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Original papers

Feces from wild *Triatoma dimidiata* induces local inflammation and specific immune response in a murine model

Victor Monteon, Ivan May-Gil, Luis Nuńez-Oreza, Ruth Lopez

Centro de Investigaciones Biomédicas, Universidad Autonoma de Campeche, Patrico Trueba s/n, Campeche 24090, Mexico

Corresponding Author: Victor Monteon; e-mail: victormonteon@yahoo.com.mx

ABSTRACT. In endemic regions for *Triatoma dimidiata* the vector for Chagas disease, subjects can be in contact with insect`s feces several times through a lifetime. The triatomine's digestive tract is colonized by diverse but few dominant genera of microorganisms. The immune responses to microbiota feces are poorly known in mammal hosts. The goal of this paper is to describe the local inflammation at the port of inoculation and the humoral immune response in a murine model mimicking natural contamination of feces from wild *Triatoma dimidiata* and its identification of bacterial community. Feces from twenty *T. dimidiata* insects captured in peridomestic and domestic ecotopes were used for bacteria isolation and phenotypic identification. Five microliters of whole feces or bacteria isolated colonies were used for intradermal inoculation of mice for detection of humoral immune response and local inflammation at the inoculation site. The bacterial community identified corresponded to *Kytococcus*, *Brevibacillus*, *Kocuria*, *Chryseobacterium*, *Pantoe*, *Proteus*, *Burkholderia*, *Acinetobacter* and *Stapylococcus*. The local inflammation at the inoculation site was dominated by neutrophils infiltration, and specific seric IgG immune response was recognized against whole feces as well as *Burkholderia*, *Acinetobacter and Staphylococcus* isolates. In conclusion, feces from *T. dimidiata* were colonized by few culturable microorganism genera that are able to induce local inflammation and IgG immune response in a murine model.

Key words: *Triatoma dimidata*, gut microbiota, Chagas disease, *Trypanosoma cruzi*

Introduction

Since the pioneer works carried out on *Rhodnius prolixus* and *Panstrongylus megistus* that reported bacterial colonization of the triatomines's gut [1–2] there has been increased publication to identify triatomine gut microbiome, using culturing and non-culturing based methods. In recent studies, using non-culturing based methods, have been reported in *Rhodnius pallescens* and *Triatoma maculata* bacteria genera such as *Williamsia*, *Kocuria*, *Dietzia*, *Aeromonas*, and *Pelomonas* as the most prevalent [3]; whereas, in *Dipetalogaster maximus*, *Panstrongylus megistus*, *Triatoma infestans*, *Triatoma vitticeps*, *Rhodnius prolixus and Rhodnius neglectus* the most prevalent genera recognized are *Candidatus*, *Rohrkolberia*, *Arsenophonus*, *Serratia*, *Rhodococcus*, *Enterococcus faecalis*, *E. liquefaciens, Pseudomonas* sp., *Enterococcus* sp., *Streptococcus* sp., *Staphylococcus aureus*, *Corynebacterium* and *Micrococcus* sp. [4–5]. One of the main efforts have been made to understand the insects' immune response against microbiota including *Trypanosoma cruzi* and if the vectorial capacity may be affected by the microbiome [6–9]. Most of these previous studies were done using non-culturing based methods, this approach although very sensitive is not the option for keeping the consortium alive.

Other important Chagas disease vector with a wide geographical distribution is *T. dimidiata*; the vector is spread from Mexico to the northern region of South America. In Mexico, *T. dimidiata* represents an important vector involved in selvatic

and peridomestic cycle transmission. In spite of its large geographical distribution and epidemiological importance, only one very recent report do exist for *Triatoma dimidiata* microbiome using non-culturing based method [10]. They are described as the most abundant orders Bacillales, followed by Actinomycetales, Enterobacteriales and Burkholderiales, but till date there is no work on the immune response that microbiota can induce in the mammals.

The rationale for this work: If the hematophagous triatomines deposit feces when they are feeding, then it is possible for an immune response against inoculated microbiota. In endemic zone for Chagas disease, the abundance of hematophagous triatomines can be high, but likely only one third is infected with *T. cruzi*. This last condition may open three possibilities: 1) the subjects can be exposed several times to triatomines feces before getting infected with *T. cruzi*; 2) the subjects can be infected since the first time exposition to triatomine bites; 3) the subjects can be exposed only to triatomine's feces free of *T. cruzi* infection. The last two options are of low probability, but in the three options, the microbiota feces will be present.

In a recent work, we demonstrated that mice inoculated several times with *T. dimidiata* feces free of *T. cruzi* contamination and challenge after with *T. cruzi* metacyclic trypomastigotes helped to control parasitemia [11].

The goal of the present work is to identify the bacteria genera in *T. dimidiata* feces using culturing based methods and determine the immune response in mice inoculated intradermally with a small volume of feces.

