The molecular interplay of the establishment of an infection – gene expression of ***Diaphorina citri*** **gut and** ***Candidatus*** **Liberibacter asiaticus**

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Abstract (max 300 words)

Candidatus Liberibacter asiaticus (CLas) is one the causative agents of greening disease in citrus, an unccurable, devastating disease of citrus worldwide. CLas is vectored by *Diaphorina citri*, and the understanding of the molecular interplay between vector and pathogen will provide additional basis for the development and implementation of successful management strategies. We focused in the molecular interplay occurring in the gut of the vector, a major barrier for CLas invasion and colonization. We investigated the differential expression of vector and CLas genes by analyzing a *de novo* reference metatranscriptome of the gut of adult psyllids fed of CLas-infected and healthy citrus plants for 1-2, 3-4 and 5-6 days. CLas regulates the immune response of the vector affecting the production of reactive species of oxygen and nitrogen, and the production of antimicrobial peptides. Moreover, CLas overexpressed *peroxiredoxin* in a protective manner. The major transcript involved in immune expression was related to melanization, a *CLIP-domain serine protease* we believe participates in the wounding of epithelial cells damaged during infection, which is supported by the down-regulation of *pangolin*. We also detected that CLas modulates the gut peristalsis of psyllids through the down-regulation of *titin*, reducing the elimination of CLas with faeces. The up-regulation of the neuromodulator *arylalkylamine N-acetyltransferase* implies CLas also interferes with the double brain-gut communication circuitry of the vector. CLas colonizes the gut by expressing two *Type IVb pilin flp* genes and several chaperones that can also function as adhesins. We hypothesized biofil formation occurs by the expression of the cold shock protein of CLas. We also describe the interplay during cell invasion and modification, and propose mechanisms CLas uses to invade the host hemocel. We identified several specific targets for the development of strategies directed to interfere with the successful utilization of the psyllid vector by this pathogen.

Keywords: host regulation, effector proteins, host – pathogen interactions, pathogen's ecology, infection strategies, host response to infection

Author Summary

Huanglongbing (HLB) or greening is an incurable disease causing severe damage to citrus production, making citrus industrial activity unsustainable in several countries around the world. HLB is caused by three species of *Candidatus* Liberibacter. *Ca.* L. asiaticus (CLas), vectored by the psyllid *Diaphorina citri*, is the prevalent species. Attempts to apply new technologies in the development of strategies for disease and pest management are been made. However, we still miss basic information on this system to efficiently apply the current technologies and envisage the implementation of new approaches for pest control, despite the relevant scientific contribution available. One major gap is regarded to the molecular interplay between CLas and its vector. We focused our attention in the molecular interplay occurring at the first relevant interaction of CLas and *D. citri*, represented by the gut barrier. We report the transcriptional activity of CLas during the invasion and establishment of the infection in the gut of the vector, as well as the transcriptional activity of the vector in response to the infection. We identified several host genes that are targeted and regulated by CLas as well as several CLas genes that are promising targets for the application of new management strategies.

1. Introduction

Microbes often establish different interactions with metazoans depending on the evolutionary history shared with their hosts. There are several examples of benefitial associations with multicellular hosts resulting from distinct ecological interactions with their hosts and associated trophic levels. But a concurrent number of interactions are also known to be noxious to multicellular hosts due to the development of a range of pathologies (Baumann 2005, Webb et al. 2006, Masnfield et al. 2012, Quigley 2013, Lu et al. 2020)

Viruses are certainly the most common microbes infecting prokaryotes and eukaryotes (Fauci 2001, Weinbauer 2004), and the year long pandemics caused by the Sars-CoV2 virus is overshadowing other threatining pathogens to animals and plants. Bacteria are widely associated with multicellular organisms, causing devastating diseases. Bacteria-causing diseases in agricultural systems are a risk to food security, causing severe losses in animal and plant production (Mansfield et al. 2012, Abebe et al. 2020).

Bacterial plant pathogens are also adapted to infect and propagate in insect tissues, as many insects are used as vectors by plant pathogens to infect new host plants and spread themselves in the environment. Plant pathogenic bacteria vectored by insects that use persistent, circulative, propagative mode of transmission require plastic phenotypes to interact with the different enviroments represented by sieves and tissues of the host plant, and the diverse environments faced in the gut lumen, hemocele and different tissues of the vector insect during the processes of acquisition and vector competence development (Huang et al. 2020). The capability of these pathogens to infect completely different hosts (plant and insect), and yet to depend on the shuttle host (insect) to locate and collect the pathogen in a diseased plant, and later transport it to

new, health host plants for the establishment of new infections requires a set of strategies devoted to manipulate the host insect (Perilla-Henao & Casteel 2016, Mauck et al. 2018). The understanding of the interactions pathogens and hosts have at their molecular level during the processes of host invasion and infection establishment is required for a fully comprehension of the mechanisms involved in guaranteeing the host – pathogen association. Such mechanisms of close interactions in the associations of host and pathogens represent potential new targets for the development of new technologies and/or use of existing technologies to interfere with the successful association pathogens establish with their hosts.

