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ISOLATION AND IDENTIFICATION OF BACTERIAL DIVERSITY FROM CARDIAC GLYCOSIDES RICH INTESTINE OF THE *Poekilocerus pictus* (FABRICIUS, 1775)

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Abstract- *Poekilocerus pictus* is a voracious feeder of *Calotropis procera* and *Calotropis gigantia*, sequestering the cardiac glycosides in the body tissues. In search of relationship between the insect's intestinal microorganisms and cardiac glycosides, microorganisms from the intestines of *P. pictus* were isolated, cultured at different pH, and biochemically characterized. Bacterial species identified on the basis of morphological, cultural, biochemical tests and 16S rDNA sequence analysis were *Pseudomonas sp, Pseudomonas aurogenosa*, *Lysinibacillus sphaericus, Bacillus cereus, and Enterococcus sp.* These bacterial isolates, grown in the presence of the cardiac glycosides at different pH, would be promising candidates in pursuit of understanding the relationship with reference to species specificity. Association of bacteria within the intestine allowing insect to withstand concentration of cardiac glycosides, which are taken up by the insect from host plant *C. procera* or *C. gigantia* during feeding habit is discussed.

Keywords- *Poekilocerus pictus*, *Calotropis sp*., Intestinal microflora, cardiac glycosides

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Introduction

Calotropis procera and *Calotropis gigantia* are known to contain cardiac glycosides in the latex [1]. Leaves, stems and roots contain calotropagenin glycosides/derivatives [1], cardenolides, flavonoids, and saponins [2]. The methanolic calotropis extract consists of phenolic compounds and cardiac glycosides which are antimicrobial and anti-cancerous in nature [3-6].

Poekilocerus pictus is a painted grasshopper, belonging to order orthoptera of *Insecta,* which feeds on cardiac glycosides containing plants, *Calotropis procera and Calotropis gigantia*. The cardiac glycosides are sequestered in the body tissues of *P.pictus*. Intestinal microorganisms are known to inhabiting within different orders of insect; these microbes have been known to play an important role in the digestion and absorption of a species specific diet [7,8].

The roles for these microorganisms are also associated with the physiology and insect growth [9-11]. Microorganisms play an important role in the development of many insects. Despite knowing the importance of the microbial association with insects; relatively few studies elucidated their components and roles in the interactions and ecological association. Studies on insect gut microbiota mainly concentrate on the contribution of microbial endosymbionts to the host's nutritional homeostasis [11]. We sought to search existence of new microorganisms from cardiac glycosides rich midgut environment of insect. Six bacterial isolates were found to be adapted to cardiac glycosides. To the best of our knowledge this is the first report on isolation of microorganisms from the insect *P. pictus* midgut. From morphological, cultural studies, biochemical tests and 16S rDNA sequence analysis they were named as *Pseudomonas sp, Pseudomonas aurugenosa*, *Lysinibacillus sphaericus, Bacillus cereus and Enterococcus sp.* When these isolates were challenged for growth in the presence of cardiac glycosides only a few bacterial isolate succeed to growth in the presence of cardiac glycosides and at different pH. We propose that these isolates would be promising candidates to dissect relationship between intestinal microflora and cardiac glycoside diet of *P. pictus*.

Material and Methods

Collection of *P. pictus*

Adult and juveniles *Poekilocerus pictus* (both male and females) were collected from *Calotropis* plants and they were fed everyday with *Calotropis procera* leaves, which was helpful for longevity. Two to three week old *P. pictus* were reared at 25ºC -30ºC and after 7-8 weeks adult grasshoppers were dissected.

Calotropis Methanolic Extract (CME)

The shade dried and pulverized aerial parts of *Calotropis procera* and *Calotropis gigantia* were extracted in methanol in a soxhlet apparatus (Borosil, India) at 60°C. The extract was concentrated under vacuum (reduced pressure, Rotavac), dried in desiccators,

and stored at 0°C for further use. This extract was labelled as methanol extract of *Calotropis* (CME)[12].

