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# Growth Control of Standard L.monocytogenes and L.monocytogenes Spiked in Goat Milk by Natural products, Antibiotics and Lactic Acid Bacteria

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**Abstract**

*Listeria monocytogenes is an important pathogen in medical and veterinary sciences. It is ubiquitous in the farm and food industrial environment and therefore control of this bacterium is extremely difficult. In the present study growth control of this bacterium was studied using crude extracts of five different plants (Terminalia arjuna, Terminalia chebula, Cinnamomum zeylanicum, Citrus sinensis, Ficus religiosa), Antibiotics (Ampicillin, Chloramphenicol, Gentamycin, Penicillin, Tetracyclin and Vancomycin ), Standard Lactic acid bacteria (Lactobacillus casei, Lactococcus lactis subspecies cremoris, Lactobacillus delbrueckii, Lactobacillus acidophilus and LAB isolates from goat milk. Growth control of Listeria spiked in raw goat milk was also studied so as to determine the effect of raw goat milk environment on L.monocytogenes, i.e. whether Listeria becomes more resistant or more sensitive to the controlling agents under study. L.monocytogenes spiked in raw goat milk was found to be more sensitive to controlling agents as larger inhibition zones were observed as compared to the standard L.monocytogenes. Ethanolic extract of Cinnamomum zeylanicum, Terminalia arjuna and LAB isolates from goat milk were found to be most effective in controlling L.monocytogenes growth.*

**Key words: Lactic acid bacteria, goat's milk, alcoholic extract**

## Introduction

*Listeria monocytogenes* is a small, motile, gram positive, nonspore forming, extremely resistant, diptheroid coccobacillus that grows under a wide temperature range 4-40 $^0$C. Listeriosis has been recognized as an emerging food borne bacterial infection and a nagging public health hazard (Farber & Peterkin, 1991). It can cause serious invasive illnesses, mainly in certain well defined high risk groups, including elderly and immunocompromised patients, pregnant women, newborns and infants. *L.monocytogenes* primarily causes abortion, septicemia or meningitis. The public health importance of listeriosis is not always recognized, particularly because listeriosis is relatively a rare disease compared with other common food borne illnesses.

However because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food borne illnesses, ranking second after salmonellosis. The problem of human Listeriosis due to consumption of contaminated foods has increased worldwide (Bortolussi, 2008). Therefore controlling Foodborne listeriosis is an important task. Various antibiotics like Penicillin, Florfenicol, Ampicillin, Ceftiofur, Erythromycin and Tetracycline are being used presently for this purpose. *Listeria monocytogenes* is resistant to Ceftiofur but susceptible to other antibiotics (Aarestrup *et al*., 2007). The spread of drug resistant pathogen is one of the most serious threats to successful treatment of microbial diseases. Down the ages, plant extracts and essential oils have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases. Besides this extracts of natural products and medicinal plants may be of value as a novel means for controlling zoonotic pathogen such as *Listeria*. These natural antimicrobials are being used to increase antibiotic susceptibility of drug resistant bacteria (Palaniappan & Holley, 2010).

Lactic acid bacteria are heterogeneous group of bacteria that are generally regarded as safe for use in food and food products (Rodriguez *et al*., 2000). They are able to inhibit spoilage and pathogenic bacteria due to production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins (Hoover, 2000; Lindgren & Doborogosz, 1990). Many Lactic acid bacteria produce proteinaceous antimicrobial bacteriocins, some of which could provide valuable alternatives to traditional therapeutic antibiotics for the treatment of infectious diseases.

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## Material and Methods

**Bacteria.** Standard strains of *Listeria monocytogenes* (MTCC-1143), *Lactobacillus casei* (MTCC-1423), *Lactococcus lactis* subspecies *cremoris* (MTCC-1484), *Lactobacillus delbrueckii* (MTCC-911), *Lactobacillus acidophilus* (MTCC-440) were procured from MTCC Chandigarh.

**Reviving of the standard strains.** *Listeria monocytogenes* (MTCC-1143) *&* *Lactobacillus acidophilus* (MTCC-440) were maintained in BHI broth. *Lactobacillus casei* (MTCC-1423) was revived in MRS broth while *Lactococcus lactis* subspecies *cremoris* (MTCC-1484) & *Lactobacillus delbrueckii* (MTCC-911) were revived in skimmed milk broth.

