Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 3 Issue 12 December 2021

Research Article

Antimicrobial Activity of Fermented Milk Containing Various AqueousHerbal Extracts

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Received: October 12, 2021

Published: November 09, 2021

Abstract

Bovine milk has been an inordinate source of nutrition for majority of vegetarian population. The milk obtained from the "Himachali Pahari cow" of the hill region has been a boon not only in providing health benefits but also in improving the socio-economic status of the farming community. However, due to inaccessibility and geographical barriers, benefits of the Himachali Pahari cow milk are limited to certain regions only. Hence, the present study was conducted with the objective to investigate the antimicrobial potential of the fermented milk supplemented with various herbal extracts of harad (*Terminalia chebula*), baheda (*Terminalia bellirica*), amla (*Emblica officinalis*) and arjuna (*Terminalia arjuna*). Antimicrobial activity of herbal extracts supplemented fermented milk was evaluated against *Bacillus cereus*, *Escherichia coli, Staphylococcus aureus, Rhodococcus equi,* and *Shigella flexneri*. The fermented milk supplemented with aqueous harad extracts was found to be most effective against *B. cereus* and *R. equi*. Pepsin digested fermented milk supplemented with amla and arjuna exhibited comparable antimicrobial activity against *R. equi*. Results of the present study indicated that supplementation of fermented milk with various aqueous herbal extracts can enhance its antimicrobial potential and can be useful in boosting the immunity against various microbial infections.

Keywords: Antimicrobial Activity; Fermented Milk; Herbal Extracts; *Escherichia coli; Staphylococcus aureus; Shigella flexneri*; *Bacillus cereus*; *Rhodococcus equi*

Introduction

Today, the foods are intended not only to content the starvation and to provide essential nutrients, but also to thwart nutrition-related diseases and improve consumer's health [1,2]. Dairy products account for around 25–30% of the average person's diet. Milk and milk products are nutrient-dense foods that are high in oleic acid, conjugated linoleic acid, omega-3 fatty acids, vitamins, minerals, and bioactive compounds such as antioxidants [3].

Milk is an important source of nutrition and a complete food which supplies almost all the nutrients for growth and maintenance of the human body. Milk is a well-known nutritious food that provides energy, proteins, lactose, lipids, amino acids, creatinine, vitamins, and minerals. Moreover, milk also provides calcium, phosphorus, riboflavin, vitamin A, ascorbic acid and thiamine etc. [4]. Apart from its nutritional value, interest has arisen in the ability of milk to deter bacteria and how the knowledge acquired can be applied to human health and develop functional foods. A number of proteins present in milk under numerous circumstances exhibit antimicrobial activity.

Fermented milk products are consumed all over the world because of their nutritive and health-promoting properties. Various bioactive compounds are released during fermentation process.

Fermentation increases digestibility and bioavailability of proteins and minerals and also activates many bioactive peptides from their inactive form. Antimicrobial proteins and peptides found in milk are capable of killing and inhibiting a wide range of bacteria. These proteins exhibit antibacterial properties which make the former as ideal candidates for use in a variety of applications, including prevention of mastitis in cattle and improving human health. Strong evidence suggests that certain probiotic strains can confer resistance against infection with enteric pathogens [5]. A number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products [6]. Antioxidants from natural sources are more superior to those produced chemically, because some synthetic antioxidants may have mutagenic and carcinogenic effects [7]. Thermal processing of milk increases its total phenol, antioxidant, and antimicrobial activities [8-11].

Fermentation of milk with lactic acid bacteria, such as *Lactobacilli* or *Bifidobacteria* generate products [12], which are easily digestible to persons with milk allergies and lactose-intolerance. Consumption of fermented milk has been effective against several health disorders such as diarrhoea, hay fever, arthritis, asthma, biliary disorders, constipation, stomach flu, gastroesophageal reflux disease, hypertension, and hypercholesterolemia [13].

Medicinal plants and their extracts have a long history of utilization as natural remedies for curing health-related difficulties including metabolic disorders such as insulin-resistance, obesity and diabetes etc. [14]. Plants contain a range of bioactive compounds, namely flavonoids, saponins, alkaloids, polyphenols etc. which are responsible for various remedial properties. Medicinal plants rich in natural antioxidants and phenolics are progressively incorporated in dairy foods as additives to improve nutritional and therapeutic properties. In recent years, fermented milk products have gained popularity to prevent some modern age diseases such as cardiovascular diseases (CVDs), Type-2 diabetes and obesity. So, there is a need to explore bioactive potential of herbal-supplemented fermented milk to develop functional foods with additional health benefits.

