47 "North latitude. The annual mean rainfall ranges from 600 to 850 mm. The annual average temperature ranged from 12 ^{0}C to 27 ^{0}C.

Research Design

The study involved laboratory based investigation aimed to investigate the Quality of milk in the basis of physio-chemical Characterstics of raw cow milk collect from small-scale milk producers, local venders and cafeterias in Adewa central zone of Tigray. A total of seventy raw milk samples was collected in morning and afternoon from local venders, Cafeterias and small-scale milk producers from selected four kebeles based on their dairy potential.

Data sources and collection procedures

A total of seventy samples of raw cow milk was collected in morning and afternoon from three different sampling point's namely local venders, Cafeterias and small scale milk producers from purposefully selected four kebeles. Fifteen local venders, twenty cafeterias and thirty five small scale local milk producers based on the interview were selected at each kebele. All the samples was collected using proportional random sampling methods. During the collection time raw milk samples were aseptically collected from the bulk milk container of producers, local venders and cafeterias and small scale milk producers and placed into sterile bottles then samples were labeled and transported to Mekelle University Animal Production and Technology Laboratory in an icebox at 4°C for physio-chemical analysis.

Physico-chemical analysis

Analyses of physicochemical properties of cow milk were done at Mekelle University Animal Production and Technology Laboratory using a Lacto scan (model number SL30 and brand name Sri Balaji Made in India Instruments) to determine the percentage composition added water, specific gravity/density, titratable acidity/lactic acid, freezing points, pH, lactose, protein, fat, ash and total solid(ST).

SNF (Solid not fat)

To know the amount of this Solids-not-fat (SNF) content was determined by subtracting percent fat from TS present used by this formula to calculate the following formula: Solids-not-fat = Total solids – fat

Statistical analysis

The data collected from physiochemical properties of milk among small scale milk producers, local venders and cafeterias was analyzed by using analysis of variance (ANOVA) used the General Linear Model (GLM) procedure of (SAS, 2012). The sample mean were compared using Duncan Multiple Range Test Mean (DMRT) tests.

Result and Discussion

Physical quality of cow milk

Milk in normal state has unique physic-chemical properties, which are used as quality indicators. It appears that the study conducted in Adewa observed differences in the temperature of raw milk samples collected from various sources, including small-scale milk producers, cafeterias, and local vendors Table 1. The findings suggest that the mean temperature of the milk samples varied significantly ($P < 0.05$) among these sources, with milk from small-scale producers having a higher temperature compared to milk from cafeterias and local vendors. The higher temperature of milk from small-scale producers could be attributed to variations in milk handling equipment and techniques. The study suggests that the lack of cooling systems and insufficient use of refrigerators among milk producers in the area contributed to the elevated temperature of the milk samples collected (Eshetu et al. 2019). Proper milk handling practices and adequate cooling systems to maintain the quality of raw milk, as temperature can impact its physicochemical properties and overall quality.

The PH values of milk samples collected from small scale milk producers were significantly higher than cafeterias and local vendors's sample (Table1). Milk pH is used as an indication of milk hygiene and it usually ranges between d"6.5 and e"6.7. The normal PH of milk was an important indicator of growth of bacteria in the milk sample. Cooling of milk reduces the risk of growth of bacteria while high milk temperature considered as favorable for the growth of bacteria in milk (Moatsou and Moschopoulou, 2014). According to the results obtained in the present study, pH of milk samples from cafeterias (6.40) and local vendorss (6.27) were not within the normal ranges. In general, the pH of milk samples collected from cafeterias and local vendors was significantly lower ($P < 0.05$) than the pH of milk obtained from small scale milk producers (Table1).

The specific gravity of raw milk samples collected from different sources in Adewa was measured, with the following values

obtained: 1.026 for cafeterias, 1.022 for local vendors, and 1.032 for small-scale milk producers. These values exhibited a statistically significant difference among the sources, as indicated in Table 1. according to a study by Janštová et al. (2011), the specific gravity of normal milk typically ranges from 1.028 to 1.033 grams per milliliter. In the current study, the specific gravity of milk samples from small-scale milk producers was within this range and comparable to findings by (Tamime, 2009). Furthermore, O'Connor (1993), suggests that a higher specific gravity value may indicate the skimming off of fat from the milk, while a lower value than the normal range could suggest the addition of water.Based on this information, the specific gravity measurements in the Adewa study indicate variations in the composition of raw milk samples from different sources, potentially reflecting differences in milk quality, such as fat content or water adulteration. These findings underscore the importance of monitoring and regulating milk quality to ensure consumer safety and trust in dairy products.

The titratable acidity of raw milk samples collected from various sources in Adewa was analyzed, revealing significant differences among samples from small-scale milk producers, cafeterias, and local vendors, as indicated in Table 1.Milk samples obtained from cafeterias and local vendors exhibited a titratable acidity value exceeding 0.18%, suggesting prolonged exposure to room temperature and poor handling practices prior to sale and consumption. Additionally, these samples showed significantly higher titratable acidity compared to those from small-scale milk producers. The elevated titratable acidity in milk from cafeterias and local vendors may be attributed to bacterial growth and multiplication during milk transportation and storage before sale. This observation aligns with the findings of Yilma and Faye (2006), who reported even higher titratable acidity (0.27%) in milk samples collected from dairy shops in the central highlands of Ethiopia. These results emphasize the importance of proper milk handling and storage practices to maintain milk quality and minimize bacterial contamination, ultimately ensuring consumer safety and satisfaction with dairy products.

Freezing point of milk is usually in the range of -0,512 and -0,550 with an average of -0,522. The freezing point of milk is a physicochemical property that is closely correlated with several other key characteristics of milk. As the concentration of total solids increases, the freezing point of milk decreases. This is because dissolved solids, such as lactose, proteins, and minerals, lower the freezing point of a solution. Therefore, milk with higher total solids content typically has a lower freezing point. Proteins, particularly casein and whey proteins, contribute to the total solids content of milk and can lower the freezing point. Additionally, changes in protein composition or denaturation

Table 1: Physical properties of cow milk obtained from small scale milk producers, cafeterias and local vendors

Physical Qualities	Milk Source			
	Small scale milk producers (n=35)	Cafeterias (n=20)	Local vendor (n=15)	Overall Mean
Temp. (^{0}C)	28.500^{a}±0.06	26.6^{b} ±0. 00	24.5^{c}±0.00	26.530±0.03
pH value	6.640^{a}±0.03	6.40^{b} ±0. 00	6.27^{c} ±0. 007	6.440±0.03
SG	1.032^{a}±0.04	1.026^{b} ±0. 00	1.022^{c} ±0. 00	1.028±0.03
TA (%LA)	0.165^{b}±0.01	0.19^{a}±0. 004	0.28^{a}±0. 006	0.121±0.05
FP	-0.530^{b}±0.07	-0.48^{a}±0. 009	-0.49^{a}±0. 001	-0.515±0.00

Means followed by different superscript letters within a row are significantly different ($P < 0.05$), Temp. = Temperature, SG = Specific gravity, TA= Titratable acidity, FP= Freezing point and n= number of samples

Table 2: Chemical properties of cow milk obtained from small scale milk producers cafeterias and local vendors

Chemical composition	Milk Source			
	Small scale milk (producers) (n=35)	Cafeterias (n=20)	Local vendor (n=15)	Over all Mean
TS (%)	12.84^{c} ±0. 05	14.81^{a}±0. 003	14.1±0.002^{b}	13.67±0.05
Fat (%)	3.98^{bb} ±0. 04	4.63^{aa} ±0. 005	4.12^{ab} ±0. 008	4.20±0.055
SNF (%)	8.86^{b} ±0. 01	10.2^{aa}±00	9.94^{a}±0. 003	9.46±0.04
Protein (%)	3.69^{b} ±0. 03	4.24^{aa}±0. 005	4.41^{a} ±0. 003	4.0±0.05
Salt (%)	0.58^{b} ±0. 06	0.73^{aa}±0. 0013	0.74^{a} ±0. 002	0.66±0.07
Lactose (%)	4.58^{b} ±0. 01	5.20^{aa} ±0. 002	4.78^{a} ±0. 0028	4.80±0.015

Means followed by different superscript letters within a row are significantly different ($P < 0.05$), TS = Total solid, SNF= Solid Not-Fat and n= number of samples

may impact the freezing point of milk. Changes in acidity levels, indicated by pH and titratable acidity, can also influence the freezing point of milk. Increased acidity, resulting from microbial spoilage or other factors, can lead to a lower freezing point due to the presence of acidic compounds in the milk (Zagorska and Ciprovica, 2013). Monitoring the freezing point of milk can provide valuable information about its quality, freshness, and suitability for various dairy processing applications The mean value the current study milk sample freezing point of the study area was -0.515±0.00 this means the milk collected from the milk is within the standard of freezing point of milk.

Chemical Composition of cow milk

Protein content of milk obtained from small scale milk producers, cafeterias and local vendors was 3.69±0.03, 4.24±0.005 and 4.41±0.003, respectively (Table2). There was difference (P>0.05) among milk samples in the three areas. This might be due to the combination of the samples from different sources. The average protein content of raw milk obtained from small scale milk producers, cafeterias and local vendors was higher than the earlier findings of Terfa (2014), who reported a protein content of 3.48% for milk produced in dairy farms. The overall mean protein content 4.0±0.05 percent obtained in the current study was higher than the protein content of 3.1 percent reported for Zebu cows' milk (O'Connor, 1995). Correspondingly, Negash et al. (2012), reported lower protein content (3.46±0.04%) for milk samples collected from householdss producing local and crossbred cows. According to European Union quality standards the fresh whole milk, total protein content should not be less than 2.9%. Therefore, the average protein content (4.0±0.05) observed from three areas was within the recommended standards.

There was significant difference (P > 0.05) in fat content observed among the samples collected from three study areas (Table2). The fat content was significantly affected by the factors like feed, parity, breed and stage of lactation. Fat content was highest in cafeterias (4.63±0.005) than in local venders (4.12 ±0.008) and small scale milk producers (3.98±0.04) (Table2). The mean value of the three areas (4.20±0.055) were greater than the earlier findings of Janštová et al. (2011), who reported a fat content of 3.79±0.18% for milk produced in dairy farms. On the other hand, the average fat content fresh (raw) whole cow milk obtained in this study (4.20±0.055) was lower than the earlier finding of Ketema et al. (2018) who reported a fat content of 5.46 ±0.51% for milk samples collected from Walmera District of Oromia Region household producing local cows. According to Teklemichael (2012) The Food and Drug Administration (FDA) require not less than 3.25% milk fat for fluid whole milk. According to Terfa (2014), consequently, the average fat content (4.0±0.05) observed from three areas was within the recommended standards. Fat of milk is unquestionably the most valuable constituent of milk. Milk having a fair amount of fat is more valuable as a food than milk which is poor in fat (Bishoftu, 2016).

The Total Solids (TS) component of milk is a crucial indicator of its overall quality and nutritional value. TS represents all the non-water components present in milk, including proteins, fats, lactose, minerals, and other solids Kennelly et al. (2000) and Bezie (2019). In the current study the data indicate the presence of the significant differences (P < 0.05) in the SNF content among milk samples collected from small scale milk producer's cafeterias and local vendors. The SNF content of milk collected from small scale milk producers cafeterias and local vendors were 8.86±0.01and 10.2±0.00 and 9.94±0.003 (Table 2). The difference observed in the SNF content of milk could be due to the difference in the feeding practice, season, milking method and lactation period (Lingathurai et al. 2009). The SNF content the three areas (9.46±0.04) obtained in the current study was lower than the result obtained by Nigussie and Seifu (2007) who reported SNF contents of 10.7% for cows' milk in Kombolcha woreda. However, this value is greater than the finding reported by Teklemichael (2012) and Debebe (2010) for milk obtained from dairy farms (8.75%) in Dire Dawa town and the minimum (8.3 ± 0.36%) and maximum (8.7 ± 0.36%) SNF content of raw cow's milk obtained from street-vendors and milk producers in and around Addis Ababa. According to European Union quality standard the raw whole cow milk SNF content should not be less than 8.5% (Tamime, 2009). Accordingly, the average SNF content (9.46±0.04) observed for three milk samples were within the recommended standard. This suggests that the milk samples analyzed in this study possess adequate total solids content, indicating good nutritional quality. Higher TS content indicates a greater concentration of essential nutrients, such as proteins, fats, and minerals, making the milk more nutritious (Kittivachra et al. 2006). Dairy processors often prefer milk with specific TS levels for different products. For example, milk with higher TS content is preferred for cheese-making, as it yields higher cheese yields and better texture. Conversely, milk with lower TS content may be more suitable for fluid milk products like pasteurized milk or flavored milk (Abd El-Gawad and Ahmed, 2011). The TS content significantly impacts the flavor and texture of milk. enhances the sensory experience of consuming milk and is often preferred by consumers (Yayota et al. 2013). Monitoring and optimizing TS content are essential for ensuring the production of high-quality milk that meets consumer preferences and regulatory standards.

The salt content of milk, also known as the mineral content, is an important aspect of its overall composition and nutritional value (Zamberlin et al. 2012). In your study, the overall mean salt content obtained was reported as 0.66±0.07. Additionally, you observed that milk samples collected from local vendor and cafeterias had significantly higher salt content compared to samples obtained from small-scale milk producers.Overall, while the salt content of cow's milk typically remains relatively constant within a certain range (around 0.7% to 0.8%), variations can occur due to factors such as breed, stage of lactation, feed, interval between milkings, completeness of milking, and the age and health status of milking cows (O'Connor, 1995). Understanding these factors is important

for interpreting variations in milk composition and ensuring the production of high-quality milk for consumption and processing into dairy products.

The lactose content of milk is an essential component that contributes to its taste, texture, and nutritional value (Pereira, 2014) and (Guetouache et al. 2014). In this study, observed that the lactose content was significantly higher in milk from cafeterias compared to milk from small-scale milk producers and local vendors. Lactose content was significantly higher ($P<0.05$) in cafeterias than milk from small scale milk producers and local vendors (Table 2). Differences in storage conditions between cafeterias and other sources may impact lactose content. Cafeterias may have different storage practices or conditions that influence the microbial activity and enzymatic processes occurring in milk, leading to variations in lactose content. The average lactose content of the three areas was 4.80±0.015% which was higher than the result obtained by Teklemichael (2012), who reported lactose contents of 4.43±0.06% for cows' milk in Shashemene town southern Ethiopia. According to European Union quality standard for unprocessed cow milk whole milk lactose content should not be less than 4.2%. There for average lactose 4.80±0.015% content observed from the three milk samples were within the recommended standard. Regarding the comparison with previous studies, this findings show that the average lactose content (4.80±0.015%) from the three milk sources was higher than the results reported by Teklemichael (2012) for cows' milk in Shashemene town, southern Ethiopia. This difference could be attributed to various factors such as breed differences, feeding practices, and environmental conditions between the study areas. The average lactose content observed in this study met the European Union quality standard for unprocessed cow milk, which specifies that the whole milk lactose content should not be less than 4.2%. This suggests that the milk samples analyzed in your study meet the recommended standard for lactose content, indicating good quality and nutritional value.

Conclusion

The study on the physicochemical and chemical composition of cow milk from different sources in Adewa, Tigray, Ethiopia, provided valuable insights into the quality of milk available in the region. Generally, variations were observed in the physicochemical of cow milk from different sources of the milk samples met quality standards. However, differences among sources are the importance of proper milk handling, storage, and management practices to ensure consistent quality and safety of milk for consumers.

Conflicts of interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Effect of interaction of herb (*Pueraria tuberosa*) components with milk constituents on properties of milk

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Abstract: Milk is a carrier which has been effectively used to deliver phytochemicals for targeted health benefits in the traditional Indian systems of medicine, particularly Ayurveda. The model system consisted of cow milk fortified with aqueous extract of *Pueraria tuberosa* (@ 0.4%) on the basis of sensory evaluation by using 9-point hedonic scale. Effect of addition of herb on compositional and storage stability of control and experimental milk was investigated. The interactive effect of milk proteins with *Pueraria tuberosa* extract (@0.4%) was also studied by SDS-PAGE and urea-PAGE electrophoresis. The addition of *Pueraria tuberosa* to milk resulted in no change in composition, increase in phenol content and decrease in pH (during successive period of storage) as compared to control. Electrophoretic pattern of sodium caseinate and whey protein concentrate containing 0.4% herb extract showed that the band width changed in terms of height and raw volume. It can be concluded that addition of *Pueraria tuberosa* to milk at 0.4% concentration altered the functional properties of milk which could be due to interaction of components of *Pueraria tuberosa* with milk constituents.

Keywords: Milk, *Pueraria tuberosa* extract; Compositional analysis; Electrophoresis

Introduction

Pueraria tuberosa commonly known as Vidarikand and Indian Kudzu, belongs to *Fabaceae* family. The plant's tuber has very widely used as an active component in various formulations of Indian system of medicine (Ayurveda). It has been used as an aphrodisiac, cardiotonic, diuretic, galactogogue, hypolipidemic, immune booster, anti-inflammatory, antioxidant, antiageing and spermatogenic in various Ayurvedic formulations. The major active components in the tuber of *Pueraria tuberosa* are isoflavonoids viz. puerarin, genistein, daidzein, tuberosin and flavanoids (Maji et al. 2014).

Milk is one such carrier which has been effectively used to deliver phytochemicals for targeted health benefits of the traditional Indian system of medical sciences (Veena et al. 2015; Sawale et al. 2020). Addition of herbs or its extracts to milk and subsequent processing treatments however poses a definite challenge as possibilities exist for varying degree of interactions among the major and minor biomolecules of milk and bioactive compounds of herbs (Sawale et al. 2019). Such interactions could have a beneficial effect but sometimes it may also lead to certain practical difficulties if they modify properties of foods.

The isoflavonoids of *pueraria tuberosa* could interact with milk proteins viz., bovine serum albumin (Cao and Liu 2009), casein micelle (Xi and Guo 2008) and â-lactogobulin as has been reported in case of certain food and drug preparation containing soya isoflavonoids. Unavailability of such data on stability of bioactive molecules of *Pueraria tuberosa* in model dairy food systems and their interaction with milk constituents is a determinant to establish efficacy of their use as nutraceutical in functional dairy foods. Hence, the present study was aimed to investigate the effect of herb (*Pueraria tuberosa*) in an herb-milk model system.

Materials and Methods

Materials

Raw cow milk was obtained from the Livestock Research Centre of the ICAR-National Dairy Research Institute, Karnal, India. A freeze-dried hot water extract of herb (*Pueraria tuberosa)* was procured from National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh, India. Sodium caseinate and whey protein concentrate was purchased from Thomas Baker Pvt. Ltd., Mumbai, India. Molecular weight markers (205 KDa to 3.5 KDa) were purchased from Genei, Bengaluru, India. All other reagents used in this study were of analytical grade.

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Preparation of hot water extract of herb

The tubers of *P. tuberosa* were bought from local market and authenticated. They were deposited in the departmental herbal drug museum of the Pharmacognosy Division, NBRI, Lucknow for future reference. Figure 1 represents the procedure for preparation of hot water extract of herb. The coarse air-dried (40–50°C), powdered tuber (500 g each) of *P. tuberosa* was extracted with hot water by heating on a boiling water bath. The respective extracts were pooled, filtered, concentrated at reduced temperature (below 55°C) by rotary evaporation (Büchi, USA), and lyophilized (Freezone 4.5, Labconco, USA) under high vacuum (133 × 104 mbar) at "40 ± 2°C to yield the hot water (112.0 g) extract with 22% yield.

Separation of puerarin by high performance thin layer chromatography (HPTLC)

Dried hot water extracts in 10 mg/ml concentration was prepared for analysis. Puerarin (1 mg/ml, as marker compound) was used as standard. A Camag HPTLC system (Muttenz, Switzerland) comprising of a Linomat 5 automatic applicator, Camag TLC scanner 3 and win-CATs version 4 software was used. Precoated silica gel-60 F_{254} plates (0.2 mm thickness, Merck) on aluminium sheets were used as adsorbent layers. 2 µl of standard and 10 µl of sample solutions were applied and the plate was developed using ethyl acetate: methanol: water (10:1:1) as developing solvent. The plate was visualized under UV at wavelength of 254 nm and 366 nm. The presence of puerarin was simultaneously identified in the hot water extracts.

Preparation of *Pueraria tuberosa*-milk model

Lyophilized extract of *Pueraria tuberosa* was crushed using pestle and mortar with a small quantity of milk and then the mixture was added to bulk milk. Herb extract was added @ 0.4% in milk was observed to be an optimum level based on preliminary sensory evaluation for colour, flavour and consistency by 9-point hedonic scale. The fortified milk was then subjected to pasteurization treatment (63ºC/30 min) before further analysis.

Compositional analysis

Total solid, protein, lactose and ash content of control and experimental milk samples were determined as per the AOAC (2000) method. Fat content was determined by the Gerber method (IS 1981). Total phenolic content of milk samples was analyzed by Folin-Ciocalteu method (Kahkonen et al. 1999).

Interaction studies by electrophoresis

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to know the interaction between the milk protein and herb extract. SDS-PAGE was run for both control and *Pureria tuberosa* added milk samples, but the visibility of bands was poor and overlapping each other because of the interfering substances in the milk. Hence, sodium caseinate and whey protein concentrate were used to study the interactive effect. Sodium caseinate (2.4%) and whey protein concentrate (0.7%) (with or without *Pueraria tuberosa* extract @ 0.4%) were analyzed by the Urea-PAGE in polyacrylamide gels was performed according to the method of Andrews (1983) and by SDS-PAGE was performed by standard method of Laemmli (1970) with direct staining using Coomassie Brilliant Blue G-250.

The gels were analysed by using ImageAide gel analysis software (Spectronics Corporation ImageAide for Windows) and the densitograms were then drawn. The results were expressed in terms of height and raw volume (area under the curve) of each band in the lane.

Storage study

Storage of control and experimental milk samples were done at refrigerated temperature (6-8°C) for 5 days and samples were analyzed on every day of storage for pH and acidity. The pH and acidity of control and experimental milk sample was determined (IS 1981).

Statistical analysis

The entire experiments were replicated three times. All statistical analyses were performed using SYSTAT 6.0.1 software. Results are presented in means ± standard error of mean (SEM), and statistical significance was set at $p<0.05$. The t-test was used to determine the main effects of treatments.

Results and Discussion

Identification of peurarin by HPTLC

Figure 1 shows the HPTLC chromatograms of the *Pueraria tuberosa* hot water extract and peurarin standard visualized under UV at 254 and 366 nm. The identity of puerarin in hot water extract of herb was confirmed by comparison of its spectrum and retardation factor (R_f) with the authentic standard. Since the health benefits of the nutraceuticals or functional foods containing different botanicals is due to the presence of the phytoconstituents, it is important to have a biological marker and also to be able to associate that biological marker with the quality of life (Veena et al. 2014). Tubers of *Pueraria tuberosa* are rich in various isoflavonoids including puerarin. The Puerarin is the major isoflavanoid present in *Pueraria tuberosa* and demonstrated to have antioxidant and immunomodulatory activity (Pandey et al. 2007). Puerarin present in Indian Kudzu, possess a cardioprotective activity and give rotection against stress induced myocardial ischemia (Verma et al. 2009).

Compositional analysis

Fat, protein, lactose, ash and total solids content of experimental milk did not differ significantly (P>0.05) compared to control (Table 1). However, significant difference (P<0.05) in phenolic content was observed between control and *Pueraria tuberosa* added milk. Significant difference was also found between aqueous solution of *Pueraria tuberosa* extract (@0.4%) and *Pueraria tuberosa* added milk (Table 1). After pasteurization also concentration of polyphenol increased significantly (p<0.05) in *Pueraria tuberosa* added milk. The present result was corroborated with the study of Gad and Abd El-Salam (2010). They reported that the addition of green tea, rosemary extract, to skim milk significantly increased the phenol content and antioxidant activity of skim milk and they were increased on heat treatment (65°C/30 min) of skim milk. It could be due to excess phenolic compounds released as the breaking of the bonds between polyphenols and milk protein in the complexation compound (Rohn et al. 2004). Decrease in phenolic content of *Pueraria tuberosa* added milk in comparison to an aqueous solution of the extract itself could therefore be related to the polyphenol present in *Pueraria tuberosa* which might have interacted with milk protein and chelated metals.

