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INCIDENCE AND RELATIVE ABUNDANCE OF LACTIC ACID BACTERIA IN RAW MILK OF BUFFALO, COW AND SHEEP

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ABSTRACT

The study was intended to isolate lactic acid bacteria with promising potential for technological exploitation in dairy products application, and to determine their incidence and relative abundance in raw milk samples of buffalo, cow and sheep origin. In all, 79 identifiable isolates were obtained from 120 samples (40 from each species), thus the overall incidence of lactic bacteria in milk was 66 percent. On the basis of various morphological characteristics the isolates were differentiated into six groups; each group with common and differential characteristics of physiological / biochemical nature was finally identified up to species level. Percent incidence of lactic isolates was highest in cow milk (30/40 = 75%), followed by buffalo milk (27/40 = 68%) and sheep (22/40 = 55%). It was found that buffalo milk contained 5 species viz., *Lactobacillus acidophilus* (25%), *Lactobacillus delbrueckii ssp. bulgaricus* (21%), *Lactococcus lactis ssp. cremoris* (21%), *Lactococcus lactis ssp. lactis* (19%), and *Streptococcus thermophilus (*14%*).* Four species identified in cow milk samples were, *Streptococcus thermophilus* (34%), *Lactococcus lactis ssp. lactis* (28%), *Lactobacillus delbrueckii ssp. bulgaricus* (28%) and *Lactococcus lactis ssp. cremoris* (10%). Isolates of sheep milk were identified as *Lactococcus lactis ssp. lactis* (36%), *Lactococcus lactis ssp. cremoris* (32%), *Lactobacillus acidophilus* (22%) and *Leuconostoc spp.* (10%). Only two species, *Lactococcus lactis ssp. lactis* and *Lactococcus lactis ssp. cremoris* were found common in all the three types of milk whereas *Leuconostoc spp.* was unique to sheep milk only.

Key Words: lactic acid bacteria, raw milk, buffalo, cow, sheep

INTRODUCTION

Variety of microorganisms including yeasts, molds and bacteria are present in raw milk. However, among these organisms, only the lactic acid bacteria (LAB) have the property of producing lactic acid from milk sugars by the process of fermentation and thus LAB constitute the predominant microflora of milk. These bacteria are responsible for most of the physiochemical and aromatic transformations intrinsic to fermented dairy products (Ogier *et al*, 2002). The LAB consume natural milk sugars and release lactic acid that increases acidity, rendering milk proteins, especially casein, to denature and tangle into a solid mass or curd. They also cause the release of bioactive compounds which contribute to important physical, chemical and therapeutic properties of fermented milk products (Van-Neil *et al*, 2002; Otles and Cagindi, 2003).Interest in these microbes, their various species, biotypes and strains is primarily because of their biotechnological potential of efficient biotransformation essential for dairy product preparation (Desmasures *et al*, 1997 and Leisner *et al*, 1999).

In dairy industry these organisms are known as starter cultures, and their essential roles are, firstly production of lactic acid which imparts a distinctive fresh and acidic flavor during manufacture of fermented milks. Lactic acid is also important in cheese making during the process of coagulation and texturization of curd. Secondly, for the production of volatile compounds (e.g. diacetyl and acetaldehyde etc) contributing towards aroma of dairy products. Thirdly, the starter cultures may possess proteolytic and lipolytic activities which may be desirable especially during the maturation of certain types of cheese. Lastly, acidic condition in these products prevents the growth of pathogens, as well as many spoilage organisms (Tamime, 1981).

Starter culture of desirable characteristics is the first limiting factor for the modern dairy industry and the success story of fermented dairy products revolves around collection / repository of well defined bacterial strains. Such collections are fundamental for successful starter culture management programs which are based upon rotation of different strains/biotypes. Hence, there is constant demand for new isolates so that new strain combinations can be made to avoid culture failure and to introduce new varieties of fermented dairy products. This study was undertaken to explore and search biotypes and strains of LAB of indigenous origin. Raw milk of different domestic animals, being natural niche and habitat of lactic bacteria, was used for strain hunt so that bacterial repository of wider biological diversity can be established. Apart from this, the study was also intended to determine relative incidence and abundance of various dairy starter species in raw milk of buffalo, cow and

sheep origin so these potential habitats can be identified for different starter species.

MATERIAL AND METHODS

Collection of samples: From the surroundings and within the capital city (Islamabad) 120 milk samples (40 each of buffalo, cow and sheep milk), from different sources like public dairy farms, peri-urban dairies, individual small holders and milk producers were collected during the period, October, 2004 to May, 2005. For this purpose udders / teats were washed and dried before sample collection. Milk was collected in sterile pouches. The samples were transferred to the laboratory in chilled condition (10 – 12°C) for microbial study.