**Isolation of lactic acid bacteria from goat milk.** Samples of raw goat milk were collected from various areas of Agra city. MRS agar and broth were used for culture of LAB (De Man *et al*., 1960). Loop full inoculum from enriched MRS broth was streaked on MRS Agar plates. Plates were incubated for 24 hrs at 37 $^0$C and examined for typical colonies. Well isolated, whitish colonies were picked from each plate and transferred to 1 ml MRS broth & incubated for 18 hrs at 37$^0$C. Enriched cultures were further double enriched in 3 ml MRS broth for 24 hrs at 37$^0$C & stored at 4$^0$C for further characterization.

**Identification of LAB isolates.** Determination of the isolates was performed according to their morphological, physiological and biochemical characteristics (Banson, 1990 and Agrawal *et al*., 2003). Initially all the isolates were examined by Gram staining and for catalase production. VP-test, gas production form glucose, hot-Loop test and fermentation of sugars were applied to distinguish the morphological, physiological and biochemical characteristics of the isolates.

***Listeria monocytogenes* spiked in goat milk.** 100 µl of *L.monocytogenes* culture (MTCC-1143) was inoculated in test tube containing 900 µl of raw goat milk. Growth of *L. monocytogenes* was observed after 24 hrs

**Checking antibiotic susceptibility.** Antibiotic susceptibilities were determined by using disk diffusion method. Susceptibility of *Listeria monocytogenes* spiked in the goat milk sample and standard *Listeria monocytogenes (*MTCC 1143) to the following antibiotics were determined: Tetracyclin, Gentamycin, Chloramphenicol, Penicillin, Ampicillin, Vancomycin. Muller-Hinton agar plates were prepared and inoculated with the test organism. Turbidity of the test inoculums was matched with the tube No. 4 of the McFarland standard series (~ 12x10$^8$ cells/ml). 1 ml of this was spread on MHA agar & antibiotic disks of 25 µg were placed on it. Zones of inhibition around the antibiotic disk were measured after incubation for 24 hrs at 37$^0$C.

**Preparation of crude extracts of Natural products.** Extracts were prepared separately in the conical flasks by ethanolic extraction by soaking 25 g of dried plant part in 100 ml of 70% ethyl alcohol & aqueous extraction by soaking 25 g of dried plant part in 100 ml distilled water for 72 hrs at ambient temperature. The mixture was then filtered and the filtrate obtained was kept in refrigerated conditions until use.

**Checking Antimicrobial activity of Plant extracts.** The screening of the alcoholic and aqueous extracts of these plants for anti-listerial activity was performed using well diffusion method. MHA agar was prepared and 200 µl of test culture (12x10$^8$ cell/ml) was inoculated.

**Detection of antagonistic activity of isolated Lactic acid bacteria from goat milk against *L.monocytogenes.*** Isolated colonies of LAB isolates were screened for antilisterial activity by using well diffusion method as described by Spelhaug and Harlander (1989) with several modifications. Muller Hinton Agar (MHA) was prepared and 200µl of *Listeria monocytogenes* culture (12x10$^8$ cells/ml) was inoculated. When media was solidified wells were made & in each well 40µl of isolated LAB cultures (12x10$^8$ cells/ml) were added. Inhibition

## Results and Discussion

**Identification of LAB isolates from goat milk: -** The isolates were identified by biochemical and physiological characteristics. 60% belonged to the genus *Lactococcus*, 20% to *Pediococcus*, 10% to *Leuconostoc* and *Enterococcus* (Table 1, 2).

**Table 1. Morphological, physiological and biochemical characteristics of isolated Lactic acid bacteria**

| S. No. | LAB isolates | Cell morphology | Gram staining | Spore formation | Catalase activity | VP test | Gas production | Hot loop test |
|---|---|---|---|---|---|---|---|---|
| **1** | A7d | Coccus (single) | + | - | + | + | - | - |
| **2** | A12b | Diplococci | + | - | - | + | - | - |
| **3** | B7a | Cocci in pairs | + | - | - | + | - | - |
| **4** | D2a | Cocci in pairs | + | - | - | - | - | - |
| **5** | N2c | Diplococci | + | - | - | - | + | + |
| **6** | N3c | Cocci in pairs | + | - | - | - | - | - |
| **7** | T3c | Cocci in tetrads | + | - | - | + | - | - |
| **8** | T4b | Cocci in tetrads | + | - | - | - | - | - |
| **9** | T5d | Cocci in tetrads | + | - | - | + | - | - |
| **10** | T7c | Coccus (single ) | + | - | - | + | - | - |