Aqueous extracts of *T. arjuna* bark exhibits significant antibacterial activity against *E. coli*, *Klebsiella aerogenes, Proteus vulgaris,* and *Pseudomonas aerogenes* [15]. Antimicrobial properties of herbs and spices can be used positively to suppress spoilage microbial growth and pathogenic bacteria in dairy products [16].

Antimicrobial activity of crude and methanol extract of *T. bellerica* dry fruit was tested by disc diffusion method, against 9 human microbial pathogens [17]. Crude aqueous extract of dry fruit at 4 mg concentration showed zone of inhibition ranging from 15.5-28.0 mm. *S. aureus* was found to be highly susceptible forming a largest zone of inhibition, suggesting that *T. bellerica* was strongly inhibitory towards this organism. These pathogens were vastly sensitive to the methanol-extract, forming 14.0 to 30.0 mm wide zones of inhibition. This suggests that the methanol extract of *T. bellerica* was more effective than crude extract against most of the microbes except enteropathogenic *E. coli* and *P. aeruginosa*. Hence, phytochemicals in *T. bellerica* dry fruits have a broad-spectrum antimicrobial activity [18].

Notably, *T. arjuna* bark has been commonly used for a number of purposes in traditional medicine system. Powdered bark of *T. arjuna* has been used by ancient health specialists to treat "hritshool" (angina) and other cardiovascular disorders [19].

Antibacterial activity of bark, stem, root, leaf, and fruit aqueous extracts of *T. arjuna* was assessed on selected Gram positive and Gram-negative bacterial strains. Phytochemical extracts from different parts of the plants demonstrated substantial antibacterial activity against tested microbial strains; however, inhibitory extract activities were based on plant component and the test organism. Results indicated that antimicrobial activity of *T. arjuna* phytochemical extracts was based on concentration (1.0 mg/disc and 5.0 mg/disc) of the tested bacterial strains. Additionally, the results showed that *T. arjuna* bark extracts could be used as a potential source of antimicrobial agents against tested bacterial strains [20].

Phenolic compounds of herbs and spices are effective substitutes as artificial antimicrobials used in food processing. In order to prevent the growth of certain pathogenic bacteria *viz*., *S. aureus, Salmonella enteritidis and Listeria monocytogenes,* the phenolic phytochemicals such as tea catechins, ferulic acid, ellagic acid and coumaric acid have been successfully used [21].

T. chebula is a popular medicinal plant since ancient times due to its broad-spectrum medicinal values such as treatment of enteric disorders. Water and as well as organic solvents, namely methanol, ethanol, ethyl acetate and chloroform extracts of its leaves have been analyzed for antibacterial activities against four different enteropathogenes, namely *E. coli*, *Salmonella* sp., *Shigella* sp. and *Vibrio cholerae* along with *Saccharomyces cerevisiae*. The analysis was carried out by taking the extracts at a concentration of 10 mg/ml, and their antimicrobial activities were documented by estimating the zones of inhibition by disc-diffusion method [22].

Lactobacillus acidophilus and rosella extract were used to produce goat milk-based yoghurt. Yoghurts were distinguished by increased antimicrobial activity and high bacterial selectivity, resulting in the production of strong antagonistic metabolites such as antimicrobial peptides (AMPs) or organic acids against Gram-positive and Gram-negative bacteria (*B. cereus, E. coli, S. aureus,* and *S. typhii*) [23].

E. officinalis is traditionally used as a medicinal plant. *E. officinalis* fruit have been tested against various pathogens, such as *E. coli, S. aureus* and *S. typhi,* by the agar well diffusion system [24]. Maximum antibacterial activity was observed against *S. aureus* by methanolic extract. The result obtained from this study revealed that plant-extracts show maximum inhibition against Gram-positive bacteria rather than Gram-negative bacteria which may be primarily due to the difference in bacterial cell wall composition. These findings support the scientific rationale of conventional usage of *E. officinalis* phytochemicals as antimicrobials against a wide range of microbes. Disc-diffusion method, due to being a simple and cost-effective method for testing a wide range of microorganisms and antimicrobial agents [25] was used in present study.

