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SOMA PAL SAHA

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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SOMA PAL SAHA*
Department of Microbiology, Darjeeling Govt. College, Darjeeling 734101, West Bengal

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Milk has an outstanding nutritional quality but it is an excellent medium for bacterial growth and an important source of bacterial infection when consumed without pasteurization. On the basis of morphology and utilization of carbon source three frequently available microbial contaminants have been isolated from eight different raw milk samples of Kolkata. The organisms were partially characterized. Attempt has been made to inhibit those bacteria and to increase the shelf-life of raw milk using six different natural antimicrobials, *Aloe vera* juice, *Syzygium aromaticum* (clove) oil, aquatic extract of *Ocimum* (tulsi) and *Curcuma longa* (turmeric), raw honey, and whey of *Lactobacillus* yogurt. Honey was used directly whereas others were applied as their lyophilized extract (1/10 volume reduced). The inhibition of microorganisms was determined following agar cup assay and the extent of efficacy of antimicrobial agents appeared as clove> honey> tulsi> whey >turmeric> aloe for all, except the Type II bacteria. The antibiotic resistant Type I, Type II and Type III bacteria revealed growth inhibition 64.75, 69.49 and 83% respectively, in NB with clove oil (0.1%, v/v) after 10h. The bactericidal activity of clove oil (0.1%) and diluted filter sterilized honey (1%, v/v) were comparable with those of ampicillin (100-200 μg/ml) and streptomycin (100 μg/ml) against the contaminants in filter sterilized raw milk.

Key words: Milk borne microorganisms, natural antimicrobial agents

INTRODUCTION

Since milk contains all the nutrients required for the growth of microorganisms it serves as a very good medium for their growth. Hence, milk and products derived from milk may harbor a variety of microorganisms which can be classified into two main groups: pathogenic and spoilage organisms, although some may play a dual role (e.g. *Bacillus cereus*). Spoilage of raw milk may caused by heterofermentatives such as coliforms, *Mycobacterium* and Micrococci that ferments lactose to equal ratios of lactic acid, CO_2, ethanol and volatile fatty acids. Hydrolysis of milk proteins may also occur by *Pseudomonas*, *Bacillus*, *Proteus* and *Streptococcus liquifaciens* and lypolytic action is caused by *Pseudomonas, Achromobacter lipolyticum, Serratia, Alcaligenes, Chromobacterium, Flavobacterium*, *Enterobacter, Candida lipolytica etc.* (Martins *et al*, 2006).

Numerous effort using natural antimicrobioals have been conducted to prevent growth of spoilage and pathogenic microorganisms in foods (Tiwari *et al,* 2009). Limited researches have been reported regarding the preservation of milk using those agents (Krushna *et al,* 2007). Main natural compounds are essential oils derived from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes obtained from animal sources (lysozyme, lactoferrin etc) bacteriocins from microbial sources (nisin, natamycin), organic acids (sorbic, propionic, citric acid) and naturally occurring polymers (chitosan etc.). Due to their antibacte-

*Email : spalsaha44@yahoo.co.in

rial, antifungal and antiviral activity, phenolic compounds and antioxidant biomolecules were the subject of anti-infective research for many years (Szabo *et al,* 2010; Cetin-Karaca and Newman, 2015). These activities suggested that phenolic compounds can be used as chemotherapeutic agents, food preserving agents and disinfectants. They can affect the growth and metabolism of bacteria, activating or inhibiting the microbial growth according to their constitution and concentration (Nazzaro *et al,* 2013). Usually, these compounds with phenolic groups are more inhibitory against Gram-positive than Gram-negative bacteria (Marino *et al,* 2001). The alcoholic extract of *Aloe vera* (Nejatzadeh-Barandozi, 2013), *Ocimum*, tulsi (Rathnayaka, 2013), Turmeric (Gupta *et al,* 2015) clove oil (Sheeladevi and Ramanathan, 2012) were reported for preservation of different food products. Honey is well known for its potent activity of against antibiotic-resistant bacteria (Mahendran and Kumarasamy, 2015) due to H_2O_2, methylglyoxal and bee defensin peptides etc. Whey of fermented milk products has been reported as a potential antioxidant (Khan *et al,* 2015) which contains whey proteins, lactoferrin, α-lactalbumin (α-LA), glycomacropeptide, immunoglobulins etc. It has a relatively broad spectrum activity against various Gram positive bacteria. Moreover, it is particularly effective against heat-resistant bacterial spores of *Clostridium botulinum* and against food-borne pathogens such as *L. mococytogenes, S. aureus,* or *B. cereus* (Kumari and Vij, 2015) and against antibiotic resistant *Helicobacter pylori* (Sachdeva *et al,* 2014) too.