Materials and Methods

Insects, feces collection and culture. Capture of triatomines was carried out between March to June that corresponds to dry season. Twenty adult insects were captured in the periphery of the urban city of Campeche, Mexico in the peridomestic area. All insects were classified as *Triatoma dimidiata* according to Lent and Wygodzinsky [12]. Once in the laboratory, they were fed with mouse blood for 10 to 15 minutes after that, insects were moved to a sterile Petri dish in order to pick up the feces/urine with a sterile tip. When two or more triatomines were worked together always they were worked individually. The feces were cultured in the blood-agar plate and the inoculum dispersed in order to get separate and individual colonies. The plates were incubated at 37°C for 24h or up to 72h for those of low growth rate. Each isolated colony was further streaked on a new blood-agar plate and incubated for 48h at 37°C to confirm homogeneity and uniqueness colony morphology. Finally, they were kept at -70°C in a mixture of glycerol and LB (Lauria broth) media.

Bacteria isolation and phenotypic identification. Each individual colony/isolate was cultured on blood-agar plate for colony morphology feature and hemolysis, after that a single colony was picked up for Gram staining, MacConkey growth, catalase and oxidase activity and biochemical analysis using API™ systems (API 20; API E; API NE and API CH50) following manufacturer's instructions or VITEK 2 Automated Identification System (bioMérieux, Marcy l'Etoile, France). Motility was confirmed on a fresh bacterial suspension and observed under microscopy examination. Identification was obtained with the numerical profile using the database (V7.0) in the apiweb™ following manufacturer's instructions. The tests were all carried out at least in duplicate.

16S rRNA gene sequencing. A single colony of pure bacteria was subcultured on a tryptic soy agar with 5% sheep blood. Thereafter, a small inoculum was cultured in Luria broth for 24 h. The cells were pellet and total DNA was isolated using a Wizard Genomic DNA Purification Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). The primers used to amplify the internal fragments of the 16S rRNA gene were: Unifor CTYAAAKRAAAATTGRCGGRRSSC; Unirev CGGGCGGTGTGTRCAARRSSC. After the PCR reaction, the amplified fragment was incised by agarose gel electrophoresis. The fragment was sequenced and their sequences were compared to sequences in GenBank using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Mice inoculation, histology. A total of twelve Balb/c mice were intradermally inoculated into the hind pad via injection with 500 µl insulin syringe contained 5 µl of feces or in particular cases with four independent isolates previously identified as mentioned above. Whole feces were a pool of ten triatomines. In the case of feces isolates, twenty ml of Luria broth (LB) was inoculated with one colony and left for 24 h at 37°C and after centrifugation, the pellet was adjusted to 0.5 McFarland units. Mice were inoculated with 5 µl of 1:100 PBS dilution