P. pictus Midgut Removal and Isolation of Microorganisms

For dissection of *P.pictus,* the procedures described by Lynn D.E. [13,14] were adopted with some modifications. The isolated midgut was opened with the help of microscissor in TC100 medium (Himedia, India), mildly washed with saline (Himedia, India) and chopped into several pieces. The chopped midgut was resuspended in 10ml saline.

Isolation of microorganisms: The midgut suspension was inoculated in nutrient broth (Himedia, India) and was incubated at 28ºC for 72 hrs. The 72 hrs growth suspension was diluted to 10^{-4} and spread on the nutrient agar (Himedia, India) plates (Two plates for each pH 5, pH6, pH7, pH8, pH9 and pH10). These plates were then incubated at 28ºC for four days for the appearance of colonies.

General Morphology and Biochemical Tests for Microorganisms

All the colonies from Nutrient agar (Himedia, India) plates were subculture and stored on agar plates and slants (Nutrient agar with pH 5 to 10). Primary phase contrast microscopy was used to observe general morphology. The cultural studies performed including study of colony with reference to size, shape, colour, margin, opacity, elevation, and consistency and Individual bacterial morphology of isolates was studied by Gram staining and Phase contrast microscopy.

The physiological tests performed according to Franzetti & Scarpellini [15]: Acid production from 0.5% sucrose, rhamnose, D-xylose was tested in sugar base medium supplemented with phenol red as pH indicator (Himedia, India). Biochemical reaction mechanisms were studied by recording observations of the above mentioned after 24 hrs., 48 hrs., and 72 hrs. incubation at 37°C. Parallel to biochemical studies in broth with pH indicator biochemical test strips were also used for the carbohydrates fermentation tests (Himedia, India). Growth at 4°C, 10°C and 41 °C was carried out. The oxidative or fermentative acid production from carbohydrate test was considered [16], Dextran formation were tested on agar medium containing 5% sucrose (Himedia, India), and gelatinase production was tested on a medium containing 1% tryptone, 0.1% yeast extract, 0.3% meat extract, 0.3% gelatin, and 1.5% agar, to which Frazier solution was added after 48 hr incubation. The enzymatic activities tested includes: lipase production [17], pectinolytic activity [18], starch hydrolysis [19], lecithinase production (Nutrient Agar plates plus 5% v/v egg-yolk emulsion) and proteolytic enzyme production(Nutrient Agar plates containing 10% skim milk powder). Pigment production was examined on tryptic soy agar (Himedia, India), Fluorescent and phenazine pigment production [20], accumulation of poly-b-hydroxybutyrate [21] were studies. Existence of flagella was indirectly estimated by studying motility with hanging drop technique and motility medium plates (Sulphur Indole Motility Medium) that were incubated for overnight at 30ºC. Tests prescribed by Himedia, India for discrimination of Gram positive and Gram negative bacteria with the biochemical test strips were also considered and performed in accordance with the instructions provided by the manufacturer. Other physiological parameters such as their sensitivity to Ouabain, Digitoxin and cardiac glycosides containing CME were also studied. Growth was determined at 28ºC and the cultures were stored for further characterization.

Screening of the Bacterial Cultures on Cardiac Glycosides

Bacterial isolates were inoculated in Minimal medium (Himedia, India) containing (10% w/v) CPME/ Digitoxin/ Ouabain. Only few of the isolates grew in the presence of the cardiac glycosides. These isolates were then used for further study.

Chemo Sensitivity Test

Bacterial isolates were spread on the minimal medium plates. The 6 mm discs were placed evenly on the plates. The Digitoxin at concentration 2 mg/ml, 5 mg/ml, 10 mg/ml, 25 mg/ml, and 50 mg/ml was loaded on the disc with micropipettes. The plates were inoculated at room temperature for 24 h for appearance of zone of exhibition around the disc containing Digitoxin.