Inoculation, screening, isolation etc: The samples were screened by inoculating them on solid media on the basis of Gram's staining (Cappuccino and Sherman, 1996; Lois, 1996), and catalase test (Collins and Lyne, 1980). As LAB are invariably Gram positive and catalase negative (Sharpe, 1976) hence only Gram positive and catalase negative colonies were selected for further study / identification at genus and species level. After screening the samples obtained, which were either cocci or rods, were inoculated on M-17 or MRS (de Man, Ragosa, and Sharpe) agar respectively (both from Oxoid, UK) for selective isolation at earlier stage and were incubated at 37°C unless otherwise required for complementary test at 10°C or 45°C. A total of 79 isolates of LAB were selected randomly from pinpoint colonies and then subcultured on nutrient agar (Oxoid, UK) after selective growth on M-17 or MRS or M-17 agar. Isolates were stored in a protective bead storage system ('Microbank', Pro-Lab Diagnostics) at – 25°C.

For presumptive identification rehydrated MRS broth (Oxoid, UK) was prepared according to the manufacturer's instructions. The pH of the broth was adjusted to 5.20 and 4.58 using 1.0 M HCl to obtain the pH-modified/adjusted agar. This modification/adjustment was done to isolate specific LAB selectively and identify among the rod shaped species, as discussed by Tharmaraj and Shah (2003).

Characterization and final identification: Before being tested, the isolates were subcultured overnight twice on M-17 and MRS agar at 37°C. The physiological tests conducted were carried out by growth in MRS (for rods) or M-17 (for cocci) broth with 4.5% or 6.5% NaCl concentration and similarly growth in respective broth at 10°C and 45°C and pH 9.6. The biochemical tests included were sugar fermentation and ammonia production. Identification was done by comparison of the results with those described and discussed by Sharpe (1976), Tamime (1981) and Cogan (1996).

RESULTS AND DISCUSSION

As the taxonomy of LAB is a fast growing and expanding subject there is confusion of terminology and sometime it varies from author to author. For sake of discussion we will stick to new taxonomy; however for clarity, the new and old nomenclature with abbreviations are given in Table 1.

Morphology and colony characteristics: By using MRS medium at pH 5.20 two distinct types of colonies were observed. One of the types having had rough colony surface, relatively smaller size (0.1 - 0.5 mm) with entire margin and dull white color was placed in Group I (Table 2). The other type having had cottony rough surface, relatively larger size (1.0 mm) with irregular margin and whitish color was placed in Group II. The former type was identified as *Lb. acidophilus* and the latter was identified as *Lb. delbrueckii ssp. bulgaricus* the identification of which was further confirmed by growing the selected isolates on the same media but at pH 4.58. Because of these distinct colony characteristics and their differential growth at different pH on the same media the identification of the two was presumed (Table 2) as *Lb. acidophilus* and *Lb. delbrueckii ssp. bulgaricus*. Further to that, the final confirmation was carried out by physiological and biochemical tests as shown in Table 3 and 4.

Physiological characteristics: The samples grown on M-17 selective medium were identified as Gram positive and catalase negative cocci. As the apparent colony characteristics of cocci as a group (Table 2) are relatively similar, their differentiation at genus and species level was carried out by studying their physiological characteristics such as growth at different pH and incubation temperatures and tolerance to different salt levels (Table 3).

All the isolated cocci were found capable to grow at 37ºC. However, when later subjected to series of tests for their growth behavior at 10ºC and 45ºC they were found to be either mesophilic or thermophilic; the reason behind could be the environmental conditions as normal ambient temperature of this region/city (15 to 35ºC) supports the growth of either mesophilic or thermophilic isolates / strains of lactic acid producing bacteria. It was found that rods in Group I and II both were unable to grow at 10ºC, but were capable to grow successfully at 45ºC, and were identified as thermophiles. When cocci were subjected to these incubation temperatures, it was found that cocci in Group III, IV and VI were able to grow at 10ºC only and not at 45ºC and thus were identified as mesophiles. The cocci in Group V, however, grew well at 45ºC and therefore, were thermophilic in nature.

High pH was observed to be an unfavorable condition for all isolates because none was able to grow

at pH 9.6. Due to importance of varying temperatures and pH conditions in identification of LAB, it is possible that differential / controlled change of pH and temperature in specific increments can be an effective tool for characterization and comparative physiological studies of LAB. Ability of isolates to tolerate different saline conditions was also examined. Isolates were inoculated in media with added common salt at the rate of 4.5 and 6.5 percent. The concentration of 4.5 percent was found tolerable to all isolates except the cocci of Group IV and Group V. Similar observations were recorded by Kobayashi *et al (*2004) who found that both high and low saline concentrations, temperature and pH values had significant impact on the growth of LAB. Guessas and Kihal (2004) also supported this observation.