**Table 2. Biochemical characteristics of the isolated LAB: Acid production by utilization of different carbon sources**

| S. No. | LAB isolates | Arabinose | Galactose | Glucose | Lactose | Maltose | Mannose | Melibiose | Melezitose | Raffinose | Salicin | Sucrose | Trehalose | Xylose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | A7d (*Lactococcus*) | - | + | + | - | - | + | + | + | + | + | + | + | + |
| **2** | A12b(*Enterococcus*) | - | - | + | - | + | + | + | + | + | + | - | + | + |
| **3** | B7a (*Lactococcus*) | - | + | + | - | - | + | + | + | + | + | - | + | + |
| **4** | D2a (*Lactococcus*) | - | + | + | - | + | + | + | + | + | + | - | + | + |
| **5** | N2c (*Leuconostoc*) | - | + | + | - | - | - | - | + | + | + | - | + | + |
| **6** | N3c (*Lactococcus*) | - | - | + | - | + | + | + | + | + | + | - | + | + |
| **7** | T3c (*Pediococcus*) | - | + | + | - | + | + | + | + | + | + | - | + | + |
| **8** | T4b (*Pediococcus*) | - | + | + | - | - | + | + | + | + | + | - | + | + |
| **9** | T5d (*Lactococcus*) | - | + | + | - | - | + | + | + | + | + | - | + | + |
| **10** | T7c (*Lactococcus*) | - | + | + | - | - | + | - | + | + | + | - | + | + |

**Antibiotic susceptibility: -** *Listeria monocytogenes* was found to be susceptible to all the tested antibiotics. Penicillin, Ampicillin and Vancomycin exhibited excellent antibacterial activity. The sensitivity to antibiotics, of standard *Listeria monocytogenes* & the one which is spiked in goat milk sample was found to be different. Larger inhibition zones were recorded for all antibiotics against standard *L.monocytogenes* than the one which was spiked in goat milk sample except for Chloramphenicol (Table 3, Fig 1). The biggest inhibition zones were recorded for Penicillin, Ampicillin & Vancomycin against standard *L.monocytogenes*. This suggested that these antibiotics are very efficient in controlling listeriosis. Larger inhibition zones were recorded for all antibiotics against standard *L.monocytogenes* except Chloramphenicol which exhibited larger zone of inhibition against *L.monocytogenes* isolated from the spiked goat milk.

Chloramphenicol (Table 3, Fig 1). The biggest inhibition zones were recorded for Penicillin, Ampicillin & Vancomycin against standard *L.monocytogenes*. This suggested that these antibiotics are very efficient in controlling listeriosis. Larger inhibition zones were recorded for all antibiotics against standard *L.monocytogenes* except Chloramphenicol which exhibited larger zone of inhibition against *L.monocytogenes* isolated from the spiked goat milk.

**Table 3. Antibiotic susceptibility of *L.monocytogenes***

| S.No. | Antibiotic target | Antibiotic | Diameter of inhibition zone (mm) against *L.monocytogenes* isolated from the spiked goat milk (A7 + *Listeria*) | Diameter of inhibition zone (mm) against *Listeria monocytogenes* (MTCC-1143) |
|---|---|---|---|---|
| 1. | Protein synthesis | Tetracyclin | 15 mm | 18 mm |
| | | Chloramphenicol | 20 mm | 16 mm |
| | | Gentamycin | 15 mm | 20 mm |
| 2. | Cell wall synthesis | Penicillin | 18 mm | 30 mm |
| | | Ampicillin | 12 mm | 30 mm |
| | | Vancomycin | 25 mm | 29 mm |