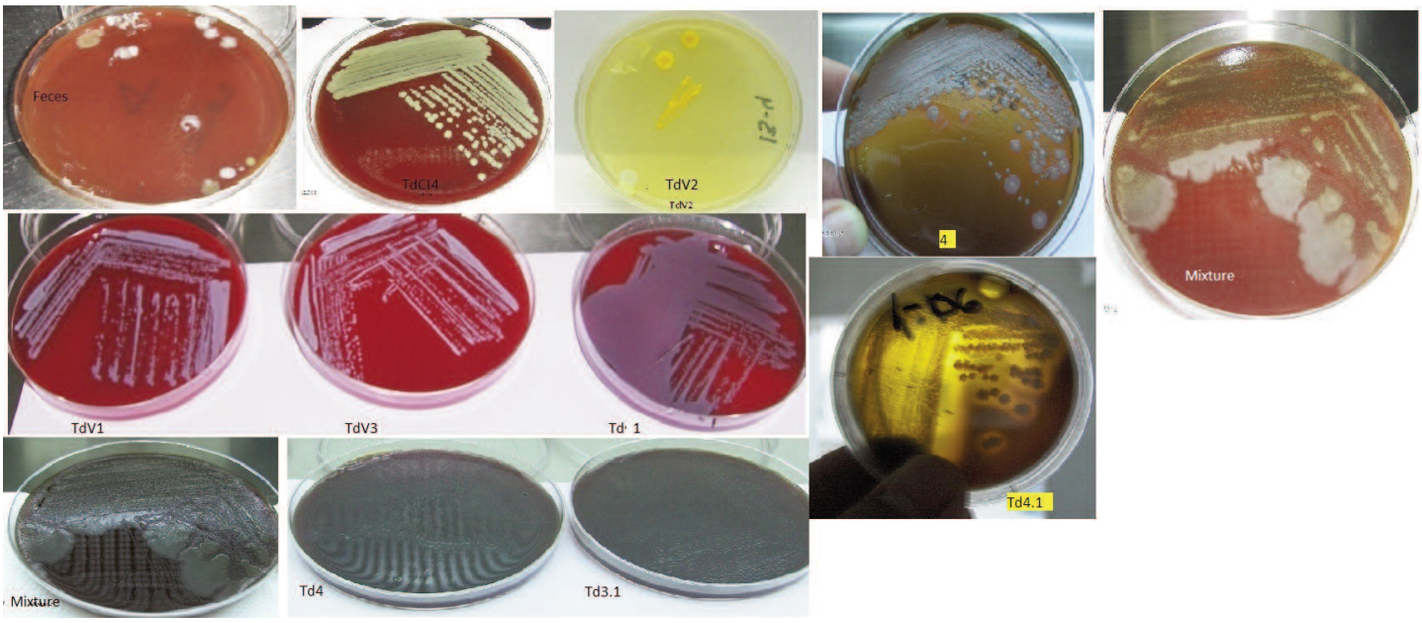

Fig. 1. Representative images of culture plates
In the upper left a blood-agar plate inoculated with whole feces of *Triatoma dimidiata* specimen. Each plate correspond to different successful isolates, whereas Mixture correspond to unsuccessful isolate.

bacteria suspension. Mice were sacrificed at 4h, 48h and 30 days after feces or isolates inoculation. Skin biopsies from the inoculation site were fixed in formalin 10% and processed for Hematoxylin-Eosin (HE) technique stain. Five-micrometre-thick slices were used for H.E and observed under an optical microscope.

The experimental protocol followed the guidelines of the institutional care and research committee, according to (NORMA Oficial Mexicana NOM-062-ZOO-1999 and University of Campeche) under the registry 2014-FI.

Serology. The blood sample was obtained from cardiac puncture of animals at 30 days post inoculation, the sample was centrifuged at 1500 g for 10 minutes and the serum sample was kept at 70°C until use. On the Dot-ELISA assay, we used square pieces of PVDF membrane coated with 5 µl of feces antigen. The antigen was obtained as follows: One hundred µl of feces from ten triatomines were spun down at 5000 g for 20 minutes, the pellet was resuspended in PBS and washed three times as above. Ten µl of suspension bacteria was diluted in 1 ml of carbonate/ bicarbonate buffer pH 9.6 to coating PVDF membrane for 1 h at 37°C. The pieces of membranes were incubated individually in 24 well plate dish. Next, the membrane was blocked with PBS-Tween 20 0.01%-skimmed milk 2% (PBS-T-M) for 1 h at 37°C. After three washes with PBS-T-M, twenty microliters of serum sample was added for 1 h at 37°C. After extensive washes with PBST, the pieces of membranes were incubated 100 µl with anti-mouse IgG conjugate peroxidase 1:5,00 for 1 h at 37°C. Then, the membranes were washed as above and finally, the reaction was revealed with DAB for ten minutes. In all assays, blank and negative control were included. For western blotting, four bacteria isolates were chosen. Each colony was cultured in 40 ml of LB media for 24 h, the pellet was collected, washed with PBS and resuspended in 5 ml. The bacteria suspension was sonicated at 100 watts for 30 seconds on ice. This cycle was repeated ten times and the cell lysis was verified by direct observation under a light microscope. Then, the sample was centrifuged at 12000 g for 10 minutes, the supernatant was recovered and protein concentration was determined (The samples were kept at -70°C until use). The SDS-PAGE electrophoresis was as follows; the gel acrylamide concentration at 10%, protein crude bacteria extraction at 40 µg/lane and electrophoresis run conditions at 120 V for 45 minutes on bed ice. A membrane of PVDF was used for transferring protein in a mini-protean system (Bio-Rad). The samples were transferred at 40 volts, 225 mA for 16 h. The membrane was blocked with PBS-T-M for 40 minutes, then serum samples diluted at 1:800 in PBS-T-M and incubated for 40 minutes at room temperature. After extensive washes with PBS-T, the strip was incubated with anti-mouse IgG

Table 1. Principal phenotypic characters of bacteria isolates of *Triatoma dimidiata* feces

Number	Isolate code	Gram	shape	colony color	hemolysis	Mac Conkey	lac	catalase	oxidase	motility	Genera	Probability
1	Td1	–	Rod	grey	–	+	+	+	+	+	*Burkholderia*	95%[a]
2	2	–	cocci–rod	white	+	+		+	–	–	Mixture	ND
3	3	–	Cocci		–	+	–	+	+	–	Mixture	ND
4	4	+	cocci–rod	grey	+	+	–	+	–	–	*Acinetobacter*	93%[a]
5	4.1	+	Cocci	whitish	+	–	–	+	–	–	*Staphylococcus haemolyticus*	87%[a]
6	1.1	+	cocci-rod	grey	+	+	+	+	–	+	Mixture	ND
7	Td3.1	+	Cocci	dark	+						Mixture	ND
8	Td3.2	–	Rod	transparent	–	+	–	+	–	+	*Proteus*	98%[a]
9	TdV1	+	Cocci	grey	+	–	–	+	–	+	*Staphyloccus sciuri*	96%[b]
10	TdV2	–	rod-cocci	yelow	–	+	+	+	–	+	Mixture	ND
11	TdV3	+	Cocci	cream	–	–	+	+	–	+	*Staphyloccocus sciuri*	96%[b]
12	TdCI1	+	Cocci	yelow	+	–	–	+	–	+	*Kytococcus*	99%[b]
13	TdCI2	–	Rod	whitish	–	+	–	+	–	+	*Brevibacillus*	98%[b]
14	TdCI3	+	Cocci	yellow	–	–	–	+	–	–	*Kytococcus*	99%[b]
15	TdCI4	+	Cocci	cream	+	–	–	+	+	–	*Kocuria*	99%[b]
16	Td1.1	–	Rod	white	–	–	–	+	–	+	Mixture	ND
17	TdC1	–	cocci-rod	whitnish	–	+	+	+	–	+	*Pantoea*	98%[b]
18	TdC2	+	Cocci	transparent	–	+	+	+	–	+	*Kytococcus*	99%[b]
19	Td4	–	Rod	transprent	-	+	–	+	–	+	*Proteus*	99 %[b]
20	T2	–	Rod	yellow	Sequence	ND	ND	ND	ND	ND	*Chryseobacterium*	98%[c]
21	T3	–	Rod	yellow	Sequence	ND	ND	ND	ND	ND	*Chryseobacterium*	98%[c]