In this study, the objectives were to evaluate the quality and the microbiological status of raw milk marketed at Kolkata and the effectiveness of six natural and traditional antimicrobial agents, *Aloe vera* juice, *Syzygium aromaticum* (clove) oil, aquatic extract of *Ocimum* (Tulsi) and *Curcuma longa* (turmeric), fresh raw honey, and whey of *Lactobacillus* yogurt to inhibit the milk -borne isolates and to increase the shelf-life of raw milk.

MATERIALS AND METHODS

Collection of milk samples

The raw milk samples (as it is sold in the market) were collected in sterile 100 ml capped containers. The samples, collected on the same day were transported to the laboratory in an insulated ice box and kept under refrigerator temperature till the experiments were performed.

Determination of milk grade

The milk samples were tested to evaluate their grade using methylene blue (MB) dye solution (EMERK). The sample (10 ml) was taken in sterile test tube and 0.1 ml of the dye solution was added to it to make the sample blue and the time for dye reduction (colorless) during incubation was noted. Boiled milk with dye was considered as control. For gradation of the milk samples the standard chart of *Classification of milk* (APHA) was followed.

Enumeration of microbes in milk samples and their characterization

Enumerations of milk-borne microbes was done using milk-agar (MA) medium following serial dilution and spread plate techniques. The colony forming units, CFU were counted after 48 h of incubation at 37^0C. The isolated organisms were characterized by their morphology, gram nature, motility, capsulation and sporulation. The experiments of utilization of carbon sources, nitrate reduction, catalase reaction, casein hydrolysis, antibiotic sensitivity, fermentation by the isolates were also determined following the standard microbiological methods.

Application of natural antimicrobial agents

Six different natural agents, *Aloe* juice, clove oil, aquatic extract of tulsi and turmeric, raw honey, and whey of yogurt. *Aloe* leaves (50 g) was taken and gel was scrapped from it. Tulsi leaves and turmeric (50 g each) were crashed and aquatic extract (as only the aquatic extract can be applicable for milk consumption) was made using a total of 50 ml of distilled water for individual agents. The extract of aloe, tulsi, turmeric and whey (50 ml) lyophilized to reduce the volume (1/10th). Clove oil was purchased from Jan Bazar market, Kolkata and raw honey was collected from local honey collector of Sundarban.

To study antimicrobial activity, each of the prepared agents was used (100-400 μl per well) following standard method of agar-cup assay and growth inhibition of isolates by the agents was determined in nutrient broth. Attempt had been made to compare bactericidal ability with those of ampi-

cillin and streptomycin against the isolates using the agents (0.1%, v/v) in filter sterilized raw milk.

RESULTS AND DISCUSSION

The time period required for MB reduction in raw milk samples, were 30 to 300 minutes and thus all of the samples appeared poor to fair grade milk (Table 1). The quality of milk samples, represented by the viable cells count (ranging 130 X 10^7 to 170 X 10^9 cells per ml) on MA also showed the similar results. Most of the raw milk samples available in open market showed quick MB reduction due to the presence of more oxidoreductase enzymes synthesized by high microbial load and rapid oxygen consumption by the milk-borne microorganisms.

On the basis of colony morphology three most frequently occurred bacterial strains were isolated which were common in all samples. The organisms (Fig.1) were characterized and represented in Table 2. A variety of carbon substrates were found utilizable, however, lactose and mannitol appeared as the preferred fermentable sugar by all of the isolates. Organisms also showed a high degree of resistance to the common antibiotics.