Isolation of Genomic DNA and Amplification of 16SrDNA Region

The mid-log phase cultures of selected isolates were used for PCR amplification of 16S rDNA region. The cultures were centrifuged at 6000 X gravity and washed twice before DNA isolation. Total Genomic DNA isolation was carried out with genomic DNA isolation kit (Life Technol. Inc. USA). The isolated and purified genomic DNA was used as template for PCR amplification.

The PCR program used was as follows: 95ºC for 2 min followed by 29 cycles of 94ºC for 30 sec, 52ºC for 30 sec, 72ºC for 1 min, final extension step at 72ºC for 5 min was carried out for amplicon maturation. Purified amplicons were then used in deciphering 16S rDNA nucleotide sequence.

DNA sequencing: Forward and reverse DNA sequencing reactions of PCR amplicons were carried out with 8F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTG TTACGACTT-3') primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer at Xcelris genomics Pvt. Ltd. (India).

DNA Sequence Data Analysis

The 16S DNA sequences were analyzed initially using the BLASTn search with *http://www.ncbi.nlm.nih.gov/blast* search engine. CHECK_CHIMERA program of http://www.ncbi.nlm.nih.gov/blast/blast.cgi was used for the chimeric artifacts. The nucleotide information obtained from this endeavour was then deposited with Genbank.

Data Analysis and Phylogenetic Tree Construction

All of the 16S rDNA sequences were analysed by using BLASTn search engine at http://www.ncbi.nlm.nih.gov/blast. The evolutionary history for these nucleotide sqeuences was inferred using the Neighbor-Joining method [22]. To represent the evolutionary history of the taxa analyzed the bootstrap consensus tree inferred from 1000 replicates was taken [23]. The evolutionary distance was computed using the Kimura 2-parameter method [24] and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted using MEGA4 as described in Tamura et al [25].

Bacterial Growth in Minimal Medium Containing Cardiac Glycosides

All isolates were grown for 15 days without any antibiotics or cardiac glycosides minimal medium at 28ºC. These cultures were then used as inoculums to further challenge them to grow in minimal medium containing cardiac glycosides namely; CPME (0 to 30 % w/

v), Ouabain (Merck, USA) (0-30%w/v) and Digitoxin (Sigma, USA) (0-30 % w/v), incubation was carried at 28ºC for 48 hrs and optical density was measured at 660nm. As the minimal medium per se did not carry any carbon source, it became essential to estimate as to whether the growth observed in culture is a result of use of cardiac glycosides as sole carbon source. To address this, residual cardiac glycosides concentration from growth medium was estimated by Kedde's reagent at 24hr and 48 hr post inoculation.

Results and Discussion

For the isolation of the microorganisms, intestine of adult *P. pictus* was removed and dissected. Spread plate method was used for isolation of midgut inhabitant bacteria on nutrient agar (NA) plate with wide range of pH such as; pH 5, pH6, pH7, pH8, pH 9.6, and pH10. Total 181 single colonies from nutrient agar were selected. Colonies exhibiting minor variations were considered as likely by dissimilar bacterial isolates and the use were picked for further study. With this subtraction screen of 181 total colonies appeared on various NA plates at different pH only 57 qualified for further testing. Cultural studies extending with reference to colony size, shape, color, margin, opacity, elevation, and consistency were recorded [Table-1], [Table-2], [Table-3]. [Table-2], shows Gram's character (phase contrast microscopy) and results of hanging drop preparation on these 57 bacterial isolates. [Table-2], [Table-3], [Table-4], [Table-5]. It is evident from [Table-2] amongst 57 majority of these isolates were Gram negative. These isolates were also screened for excellent utilization of cardenolides containing nutrient medium.