Biochemical characteristics of rods and cocci: All isolates were tested for fermentation of glucose, fructose, lactose, mannitol, sucrose and maltose. Lactose, fructose and glucose were utilized by all strains as carbon source to produce lactic acid. The identification was then done by differences in the fermentation profile of the rest of sugars and related compounds. Group I and II both were thermophilic rods. It was found that rods in Group I fermented all the above mentioned compounds except mannitol; however, the group II rods were unable to ferment maltose, sucrose and mannitol. Moreover, isolates of both the groups were unable to produce ammonia from arginine (Table 4).

All the cocci were placed in four groups (Group III to VI). The cocci of Group III were able to ferment glucose, fructose, lactose and maltose while a variable reaction was revealed for sucrose. The isolates of this group were also positive for production of ammonia from arginine. The isolates in Group IV were only able to ferment glucose, fructose and lactose and were negative for all other tests. The isolates in Group V were able to ferment glucose, fructose, lactose as well as sucrose, but were negative for the rest of the biochemical tests. The isolates in Group VI fermented glucose fructose and lactose, and were negative for all other tests.

Final identification and confirmation: The results were compared with Sharpe (1976), Tamime (1981) and Cogan (1996), for identification and confirmation.

Rods: On the basis of morphological and biochemical characteristics bacilli of Group I, capable to grow at 45ºC, but not at 10ºC and capable of fermenting all carbohydrates except mannitol and unable to produce ammonia from arginine were identified as *Lactobacillus acidophilus* (Table 4). On the other hand bacilli of Group II were unable to grow at 10ºC. Moreover, these isolates were also capable to ferment glucose, fructose and lactose, but were incapable to ferment mannitol, sucrose and maltose and didn't produce ammonia from arginine; therefore these were identified as *Lactobacillus delbrueckii ssp. bulgaricus* (Table 4).

Cocci: Cocci from Group III were able to grow at 10º C and 37º C, but not at 45º C. They were also unable to tolerate 6.5% salt concentration and pH 9.6. They were able to ferment all sugars except mannitol and were identified as *Lactococcus lactis ssp. lactis*, while cocci of Group IV were identified as *Lactococcus lactis ssp. cremoris* (Table 4). The two species of cocci are closely related. The difference found between these two species was that *Lactococcus lactis ssp. cremoris* was unable to tolerate 4.5% salt concentration while *Lactococcus lactis ssp. lactis* was able to grow at same salt concentration. In addition, the *Lactococcus lactis ssp. lactis* has the ability to produce ammonia from arginine while *Lactococcus lactis ssp. cremoris* did not possess this character.

Group V cocci in chains showed growth at 37ºC and 45ºC, but not at 10ºC. These were able to ferment all sugars except mannitol and maltose and were identified as *Str. thermophilus.* On the other hand chains and pairs of cocci placed in Group VI were identified as *Leuconostoc* due to their ability to grow at 10ºC and 37ºC, but not at 45ºC. This species was capable to ferment all sugars except mannitol, maltose and sucrose and was incapable to produce ammonia from arginine (Table 4).

Relative abundance of different isolates and species: Out of 79 total isolates obtained from 120 samples (40 from each species) rods were placed in two groups; Group I included 12 isolates and Group II 14 isolates. Cocci were categorized into four groups among which Group III comprised of 21 isolates and 16 isolates were placed in Group IV. Group V and Group VI included 14 and 2 isolates respectively (Table 5).

In total four genera and six bacterial species from three types of raw milk were identified during the course of study. The frequency distribution and relative abundance of various species have been shown in Fig 1, 2 and 3.

Buffalo Milk: Total five LAB bacterial species were identified from the 27 isolates obtained from buffalo milk. The species identified were, *Lb. acidophilus* (25%), *Lb. delbrueckii ssp. bulgaricus* (21%), *Lc. lactis ssp. cremoris* (21%) *Lc. lactis ssp. lactis* (19%) and *Str. thermophilus* (14%). *Lb. acidophilus* was found relatively dominating species of buffalo milk (Fig 1).

Cow Milk: Among 30 LAB isolates of LAB obtained from cow milk, four bacterial species were identified, which included *Str. thermophilus* (34%), *Lb. delbrueckii ssp. bulgaricus* (28%), *Lc. lactis ssp. lactis* (28%) and *Lc. lactis ssp. cremoris* (10%). *Str. thermophilus* was found relatively dominant and abundant species of cow milk (Fig 2).