The antimicrobial activity of natural agents were tested against the two gram positive and one gram

Table 1 : Determination of raw milk quality following MB reduction and viable cell count method on milk agar plate

Raw milk sample	MB reduction time, min[a]	Grade	Viable bacterial count/ ml[b]
Sample 1	80	Poor	214 X 10^8
Sample 2	130	Fair	196 X 10^8
Sample 3	240	Fair	340 X 10^7
Sample 4	30	Very Poor	170 X 10^9
Sample 5	240	Fair	210 X 10^8
Sample 6	180	Fair	160 X 0^8
Sample 7	300	Fair	130 X 10^7
Sample 8	260	Fair	235 X 10^8

[a] MB reduction at 37°C; [b] viable count was taken after 48h of incubation at 37°C.

Table 2 : Characterization of bacterial strains isolated from raw milk samples

Features*	Type I	Type II	Type III
Colony morphology	Small, flat, transparent	Small, dark yellow thick	Large, white, flat
Gram nature	Negative	Positive	Positive
Shape	Bacilli	Short bacilli	Bacilli
Size	1.5 μm X 2.2 μm	1.2μm X 1.6 μm	1.2 μm X 2.5 μm
Motility	+	+	+
Capsulation	-	+	-
Sporulation	-	-	+
Casein degradation	+	+	+
Nitrate reduction	+	-	+
Catalase reaction	+	+	-
Utilizable carbon sources	Glucose, galactose, lactose, sucrose mannitol	Glucose, galactose, lactose, sucrose acetate, mannitol	Glucose, galactose, lactose, sucrose
Non-utilizable carbon sources	Maltose, citrate, acetate, malate, starch	Maltose, citrate, malate, starch	mannitol, starch
Fermentable sugar	Lactose, Mannitol	Lactose, Mannitol	Maltose, citrate, acetate, malate,
Resistance to antibiotics, 200 μg/ml (Disc diffusion method)	Penicillin, Ampicillin methicillin, erythromycin, vancomycin, oxytetracyclin	Penicillin, methicillin, Streptomycin vancomycin Oxytetracyclin	Lactose, Mannitol Penicillin, methicillin erythromycin, Streptomycin vancomycin, oxytetracyclin
Sensitive to 200 μg/ml	Streptomycin	Ampicillin,	Ampicillin

Features of the organisms were determined following the methodology described by Cappuccino and Sherman (2005).

Table 3 : Determination of optimum effective volume (il) of working natural antimicrobial extract per well against the bacterial strains isolated from raw milk

Natural antimicrobial agents	Reported antimicrobial chemicals	Type I *		Type II *		Type III*	
		µl/well	D**, mm	µl/well	D**, mm	µl/well	D**, mm
Aloe extract	Non-flavonoid polyphenols, pyrocatechol, coumaric acid, ascorbic acid, cinnamic acid, anthraquinoneglycoside as Barbaloin and isobarbaloin -	300	20	-	-	300	22
Clove oil	Eugenol acetyleugenol, chavicol, acetyl salicylate and humulenes	100	30	200	32	100	37
Honey (raw)	H_2O_2, methylglyoxal, Bee defensin 1	100	28	-	-	300	33
Tulsi extract	Ursolic acid flavonoids like apigenin, polyphenols, anthocyanins and luteolin, eugenol, thymol, sesquiterpene alcohols	300	25	400	22	300	26
Turmeric extract	curcumin, bisdemethoxycurcumin and demethoxycurcumin	300	21	400	27	300	24
Yogurt whey	Lactalbumin, lactoferrin, glycomacropeptide nisin, vanillin bacteriocin,	300	23	-	-	400	25

*The purified bacterial culture (0.1ml) was spreaded onto the MA medium. ** Diameter, zone of inhibition, mm measured after 24h of incubation at 37^0C.

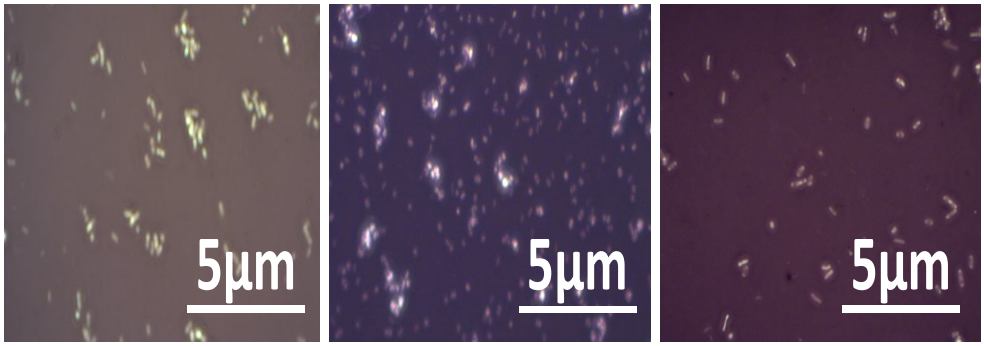

Fig. 1 : Bacterial strains (after negative staining) isolated from raw milk samples