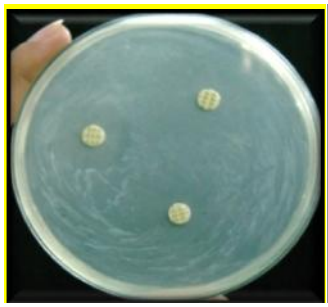

**Fig 1. Antibiotic susceptibility of *L.monocytogenes***

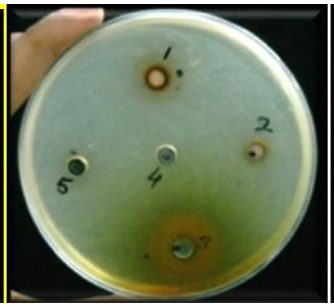

**Fig 2. Inhibition of *L.monocytogenes* by ethanolic plant extracts**

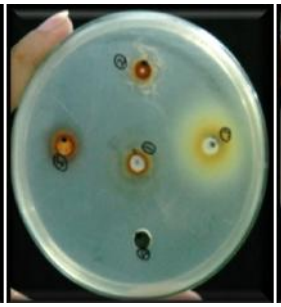

**Fig 3. Inhibition of *L.monocytogenes* by aqueous plant extracts**

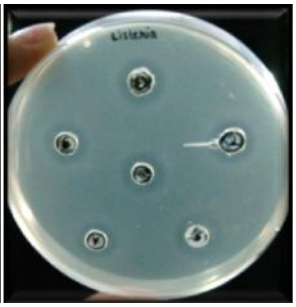

**Fig 4. Innhibition of *L.monocytogenes* by LAB isolates from goat milk**

**Table 4. Inhibition of *L.monocytogenes* by the extracted ethanolic and aqueous plant extracts**

| S.No | PLANT EXTRACTS | | Diameter of inhibition zone (mm) against *L.monocytogenes* isolated from the spiked goat milk(A7 + *Listeria)* | *Diameter of inhibition zone (mm) against Listeria monocytogenes* (MTCC-1143) |
|---|---|---|---|---|
| 1. | *Terminalia arjuna* | Aqueous | 10 mm | 11 mm |
| | | Alcoholic | 14 mm | 13 mm |
| 2. | *Cinnamomum zeylanicum* | Aqueous | 9 mm | 9 mm |
| | | Alcoholic | 15 mm | 10 mm |
| 3. | *Terminalia chebula* | Aqueous | 23 mm | 16 mm |
| | | Alcoholic | 26 mm | 20 mm |
| 4. | *Citrus sinensis* | Aqueous | - | - |
| | | Alcoholic | 7 mm | 8 mm |
| 5. | *Ficus religiosa* | Aqueous | 7 mm | 7 mm |
| | | Alcoholic | 9 mm | 11 mm |

* Data are mean of two replications & Includes diameter of well (6 mm).

**Antimicrobial activity of Plant extracts: -** The antibacterial activity of aqueous and ethanolic crude extracts of five plants against standard *Listeria monocytogenes* & against the *Listeria* spiked in goat milk sample is summarized in Table 4. The results revealed that selected plant extracts showed antibacterial activity with varying magnitudes. *Terminalia chebula* showed maximum antibacterial activity against *Listeria monocytogenes*. Except for aqueous extract of *Citrus sinensis* all the plant extracts demonstrated remarkable antibacterial activity (Fig 2, 3). Compared to standard *L.monocytogenes* (MTCC-1143), *L.monocytogenes* spiked in goat milk sample was found to be more sensitive to all crude plant extracts as larger inhibition zones were observed. The present study shows that alcoholic extracts were more efficient in controlling *L.monocytogenes* growth, presumably because of difference in extraction ability of specific active ingredients by the two solvents used (Lino & Deogracious, 2006). Parekh *et al* (2005) also reported difference in antibacterial activity when two extraction methods were used.

**Antagonistic activity of isolated Lactic acid bacteria from goat milk against *L.monocytogenes:-*** The antagonistic activity of 11 LAB isolates was determined against standard *Listeria monocytogenes* & against the *Listeria* spiked goat milk sample. Results revealed that *L.monocytogenes* surviving in goat milk was more sensitive than the standard *L.mono*cytogenes as larger zones of inhibition were observed in case of *L.monocytogenes* isolated from the spiked goat milk sample (Table 5, Fig 4). In our study all the coccoid LAB show the maximum antilisterial activity.