The feces were cultured in blood-agar plate for at least 72h at 37°C. The phenotypic identification was carried out with different procedure: a - VITEK 2; b - API Rapid ID 32 Strep; API 20NE; API 20E; API Staph; API 50 CH; c - 16S rRNA gene sequencing; ND - not determined.

peroxidase at 1:1000 for 40 minutes, followed by extensive washes as mentioned above, the reaction was revealed with a Tris buffer pH 7.6 with 0.01% of H_2O_2, and diaminobenzidine 0.01% for 3 minutes, the reaction was stopped by washing with water. In all assays, blank and negative serum sample was included.

Results

The culture of feces on blood-agar plate allowed growth of four to five different colonies for each *T. dimidiata* specimen, overall, twenty one colonies with apparently different colony morphology was obtained, but in six cases it was not possible getting purified isolates with a unique microscopic profile, in spite of several times in trying to get pure microorganism (Fig. 1). In Table 1 the principal phenotypic characters are summarized; the ration of Gram+ and Gram – isolates was similar, but some of them are Gram-variable, also there were hemolytic isolates and others with the ability to oxidized blood culture. Either the identification was done with API^{TM} systems (API 20; API E; API NE and API CH50) or VITEK 2 analysis (Fig. 2) or only in two

Table 2. Sequencing of T2 and T3 isolates

>T2
>T3
16S rRNA gene sequencing

CATCTGCAGTTTACGATTCGCCCTTCGGGCGGTGTGTACAGAGCCGGGAACGTATTCACC
GCGCCATGGCTGATGCGCGATTACTAGCGATCCCAGCTTCATAGAGTCGAGTTGCAGACT
CCAATCCGAACTGAGACCGGCTTTCGAGATTTGCATCACATCGCTGTGTAGCTGCCCTCT
GTACCGGCCATTGTATTACGTGTGTGGCCCAAGACGTAAGGGCCGTGATGATTTGACGTC
ATCCCCACCTTCCTCTCTACTTGCGTAGGCAGTCTCACTAGAGTCCCCAACTGAATGATG
GCAACTAGTGACAGGGGTTGCGCTCGTTGCAGGACTTAACCTAACACCTCACGGCACGAG
CTGACGACAACCATGCGGCACCTTGAAAATTGCCCGAAGGAGGATCTATTTCTAAATCTG
TCAATTCCCATTTAAGTCTTGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATAAT
CCACCGCTTGTGGGCCCTCCGTCAATTCCTTTGAGAAGGGCGAATTCGCGGCCGCTAAAT
TCAATTCGCCCTATAGTGAGTCGTATTACATTCACTGGCCGTCGTTTTACACGTCGTGAC
TGGGAAACCCTGCGTACCCAACCTAATCGCTGCAGCAATCCCCTTCGCAGCTTGCGTATA

Chryseobacterium sp. FBF-7 16S ribosomal RNA gene, partial sequence	98%	FJ938215.1
Elizabethkingia miricola strain L99 16S ribosomal RNA gene, partial sequence	98%	EU375848.1
Chryseobacterium sp. LC 16S ribosomal RNA gene, partial sequence	98%	EF587903.1

cases,16S rRNA gene sequencing was used (Table 2).

Among bacteria classification, they corresponded to Actinobacteria-Actinomycetales (*Kytococus* and *Kocuria*), Firmicutes-Paeni bacillaceae (*Brevibacillus*), Firmicutes-Staphylococcaceae (*Staphylococcus haemolyticus* and *S. sciuri*), *Bacteriodetes* (*Chryseobacterium*), Proteobacteria-Enterobacteriaceae (*Pantoe* and *Proteus*), Beta-Proteobacteria (*Burkholderia*), Gamma-bacterias-Pseudomonadales (*Acinetobacter*). The probability of the accuracy in identification oscillates between 87% to 99% (Tables 3–6).