Electrophoresis (Urea-PAGE and SDS-PAGE)

To investigate the interactive effect of milk proteins with *Pueraria tuberosa* extract, sodium caseinate and whey protein concentrate (WPC) were used for urea-PAGE and SDS-PAGE and change in band intensity was measured using Image Aide gel analysis software. Figure 2 shows the urea-PAGE patterns of sodium caseinate and WPC containing 0.4% herb extract. The electrophoretic pattern of with and without addition of herb extract did not show any difference in band pattern i.e there was no difference in mobility based on charge of the proteins, but the intensity (width) of band differed. Table 3 represents the effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC in terms of height and area under curve (raw volume). From Table 3, it could be observed that the height as well as raw volume decreased in sodium caseinate+*Pueraria tuberosa* extracts and WPC+*Pueraria tuberosa* extract as compared to sodium caseinate and WPC lanes, respectively. Therefore it could be inferred that the addition of aqueous extract of *Pueraria tuberosa* to milk and subsequent pasteurization led to formation of protein-polyphenol (isoflavons) complexes. Brown and Wright (1962) studied the tea polyphenol/milk protein system. They concluded that the possibility of hydrogen bond formation by performing electrophoresis on membrane filters in the presence of urea which is well known agent for breaking hydrogen bonds. The absence of complex formation in the presence of urea would suggest a mechanism involving hydrogen bonds. They also reported that the protein patterns are unchanged by the presence of the tea polyphenols. In the presence of the urea there was no precipitation of α-lactalbumin or β-lactoglobulin on addition of

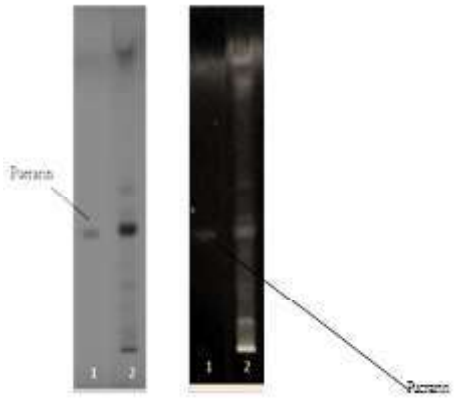

Fig.1 HPTLC Profiles of hot water extracts of *Pueraria tuberosa* using Puerarin as standard visualized under A) UV (λ at 254 nm); B) UV (λ at 366 nm) (Track 1 - Puerarin standard; Track 2 - Hot water extract (Solvent

Table 1: Compositional parameter of control and milk added with *Pureria tuberosa*

Constituents	Control	Milk added with PT	Aqueous solution of PT (0.4%)
Fat (%)	3.80±0.26[a]	3.73±0.30[a]	
Total Solids (%)	13.14±0.08[a]	13.33±0.06[a]	
Protein (%)	3.35±0.03[a]	3.36±0.02[a]	
Lactose (%)	3.93±0.06[a]	3.99±0.10[a]	
Ash (%)	1.12±0.02[a]	1.123±0.02[a]	
Phenol content (µg gallate eq/ml)	141.56±1.686[a]	181±16.22[b]	265.55±0.33[c]

Results are expressed as Mean±SEM (n=3). The values with same superscripts (a) in each row did not differ significantly (p>0.05), PT- *Pueraria tuberosa*

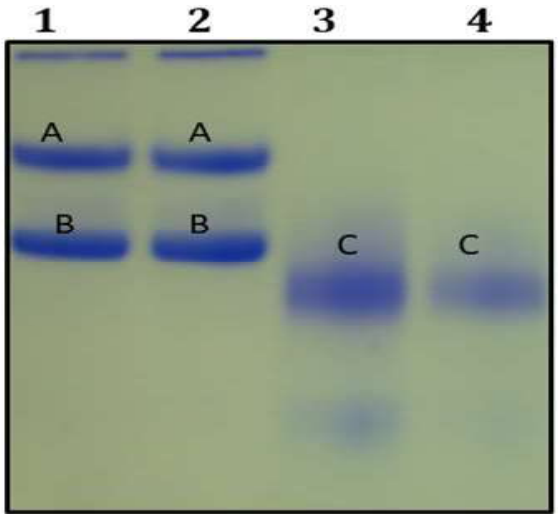

Fig 2. Urea-PAGE pattern of sodium caseinate and WPC added with *Pueraria tuberosa* separated on 12% gel. Lane 1 - Sodium caseinate; Lane 2 - Sodium caseinate+ *Pueraria tuberosa* herb extract, Lane 3 - WPC, Lane 4 - WPC+ *Pueraria tuberosa* herb extract. (A -β –Casein, B - α-Casein, C - β-Lactoglobulin)

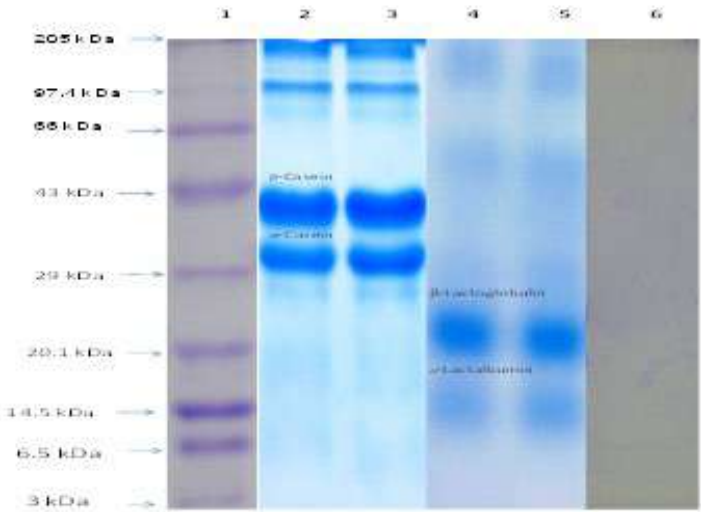

Fig. 3 SDS-PAGE patterns of Sodium caseinate and WPC added with *Pueraria tuberosa* separated on 15% gel. Lane 1 - Molecular weight standards ranging from 205 KDa to 3.5 KDa, Lane 2 - Sodium caseinate, Lane 3 - Sodium caseinate + *Pueraria tuberosa* extract, Lane 4 - WPC, Lane 5 - WPC + *Pueraria tuberosa* extract, Lane 6 - *Pueraria tuberosa* extracts

Table 2: Effect of storage period on pH and titratable acidity of control and *Puraria tuberosa* added milk

Parameter	Types of milk	Storage days (at 6–8ºC)					
		0	1	2	3	4	5
pH	Control	6.72 ± 0.03^{aA}	6.67 ± 0.05^{bA}	6.54 ± 0.03^{cA}	6.32 ± 0.03^{dA}	6.24 ± 0.03^{eA}	6.09 ± 0.03^{fA}
	Milk added with PT	6.65 ± 0.05^{aB}	6.48 ± 0.03^{bB}	6.30 ± 0.03^{cB}	5.12 ± 0.03^{dB}	6.091 ± 0.03^{eB}	5.99 ± 0.03^{afB}
Acidity (% lactic acid)	Control	0.135 ± 0.001^{A}	0.151 ± 0.002^{Al}	0.168 ± 0.001^{Ac}	0.17 ± 0.001^{Ad}	0.178 ± 0.001^{Ae}	0.20 ± 0.001^{Af}
	Milk added with PT	0.153 ± 0.001^{B}	0.178 ± 0.001^{Bl}	0.182 ± 0.003^{Bc}	0.19 ± 0.003^{Bd}	0.12 ± 0.001^{Be}	0.25 ± 0.0005^{B}

Results are expressed as Mean± SEM with different superscripts in each row (a, b, c, d, e, f) and in each column (A, B) differ significantly ($P<0.05$) ($n=3$). PT- *Pueraria tuberosa*

Table 3: Effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC by urea-PAGE

Track No	Lane 1 Sodium caseinate		Lane 2 Sodium caseinate+ *Pueraria tuberosa*		Lane 3 WPC		Lane 4 WPC+ *Pueraria tuberosa*	
	Height	Volume	Height	Volume	Height	Volume	Height	Volume
1	71.254	982551.00	67	804078.25	45.170	4364145.61	27.67	1914389
2	77.55	1320863.75	72	1123163				

the tea infusion and in all cases none of the brown colour was seen to move with the protein. Membrane filter electrophoresis in phosphate buffer (pH 6.7) and 7 M with respect to urea indicates that the milk protein/tea polyphenol interactions are at least initiated by the formation of hydrogen bonds. In a similar study, Chapon et al. (1961) studied beer polyphenol and protein interactions. It was concluded that beer polyphenols formed complexes with proteins through the formation of hydrogen bonds.

Figure 3 represents the SDS-PAGE electrophoretic patterns of sodium caseinate and WPC containing 0.4% herb extract. It is

Table 4: Effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC by SDS-PAGE

Track No	Lane 2 Sodium caseinate		Lane 3 Sodium caseinate+ *Pueraria tuberosa*		Lane 4 WPC		Lane 4 WPC+ *Pueraria tuberosa*	
	Height	Volume	Height	Volume	Height	Volume	Height	Volume
1	18.650	114132.84	19.957	125724.06	23.614	1223801.13	73.104	1233715.5
2	129.684	2266318.75	133.645	2388853.5				
3	116.727	1735054.88	118.817	1863118.75				

obvious that native casein was resolved into two major bands (α_s- and β-casein). In addition, some aggregates were also observed. The results are in accordance with the observation of Chobert et al. (2007), who reported that bovine casein separated into two major bands along with some aggregates. In the present study, a slight difference in band intensity was observed. There was no extra band resolved in sodium caseinate + *Pueraria tuberosa* and WPC+ *Pueraria tuberosa* lane as compared to sodium caseinate and WPC lanes. In pure *Pueraria tuberosa* extract (0.4%) (Lane 6), no band was observed. Gel analysis software Image Aide, measured the height and area under curve (raw volume) of each lane of SDS-PAGE gel represented in Table 4. Results revealed that, the band height and raw volume was increased in sodium caseinate+*Pueraria tuberosa* as compared to sodium caseinate alone. Puerarin is an active component in *Pueraria tuberosa* which could have interacted with micelle of identical positive charged head groups and varying tail length, affinity of micelle being more toward greater chain length. Similarly, there was an increase in the height and raw volume in WPC+ *Pueraria tuberosa* as compared to WPC alone. Xi and Guo (2008) reported that puerarin (methanol extract) can bind with blood serum albumin (BSA) at 20-30°C and decrease binding stability with increased temperature and the presence of Cu^{2+} and Fe^{3+} ions increased the binding constants and the number of binding sites of the puerarin–BSA complex.

Storage study

The control and experiment milk samples stored at 6-8°C were evaluated for pH and acidity every day during storage. pH of control and Pueraria tuberosa extract added milk differed significantly (P>0.05) during the entire period of storage. A significant (P<0.05) decrease in pH of milk added with *Pueraria tuberosa* extract and control was noticed during the entire period of storage (Table 2). The decrease in pH of *Pueraria tuberosa* added milk sample was sharper than the control. The acidity of *Pueraria tuberosa* added milk was increased from 0.15 to 0.25% lactic acid (LA) during 5 days of storage (Table 2). A significant increase in titratable acidity was noticed in control as well as *Pueraria tuberosa* added milk samples during storage. Also, significant difference (P<0.05) was observed in titratable acidity between control and *Pueraria tuberosa* added milk throughout the storage period. After 5 days, both the samples were curdled. The results are in accordance with Petrotos et al. (2012) who observed that the rapid drop of pH of olive fruit polyphenol-milk system during fermentation of milk (during successive period storage). A decrease in pH and increase in titratable acidity with increasing storage period was observed in control as well as milk fortified with flaxseed oil, phytosterols and polydextrose (Nagarajappa and Battula 2017). The bioactive compound of *Pueraria tuberosa* might be responsible for growth of bacteria; hence, more drop in pH of milk during storage.

Conclusions

The addition of *Pueraria tuberosa* to milk resulted in no significant change in the proximate composition but increase in phenol content as compared to control. A decrease in pH and increase in acidity was observed in both samples during storage period. The electrophoretic pattern showed that herb components were interacted with the milk proteins. These interactions could alter the different properties of milk. *Pueraria tuberosa* might be responsible for enhance the health benefits due to release of phenol and faster reduction of pH of milk which possibly provide scope for reduction in production time for the fermented products. Furthermore, there is scope to develop *Pueraria tuberosa* fortified dairy products and also need to verify the beneficial effect of interaction of herb components and milk constituents by using animal/ human studies.

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RESEARCH ARTICLE

Optimisation of the *Shrikhand* incorporated with lemongrass *(Cymbopogon citratus)* distillate

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Abstract: *Shrikhand* is a fermented milk product prepared from Chakka, a finely ground sugar and flavouring ingredient. Fermentation is a process of adding value to raw materials by converting them with microorganisms and enzymes into a variety of products with different nutritional and sensory qualities. The purpose of this study was to standardize the optimum level of lemongrass distillate in the manufacturing of *Shrikhand*, with the major goal of evaluating the product organoleptically, physico-chemically, and microbiologically. *Shrikhand* was prepared with lemongrass distillate replacing sugar with jaggery powder (28%) used as a sweetener in *Shrikhand*. Different levels of lemongrass distillate viz. 0.5%, 1% and 1.5% were tried and 28% jaggery powder was used as a sweetener. *Shrikhand* with 0.5% lemongrass distillate (T_1) showed better sensory attributes as compared to others. The optimized product contained 38.24% carbohydrate, 8.34% protein, 11.90% fat, 0.99% ash, 59.37 % total solids, 40.63%moisture, 0.99% titratable acidity, 94.48% anti-oxidant activity, 0.04% crude fiber, 2.28mg vitamin C and 282.07 kcal Energy. Thus, product acceptability as judged by sensory evaluation, was rated as $T_2>T_1>T_0>T_3$.

Keywords: Chakka, lemongrass distillate, *Shrikhand*, Antioxidant activity, Jaggery

Introduction

Presently, Dairy is the single largest agricultural commodity contributing 5 per cent of the national economy and employing more than 8 crore farmers directly. India is ranked 1st in milk production contributing 23 per cent of global milk production. Milk production in the country has grown at a compound annual growth rate of about 6.2 per cent to reach 209.96 million tons in 2020-21 from 146.31 million tonnes in 2014-15 (Economic Survey 2022). This increase in milk production represents sustained growth in TIDPs (Traditional Indian Dairy Products) to meet the requirement of the growing population. The market for TIDPs is the second-highest after fluid milk, and accounts for 95 % of all the milk-based products consumed (Rasane et al. 2015).The milk and milk products are so valued that the National Institute of Nutrition of India (NIN, 2011), and US Department of Agriculture (USDA 2022), and the Food and Agriculture Organization (FAO 1990), have recommended it in their dietary guidelines. TIDPs are highly valued in society due to their social, economic, religious, medicinal, and cultural significance (Rasane et al. 2015). Currently, there is a lack of reliable data about the exact quantity of milk used for the production of TIPs (Sanyal 2020).

Shrikhand is a semi-solid, sweetish-sour fermented milk product made from Dahi. Whey is drained from Dahi to give Chakka, which is then mixed with sugar, flavour, color, and spices to form a soft homogeneous mass. *Shrikhand* is a popular dessert that is served as part of a festive meal. *Shrikhand* is known for its high nutritive, characteristic flavour, taste, palatable nature and possible therapeutic value. It is very refreshing particularly during summer months. It can be recommended as health food for specific patients suffering from obesity and cardiovascular disease (Swapna and Chavannavar 2013).

Fermented milk products have a long history of use in human nutrition. Protein, vitamins, and minerals are abundant in fermented milk products. Constipation, diarrhea, acidity, gastroenteritis, gingivitis, tumor genesis, hypercholesterolemia,

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and other disorders have been claimed to be mitigated successfully by consumption of fermented milks (Hadjimbei et al. 2022). Different colorants and flavouring agents are commonly used to improve the colour, flavour, and overall acceptability of milk products. Charoli, nutmeg, cardamom, and saffron were once used to enhance the flavour and acceptance of *Shrikhand*. However, multiple attempts have been made to mix various additives into *Shrikhand* in order to accommodate the growing desire in diversifying food products in order to appeal to a wider spectrum of consumers.

Lemongrass (*Cymbopogon citratus*) is an aromatic perennial tall grass with rhizomes and densely tufted fibrous root. It has short underground stems with ringed segments, coarse, green slightly leathery leaves in dense clusters (Figueirinha et al. 2008).

Lemongrass is used to flavour tea and in dishes like kadha, a traditional herbal soup used to treat coughs, colds, and other ailments. It's also high in -ione, which is used to make synthetic vitamin A, and - ionone, which is the key flavouring ingredient. Citral, an aromatic molecule also known as lemonal, is the major chemical component found in lemongrass. It's an antibacterial, which means it may kill or prevent microbes from growing. Citral also contains anti-fungal properties. It also has a positive effect on the body's ability to use Vitamin A.

Lemongrass has high antioxidant capacity and free radical scavenging effect of hydro-alcoholic extract of *Cymbopogon citratus* was established (Rao et al. 2009). Mirghani et al (2012) collected lemongrass leaves from Kuala Lumpur state of Malaysia for evaluating the antioxidant potential of its oil. The results showed that the phenolic concentration in lemongrass oil was 2100.769 mg/l GAE, DPPH scavenging activity for lemongrass stalk was 89.5% and highest degree of inhibitory activity in anti-diabetic tests was found as 89.63%.

Jaggery contains considerable amount of ferrous salts (iron), which are good for health, particularly for those who are anemic or lack iron. Jaggery is very good as cleansing agent. It cleans lungs, stomach, intestines, oesophagus and respiratory tracts. Jaggery helps to prevent asthama, cough, cold, congestion in chest etc. Jaggery is supplied to the workers to prevent them from dust allergies (Shrivastav et al. 2016). Jaggery is far complex than sugar, as it is made up of longer chains of sucrose. Hence, it is digested slower than sugar and releases energy slowly and not spontaneously. Jaggery is generally called as "medicinal sugar" because of its use in Ayurveda as well as its comparison with honey (APEDA 2016). The objective of this research is to standardize the process of production of Shrikhand with different concentrations of lemongrass distillate and keeping jaggery concentration constant, along with evaluation of the physico-chemical, microbiological, and sensory properties of the final product. The final product's cost and shelf life were also evaluated.

Materials and Methods

Raw materials

Milk and other items

The whole fresh and clean standardized fresh milk was procured from Ajmer Saras Dairy plant having 6.0 percent fat and 9 percent SNF. Lemongrass and jaggery powder were procured from local market

Preparation of lemongrass distillate

Lemongrass leaves were freshly collected and rinsed using water to wash away any debris and dust. The leaves were broken up into little pieces and put through a grinder.100 g of the ground leaves added with 1000 ml of distilled water were transferred to 2000 ml round bottom flask and mixed. After the mixture was boiled, the vapours were condensed in a 100 ml conical flask after being collected over an ice bath. Using aluminum foil, the flask was quickly sealed tightly before being placed in the refrigerator. To determine the amount of leaves to be used, the amount of water to be utilized, the amount of distillate to be collected, etc., preliminary trials were conducted. From 100 g of lemongrass leaves, it was found that roughly 35 ml of distillate collection was sufficient to extract the majority of the aromatic components (Sutariya and Rao 2015).

Preparation of control sample

The control sample was prepared according to De (2013) with a slight modification. The milk procured (4-7°C) was heat treated at 85°C for 30 min and used for dahi (yoghurt) preparation.

Preparation of experimental lemongrass *Shrikhand*

The experimental *shrikhand* from dahi (yoghurt) was prepared by the process as shown in Figure 1. The *shrikhand* samples thus prepared were packaged in polystyrene cups and stored at 4-5°C.

Sensory analysis

Optimized *Shrikhand* with lemongrass distillate was evaluated for organoleptic properties by using 9-point Hedonic scale designed and described by Munoz and King (2007).

Microbiological analysis

Yeast and mold count and coli form counts were determined as per procedure laid down in BIS (1981).

Physico-chemical analysis of control and experimental *Shrikhand*

Total solids and moisture was measured using the procedure outlined in AOAC (2005).

The technique, as defined in AOAC (2005) for cheese, was used to determine fat. *Shrikhand's* total nitrogen/protein was evaluated using the Semi Micro Kjeldahl technique (IS: 1479 Part II 1961). The ash content of all the samples was evaluated using the IS: 1479 Part II technique (1961) mentioned for milk. *Shrikhand's* acidity was determined using a method published in IS: 1166 (1986) for condensed milk. The carbohydrate content was determined using the differential technique (AOAC 2005).

The pH was measured using a digital pH meter in accordance with the AOAC guidelines (2005).

Antioxidant activity

The antioxidant activity of control and experimental *Shrikhand* was done using DPPH (*2, 2-diphenyl-1-picrylhydrazyl*) method reported by Chanda and Dave (2009) using stable DPPH. The results were expressed as percent inhibition using the formula.

DPPH inhibition percentage (%*I*) = A0 - AS/A0 × 100

Where, A0 is the absorbance of the control, AS is the absorbance in the presence of the sample.

Crude fiber, vitamin C, Ca, P and energy contents were determined as per AOAC (2005).

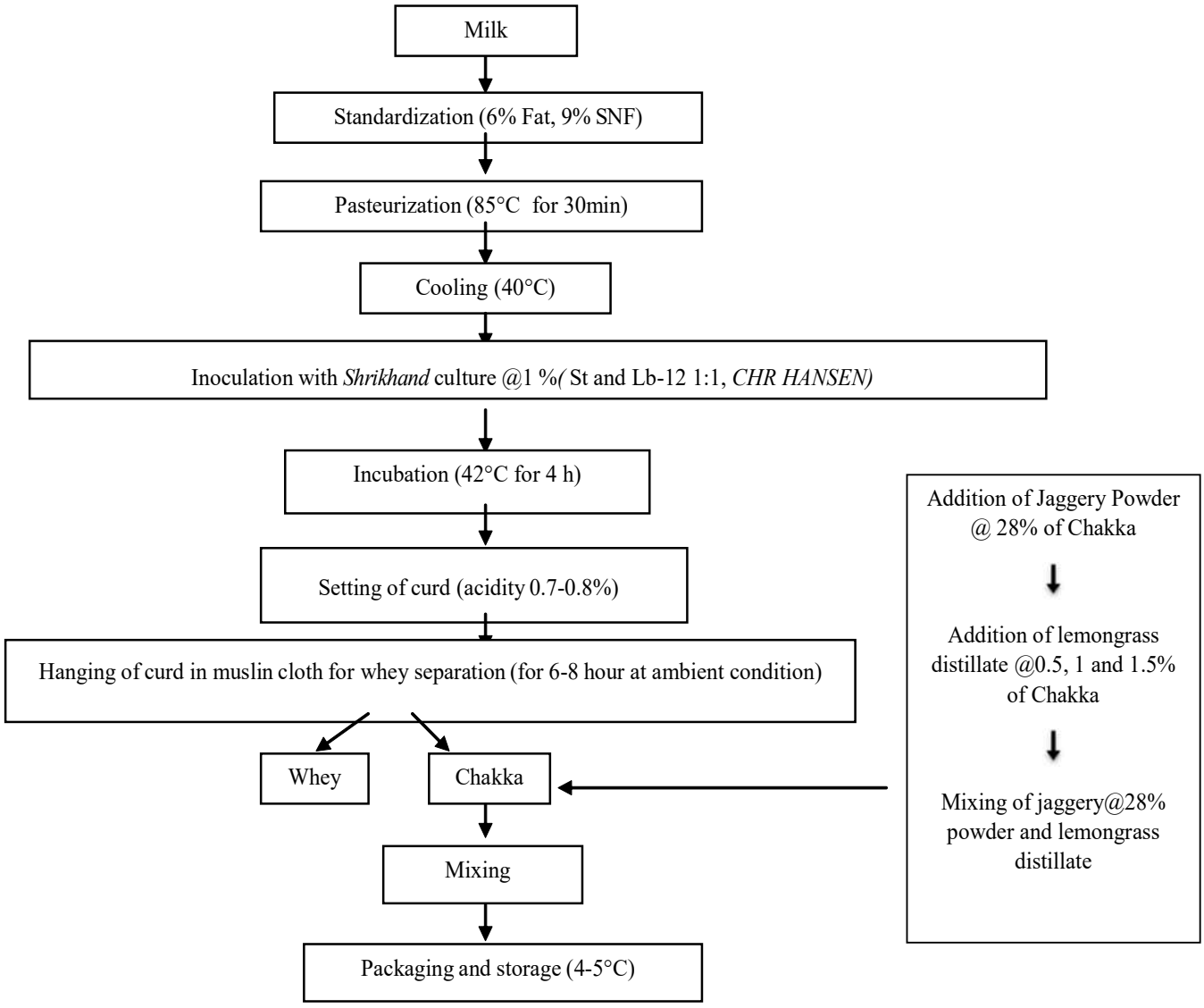

Fig. 1 Process flow chart for the manufacturing of experimental *Shrikhand* (Source: De 2013 with slight modification)

Statistical analysis

WASP software and Excel software were used to analyze the data using Analysis of Variance (ANOVA) at a 5% level of significance and critical difference (C.D).

Cost estimation

The cost estimation of the formulated product was done to compare it with the options available in the market.

Result and Discussion

Optimization of lemongrass distillate

Optimization of experimental *Shrikhand* was based on sensory assessments. Table1 shows the data of sensory analysis of control and experimental *Shrikhand* with different level of lemongrass distillate. It was noticed that the increasing concentration of lemongrass distillate first increased the acceptability of sensory parameters such as flavor, body and texture and overall acceptability and then decreased them. Therefore, *Shrikhand* with low level of lemongrass distillate i.e., 0.5% was most preferred. At 1% and 1.5%, level of lemongrass distillate, the scores though in acceptable range, were less because of acrid smell and astringent flavor of lemongrass distillate. Hence, *Shrikhand* with 0.5% lemongrass distillate and 28% of jaggery powder was acceptable in sensory parameters without causing adverse effect and considered as optimized lemongrass *Shrikhand*. (Sameem et al. 2018) also reported similar trend in dragon fruit pulp *Shrikhand*, where with increased concentration of dragon fruit pulp (0-9%), decreased sensory score are given by the panelists.

Proximate composition of control and experimental samples of *Shrikhand*:

The proximate analysis result of control and experimental *Shrikhand* is shown in Table 1. There were significant differences observed in the carbohydrate content of control and experimental *Shrikhand*. Its value ranged from 39.74% to 37.26% for different treatments. After adding lemongrass distillate in Chakka and keeping the jaggery powder constant, the carbohydrate content from the final product was reduced. Protein of *Shrikhand* samples ranged between 8.15%- 8.80% for different treatments. The fat content ranged between 12.22% - 11.31%. Ash content of the control and experimental *Shrikhand* treatments ranged between 0.95%- 1.10%; ash content represents minerals present in food. Jaggery contains significant amounts of minerals (Jabeen Begum 2023). Total solid percentage ranged between 61.06%-58.27%, the declining trend in control and experimental *Shrikhand* can be attributed to the fact that addition of lemongrass distillate and jaggery powder significantly decreases the total solid percentage as it contains more moisture. There were significant differences observed in the moisture content of control and experimental *Shrikhand* treatments. Its value ranged from 38.97%- 41.73%. The moisture content from the control product increased due to addition of lemongrass distillate. Titratable acidity of control and experimental *Shrikhand* treatments was observed in increasing manner ranging from 0.97%-1.02%. Titratable acidity is inversely proportional to pH. It values ranged from 4.71- 4.58. The difference was significant, indicating significant effect of treatments on pH. The crude fiber percentage of the control was found to be nil, whereas crude fiber content in the different treatments of experimental lemongrass *Shrikhand* was found to be 0.04, 0.05 and 0.08. However, the jaggery could be source of fibre in the experimental samples. The energy value for the experimental and control treatments was 280.92±0.53 to 282.40±0.03kcal/100g. The results regarding the increase and decrease in proximate composition of experimental lemongrass *Shrikhand* and control are similar to the findings of Sameem et al (2018) and Masih et al (2020).

Antioxidant activity

The value represented in Table 1 of antioxidant activity in term of DPPH (% Radical scavenging activity) was found to be 92.95% for the control and 94.48%, 95.44% and 97.24% for experimental treatments respectively. When the result of control *Shrikhand* and experimental *Shrikhand* treatments were compared, increased antioxidant activity percentage of experimental *Shrikhand* treatments was found because of lemongrass distillate and jaggery powder, as they have good free radical scavenging property. Methanol, MeOH/water extracts, infusion and decoction of *Cymbopogon citratus* were shown to have free radical scavenging effects by measuring the bleaching of the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical, scavenging of the superoxide anion and inhibition of the enzyme xanthine oxidase and lipid peroxidation in human erythrocytes (Cheel et al. 2005).

Vitamin C

Table 1 shows the Vitamin C content of control and experimental *Shrikhand* treatments. Vitamin C content in experimental *Shrikhand* treatments was 2.28 mg/100g, 2.46 mg/100g and 2.65 mg/100g which was higher than that of the control sample (1.90 mg/100g). Jaggery added could be the source for vitamin C content. Plant-based foods include a significant amount of polyphenols and flavonoids, which have antioxidant and nutritional benefits. Increase in Vitamin C content relates to the presence of lemongrass distillate.

Calcium

The results shown in Table 1, revealed that the average of calcium value were 107.90mg/100g for control and 108.09, 108.39 and 108.61 for experimental samples (T_1, T_2 and T_3), respectively. The calcium contents of experimental samples could be traced to the added jaggery which contains significant amounts of calcium (Jabeen Begum 2023).