*Lactococcus lactis* subspecies *cremoris* (MTCC-1484) & Lactobacillus *acidophilus* (MTCC-440) were found to be ineffective while *Lactococcus lactis* subspecies *cremoris* (MTCC-1484) & *Lactobacillus acidophilus* (MTCC-440) showed antagonistic activity against *Listeria.* Compared to the standard LAB, isolates from goat milk were found to be more efficient in controlling growth of *Listeria.* In raw goat milk, most of the antilisterial LAB isolates were identified as *Lactococcus*. Tserovska *et al*., 2002 also reported similar results. LAB isolates exhibited same results against *L.monocytogenes* isolated from the spiked goat milk & standard *L.monocytogenes* except isolate D2a, T5d (*Lactococcus*). For these two isolates the biggest inhibition zone was recorded against *L.monocytogenes* isolated from the spiked goat milk. Among standard LAB *Lactobacillus casei* (MTCC-1423) & *Lactobacillus delbrueckii* (MTCC-911) were found to be active against *L.monocytogenes*.

**Table 5. Inhibition of *L.monocytogenes* by standard LAB and LAB isolated from goat milk**

| S.No. | Lactic acid bacteria | Diameter of inhibition zone (mm) against *L.monocytogenes* isolated from the spiked goat milk (A7 + *Listeria*) | Diameter of inhibition zone (mm) against *Listeria monocytogenes* (MTCC-1143) |
|---|---|---|---|
| 1. | A12b (*Enterococcus*) | 10 mm | 11 mm |
| 2. | A7d (*Lactococcus*) | 9 mm | 8 mm |
| 3. | B7a (*Lactococcus*) | 8 mm | 12 mm |
| 4. | D2a (*Lactococcuss* | 12 mm | 9 mm |
| 5. | N2c (*Leuconostoc*) | 7 mm | 10 mm |
| 6. | N3c (*Lactococcus*) | 12 mm | 13 mm |
| 7. | T3c (*Pediococcus*) | 12 mm | 10 mm |
| 8. | T4b (*Pediococcus*) | 12 mm | 12 mm |
| 9. | T5d (*Lactococcus*) | 13 mm | 11 mm |
| 10. | T7c (*Lactococcus*) | 12 mm | 12 mm |
| 11. | *Lactobacillus casei* | 10 | 9 |
| 12. | *Lactococcus lactis* | - | - |
| 13. | *Lactobacillus acidophilus* | - | - |
| 14. | *Lactobacillus delbrueckii* | 10 | 10 |

## References


Agrawal RK, Bhilegaonkar KN, Singh DK, Kumar A, Rathore RS. 2003. Laboratory Manual for the isolation and identification of Foodborne pathogens. 1st ed.

Banson HJ. 1990. Microbiological Applications, a Laboratory Manual in General Microbiology. 5th ed.

De Man Rogosa JC, Sharp ME. 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23: 130-135.

Hoover DG. 2000. Microorganisms and their products in the preservation of foods. The Microbiological safety and quality of food. 251-276.

Lino A, Deogracious O. 2006. The in vitro antibacterial activity of Annona senegalensis, Securidacca longipendiculata and Steanotaenia araliaceaugandan medicinal plants. Arican Health Sci..6: 31-35.

Lindgren SE, Doborogosz WJ. 1990. Antagonistic activities of Lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 7(1-2): 149-163.

Farber JM, Peterkin PI. 1991. Listeria monocytogenes, A Foodborne pathogen. Microbiol Rev.. 55(3):476-511.

Bortolussi, R. 2008. Listeriosis: a primer. CMAJ, PRACTICE. 179: 795-797.

Aarestrup FM, Knochel S, Hasmant, H. 2007. Antimicrobial susceptibility of Listeria monocytogenes from food products. Foodborne Pathogens and Disease. 4(2): 216-221.

Palaniappan K, Holley RA. 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. Int. J. Food Microbiol. 140(2-3): 164-168.

Parekh J, Jadeja D, Chanda S. 2005. Efficacy of aqueous and methanolic extracts of medicinal plants for potential antibacterial activity. J. Turk. Biol.. 29: 201-203.

Rodriguez E, Gonzalez B, Gaya P, Nunez M, Medina M. 2000. Diversity of bacteriocins produced by Lactic acid bacteria isolated from raw milk. Int. Dairy J. 10(1-2): 7-15.

Spelhaug SR, Harlander SK. 1989. Inhibition of Foodborne bacterial pathogens by bacteriocins from Lactococcus lactis and Pediococcus pentosaceous. J. Food Prot. 52: 856-862.

Tserovska L, Stefanova S, Yordanova T. 2002. Identification of Lactic acid bacteria isolated from Katyk, goat's milk and cheese. J. culture collection. 3: 48-52.