Feces inoculated into the dermis at 4h and 48h induces an inflammatory reaction with a predominance of neutrophils with particulate material retained in the dermis; at 30 days, post-

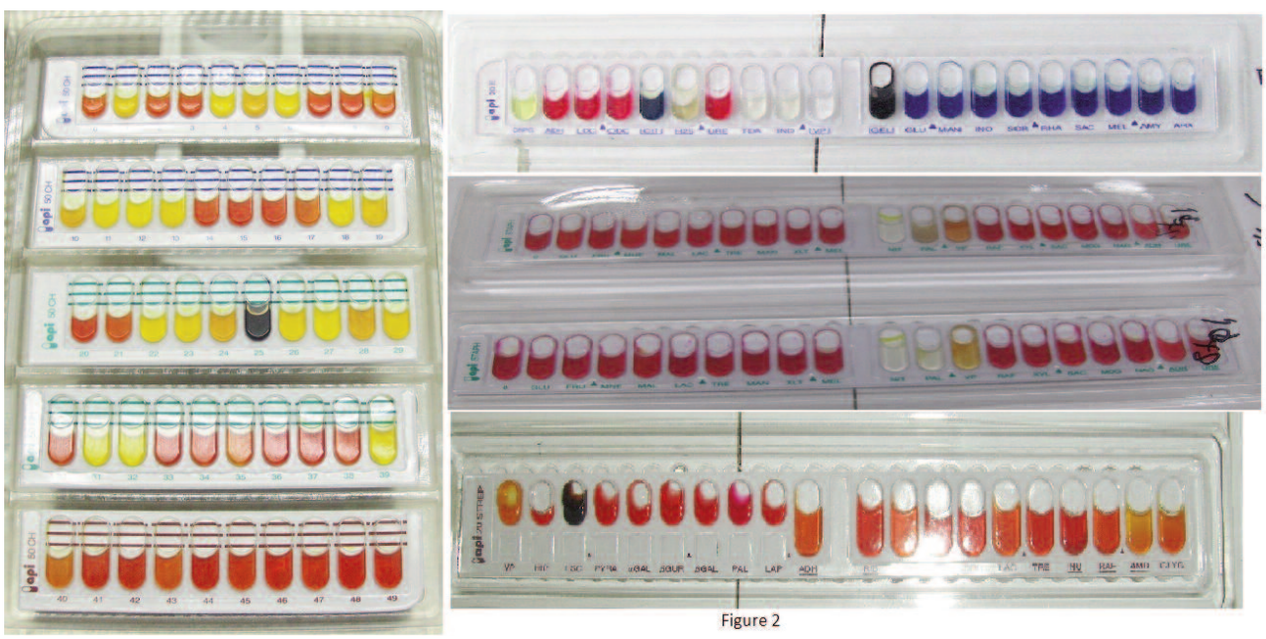

Fig. 2. Representative Api Gallery used

Table 3. Identification of bacteria isolates using GN (VITEK 2)

	Burkholderia	*Acinetobacter*	*Proteus*
Isolate code	1	4	Td3.2/Td4
APPA	–	–	–
H_2S	–	–	+
BGLU	–	–	–
ProA	–	–	–
SAC	–	–	–/+
ILATk	–	+	+
GlyA	–	–	–
O129R	–	–	–
ADO	–	–	–
BNAG	–	–	+
dMAL	–	–	+
LIP	–	–	+
dTAG	–	–	–
AGLU	–	–	–
ODC	–	–	–/+
GGAA	–	–	–
PyrA	–	–	–
AGLTp	–	+	–
dMAN	–	–	–/+
PLE	–	–	+
dTRE	+	–	+
SUCT	+	+	–
LDC	–	–	–
IMLTa	–	+	–
IARI	–	–	+
dGLU	+	+	–
dMNE	–	–	+
TyrA	+	+	–
CIT	–	–	+
NAGA	–	–	–
IHISa	–	–	–
ELLM	–	–	–
dCEL	–	–	–
GGT	–	–	+
BXYL	–	–	–
URE	–	–	–
MNT	–	–	–
AGAL	–	–	–
CMT	+	+	+
ILATa	–	+	–
BGAL	–	–	–
OFF	–	–	+
BAlap	–	–	–
dSOR	–	–	–
5KG	–	–	–
PHOS	+	–	+
BGUR	–	–	ND
Probability	95%	93%	98%

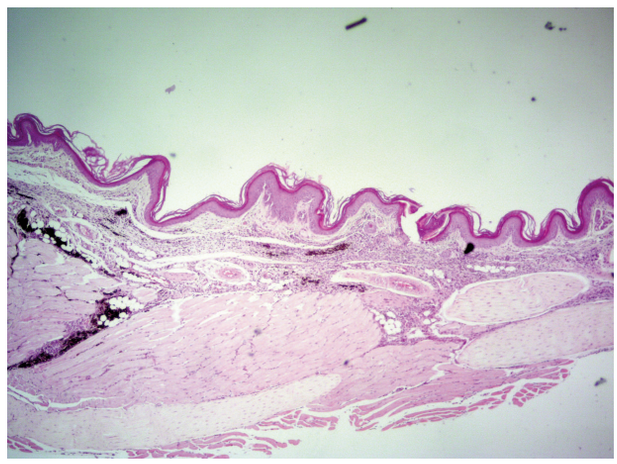

Fig. 3. Histology section at the inoculation site of feces in the dermis
Dark material correspond to 5–10 µl of *Triatoma dimidiata* feces inoculated intradermal. Inflammatory cells can be seen in the dermis at 4 h post inoculation. Magnification using 10× objective.

