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Larval Food Analysis and Qualitative Determination of Exoenzyme-Producing Gut Bacteria in Adult Ceratopogonid Midges (Diptera)

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ABSTRACT

Biting midges are small nematocerous Diptera. *Culicoides* and *Dasyhelea* are two important genera of the family Ceratopogonidae. Larvae of *Culicoides innoxius* and *Dasyhelea aprojecta* are found in the semiaquatic moist habitat. The larvae feed on the small debris and habitat substrata. The materials consumed by these larvae aid in their development to become adult. The nutritional evaluation of the food material of larvae of *C. innoxius* and *D. aprojecta* was carried out to know the essential elements for their development. In the case of adult *Culicoides,* many species are hematophagous. However, the adult midges of the genus *Dasyhelea* are dependent on nectar and honeydew. Along with their digestive enzymes, exoenzyme-producing gut associated bacteria have also an important role in the digestion of these food materials. Digestion and metabolism of these food materials aid in insect maturation, immunity, reproduction, maintaining diapause, etc. Qualitative determination of the gut associated bacteria of adult *C. innoxius* and *D. flava* was accomplished to infer the role of bacteria supplementing the digestive enzymes.

Keywords: *Culicoides, Dasyhelea*, Larval food material, Proximate composition, Exoenzyme-producing gut bacteria, Qualitative determination.

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INTRODUCTION

Family Ceratopogonidae is an enormously diverse group of small nematocerous midges, usually known as biting midges. In this family along with some other genera *Culicoides* Latreille [1], and *Dasyhelea* Kieffer [2], are important in having medico-veterinary and economic significance respectively. Many species of *Culicoides* are vectors of pathogenic protozoans, viruses, and filarial nematodes [3-5] causing Akabane, Blue Tongue disease, etc. [6, 7]. Some members of the genus *Dasyhelea* are pollinators of cocoa and rubber trees [8, 9]. In the case of female *Culicoides*, the adult stage is hematophagous, feeding on vertebrate blood, though there is report of natural sugar feeding habit also [10]. The larvae of some species of *Culicoides* are trophic generalists [11]. Adult stages of both the sexes belonging to the genus *Dasyhelea* rely on nutrition from the honeydew and nectar [12], larval stages of this genus are primarily herbivorous actively feeding upon the plant and animal debris [13-15]. However, there is also evidence of carnivorous feeding habit of *D. pseudoincisurata* Waugh and Wirth, [16, 17].

Insects harbor a broad variety of microorganisms in their gut which help in numerous physiological functions [18]. There is also role of autochthonous bacteria in production of digestive enzymes for plant-derived polymers [19]. Digestion of lipid and protein may also be contributed by these microorganisms [20].

This article aims to evaluate the composition of food materials ingested by the larvae during

active feeding stage. So far, no investigation has been made for qualitative determination of autochthonous enzyme-producing gut bacteria of adults *Culicoides* and *Dasyhelea* which we have also done in this study.

MATERIALS AND METHODS

Analysis of larval food **(Figures 1a-1c)**

A site (Site: India, West Bengal, Nadia, Krishnanagar) was selected for the collection of food material. Rotting banana stems were found at the collection site and the presence of flying adults confirmed the breeding habitat. Two types (Sample 1 and Sample 2) of the rotting stems were cut out with the help of a fine knife, bagged in 45×40 cm clear plastic packets, and brought to the laboratory keeping the packets airtight. The samples were kept in a 30 cm × 25 cm × 4 cm plastic tray with a little amount of water just to make the sample moistened. A small amount of sample was taken and examined carefully for the presence of any larva. Some larvae were observed underneath the Olympus SZX 16 microscope (Japan) and found consuming their habitat substrata which are the upper thin membranous leaflets of the stem in both the samples. Few larvae were isolated and reared up to adult emergence. Upon the adult eclosion, the midges were identified under Carl Zeiss Stemi 2000 Stereozoom microscope (Germany) and confirmed their belonging to the genera *Culicoides* and *Dasyhelea*. Further species-level identification was confirmed after mounting in glass slides and observing under a Wild Leitz GMBH Trinocular microscope (Portugal). The upper thin membranous layer of both samples was isolated carefully by forceps, washed thoroughly in tap water, and sun dried for 3–4 days. The two dried samples (Sample 1 and Sample 2) were then put into a mixer grinder, and completely pulverized. The ground sample 1 and sample 2 were then stored in zipper plastic packets. Estimation of crude lipid, crude fiber, ash content (minerals), crude protein content, total free amino acid, and free fatty acid content of the food material of *Culicoides* and *Dasyhelea* larvae was done by proximate composition analysis. Proximate composition of the samples were analyzed following the standard methods of the Association of Official Analytical Chemists (AOAC)[21] – crude protein by micro Kjeldahl digestion and distillation, crude lipid by extracting the residue with 40-60°C petroleum ether in a Soxhlet apparatus (Pelican, Chennai, India), crude fiber as loss on ignition of dried lipid-free residue following digestion with 1.25% H_2SO_4 and 1.25% NaOH and ash content by ignition at 550°C (6 hrs) in a Muffle furnace to constant weight. Total free amino acid and fatty acid contents were also measured [22, 23].