Candidatus Liberibacter are bacteria posing serious threat to food security worldwide by causing diseases to potatoes and citrus. Citrus is certainly the most severely damaged crop by *Ca.* Liberibacter infections, as three different species are known to infect citrus plants (*Ca.* L. asiaticus, *Ca.* L. americanus and *Ca.* L. africanus), causing the incurable greening or huanglongbing (HLB) disease (Bové, 2006; Graça et al., 2016; Tomaseto et al., 2019; Bassanezi et al., 2020). Candidatus Liberibacter did not make to the top 10 plant pathogenic bacteria (Masnfield et al. 2012), but HLB has caused a tremendous impact in the citrus industry and led to the complete elimination of citrus orchards and the interruption of citrus production in what used to be highly productive citrus centers (Dala-Paula et al. 2019, Bassanezi et al. 2020, Singerman & Rogers 2020).

Candidatus Liberibacter asiaticus (CLas) is the most spread in the world, currently infecting citrus in the major producing areas (CABI/EPPO 2017, Ajene et al. 2020, Wulff et al. 2020). In plants, *Candidatus* Liberibacter reside exclusively in the phloem sieve tubes (Jagoueix et al., 1994; Bové & Garnier, 2003; Bové, 2006; Bendix & Lewis, 2018), but it will infect several tissues of their host vector insect (Ammar et

al., 2011a; Ammar et al., 2017; Kruse et al., 2017; Ammar et al., 2019). CLas is vectored by the worldwidely distributed Asian Citrus Psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Bové, 2006).

Despite the importance of this disease to the worldwide citriculture and all of the investments and efforts of the scientific community to understand the interactions of the pathogen with its vector, much of the mechanisms involved in the pathogen-vector interplay remain unknown. We learned that CLas is transmitted from host plant to host plant by establishing a persistent, propagative transmission mode with its vector, a transmission mode that is very common to plant viruses vectored by aphids and whiteflies (Gray et al., 2014; Ammar et al., 2016; Kruse et al., 2017). We also learned CLas infects salivary glands and the midgut of vectors, tissues that are often recognized as natural barriers to circulative, propagative pathogens as they can prevent translocation of pathogens within the vector host (Ammar et al., 2011 a; Ammar et al., 2011 b; Ammar et al., 2016; Ammar et al., 2020). More recently, new information on the higher efficiency of adults than psyllid nymphs as vectors of CLas has been reported, with adults displaying a higher successful rate of infection of healthy citrus plants than nymphs (Ammar et al. 2020). Nevertheless, little information at the physiological and molecular level on the interface of the interactions of CLas with key vector tissues is available (Molki et al., 2019).

The limitations in the availability of efficient, cost-effective strategies for the management of the vector and the disease prompted a large number of investigations for the exploitation of new technologies, and promising results were mainly obtained with RNAi-based approaches (Santos-Ortega & Killiny, 2018; Lu et al., 2019a; Lu et al., 2019b; Yu & Killiny, 2020).

In here we focused on investigating the metatranscriptome of the gut of adults of *D. citri* feeding on CLas-infected citrus plants for different periods of time in order to identify genes of CLas that are expressed during the colonization of the gut of adults and the differential gene expression in the gut of psyllids over time against psyllids feeding on healthy, CLas-free citrus plants. Our major goal was to understand the dynamics of CLas gene expression during psyllid colonization and the response mechanisms that were activated in the gut epithelium of psyllids when exposed to *Ca*. Liberibacter asiaticus cells. We believe our data represents a source of very specific targets for the development and implementation of new strategies of psyllid/disease control using RNAi and/or gene editing technologies such as the CRISPR-Cas9 system.