Phosphorus

The phosphorus content in control sample and experimental treatments was 60.88(mg/100g) and 61.24, 61.63 and, 61.90(mg/100g) for T_1, T_2 and T_3, respectively. The nutritional importance of the *Shrikhand* is increased by its higher mineral content. The quality of milk used for the preparation of Chakka may be attributed to the higher content of the mineral. According to Boghra, the Chakka's mineral composition could significantly change as a

Table 1: Physico-chemical, microbial and sensory analysis of control and experimental lemongrass *Shrikhand*

Parameters	Treatments				C.D	F VALUE
	T_0	T_1	T_2	T_3		
Physico-chemical analysis						
Carbohydrate (%)	39.74±0.10	38.24±0.02	37.51±0.05	37.26±0.02	0.09	1337.84
Protein (%)	8.15±0.03	8.34±0.06	8.64±0.06	8.80±0.04	0.09	111.46
Fat (%)	12.22±0.05	11.90±0.10	11.54±0.03	11.31±0.06	0.10	144.28
Ash (%)	0.95±0.01	0.99±0.02	1.06±0.02	1.10±0.02	0.02	67.57
Total Solid (%)	61.06±0.07	59.37±0.02	58.55±0.04	58.27±0.03	0.08	2346.91
Moisture (%)	38.97±0.03	40.63±0.15	41.45±0.08	41.73±0.07	0.15	388.44
Titratable acidity (%) LA#	0.97±0.01	0.99±0.00	1.00±0.01	1.02±0.00	0.02	11.52
pH	4.71±0.01	4.66±0.01	4.64±0.03	4.58±0.03	0.04	22.84
Anti-oxidant activity(%Radical scavengingactivity)	92.95±0.33	94.48±0.20	95.44±0.43	97.24±0.45	0.44	157.96
Crude fiber (%)	0.00±0.00	0.04±0.01	0.05±0.01	0.08±0.01	0.02	37.84
Vitamin C(mg/100g)	1.90±0.02	2.28±0.01	2.46±0.01	2.65±0.02	0.05	1433.04
Energy(kcal/100g)	280.92±0.53	282.07±0.02	282.22±0.03	282.40±0.03	0.40	26.40
Calcium(mg/100g)	107.90±0.02	108.09±0.04	108.39±0.03	108.61±0.03	0.04	657.74
Phosphorus(mg/100g)	60.88±0.03	61.24±0.03	61.63±0.03	61.90±0.02	0.06	979.31
Microbial analysis						
Yeast and mould count (cfu/g)	19.00±0.48	20.60±0.74	21.20±0.80	22.00±0.40	1.17	3.82
Coliform count (cfu/g)	NIL	NIL	NIL	NIL	NIL	
Sensory analysis						
Colour and appearance	7.20±0.40	8.20±0.40	8.60±0.48	8.40±0.48	0.59	10.5455
Flavour	7.60±0.48	8.40±0.48	7.80±0.40	7.00±0.63	0.74	5.71429
Body and texture	8.60±0.17	9.32±0.11	8.70±0.12	8.64±0.10	0.21	25.8502
Overall acceptability	8.42±0.33	8.70±0.21	8.08±0.24	7.70±0.22	0.38	11.9463

- Results are mean of five determinations ± SD (standard deviation)
- #as Lactic Acid. T_0 – Control; , T_1- Shrikhand with 0.5% lemongrass distillate; , T_2- Shrikhand with 1.0% lemongrass distillate; and T_3 - Shrikhand with 1.5% lemongrass distillate

Table 2: Storage related changes in overall acceptability and yeast and mould count of control and experimental lemongrass *Shrikhand*

Treatment	Storage period (Days)					
	Overall acceptability					
	0	3	6	9	12	15
T_0	8.50	8.40	8.40	8.00	7.50	7.00
T_1	8.70	8.50	8.20	8.20	8.00	7.50
T_2	8.08	8.00	8.00	8.00	7.50	7.00
T_3	7.70	7.50	7.50	7.00	7.00	6.00
	YMC					
T_0	19.00	20.00	22.00	25.00	28.00	31.00
T_1	20.60	22.00	24.00	26.00	28.00	30.00
T_2	21.20	22.00	24.00	27.00	29.00	30.00
T_3	22.00	22.00	23.00	25.00	27.00	30.00

Table 3: Cost estimation of control and experimental lemongrass *Shrikhand*

S. No.	Particulars	Cost(Rs/ kg)	T_0		T_1		T_2		T_3	
			Qty	Amt. (Rs)	Qty	Amt. (Rs)	Qty	Amt. (Rs)	Qty	Amt. (Rs)
1.	Milk (g)	56	1000	56	1000	56	1000	56	1000	56
2.	Chakka obtained(g)	-	350	-	350	-	350	-	350	-
3.	Jaggery powder (g)	140	100	14	100	14	100	14	100	14
4.	Lemongrass (1000 g)	200	-	-	2.26	0.45	4.54	0.91	6.75	1.35
5.	*Shrikhand* culture	-	--	1.8	-	1.8	-	1.8	-	1.8
6.	Total productobtained (g)	-	450		452.26		454.54	-	456.75	-
7.	Miscellaneous	-	-	10	-	10	-	10	-	10
8.	Labour charges	-	-	10	-	10	-	10	-	10
9.	Total cost of product obtained (Rs)	-	-	91.8	-	92.25	-	92.71	-	93.15
10.	Total cost, Rs per kg	-	-	204	-	205	-	206.02	-	207
11.	Total cost Rs per 100g	-	-	20.4	-	20.5	-	20.60	-	20.7

result of the fermenting process. The calcium contents of experimental samples could be traced to the added jaggery which contains significant amounts of phosphorus (Jabeen Begum 2023).

Microbial analysis

Yeast and mold count and coliform counts are the basic tests for food safety from a microbiological standpoint. The result of yeast and mold count and coliform counts of control and experimental *Shrikhand* treatments are shown in Table 1. The coliform count was nil because of the properly maintained hygienic conditions during the preparation of the treatments, whereas yeast and mold growth was observed.

Storage study

The overall acceptability scores including sensory parameters of the control and experimental lemongrass *Shrikhand* during the storage at 4-5°C are shown in Table 2. The mean scores of overall acceptability also showed a significantly decreasing trend with increasing storage days for both control as well as treatment samples. Devi et al (2018) added mango pulp (25% on chakka basis) in *Shrikhand* and observed that a significant effect of storage was observed on the entire sensory parameters.

Microbial analysis of control and developed *Shrikhand* during storage

On days 3, 6, 9, 12, and 15 of storage, the amount of yeast and mould increased. The rise in titratable acidity of different treatments may be responsible for the increase in the number of yeast and mould. Increased yeast and mould counts are signs that dairy products are deteriorated as shown in Table 2.

Cost estimation

The calculation for cost estimation was done for 1000g of control and experimental *Shrikhand*. Cost analysis calculation are shown in Table 3. The cost for control and experimental *Shrikhand* treatments (developed product) were 204 (T_0), 205(T_1), 206.02(T_2) and 207(T_3) per 1000g. The cost of developed lemongrass *Shrikhand* was lower as compared to market *Shrikhand* as these costs did not include state taxes, sale commission, etc. The findings of the present cost investigation are similar to Waghmare et al (2019) in ginger powder *Shrikhand*, where cost of most acceptable quality ginger *Shrikhand* (T_2) was Rs.153.1 per kg.

Conclusion

It can be concluded from the results obtained that the *Shrikhand* can be successfully prepared by using milk, jaggery (added @28% of Chakka) and lemongrass distillate. The product prepared was found to have high anti-oxidant activity, energy value and vitamin C content, owing to the jaggery and lemongrass distillate. The developed product can be stored up to 15 days at 5°C. As a result, we can assert that our product not only offers significant health advantages but also is affordable and suitable for consumption by people from all socio-economic groups.

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RESEARCH ARTICLE

Effect on quality of *paneer* using unripe mango powder as a natural coagulant

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Abstract: Paneer, an important traditional dairy product is manufactured by different types of coagulants, however, use of natural coagulants reported are few. In this study, unripe mango powder was used as the natural coagulant in paneer manufacturing. Three variables employed were unripe mango powder (12-16 g/kg), coagulation temperature (70 to 85 °C), and fat content of milk (2.00 to 4.50 %); These variables were studied using Face-centered central composite design in design expert 13.0.5.0. The proximate compositional analysis of the optimized paneer revealed 59.55 ± 0.16 % moisture, 14.68 ± 0.03 % fat, while protein content was 18.94 ± 0.06 %, total carbohydrate content 4.76 ± 0.23 %, ash content 2.07 ± 0.04 % and fiber content 0.5 %. The physicochemical properties studied include titratable acidity (0.43 ± 0.02 % LA), pH (6.0 ± 0.01), water activity (0.98 ± 0.01) and Free fatty acid content (0.16 ± 0.03 %). The rheological characteristics of the developed paneer indicated values for hardness as 7.29 ± 0.60N, springiness 5.83 ± 0.15mm, cohesiveness 0.31 ± 0.01, chewiness of 13.23 ± 1.57Nmm, gumminess of 2.27 ± 0.22N, and adhesiveness of 0.03 ± 0.01Nmm. Fresh paneer sample had APC (Aerobic Plate Count) of 4.72 ± 0.47 $\log_{10}$ cfu/g, while Yeast and Mold count and Coliform count were absent/g. The sensory scores for the optimized paneer were 43.80 ± 0.51 for flavour (out of 50), 30.15 ± 0.77 for body and texture (out of 35), 8.16 ± 0.11 for colour and appearance (out of 10) and 87.11 ± 1.20 for the total sensory score (out of 100). The yield of the optimized paneer obtained was 17 %.

Keywords: Paneer, unripe mango, natural coagulant, coagulation, reconstitution, texture profile

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Introduction

Paneer is the omnipresent traditional Indian dairy product in the country. Acceptable in all households, celebrations without paneer are considered to be incomplete. Paneer consumes 7 per cent of the total milk production of India. Indian paneer market valued [1] 494 billion in 2022. Between 2023- 2028, It is expected that the paneer market will grow at a CAGR of 15.7 per cent or to [1] 1,173 billion (IMARC,2023). FSSAI (2020) has defined and classified Paneer. It consists of a pleasant mild acidic nutty and sweet flavour with a smooth and compact body and texture; white colour with a greenish tinge.

In Mango, a bio active component, Mangiferin serves as potential antioxidant, anti-lipid peroxidant, immunomodulator, cardiotonic, and hypotensive, helps in wound healing and acts as an antidegenerative and anti-diabetic compound. Another compound glucosyl xanthone also acts as a polyphenolic antioxidant. The mango fruit is regarded as energising and refreshing. Mango can be used in the treatment of different types of ailments viz. piles, leucorrhoea, haemorrhage, bronchitis, cough, asthma, hypertension, rheumatism and insomnia. It also functions as an antiseptic, stomachic, laxative, vermifuge, tonic and diuretic (Lauricella et al. 2017).

Mango acidity varies to the content of citric (0.13 to 0.71% fresh weight (FW)) and malic acids, although common organic acids are also found like oxalic, succinic and pyruvic as well as tartaric, muconic, galipic, glucuronic, and galacturonic acids as well as ascorbic acid (Maldonado-Celis et al. 2019). Unripe mango consists of 36.4 mg ascorbic acid, 1.6g dietary fibre, 0.111mg copper and 0.16 mg iron per 100 g , which all lack in the milk so the addition of unripe mango can increase the ascorbic acid, fibre, copper and iron content of milk (USDA, 2019). Thus mango in the form of unripe mango powder, with its medicinal and therapeutic

properties can be a potential coagulant in the manufacture of Paneer.

Materials and Methods

Materials

Raw milk was procured from Anubhav dairy, Anand and Unripe mango (Rajapuri) powder was procured from Aum Agri Foods Pvt. Ltd., Vadodara.

The basic process of unripe mango powder coagulated paneer preparation.

Fresh good quality raw milk was procured from Anubhav Dairy. It was filtered and standardized as per the runs suggested by design software 13.0.5.0. The milk was poured into the S.S. vessel and the heating of milk inside the vessel was carried out by indirect heating. The milk was heated up to 90°C for 10 minutes and then the temperature of the milk was lowered to coagulation temperature. At this stage, 10% of the mango powder solution prepared by rehydration and reconstitution with distilled water was brought to the coagulation temperature and added to milk as a coagulant. After holding the coagulated curd for 4-5 minutes, the whey was separated by straining. Transfer of the coagulum into the S.S. paneer hoops and subjected for pressing at 6 kg/cm^2 for 30 mins. After de-hooping, the paneer block was submerged in pasteurised cold water (2-4°C) for two hours. The yield of paneer was measured and it was packed in multi layer (12µ polyester+ 50µ LD/LLDPE) pouches and stored at 7±1°C.

Experimental Design

The Face-centered central composite design was used for designing the experimental combination. The experiment was designed using the software Design Expert version 13.0.5.0 (Table 4). The independent variables were the rate of addition of unripe mango powder (12 to 16 g), coagulation temperature (70 to 85°C) and fat content of milk (2 to 4.5%). These ranges were entered into Design Expert 13.0.5.0. It suggested treatments involving various combinations or levels of the chosen process parameters and based on these data and the number of the selected process parameters or factors, a total of 20 trial combinations were suggested by the Statistical programme.

Analysis

Compositional analysis

The moisture and fat content of the developed paneer was evaluated as per FSSAI (2022). Total protein content was determined by using the micro Kjeldhal method given by AOAC (1980). The ash content was measured according to Indian Standards (1981).

Physico-chemical analysis

The titratable acidity of unripe mango powder coagulated paneer was analysed by using the method suggested by Ahmed and Bajwa (2019). pH was determined by a digital pH meter by following the process given by Franklin and Sharpe (1963) for cheese. The water activity of the paneer sample was determined using the Rotronic Hygroskop Model: Hygrolab-3 (M/s. Rotronic ag, Switzerland) connected to a sensing element (AW-DIO) with a measuring range of 0-100% relative humidity at 25°C. For the analysis of free fatty acids method given by Deeth et al. (1975) was followed. The tyrosine value of paneer was determined by a method suggested by Lowry et al. (1951).

Texture profile/Rheological analysis

Using a Food Texture Analyzer of Lloyd Instruments LRX Plus material testing machine, England, equipped with a 0-500 kg load cell, five samples from each of the experimental paneer were subjected to uniaxial compression to 40% of the initial sample height. The cross-head speed was 50 mm/min, the trigger was 10 gf, and a 40 per cent force was applied to obtain the force-distance curve for a two-bite deformation cycle. To study the paneer‘s textural attributes samples were held for 1 hour at 23°C and 55% RH.

Sensory Evaluation

More than 8 judges were chosen for the paneer’s organoleptic assessment. Using the 100-point scale outlined in Indian Standards (IS 15346, 2003), the paneer samples were assessed. The judges were also requested to give criticism for each attribute of the samples.

Microbiological analysis

All of the paneer samples were examined using the BIS (Indian Standards 5550, 2005) method with minor modifications for the Aerobic Plate Count (APC), Coliform count, and Yeast and Mold count.

Statistical analysis

The experiment was designed and responses were analysed using software design expert version 13.0.5.0. All the compositional, Physicochemical, Rheological, Sensory evaluation and microbial analyses done for optimized products obtained by the software were repeated seven times. FCCD independent variables were the addition of unripe mango powder (g/kg of milk), temperature of coagulation (°C) and fat content of the milk (%).

Results and Discussion

The effect of the variables on the unripe mango powder coagulated paneer and the responses and actual values observed are reported in Table 3. A quadratic model was fitted for all the responses. R^2 > 0.80 for sensory attributes is statistically adequate for developing a model or equations (Henika,1982). In the present study R^2 was > 0.80 for all sensory responses as well as moisture and yield was observed. The probability value (p) showed the adequacy of the models so used to describe the effect of variables on different responses. The effect of the rate of addition of unripe mango powder, the temperature of coagulation and the fat content of the milk on the responses (Flavour, Body and Texture, Colour and Appearance, Total score, Yield and Moisture) are shown in the equations below and Table 2. The sign and magnitude of coefficients indicate the effect of the variable on the responses. The models thus developed with coded variables are as follows:

Flavour = $42.18 - 0.8129A - 0.0476B - 1.23C + 0.0119AB + 0.3810AC - 0.4048BC - 0.1937A^2 - 1.48B^2 + 0.0838C^2$.

Body and Texture = $29.64 - 0.6899A - 0.8280B - 1.09C - 0.0655AB + 0.2798AC - 0.3274BC - 1.19A^2 + 0.7155B^2 - 0.5617C^2$.

Body and Texture = 29.64- 0.6899A -0.8280B -1.09C -0.0655AB + 0.2798AC -0.3274BC - $1.19A^2$ +$0.7155B^2$ -$0.5617C^2$.

Table 1: Partial Coefficient of Regression Equations of Suggested Model for Sensory Scores, Yield and Moisture of Paneer

	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
Flavour Score	42.18	-0.8130*	-0.0476	-1.2267*	0.0119	0.3810	-0.4048	-0.1937	-1.4844*	0.0838
Body & Texture Score	29.64	-0.6899*	-0.8280*	-1.0892*	-0.0655	0.2798	-0.3274	-1.1892*	0.7155	-0.5619
Colour & Appearance Score	7.78	-0.1054	-0.1071	-0.4977*	0.0223	0.1384	-0.2009	0.0051	-0.4681	-0.2657
Total Score	84.60	-1.6081*	-0.9827	-2.8136*	-0.0313	0.7991	-0.9330	-1.3778	-1.2370	-0.7436
Yield	16.94	-0.5690	0.1290	2.336**	0.5513	0.0613	-0.2862	1.2290	-0.1709	0.3540
Moisture	53.70	-0.782	-0.031	4.658**	0.7175	0.0375	0.0225	3.2027*	0.4516	0.3768

*Significant at 5 per cent level (Pd”0.05), **Significant at 1 per cent level (Pd”0.01),

Note- A, B and C refer to the three factors studied viz. unripe mango powder, coagulation temperature and fat per cent of milk respectively

Table 2: Experimental Design Matrix and Sensory Scores, Yield and Moisture of Paneer

Run	Coagulant (g/kg)	Coagulation Temperature (°C)	Fat (%)	Flavour Score (50)	Body and Texture Score (35)	Colour and Appearance (10)	Total Score* (100)	Yield (%)	Moisture (%)
1	14	70	3.25	40.00	30.47	7.25	82.72	16.80	53.13
2	14	85	3.25	41.43	30.71	7.43	84.57	17.60	54.36
3	16	70	2	40.57	30.00	7.36	82.93	13.70	50.00
4	12	70	4.5	40.57	29.71	7.00	82.29	22.00	63.32
5	14	77.5	2	42.94	29.88	7.58	85.40	16.15	52.00
6	14	77.5	3.25	42.43	29.65	7.71	84.79	18.00	55.96
7	16	70	4.5	39.14	27.71	6.57	78.43	20.00	60.55
8	14	77.5	3.25	41.57	29.69	7.86	84.12	17.60	54.82
9	12	77.5	3.25	42.26	28.88	7.50	83.64	18.30	57.56
10	16	77.5	3.25	41.75	28.50	8.13	83.38	18.90	58.00
11	14	77.5	3.25	42.67	29.83	7.83	85.33	16.15	52.10
12	14	77.5	3.25	41.50	29.69	7.42	83.61	15.90	51.50
13	12	85	4.5	39.00	26.00	6.00	76.00	21.00	62.20
14	16	85	4.5	38.14	25.86	6.14	75.14	20.10	60.99
15	14	77.5	3.25	42.43	29.00	7.86	84.29	16.15	52.30
16	14	77.5	3.25	42.43	29.00	7.86	84.29	16.15	52.00
17	16	85	2	40.67	27.33	7.25	80.25	16.05	51.66
18	14	77.5	4.5	41.63	28.75	7.50	82.88	19.30	59.16
19	12	70	2	43.00	31.00	7.86	86.86	17.05	54.23
20	12	85	2	43.57	30.71	8.14	87.43	16.09	51.71

* Total score also includes package score (5)

Body and Texture = 29.64- 0.6899A -0.8280B -1.09C -0.0655AB + 0.2798AC -0.3274BC - 1.19A^2 +0.7155B^2 -0.5617C^2.

Colour and Appearance = 7.78 -0.154A -0.1071B -0.4976C + 0.0223AB +0.1384AC - 0.2009BC +0.0051A^2 - 0.4681B^2 -0.2657C^2.

Total Score = 84.60 -1.60A -0.9827B -2.81C -0.0312AB +0.7991AC -0.9330BC -1.38A^2- 1.24B^2 -0.7436 C^2.

Yield = 16.94 - 0.5690A + 0.1290B +2.34C +0.5512AB +0.0612AC- 0.2863BC +1.23A^2 -0.1709B^2 +0.3541C^2.

Moisture = 59.04- 0.7820A -0.0310B +4.66C +0.7175AB +0.0375AC +0.0225BC + 3.20A^2 – 0.8323B^2 + 0.9827C^2.

Sensory scores of developed paneer for flavour varied from 38.14 to 43.57 out of 50, Body and texture varied from 25.86 to 31.00 out of 35, Colour and appearance varied from 6.00 to 8.14 out of 10, Total score varied from 75.15 to 87.43 out of 100. Similarly, Moisture ranges from 50.00 to 63.32 per cent and yield varied from 13.70 to 22.00 % as shown in Table 2.

The Flavour scores (Figure 1) of the paneer was affected significantly (P<0.05) in a negative way by unripe mango powder and fat content linearly and coagulation temperature at quadratic levels (Table 1). The flavour score of paneer ranged from 38.14 to 43.57 (out of 50). The paneer prepared by using unripe mango powder at the rate of 12 g/kg of milk and coagulation temperature at 85p C, having 2% fat in milk was rated the best for its flavour score by the selected panellists. However, paneer prepared by using unripe mango powder at the rate of 16 g/kg of milk at 85p C of coagulation temperature having 4.5% fat in milk was rated the lowest for its flavour score. Joseph and Rao (2019) though in different types of paneer, reported a similar decreasing trend for flavour. They observed that when an increase in the amount of lemongrass oil (in milk) from 0.015 to 0.025% and varying levels of incorporation of crushed extract of the lemongrass in milk from 4 to 6% resulted in a significant decrease in the flavour score of the paneer. Khandagale et al. (2022) prepared herbal paneer using turmeric (0.1%) and black pepper (0.1 to 0.3%) and observed that as the amount of turmeric and black pepper increased the flavour score decreased.

The body and texture of paneer was significantly (P<0.05) negatively impacted by unripe mango powder, coagulation temperature and fat content of milk. Unripe mango powder also had a significant (P<0.05) negative impact at quadratic levels (Table 1) .The body and texture score of paneer ranged from 25.86 to 31.00 (out of 35). The paneer prepared with unripe mango powder at 16 g/kg milk and 85p C coagulation temperature for coagulation of milk and 4.5% fat milk received the lowest body and texture score, whereas paneer prepared using unripe mango powder at the rate of 12 g/kg milk, 70p C coagulation temperature and 2.00 % fat milk was rated the best for body and texture score by the panellists. Yashvantha et al. (2020) reported the addition of lemon rinds in paneer and such additions lead to a significant (P<0.05) negative effect on the body and texture score of the paneer. They also observed that at 75 p C coagulation temperature, paneer had a better body and texture score compared to other coagulation temperatures in the study i.e. at 70p C, 80p C and 85p C.

The colour and appearance score of the paneer as depicted in Table 2, was negatively significantly (P<0.05) affected by the fat content of the milk. The colour & appearance score of paneer ranged from 6.00 to 8.14 (out of 10). Paneer prepared from unripe mango powder at the rate of 12 g/kg milk, 85 p C coagulation temperature and 4.5% fat in milk received the lowest score, whereas paneer prepared by using unripe mango powder at the rate of 12 g/kg milk, 85 p C coagulation temperature for coagulation of milk and 2.00 % fat in milk was rated superior in relation to the colour and appearance score by the panellists. Paul et al. (2018) observed that the addition of herbal extract of mint and ginger in paneer reduced the colour and appearance score significantly compared to the control paneer. As the fat content increased in basil incorporated paneer the score of colour and appearance decreased.

Figure 2 depicts the effect on total score. It got impacted in a significant (P<0.05) negative way by unripe mango powder and the fat content of the milk (Table 1). The total score of paneer ranged from 75.14 to 87.43 (out of 100). The paneer prepared with unripe mango powder at the rate of 16 g/kg milk, 85 p C coagulation temperature and 4.5% fat in milk had the lowest total score, whereas paneer prepared by using unripe mango powder at the rate of 12 g/kg milk, 85 p C coagulation temperature for coagulation of milk and 2.00 % fat in milk was rated the best and recived highest total score by the panellists. A similar negative trend for total score was observed by Yashvantha et al. (2020) for paneer containing lemon rind in lemon-flavoured paneer. Paul et al. (2018) also reported that overall acceptability score decreased in basil paneer when the concentration of basil increased from 1 to 1.5 % and fat per cent increased from 1.5 to 2.5 %.

As indicated in Table 2, yield and Moisture content of paneer was significantly (p<0.05) impacted by the fat content of the milk linearly. Moisture content was affected by unripe mango powder significantly at quadratic levels (Table 1). The values of the yield of paneer ranged from 13.70 to 22.00 %. However Yield values higher than 20.0 % had the moisture content higher than the legal limit. The paneer prepared by using unripe mango powder at the rate of 16 g/kg milk, a coagulation temperature of 70°C and fat per cent of milk 2% had lowest yield. The paneer prepared by using unripe mango powder at the rate of 12 g/kg milk, a coagulation temperature of 70 °C and fat per cent of milk 4.5% had highest value of yield. The moisture content of paneer ranged from 50.00 to 63.32 per cent. The paneer prepared by using unripe mango powder at the rate of 16 g/kg milk, a coagulation temperature of 70°C and 2% fat in milk had lowest moisture. The

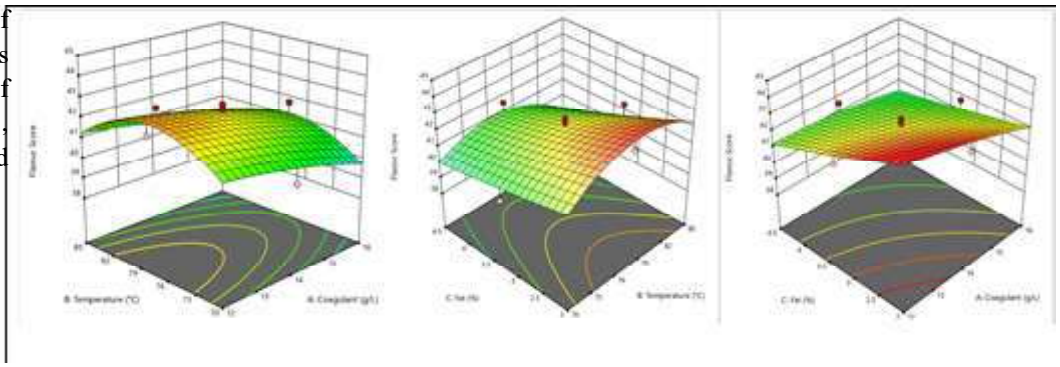

Fig. 1 Response Surface of Flavour Score (Out Of 50) as Influenced by Varying Levels of Unripe Mango Powder (A), Coagulation Temperature (B) and Fat per cent of Milk (C)

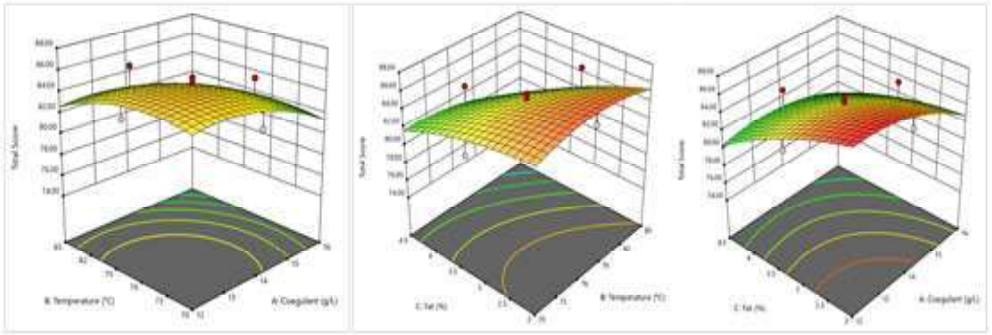

Fig. 2 Response Surface of Total Score (Out Of 100) as Influenced by Varying Levels of Unripe Mango Powder (A), Coagulation Temperature (B) and Fat per cent of Milk (C)

Table 3: Comparison of Predicted v/s Actual Values of Responses Used for Process Optimization for Paneer Manufacture

Response	R^2	Predicted Value *	Actual Value @	Cal. t-Value#
Flavour Score (Out of 50)	0.89	43.98	43.80	0.70
B & T Score (Out of 35)	0.82	30.42	30.15	0.67
C & A Score (Out of 10)	0.80	8.13	8.16	0.56
Total score** (Out of 100)	0.86	87.526	87.50	0.052
Yield %	0.88	17	17.05	1.34
Moisture %	0.90	59.56	59.40	0.94

** Total score includes maximum package score of five
* Predicted values of Design Expert 13.0.5.0 package
@ Actual values are average of five trials for optimised product
# t-values found non-significant at a 5 per cent level of significance
Tabulated t-value = 2.78 (cal. t-value less than tabulated value)

paneer prepared by using unripe mango powder at the rate of 12 g/kg milk, a coagulation temperature 70°C and fat per cent of milk 4.5% had highest moisture content. Thus signifying the negative impact of unripe mango powder and fat content of milk on moisture content of paneer.

Optimization of independent variables

Optimization of the process for the manufacture of paneer using unripe mango powder as a coagulant was carried out to determine the best possible combination(s) of the rate of addition of unripe mango powder, coagulation temperature and per cent fat content of milk, which leads to the most acceptable product in terms of compositional, physicochemical, rheological characteristics, sensory and microbial attributes. The optimum levels as suggested by the software Design expert 13.0.5.0 for the rate of addition of unripe mango powder was 12.70 g/kg of milk, coagulation temperature 76.0°C and fat content of milk was 2.00% with a desirability of 1.00. The predicted and actual response values (obtained after making the product using the optimum level of ingredients) have been presented in Table 3 from which it can be observed that both the values were statistically at par, suggesting the levels of ingredients recommended fits well in the model.

The proximate chemical composition, physicochemical characteristics, rheological values, sensory scores and microbial attributes for the optimized paneer samples are delineated in Table 4. As depicted in Table 4, the developed paneer had 18.94 ± 0.06 % protein and 0.50 % fiber content (calculated value as obtained

from values of mango powder). Titratable acidity was 0.43 ± 0.02 (% LA) and pH was 6.00 ± 0.01. It had water acitivity value of 0.98 ± 0.01, while Free Fatty Acids content (as % Oleic acid) was 0.16 ± 0.03. Rheological characteristics was affected by coagulant (mango powder) and hardness, guminess and chewiness decreased and had values 7.29 ± 0.60 (N), 2.27 ± 0.22 (N) and 13.23 ± 1.57 (Nmm), respectively; while adhesiveness was 0.03 ± 0.01 (Nmm).