Qualitative determination of exoenzyme-producing ability of the gut-associated bacteria in adult midges **(Figures 1d-1g)**

Battery operated UV light trap (8 W) was used for collecting the biting midges (Site: India, West Bengal, Purba Bardhaman, Burdwan, Keshtopur). The midges were collected in phosphate buffer saline. The collected insects were brought to the laboratory and sorted out quickly to avoid postmortem changes in physiology using a stereozoom microscope Carl Zeiss Stemi 2000 with the help of a fine "000" brush. The species were identified after mounting the genitalia on glass slides [24]. Midges of the genera *Culicoides* and *Dasyhelea* were separated and stored in plastic vials filled with phosphate buffer saline. For each species, the sample processing was performed separately. Samples of 0.05 g were weighed in Mettler Toledo Model - JS1203C/A71 (Switzerland) and washed thoroughly with distilled water. As a surface sterilizing agent 70% ethanol was used. The midges were beheaded beneath the stereozoom microscope using a fine needle and taken in an autoclaved plastic container of 2 ml and sterilized distilled volume of 20 times (i.e. 1 ml) of the weighed sample. IKA T10 basic ULTRA-TURRAX Model - T 10B (India) was used to homogenate the sample. The homogenized samples were stored in a refrigerator at 4°C in an autoclaved plastic container. The homogenates were serially diluted (1:10) with sterilized distilled water [25]. A total of three-step dilution 10^{-1}–10^{-3} was prepared for each species. In order to determine the culturable heterotrophic autochthonous aerobic/ facultative anaerobic microbial population, nutrient agar plates were prepared. The plates were stored in a refrigerator (4°C) until further study. A plate for each species having well-separated colonies with

distinguished growth is selected to prepare pure culture to get different bacterial isolates. After that, the colony characteristics of each isolate from pure culture were recorded and the colonies were transferred to new culture plates which were stored at 4°C in a refrigerator. Gram staining and endospore staining of bacteria were done. After that measurements of the bacteria were taken underneath compound light microscope (Wild Leitz GMBH, Portugal) using ocular and stage micrometer. For enzymatic analyses bacterial isolates were grown on different culture media and stained: for amylase – cultured in starch agar then stained by Lugol's Iodine; for protease – cultured in agar with gelatin, peptone, and yeast extract then treatment by HCl + $HgCl_2$; for cellulase – cultured in bacteriological agar with Carboxymethylcellulose (CMC), then stained by Congo Red; for lipase – cultured in Tributyrin agar and Trybutyrin base. The plates were inspected for any halo zone, if present the zone characteristics were recorded.