2. Results

2.1. *De novo* transcriptome assembly

Sequencing from libraries of the gut of uninfected and CLas-infected nymphs and adults of *D. citri* yielded 395,151,161 reads, with an average of 16,464,632 reads/library. The use of the resulting 385,677,949 trimmed, quality-filtered reads (average 16,069,915 reads/library) allowed the *de novo* assembly of a transcriptome with 260,612,776 nucleotides (260 Mb), with transcripts with an average size of 481 bp and a N50 of 2,095 bp. The assembly resulted in 248,850 transcripts with an average of 39.9% GC content. Annotation of the transcriptome allowed the putative identification of 90,531 transcripts (36.4%), of which 52,081 transcripts were allocated to different gene ontology categories (S1 Fig). Additional filtering using the highest score hit after BlastX allowed the identification of 66,993 transcripts belonging to *D. citri*, 807 belonging to *Ca.* Liberibacter, and 1967 to the secondary symbiont *Wolbachia*, among others (S2 Fig).

175

176 **2.2. CLas gene expression in the gut of adults of *Diaphorina citri***

177 Since we could not detect relevant read countings against transcripts belonging
178 to *Ca*. L. asiaticus when using samples obtained from nymphs, only samples collected
179 at the adult stage were were subjected to differential expression analysis. Gene
180 expression analysis recognized 807 transcripts belonging to *Ca*. L. asiaticus in the gut of
181 adult psyllids fed on CLas-infected citrus plants in at least one of feeding exposure
182 periods analyzed for adults (1-2 d; 3-4 d; 5-6 d), with no CLas-related transcripts being
183 identified in gut samples from insects fed on healthy plants (*CLas*$^-$).

184 The detection of gene expression of *Ca.* L. asiaticus in adult psyllids increased in
185 adults feeding on CLas-infected plants for 1-2 d to 5-6 d. Thus, 725 transcripts of CLas
186 were expressed in *A1CLas*$^+$, 766 in *A2CLas*$^+$ and 804 in *A3CLas*$^+$ (Fig 1). One
187 transcript was expressed only in *A1CLas*$^+$ and *A2CLas*$^+$, 22 in *A1CLas*$^+$ and *A3CLas*$^+$,
188 and 63 in *A2CLas*$^+$ and *A3CLas*$^+$. We also identified CLas genes that were exclusively
189 expressed at the early (1-2 d, *A1CLas*$^+$= 1 transcript, DN14421_c0_g2_i2 = nicotinate-
190 nucleotide adenylyltransferase), intermediate (3-4 d, *A2CLas*$^+$= 1 transcript,
191 DN18703_c1_g1_i24 = Flp type IVb pilin) and late stage (5-6 d, *A3CLas*$^+$= 18
192 transcripts) after adult started feeding on CLas-infected citrus plants (Fig 1). Most of
193 these transcripts were represented by several isoforms of a gene, with different isoforms
194 expressed in more than one of the sampled feeding times. Only *A3CLas*$^+$ adults had
195 transcripts represented by unique isoforms of a gene specifically expressed in their gut
196 (Table 1).

197 **Table 1.** Single isoforms of *Candidatus* Liberibacter asiaticus transcripts exclusively
198 expressed in the gut of adult psyllids after 5-6d of feeding (*A3CLas*+) on CLas-infected
199 citrus plants.
200

Transcript ID	Annotation	TPM count

DN1443_c0_g1_i1	hypothetical protein PSGCA5_05	58.77
DN15414_c0_g1_i2	pyridoxine 5'-phosphate synthase	5.33
DN25987_c0_g1_i1	hypothetical protein	30.77
DN32802_c0_g1_i1	anti-repressor protein	38.31
DN39169_c0_g1_i1	hypothetical protein BWK56_05555	69.16
DN44737_c0_g1_i1	threonine--tRNA ligase	168.28
DN47430_c0_g1_i1	flagellar basal body rod protein FlgF	54.15
DN55271_c0_g1_i1	phage related protein	10.66
DN62541_c0_g1_i1	flagellar basal body P-ring protein FlgI	31.40
DN64078_c0_g1_i1	putative phage-related acetyltransferase	43.56

Pairwise gene expression analysis of CLas in the gut of adult psyllids after different periods of feeding on CLas-infected citrus plants revealed a high number of differentially expressed CLas genes in *A3CLas*$^+$ as compared to *A1CLas*$^+$ and *A2CLas*$^+$ adults (S1 Table). One-hundred transcripts out of the over 700 transcripts detected in the three sampling times differed in their abundance (S1 Table). Differences in the level of expression were detected for 80 transcripts when comparing *A1CLas*$^+$ and *A3CLas*$^+$ and 20 transcripts in comparisons of *A2CLas*$^+$ and *A3CLas*$^+$ (S1 Table). CLas expression was always higher in *A3CLas*$^+$ when compared to the others. No differences in gene expression between *A1CLas*$^+$ and *A2CLas*$^+$ were detected (S1 Table).