Table 1- Growth of bacterial isolates on cardiac glycosides containing medium (10% w/v). *Where* the pHs indicates the pHs of the medium from which microorganisms were isolated

	pH5	pH6	pH7	pH8	pH9.6	pH10
Initial Bacterial isolates	12	12	17	20	53	67
Isolates Grown on cardiac glycosides Containing minimal medium (HiMedia, India) 10% (w/v)	1	1	4	3	6	4

Amongst 57 when challenged with cardenolides only 27 isolates exhibited good growth in the presence of cardenolides and these isolates after second sieve were considered for further studies [Table-1]. [Table-3] shows that out of 27, fifteen (55.5%) of the isolates were Gram negative, ten were Gram positive either coccobacilli (37.0%) or two Gram positive cocci (7.40%). All of these 27 bacterial isolates were able to consume carbon aerobic as well as anaerobic, demonstrating as to they belong to facultative anaerobic group. Most of the bacterial isolates had circular and convex colonies with entire margin, opaque and some were found pigmented. Most of the isolates were found to be highly motile it is apparent from Craigs test as well as hanging drop method.

Out of fifteen gram negative isolates, Isolate PPBPH5 and KRK6 were grown in cardiac glycosides containing minimal medium. The PPBPH5 was isolated from the Nutrient agar of pH 5 and KRK 6 was isolated from nutrient medium of pH 6. The natural fluorescence, biochemical tests [Table-2], and 16S rDNA sequence homology result shows relations with family *pseudomonadaceae* [Fig-1](a &b). The BLASTn analysis shows homology of PPBPH5 and KRK6 to *Pseudomonas* sp. and *Pseudomonas aeruginosa*, respectively. Organisms *Pseudomonas aeruginosa* has been known to exhibit resistance to antibiotics and disinfectants [15]. Ability to produce siderophore by this isolate was confirmed by ferric chloride test. It can grow on a wide range of substrates and alter its properties in response to changes in the environment [15]. This mechanism of resistance results from mutational changes in target enzymes, which eventually results in maintenance of their vital role in cell metabolism but still it shows resistance to the action of selective inhibition by antibiotics [15].

In view of the high midgut alkalinity, it is reasonable to assume that at least some of the bacteria in the midgut are alkalophilic. Along with pseudomonas sp, several *Bacillus spp*. have previously been identified in insects. Phenotypically, the genus *Bacillus* is a large and heterogeneous collection of aerobic, rod-shaped, Gram-positive, endospore-forming bacteria [26,27]. The isolates BPH31 [Fig-1](c) to BPH33 were isolated from nutrient medium at pH10. All of these three bacterial isolates were motile and Gram-positive coccobacillary rods. Phylogenetically BPH31 is a close relative of *Lysinibacillus sphaericus* [Fig-1] that grows at 10ºC, 28ºC and 40ºC. *Lysinibacillus sphaericus* is an important organism to study as it can be used as an insecticidal toxin that controls mosquito growth. The BPH31 is dominant species of *Poekilocerus pictus* midgut and shows urease activity.

Table 2- Biochemical tests for the *Pseudomonas* sp. isolates

Characteristics	*Pseudomonas* sp PPBPH5	*Pseudomonas* sp KRK6
Grams nature	-	-
Denitrification	+	+
Indole	-	-
Glucose	+	+
Ammonia from arginine	-	-
Urease	-	-
Gelatine	+	+
Assimilation of		
Glucose	+	+
Arabinose	-	-
Mannose	-	-
Mannitol	-	-
Maltose	-	-
Gluconate	+	+
Citrate	+	+
Catalase	+	+
Oxidative in of medium	+	+
Fluerescent pigment	+	+
Pyocyanin production	+	+
Growthat 4ºC	-	-
10ºC	-	+
45ºC	-	+
Growth in		
6.5% NaCl	-	+
0.1% Methylene blue	-	+
Motility	-	-
Voges-proskauer	-	-
Alkaline phosphatase	-	+
Egg yolk reaction	-	-
Pectinolytic activity	-	-
Proteolytic activity	-	-

Isolate BPH32 and BPH33 were found to be the close relative of *Bacillus cereus sp* [Fig-1](d&e). The BPH33 appears as big rods nonmotile in nature whereas BPH32 are gram-positive coccobacillary nonmotile rods. Previous report on the growth of *Bacillus sp* on herbal extracts shows that the herbal products possess bacterio-

static effect on the *Bacillus cereus* [28]. The optimum growth of BPH31 to BPH33was observed at 30ºC at pH10. Isolate BPH31 and BPH32 show amylase, deaminase, casienase and catalase activity.