Materials and Methods

Collection of milk

Milk samples of *Himachali Pahari* cow were collected from surrounding area of Palampur, District Kangra, Himachal Pradesh. The pH and total titratable acidity of milk were determined, and then milk containers were stored in a freezer at -20°C for further use [26].

Collection of herbal plant material

The bark of *T. arjuna* (arjuna) and fruits of *T. bellerica* (baheda), *T. chebula* (harad) and *E. officinalis* (amla) were collected from the surrounding areas of Palampur (as above), and processed as per [26].

Water extraction of herbal plants

Dried powder of *T. chebula, T. bellerica, T. arjuna* and *E. officinalis* (10g) was extracted in distilled water as described in [26].

Preparation of starter culture

Starter culture was prepared by using *Lactobacillus rhamnosus* (347) bacteria, purchased from National Collection of Dairy Cultures (NCDC), ICAR-National Dairy Research Institute, Karnal (Haryana) using the method described by [26].-

Preparation of fermented milk containing herbal water extracts

Fermented milk was prepared by adding 10 ml of various herbal water-extracts into 85 ml of fresh boiled milk of *Himachali Pahari* cow and 5 g of starter culture as per the method described by [26].

In vitro enzymatic digestion of fermented milk containing aqueous herbal extracts

In vitro enzymatic digestion protocol [27] with modifications was used. Yogurt sample (10 ml) was taken. Undigested and digested samples were centrifuged at 12,000 rpm for 30 min., and the harvested supernatant was stored at -20°C for further analysis [26].

Protocol for evaluation of antimicrobial activities

Antimicrobial activities of all samples and its *in vitro* digested samples have been evaluated by disc diffusion method. This method was given by [28]. The cultures were grown in 5 ml nutrient broth at 37°C for 24 hours. A 24-hour old culture (100 µl) was spread uniformly and aseptically onto solidified nutrient agar plate. Then sterile discs of size 10 mm (HiMedia, Mumbai, India) were placed aseptically on nutrient agar. Four discs were used for each petri plate. Applied 100 µL of sample directly on disc and left undisturbed for 30 min. at room temperature. Agar plates were then incubated at 37°C for overnight. Streptomycin (10 µg) was used as positive control, and nutrient broth was taken as negative control. Zone of inhibition was measured in mm. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to a particular antimicrobial compound.

Results and Discussion

In our research, disc-diffusion method was used to detect antimicrobial activity in fermented milk containing aqueous herbal extracts and its *in vitro* digested milk. Antimicrobial activity in various fermented milk containing aqueous herbal extracts of *Himachali Pahari* cow was recorded against different microorganisms including *B. cereus*, *E. coli, S. aureus, R. equi,* and *S. flexneri*. The results of antimicrobial activity are depicted in figures 1, 2, 3, 4, and 5.

Antimicrobial activity against *B. cereus,* a Gram-positive, facultative anaerobe that causes food-borne illness in humans, was tested. Antimicrobial activity in the pepsin-digested fermented milk in control as well as in all fermented milk samples containing aqueous herbal extracts (Figure 1) was studied. Among all the herbal-supplemented fermented milk and its *in vitro* digested samples, *baheda* exhibited detectable zone of inhibition in all the samples. Maximum inhibition was detected in pepsin-digested samples (15 mm) (Figure 1).

T. bellerica fruit extract has been reported to possess strong antimicrobial activity against *B. cereus* [18,29]. Aqueous extract of arjuna exhibited maximum antimicrobial activity against *B. cereus* (15 mm), followed by harad (14 mm), and amla (12 mm). Antimicrobial activity of baheda was not very clear. Harad has been described to be an effective antibacterial herbal therapeutic against a variety of Gram-positive bacteria [29-32]. *T. arjuna* bark has been reported to be a good source of antioxidants and high antimicrobial activity [33]. *E. officinalis* has also been reported to be a strong antibacterial agent against Gram-positive bacteria [29,34-35].

Antimicrobial activity against *B. cereus* was observed in pepsin-digested samples only. No antimicrobial activity was observed in milk, fermented milk (undigested) and overnight digested samples containing aqueous herbal extracts. Streptomycin sulphate showed inhibition zone 17 mm against *B. cereus.*

Antimicrobial activity against *E. coli*, that is common the lower intestine and causes diarrhea, was noted. The result exposed that no antimicrobial activity was noted in all samples of fermented milk containing different aqueous herbal extracts and the *in vitro* digested samples (Figure 2). *T. bellerica* fruit has been shown to decrease pathogenicity of Gram-negative motile bacteria such as *S. typhi*, *S. typhimurium*, *E. coli* and *P. aeruginosa* by rendering them less motile [18]. Streptomycin sulphate showed 20 mm inhibition zone against *E. coli*.