Aerobic plate count of the fresh paneer sample was 4.72 ± 0.47 (Log10 cfu/g) and was well within the legal limit as prescribed by FSSA specifications. Yeast and Mold as well as Coliform was absent per g of fresh paneer sample.

Conclusion

It has been observed that all the study parameters including unripe mango powder as coagulant (12.70 g/kg of milk), coagulation temperature (76°C) as well as fat content of the milk (2%) play a significant role in obtaining paneer of comparable quality and acceptability. The optimized paneer had yield of 17.0 % and it had calculated fiber content of 0.5 %. The developed paneer was found to have higher values of vitamin C, iron, copper and fiber content as compared to control paneer and such effect could be due to the presence of vitamin C (48.77 mg/100gm), iron (258.80 mg/kg), copper (5.15 mg/kg) and crude fiber (6.37 %) in unripe mango powder. It can be concluded that the unripe mango powder can be successfully employed as an alternative natural coagulant in the manufacture of paneer.

Table 4. Proximate Chemical Composition, Physicochemical Properties, Rheological, sensory attributed and Microbiological Quality of Paneer Manufactured by Standardized Process

Compositional Attributes	Paneer
Moisture (%)	59.55 ± 0.16
Fat (%)	14.68 ± 0.03
Total protein (%)	18.94 ± 0.06
Total carbohydrate (%)	4.76 ± 0.23
Ash (%)	2.07 ± 0.04
Fiber content (%) (Calculated value)	0.50
Vitamin C (mg/100gm)	48.77
Copper (mg/kg)	5.15
Iron (mg/kg)	258.80
Physico-Chemical Properties	
Titratable Acidity (% LA)	0.43 ± 0.02
pH	6.00 ± 0.01
Water Activity(a_w)	0.98 ± 0.01
Free Fatty Acids (% Oleic acid)	0.16 ± 0.03
Rheological Characteristics	
Hardness (N)	7.29 ± 0.60
Springiness (mm)	5.83 ± 0.15
Cohesiveness	0.31 ± 0.01
Chewiness (Nmm)	13.23 ± 1.57
Gumminess (N)	2.27 ± 0.22
Adhesiveness (Nmm)	0.03 ± 0.01
Sensory Attributes	Score
Flavour Score (out of 50)	43.80 ± 0.51
Body and Texture Score (out of 35)	30.15 ± 0.77
Colour and Appearance Score (out of 10)	8.16 ± 0.11
Package Score (out of 5)	5.00 ± 0.00
Total Score (out of 100)	87.11 ± 1.20
Microbial Count	
Aerobic Plate Count (Log_{10} cfu/g)	4.72 ± 0.47
Yeast and Mold Count (cfu/g)	Absent/g
Coliform Count (cfu/g)	Absent/g

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RESEARCH ARTICLE

Isolation and Characterization of Methicillin Resistant *Staphylococcus aureus* (MRSA) from milk samples

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Abstract: This study reports the isolation and molecular characterization of methicillin-resistant *S. aureus* (MRSA) from sub-clinical mastitic milk samples of cows and does followed by the detection of multi-drug resistance profile of the isolates. The occurrence of MRSA was confirmed phenotypically as well as by molecular detection of the *mecA* gene. A total of 165 suspected mastitic milk samples were collected from cows (116) and does (49) in 2022 in Uttarakhand India. Out of 165 samples, 107 (64.8%) were positive for *S. aureus* (74 from cow and 32 from does samples) and 21 (19.6%) were positive for MRSA out of 107 (15 cow and 6 does samples). All the MRSA isolates were multidrug resistant. However, both MRSA and non-MRSA isolates were most sensitive to Chloramphenicol and complete resistance was observed against eight antibiotics including methicillin. The present study also revealed the resistance of MRSA isolates to antibiotics not used in veterinary medicine earlier. Our findings also highlights the detection of a significant percentage of MRSA in sub-clinical mastitic milk samples for the first time in the state of Uttarakhand, India; growing trends of multidrug resistance due to indiscriminate use of antibiotics and possible diffusion into the human food chain.

Keywords: Antibiotic resistance, sub-clinical mastitis, *mecA* gene, MRSA

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Introduction

Staphylococcus aureus causes several infections in domestic animals but mastitis is the most significant one from dairy economy and public health point of view (Peacock and Paterson 2015). The *S. aureus* infections become even more complicated with the emergence of methicillin resistant *S. aureus* (MRSA). Though, methicillin is no longer used clinically, it represents the resistance against most of the β-lactam antibiotics in addition to some other alternative antimicrobials used commonly in clinical settings (Peacock and Paterson 2015; Begum and Mir 2023). The emergence of MRSA is associated with the acquisition of large mobile genetic element – staphylococcal cassette chromosome *mec* encoding a low-affinity penicillin binding protein having lower affinity for all β-lactam antibiotics which are mostly used in dairy animal mastitis treatment (Mahanti et al. 2020; Girmay et al. 2020; Derib et al. 2017; Srednik et al. 2019). Because of the strain exchange between humans and animals, the epidemiology of MRSA infections is quite complex (Parisi et al. 2016). Initially MRSA was confined to hospital and community settings, but now the human originated strains have adapted to livestock resulting in livestock-associated MRSA (LA-MRSA) (Mahanti et al. 2020; Price et al. 2012). The shedding of organisms in milk without organoleptic changes, predominance of backyard system of cattle rearing involving direct contact with animals, and continuous detection of MRSA in meat and milk (Mahanti et al. 2020; Giovanni et al. 2020; Carfora et al. 2016) have raised serious public and veterinary health concerns. Though, not reported in India, yet there are the reports showing MRSA transmission between humans and animals (Ferreira et al. 2011; Christaine et al. 2015; Smith, 2015).Therefore, there is a requirement of strict monitoring of bacterial populations at the human-animal interface. Owing to the potential zoonotic transmission of MRSA, there is a need of surveillance of antibiotic sensitivity profile, genotypic detection of pathogenic strains, and detection of cross resistance to other antibiotics to devise and adopt new therapeutic approaches (Mistry et al. 2016). The present study was carried out by using the samples collected from the suspected cases of mastitis presented at the veterinary clinical complex of College of Veterinary and Animal Sciences GBPUAT, Pantnagar and around Pantnagar campus. Therefore, this study was conducted to determine the prevalence of *S. aureus* induced mastitis by cultural

and biochemical tests followed by detection of MRSA among the Staphylococcal isolates by antibiotic sensitivity testing and confirmation by detection of *mecA* gene by PCR technique.

Materials and Methods

Sampling

During 2021-22, 165 milk samples of each 10-15 mL volume were collected from 116 cows and 49 goats from Veterinary clinical complex and around Pantnagar campus. All the samples were collected from the suspected cases of mastitis and confirmed by California mastitis test. The milk samples from the affected animals were collected directly in sterile vials from all the teats equally and mixed. Before collection, first few strips of milk were discarded from each teat to prevent the contamination. The milk samples were transported to laboratory by maintaining cold chain and stored at 4 °C till further processing.

Isolation and identification of S. aureus

For isolation and maintenance of *S. aureus*, the method of Missiakas et al. (2013) was used with suitable modifications. The milk samples were inoculated into Brain heart infusion (BHI) broth (HiMedia, India) and incubated at 37 °C for overnight enrichment followed by subsequent inoculation on BHI agar (HiMedia, India). The colonies showing different morphologies were picked up and subjected to Gram staining; and the Gram positive colonies with bunch of grape appearances were further inoculated into selective media – Manitol salt agar (MSA) and Baird parker agar (BPA) for further identification. The pure culture of selected colonies of golden yellow colour on MSA and jet black colour with clear surrounding halo on BPA were further streaked on nutrient agar slant (HiMedia, India), incubated at 37 °C for 18-24 hrs, and stored at 4 °C for further biochemical confirmation. Also, the motility of the isolated bacterial isolates was checked by hanging drop method.

Biochemical confirmation of S. aureus

Pure culture isolates of *S. aureus* were further confirmed by various biochemical tests – catalase test, oxidase test, urease test, nitrate reduction test, indole test, methyl red test, Voges-prausker test, citrate utilization test, and fermentation of different sugars (glucose, sucrose, lactose, maltose, mannitol, mannose, trehalose and xylose) (Holt et al. 1994). The biochemical tests were conducted by using reference strain of *S. aureus* ATCC 25923 (Hi-Media) for confirmation of the species.

Antimicrobial susceptibility testing of S. aureus

Culturally and biochemically confirmed pure isolates of *S. aureus* were subjected to antimicrobial susceptibility testing by the agar disc diffusion method (Bauer et al. 1966) on Mueller-Hinton agar after adjusting the turbidity of the bacterial cultures to 0.5 Mc Farland turbidity standard. *Staphylococcus aureus* (ATCC 6538), standard methicillin-susceptible *Staphylococcus aureus* (MSSA, ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591) were used as reference strain for conducting the test. The isolates were tested against 17 different antibiotics (Hi-Media, India) of standardized concentrations (Table 1).The diameter of zone of inhibition around each antibiotic disc were measured to determine the sensitivity of the *S. aureus* isolates against each antibiotic following CLSI guidelines.

Molecular identification and confirmation of methicillin resistance in S. aureus

A loopful of *S. aureus* colonies from the nutrient agar slant were inoculated into BHI broth and incubated at 37°C overnight. The DNA extraction of *S. aureus* was done as per Hassanzadeh et al. (2016) with suitable modifications. The bacterial broth cultures were subjected to centrifugation at 10,000 rpm for 5 minutes and the pellets obtained were re-suspended in 500 µl of sterile phosphate buffer saline (PBS)solution (pH 7.4). The bacterial pellets were washed thrice with sterile PBS (pH 7.4) solution by centrifugation and finally re-suspended in 400 µl of nuclease free water (NFW). The suspension was boiled in a water bath for 10 minutes followed by snap chilling on ice for 10 minutes and centrifugation at 8,000 rpm for 10 minutes. The supernatant was collected in a sterile eppendorf tube and used as template for PCR reaction. Purity of the extracted DNA of each sample was measured by Nano Drop spectrophotometer (Thermo scientific) at OD 260/280.

The determinant of methicillin resistance in *S. aureus* is *mecA* gene which codes for Penicillin binding protein 2 (PBP-2). For the detection of *mecA* gene in the *S. aureus* isolates, the forward and reverse oligonucleotide primer sequences used in this study were 5'ACTGCTATCCACCCTCAAAC3' and 5'CTGGTGAAGTTGTAATCTGG3', respectively, with the product size of 169 bp (Oliveira and de Lencastre, 2002). The reaction mixture used in PCR for the detection of *mecA* gene was prepared by using PCR master mix (Thermo Scientific) following the manufacturer's instructions. Final reaction mixture of 25µl was prepared by taking 12.5 µl of 2x PCR master mix (Thermo Scientific), 1 µl each of forward and reverse primers (10 µM), 5 µl of template DNA, and 5.5 µl of NFW.

Amplification parameters as described for *mecA* PCR were used with suitable modifications (Arakere et al. 2005). The amplification cycle consisted of denaturation at 95 °C for 5 minutes, followed by 40 cycles of initial denaturation at 94°C for 30 seconds, annealing at 57 °C for 1 min and extension at 72ºC for 1 minute, followed by final extension at 72ºC for 7 min and holding at 4°C. The confirmation of PCR product was done by electrophoresis of amplified products in 1.2 % agarose gel using 1x Tris acetate-EDTA (TAE) buffer in horizontal electrophoresis unit. For each sample 10 µl PCR product was mixed with 2 µl gel loading dye

blue (6x) (Biolabs, #B7021S) and 5 µl of each mixed product were loaded in different wells. A positive (ATCC 25923) and negative control PCR product were also loaded in between the wells of samples. 5 µl of GeneRuler 100 bp plus DNA ladder (Thermo Scientific, SM0323) was also loaded in ladder lane.

Results and Discussion

Cultural and staining characteristics of bacterial isolates

Gram-staining of the bacterial colonies from culture media revealed the presence of Gram positive round shaped cocci, arranged in clusters or bunch of grape appearance. Motility test by hanging drop method revealed that the organisms are non-motile. On MSA, yellow colonies with yellow zones were observed after overnight incubation at 37°C due to the fermentation of mannitol and production of an acidic byproduct that causes the phenol red in the agar to turn yellow (Fig 1a). On BPA, black colonies surrounded by opaque halos due to tellurite reduction and lecithinase break down of the egg yolk in the media (Fig 1b) were observed. Similarly, on triple sugar iron agar both slant and butt of media were completely yellow coloured because of the drop in pH due to the fermentation byproducts of glucose, lactose, and sucrose in the medium. There was no blackening of the media as well as no gas produc-tion. All these characteristics features were indicative of *S. aureus*. However, among 165 confirmed cases of mastitis; 75 cases of cows and 32 cases of goats (107 = 64.8%) were positive for *S. aureus*. The remaining mastitis cases were of non-staphylococcal origin.

Biochemical profile of bacterial isolates

All the 107 pure culture isolates of *S. aureus* identified by Gram staining were positive for catalase, urease, nitrate, methyl red, voges-prausker, and citrate utilization tests while indole test and oxidase test were negative for all the isolates. The total 107 isolates fermented glucose, sucrose, maltose, lactose, mannitol, mannose, sucrose, and trehalose; but not xylose which confirm the presence of *S. aureus* microorganism.

Antibiogram of S. aureus

Among the 17 antibiotics tested, *S. aureus* was most sensitive to chloramphenicol followed by nitrofurantoin, vancomycin, and gentamicin (Fig 2; Table 1). Intermediate sensitivity was shown against gentamicin, vancomycin, tetracycline, enrofloxacin, bacitracin, penicillin G, and ciprofloxacin in the decreasing order. However, complete resistance was shown by *S. aureus* isolates against methicillin, trimethoprim, oxacillin, erythromycin, ceftriaxone, amoxycillin, azithromycin, and ampicillin.

Confirmation of MRSA by detection of *mecA* gene

Out of 107 confirmed pure isolates of *S. aureus*, 21 (19.6%) were positive for *mecA* gene expression with a product size of 169 kb (Fig 3, Table 2) (15 cow sample and 6 goat sample isolates) which confirms the presence of MRSA in mastitis cases of the study area. The overall prevalence of MRSA observed in this study was 12.7%. All the 21 confirmed MRSA isolates were resistant to at least four antibiotics tested and all the isolates showed resistance against the methicillin and oxacillin. This confirms the prevalence of multi-drug resistant MRSA in the mastitic animals of the study area and strong concurrence was observed between phenotypic and genotypic resistance.

Wide range of *Staphylococcus* species have been found to be associated with mastitis in dairy animals in addition to *S. aureus*

Table 1: Antibiotic sensitivity testing

S. No.	Antibiotics	Concentration/disc	Zone of inhibition (mm)*	Susceptibility
1.	Chloramphenicol	30 mcg	25 mm	S
2.	Vancomycin	10 mcg	17 mm	I
3.	Tetracycline	10 mcg	16 mm	I
4.	Penicillin G	10 units	13 mm	I
5.	Methicillin	30 mcg	-	R
6.	Bacitracin	10 units	15 mm	I
7.	Trimethoprim	30 mcg	-	R
8.	Gentamicin	10 mcg	17 mm	S
9.	Oxacillin	5mcg	-	R
10.	Erythromycin	15 mcg	-	R
11.	Ceftriaxone	10mcg	-	R
12.	Amoxycillin	30 mcg	-	R
13.	Azithromycin	15 mcg	-	R
14.	Nitrofurantoin	100 mcg	20 mm	S
15.	Ciprofloxacin	10 mcg	10 mm	I
16.	Ampicillin	25 mcg	-	R
17.	Enrofloxacin	10 mcg	16 mm	I

S: Susceptible, I: Intermediate, R: Resistant

* Represents mean values of samples against each antibiotics

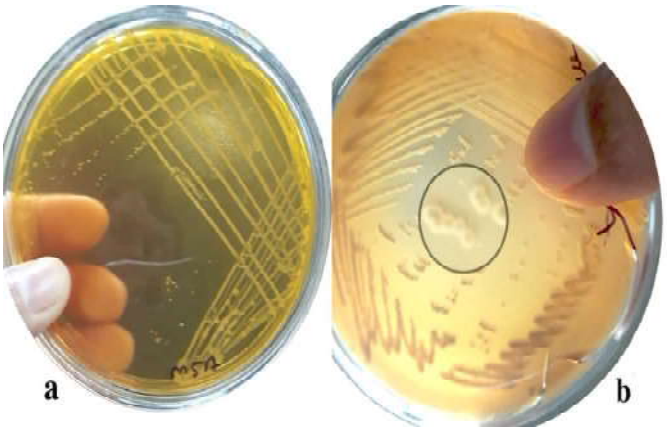

Fig. 1 a) Yellow colonies of *S. aureus* on MSA
b) Black colonies of *S. aureus* surrounded by opaque halos

Fig 2: Antibiotic sensitivity of *S. aureus* against different antibiotics

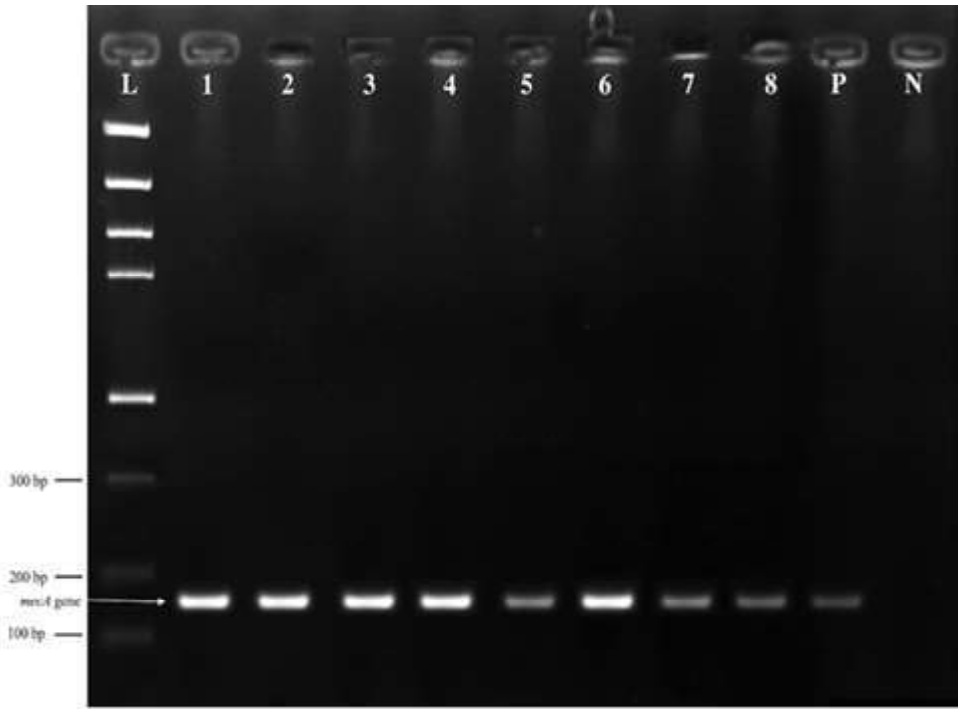

Fig. 3 Gel electrophoresis analysis of PCR amplification product showing presence of *mecA*gene (169 bp) in all the phenotypically identified MRSA isolates

L: Ladder(GeneRuler 100 bp plus DNA ladder)

1-8: MRSA isolates(All cow isolates)

P: Positive control(ATCC 25923)

N: Negative control

and have been reported to carry the genetic determinants of methicillin resistance (Shrivastava et al. 2017; Febler et al. 2010). On the other hand, various researcher have reported the occurrence of MRSA strains sensitive to oxacillin (Mistry et al. 2016; Pu et al. 2014; Pournaras et al. 2013; Kumar et al. 2013) which has put a question mark on the reliability of phenotypic tests in the detection of MRSA in food animals. Therefore, accurate identification of *Staphylococcus* species carrying methicillin resistance determinant by employing phenotypic tests as well as by molecular assays simultaneously cannot be overlooked.

In the present study, isolates *S. aureus* were observed as Gram-positive, round shaped cocci, arranged in clusters or bunch of grape appearance with yellowish colonies on MSA, black colonies surrounded by opaque halos on BPA, and there was production of acidic butt as well as slant in triple sugar iron agar. All the pure culture isolates obtained in this study revealed typical biochemical characteristics of *S. aureus*. Similar staining, cultural, and biochemical characteristics of *S. aureus* were also reported earlier (Habib et al. 2015; Konuku et al. 2012). This study revealed very high prevalence (64.8%) of *S. aureus* in mastitic milk of cattle and goat. However, owing to different animal

Table 2: MRSA prevalence in milk

Species	No. of samples collected	No. of samples positive for *S. aureus*	No. of samples positive for mecA gene	Over all prevalence of MRSA
Goat milk	49	32 (65.30%)	15 (20%)	21/165x100
Cow milk	116	75(64.65%)	6 (18.75%)	= 12.70%
Total	165	107 (64.8%)	21 (19.6%)	

husbandry practices, animal health delivery systems, sample collection procedures, study areas, and bacterial isolation techniques, the prevalence of *S. aureus* in milk and milk products vary widely from 7.0% to 84.0% (Mahanti et al. 2020; Girmay et al. 2020; Giovanni et al. 2020; Ananya and Pranab, 2015). In India, a detailed survey of the existing literature on mastitis infection in dairy animals from northern and north-eastern regions revealed very high prevalence of *S. aureus* (47.86% – 83.72%) which is comparatively higher than other Asian countries.

The worrisome aspect of mastitis infection in India is the much higher prevalence of *S. aureus* and thus, the consequent possibility of widespread MRSA in animals as well as humans. Though, there is no evidence of MRSA anthropozoonosis in India, the probability cannot be ruled out because the zoonotic transmission of MRSA between farm animals and humans have been reported outside India (Ferreira et al, 2011; Christaine et al. 2015; Smith, 2015; Pillai and Reji 2023). In this study, 19.6% of *S. aureus* were positive of *mecA* gene and the prevalence of MRSA in sub-clinical mastitic milk was 12.7%. These findings are alarming in highlighting the dangers of collecting milk from diseased animals as these *mecA* possessing *S. aureus* may enter the human food chain. In India, the prevalence of MRSA in dairy animals based on the molecular detection of genetic determinants of methicillin resistance fall in a wide range of 5.4% to 29.41% (Mahanti et al. 2020; Shrivastava et al. 2017; Kumar et al. 2010; Chandrasekaran et al. 2014; Kutar et al. 2015). The variation in the prevalence of *mecA* gene may be as a result of differential selection pressure imposed by the differences in the preference of antibiotic use in different places. In Turkey, MRSA prevalence of 9% was reported from buffalo milk and milk products by detection of *mecA* gene determinant (Saka and Terzi Gulel, 2018) and in Italy 4% dairy farms and 3% bulk tank milk samples tested positive for MRSA (Giovanni et al. 2020).

In this study, all the isolates of *S. aureus* were most sensitive to chloramphenicol followed by nitrofurantoin, vancomycin, and gentamicin. Complete resistance was observed against methicillin, trimethoprim, oxacillin, erythromycin, ceftriaxone, amoxycillin, azithromycin, and ampicillin. All the phenotypically identified MRSA possessed *mecA* gene determinant and this concurrence between phenotypic and genotypic detection was reported by other researchers too (Mahanti et al. 2020; Karmakar et al. 2016; Bhattacharyya et al. 2016). Even resistance was shown against the antibiotics which are not used in treatment of dairy animals, such as azithromycin, which indicates the environmental transfer of resistance genes from human to animal settings. In fact, resistance against the antimicrobials can be found which are never used in animal settings (Gebreyes et al. 2006; Mollenkopf et al. 2014) and similarly a tobramycin resistant MRSA was detected in buffalo milk, an antibiotic which is rarely used in veterinary medicine (Giovanni et al. 2020). However, all the *S. aureus* isolates were completely susceptible to chloramphenicol and nitrofurantoin because they are used rarely in animals and similar sensitivity pattern was observed earlier (Mahanti et al. 2020). Furthermore, the most worrisome finding of this study was that, all the MRSA isolates were multidrug resistant with resistance to at least four antibiotics tested. This multidrug resistance have been reported earlier (Mahanti et al. 2020; Girmay et al. 2020; Carfora et al. 2016; Bhattacharyya et al. 2016) and is emerging at a much rapid rate now.

Conclusion

This study revealed high prevalence of *S. aureus* as a major cause of sub-clinical mastitis in dairy animals and detection of MRSA isolates for the first time in the state, Uttarakhand, India. Though, chloramphenicol and nitrofurantoin were most effective antibiotics, all the MRSA isolates were multidrug resistant. Detection of multidrug resistant MRSA in milk samples represents a serious food safety and public health concern because of the possibility of diffusion of methicillin resistance genes into human health settings via adulterations in food chain. This study also points out the potential transfer of resistance genes from humans to animals because antibiotic resistance of *S. aureus* isolates against the antibiotics not used in veterinary medicine were observed and thus, warrants the judicious use of antimicrobials in animal and human health systems, simultaneously.

Conflict of interest

The authors declare no conflict of interest of any kind arising out of this manuscript

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RESEARCH ARTICLE

Effect of non-genetic factors on linear type traits score in Sahiwal cattle

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Abstract: The aim of the present investigation was to determine if environmental or non-genetic variables may affect linear type scores in Sahiwal cows. For this purpose, 246 observations were obtained from 23 linear type traits on 150 Sahiwal cattle from 2019 to 2021. Data was analyzed by the least squares technique to examine the effect of non-genetic factors on conformation traits. All the data was analyzed by using univariate analysis of variance in the GLM of SPSS. In this study, the season had no statistically significant impact on linear type traits. Parity had a significant ($p<0.05$) effect on chest width, body depth, rump width, fore udder attachment, udder depth, rear udder width, central ligament, fore teat length and rear teat length. Stage of lactation had a significant ($p<0.05$) effect on body depth, body condition score, rear udder height and rear udder width. According to the study's findings, the parity, and lactation stages were significant drivers of variance for most features of the linear type. To minimize known animal differences and achieve accurate estimates of the attributes, data must be adjusted for these effects.

Keywords: Linear type traits, Parity, Sahiwal cows, Season of scoring, Stage of lactation

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Introduction

To fulfil the demands of future production and reproduction, the dairy sector is faced with the unique problem of continually enhancing the performance of dairy cows. Selection of the best animals forms the cornerstone of advances (Getu and Misganaw, 2015). By incorporating the research on direct information on herd life and indirect information derived from conformation features, selection accuracy may be improved Miglior et al. (2017). Dairy animals' survival is affected by various genetic and non-genetic factors. Genetic factors include higher milk production, normal cyclicity and calving, accuracy of conception, maintaining adequate body condition and resistant metabolic diseases. Non-genetic factors include housing design, stall size, bedding materials and affordability of heifer replacement. The bodily components of a dairy cow that enable her to produce milk and those features that are either directly or indirectly connected to one another are referred to as linear type traits. Culling decisions Kern et al. (2014), longevity Török et al. (2021), and milk production Campos et al. (2015) are all impacted by these features both directly and indirectly. Cattle breeders can spot genetically predisposed functional and structural weaknesses as well as prospective issues brought on by improper breeding practices by monitoring and measuring specific parts of each animal Kumar et al. (2023). As an indirect selection criterion for herd life, conformation attributes have been applied Vukasinovic et al. (2002). In a nutshell, the linear type trait is an important selection criterion for animal breeding. Linear type attributes depend on non-genetic factors including season, parity, and lactation stage, which might be categorized as elements with observable impacts. The quantifiable results may be useful in developing future cattle development programs Javed et al. (2013). To reduce known environmental variations between animals and reliably estimate breeding values, performance records of the animals in these programs should be corrected for the environmental causes of variation Güler et al. (2018). For dairy cows, conformation documentation is not routine in India. However, there is growing motivation among farmers to widen breeding aims and incorporate additional economically significant traits, particularly conformation aspects. Therefore, the present research was carried out to determine the extent to which non-genetic variables

influence the linear type features in Sahiwal cows at an organized farm in India.