Sheep Milk: In all, 22 LAB isolates were obtained from sheep milk. They were identified as *Lc. lactis ssp.lactis* (36%), *Lc. lactis ssp. cremoris* (32%), Lb. *acidophilus* (22%) and *Leuconostoc spp* (10%). *Lc. lactis ssp. lactis* was found to be the dominant species of sheep milk (Fig.3). Moreover, Leuconostoc species was isolated only from sheep milk and its low frequency is probably due to their inability to compete with other LAB in mixed cultures environment (Togo *et al*, 2002).

In Rawalpindi region Toqeer *et al* (2002) isolated *Lc. lactis ssp. cremoris, Lc. lactis ssp. lactis* and *Lb. acidophilus* from camel milk and reported that *Lb. acidophilus* grew relatively more rapidly in camel milk. Similarly *Lc. lactis ssp. lactis* and *Str. thermophilus* were found as dominant microflora of raw milk samples, collected by Al-Sheikhli and Abood (1979). In addition to above mentioned microbial assemblage (Table 5), Naeem and Rizvi (1983) also reported presence of *Lb. helveticus, Lb. casei* and *Lb. acidophilus* in different samples of dahi collected from Lahore city. Togo *et al* (2002) reported isolation and identification of potential lactic starters from unusual indigenous niche for LAB i.e. opaque beer (Chibuko).

Fifty samples of indigenous dahi were collected randomly from the local market of Rawalpindi/Islamabad by Masud *et al* (1991) to determine the incidence of lactic acid bacteria. The micro-organisms isolated were *Lactobacillus bulgaricus* (86%), *Streptococcus thermophilus* (80%), *Streptococcus lactis* (74%), *Lactobacillus helveticus* (34%), *Streptococcus cremoris* (30%), *Lactobacillus casei* (20%) and *Lactobacillus acidophilus* (14%) respectively. The results of the present study revealed that indigenous dahi contains mixtures of lactic acid bacteria and thus the quality of dahi may vary with the species type of starter culture used for inoculation.

Conclusions: From the observations noted above on relative abundance of different LAB in different types of raw milk few generalizations can be made, although there are no definite conclusions. Buffalo milk is likely to have less relative abundance (27/40 = 68%) of LAB than cow milk (30/40 = 75%) and the sheep milk further less (22/40 = 55%). Cow milk is more likely to contain yoghurt starter species, whereas cheese starter strains are likely to be found in sheep milk. LAB microflora of buffalo milk is mixed type and is likely to be good as butter starter. This is perhaps due to predilection of lactic bacterial consortia for specific and distinct niches provided by the milk of different species. More importantly, it also seems that LAB predominate in raw milk samples universally and the variations in microflora seem primarily due to geographical, environmental and milk compositional differences among different milk species.

Table 1: New & old taxonomy of LAB with their abbreviations reported in this study

New	Old	General Abbreviations
Lactobacillus delbrueckii ssp. bulgaricus	*Lactobacillus bulgaricus*	*Lb. delbrueckii ssp. bulgaricus*
Lactobacillus acidophilus	*Lactobacillus acidophilus*	*Lb. acidophilus*
Streptococcus thermophilus	*Streptococcus salivarius ssp. thermophilus*	*Str. thermophilus*
Lactococcus lactis ssp. cremoris	*Streptococcus cremoris*	*Lc. lactis ssp. cremoris*
Lactococcus lactis ssp. lactis	*Streptococcus lactis*	*Lc. lactis ssp. lactis*
Leuconostoc spp.	*Leuconostoc spp.*	*Leuc. spp.*

Table 2: Morphological characteristics of isolated bacterial groups

CHARACTERISTICS	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
	Rods		Cocci			
Colony surface	Rough	Cottony, rough	Smooth	Smooth	Smooth	Slimy
Colony size	0.1– 0.5 mm	1.0 mm	0.1– 0.5 mm	0.1– 0.5 mm	0.1– 0.5 mm	0.5 – 1.0 mm
Colony margin	Entire	Irregular	Entire	Entire	Entire	Undulate
Colony color	Dull white	White	White	White	White	White
Cell morphology	Medium to short rods	Long rods in chains	Chains	chains	chains	Chains/ pairs
Presumptive identification	*Lactobacillus*	*Lactobacillus*	*Lactococcus*	*Lactococcus*	*Streptococcus*	*Leuconostoc*

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Table 3: Physiological characteristics of isolated bacterial species