Antimicrobial activity against *S. aureus*, a Gram-positive, round-shaped bacterium, frequently found in the upper respiratory tract and on the skin and causes skin infections, heart valve infections, and bone infections, was evaluated. Highest antimicrobial activity was found in aqueous harad extract (18 mm) followed by aqueous amla extract (13 mm). *T. chebula* fruit extract showed antimicrobial activity against *S. aureus* and the compounds responsible for this activity were gallic acid and its ethyl ester [36]. *E. officinalis* fruit extract has shown antimicrobial activity against *S. aureus* [37]. No antimicrobial activity was observed in milk and fermented milk containing aqueous herbal extracts and corresponding *in vitro* digested samples. Streptomycin sulphate showed inhibition zone of 20 mm diameter against *S. aureus* (Figure 3).

Antimicrobial activity against *R. equi,* a Gram-positive facultative intracellular pathogen that is a common cause of bronchopneumonia in foals, was evaluated. Maximum antimicrobial activity was observed in the pepsin-digested fermented milk in control as well as in all the pepsin-digested samples containing aqueous herbal extracts (Figure 4). Among all the samples, amla and arjuna pepsin-digested fermented milk exhibited comparatively same antimicrobial activity (17 mm) followed by harad and baheda pepsin-digested fermented milk samples (15 mm). Maximum antimicrobial activity was seen in aqueous arjuna extract (16 mm) followed by amla and harad having almost similar activity (13 mm).

No antimicrobial activity was seen in milk, fermented milk containing different aqueous herbal extracts and its overnight-digested samples. Streptomycin sulphate showed inhibition zone 22 mm diameter against *R. equi* (Figure 4).

Antimicrobial activity against *S. flexneri,* Gram-negative bacteria that causes diarrhoea in humans, was tested. The result exhibited that no antimicrobial activity was seen in all the samples of fermented milk containing different aqueous herbal extracts and corresponding *in vitro* digested samples (Figure 5). *T. bellerica* fruit has shown to reduce the pathogenicity of Gram-negative motile bacteria by rendering them less motile [18]. Streptomycin sulphate showed inhibition zone 21 mm against *S. flexneri.*

Figure 1: Details: Antimicrobial activity against B. cereus in digested samples of fermented milk containing aqueous herbal extracts. Plate 1-Control: A- Milk, B- Fermented milk (without aqueous herbal extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 2-Harad: A- Aqueous harad extract, B- Fermented milk (with aqueous harad extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 3-Baheda: A- Aqueous baheda extract, B- Fermented milk (with aqueous baheda extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 4-Amla: A- Aqueous amla extract, B- Fermented milk (with aqueous amla extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 5-Arjuna: A- Aqueous arjuna extract, B- Fermented milk (with aqueous arjuna extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 6: A- Nutrient Broth (-ve Control), B- Streptomycin Sulphate (+ve Control).

Figure 2: Details: Antimicrobial activity against *E. coli* in digested samples of fermented milk containing aqueous herbal extracts. Plate 1-Control: A- Milk, B- Fermented milk (without aqueous herbal extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 2-Harad: A- Aqueous harad extract, B- Fermented milk (with aqueous harad extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 3-Baheda: A- Aqueous baheda extract, B- Fermented milk (with aqueous baheda extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 4-Amla: A- Aqueous amla extract, B- Fermented milk (with aqueous amla extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 5-Arjuna: A- Aqueous arjuna extract, B- Fermented milk (with aqueous arjuna extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 6: A- Nutrient Broth (-ve Control), B- Streptomycin Sulphate (+ve Control).

Figure 3: Details: Antimicrobial activity against *S. aureus* in digested samples of fermented milk containing aqueous herbal extracts. Plate 1-Control: A- Milk, B- Fermented milk (without aqueous herbal extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 2-Harad: A- Aqueous harad extract, B- Fermented milk (with aqueous harad extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 3-Baheda: A- Aqueous baheda extract, B- Fermented milk (with aqueous baheda extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 4-Amla: A- Aqueous amla extract, B- Fermented milk (with aqueous amla extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 5-Arjuna: A- Aqueous arjuna extract, B- Fermented milk (with aqueous arjuna extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 6: A- Nutrient Broth (-ve Control), B- Streptomycin Sulphate (+ve Control).