Materials and Methods

Experimental animals and area

Data on linear type traits measured and documented from the herd of all lactating Sahiwal cows maintained at Livestock Research Complex, ICAR-National Dairy Research Institute, Karnal (Haryana), India. This institute is situated at latitude 29° 42 N' and longitude 72° 54' E on the Indo-Gangetic alluvial plains, 250 meters above sea level. The average annual rainfall is 860 mm, most of which falls between June and August, during the monsoon season. There is a 41 to 85 per cent relative humidity range. A subtropical climate creates temperature extremes in winter (2°C) and summer (45°C) in Karnal. The experimental animals were kept in a system of loose housing. The paddock, which was big, open, and brick paved with a drainage in between covered and open area with an acceptable slope for better drainage. For all lactating animals in the herd, the feeding management practices and feed components (ICAR 2013) were the same. To give the lactating animals the nutrients they needed, finite amount of concentrates and ad libitum green fodders were offered.

Collection and classification of data

In this study, 246 observations of 150 Sahiwal (*Bos indicus*) cows were gathered. Measurements on type traits were recorded one to two hours before the evening milking at three stages of lactation (20-50 days, 90-130 days and 180-200 days). Body measurements were recorded as per the recommendations made by International Committee for Animal Recording by three classifier (ICAR, 2018). Twenty-three linear type traits were scored on a scale of 1-9 scoring system. These 23 linear type traits included 7 body measurements traits, 6 udder measurements traits, 4 teat measurements traits and 6 visual observation linear type traits generated for each animal. All the measurements were recorded for each animal when standing evenly on her feet and for the same person to avoid the between-scorer effect. Measurements on type traits were recorded one to two hours before the evening milking at three stages of lactation (20-50 days, 90-130 days, and 180-200 days). The non-genetic factors incorporated were the effect of parity (1^{st}, 2^{nd}, 3^{rd}, $4^{th,}$ and 5^{th}), stage of lactation (1^{st}, $2^{nd,}$ and 3^{rd}) and season viz., summer (March-June), rainy (July-September), autumn (October-November), winter (December-February).

Statistical analysis

Least squares analysis of variance for unequal and non-orthogonal data was done to study the effect of non-genetic factors. All the data were analyzed by using univariate analysis of variance in the GLM of SPSS. The model was used under the presumption that all of the factors that were put into it are linear, independent, and additive. The following is the analytical model for the influence of non-genetic factors on linear-type traits:

$$Y_{ijkl} = \mu + L_i + P_j + S_k + e_{ijkl}$$

Where,

Y_{ijkl} = 1^{th} observation of cow in i^{th} stage of lactation, j^{th} parity and k^{th} season at scoring

μ = Overall mean

L_i = Fixed effect of i^{th} stage of lactation (i= 1 to 3)

P_j = Fixed effect of j^{th} parity (j= 1 to 5)

S_k = Effect of k^{th} season at scoring

e_{ijkl} = Random error NID (0, $\acute{o}^2_e$)

Results and discussion

Tables 1-4 are showing the least squares means and associated standard errors for the linear type traits of Sahiwal cows for various seasons, parities, and lactation stages.

Effect of season of scoring

The season had no statistically significant impact on the linear type traits in the current study (Tables 1 to 4). The results of prior research by Kumar et al. (2023) and Sharma et al. (2022), which revealed that the season had no significant impact on teat-type features, are in agreement with this study. The observed season had no significant impact on the udder and teat-type features in Sahiwal cows, according to Togla et al. (2021). In contrast, Erdem et al. (2017) discovered that the season had a significant ($p<0.05$) impact on the udder cleft, dairy form (angularity), body depth, rump angle, rump width, teat length, teat placement, udder depth, and rear udder height, whereas the effect of season on fore udder attachment was highly significant ($p<0.01$).

Effect of parity

Parity had a significant ($p<0.05$) effect on chest width, body depth, rump width, fore udder attachment, udder depth, rear udder width, central ligament, fore teat length and rear teat length in Sahiwal cows has been presented in Table 1 to 4. Up to the fourth parity, chest width and body depth significantly increased, but after that, they started to decline until the fifth parity. Wider-chested cows with high scores are often preferred because they provide the heart and lungs more room to operate properly. Greater body capacity is related to a bigger digestive system, which is associated with ingesting more feed and fodder for producing more milk (Roy et al. 2020). These results are in agreement with

Erdem et al. (2017) who revealed that parity had a significant ($p<0.05$) influence on body depth in Sahiwal cattle. Similarly, according to Yanar et al. (2018), the influence of parity was a significant ($p<0.01$) source of variation for chest width, but not for body depth ($p<0.05$), however, Güler et al. (2020) reported the effect of parity had significant ($p<0.01$) source of variation for chest width and body depth. Up to the third parity, the rump width increased, but for the fourth and fifth parities, it decreased. Similarly, Marinov et al. (2015); Erdem et al. (2017) and Güler et al.

Table: 1 Least squares mean of average score points (ASP) for linear type traits (ST, CW, BD, RW, RA and ANG) under 1- 9 score system

Effects	Stature (ST)	Chest width (CW)	Body depth (BD)	Rump width (RW)	Rump angle (RA)	Angularity (ANG)
Overall (246)	5.82±0.10	4.69±0.11	5.31±0.11	5.51±0.11	4.42±0.10	4.82±0.11
Season of scoring						
Winter (63)	6.01±0.20	4.76±0.21	5.19±0.21	5.50±0.21	4.25±0.19	5.08±0.21
Summer (33)	5.81±0.27	4.77±0.28	5.35±0.28	5.17±0.28	4.42±0.26	5.19±0.28
Rainy (94)	5.68±0.16	4.49±0.16	5.19±0.17	5.52±0.17	4.64±0.15	4.52±0.17
Autumn (56)	5.81±0.22	4.76±0.22	5.51±0.23	5.83±0.22	4.36±0.20	4.52±0.22
Parity						
P1 (45)	5.66±0.26	3.87^{a}±0.26	4.26^{a}±0.27	4.63^{a}±0.27	4.29±0.24	4.69±0.26
P2 (71)	5.71±0.18	4.44^{ab}±0.19	5.30^{ab}±0.19	5.49^{b}±0.19	4.48±0.17	4.65±0.19
P3 (53)	5.84±0.23	5.18^{bc}±0.23	5.69^{b}±0.24	5.95^{b}±0.24	4.64±0.22	4.37±0.24
P4 (45)	5.87±0.23	5.29^{c}±0.23	6.06^{b}±0.24	5.85^{b}±0.23	4.41±0.21	4.95±0.23
P5 (32)	6.06±0.28	4.80^{bc}±0.29	5.31^{b}±0.30	5.78^{b}±0.29	4.33±0.27	5.35±0.29
Stage of lactation						
L1 (95)	6.02±0.16	4.72±0.16	4.90^{a}±0.17	5.42±0.17	4.26±0.15	4.85±0.16
L2 (76)	5.79±0.19	4.84±0.20	5.25^{a}±0.20	5.55±0.20	4.61±0.18	4.79±0.20
L3 (75)	5.66±0.20	4.49±0.20	5.79^{b}±0.21	5.58±0.21	4.40±0.19	4.80±0.20

Means with different superscripts (a,b,c) indicates significant difference ($p<0.05$) in the same column

Table: 2 Least squares mean of average score points (ASP) for linear type traits (RLRV, RLS, FA, BCS, LM and FUA) under 1- 9 score system

Effects	Rear legs rear view (RLRV)	Rear leg set (RLS)	Foot angle (FA)	Body condition score (BCS)	Locomotion (LM)	Fore udder attachment (FUA)
Overall (246)	5.08±0.07	4.67±0.13	4.91±0.12	6.33±0.10	4.84±0.08	4.71±0.13
Season of scoring						
Winter (63)	5.31±0.13	5.01±0.26	4.74±0.23	6.60±0.19	5.05±0.14	4.50±0.26
Summer (33)	4.97±0.18	4.50±0.35	5.09±0.31	6.33±0.25	4.67±0.19	4.70±0.34
Rainy (94)	5.02±0.11	4.53±0.21	4.72±0.18	6.12±0.15	4.89±0.11	5.21±0.20
Autumn (56)	5.03±0.14	4.65±0.28	5.11±0.24	6.27±0.20	4.74±0.15	4.40±0.27
Parity						
P1 (45)	5.43±0.17	4.29±0.33	4.90±0.29	6.40±0.23	4.92±0.18	5.24^{b}±0.32
P2 (71)	4.91±0.12	5.07±0.24	4.53±0.21	6.33±0.16	4.97±0.13	5.05^{b}±0.23
P3 (53)	4.96±0.15	4.91±0.30	5.02±0.26	6.59±0.20	4.62±0.16	4.86^{b}±0.29
P4 (45)	5.07±0.15	4.83±0.29	4.86±0.25	6.35±0.20	4.91±0.16	4.50^{ab}±0.28
P5 (32)	5.02±0.19	4.30±0.37	5.30±0.32	6.06±0.25	4.72±0.20	3.86^{a}±0.36
Stage of lactation						
L1 (95)	5.01±0.10	4.74±0.21	4.97±0.18	6.00^{a}±0.14	4.95±0.11	4.55±0.20
L2 (76)	5.29±0.13	4.67±0.25	4.68±0.22	6.25^{ab}±0.17	4.91±0.13	4.69±0.24
L3 (75)	4.94±0.13	4.61±0.26	5.09±0.22	6.75^{b}±0.18	4.65±0.14	4.89±0.25

Means with different superscripts (a,b,c) indicates significant difference ($p<0.05$) in the same column

(2020) reported the effect of parity had a significant ($p<0.01$) source of variation for rump width. The body growth of an animal with increasing age and subsequent parturition might be a reason for increasing the distance between two pin bones. A higher mean score for fore udder attachment indicated that strong attachment of the udder to the ventral body wall, whereas loose attachment of the udder to the ventral body wall is indicated by a lower mean score of fore udder attachment. Fore udder attachment was stronger in 1st parity and then decreased with the advancement of parity. These findings support earlier research studies like Petkov and Stoyanova (2006) also reported a significant impact of parity on fore udder attachment in Black and White cows. Up to the sixth parity, there was a rise in the linear score for rear udder width. The results of rear udder width for parity effect are consistent with Sharma et al. (2022), who found that parity had a significant ($p<0.05$) impact on Sahiwal cows' rear udder width. Because of the udder's growth starting with the first lactation and continuing afterwards, the parity discrepancies were validated (Pawar et al. 2012). This was also the time when the production of cows attained a peak around 6th parity, when an animal was 8- to 9 years old and gained adult size. From the first calving onward, the value of the udder depth linear score was steadily dropping. The results of the present study were consistent with Sharma et al. (2022), who showed that parity had a significant ($p<0.05$) impact on udder depth in KF and Sahiwal cattle. The effect of parity on the central ligament was found to be significant ($p<0.05$). These findings support earlier research by Togla et al. (2021), which revealed that parity had a significant ($p<0.05$) impact on udder cleft in Sahiwal cows. Parity was found to significant ($p<0.05$) effect on fore teat length and rear teat length. This trait showed an increasing trend with the advancement of parity. These findings are consistent with previous research from several workers who indicated that foret teat length in Sahiwal cows was significantly ($p<0.05$) affected by parity (Kumari et al. 2022; Sharma et al. 2022). In another study, fore and rear teat length in multiparous cows with more than three parities increased by 0.55 and 0.44 cm, or 9.61 and 9.32%, compared to primiparous cows (Kuczaj, 2003). Age-related increases in teat length may be explained by the negative effects of machine milking. Stature, rump angle, angularity, rear leg rear view, rear legs set, foot angle, BCS, and locomotion were the only variables for which parity was not a significant source of variation ($p<0.05$) and these results concur with the report of Khan and Khan (2015) on Sahiwal cows.

Effect of stage of lactation

As shown in Tables 1 to 4, the stage of lactation had a significant ($p<0.05$) impact on the Sahiwal cow's body depth, body condition score, rear udder height, and rear udder width. In the later phases of lactation, the cows had a deeper body, better body condition, and a shallow udder. These findings are somewhat consistent with those of (Khan and Khan 2015), who found that along with body depth, Sahiwal cows' stature increased from the first to the

Table: 3 Least squares mean of average score points (ASP) for linear type traits (RUH, UD, RUW, CL, FTL and RTL) under 1- 9 score system

Effects	Rear udder height (RUH)	Udder depth (UD)	Rear udder width (RUW)	Central ligament (CL)	Fore teat length (FTL)	Rear teat length (RTL)
Overall (246)	5.55±0.12	5.23±0.08	4.27±0.11	3.97±0.09	3.82±0.12	3.96±0.11
			Season of scoring			
Winter (63)	5.60±0.23	5.33±0.15	4.23±0.21	4.27±0.18	4.17±0.24	4.43±0.21
Summer (33)	5.88±0.30	5.17±0.21	4.41±0.28	3.44±0.24	3.64±0.32	3.82±0.28
Rainy (94)	5.38±0.18	5.34±0.12	4.19±0.16	4.00±0.14	3.53±0.19	3.80±0.16
Autumn (56)	5.36±0.24	5.07±0.16	4.22±0.22	4.11±0.19	3.96±0.25	3.81±0.22
			Parity			
P1 (45)	5.87±0.29	6.58^{c}±0.19	3.86^{a}±0.26	3.24^{a}±0.23	3.18^{a}±0.30	3.53^{a}±0.26
P2 (71)	5.89±0.21	5.34^{b}±0.14	4.14^{ab}±0.19	3.70^{ab}±0.16	3.42^{ab}±0.21	3.64^{ab}±0.19
P3 (53)	5.57±0.26	5.14^{b}±0.17	4.24^{ab}±0.24	4.33^{bc}±0.20	3.79^{abc}±0.26	4.07^{ab}±0.23
P4 (45)	5.14±0.25	4.79^{ab}±0.17	4.45^{ab}±0.23	4.26^{bc}±0.20	4.35^{bc}±0.26	4.48^{b}±0.23
P5 (32)	5.23±0.32	4.20^{a}±0.22	4.67^{b}±0.29	4.42^{c}±0.25	4.62^{c}±0.33	4.14^{ab}±0.29
			Stage of lactation			
L1 (95)	5.92^{b}±0.18	5.20±0.12	4.68^{b}±0.17	3.96±0.14	3.81±0.18	4.15±0.16
L2 (76)	5.52^{a}±0.21	5.32±0.15	4.27^{ab}±0.20	3.90±0.17	3.88±0.22	3.94±0.20
L3 (75)	5.18^{a}±0.22	5.17±0.15	3.82^{a}±0.21	4.04±0.18	3.90±0.23	3.80±0.20

Means with different superscripts (a,b,c) indicates significant difference ($p<0.05$) in the same column

last lactation stage. Body depth was found to differ significantly ($p<0.05$) between different stages of lactation in Sahiwal cows. These results concur with those of Yanar et al. (2018), who found that the lactation stage was a significant ($p<0.05$) source of variation for body depth in Simmental cows. Animals tend to score better from mid to late lactation since the body depth and body size traits are directly related to body weight. These findings are supported by the observation that cows exhibited a negative energy balance during the beginning of lactation, which also happened to be the time of lactation peak Esteves et al. (2004). The effect of the stage of lactation on body condition score was found to be significant ($p<0.05$). These results are in agreement with the previous studies by Marinov et al. (2015) who reported that in Holstein cattle, the mean of BCS was significantly ($p<0.05$) affected by stage of lactation. These results could be due to cows having a lower body condition score during the early lactation stage (negative energy balance). The least square means score for rear udder height were consistent with those of Salam and Zia-ul-Haq (2016) and Togla et al. (2021), who found that the lactation stage had a significant ($p<0.05$) impact on the rear udder height of Sahiwal cows. Several researchers have reported that the stage of lactation had a significant ($p<0.01$) impact on the height of the rear udder in Holstein, Simmental, and Brown Swiss cows (Liu et al. 2014; Erdem et al. 2017; Yanar et al. 2018; Güler et al. 2020). These findings might be a result of the ligaments becoming weaker and the mammary glands contracting as the lactation stage progresses. In this study, the rear udder width decreased steadily from the first to the third phase of lactation. These results concur with those of Mazza et al. (2013), who also reported that rear udder width decreased significantly ($p<0.01$) as lactation progressed in Valdostana cattle. This study found that there was not a significant variation in udder diameter and teat circumference across lactation stages. However, this conclusion conflicts with that of Kumar et al. (2023), who found that the lactation stage was a significant source of variance for udder diameter and teat circumference in Sahiwal cows. Additionally, given the impacts of stages of lactation on udder and teat measurements, we may draw an inference from the current data that the stage of lactation does not necessarily affect all udder and teat measurements.

Table: 4 Least squares mean of average score points (ASP) for linear type traits (TC, TD, UB, FTP and RTP) under 1- 9 score system

Effects	Teat circumference (TC)	Teat diameter (TD)	Udder balance (UB)	Fore teat placement (FTP)	Rear teat placement (RTP)
Overall (246)	4.21±0.13	4.11±0.13	5.24±0.13	4.40±0.09	6.36±0.96
			Season of scoring		
Winter (63)	4.10±0.25	4.22±0.25	4.82±0.25	4.28±0.18	6.38±0.18
Summer (33)	4.03±0.33	3.77±0.33	5.25±0.33	4.34±0.24	6.64±0.24
Rainy (94)	4.24±0.19	4.22±0.19	5.63±0.20	4.51±0.14	6.18±0.14
Autumn (56)	4.45±0.26	4.22±0.26	5.24±0.26	4.46±0.19	6.26±0.19
			Parity		
P1 (45)	4.05±0.31	3.76±0.31	4.96±0.31	4.18±0.23	6.22±0.22
P2 (71)	4.30±0.22	4.04±0.22	5.46±0.22	4.38±0.16	6.29±0.16
P3 (53)	4.31±0.28	4.26±0.28	5.10±0.28	4.39±0.20	6.34±0.20
P4 (45)	4.18±0.27	4.51±0.27	5.20±0.28	4.78±0.20	6.74±0.20
P5 (32)	4.23±0.34	4.03±0.34	5.49±0.35	4.26±0.25	6.33±0.25
			Stage of lactation		
L1 (95)	4.45±0.19	4.35±0.19	5.14±0.20	4.33±0.14	6.62±0.14
L2 (76)	3.96±0.23	3.98±0.23	5.19±0.23	4.44±0.17	6.51±0.17
L3 (75)	4.21±024	4.01±0.24	5.41±0.24	4.43±0.17	6.02±0.17

Means with different superscripts (a,b,c) indicates significant difference ($p<0.05$) in the same column

Conclusion

The result of our study showed a considerable effect of environmental factors on the linear type traits of Sahiwal cattle. While parity and stage of lactation were the main sources of environmental factors affecting the linear type traits. In livestock improvement programs that will be carried out on conformation traits of Sahiwal cattle, the performance record of animals has to be adjusted for the significant environmental source of variation in order to decrease the known environmental difference between animals and to accurately estimate breeding values.

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RESEARCH ARTICLE

Estimation of direct and maternal covariance of production efficiency traits in Murrah buffalo

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Abstract: The study employed data collected from 662 Murrah buffaloes meticulously documented over a 24-year period (1996-2019). The data were sourced from historical pedigree records maintained at the buffalo farm of the Department of Livestock Production and Management (LPM) at Lala Lajpat Rai University of Veterinary and Animal Sciences in Hisar. The average values under univariate animal model for production efficiency traits *viz.* MCI and MSC were 4.84 kg/day and 1.33 kg/day, respectively. Six univariate animal models were utilized to compute (co)variance components and heritabilities for traits related to first lactation production efficiency. Among these models, Model 1 was identified as the most suitable for calculating milk yield per day of calving interval (MCI), while Model 2 proved optimal for milk yield per day of age at second calving (MSC). Maternal effects were observed to influence MSC (ranging from 0.09 to 0.22). The heritability estimates for the production efficiency traits, namely MCI and MSC, were 0.35±0.12 and 0.15±0.09, respectively. The breeding values for production efficiency traits ranged from 0.46 kg/day for MCI to 0.63 kg/day for MSC. A significant and positive genetic correlation between additive and maternal effects was identified, ranging from 0.41 to 0.98. This suggests a consistent and strong interaction between genetic factors inherited from the dam. Rank correlation of breeding values across all six models ranged from 0.71 (non-significant) to 1.00 for MCI and MSC. MCI exhibited positive genetic and phenotypic trends, showing annual increases of 0.009±0.005 kg/day and 0.148±0.018 kg/day, respectively. In contrast, MSC displayed a very low negative genetic trend (-0.001±0.001 kg/day), while a positive phenotypic trend was observed at 0.047±0.006 kg/day per year. These trends indicate that both selection and management practices are concurrently contributing to the improvement of production efficiency traits.

Keywords: Production efficiency traits; Univariate animal model; Maternal effects; Spearman's rank correlation; Genetic trends and phenotypic trends

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Introduction

India holds the top position globally in milk production, boasting the largest buffalo population featuring premier breeds such as Murrah, Nili-Ravi, Bhadawari, and Surti. The Murrah buffalo, particularly, serves as the focal point of the dairy industry and is utilized as an improving breed not only in India but also in several other countries, as indicated by NBAGR (2006). Indigenous and non-descript buffaloes contribute significantly, accounting for 45% of India's total milk production, reaching 89 million tonnes (DAHD, 2022-23). A linear animal model incorporates both direct and maternal genetic effects, featuring covariance between them, along with a maternal permanent environmental effect. Additive maternal effects, as defined by Schutz et al. (1992), encompass any influence from a dam on its offspring, excluding the effects of directly transmitted genes affecting the offspring's performance. Maternal effects comprise additive genetic maternal effects and environmental maternal effects. The maternal environment primarily encompasses factors like the mother's milk yield, mothering ability, and the uterine environment. The maternal genotype influences the phenotypic expression of the offspring through its genotype for maternal effects and its direct additive genes for growth. Exclusion to consider maternal genetic effects in the statistical model results in biased upward heritability estimates and diminished realized efficiency of selection compared to expectations, as demonstrated by Rashidi et al. (2008), Jafaroghliet al. (2010), Eskandarinasabet al. (2010), and Prakash et al. (2012).

Genetic and phenotypic trends indicates the improvement in overall productivity over an entire year or period, depends upon the time taken into account. Annual rate of change in productive and reproductive traits reveal the quantitative appraisal and genetic gain per year. Keeping the environmental trends aside, we can measure the change in performance of the population for

an economic trait per year or period, can estimate the phenotypic and genetic trends. The genetic and phenotypic trends were estimated by regressing breeding value and phenotypic value of the trait on year of the birth of the bull/animal, respectively. Several researchers (Yadav et al. (1983), Sahana and Sadana (1998), Chakraborty et al. (2010), Singh et al. (2011) and Chakraborty and Dhaka (2012) reported genetic and phenotypic trends in various traits in Murrah buffaloes.The present study is conducted to know the impact of additive and maternal influences on production efficiency traits and to determinethe progress through phenotypic and genetic trends is necessary to assess the effectiveness of selection within a population over time.

Materials and Methods

Source of data

The research dataset includes information from 662 Murrah buffaloes meticulously recorded over a span of 24 years (1996-2019). These invaluable data, pertaining to crucial production efficiency traits, were sourced from historical pedigree-sheets maintained at the buffalo farm within the Department of Livestock Production and Management (LPM) at LalaLajpat Rai University of Veterinary and Animal Sciences, Hisar. The research site, Hisar, is characterized by a semi-arid, sub-tropical climate and is geographically situated at 29° 10' N latitude, 75° 40' E longitude, with an altitude of 215.2 meters.

Traits under study

The production efficiency traits under this study included were milk yield per day of calving interval (MCI) and milk yield per day of age at second calving (MSC) of first lactation. Milk yield per day of calving interval was calculated by dividing total lactation milk by calving interval. Milk yield per day of age at second calving was the daily outcome of milk yield up to the second calving. It was calculated by dividing the total lactation milk yield by total age in days up to second calving. Buffaloes with lactation periods shorter than 150 days or yielding less than 500 kg of milk were excluded, as were those flagged as outliers based on abnormal records such as abortion, mastitis, and chronic illnesses. The study's herd maintained a male to female ratio of 1:50.

Statistical analyses

Estimations of (co)variance components and heritabilities for various traits were computed using a set of univariate animal models. The analysis utilized the average information restricted maximum likelihood (AIREML) algorithm implemented with WOMBAT software (Meyer,2007). The primary focus was on first lactation production efficiency traits. To estimate maternal (co)variance components, six distinct models were applied. Each trait underwent a univariate analysis in which variance and (co)variance components were estimated. The models considered or excluded maternal components, providing a comprehensive exploration of the trait characteristics.

Model 1: $y = X\hat{a} + Z_1a + e$

Model 2: $y = X\hat{a} + Z_1a + Z_2m + e$ **with** $Cov(a, m) = 0$

Model 3: $y = X\hat{a} + Z_1a + Z_2m + e$ **with** $Cov(a, m) = A\sigma_{am}$

Model 4: $y = X\hat{a} + Z_1a + Z_3c + e$

Model 5: $y = X\hat{a} + Z_1a + Z_2m + Z_3c + e$ **with** $Cov(a, m) = 0$

Model 6: $y = X\hat{a} + Z_1a + Z_2m + Z_3c + e$ **with** $Cov(a, m) = A\sigma_{am}$

Where, y = n × 1 vector of observations for each trait; X = Incidence matrix that relates data to the unknown vector of fixed effects â; Z_1 = Incidence matrix that relates unknown vector of direct (a) breeding values, to y; Z_2 = Incidence matrix that relates unknown vector of maternal (m) breeding values, to y; Z_3 = Incidence matrix that relates unknown additional random vector of permanent maternal environmental effects (c) to y; e = Unknown vector that contains random residuals due to environmental effects; A = Numerator relationship matrix; $\acute{o}_{am}$ = Covariance between direct and maternal additive genetic effects.

Heritability (h^2) was obtained in all the models; however, maternal heritability (m^2) was estimated in Model 2, 3, 5 and 6. Maternal permanent environmental (c^2) was evaluated in Model 4, 5 and 6 whereas correlations between direct and maternal additive genetic (r_{am}) components were obtained under Model 3 and 6.

Assumptions of the model were:

$V(a) = A\sigma^2_a$, $V(m) = A\sigma^2_m$, $V(c) = I\sigma^2_c$, and $V(e) = I\sigma^2_e$

Where, I = Identity matrix, σ^2_a = Direct additive genetic variance, σ^2_m = Maternal additive genetic variance, σ^2_c = permanent environmental variance, σ^2_e = Residual variance

Estimated (co) variance components were used to obtain,

$(h^2=\sigma^2_a/\sigma^2_p)$ = Heritability

$(m^2=\sigma^2_m/\sigma^2_p)$ = Maternal heritability

$(c^2=\sigma^2_c/\sigma^2_p)$ = common environmental variance as a proportion of phenotypic variance

$[r_{am} = (\sigma_{am}/\text{”}\sigma^2_a \times \sigma^2_m)]$ = Direct-maternal genetic correlation

$(h^2_t=(\sigma^2_a+0.5\sigma^2_m+1.5\sigma_{am})/\sigma^2_p)$ = Total heritability (Willham,1980)

$(t_m = (¼)\ h^2 \pm m^2 \pm c^2 \pm r_{am}\ m^2\text{”}h^2)$ = Maternal effect across year repeatability for dam performance (Willham, 1972)

Identification of optimum model for different traits-

The model possessing the highest log-likelihood, deemed the 'best' model, underwent a comparison with every other model using the AIC criteria. The goal was to identify the simplest model whose log-likelihood did not significantly differ from that of the 'best' model. The comparison among all models was executed through the likelihood ratio test (LRT). The statistical measure for the likelihood ratio test (LR_{ij}) for sequentially reduced models (Rao, 1973) is as follows:

$$\mathbf{LR_{ij} = -2\log_e (L_j/L_i) = 2\log_e L_i - 2\log_e L_j}$$

Where,

L_i = Maximum likelihood for the complete model (with the maternal effect);

L_j = Maximum likelihood for the reduced model (without the maternal effect).

An effect was considered to have a significant impact if its inclusion resulted in a noteworthy increase in log-likelihood compared to a model that omitted it. Statistical significance, determined at $P<0.05$, involved assessing differences in log-likelihood values. This comparison was conducted against a Chi-square distribution with degrees of freedom equal to the variance in the number of (co)variance components fitted for the two models.Spearman's rank correlation and Pearson correlation of the estimated breeding values for first lactation production efficiency traits were computed using IBM SPSS Statistics version 23.