CHARACTERISTICS	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
Growth at 10ºC	-ve	-ve	+ve (mesophilic)	+ve (mesophilic)	-ve	+ve (mesophilic)
Growth at 37ºC	+ve	+ve	+ve	+ve	+ve	+ve
Growth at 45ºC	+ve (thermophilic)	+ve (thermophilic)	-ve	-ve	+ve (thermophilic)	-ve
Growth at pH 9.6	-ve	-ve	-ve	-ve	-ve	-ve
Growth in 4.5% NaCl	–	–	+ve	-ve	-ve	+ve
Growth in 6.5% NaCl	–	–	-ve	-ve	-ve	-ve
Presumptive identification	*Lb. acidophilus*	*Lb. delbrueckii ssp. bulgaricus*	*Lc. lactis ssp. lactis*	*Lc. lactis ssp. cremoris*	*Str. thermophilus*	*Leuc. spp*

Table 4: Biochemical characteristics of isolated bacterial species

SUGARS	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
Glucose	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	-ve	-ve	-ve	-ve	-ve	-ve
Sucrose	+ve	-ve	±	-ve	+ve	-ve
Maltose	+ve	-ve	+ve	-ve	-ve	-ve
NH_3 from arginine	-ve	-ve	+ve	-ve	-ve	-ve
Final confirmation	*Lb. acidophilus*	*Lb. delbrueckii ssp. bulgaricus*	*Lc. lactis ssp. lactis*	*Lc. lactis ssp.cremoris*	*Str. thermophilus*	*Leuc. spp*

Table 5: Relative abundance of isolates of various species of LAB in milk samples (N = 120)

Group	Species	Buffalo Milk	Cow Milk	Sheep Milk	Total
I	*Lb. acidophilus*	7	-	5	12
II	*Lb. delbrueckii ssp. bulgaricus*	5	9	-	14
III	*Lc. lactis ssp. lactis*	5	8	8	21
IV	*Lc. lactis ssp.cremoris*	6	3	7	16
V	*Str. thermophilus*	4	10	-	14
VI	*Leuc. spp*	-	-	2	2
	Total	27	30	22	79

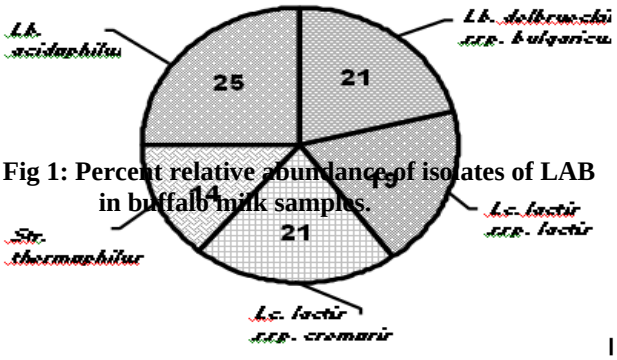

Fig 1: Percent relative abundance of isolates of LAB in buffalo milk samples.

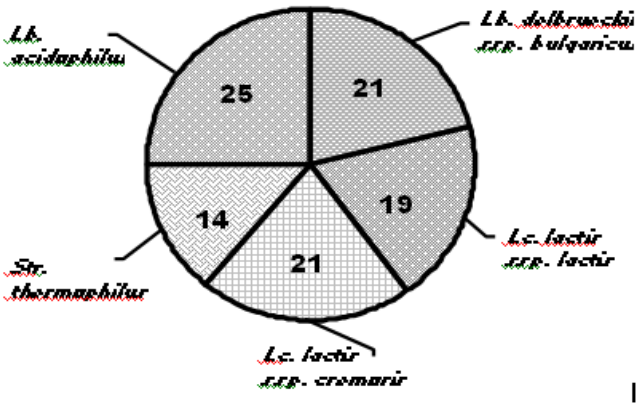

Fig 2: Percent relative abundance of isolates of LAB in cow milk samples.

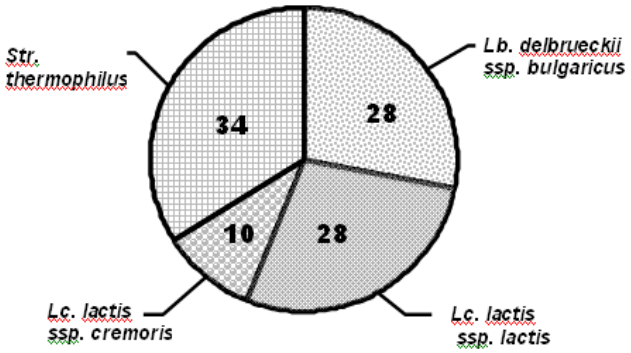

Fig 3: Percent relative abundance of isolates of LAB in sheep milk samples.

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