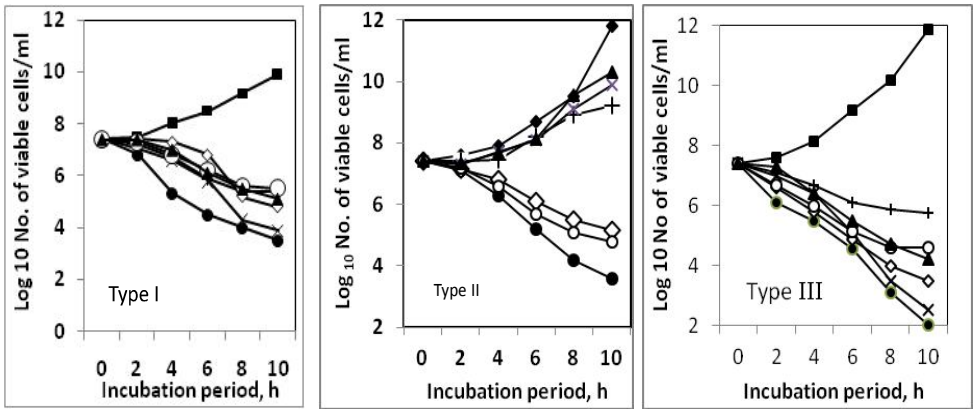

Fig. 2 : Effects of natural agents (0.1%, v/v) on the growth of milk-borne organisms, Type I, Type II and Type III in nutrient broth medium. Symbols: Control, growth in NB (-■-), NB with aloe extract (-+-), clove (-Ï%-), honey (-×-), tulsi (-Ê%-), turmeric (-O-), and yogurt whey (-▲-).

negative milk-borne bacterial strains on MA and the zone of inhibition was noted. The optimum effective volume of the all extracts, honey and whey were determined depending on the inhibition zone diameters and clove oil showed the best result for each isolates (Table 3).

Type II organism was less inhibited by aloe, honey and whey; where as other two strains showed susceptibility to all and extent of efficacy appeared as clove> honey> tulsi> whey >turmeric> aloe. The organisms, having resistance to common antibiotics, were found to be inhibited by the used agents of plant or microbial origin and this has been attributed by the respective antimicrobial chemical constituents listed in the Table 3. Similar approach for inhibition of antibiotic resistant pathogens *S. aureus, K. pneumoniae*, *P. aeruginosa, E. coli etc.* has been made by Joshi *et al,* (2011) using alcoholic extract of tulsi, clove and neem.

As the inhibition produced by the agents depends on variable diffusibility in agar medium, the antibacterial property was also tested in the broth medium. Data revealed that maximum 64.75, 69.49 and 83% of growth inhibition occurred by clove oil (0.1%, v/v) for streptomycin resistant Type I and ampicillin resistant Type II and Type III bacteria respectively (Fig.2). Raw honey also appeared as the next choice for Type I and III and turmeric for Type II bacteria.

In filter-sterilized raw milk addition of clove oil (0.1%, v/v) resulted in no growth of the isolates after 24h of incubation at 37^0C and thereafter, when it was subcultured on MA media. Similar bactericidal action of streptomycin (100 μg/ml) was noted against Type I bacteria and that of ampicillin (100 and 200 μg/ml, respectively) for Type II and Type III strains. Like earlier reports (Krushna *et al,* 2007; Chen *et al,* 2012), ten times diluted filter sterilized raw honey (1%, v/v) also revealed complete inhibition of catalase negative Type III strain due to H_2O_2 activity and partial inhibition of catalase positive Type I and II strains by other non-peroxide compounds.

Use of the natural antimicrobials in milk could reveal fewer side effects, better patient tolerance, relatively less expensive and acceptance due to long history of use. The present findings support the traditional knowledge and it is a preliminary, scientific, validation to promote proper use of such resources.

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