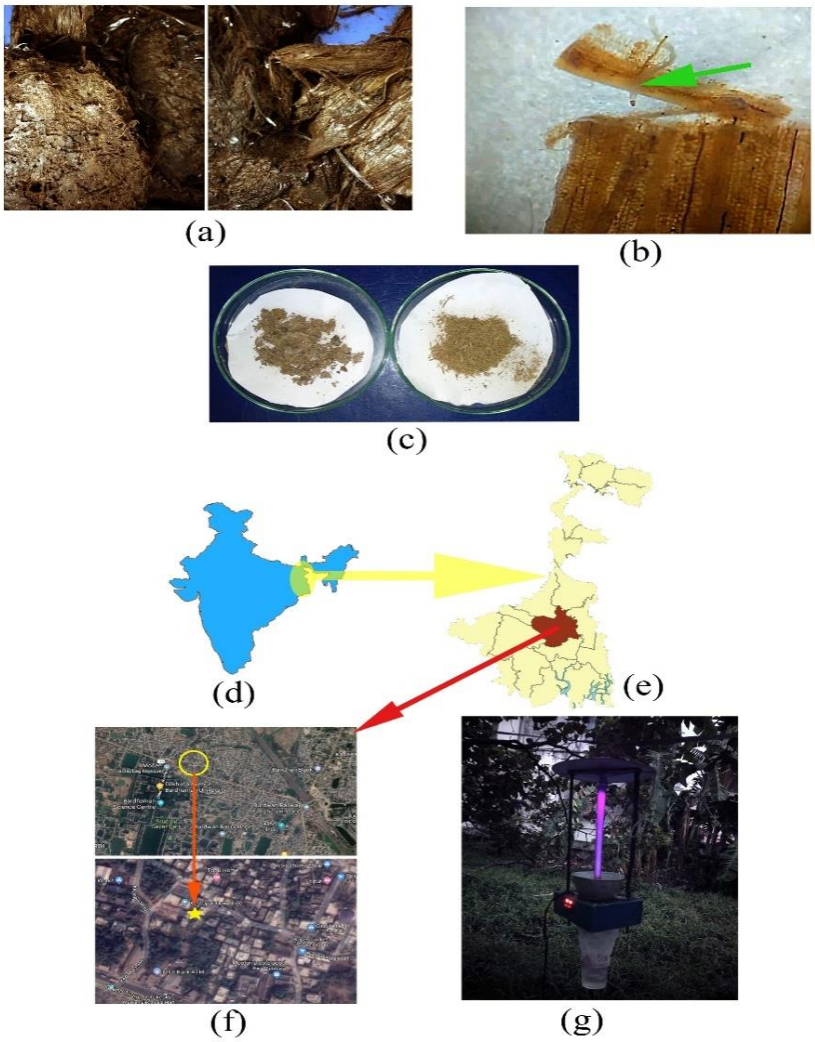


Figure 1. a) Habitat substrata of Sample 1 (left) and 2 (right); b) Larva of biting midge in the habitat substratum; c) pulverized Sample 1 (left) and 2 (right); d) Map of India; e) Map of West Bengal showing the collection site district; f) Collection site of adult biting midges (Google Map: Imagery ©2022 Maxar Technologies, Imagery ©2022 CNES / Airbus, Maxar Technologies, Map data ©2022); g) collection of adult biting midges by UV light trap.

RESULTS AND DISCUSSION

Analyses of larval food

The larvae procured from the habitats were identified as *Culicoides* (*Hoffmania*) *innoxius* Sen and Das Gupta, [26] and *Dasyhelea* (*Pseudoculicoides*) *aprojecta* Brahma, Chatterjee and Hazra, [27] upon adult emergence. The result of larval food content (habitat substrata) analysis is depicted in **Table 1**.

Table 1. Proximate composition of Sample 1 and Sample 2 (g/100g) consumed by the *Culicoides innoxius* and *Dasyhelea aprojecta*larvae respectively. Data expressed as mean ± standard error (n = 3).

Sl. No.	Nutrients	Sample 1	Sample 2
1.	Crude Lipid	5.24±0.14	9±0.02
2.	Crude Protein	13.14±0.35	11.74±0.32
3.	Crude Fiber	35.96±0.92	37.63±1.01
4.	Ash content (minerals)	4.01±0.11	0.79±0.01
5.	Nitrogen Free Extract (NFE)	41.63±1.02	40.82±1.3
6.	Free amino acids (FAA)	0.88±0.01	0.71±0.01
7.	Free fatty acids (FFA)	0.77±0.01	0.61±0.001

Qualitative determination of exoenzyme-producing ability of the gut associated bacteria in adult midges **(Figure 2)**

The species used for enzymatic determination are identified as females of *Culicoides* (*Hoffmania*) *innoxius* Sen and Das Gupta, [26] and females of *Dasyhelea* (*Prokempia*) *flava* Carter, Ingram and Macfie, [28].

In the case of both species colonies of the concentration 10^{-1} were overlapped and uncountable. Colonies of concentration 10^{-2} were separated with distinct margins and variations, so they were selected for preparation of pure culture (In both *Culicoides innoxius* and *Dasyhelea flava* 10^{-2} concentration produces eight morphologically distinct colonies) **(Table 2**). Least numbers of colonies were observed in concentration 10^{-3}.