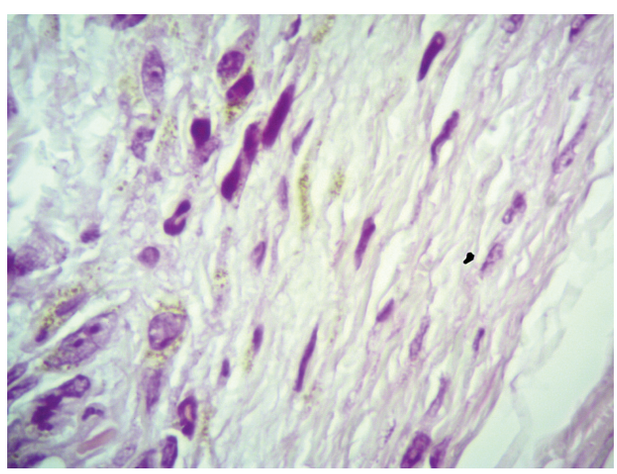

Fig. 4. Histology section at the inoculation site of feces at 30 days post inoculation
Dark material yet persist in some macrophages. Magnification using 100× objective.

inoculation macrophages can be seen yet with material ingested. This time corresponded to the last experimental time (Figs 3 and 4). Also, similar inflammatory reaction was observed with isolates corresponding to *Burkholderia*, *Acinetobacter and Staphylococcus* (data not shown).

The inoculation of feces induces a specific humoral IgG response in mice at 30 days post-inoculation; a clear positive reaction in the dot-ELISA assay can be detected when used as antigen the whole feces as indicated in material and methods (Fig. 5). When this same serum was used with pure bacteria isolated, such as *Burkholderia*, *Acinetobacter and Staphylococcus* and analyzed by western blotting; we observed a positive reaction that

Table 4. Identification of bacteria isolates using GP (VITEK 2)

	Staphylococcus haemolyticus
Isolate code	4.1
AMY	+
APPA	–
LeuA	–
AlaA	–
dRIB	+
NOVO	–
dRAF	+
OPTO	+
PIPLC	–
CDEX	–
ProA	–
TyrA	–
ILATk	+
NC6.5	+
0129R	+
dXYL	–
AspA	–
BGURr	–
dSOR	–
LAC	+
dMAN	+
SAL	–
ADH1	+
BGAR	–
AGAL	–
URE	–
NAG	+
dMNE	–
SAC	+
BGAL	+
AMAN	–
PyrA	+
POLYB	–
dMAL	+
MBdG	–
dTRE	+
AGLU	+
PHOS	–
BGUR	+
dGAL	+
BACI	–
PUL	–
ADH2s	–
Probability	87%

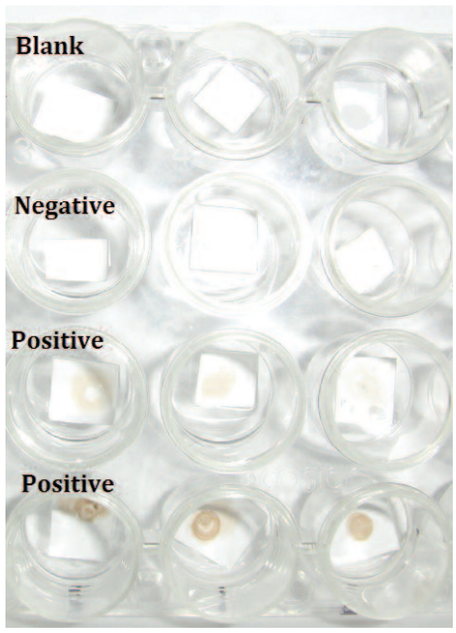

Fig. 5. Representative image of Dot-ELISA with whole feces as antigen
From left to right serum samples diluted at 1:10; 1:50 and 1:100, respectively. Blank and pre-immune serum gave negative reaction.

recognized several antigens between 10–75 KDa (Fig. 6). This finding confirms that bacteria presents in the feces are able to induce a specific immune response in a murine model.

Discussion

Triatomines are usually abundant in regions that are endemic to Chagas disease, and inhabitants can be in contact with feces more than once in their lifetime. It is accepted that triatomine's feces can gain access to the body via bite puncture or oral mucosa. The amount of fluid (feces-urine) that enter into the host may result in a matter of controversy, but an adult *T. dimidiata* can excrete after meal between 10–100 µl or feces/urine within a period of 1h (data not shown). It is likely that the volume of feces entering the human body should be minute. The above rationale, made us to use 5–10 µl inoculum in our experiment.