Figure 4: Details: Antimicrobial activity against R. equi in digested samples of fermented milk containing aqueous herbal extracts.Plate 1-Control: A- Milk, B- Fermented milk (without aqueous herbal extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 2-Harad: A- Aqueous harad extract, B- Fermented milk (with aqueous harad extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 3-Baheda: A- Aqueous baheda extract, B- Fermented milk (with aqueous baheda extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 4-Amla: A- Aqueous amla extract, B- Fermented milk (with aqueous amla extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 5-Arjuna: A- Aqueous arjuna extract, B- Fermented milk (with aqueous arjuna extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 6: A- Nutrient Broth (-ve Control), B- Streptomycin Sulphate (+ve Control).

Figure 5: Details: Antimicrobial activity against *S. flexneri* in digested samples of fermented milk containing aqueous herbal extracts. Plate 1-Control: A- Milk, B- Fermented milk (without aqueous herbal extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 2-Harad: A- Aqueous harad extract, B- Fermented milk (with aqueous harad extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 3-Baheda: A- Aqueous baheda extract, B- Fermented milk (with aqueous baheda extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 4-Amla: A- Aqueous amla extract, B- Fermented milk (with aqueous amla extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 5-Arjuna: A- Aqueous arjuna extract, B- Fermented milk (with aqueous arjuna extract) Undigested, C- Pepsin Digest, D- Overnight Digest, Plate 6: A- Nutrient Broth (-ve Control), B- Streptomycin Sulphate (+ve Control).

S. No.	Sample	Treatments		Inhibition zone (mm)				
			Bacillus cereus	***Escherichia coli***	***Staphylococcus aureus***	***Rhodococcus equi***	***Shigella flexneri***	
1	Control	A	Milk	--	--	--	--	--
		B	Fermented milk (without herbal extract) undigested	--	--	--	--	--
		C	Pepsin digest	14	--	--	16	--
		D	Overnight digest	--	--	--	--	--
2	Harad	A	Aqueous harad extract	14	--	18	13	--
		B	Fermented milk (with aqueous harad extract) undigested	--	--	--	--	--
		C	Pepsin digest	15	--	--	15	--
		D	Overnight digest	--	--	--	--	--
3	Baheda	A	Aqueous baheda extract	--	--	--	--	--
		B	Fermented milk (with aqueous baheda extract) undigested	--	--	--	--	--
		C	Pepsin digest	15	--	--	15	--
		D	Overnight digest	--	--	--	--	--

4	Amla	A	Aqueous amla extract	12	--	13	13	--
		B	Fermented milk (without aqueous amla extract) undigested	-	--	--	--	--
		C	Pepsin digest	14	--	--	17	--
		D	Overnight digest	--	--	--	--	--
5	Arjuna	A	Aqueous arjuna extract	15	--	--	16	--
		B	Fermented milk (with aqueous arjuna extract) undigested	--	--	--	--	--
		C	Pepsin digest	14	--	--	17	--
		D	Overnight digest	--	--	--	--	--
6	Antibiotic control	A	Streptomycin sulphate (+ ve control)	17	20	20	22	21
		B	Nutrient broth (-ve control)	--	--	--	--	--

Table 1: Antimicrobial activity against different bacterial cultures in fermented milk containing aqueous herbal extracts and it's *in vitro* digested samples.

Conclusion

Maximum antimicrobial activity (18 mm diameter of zone of inhibition) was observed in aqueous harad extract against *S. aureus*. Fermented milk containing different aqueous extracts of herbal plants exhibited antimicrobial activity against *Bacillus cereus* and *R. equi*. Highest antimicrobial activity against *R. equi* was observed in pepsin-digested samples of fermented milk containing aqueous arjuna (17 mm diameter of zone of inhibition) and aqueous amla (17 mm diameter of zone of inhibition) extracts. However, no antimicrobial activity was noticed in fermented milk containing various aqueous herbal extracts against other bacterial cultures such as *E. coli, S. aureus* and *S. flexneri*.

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