Only six isolates of *Culicoides innoxius* and *Dasyhelea flava* each (Cu_{1-3}, Cu_{5-7}; Da_{1-3}, Da_{5-7}) were taken for downstream analysis for better growth in pure culture. Among the six isolates retrieved from both the species (Cu_{1-3}, Cu_{5-7}; Da_{1-3}, Da_{5-7}) two isolates (Cu_3, Cu_7) were found as Gram-positive while the other four (Cu_1, Cu_2, Cu_{5-6}) were found to be Gram-negative in the case of *Culicoides innoxius,* and three isolates (Da_{1-3}) were found as Gram-positive while other three (Da_{5-7}) were found as Gram-negative in the case of *Dasyhelea flava*. All the isolates belonging to *Culicoides* are bacilli and isolates belonging to *Dasyhelea* consist of a mixture of cocci form (Da_1) and bacilli (Isolates Da_{2-3}, Da_{5-7}). The length and width of bacilli retrieved from *Culicoides* range from 1.4–2.76 µm and 0.60–0.74 µm respectively **(Table 3)**; the length and width of bacilli retrieved from *Dasyhelea* range from 1.82–2.8 µm and 0.69–0.74 µm respectively and the diameter of cocci form ranges between 0.62 and 0.64 µm **(Table 3)**. All six isolates from both species were found as non-endospore-forming bacteria.

Determination of enzyme-producing bacteria in adult Culicoides innoxius **(Figures 2a–2d; Tables 4 and 5)**

Amylase*.* Halo zones were observed at the periphery of colonies Cu_{1-7}. So, six isolates were found as amylase positive.

Protease*.* Halo zones were observed at the periphery of colonies Cu_{1-3} and Cu_{5-7}. So, six isolates were found as protease positive.

Cellulase*.* Halo zones were observed at the periphery of colonies Cu_{1-2}. So, two isolates were found cellulase positive.

Lipase*.* Halo zones were observed at the periphery of colonies Cu_{1-3} and Cu_{5-7}. So, six isolates were found as lipase positive.

Determination of enzyme-producing bacteria in adult *Dasyhelea flava* **(Figures 2e–2h; Tables 4 and 5)**

Amylase*.* Halo zones were observed at the periphery of colonies Da_{1-3} and Da_{6-7}. So, four isolates were found as amylase positive.

Protease*.* Halo zones were observed at the periphery of colonies Da_1 and Da_{6-7}. So, three isolates were found as protease positive.

Cellulase*.* No halo zone was observed around the periphery of any colony. Hence, all six isolates ($Da_{1–3}$ and $Da_{5–7}$) were found cellulase negative.

Lipase*.* Halo zones were observed at the periphery of all colonies ($Da_{1–3}$ and $Da_{5–7}$) So, six isolates were found as lipase positive.

In past, nutritional and chemical properties of the larval habitats of certain species of *Culicoides* were evaluated, and categorization of the species of *Culicoides* was done based on inhabiting high nutrients, moderate to high nutrients, wide nutrient range, moderate to low nutrient, and low nutrient zone [29]. Larvae of *Dasyhelea* feed on algae and fungi [13]. But carnivorous feeding habit of *D. ampullariae* Macfie, [30] was also reported [31] which consumes mosquito larvae. There was no record regarding the nutrient contents in the habitat substrata which are consumed by the larvae of *Dasyhelea* before this study. The larval stages of *Culicoides* and *Dasyhelea* actively feed upon the debris, organic matter, and substrata present in their habitat. In this study, proximate composition analysis reveals the number of

different nutrients in the food content. High crude fiber quantity has been found in both samples. Moderate quantities of crude protein and crude lipid are also observed which may be crucial for larval maturation. High value of Nitrogen Free Extract (NFE) corresponds to a high amount of carbohydrate which may be important for larval growth. Both types of habitat substrate samples reflect low Free Amino Acids (FAA) and low Free Fatty Acids (FFA) quantity referring to their little requirement for larval development. Finally, a low to moderate amount of minerals (ash content) may be sufficient for sustaining metabolic activity in the larval stages of the *Culicoides innoxius* and *Dasyhelea aprojecta*.

Table 2. The colony characteristics of eight bacterial isolates from *Culicoides innoxius* and *Dasyhelea flava*.