The gut insect's microbiota contains relatively few microbial species as compared to mammalian guts, their presence results in many cases that are a potentially beneficial to the host [13]. Bacteria microbiota in triatomines has been documented using two approaches: culture-dependent and culture-independent methods. Both methods can offer different advantages, the analysis of metagenomic DNA of 16S rDNA is able to identify

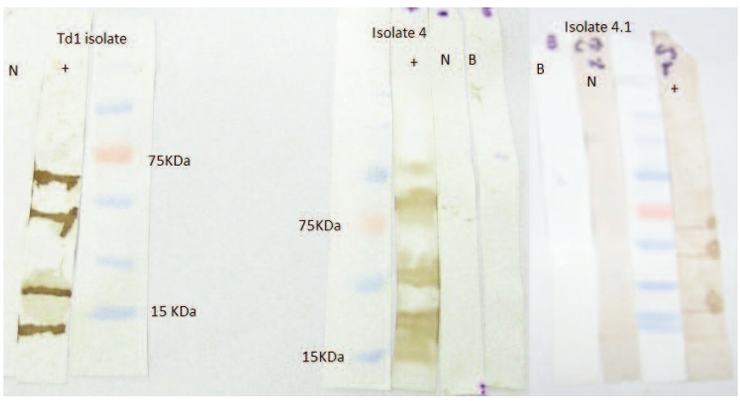

Fig. 6. Representative Western blot using as antigen three different isolates
Positive sera corresponded to a serum sample of mice immunized with whole feces. Negative correspond to pre-immune serum sample and Blank without serum. Serum sample dilution at 1:50.

a high number of microorganism, even those uncultured, but the culture-dependent method can allow to isolate and maintain the strain for further *in vivo* studies. In our case, we used the culture-dependent method and phenotypic identification means.

Among bacteria identified in *T. dimidiata* corresponded to Actinobacteria-Actinomycetales (*Kytococus* and *Kocuria*), Firmicutes-Paenibacillaceae (*Brevibacillus*), Firmicutes-Staphylococcaceae (*Staphylococcus haemolyticus* and *Staphylococcus sciuri*), *Bacteriodetes* (*Chryseobacterium)*, Proteobacteria-Enterobacteriaceae (*Pantoe* and *Proteus*), Beta-Proteobacteria (*Burkholderia*) and Gammabacterias-Pseudomonadales (*Acinetobacter*).

Although, we are still far from understanding the relationship between the above complex consortion, epimastigotes and metacyclic trypomastigotes of *Trypansoma cruzi* inside the vector, even what happens when they are inoculated in mammals. This work may be the basis in understanding this complex interaction.

As far as we know, this is the first report that describes the cultivable gut microbiota of *T. dimidiata*. During the review process of the present paper, a published a work about microbiota in *T. dimidiata* [10] appeared. They reported genus such as *Rhodococcus*, *Corynebacterium*, *Actinomycetospora*, *Bacillus*, *Staphylococcus* and *Anoxybacillus*. In our data, we did not find *Rhodococcus*, *Corynebacterium*, *Actinomycetospora* and *Anoxybacillus* genus instead we identified *Kytococus*, *Kocuria*, *Chryseobacterium*, *Burkholderia*, *Brevibacillus* and *Pantoe*. Some of these bacteria groups

Table 5. Identification of bacteria isolates using API Staph/CH 50

Isolate	*Staphylococcus sciuri* TdV1/TdV3	*Kytococcus* TdCI1/TdC2 /TdCI3	*Kocuria* TdCI4
GLY	+	+	ND
ERY	–	–	ND
DARA	–	+	ND
LARA	+	+	ND
RIB	+	+	ND
DXYL	+	+	ND
ADO	–	+	ND
MDX	–	–	ND
GAL	+	+	–
GLU	+	+	+
FRU	+	–	+
MNE	+	+	+
SBE	+	+	ND
RHA	–	+	ND
DUL	–	+	ND
IND	–	+	ND
MAN	+	–	–
SOR	+	–	–
MDM	+	–	ND
MDG	–	+	–
NAG	–	+	–
AMY	+	–	ND
ARB	+	+	ND
ESC	+	+	+
SAL	+	+	ND
CEL	+	+	ND
MAL	+	–/+	+
LAC	+	–/+	+
MEL	+	–/+	+
SAC	–	–/+	+
TRE	+	–	–
INU	+	ND	–
MLZ	–	ND	ND
RAF	–	–	+
AMD	+	ND	+
GLYG	–	ND	+
XLT	+	–	–
GEN	–	ND	ND
TUR	+	ND	ND
LYX	+	ND	ND
TAG	–	ND	ND
DFUC	–	ND	ND
LFUC	+	ND	ND
CARL	–	ND	ND
LARL	–	ND	ND
GNT	–	ND	ND
2KG	–	ND	ND
5KG	–	ND	ND
VP	–	+	+
ADH	+	+	+
URE	+	+	–
NIT	ND	–	–
PAL	ND	–	–
XYL	ND	+	+
Probability	96%	99%	99%