Table 3- Biochemical tests for the *Bacillus* sp. isolates

Biochemical Tests	*Bacillus* sp .BPH32	*Bacillus* sp. BPH33
Gram's nature	+	+
Motility	+	+
Catalase production	+	+
Parasporal bodies	-	-
Pigment	-	-
Lipid globules in protoplasm	+	+
Citrate utilization	+	+
Anaerobic growth	+	+
VP reaction	+	+
Growth at 50˚C	-	-
Growth at 60˚C	-	-
Growth in 7% NaCl	+	+
Nitrate reduction	+	+
Casein hydrolysis	+	-
Starch hydrolysis	+	+
Gelatin hydrolysis	+	+
Tyrosine hydrolysis	+	+
Indole	-	-
Urease	-	-
Lecithinase	+	-
Lecithovitellin	+	+
Acid from		
Glucose	+	+
Mannitol	-	-
Xylose	-	-
Arabinose	-	-
Maltose	+	+
Trehalose	+	+
Glycerol	+	+
Sucrose	+	+
Lactose	-	-
Inositol	-	-
Sorbitol	-	-

Isolate BPH34 was recovered from Nutrient agar plates at pH 7, shows phylogenetic relations with *Enterococcus sp*. [Fig-1](f). The usual ecological niche for *Enterococcus* species is the intestines of humans and other animals. The *enterococci* could also be found free-living in soil, on plants, or in dairy products [29-31].The BPH34 is a Gram-positive cocci which tolerate a wide range of temperature 10°C - 45°C shows optimum growth at 30ºC.The genus *Enterococcus* consists gram-positive cocci, catalase negative, usually facultative anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts, and 0.1% methylene blue milk and at pH 9.6. They grow at 10°C and 45°C and can resists upto 20% w/v Cardiac glycosides.

The 16S rDNA region sequences of all the isolates were submitted to GenBank. GenBank Ids- *Enterococcus sp.BPH34* HM771263, *Bacillus cereus BPH33* HM771262, *Bacillus cereus BPH32* HM771261, *Lysinibacillus sphaericus BPH31* HM771260, *Pseudomonas aeruginosa KRK6* HM366592, *Pseudomonas sp. PPBPH5* HM366591.

Table 4- Biochemical tests for the *Enterococcus* sp. isolates

Characteristics	*Enterococcus* sp.BPH34
Gram's nature	Positive
L-Arabinose	+
Cellobiose	+
Dextrin	-
Galactose	+
D glucose	+
Glycerol	+
Glycogen	-
Lactose	+
Maltose	+
Mannitol	+
Ribose	+
Starch	-
Sucrose	+
D-xylose	-
Growth at	
4ºC	-
10ºC	+
45ºC	+
pH4	-
pH9.6	+
Growth in 6.5%NaCl	+
0.1% methylene blue milk	+
0.01% tetrazolium	+
Gelatin liquefaction	-
β-Galactosidase	+
Catalase	-
H2S production	-
Alpha hemolysis	+
Beta hemolysis	-
Motility	-
Voges-Proskauer	+
Yellow pigment	-
Alkaline phosphatase	-
Arginine dihydrolase	+
a-Galactosidase	+

Chemo Sensitivity Test

In brief bacteria were spread inoculated onto minimal agar plates. Digitoxin discs were prepared for 2 mg/ml, 5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml. Discs were placed with appropriate spacing on spread inoculated plates. Plates were incubated for appearance of growth surrounding disc. Observations after 24 hours showed that there was no growth in the plate any area except periphery of digitoxin disc. Zone of exhibition was calculated, all of the isolates could grow well with various concentrations of digitoxin, indicating indeed growth was at the cost of digitoxin utilization. Results shown in [Fig-2] demonstrate exhibition of chemoresistance of intestinal bacteria to digitoxin. A non parametric statistical analysis performed indicated difference seen with digitoxin as compared to without digitoxin is significant ($p < 0.2463$). The significant differences were observed near the digitoxin containing discs and non digitoxin containing discs.