Characteristics	Isolate 1		Isolate 2		Isolate		Isolate 4		Isolate 5		Isolate 6		Isolate 7		Isolate 8	
	(Cu_1)	(Da_1)	(Cu_2)	(Da_2)	(Cu_3)	(Da_3)	(Cu_4)	(Da_4)	(Cu_5)	(Da_5)	(Cu_6)	(Da_6)	(Cu_7)	(Da_7)	(Cu_8)	(Da_8)
Form	Circular	Circular	Circular	Circular	Circular	Irregular	Circular	Circular	Irregular	Acute circular	Acute circular	Acute circular	Circular r	Circular	Irregular	Irregular
Size	Punctiform	Punctiform	14-15mm	10-15mm	10-12mm	10-15mm	10-15mm	20-27mm	Punctiform	Punctiform	12-15mm	14-15mm	20-24mm	28-30mm	10-14mm	10-11mm
Elevation	Flat	Raised	Flat	Flat	Flat	Flat	Flat	Flat	Raised	Raised	Ra999 999	Raised	Raised	Raised	Flat	Flat
Margin/Border	Undulate	Entire	Entire	Entire	Undulate	Undulate	Irregular	Irregular	Entire	Entire	Entire	Entire	Entire	Entire	Irregular	Irregular
Surface	Rough	Smooth	Smooth	Smooth	Rough	Rough	Dull	Dull	Glistening	Glistening	Glistening	Glistening	Glistening	Rough	Rough	Rough
Opacity	Opaque	Opaque	Opaque	Opaque	Semitrans lucent	Semitrans lucent	Translucent	Translucent	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Semitrans Lucent	Semitrans Lucent
Color	Yellowish white	Yellow	White	White	White	White	Dull white	Dull white	Yellow	White	Yellow	Bright yellow	Yellowish white	Yellowish white	Dull white	Grey

Table 3. Characteristics of bacteria from pure cultures and their endospore-forming ability.

Isolates	Gram Positive/ Negative	Bacilli/ Cocci	Length/ Diameter (µm)	Width (µm)	Endospore
Cu_1	Negative	Bacilli	1.42	0.70	Absent
Cu_2	Negative	Bacilli	1.80	0.64	Absent
Cu_3	Positive	Bacilli	1.80	0.62	Absent
Cu_5	Negative	Bacilli	1.84	0.60	Absent
Cu_6	Negative	Bacilli	2.74	0.74	Absent
Cu_7	Positive	Bacilli	1.76	0.72	Absent
Da_1	Negative	Cocci	0.64	-	Absent
Da_2	Positive	Bacilli	1.84	0.73	Absent

Da_3	Negative	Bacilli	2.76	0.69	Absent
Da_5	Negative	Bacilli	2.80	0.70	Absent
Da_6	Positive	Bacilli	1.84	0.74	Absent
Da_7	Positive	Bacilli	1.82	0.73	Absent

Table 4. Extracellular enzyme producing ability of different isolates ("+" = positive, "-" = negative).

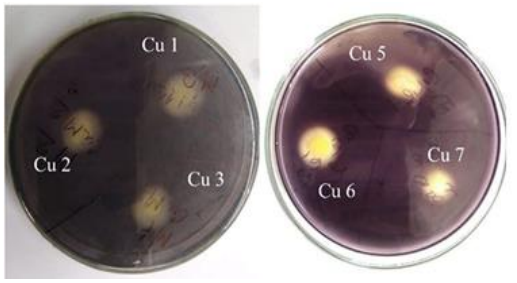
	Amylase	**Protease**	**Cellulase**	**Lipase**
Cu_1 / Da_1	+ / +	+ / +	+ / -	+ / +
Cu_2 / Da_2	+ / -	+ / -	+ / -	+ / +
Cu_3 / Da_3	+ / +	+ / -	- / -	+ / +
Cu_5 / Da_5	+ / -	+ / -	- / -	+ / +
Cu_6 / Da_6	+ / +	+ / +	- / -	+ / +
Cu_7 /Da_7	+ / +	+ / +	- / -	+ / +

Table 5. Halo zone characteristics (Diameter: 1-4mm = +, 5-8mm = ++, 9-12mm = +++, 13-16mm = ++++, 17-20mm = +++++ and 21-24mm= ++++++; margin: very diffused = VD, diffused = D, prominent = P, – = negative).