Estimation of Genetic and Phenotypic Trends:

Estimation of Genetic Trends

The genetic trends of different traits were estimated by taking regression of weighted average of sire's estimated breeding value (WAEBV) for each year on year. The WAEBV for the k^{th} year was calculated as follows:

$$\mathbf{\Sigma n_{ik} S_i / n_{.k}}$$

Where,

n_{ik} = number of daughters of sire i (i =1,2,3,4…….n) in year k

S_i = estimated breeding value of sire i.

$n_{.k}$ = total no. of daughters of n sires in year k.

Estimation of Phenotypic Trends

Phenotypic trends for each trait were estimated as linear regression of performance of population on year.The standard error for of linear regression required for estimating phenotypic and genetic trends was estimated using formula given by Falconer (1991).

$$\text{S.E. (b)} = \sqrt{\ } [\{1/(N-2)\}\ \{(\sigma^2_x / \sigma^2_y) - b^2\}]$$

Where,

N = number of period observations of x and y,

$\sigma^2_{y=}$ variance of y,

$\sigma^2_{x=}$ variance of x and

b = regression coefficient of y on x.

Results and Discussion

The average values under univariate animal model using WOMBAT software for production efficiency traits *viz.* MCI and MSC were 4.84 kg/day and 1.33 kg/day, respectively (Table 1). Average value of MCI were close to the findings of Godara (2003), Chakraborty et al. (2010) and Patil et al. (2018) but lower values were reported by Kumar et al. (2000) and Suresh et al. (2004), however, higher estimates ranging from 13.7 to 26.4 kg/day were obtained by Arbel et al. (2001) and Zambianchi et al. (1997) in Holstein cows. The heritability estimates of the production efficiency traits *viz.* MCI and MSC were 0.35±0.12 and 0.15±0.09, respectively. MCI and MSC were found moderate and low heritable respectively; which were in accordance with the findings of Chakraborty et al. (2010b) and Patil et al. (2018). The range of breeding values of production efficiency traits was 0.46 kg/day for MCI and 0.63 kg/day for MSC.

The analysis of variance components and genetic parameters for milk yield per day of calving interval (MCI) revealed varying estimates under different models (Table 2). In Model 1, the additive genetic variance (σ^2_a) was estimated at 1.50, which remained consistent in Models 2 and 3. However, Model 4 showed a slight increase to 1.53, and this remain persisted in Models 5 and 6. The maternal genetic variance (σ^2_m) ranged from 1.43 to 1.80 across the models, indicating some variability. The heritability

Table 1: Sum model values for production efficiency traits

Particulars	MCI	MSC
No. of animal IDs in data file	662	662
No of sires	169	169
No of sires with records & progeny in data	120	120
No of dams with progeny in data	105	105
Mean	4.84	1.33
Standard Deviation	1.51	0.59
Minimum	0.1	0.2
Maximum	9.52	4.83

(h^2) exhibited fluctuations from 0.26 to 0.41, suggesting that genetic factors contribute moderately to the variation in MCI which were in accordance with the findings of Chakraborty et al. (2010) and Patil et al. (2018) in Murrah buffaloes and Zambianchi et al. (1997) in Holstein cows. The proportion of the variance attributed to the common environment (c^2) ranged from 0.21 to 0.32, indicating a notable environmental influence. High and positive genetic correlation between the additive effect and materanl effect varied from 0.41 to 0.67, suggesting that there is a consistent and strong interaction between the genetic factors inherited from the dam. The range of genetic correlations observed across models highlights the importance of considering both additive and maternal genetic effects in breeding programs aimed at improving milk production in the given population. The estimates of the permanent environment variance (σ^2_p) ranged from 3.77 to 5.81, indicating substantial overall variability in Milk yield. The log-likelihood values varied among models, with Model 1 showing a significant difference compared to other models, as denoted by the double asterisks (**). Scanty information was available on the use of different animal model on MCI and MSC. The observation in these estimates provide insights into the genetic and environmental factors influencing milk yield per day of calving interval.

Based on loglihood value, model 3 outcame as appropriate model for MSC but there was no significant difference reported between the model 2 and model 3. Therefore, model 2 was taken to be the best model. This model constituted direct additive and maternal components the direct additive and maternal variance were 0.12 and 0.14, respectively. And the direct additive and maternal heritability for this model were 0.08 and 0.19, respectively. This model had lowest heritability among different models, ranged from 0.07 to 0.15 (Table 3). The additive genetic variance (σ^2_a) ranged from 0.08 to 0.15 across models, with Model 4 exhibiting the highest estimate. Maternal genetic variance (σ^2_m) varied between 0.10 and 0.17, reaching its peak in Model 5. The

Table 2: Estimates of co(variance) components and genetic parameters for Milk yield per day of calving interval

Trait	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
σ^2_a	1.50	1.50	1.41	1.53	1.53	1.53
σ^2_m		1.80	1.52		1.63	1.43
σ_{am}			0.98			0.61
σ^2_c				1.22	1.22	1.22
σ^2_e	0.98	0.50	0.61	1.02	1.02	1.02
σ^2_p	3.99	3.81	5.64	3.77	5.40	5.81
h^2	0.35	0.39	0.30	0.41	0.28	0.26
m^2		0.47	0.35		0.30	0.25
r_{am}			0.67			0.41
c^2				0.32	0.23	0.21
h^2_t	0.38	0.63	0.65	0.41	0.43	0.54
t_m	0.09	0.57	0.64	0.43	0.59	0.63
Log-L	-681.00**	-760.20	-769.16	-742.87	-696.69	-692.26

** denotes significant difference among LRT value

Table 3: Estimates of co(variance) components and genetic parameters for Milk yield per day at age of second calving

Trait	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
σ^2_a	0.11	0.12	0.11	0.15	0.11	0.08
σ^2_m		0.14	0.12		0.17	0.10
σ_{am}			0.01			0.12
σ^2_c				0.11	0.10	0.10
σ^2_e	0.21	0.18	0.18	0.20	0.18	0.18
σ^2_p	0.27	0.25	0.34	0.26	0.28	0.35
h^2	0.15	0.08	0.09	0.14	0.07	0.08
m^2		0.19	0.09		0.22	0.12
r_{am}			0.98			0.98
c^2				0.04	0.004	0.004
h^2_t	0.39	0.76	0.53	0.58	0.68	0.89
t_m	0.04	0.22	0.21	0.08	0.24	0.24
Log-L	213.63	217.51**	218.55	213.74	217.08	218.17

**denotes significant difference among LRT value

Table 4: Rank correlations coefficient of entire six models of production efficiency traits

	Production Efficiency Traits					
MCI						
	1	2	3	4	5	6
1	1	0.71	0.71	0.86*	0.86*	0.86*
2	0.71	1	1.00**	0.86*	0.93**	0.90**
3	0.71	1.00**	1	0.86*	0.93**	0.84**
4	0.86*	0.86*	0.86*	1	1.00**	1.00**
5	0.86*	0.93**	0.93**	1.00**	1	0.98**
6	0.86*	0.90**	0.84**	1.00**	0.98**	1
MSC						
	1	2	3	4	5	6
1	1	0.71	0.86*	1.00**	0.71	0.71
2	0.71	1	0.95**	0.71	0.98**	0.98**
3	0.86*	0.95**	1	0.86*	0.90**	0.98**
4	1.00**	0.71	0.86*	1	0.78*	0.78*
5	0.71	0.98**	0.90**	0.78*	1	0.97**
6	0.71	0.98**	0.98**	0.78*	0.97**	1

Where *P<0.05, **P<0.01

Table 5: Genetic and phenotypic correlation of performance traits using Bi-variant animal model

Traits	MCI	MSC
MCI	-	0.51**±0.19
MSC	0.73±0.05	-

Table 6: Year wise genetic and phenotypic trends of production efficiency traits

Traits	Genetic trends (Y_G)	Phenotypic trends (Y_P)	$R^2_{G(\%)}$	$R^2_{P(\%)}$
MCI (kg/day)	0.009±0.005	0.148**±0.018	13	75
MSC (kg/day)	-0.0004±0.000	0.047**±0.006	2	71

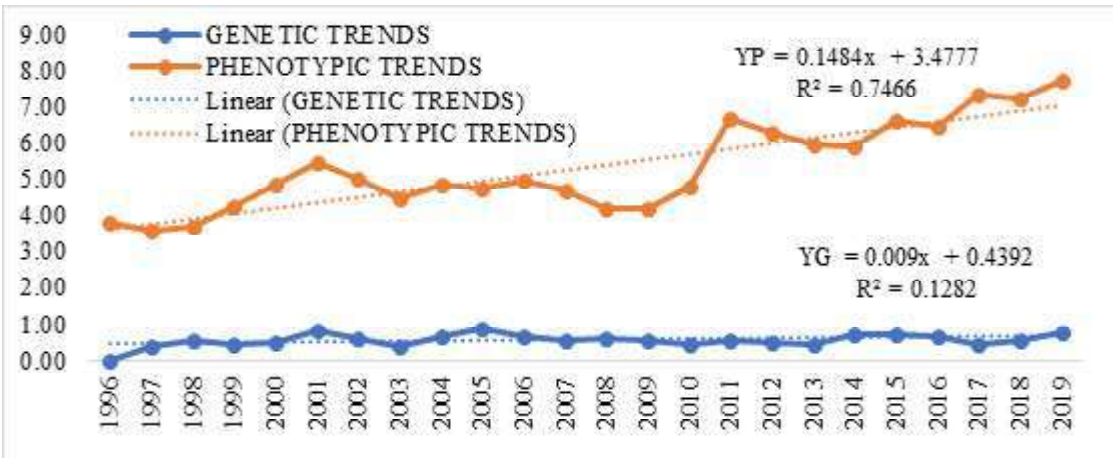

Fig. 1 Genetic and phenotypic trends of MCI

heritability (h^2) fluctuated between 0.07 and 0.15, showcasing the range of genetic influence on MSC, these estimates were comparable with Chakraborty et al. (2010) and Patil et al. (2018). Noteworthy was the consistently high genetic correlation (r_{am}) of 0.98 between additive and maternal effects across all models. Environmental components, such as the common environment (c^2), ranged from 0.004 to 0.04, indicating a subtle environmental contribution. Evaluating the significance of model fit, Model 2 emerged as the most suitable, with a log-likelihood value of 217.51. Importantly, the difference between the maximum log-likelihood value and Model 2's value was not statistically significant,

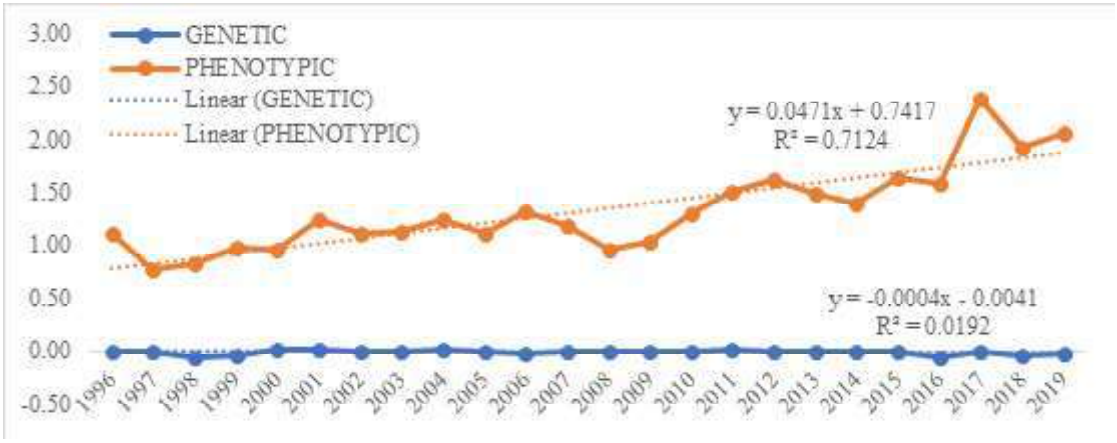

Fig. 2 Genetic and phenotypic trends of MSC

affirming the adequacy of Model 2 in capturing the underlying genetic architecture of milk yield per day at the age of the second calving. Rank correlations coefficient of the breeding values of all the 6 models used for each trait has been shown in Table 4 for production efficiency traits. The lowest and highest values of rank correlation of breeding values of all six models were ranged from 0.71 (non-significant) to 1.00 for MCI and MSC. The genetic correlation between MCI and MSC using bi-variate analysis was 0.73±0.05 and phenotypic correlation was 0.51±0.19 which was highly significant at $p<0.01$ (Table 5). Similar to the findings of Kandasamy et al. (1991), Chakraborty et al. (2010) and Patil et al. (2018).

Year wise genetic and phenotypic trends of production efficiency traits is shown in table 6. Positive genetic and highly significant ($p<0.01$) phenotypic trends were shown by MCI, as 0.009±0.005 kg/day and 0.148±0.018 kg/day annually. This indicated that selection as well as management practices going on hand in hand to raise the production efficiency traits. MSC had very low negative genetic trend (-0.001±0.001 kg/day) which was non-significant while positive and highly significant ($p<0.01$) phenotypic trend was shown by MSC as 0.047±0.006 kg/day per year, indicated the decrease in variability in this trait along the years. In contrast to this, Chakraborty and Dhaka (2012) reported negative genetic and phenotypic trends for MCI and MSC and Sahana and Sadana (1998) found negative genetic trend for MCI. However, Sharma and Singh (1992) reported positive genetic trends for MCI in Murrah buffalo. Similar to present study, Sahana and Sadana (1998) and Singh et al. (2003) reported positive phenotypic trends in Murrah buffaloes and Karan Swiss cattle.

Conclusion

The positive trends indicated the improvement in production efficiency traits over the years which pointed towards the better selection strategy, nutrition and management practices being followed at the farm.

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RESEARCH ARTICLE

Performance assessment of the dairy co-operatives in the mainland and Saurashtra-Kutch regions of the Gujarat, India

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Abstract: Dairy co-operatives in Gujarat (India) is a perfect case of local institution driving economic development. However, the discrepancies in its growth still exist across regions due to factors like social rigidity, bureaucratic procedures, inadequate local involvement, and management issues. The co-operative model completely failed once in the Saurashtra-Kutch region of the state and was re-established during 2007-09. Hence, the present study measures the performance of village-level dairy co-operatives societies (DCS) in the Mainland portion and Saurashtra-Kutch region of Gujarat over two periods (2011-14 & 2015-19) and proposes strategies to improve the performance. A total sample of 120 DSCs was taken from four milk unions, two from each region, and Iyengar and Sudarshan's approach was used for calculating the composite performance index which assigns weights inversely varying with variation in the indicator. The inverse variance-based co-operative performance index analysis of 120 DCSs across two regions suggests that the Mainland region (0.269) DCSs perform better than the Saurashtra-Kutch region (0.194) and over a period, the performance has improved to 0.354 and 0.271, respectively. Further, logistic regression-based marginal effects indicated that the probability of having high financial performance is 0.278 times greater for DCS with high physical performance than the counterpart. This suggests that poor performers should diversify and intensify input and other services delivery which will raise member participation rate, increase animal productivity, and lead to intensified dairying and higher milk procurement.

Keywords: Dairy co-operatives, Mainland, Saurashtra-Kutch, Performance, Logistic regression

Introduction

Indian dairy development has its roots in a massive dairy development program, popularly known as Operational Flood (OF) which led to the "white revolution" in India during 1970's. The OF program enabled a great transformation in the Indian dairy sector from scarcity to plenty and made the nation the world's top milk producer. The OF program was initiated in a situation when there was a dairy commodity surplus in Europe (1970), which threatened the milk deficit nascent Indian dairy industry. The menace of cheap imports from Europe, adversely affecting Indian dairying was converted into an opportunity for building a robust dairy sector (Gautam et al. 2009). The three-tier dairy co-operative structure (DCS at village level, Milk Union at district level & Milk Federation at state) initiated from the "AMUL (Anand Milk Union Limited)", was the base for this programme. Subsequently, National Dairy Development Board (NDDB) was established in 1965 with the mission to replicate the "AMUL pattern" that originated in Anand town of Gujarat all over India through the OF program. However, social rigidities coupled with several other factors have rendered the AMUL pattern less efficient in a few states, especially in Northern and Eastern India. Whereas it led to great success in Western and Southern India on account of local initiatives and state support. According to Christie (2020), the dominance of elite communities in the milk co-operatives and political arena was found to impede the involvement and leadership of the weaker section. The Planning Commission (2003) also reported misconduct and inadequate accountability of DCS employees. Further reasons reported for the non-functionality of co-operatives were the quality of animals, the cost of milk production, and the difference between the market price and price received by the farmers from DCS (Planning Commission, 2003).

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Among all the states, Gujarat is the only state where the co-operatives procure more than half (57%) of the state's total milk production (NDDB, 2023). Gujarat contributes 7.5 percent to the country's milk production by producing 17.28 million tonnes of milk annually out of a total of 230.58 million tonnes. (NDDB, 2023) The state has 22 district-level milk unions with a network of 19,853 village DCSs. Although, even within Gujarat, evident discrepancies exist in the concentration and success of dairy co-operatives across the Mainland and Saurashtra-Kutch region of the state. The Mainland region includes the Northern, Southern, and middle parts of Gujarat, while the Saurashtra-Kutch region includes all the districts present in the peninsular area of the state (NDDB, 2019). In the Mainland region, co-operatives procure around 73% of the regional milk production, while it takes only 25% of milk produced in the Saurashtra-Kutch region (AHDS, 2019). The Gujarat government has put efforts into the past to promote co-operatives-based dairying in the Saurashtra - Kutch region through the establishment of the Gujarat Dairy Development Corporation (GDDC) in the year 1975 because the Gujarat Co-operative Milk Marketing Federation (GCMMF) was not effective enough to move in this region. Hence, left the dairying potential of the Saurashtra-Kutch region untapped. In the region of Saurashtra-Kutch, the milk cooperatives failed due to insufficient milk collection, which resulted in heavy losses to GDDC on account of unpaid loans from dairies. On the order of the Board of Industrial Finance and Reconstruction (BIFR), all the assets of Dairies financed by the GDDC were handed over to NDDB (in 2005) to recover the advances made by NDDB to GDDC for the dairy co-operative's promotion in the Saurashtra-Kutch region. Later with the joint efforts of GCMMF, NDDB, and the state government, within the short span of two years (2007 to 2009), around 1600 new DCSs were created in the Saurashtra-Kutch region, procuring over one million liters of milk per day. The farmers were paid the highest ever remunerative prices that they ever received in the past and were equal to other developed milk sheds in Gujarat (Syed, 2009). However, by the entry of *Maahi*, a producer company since 2012, co-operatives are facing cut-throat competition, especially in the Saurashtra-Kutch region. Hence, assessment of the performance of these dairy co-operatives in the current competitive dairy environment is important. Also, co-operatives are the major player in milk procurement and marketing in the state and hence, their effective functioning can be translated to substantial benefits to the dairy farmers. Therefore, this study endeavored to measure the performance of DCSs over time as well across regions in terms of coverage and financial aspects.

This study analysed the physical and financial performance of DCSs falling under the Mainland and Saurashtra-Kutch region of Gujarat and classified them into three categories i.e., Good, Average and Poor based on their composite performance index value.

Data and methodology

The study is based on the secondary data collected from the village level dairy co-operative societies (DCSs) for the period from 2011-12 to 2018-19. Two milk unions (MUs) with the highest average membership per DCS were selected from the Mainland and Saurashtra-Kutch regions of Gujarat each. Khaira and Mahesana MUs from Mainland and Surendranagar (Sur'nagar) and Junagadh MUs from the Saurashtra-Kutch region were selected for the study. To avoid the over or under-representation of DCSs in the sample, a total of 120 registered DCSs working for the last ten or more years were randomly selected from all four MUs using the Probability Proportion to Size (PPS) approach (Appendix I). The selected sample of 120 DCSs entails 46, 48, 23, and 3 DCSs from Khaira, Mehsana, Sur'nagar, and Junagadh MU, respectively.

The relative performance of the DCSs was measured through a composite performance index (CPI) of 120 DCSs for two-time periods i.e. first quadrennial ending in 2014-15 and the second quadrennial ending in 2018-19. The index is based on two types of performance indicators, i.e., physical indicators and financial indicators, comprising 18 variables having a close association with the overall performance of DCSs. The physical indicators used to measure co-operatives coverage were:

The number of members per household of the village[1], The quantity of milk procured per milk pourer (including members and non-members), Annual sale (kg) of cattle feed per animal of the village, Animals treated per 100 animal population,

Artificial insemination (AI) done per 100 animal population and Score for other services provided by DCS.

The other services by DSCs include five services that are vaccination, animal insurance assistance, sale of fodder seeds, milk products, and other items (implements, edible oil, sugar, etc). Giving equal weight to all five services, the maximum score was set equal to five.

The financial indicators included:

Net income of DCS per liter of milk procured (Rs. /lit), Reserved fund of DCS per liter of milk procured (Rs. /lit), Share capital and dividend per member (Rs. per member), Total turnover per liter of milk procured (Rs./lit), Ratio of the value of milk sold to the union to value of milk procured, Ratio of local milk sale to the value of milk procured, Depreciation per liter of milk procured (Rs./lit), Salaries and incentives paid to DCS staff per liter of milk procured (Rs./lit.), Share of annual bonus to the total value of milk procured (%), Operational cost per liter of milk procured (Rs./lit), Procurement price paid per liter of milk (Rs./lit.) and Ratio of the value of milk procured in the total value of turnover.

The absolute values of financial indicators were first deflated using state GDP deflator to remove the inflation effect and to measure the progress in real terms. To draw reliable conclusions,

the indicators for index calculation were first converted at per unit level. The need for this conversion arises because generally, DCSs with a larger operational area (a village) tend to have higher absolute values of the parameters which give biased results. The index calculation used the approach of Iyengar and Sudarshan (1982) which is the generalization of the *core relative approach* of the UNDP-Human Development Index (1990). Ease in use and freedom from the restrictive assumption of linearity in the relationship of variables makes it advantageous over other methods.

The methods comprise of two steps:

Step-1: Index (Y_{id}) for each indicator and d^{th} DCS

If the variable is assumed to be positively associated with the performance

$$Y_{id} = \frac{(X_{id} - Min\ X_{id})}{(Max\ X_{id} - Min\ X_{id})}$$ where, i= 1,2,3….n & d= 1,2,3…..m

If the variable is assumed to be negatively associated with the performance

$$Y_{id} = \frac{(Max\ X_{id} - X_{id})}{(Max\ X_{id} - Min\ X_{id})}$$ where, i= 1,2,3….n & d= 1,2,3…..m

Where X_{is} represents the four-year average value of i^{th} performance variable of d^{th} DCS.

The numerator in the equation measures the extent by which the d^{th} DCS is better in the i^{th} variable as compared to the worst-performing DCS. The denominator is the range, which measures the total variation present in the variable across DCSs. All the variables except operational cost per liter and the ratio of the value of milk procured in the total value of turnover were assumed to be positively associated with the performance.

Step- 2: The physical, financial, and lastly, composite performance indices for each DCS. The weights reflecting the relative importance of variables were used.

$$W_i = \frac{k}{\sqrt{Var\ (Y_i)}}$$

Where, $k = \left[\frac{1}{\sum_{i=1}^{n} \frac{1}{\sqrt{Var(Y_i)}}}\right]$

$0 < W_i < 1$ and $W_1 + W_2 + \cdots + W_n = 1$

The weights were calculated assuming that they vary inversely as the variation in the respective performance indicators. This manner of weights assignment prevents undue dominance of any one variable having large variation, to the contribution of the rest of the indicators and hence, the distortion of inter-society comparisons. The weights used for the calculation of the index are given in Appendix II.

Physical index

$$Y_d^p = W_1 Y_{1d} + W_2 Y_{2d} + \cdots + W_6 Y_{6d}$$

Financial index $Y_d^f = W_7 Y_{7d} + W_8 Y_{8d} + \cdots + W_{18d} Y_{18d}$

Composite index

$$Y_d^c = Y_d^p + Y_d^f = W_1 Y_{1d} + W_2 Y_{2d} + \cdots + W_{18} Y_{18d}$$

The composite index Y^C_d varies from zero to one.

To validate the positive association between physical and financial performance of DCSs, we used logistic regression using Stata 12. Two categorical variables were generated by grouping the DCSs into two groups *viz*, Better performer (1) and Poor performer (0) based on both PPI and FPI using above and below average criteria. In our study, the PPI-based categorical variable was predicator while the FPI based categorical variable was taken as an unexplained variable. The logistic regression model is used when the outcome variable is dichotomous, it typically takes value 0 or 1.

The simple logistic model has the form

$$Logit\ (Y) = Natural\ \log(odds) = ln\left[\frac{\pi}{1-\pi}\right] = (\alpha + \beta X)$$ ——————————————————(1)

Taking the antilog of Equation 1 on both sides, one derives an equation to predict the probability of the occurrence of the outcome of interest as follows:

$$\pi = P\ (Y = outcome\ of\ interest, X = X_i, a\ specific\ value\ of\ X) = \frac{e^{\alpha+\beta_i X_i}}{1+e^{\alpha+\beta_i X_i}}$$ ——————————————————(2)

Where, π is a conditional probability of the form P (Y=1| X_1,...,X_s), such as the high performance of DCS. That is, it is assumed that "success" is more or less likely depending on the combinations of values of the predictor variables. The log-odds is also known as the logit transformation of π.

α and βs are the regression coefficients, Xs are a set of predictor variables, and e = 2.71828 is the base of the system of natural logarithms. α and βs are typically estimated by the maximum likelihood (ML) method which is designed to maximize the likelihood of reproducing the data given the parameter estimates.

Results and Discussion

This section discusses the extent of progress shown by the DSCs of both regions on various measures of physical and financial performance and hence, the composite performance index value over time and also how the physical and financial performance of DCSs is related to each other. The comparison of the quadrennial average of absolute values of physical parameters for two-time intervals along with percent change over time is presented in Appendix III. The DCSs of the Saurashtra-Kutch region have progressed well on the all-physical parameters compared to the Mainland region, indicating a increasing role of dairy co-operatives as an alternative channel of milk disposal for producers in the area, especially in the Sur'nagar MU. The provision of services at remunerative prices enables them to increase milk yields, reduces the cost of milk production, and encourages farmers to practice dairy farming on a commercial level with the adoption of modern package of practices. Gupta and Murthy (1985) had also concluded that the largely scattered small milk producers cannot be served better by merely procuring their milk for urban society, but the co-operatives should also integrate the production and distribution of inputs and services. However, a big difference was seen in the input services offered by the DCSs of both regions. No input services were offered by DCSs of the Saurashtra-Kutch region except the sale of cattle feed, which too was the initiative of a few DCSs on their own and was irregular. Among all the other services, the sale of milk products was the most common among the DCSs of both regions. In the Mainland region, DCSs of Khaira MU showed a greater increase in membership, milk procured quantity, and cattle feed sale than DCSs of Mahesana MU, while the latter progressed more on providing veterinary and other services than the former.