Table 5- Biochemical tests for the *Lysinibacillus sphaericus* BPH31

Characters	Lysinibacillus sphaericusBPH31
Gram's reaction	+ ve
Margin	Regular
Elevation	Convex
Surface	Dull
Pigment	No pigments
Opacity	Opaque
Cell shape	long rods
Endospore	+
Motility	Motile
Growth at 4ºC	-
10ºC	+
15ºC	+
25ºC	+
30ºC	+
42ºC	+
Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate Utilization	-
Casein hydrolysis	-
Gelatin hydrolysis	+
Starch hydrolysis	-
Urea hydrolysis	+
Nitrate reduction	-
H2S production	-
Catalase test	+
Oxidase test	+

Effect of CME on Growth of Microbes

When symbionts were cultured on plant material, the exposure to toxins is obvious. Fungal symbionts that inhabit the gut of insect would expose to toxins [32].The insects that consume plant materials would be expected to be exposed to the secondary metabolites or the toxins of plant. In primary screening, we used minimal medium with CME. The *Calotropis* extract is known as antibacterial in nature. The midgut microflora should tolerate to cardenolide content of *Calotropis* sp. In this view, the isolates were grown on CME containing medium. For these bacterial isolates, optimum growth was observed at 12 % w/v CME [Fig-3], [Fig-4], [Fig-5]. Previous studies have documented that many insects live in close association with microorganisms e.g. plant sap-sucking insects with bacterial endosymbionts [33]. In their studies Sirko & Brodzic [34] reported that many enzymatic activities known to occur in bacteria and fungi but their role in detoxifying secondary plant compounds still being suspected. The growth of microorganisms on the cardenolides proves that host insect and microorganisms of gut are resistant to cardenolides from *Calotropis* spp. These bacteria may interfere with host reproduction, which may lead to cytoplasmic incompatibility, parthenogenesis, or sex ratio distortion [35-37]. The biotransformation of cardenolides could be possible in *P. pictus.* Isolation of microorganisms from different environments offers an opportunity to obtain strains of novel potential applications in biotechnological processes. These bacterial isolates are highly elastic with reference to adaptability and thus are important. The presence of bacteria in midgut may helps in digestion and sequestrations of the cardenoldies and toxic chemicals from plant in insect body and improves the survival of *P.pictus*.