Enzymes	***Isolates***											
	Cu_1	**Da_1**	**Cu_2**	**Da_2**	**Cu_3**	**Da_3**	**Cu_5**	**Da_5**	**Cu_6**	**Da_6**	**Cu_7**	**Da_7**
Amylase	+++, D	++, VD	+++, P	–	+++, D	+, D	+++, P	–	+++, P	++, D	+++, P	+++, P
Protease	+++, P	+++, D	+++, P	–	+++, P	–	+++, P	–	+++, P	+++, D	+++, P	++++++, P
Cellulase	++, D	–	+, D	–	–	–	–	–	–	–	–	–
Lipase	++, P	++, P	++, P	+++, P	++, D	++, P	+++, P	++, P	+++, P	++. P	+++, D	+++, P

Culicoides innoxius has a potential role in spreading BTV through its blood-feeding habit in cattle [6, 7]. Adult *Dasyhelea* feeds on honeydew and sugar solutions of plants [16]. Adult biting midges may also visit flowers for nectar. Nevertheless, no information was available regarding extracellular enzyme-producing bacteria in the gut of adult *Culicoides* and *Dasyhelea*. Starch, a unique source of energy for these midges can provide the vigor for sustained flight and reproduction. In this study, six and four bacterial isolates were recorded as amylase positive in females of *Culicoides innoxius* and *Dasyhelea flava* respectively, indicating the positive role of the gut bacterial colonies in supplementing the enzyme. A high amount of the phloem sap proteins may have a role in stress and defense reactions in the phloem feeder insects [32], which also corroborates with our present study as we got three protease positive bacterial isolates from *Dasyhelea flava.* However, six protease-positive isolates indicate a protein-rich diet of *Culicoides innoxius* inferring their possible hemtophagous habit upon the vertebrate host. Cellulose is derived from D-glucose units and condenses through β- 1, 4-glycosidic linkage. As cellulose is the structural part of a plant cell wall, insects that feed on fluids of xylem or phloem cannot get access to the cellulose content. In the case of *Culicoides innoxius,* only two isolates were found as cellulase positive which leads us to assume its little dependency on cellulose rich food. However, in case of *Dasyhelea flava* no cellulase-producing bacteria was detected confirming that they feed on nectar, honeydew, etc. [12] which lack cellulose content. So, the feeding habit of adult *Dasyhelea flava* might have made their gut unfavorable for the growth of cellulase-producing bacteria. Triglycerols stored in adipose tissue are a major form of energy storage in animals. Insect adipocytes can store a large amount of lipid reserves as cytoplasmic lipid droplets [33]. Insect embryogenesis is highly dependent upon lipid metabolism and during their development, insect oocytes increase their lipid content several fold in a very short period [33]. Insect growth and reproduction, synthesis of trehalose and proline, and insect immune response rely upon lipid metabolism and lipids also provide the energy needed during diapause [34]. Fatty acids stored as triglycerides in anhydrous form can be used for energy production through β-oxidation [35].

The present study validates the vast role of lipid utilization by the *Culicoides innoxius* and *Dasyhelea flava* midges as all six isolates retrieved from both species were detected as lipase positive. Thus, gut bacteria aid in the digestion of lipid in the adult actively.



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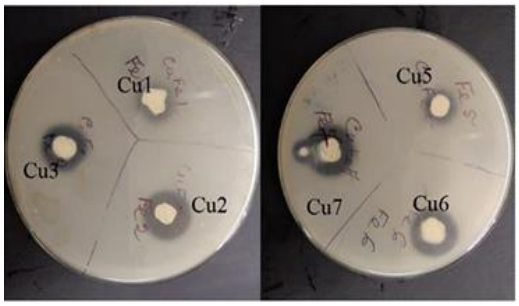

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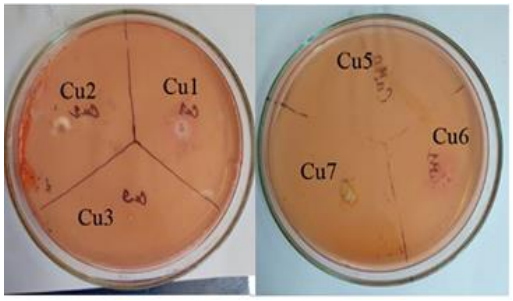