The CH 50 gallery was used as complement with staph for the identification of TdV1/TdV3 isolates

Table 6. Identification of bacteria isolates using API 20E/API 20NE/API 50 CH

	Brevibacillus	*Pantoea* spp.
Isolate code	TdCI2	TdC1
ONPG	–	+
LDC	+/–	–
ODC	+/–	–
H_2S	–	–
TDA	–	–
IND	–	–
VP	–/+	–
GEL	+	–
GLU	–	+
FRU	–	+
MNE	+	+
SBE	–	+
RHA	–	+
SAC	–	+
AMY	–	+
ARA	+	+
DUL	–	+
IND	–	+
MAN	+/–	+
INO	–	+
SOR	–	+
MDM	–	–
MDG	–	+
NAG	+	+
AMY	–	+
ARB	–	+
ESC	–	+
SAL	–	+
CEL	–	+
MAL	–	+
LAC	–	+
MEL	–	+
SAC	–	+
TRE	–	+
INU	–	–
MLZ	–	+
RAF	–	+
AMD	–	+
GLYG	–	+
XLT	–	–
GEN	–	+
TUR	–	+
LYX	–	–
TAG	–	+
DFUC	–	+
LFUC	–	+
DARL	–	+
LARL	–	+
GNT	+	–
2KG	–	–
5KG	–	–
ADH	+/–	–
URE	+/–	–
ESC	+	+
GEL	+	–
PNPG	+	ND
ARA	+/–	+
GNT	+	–
CAP	+	ND
ADI	–	ND
MLT	+	ND
CIT	+	+
PAC	+	ND
Probability	98%	98%

The three galleries were used to identification of isolates

have been documented for other triatomimes, such as *Chryseobacterium*, *Actinomyces*, *Acinetobacter* in *T. pseudomaculata* and *Staphylococcus* and *Acinetobacter* in *T. braziliensis*, but in general Proteobacteria, Actinobacteria, Firmicutes and Bacteroidete are common in triatomines [14].

Overall, the microbiota reported for different triatomines genus are similar but not identical, there is even a tendency to find a high frequency of particular bacteria genus or species in a given triatomine species. Such is the case of Actinobacteria in *Rhodnius pallescens*, *Serratia*, *Rhodococcus rhodnii* and *Dietzia* sp. in *Rhodnious prolixus* [4,5,14].

In our work, we found *S. sciuri* and *Micrococcus*

which have been associated with *Amblyomma cajennense* eggs [15], another important blood-sucking vector of a diversity of hosts, including humans. Many bacterial species found in hematophagous insects are part of the normal flora and widespread over human and animals skin such as *Kytococcus*, *Kocuria* and *Staphylococcus haemolyticus.* They are in general considered as commensal animal-associated species. To find them as part of feces'microbiota makes sense because some of these bacteria are associated with a given prey.

The approach we used for bacteria identification was based on standardized test systems such as APIH and VITEKH 2 (bioMerieux), complemented by traditional culture and microscopy methods, it is true that other platforms are available such as a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), multiple molecular marker sequencing, protein fingerprinting techniques and 16S rRNA gene sequencing. Undoubtedly, the best is 16S rRNA gene sequencing, however, there are several studies that demonstrate good agreement between the rest of the approaches [16–17], but other cases the conconcordance for some bacteria species is very low [18]. In our work, the cases with a probability 95% must be considered accurate at the genus level, for example, isolate number 1, 4 and 5, but the case 4.1 that corresponds to *Staphylococcus haemolyticus* must be verified with 16S rRNA gene sequencing.

A remarkable observation is that the bacteria genus found in our work, can be commensals of the skin surface of many animals, but it is possible that in favorable circumstances it may cause infectious diseases in humans.

The demonstration of the immune response to minute numbers of gut bacteria microbiota in animal model may be interesting in a different aspect. 1) Human beings that live in an endemic zone could be exposed several times to gut bacteria microbiota during their lifetime and as a consequence acquire a specific immune response; this immune response may be used as exposure marker or may have a possible role in modulating the *T. cruzi* infection, as we suggested recently [11]. 2) It could also be possible that gut microbiota may result in an opportunistic pathogen in the immuno-compromised human host or may lengthen the inflammatory reaction in susceptible subjects.

At the histology level, we observed that feces induce a strong local inflammation characterized by neutrophils during the first 48 h whereas, after a month, the mononuclear cells predominate in the infiltrate, even humoral specific IgG antibody response to bacteria gut microbiota can be demonstrated.

In conclusion, a minute number of gut bacteria microbiota induce an evident local inflammatory reaction and specific humoral immune response to *T. dimidiata* bacteria, in addition, bacteria genus identified corresponded to commensals common in the skin tissues.

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