Among the MUs of Mainland, the average absolute values of financial indicators (Appendix IV) for Mahesana MU are less than that of Khaira MU, which is due to a greater share of small and medium-size DCSs in Mahesana MU. It is important to understand the method and government rules governing the finance calculation of DCSs to reach valid conclusions. Before calculating the profit-loss statement, generally, the DCSs deduct the amount of price difference paid by MU to DCS and also the price difference to be paid by DCS to farmers from the income from other sources (income from sample milk sold to MU, local milk sale, profit from the sale of cattle feed, milk products, and other items). Generally, the whole amount of price difference paid by MUs to DCS is passed on to the farmers. Dairy co-operatives pay the producer on a volume (liter) basis while MUs pay the DCSs on a weight (kilogram) basis which generates positive-sum for the same quantity of milk. The MUs also pay DCSs a commission (around 3%) on the value of milk sent to it. These both also form income to the DCSs. The management board of respective DCSs decides on what amount to be passed on to the farmers as the DCS's price difference. Then the profit-loss statement is calculated and the net income that arrived is distributed among different funds as per criterion laid under by-laws of co-operatives. This fund includes reserves, share dividend, education, member's welfare, member bonus, religious, animal breed improvement, staff bonus, and co-operatives promotional funds. Hence, after allocating the required finance for these funds and working capital for society, the rest is passed on to the farmers as price difference paid by DCS. Therefore, the average absolute value of net income, reserve funds, and share dividend of DCSs are lower in the mainland region. But, in the Saurashtra-Kutch region, the quantity of milk procured by recently formed DCSs is less, and also the income from other sources like the sale of inputs or other items is almost nil. Therefore, they were hardly able to distribute the DCS's price difference to the farmers from their income and the left net income is relatively higher. These DCSs lack controlling of financial flows between the payments to the farmers and the different government funds due to meagre values of incomes. The other major reason could be illiteracy and lack of knowledge about maintaining financial records and analysing the financial statements among the management board of the societies of the Saurashtra-Kutch region. All the DSCs in the sample from the Saurashtra-Kutch region were dependent on professional accountants to prepare their financial statements. The substantial increase in the quantity of milk procured by the DCSs in the Saurashtra-Kutch region is also reflected in the increase in the absolute values of milk procured and milk sold to the MUs. However, the average value of locally sold milk has reduced in DCSs of the Saurashtra-Kutch region entirely accounted to the DSCs of Sur'nagar MU. The values of depreciation indirectly represent the magnitude of physical assets owned by DCSs. The increasing average absolute values of depreciation in DCSs of all the MUs suggest improving the financial conditions of DCSs, especially in Khaira and Sur'nagar MUs.

The perusal from table 1 indicates that on a facet of physical performance, the DCSs under Khaira MU were the best followed by Mahesana MU of Mainland region, Sur'nagar MU, and Junagadh MU from Saurashtra-Kutch region, respectively. The DCSs of the Mainland are older and more experienced than those of the Saurashtra-Kutch region, hence the co-operative coverage gap is explainable. Besides, the MUs of the Mainland region have their well-established infrastructure for the production of cattle feed and veterinary services and well-managed supply chain through DSCs, while the newly formed MUs of Saurashtra-Kutch regions lack this infrastructure and hence struggle to pass on the inputs and services to the farmers regularly through DCSs at remunerative prices. Also, on the demand side, the poor adoption of new technologies like crossbred cattle, artificial insemination results in inadequate development of the market for these inputs and services. Therefore, the producers should be educated and made aware of the new methods of production and local leaders should be given a major role in bringing change in the attitude of the producers.

Likewise, financial and composite (overall) performance index values of DCSs followed the same order as physical performance. The physical performance indices (PPI) values are smaller than financial performance indices (FPI) because the set of physical indicators is smaller than financial indicators. Over time, the performance of DCSs has improved as indicated by the increased performance index values from the period 2011-15 to 2015-19. The difference between the average PPI of two-time intervals showed that maximum progress in physical performance was made by DCSs of Mahesana MU (0.020) followed by Khaira MU (0.017), Sur'nagar MU (0.017), and Junagadh MU (0.009), respectively. While, in the case of financial performance (FPI), DCSs under Khaira MU (0.077) have progressed maximum followed by Mahesana MU (0.066), Sur'nagar MU (0.057), and Junagadh MU (0.016), respectively. The maximum progress in overall performance was seen among DCSs of Khaira MU (0.094) followed by Mahesana MU (0.077), Sur'nagar MU (0.077), and Junagadh MU (0.022), respectively. The relatively better improvement in the performance of co-operatives under the Mainland region can be accounted to the easy availability and reasonable costs of inputs and services such as cattle feed, mineral mixtures, breeding (AI), and veterinary services. The efficient and effective extension support, assured milk prices, and economic benefits such as annual bonus by the milk union are the other economically incentivizing factors. These findings are supported by earlier studies namely, Wani et al. (2015), and Bhaviskar (1998). A study by Sutar et al. (2022) also reports that governance and management support offered to members and support received from milk unions plays a significant role in the performance of the dairy co-operatives. The variation in the overall performance across DCSs measured through the coefficient of variation indicates that there is a relatively high variation in overall performance among DCSs of Khaira MU, followed by DCSs of Mahesana MU, Sur'nagar MU, and Junagadh MU. Although, this variation has reduced over the period among the DCSs of Khaira MU and Junagadh MU, while it has increased in the Sur'nagar MU and remained almost unchanged in the Mahesana MU.

Figure 1 gives a graphical presentation of CPI value-based classification of DCSs and percentage share of DCSs in the different performance categories across regions and MUs. The categorization of DCSs into good, average, and poor performance categories was done using the cumulative square root frequency approach. The increased frequency of DCSs in the higher performance category for time interval 2015-19 shows improvement in the performance of DCSs in the region or MU over time. The greater frequency of DCSs in the higher performance category indicates better performance of DCSs in the region or MU. In the time interval of 2011-15, the DCSs of the Mainland region performed better than that of the Saurashtra-Kutch region. In the Mainland region over a period around 36 % of total DCSs have shifted toward higher performance category while in the Saurashtra-Kutch region only 23% of DCSs have shifted toward higher performance category and there has not been a single DCS in the good performance category which implies that the DCSs in the Mainland region are improving at a relatively faster pace than that of Saurashtra-Kutch region.

Table 1: Average region-wise performance score DCSs

Particular	Mainland			Saurashtra-Kutch			Gujarat
	Khiara	Mahesana	Overall	Sur'nagar	Junagadh	Overall	
				2011-2015			
PPI	0.081	0.054	0.067	0.022	0.014	0.021	0.057
FPI	0.202	0.202	0.202	0.176	0.148	0.173	0.195
CPI	0.283	0.256	0.269	0.198	0.162	0.194	0.253
MAX CPI	0.556	0.372	0.556	0.245	0.193	0.245	0.556
MIN CPI	0.214	0.190	0.190	0.159	0.154	0.154	0.154
CV CPI (%)	18.93	14.12	17.54	9.85	5.97	11.31	20.99
				2015-2019			
PPI	0.098	0.074	0.085	0.039	0.023	0.037	0.075
FPI	0.279	0.259	0.269	0.242	0.164	0.233	0.261
CPI	0.377	0.333	0.354	0.282	0.186	0.271	0.336
MAX CPI	0.468	0.448	0.468	0.348	0.193	0.348	0.468
MIN CPI	0.291	0.233	0.233	0.217	0.180	0.180	0.180
CV CPI (%)	10.39	14.02	13.63	11.83	3.52	16.31	17.43

PPI is physical performance index value*; FPI* is financial performance index value; *CPI* is composite performance index value*; MAX* is maximum*; MIN* is minimum and *CV* is coefficient of variation (%)

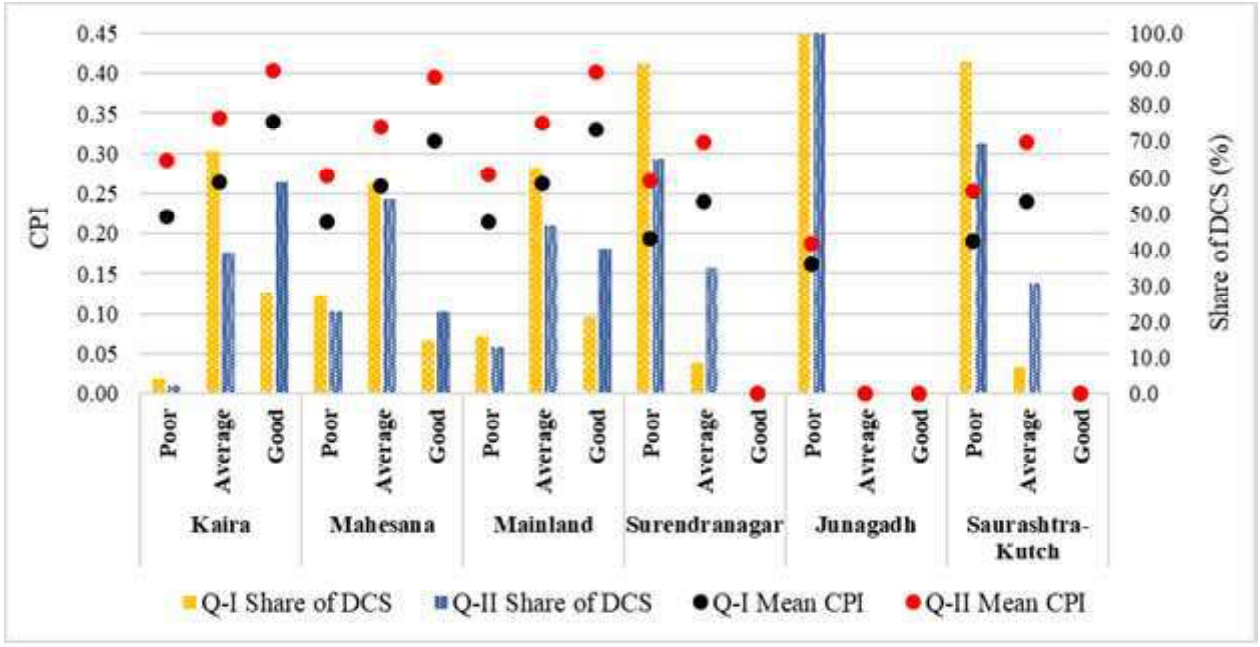

Fig 1. Classification of DCSs according to composite index value across regions

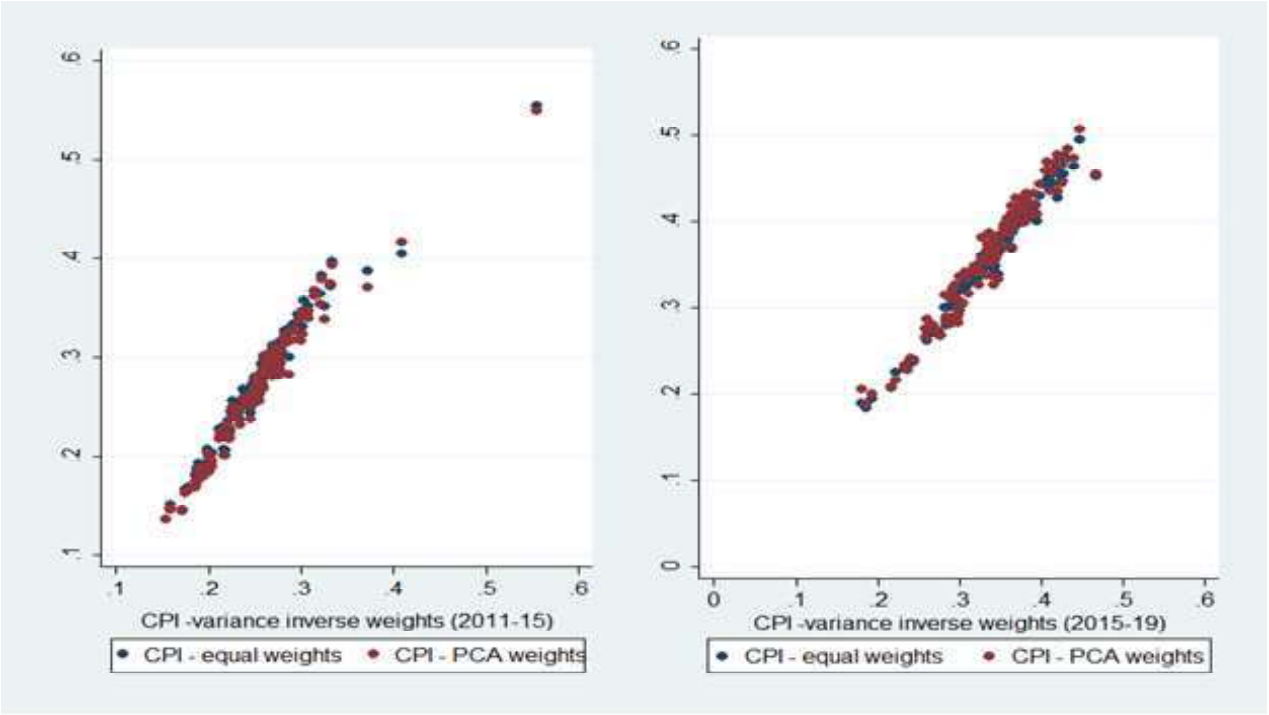

Fig 2. Robustness of CPI to different weighting schemes

Correlation coefficients: CPI- variance inverse weights and CPI-equal weights =0.9720 for 2011-15 and 0.9889 for 2015-19; CPI-variance inverse weights and CPI- PCA weights = 0.9720 for 2011-15 and 0.9754 for 2015-19

Table 2 shows the association between physical and financial performance measured through logistic regression-based Odds ratio for both time intervals. The value of the Odds ratio for the period 2011-15 indicates that for the DCS's with high physical performance, the odds of being in the high financial performance category is 1.698 times higher than DCS's having lower physical performance. The values of marginal effect indicate that the predicted probability of falling in the high financial performance group for the DCS with high physical performance is 0.128 times

Table 2: Association between the physical and financial performance of DCSs

Y (Physical performance) (0 if low & 1 if high)	2011-2015		2015-19	
	Odds ratio	ME (dy/dx) #	Odds ratio	ME (dy/dx) #
X = Financial performance (Dummy, 0 if low & 1, if high)	1.698	0.128	3.221*	0.278**
	(0.638)	(0.903)	(1.251)	(0.087)
Constant	0.545*	-	0.757	-
	(0.138)		(0.190)	

ME is marginal effects; # is for discrete change of the dummy variable from 0 to 1; * is significance level at ($p<0.05$) and ** is significance at ($p<0.01$); values in parenthesis indicates standard errores

greater than the low physical performing DCS. Similarly, for the period 2015-19, the probability of a DCS with high physical performance to fall in the high financial performance group is 0.278 times (statistically significant) higher than the counterpart. It implies that the higher physical performance of DCS leads to higher performance on the financial front. Therefore, the DCSs must focus more on the physical aspects of increasing membership, milk procurement, and provision of various technical and physical inputs and services to the members. These results are in confirmation with the study carried out in Jammu and Kashmir by Rather et al. 2016.

The robustness of CPI calculated using variance inverse weights to other weight allotment approaches like equal weights and Principal Component Analysis (PCA) based weights, was checked through correlation analysis as presented in Figure 2. The correlation coefficient values indicate that these results are unaffected by the selection of the weighting scheme.

Conclusion

The co-operatives have played a crucial role in the dairy development of the Gujarat state, although it shows a disparity in its growth across regions in the state. The results brought out the vast performance gap among the DCSs of both Mainland (0.354) and Saurashtra-Kutch (0.271) regions of the state. Despite the rejuvenation efforts made by the NDDB and the state government for a decade, the progress in the performance of DCSs in the Saurashtra-Kutch region has been slow and unsatisfactory. Further, the study reported that better physical services enhance the probability (0.278) of DCSs to have high financial performance. However, in the Saurashtra-Kutch region, farmers hardly get extension support, inputs, and animal health care services from co-operatives, and thus, a substantial market share goes to the private dairy or vendors in the local milk market. Hence, the MUs of the region should coordinate with the Directorate of Animal Husbandry Department of the state and other non-government agencies to play the role in the provision of key input services like breeding, feeding, health care, and extension services for member producers. Studies show that illiteracy and lack of democratic functioning of cooperatives limit member participation, hence the awareness activities and mandatory representation from different social groups in the management of co-operatives should be considered to improve participation. Other factors such as agricultural conditions, water availability, the adoption of new technologies, non-farm income sources, co-operatives management at the ground level, market conditions, etc. may influence the farmer's participation and co-operatives growth.

The bulk milk cooler installation at the DCS level should be done to increase the milk shelf-life so that society gets a better price from MU and reduce the double transportation cost incurred by the MUs. An intensive membership drive, with a prime focus on progressive and young farmers, will help in increasing contact with other milk producers in the future. Giving a key role to DCSs in providing subsidies for the purchase of animals as well as implements like chaff cutters, fogging systems, etc. to the producers, will also help strengthen the region's co-operatives. The development of dairying in general and dairy co-operatives, in particular, requires well-coordinated joint efforts of the animal husbandry department of the state, all the MUs of the region, and other dairy development agencies like NDDB, GCMMF.

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Supplementary files

Appendix I-IV

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RESEARCH ARTICLE

Dairy innovation portal: A web-based platform to address farmer-led innovations in the Indian dairy sector

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Abstract: The study was carried out in four districts of Haryana and Punjab states to conduct the baseline survey about developing a Dairy Innovation portal for the dairy stakeholders. A first- of-its-kind Dairy Innovation Portal was developed consisting of the details of Farmer- led Innovations resulting from the preliminary survey. The portal was developed keeping in view the diverse needs of the stakeholders in the dairying sector. The multilingual portal was equipped with innovation submission forms in farmers' native language for easy access. The portal also directs the farmers to claim Intellectual Property benefits from government organizations. A central database for documentation of farmer-led innovations in dairying will make it simple for people who want to access it and beneficial for dissemination. Futuristic vision enables this portal to cover farmer-led innovation in pan-India exploration which can bring about a radical change in the Indian Dairy Sector.

Keywords: Dairy, Dissemination, Farmer-Led Innovation, Innovation Portal, Stakeholders,

Introduction

Dairy extension education aims to give farmers the know-how they need to carry out better dairy husbandry operations, to make timely information and better practices accessible in a way that is suitable for their level of literacy and awareness, and to cultivate in them a positive attitude toward innovation and change (Benor,1984).The lack of information based on the farmers' needs and the irregular times at which it was broadcast on radio and television were the significant limitations of traditional extension approaches (Singh.et al.2020). The majority of technical staff members in the State Departments of Animal Husbandry (SDAHs) and Dairy Development Departments were unable to interact successfully with the stakeholder group and the research system (Singh.et al.2018). According to the Working Group on Agricultural Extension in 2007, there are only around 0.1 million extension workers employed, which is insufficient to meet the demands of the farming community. 1.3 to 1.5 million workers are needed for extension work. Determining how to disseminate information in a way that meets the needs of the greatest number of people at any given time while also being cost-effective and easily accessible is therefore urgently needed (Singh.et al.2020). The creation of information and communication technologies based on the internet can address this. A positive relationship was reported between high educational status, internet access and improved nutrition information (Dominic et al.2023).

Indian agriculture has been increasingly characterized by a diverse set of actors, relationships, and policies that are required for coordinated actions to benefit farmers. The dairy industry, a vital part of agriculture, is also aware of this complex web of actors at play. India is the largest milk producer in the world with a 23% share of world milk production and has been growing at an annual rate of 4.2% for the last 2 decades (USDA, 2021). The demand for animal-based calories, such as those found in meat, dairy products, and eggs, is expected to more than quadruple globally between 2010 and 2050, particularly in developing nations due to population expansion, economic development, and urbanization (Gouel and Guimbard, 2019). Dairying is crucial for food security in many developing countries as it is the primary source of income and food for a large portion of the rural poor (FAO,2011). It is also seen as one of the important sectors for reducing poverty, unemployment and income inequality. In an era of global competitiveness, the greatest problem for dairy farmers is obtaining maximum output while using scarce natural resources, which can be solved by applying dairy innovations on every farmer's farm. Application of innovations at every stage of production since from cultivation of fodder to marketing of milk is the dire need of the present day.

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Dairying in India has persisted and thrived through periods of harsh market conditions and extreme environmental stress, mostly because of the inherent inventiveness and inherent innovative capacity of its farmers. Farmers' informal experimentation results in their innovations. Farmers are encouraged to take action to solve their difficulties as a result of the limited environment and new chances. Farmers' inventions are more quickly adopted by other farmers than the findings of official research that is pushed on them since they are less expensive, more accessible, locally relevant, and tested in genuine farm situations. Small and marginal farmers engaged in dairying constantly experiment with low-cost technology to meet their needs when faced with financial issues. In order to preserve, prepare, and package a variety of perishable products for longer shelf life as well as improved market potential, farmers have also developed innovative, affordable technology. Considering the scenario, the present study was taken up to design and implement the first-of-its-kind 'Dairy Innovation Portal' which will help in the parallel diffusion of Farmer-led Innovations among the farming communities of different regions. This will also help in generating awareness among the farmers about the prevalent farmer-led innovations in the dairy sector. The need of documenting and claiming the rights for innovation for farmers has been made easy and affordable through this interactive portal.

Materials and Methods

Haryana and Punjab states were purposively selected considering their prominence in the number of innovative farmers related to the field of dairying. From each state randomly two districts were selected. As a whole, a total of four districts were selected. From each district, two blocks were randomly chosen comprising eight blocks in total. From each block,35 dairy farmers were selected comprising a total of 280 dairy farmers as sample. Apart from that 10 government R&D (research and development) persons and 10 private R&D persons related to the field of dairying were selected from the district level. So, a total of 280 dairy farmers and 80 R&D persons (both public and private) were selected as complete samples for the study. 360 samples in total were therefore chosen. The baseline data was used to design the interactive web portal. Data Repository was created in MySQL Workbench. The Portal was created using several codes using SQL Workbench. The researcher has undergone basic training to develop this portal along with valuable inputs and suggestions from the project team. The coding was done for different Farmer-led Innovations in different ways. Separate windows were created for the different web pages under development. The project team and survey visits to the individual farmers were documented in the portal. The portal can be accessed on the weblink https://no1.in.net/farm_innovations.

Results and Discussion

The Dairy Innovation Portal is a platform designed to address innovations in the dairy sector by bringing together stakeholders from various sectors including industry, academia, and government. The aim of the portal is to foster collaboration and knowledge sharing among these stakeholders to drive innovation in the dairy industry. The portal offers a range of features including a knowledge library, news and events section, innovation challenges, and a collaboration space for industry experts to connect with one another. The knowledge library is a central repository of information on the latest developments in the dairy industry, covering topics such as sustainability, nutrition, and technology.

Farmers are gradually creating new methods, putting them into practice, and improving them over time. These farmer-led innovations have not received adequate documentation or recognition over time. (Baliwada *et al.*2016). Grassroot innovations should be given priority in every sector to increase its potential area of application in the different parts of the country. So, dissemination and scientific validation part is very much essential. This portal is a first-of-its-kind novel attempt to document innovations, particularly in the Indian Dairy sector. This portal covers innovations from different aspects of the dairy industry i.e., production, processing, and management. This portal briefly covers the innovations specified to different locations as per the secondary data analysed specifically in the states of Punjab and Haryana. Additionally, the Intellectual Property Rights (IPR) on agricultural innovations are frequently disregarded. Scientists have frequently overlooked the importance of traditional knowledge and its documentation. As a result, many agricultural technologies created by creative farmers have not been made available to other farmers (TAAS, 2011).

The creation of regionally specific content is crucial and differs from region to region. The value of bilingual information retrieval systems was underlined by numerous studies in a wide range of Indian regional languages, according to Sendil (2010). Agricultural education and extension can be a key component of the transformation process to transfer technology, encourage technological learning, help farmers solve problems, and make it possible for farmers to participate more actively in the agricultural knowledge and information system. The layout, content presentation style, and gestural design must be taken into consideration before designing a portal/module (Verma *et al.*2019). The portal is equipped with Google Translator and farmers from different regions can view and contribute towards it in their native language. A separate google form is created by the developers to submit an idea/innovation by the farmers. The portal also directs the farmers/stakeholders to several innovation websites like National Innovation Foundation, Indian Patent Office, Honey Bee Network, Prolinova etc.

Fig. 1 Summary of the Innovations reported in the Dairy Innovation Portal

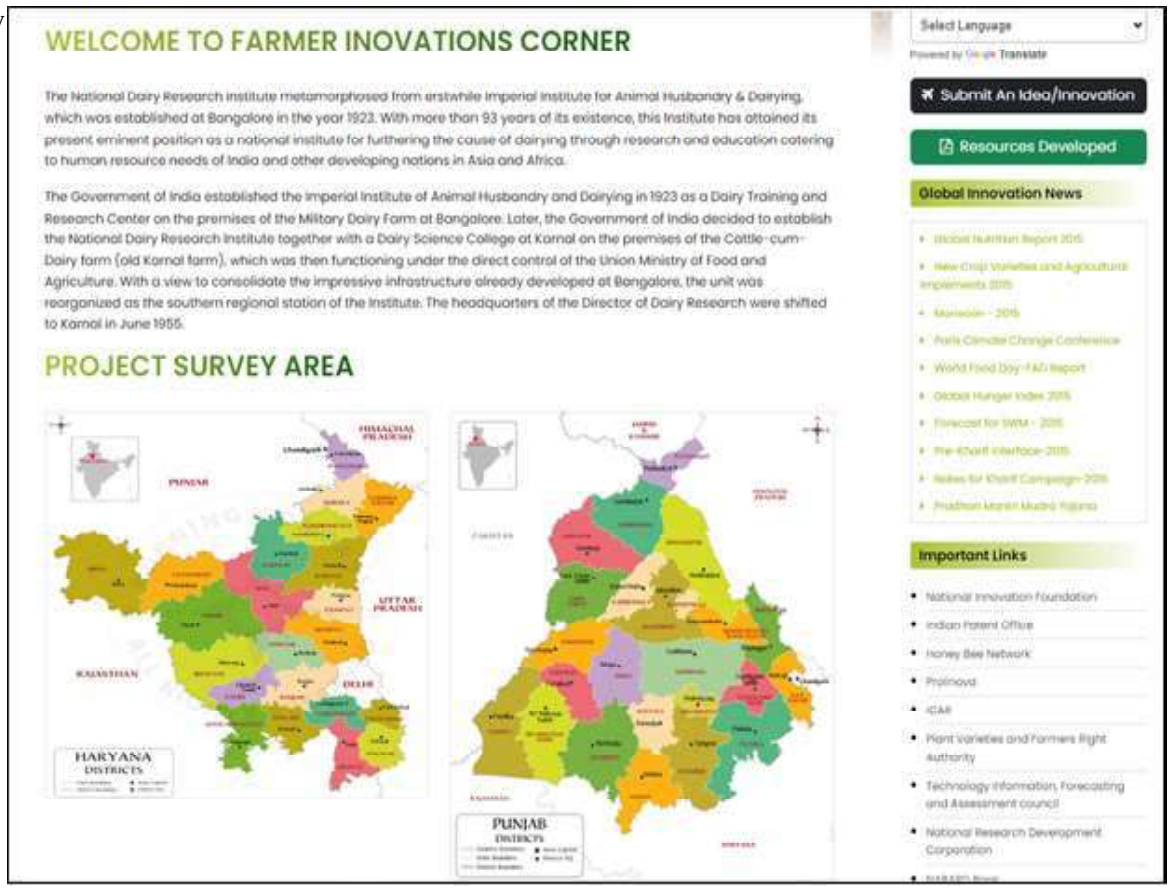

Fig. 2 Finalized Dairy Innovation Portal

Whatever the medium of communication, it should intelligently deliver information that is pertinent to the area with a strict degree of explanation. Information repositories are bringing new possibilities for data retrieval as well as new difficulties. Users worldwide can now instantly access previously unimaginable sources of knowledge thanks to the availability of online materials in a variety of different languages. The portal is equipped with different language provisions which in turn will made communication very easy for farmers. The farmers and stakeholders can view the portal in their regional language for easy access of the information. According to Keniston (2000), the creation of regionally specific content is crucial and differs

Fig. 3 Provision of reporting in different Intellectual Property registering website

Fig 4 Provision of all regional languages in the Innovation Portal for effective understanding of farmers from different regions of India

from region to region. They can see the different innovator's identity and the methodology of their innovations in their regional language. This will further develop a sense of innovativeness in the farmer to solve his own local problems.

Most of the time, grassroots inventors are completely unaware of their own innovations. According to Saraswathi et al.(2010), the growth of information repositories is leading to both new opportunities and problems for information retrieval. Users worldwide can now instantly access previously unimaginable sources of knowledge thanks to the availability of online materials in a variety of different languages. According to Mallinga et al. (2012), CD lessons were helpful in improving milk dealers' awareness of clean milk production. He said that the knowledge gain was 32.33 percent for maintaining animal sheds, 51.43 percent for management, and 32.08 percent for the system for storing and transporting milk. Meena et al. (2014) reported in their study that the created educational DVD was successful in terms of knowledge acquisition. The educational module developed for information dissemination of brucellosis disease has contributed to the increased knowledge about the disease transmission as reported by Verma et al. (2021). The majority of farmers expressed satisfaction with the content's relevance and appropriateness, as well as its usefulness in expanding knowledge, suitability of the information to the field scenario, improvement of self-confidence, and arousing of curiosity and interest.

