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Determining the bacterial population in the tissues of the slug *Laevicaulis alte* (Gastropoda: Pulmonata) and in the habitat soil from Courtallam, Tamil Nadu

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Abstract
Our study was undertaken to identify, to quantify and also to compare the bacterial populations found in six body tissues namely haemolymph, albumen gland, foot, intestine, hepatopancreas and mantle, with that of the habitat soil of slug *Laevicaulis alte*. Totally six isolates *Bacillus subtills, Citrobacter frundii, Serratia* marcescens, *Enterobacter cloacae*, *Hafnia* and *Enterobacter faecalis* were isolated from both the habitat soil and from six tissues. *Bacillus subtltis* is a gram - positive bacteria and the remaining five species are gram - negative bacteria. The study revealed the presence of large number of bacteria found in the hemolymph of *Laevicaulis alte*. This is due to mollusc-bacteria interactions occuring in natural conditions.

Keywords: *Laevicaulis alte*, hacmolymph, Bacterial isolates

1. Introduction
Many studies refer to microflora in hemolymph as causing infection against pathogens rather than as being part of the normal flora (Stewart and Robin, 1970; Rosen mark and Conklin, 1983) [23]. Mollusc – Bacteria interaction occur under natural conditions since bacteria constitute an important part of the micro flora in the environment (Oubella *et. al.,* 1994) [19]. Bacteria may constitute a substantial proportion of the diet of marine filter feeders (Zobell and Feltham, 1938) [30] and colonize their integument and gut (Mc Henry and Brick back, 1985) [14]. Thus the bivalves accumulate large numbers of gram – positive and gram – negative micro-organisms (Colwell and Liston, 1962; Murchelano and Brown, 1968) [27, 17].
Molluscs can act as vectors for the spread of certain diseases that are pathogenic for humans such as *Vibrio cholorae*, *Vibrio vulnificus* and *Vibrio Parahaemolyticus* (Colwell, 1984; Tamplin *et al*; 1982; Tamplin and Capers 1992) [27, 25, 26]. Some specificity in the uptake of bacteria by bivalves exists with preferences for *Vibrio* and *Pseudomonas*; these groups are often found in viscera, mantle tissues and hemocytes (Elston *et.al.*, 1980) [8]. Bacteria colonizing the gut or body surfaces of animals processing a moist integument may enter the tissues through this surface (Farley, 1997)
Microorganisms play important role in digestion in crustaceans, myriapods and insects as a result of fermentative processes (Anderson *et.al.*, 1983; Bigwell *et.al*., 1980; Breznale, 1975; Griffiths and Word, 1985) [1, 10]. Charrior (1990) [4], Lesel *et.al*. (1990) [12] and Walkins and Simkiss (1990) showed that in the garden snail *Helix aspersa*, facultative anaerobic gram-negative bacteria are the most abundant and that the intestine contains most of bacterial population and favourable to anerobiasis. Watkins and Sim kiss (1990) indicated that most of the snail's micro flora is picked up from the environment during feeding, without discrimination, and modified by such environmental variables as starvation and hibernation. It has been suggested that bacteria were possibly associated with snails in degrading cellulose (Lesel *et.al.,* 1990) [12].
Our study was undertaken to identify, to quantify and to compare the bacterial populations found in the six body tissues namely haemolymph, albumen gland, foot, intestine, hapatopancreas and mantle with that of its habitat soil.

Materials and methods
Specimen Collection and handling
The slugs were collected from the natural habitat and the surface of the animal was cleaned and wiped with alcohol. Haemolymph of *Laevicaulis alte* was collected in small ice-cold test tubes, by making an incision with a sharp blade along the middorsal line of the body wall of the slug. Albumen gland, foot, intestine, hepatopancreas and mantle were dissected, weighed and immediately processed for microbiological investigations.

Media Preparation
Nutrient broth was prepared as follows: the nutrition agar media was boiled to dissolve completely and centrifuged at 15 to 16K rpm (at 120º C) for 15 minutes. The medium was called at 50ºc and transferred to the sterilized petridishes and allowed to settle to ambient temperature.

Enumeration of bacteria
One ml of slug hemolymph and trace amount of habitat soil and supernatant sample of different body components were serially diluted using sterile distilled water. These dissolutions were made up to 10^{-5} dilutions. After dilution they are transferred to the petridishes by power plate technique and inoculated for 24hrs. The results were interpreted as colony forming units (CFU/ml).

Methods employed in bacterial identification
The overall methodology for bacterial identification follows

Cappuccinos *et.al.* (1999). From the nutrient broth the bacterial colonies were transferred to the trypticose soy agar slants and sim agar slants for further analysis. Bacterial populations were identified through biochemical tests and cultural characteristics of the organisms.

Results

Table I shows the bacterial isolates and total number of each bacteria in the six tissues namely haemolymph, albumen gland, foot, intestine, hepatopancreas and muscle of *Laevicaulis alte* and compared with that of its habitat soil.