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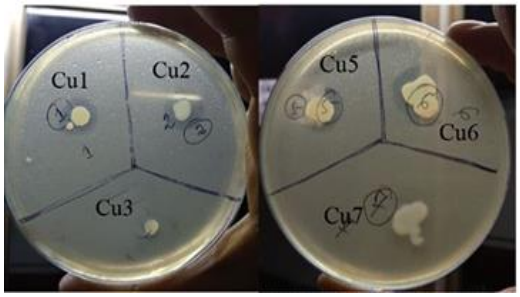

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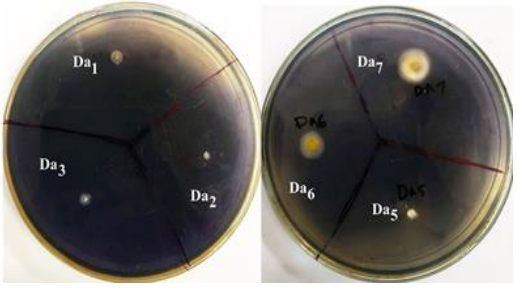

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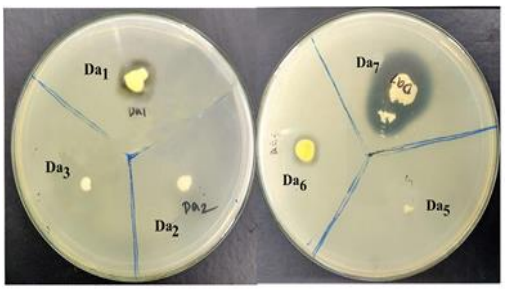

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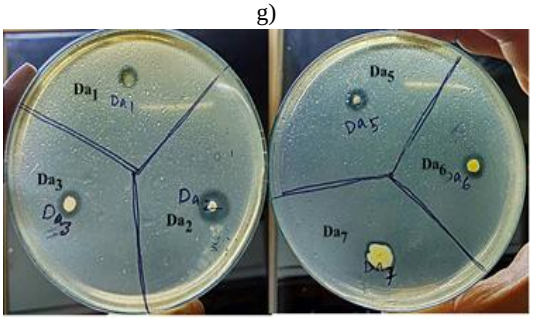

h)

Figure 2. Qualitative Determination of exo-enzyme producing autochthonous gut associated bacteria in adult *Culicoides innoxius* Sen and Das Gupta and *Dasyhelea flava* Carter, Ingram and Macfie: a) amylase; b) protease;c) cellulase and d) lipase producing bacterial determination of *Culicoides innoxius*; e) amylase; f) protease; g) cellulase and h) lipase producing bacterial determination of *Dasyhelea flava*.

CONCLUSION

The larvae of *Culicoides* are reported as trophic generalists and larvae of *Dasyhelea* are stated to usually feed on algae and fungi but the present investigation with the larvae of *Culicoides* (*Hoffmania*) *innoxius* [26] and *Dasyhelea* (*Pseudoculicoides*) *aprojecta* [27] are found to feed on plant derived organic habitat substrata. So, an attempt was made to get an idea about the overall nutrient content of the probable larval food material by analyzing the proximate composition of the substances. The result reveals the presence of a high quantity of crude fiber, moderate quantities of crude protein, crude lipid, low to moderate amount of minerals, and a high amount of carbohydrate

which may be vital for larval growth and metabolic activity. The result also refers to fewer requirements of Free Amino Acids (FAA) and Free Fatty Acids (FFA) as the value of these is very low.

Qualitative determination of exoenzyme-producing ability of the gut associated bacteria in adult *Culicoides innoxius* and *Dasyhelea flava* [28] midges indicate the presence of amylase, protease, cellulase, and lipase-producing bacteria in former*;* however, only cellulase producing bacteria are wanting in latter. Among the six bacterial isolates grown from the gut of *C. innoxius* all are positive in producing amylase, protease, and lipase*,* and only two isolates produce cellulase*.* However, in *D. flava*, all six, four and three isolates have been recorded as lipase, amylase, and protease positive respectively and no cellulase producing gut bacteria has been detected, reason may be due to their plant sap and nectar feeding habit. These gut bacteria have their role in supplementing the enzymes for digestion.

Detailed analysis of the larval food materials and association of symbiotic gut bacteria of adult biting midges will help the future workers to rear the insects in large number which will eventually help to decipher the biology of medico-veterinary important *Culicoides* spp. and economically important *Dasyhelea* spp. However, rearing of economically important *Dasyhelea* spp. will have a havoc impact on cocoa and rubber production.

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ETHICS STATEMENT: None

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