Additionally, the IPR on the ideas created by farmers are frequently disregarded. A central database for documentation of farmer-led innovations in dairying will make it simple for people who want to access it and beneficial for dissemination. The database in the portal will be updated from time to time for proper documentation in different states. Futuristic vision enables this portal to cover farmer-led innovation in pan-India exploration. Research is

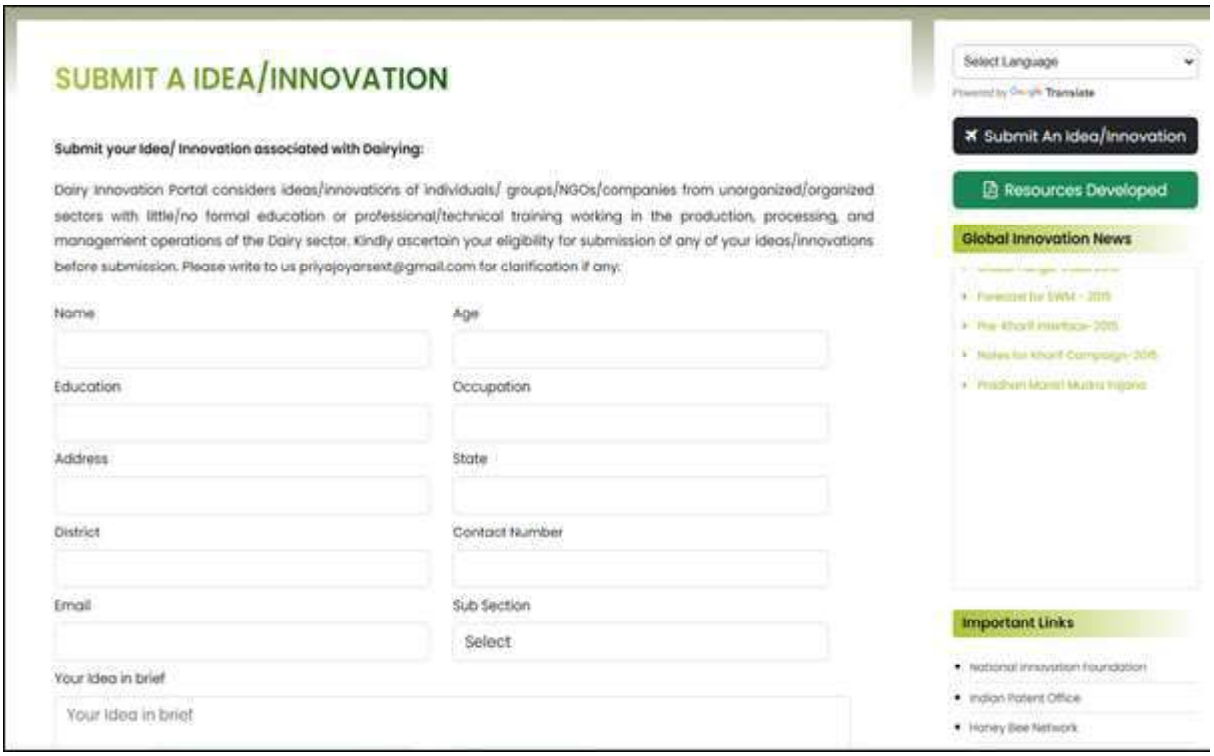

Fig. 5 Format for submission of a New Idea/ Innovation

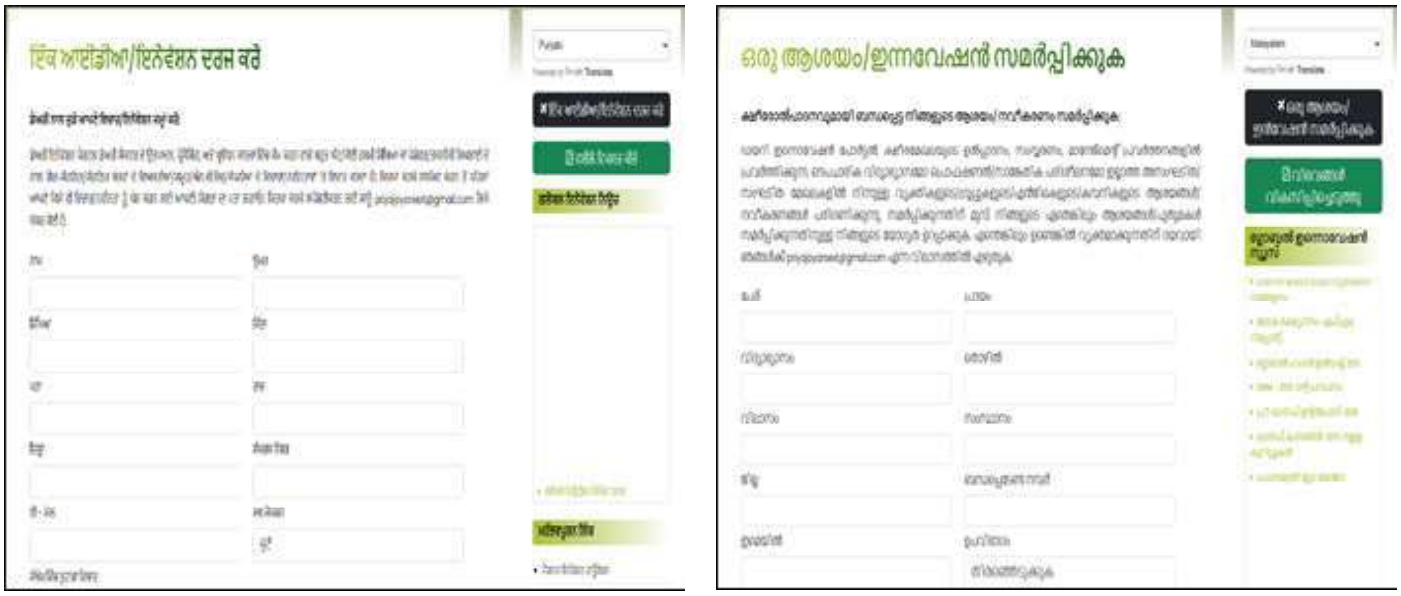

Fig. 6 Pan- India approach(multi-lingual) for exploring and reporting Farmer Led Innovations in Dairy Sector

required to determine whether industry-standard software or templates can be created to store Farmer Led Documentation data in a single database. The rewards of validating or further developing grassroots inventions may seem restricted because there are few opportunities to discover their true potential. Local innovators and bearers of traditional knowledge are under minimal, fragmented, and easily ignorable pressure to influence policies (Sci Dev, 2007). The lack of funding continues to be a barrier to the commercialization of grassroots inventions, according to Olga (2015). Farmers are not properly acknowledged as actors in the innovation system, there is little information supplied on the various knowledge sources involved or the flow of knowledge, and there is little focus on long-term effects on livelihoods (Brigidletty et al. 2012). This will facilitate access for people who are interested in the content and make it helpful for dissemination (Matthias, 2010). According to Prolinnova (2009), existing effective farmer inventions are deserving of greater distribution and do not necessarily need further study.

Conclusions

The Farmer-led innovation section in the portal provides a platform for industry stakeholders to collaborate and develop innovative solutions to pressing issues facing the dairy industry. The innovations were typically focused on sustainability, nutrition, and food safety, and participants can work together to develop new products, processes, or technologies to address these challenges. The collaboration space allows industry experts to connect with one another, share knowledge, and work together on research and development projects. This feature is particularly valuable for smaller businesses and startups who may not have the resources or expertise to undertake large-scale R&D projects on their own. Documentation of field experiences based on empirical evidence is necessary for various dairy innovations. Launching of separate network projects or All India Coordinated Research Projects (AICRP) on farmer-led innovations will have a better impact. The creativity of the innovators should be properly acknowledged and their intellectual property rights need to be protected. Front Line Demonstrations (FLD) in innovative farmer fields and agri-tourism around farmers' innovative efforts would not only generate awareness but will also help in revenue generation and motivates fellow farmers. Overall, the Dairy Innovation Portal is an innovative approach to addressing the challenges facing the dairy industry by bringing together stakeholders from across the sector to collaborate and drive innovation. The platform is helping to accelerate the development of new products, processes, and technologies that are critical to the future success of the dairy industry.

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Conflict of Interest

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Constraints impeding livelihood diversification of farmers in aspirational districts of Bihar

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Abstract: Farmers are facing a major problem of managing livelihood security because of changing environmental condition, globalization, changing economy, and soil fertility that ultimately threatens our food, economic, infrastructure and social security in the long term. Thus, this study attempted to assess the constraints in achieving the livelihood security of farmers using the Garrett ranking method. The research was conducted by interviewing 320 dairy farmers from selected districts Gaya, Khagaria, Sitamarhi and Sheikhpura. In this study different constraints have been identified under sub-headings of economic constraints, infrastructure constraints, marketing constraints, technical constraints and communication constraints. ─The study revealed that the top most economic constraint as reported by the respondent was 'high cost for balanced and nutritious food items' with Garrett mean score of 61.29. Out of seven infrastructure constraints perceived by them, first rank was assigned to the "Poor or underdeveloped marketing infrastructure (mean score 63.80)". "Malpractices by traders" was ranked as first rank by respondents among the various marketing constraints. Whereas "Lack of knowledge for fodder conservation practices during natural calamities" was reported rank one with Garrett mean score of 61.75. Asymptotic significance ($p<0.01$) was obtained from the Friedman test with chi-square value of 22.803 and degrees of freedom of value 4. These limiting constraints are global, comprehensive, integrated, and holistic and beyond the ability of farmers to manage them, thus there is a need for proactive problems-solving by the governmental intervention to overcome them.

Keywords: Livelihood security, Farmers, Diversification, Garrett score, Constraints, Sustainable

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Introduction

In developing countries like India, land-based livelihoods for small and marginalized farmers are becoming increasingly unsustainable as land is no longer able to meet the food and fodder needs of their families (Hiremath, 2007). Because of this poverty rate, the national poverty rate remained at 29.5 percent from 2011 to 2012 (Planning Commission, 2014). The small and marginal holdings constituted 86.21 per cent in 2015-16 while their share in the operated area stood 47.34 per cent in the current census as against 44.31 per cent in 2010-11 (Agriculture Census 2015-16). So, a farmers need to engage in different activities and earn enough to sustain themselves. Livelihood diversification occurs in both agricultural and non-agricultural activities. That is, Production of multiple crops or high value crops. Non-agricultural activities like starting small businesses or choosing non-agricultural livelihoods such as temporary work or migration (Khatun and Roy,2012). The different categories of activities people live for include farming, off-farm, and non-farm sources of income (Saith, 1992). The livestock sector has emerged as a key segment of distended and diversified agricultural sector in the Indian economy. Farmer's dependence on livestock besides farming as an alternative source of income is very high according to (Malathesh et al., 2009). Livestock production provides opportunities for risk coping, farm diversification and intensification as well as providing livelihood benefits to the people and society (Bossio, 2009). Forty percent of the people living below the poverty line are largely dependent on livestock (Rao, 2004). Dey et al. (2012) observed that goat rearing is still to be accepted by all classes of people in Bihar. The vast variety of livelihood diversification tactics employed by rural people underscores the fact that they operate in variety of different environments which are complex and risk prone (Chambers et al.,

1989) are often not able to develop effective strategies to diversify their sources of income due to whelming odds. This part analyzed the odds that needed immediate attention from planners as well as policy makers to secure livelihood of farmers. The odds vary from households to households and region to region. In order to streamline it, a sound strategy needs to be planned to promote large scale livelihood diversification in rural areas. In order to secure people's livelihood, it is imperative to determine the inhibiting factors so that necessary interventions may accordingly be made to create a conducive climate. Having this assumption in view, an attempt was made in this paper to identify the constraints that impede the farmers to diversify their livelihood in selected districts of Bihar.

Methodology

The study was carried out in aspirational districts of Bihar. The set of four districts Gaya, Khagaria, Sitamarhi and Sheikhpura representing different types of agro-climatic, socio-economic conditions, and having lowest per capita agricultural income of the state were selected ~~from all the zones~~ from different agro-climatic zones. Two blocks from each district and two villages from each block were selected randomly. Twenty households of dairy farmers in each village were randomly selected to constitute a total sample size of 320. The constraints in this study refer to economic, communication, infrastructural, technical, and all other factors or forces that may enable, individually or in combination, to prevent farmers from diversifying their livelihoods. Based on the available literature, surveys, and discussions with various stakeholders, a complete list of barriers to diversification was developed and grouped into broad groups based on their nature. These limitations can be broadly classified into: (i) infrastructural, (ii) economical (iii) marketing(iv) technical and(v) communication constraints. A semi-structured questionnaire was developed based upon the information acquired during the explorative research phase, and pre-tested prior to the survey. Garrett's ranking method was used to identify constraints that impede the farmers to diversify their livelihood. Garrett's formula for converting ranks into percent used was:

$$\text{Percent position} = \frac{100 * (Rij - 0.5)}{Nj}$$

Where, Rij = Rank given for i^{th} constraint by j^{th} individual;

Nj = Number of constraints ranked by j^{th} individual.

The per cent position of each rank was converted into scores referring to the table given by Garrett and Woodworth (1969). For each factor, the scores of individual respondents were added together and divided by the total number of the respondents for whom scores were added. Relevant constraints were selected on the basis of secondary and primary data. Alternatively, Kendall's W (Coefficient of Concordance) test was used to examine agreement between subjects and gives a value between 0 and 1. A nonparametric test i.e., Friedman two-way ANOVA by ranks test, as elucidated by Tripathi (2014) was also used to identify the most severe constraints among the five broad constraints faced by dairy farmers by using the following formula of difference between conditions:

$$x^2r1 = \frac{12}{Nn(n+1)} \times \sum R1^2 - 3N(n+1)atdf = n-1$$

N= number of respondents

n= number of broad constraints

$\sum R1^2$= row ranks summed up in each column, squared and then added

Results and Discussion

Economic constraints

The data presented in Table 1, revealed that under economic constraints, the top most constraint as reported by the respondent was 'high cost for balance and ~~nutricious~~ nutritious food items' with Garrett mean score of 61.29 and the second most important constraint reported by the farmers was 'low income from agricultural activities' with Garrett mean score of 60.24. It was mostly due to the problem of water scarcity and lack of irrigation sources in the study area, agricultural production was not up to mark and was responsible for affecting the livelihoods of farmers engaged in agricultural activities which affected the income and diet of respondents. As agriculture is totally dependent on seasonal phenomenon, farmers were facing many natural and unexpected calamities, which they were not expecting, thereby causing heavy losses to them.

Lack of employment opportunity at locality level, low and seasonal agro animal produce that restrict the producer-consumer direct linkage, high charges by veterinarians for treatment of animals, high premiers for livestock insurances as compared to benefits and low prices for the produce in the market was ranked as III, IV, V, VI and VII with Garrett mean score of 59.52, 55.56, 50.48, 46.09 and 42.01 respectively. The cost of agricultural inputs has risen dramatically over time, significantly raising the overall cost of farming and raising livestock; however, prices for various agricultural and livestock products have not increased proportionately, leaving farmers with significant losses in agricultural production. However, the constraint like, high interest rates paid for taking a loan from institutions that lend money was ranked last i.e., VIII with mean score of 39.23.

Infrastructure constraints

Infrastructure is a source of positive externalities in the development process. In fact, the absence of infrastructure is positively related to incidence of poverty. Many studies have found a positive relationship between the level of economic development and the quality of housing, access to basic amenities like electricity, safe drinking water, roads, etc. (Kundu 2009). The data presented in Table 2 depicts the infrastructure constraints and the rank pattern given by the respondents. Out of seven constraints perceived by them, first rank was assigned to the 'poor or underdeveloped marketing infrastructure' (mean score=63.80). Lack of animal vaccination and deworming facilities in the study area was ranked second by the respondents with the mean score of 60.55. There was a lack of proper infrastructural facilities in terms of improper market facilities, due to which farmers were facing problems; and hence, there is a need to improve them in the study area.

Distance of veterinary hospitals and non availability of VO at required time, poor or slow internet connection, poor availability of sources of irrigation facilities, and insufficient power supply was ranked as III, IV, V and VI with Garrett mean scores of 53.15, 48.27, 45.39 and 40.93, respectively. A study concluded that essential infrastructure amenities, such as irrigation supply and, employment possibilities, and market, etc, need to be improved to overcome the constraints faced by small and marginal farmers for improving the overall livelihood security of the farmers. While, Poor infrastructural facilities such as proper road, transportation facilities etc. was ranked VII with mean score of 36.98. These findings were found to be in line with the findings of Brar et. al. (2020). From the given data, it can be inferred that the absence of a good infrastructure creates problem for local dairy farmers to operate their dairy business efficiently and effectively, hence it is necessary to provide them with the basic infrastructural facilities such as training centers, facilities for dairy equipment, facilities for testing milk, facilities for milk storage and preservation, and facilities for disease diagnostics.

Marketing constraints

Marketing is one of the important aspects of running any enterprise nowadays. Organized markets are not yet developed in developing countries like India to sell the farm products, there is no market to supply neither agricultural products to customers, nor agricultural inputs to the farmers. Data in Table 3 depicts that 'malpractices by traders' with Garrett mean score of 63.53 was perceived as most serious marketing constraint and ranked I by the dairy farmers. Delayed payment of the produce to the

Table 1: Distribution of respondents according to Economic Constraints

S. No.	Statements	GMS	Rank
1	Low income from agricultural activities	60.24	II
2	Low prices for the produce in the market	42.01	VII
3	High charges by veterinarians for treatment of animals	50.48	V
4	High premiums for livestock insurances as compared to benefits	46.09	VI
5	High interest rates paid for taking a loan from institutions that lend money	39.23	VIII
6	Low and seasonal agro animal produce that restrict the producer-consumer direct linkage	55.56	IV
7	Lack of employment opportunity at locality level	59.52	III
8	High cost for balanced and nutritious food items	61.29	I

Note- *GMS- Garrett mean score

Table 2: Distribution of respondents according to Infrastructure Constraints

S.No.	Statements	GMS	Rank
1	Poor availability of sources of irrigation facilities	45.39	V
2	Distance of veterinary hospitals & non availability of VO at required time	53.15	III
3	Lack of animal vaccination & deworming facilities in the study area	60.55	II
4	Poor or underdeveloped marketing infrastructure	63.80	I
5	Poor or slow internet connection	48.27	IV
6	Insufficient power supply	40.93	VI
7	Poor infrastructural facilities such as proper roads, transportation facilities etc.	36.98	VII

Note-*GMS- Garrett mean score

producers was ranked II with Garrett mean score of 60.80. The reasons are commission agent exploitation, low prices, lack of transparency in the trading process, collusion among traders, delayed payments, and poor quality of mandi infrastructure. Late payments also forced the respondents to rely on credit and savings from their commission agent and local money lender for their daily expenses.

Distant markets from the rural areas, Lack of market information, Lack of stability in market price of farm produce was ranked as III, IV and V with Garrett mean score of 56.55, 53.48 and 42.47, respectively. Lack of market for selling of milk and live animals was ranked VI by the respondents with Garett mean score of (37.93) as perceived by the dairy farmers. Respondents in the study region were having these constraints since there were no standardized and graded quality of the produce, lack of proper information transfer, malpractices of adulteration of standard and inferior quality of goods and inadequate infrastructure amenities. Thus in order to improve market accessibility embracing new marketing strategies is required, that will help to overcome the constraints faced by the respondents. However according to Lal et al. (2015) the third major constraint was reported to be 'low price for milk in the market' as after calamity many farmers moved their focus towards dairy farming but market did not grow in that proportion.

Technical constraints

The data presented in Table 4 indicates six technical constraints and the rank pattern experienced by the respondents. Out of six constraints perceived by respondents, first rank was assigned to the 'lack of knowledge for fodder conservation practices during natural calamities" (Garrett mean score=61.75). It might be due to many reasons like lack of proper information from extension functionaries/KVKs regarding management of feed and fodder during the natural calamities and respondents were not much aware about the new technologies and techniques in the agricultural and livestock production. The other constraints perceived by the respondents in order of their importance are represented in the table in descending order.

The second rank under technical constraints was assigned to the 'lack of need based non-formal trainings and demonstrations' (mean score=55.61). Lack of knowledge about scientific livestock management practices such as health care, breed improvement and housing, with the mean score of (55.12) was ranked III by the respondents. Lack of visits by EO/VO, distance of veterinary hospitals/KVKs from village, and lack of awareness towards new practices are some of the reasons for poor knowledge of scientific management practices among the respondents. Likewise, lack of training facilities for dairy farming in the locality, lack of extension functionaries and advisory services in the locality, were ranked as IV and V with Garrett mean score of 48.38 and 42.86 respectively. Thus, there should be more organized and frequent trainings for farmers, demonstrations, and visits by the experts and veterinarian in the study area. 'Inadequate awareness on preparing balanced ration feed' was ranked VI with mean score of (38.71). Similarly, a study in Gujarat was conducted by Kathiriya et al. (2014) reported that, more than 70 percent of the respondents had difficulties in getting medical aids, lack of technical knowledge about feed,

Table 3: Distribution of respondents according to Marketing Constraints

S.No.	Statements	GMS	Rank
1	Lack of market information	53.48	IV
2	Malpractices by traders	63.53	I
3	Lack of stability in market price of farm produce	42.47	V
4	Delayed payment of the produce to the producers	60.80	II
5	Distant markets from the rural areas	56.55	III
6	Lack of market for selling of milk and live animals	37.93	VI

Note-*GMS- Garrett mean score

Table 4: Distribution of respondents according to Technical Constraints

S.No.	Statements	GMS	Rank
1	Lack of knowledge for fodder conservation practices during natural calamities	61.75	I
2.	Lack of need based non-formal trainings and demonstrations	55.61	II
3	Lack of knowledge about scientific Livestock management practices such as health care, breed improvement and housing	51.12	III
4	Lack of training facilities for dairy farming in the locality	48.38	IV
5	Lack of extension functionaries and advisory services in the locality	42.86	V
6	Inadequate awareness on preparing balanced ration feed	38.71	VI

Note- *GMS- Garrett mean score

Fig. 1- Mean Rank by Friedman test

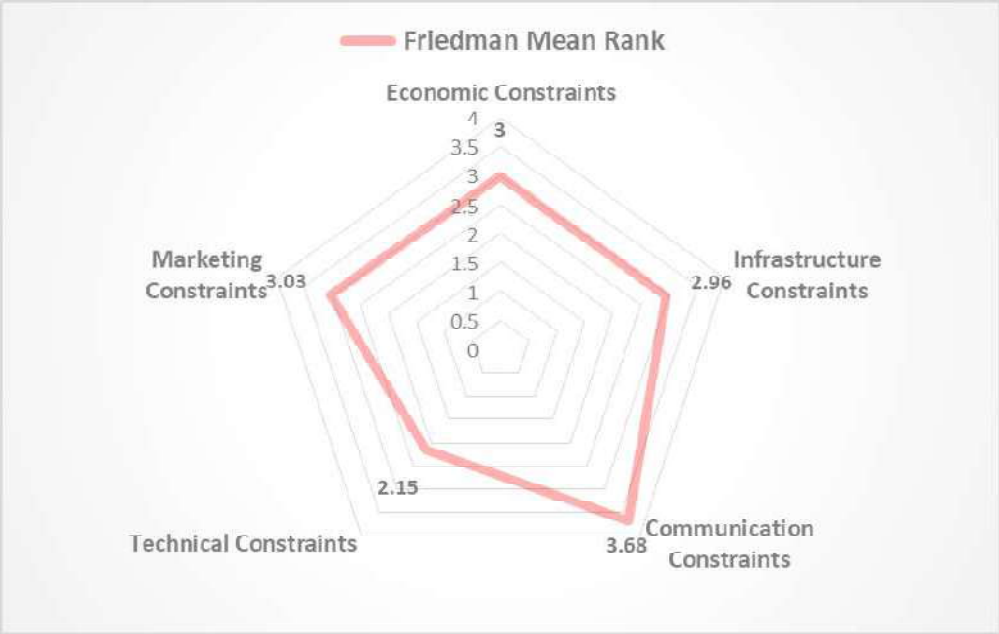

Table 5: Distribution of respondents according to Communication Constraints

S.No.	Statements	GMS	Rank
1	Poor commitment of Govt. functionaries in proper implementation of any schemes in the study area	62.34	I
2	Reluctant interest to learn any new activity of livelihood generation	53.69	III
3	Difficulty to learn term and conditions of credit agency while taking loan	40.55	VII
4	High cost of internet service provider	46.89	V
5	Lack of farmers participation in making various plans and policies	45.12	VI
6	Lack of research-extension linkage among farmers, universities and scientists	51.19	IV
7	Lack of monitoring by extension /KVK personnel in the study area	58.09	II

Table 6: Test Statistics of Friedman test

N	320
Kendall's W[a]	.049
Chi-Square	22.803
Df	4
Asymp. Sig.	.000

a. Kendall's Coefficient of Concordance

fodder and health management, lack of artificial insemination facilities at village level, lack of quality fodder and lack of medicinal facilities in the villages as major constraints in dairy farming.

Communication constraints

Sending a message from a sender to a recipient can be affected in many ways- like emotions, our cultural context, ~~our~~ medium of communication, and even ~~our~~ location. The data presented in Table 5 depicts seven communication constraints experienced and ranked by the respondents. The first rank was assigned to the 'poor commitment of government functionaries in proper implementation of any schemes in the study area' (Garrett mean score=62.34). Some government functionaries were not fair in their implementation while doing some kind of demarcation between the dairy farmers when providing some kind of government facility to them. It was also found that dairy farmers were unaware of recent advances indifferent aspects of dairy. Moreover, there was acute unawareness of government support and subsidies.

Lack of monitoring by extension /KVK personnel in the study area, reluctant interest to learn any new activity of livelihood generation, lack of research-extension linkage among farmer, universities and scientist, high cost of internet service providers and lack of farmers participation in making various plans and policies were ranked as II, III, IV, V and VI with Garrett mean scores of 58.09, 53.69, 51.19, 46.89 and 45.12, respectively. For the improvement of the general living conditions of farmers, the first and most important thing is the "economic motivation" or the "interest to learn" of the farmers themselves to improve their

own living conditions. However, many farmers in the study area lacked this motivation and wanted to continue making a living in the same conditions as before. Therefore, to overcome this limitation, farmers need external motivation and guidance to improve their overall living conditions. While, difficulty to learn term and conditions of credit agency while taking loan was ranked least important constraints VII with Garrett mean score of (40.55) by the respondents.

Result in Table 6 depicts that asymptotic significance ($p<0.01$) was obtained from the Friedman test with chi-square value of 22.803 and 4 degrees of freedom. A value of Kendall's W indicates that all subjects ranked the four methods in the same way and therefore they were in complete agreement. The Kendall's W was 0.049, which signifies a good effect size as well as moderate agreement between subjects on the preferable ordering of the methods, Hence, it can be interpreted that there was significant difference in between the different constraints faced by the dairy farmers.

The Friedman test also identified the most severe broad constraints perceived by dairy farmers. Hence, Figure 1 further revealed that the mean ranks obtained by the use of Friedman test was highest for communication constraints (3.68) which means that it was most severe constraint among all the five constraints. The mean rank of technical constraints was 2.15 which implied that it was the least severe constraints found among all the broad constraints.

Conclusion

The focus of the study was to find out the perceived constraints among dairy farmers in aspirational districts of Bihar, India. The five broad constraints with different numbers of statements under each constraint have been investigated. Friedman test and Garrett ranking were used to identify the most severe constraints and to provide ranking to the identified constraints, while appropriateness of the test was confirmed with the asymptotic significance level. It could be concluded from the above research findings that major constraints as expressed by the dairy farmers which affected their development were poor commitment of government functionaries in proper implementation of any schemes ,Poor or underdeveloped marketing infrastructure, lack of market information and lack of training to improve technical know-how. This pointed out the fact that capacity building of the dairy farmers with respect to sustainable livelihood, it is crucial to have an appropriate policy that gradually encourages and strengthens the relationship between farmers and research institutions. This research recommends the establishment of dairy cooperatives to achieve favorable prices for dairy products and the development of alternative non-farm income sources, as dairy farming alone cannot bring much social recognition to dairy farmers. To further accelerate and sustain the productivity growth in the area, infrastructure development and the growth of rural institutions are crucial.

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