There are totally six isolates namely *Bacillus subtilis, Citrobacter frundii, Serratia marcescens, Enterobacter cloacae, Hafnia spp* and *Enterobacter faecalis* have been isolated from both the habitat soil and from six tissues. Among the six isolates only three species namely *Bacillus subtilis, Citrobacter frundii* and *Serratia marcescens*are found in the habitat soil and are not found inside the six tissues. *Bacillus subtilis*is a gram-positive bacterium and remaining four species are gram-negative bacteria.

Bacillus subtilis

The density of this species is very high (20 – 23 x 10^4 CFU/gm of soil) in the habitat soil, but found lower in the albumen gland (10-14 x 10^3 CFU/ml), low in the hemolymph (4 – 6 x 10^2 CFU/ml). and very low in the muscle tissue (4 – 6 x 10^3 CFU/ml).

Citrobacter frundii

In the maximum density (20-21.5 x 10^4 CFU/ml) of *Citrobacter frundii* are found in the intestine tissue, low in the foot tissue (7 – 7.5 x 10^4 CFU/ml),and very low in the habitat soil (3 – 3.5 x 10^3 CFU/ml) and lowest in haemolymph (2.3 – 3.8 x 10^2 CFU/ml).

Serratia marcescens

In the current investigation the density of *Serratia marcescens* is very high (3 – 3.5 x 10^4 CFU/ml) in the intestine tissue and low in the foot tissue (3–3.5 x10^3 CFU/ml), hepatopancreas (2 – 2.5 x 10^3 CFU/ml) and very low in the habitat soil (2.0 –2.5 x 10^2 CFU/ml) and lowest in the hemolymph (20 – 25 x 10^2 CFU/ml).

Enterobacter cloacae

The study revealed that the maximum density (50 – 55 x 10^4 CFU/ml) of *Enterobacter cloacae* found in the hemolymph and low in the intestine tissue (17 – 21 x 10^4 CFU/ml), hepatopancreas (8 – 9 x 10^4 CFU/ml), and very low in the foot tissue (5-5.8 x 10^3 CFU/ml)and lowest (2 – 2.5 x 10^2 CFU / ml) in the muscle tissue(2 – 2.5 x 10^2 CFU / ml) Table I.

Hafnia spp

Intestine tissue of slug *Laevicaulis alte* has the highest (10 – 1.5 x 10^4 CFU/ml) number of *Hafnia spp.* Low in the habitat soil (6 – 6.5 x 10^3 CFU/ml) and very low in the foot tissue (2.5 -3 x 10^2 CFU/ml). *Hafniaspp*are absent in the other bodytissues.

Enterobacter faecalis

Only in the hemolymph *Enterobacter faecalis* (10 – 18 x 10^4 CFU/ml) is found and absent in the habitat soil and other tissues.

Discussion

Gastropod snail could serve as a reservoir of pathogens, since they concentrate bacteria (Malek and Cheng, 1974; Suresh and Mohandass, 1990).Invertebrate hemolymph and body parts contain lectins that accumulate various species of bacteria and which may act in their defense by facilitating opsonization and phagocytosis (Cheng *et al.*, 1984; Olafsen, 1986; 1988 and Renwrantz, 1986). Charrier *et al* (1998) indicated that anaerobic intestine dissections favoured the isoloation of gram-positive bacteria, reachingupto10^9 CFU g^{-1} of fresh intestinal weight. The presence of *Enterococcus cassseliflarus* in the intestine of *H. aspera* strongly suggests that lactic acid might play an important role during the digestive process of the snail.