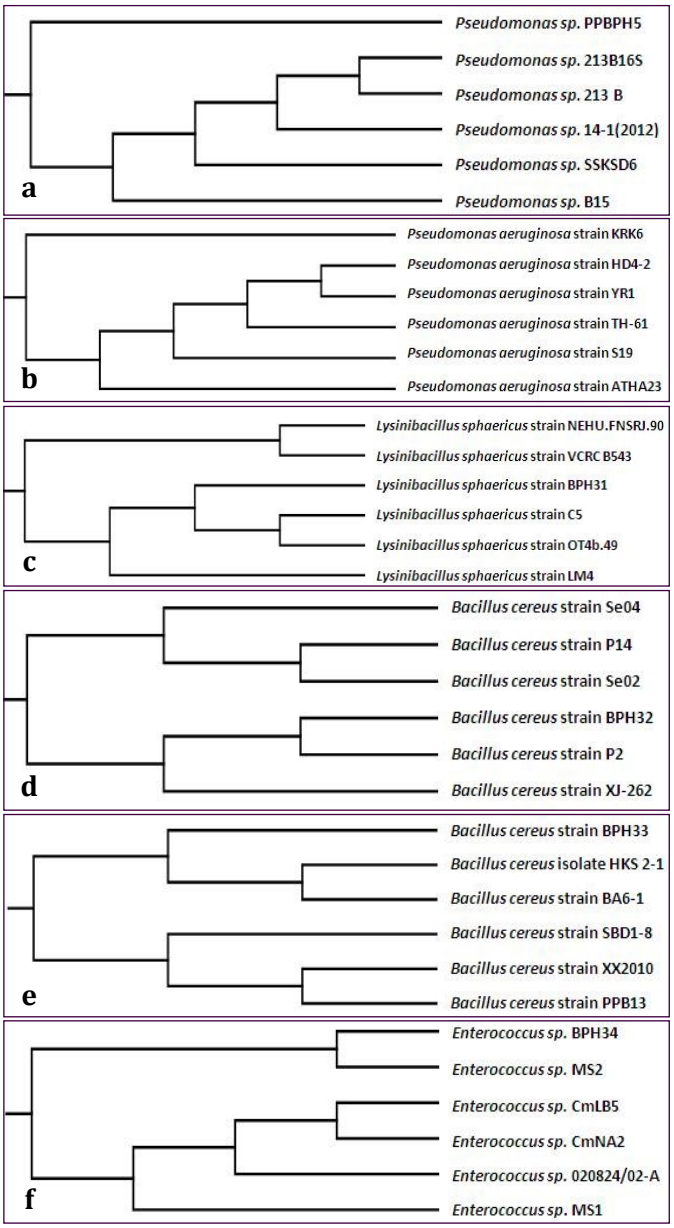

Fig. 1- Phylogenetic tree of isolates where a) *Pseudomonas sp* PPBPH5; b) *Pseudomonas aeruginosa* KRK6; c) *Lysinibacillus sphaericus* BPH31; d) *Bacillus cereus* BPH32; e) *Bacillus cereus* BPH33; f) *Enterococcus sp* BPH34

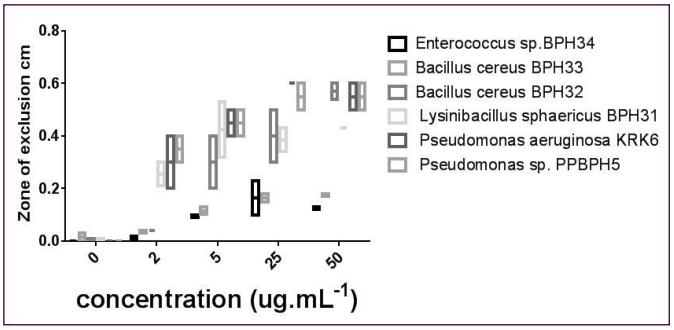

Fig. 2- Chemosensitivity assay; the Digitoxin was added to the minimal medium (HiMedia, India); the disc diffusion method was used to check the sensitivity of bacterial isolates; one way ANOVA was used to analyse the data by using Graphpad Prism5, (p=0.2463)

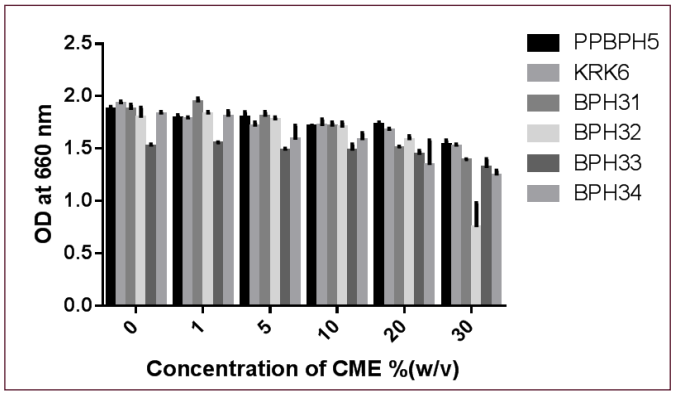

Fig. 3- Growth of microbes at various concentrations of CME; the CME was added to the minimal medium (HiMedia, India); the growth was estimated at 660nm. (The O.D. at 660nm on y axis); one way ANOVA was used to analyse the data by using Graphpad Prism5, (p=0.2463)