Biochemical and cultural characterization and identification of bacteria

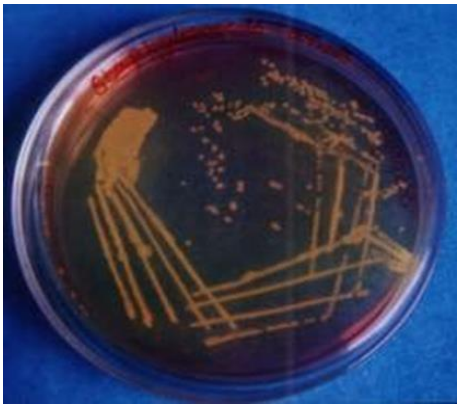

Fig 1: Bacterial colonies of the habitat soilof *Laevicaulis alte*

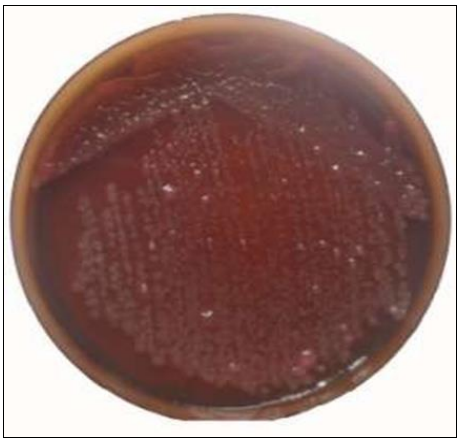

Fig 2: Bacterial colonies of the hemolymph of *Laevicaulis alte*

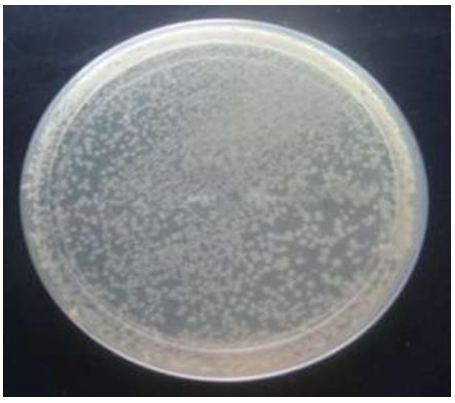

Fig 3: Bacterial colonies of the hemolymph of *Laevicaulis alte*

Table I: Comparison of bacterial density of the slug laevicaulis alte in the-habitat soil and in six different tissues

S. No	Parameters	Bacterial isolates	Bacterial density CFU/ml
1	Habitat soil	Bacillus subtilis Citrobacter freundii Serratia marcescens	20-23 X 10^4 4.5 - 6 X10^2 23- 25 X10^2
		Enterobacter cloacae	3.2 -5.5 X 10^4
		Hafnia	50.65 X 10^2
2.	Haemolymph	Serratia marcesans Citrobacter frundii	2.0-2.5 X10^3 2.3-3.5 X10^3
		Enterococcus faecalis	10-18 X 10^4
		Bacillus subtilis	4-6 X 10^2
3	Albumen gland	Bacillus subtilis Serratia marcesans	10-14 X 10^2 5-9 X 10^2
		Serratia marcesans	30-35 X 10^3
4	Foot	Citrobacter frundii Pseudomonas sp.	3-3.8 X 10^3 17-21 X 10^3
		Enterobacter cloacae	50-58 X 10^3
		Citrobacter frundii	20-21.5 X 10^4
5	Intestine	Enterobacter cloacae Serratia marcesans	17-21 X 10^4 2-3.5 X 10^5
		Hafnia	10-15 X 10^4
		Enterobactercloacae	8-9 X 10^4
6	Hepatopancreas	Citrobacter frundii	7-7.5 X 10^3
		Serratia marcesans	20-25 X 10^3
		Staphylococcus sp.	4-6 X 10^3
7	Body Muscle	Serratia marcesans Enterobactercloacae	0-25 X10^3 1-25 X10^2
		Bacillus subtilis	4-6 X 10^3

Unlike mammals, healthy invertebrates may have bacteria in their body fluid and tissues (Farley, 1977 and Stein *et al.*, 1987) [22]. The present investigaton revealed that the different body components and habitat soil contained gram-negative bacteria namely, *Enterobacter cloacae, Hafnia spp., Serratia marcesans, Citrobacter frundii, Enterococcus faecalis* and *Bacillus subtilis*. Lesel *et al. (*1990) [12] suggested that gram-negative members of Enterobacteriacae predominated in aerobically dissected snails.

According to Devries *et al.* (1991) [7], *Enterococci* are resistant to many anitibiotics, including tetracyclines and nalidixic acid. In addition, Mundt (1982) [16] isolated three *Enterococci* namely *Enterococcus faecalis, E. facium* and *E. casseliflavus* in comparable high numbers (10^7 – 10^{10} cells 8^{-1} homogenized bodies) in herbivorous insects. Cheng and Rudo (1976) [5] suggested that Oyster hemocytes get attracted to the metabolic products of gram-negative bacteria. The current study revealed that the presence of large number of bacteria in the hemolymph. The work of Tubiash *et.al.* (1975) [27] confirmed the presence of large number of bacteria in the hemolymph of blue crab *Callinectus sapidus*. The reasons may be that the bacteria gain access through diet (Zobell and Feltham, 1938) [30] integument and gut (Mc Henry and Birkbeck, 1985) [14]. Another reason may be due to that mollusc-bacteria interactions occur in natural conditions since bacteria constitute an important part of the microflora of the environments (Obella *et al*.,1994).

Turnbull (1996) suggested that *Bacillus* is a genus of gram-positive, rod- shaped bacteria and a member of the division Firmicutes. *Bacillus substilis* is one of the best understood prokaryotes, in terms of molecular biology and cell biology. Research on *Bacillus subtilis* has been at the forefront of bacterial molecular biology and cytology and the organism is a model for differentiation, gene regulation and cell cycle events in bacteria. *Enterobactor cloacae* has been used in biological control of plant disease. *Citrobacter freundii* are aerobic gram-negative *bacilli.* In the biotech industry, *Citrobacte rfrundii* produces many important enzymes. (Hillel1998)

Serratia marcenes and *Hafnia spp*. are pathogenic gram-negative, rod shaped bacteria and it belong to the family Eneterobacteriaceae. The comparative study on the bacterial isolates found in the habitat soil *Lavicaulis alte* and in the six different tissues will reveal its defense mechanism against the pathogens and also the immunological status of these organisms.

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