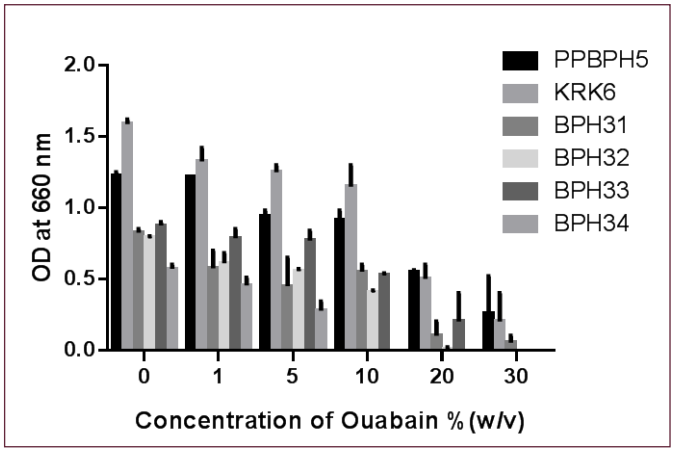

Fig. 4- Growth of microbes at various concentrations of *Ouabain* (Merck, USA); the Ouabain was added to the minimal medium (HiMedia, India); The growth was estimated at 660nm (The O.D. at 660nm on y axis); one way ANOVA was used to analyse the data by using Graphpad Prism5, (p=0.2463)

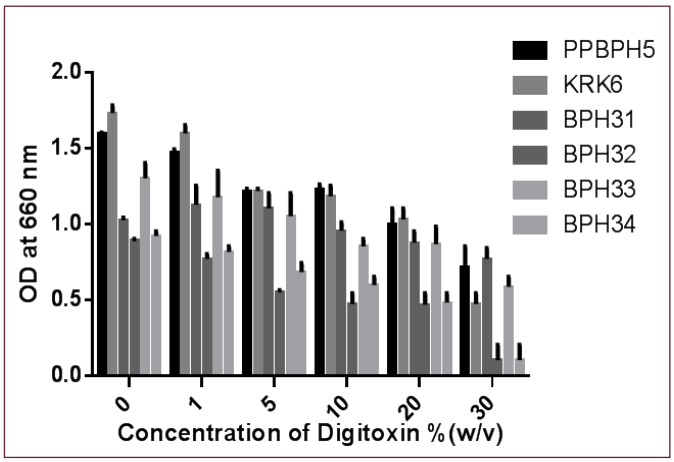

Fig. 5- Growth of microbes at various concentrations of Digitoxin (Sigma); the Digitoxin was added to the minimal medium (HiMedia, India); the growth was estimated at 660nm (The O.D. at 660nm on y axis); one way ANOVA was used to analyse the data by using Graphpad Prism5, (p=0.2463)

The microbes could be beneficial to *P.pictus* by protecting against pathogenic bacteria –exhibiting antagonism as well protect insect from toxic cardenolides. Our findings may open new window for understanding grasshopper-bacteria interaction. The presence of bacteria inside cardiac glycosides containing midgut may imply in a more specific insect-microorganism interaction than previously described.

However, the availability of microorganisms in culture may represent the best tool to study in detail the multiple factors involved in cardiac glycosides adaptation, absorption and metabolism in *P. pictus*.

Our report is for the first time indicating that eating on cardiac glycoside and cardenolides containing plants by insect is making use of its midgut normal flora bacterium. This exhibits tight ecological and environmental relation, chain between microbe, insect and a plant.

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Conflicts of Interest: None declared.

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