2.3. Differential gene expression in the gut of *CLas*$^+$ and *CLas*$^-$ adults of *Diaphorina citri*

Differential gene expression (DE) of the gut of adults of *D. citri* after feeding on CLas-infected plants for 1-2 days identified 24 DE transcripts in the gut of *CLas*$^+$ insects, 20 of them were up-regulated and 4 were down-regulated in response to CLas infection. Most of the up-regulated transcripts (11) belong to uncharacterized proteins, while nine up-regulated transcripts were identified (Table 2). After 3-4 days of feeding

on CLas-infected plants, the number of DE transcripts in the gut of *CLas*$^{+}$ as compared to *CLas*$^{-}$ adult psyllids increased to 35, most of which were up-regulated (27). Eighteen out of the 27 up-regulated transcripts, and seven out of the eight down-regulated transcripts were putatively identified (Table 2). The number of DE transcripts in the gut of adult psyllids after 5-6 days of feeding on CLas-infected plants was the highest, with 61 DE transcripts in the gut of *CLas*$^{+}$ insects, 57 (33 unknown proteins) of them were up-regulated and 4 were down-regulated when compared to *CLas*$^{-}$ adult psyllids (Table 2).

Table 2. List of genes difference expressed comparison of *Diaphorina citri* adults fed on *Candidatus* Liberibacter asiaticus infected and non-infected plants at different

Stage	DE	Annotation	Transcript ID	FC	FDR
***A1 - *CLas*$^{+}$ x *CLas*$^{-}$**	**Up-regulated**	arylsulfatase B-like isoform X1	DN19237_c6_g3_i2	20.9	$2.0e^{-2}$
		CLIP domain-containing serine protease 2-like isoform X1	DN16851_c1_g2_i2	119.9	$3.0e^{-3}$
		kinesin-like protein KIF3B isoform X4	DN10161_c0_g1_i1	55.2	$6.1e^{-3}$
		membrane metallo-endopeptidase-like 1 isoform X2	DN16798_c0_g1_i1	49.0	$4.8e^{-4}$
		methylglutaconyl-CoA hydratase, mitochondrial	DN18628_c4_g1_i2	26.7	$1.0e^{-2}$
		mitochondrial cytochrome c oxidase subunit IV	DN18774_c0_g1_i7	44.2	$2.3e^{-6}$
		nuclear speckle splicing regulatory protein 1-like	DN17904_c2_g1_i3	133.9	$4.0e^{-2}$
		uncharacterized protein LOC103515486	DN16253_c6_g2_i4	11.3	$9.64e^{-3}$
		protein phosphatase 1 regulatory subunit 21	DN19112_c0_g1_i1	36.7	0.02
		putative rna-directed dna polymerase from transposon bs	DN20114_c0_g1_i11	26.6	0.05
		uncharacterized protein LOC103508016	DN17144_c6_g5_i2	83.6	$2.93e^{-5}$
		uncharacterized protein LOC103508016	DN17144_c6_g5_i3	62.7	0.02
		uncharacterized protein LOC103518490	DN15191_c0_g1_i1	222.8	$2.03e^{-3}$
		uncharacterized protein LOC103518490	DN15191_c0_g1_i4	41.0	$6.75e^{-3}$
		uncharacterized protein LOC103519237	DN15191_c0_g1_i7	210.2	$1.06e^{-4}$
		uncharacterized protein LOC113469268	DN20635_c0_g3_i1	242.2	$1.06e^{-4}$
		uncharacterized protein LOC113469268	DN20635_c0_g1_i3	106.3	$5.87e^{-4}$
		uncharacterized protein LOC113471433	DN20585_c3_g1_i12	167.6	$5.28e^{-3}$
		uncharacterized protein PFB0145c-like	DN15546_c0_g1_i1	31.3	0.04

		uncharacterized protein YMR317W-like	DN15237_c0_g1_i1	132.6	$4.69e^{-4}$
	Down-regulated	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3	DN17549_c4_g1_i2	-32.5	$6.75e^{-3}$
		protein pangolin, isoforms A/H/I/S-like	DN15424_c0_g3_i2	-28.0	0.03
		titin isoform X7	DN21409_c5_g2_i1	-36.1	0.03
		zonadhesin-like	DN14644_c0_g1_i1	-13.0	0.03
**A2 - *CLas*$^{+}$ x *CLas*$^{-}$	**Up-regulated**	Ac1147-like protein	DN18394_c1_g1_i3	19.7	0.02
		arylalkylamine N-acetyltransferase	DN20634_c1_g1_i13	45.9	0.05
		cathepsin L1-like	DN15698_c4_g11_i2	111.4	$1.45e^{-3}$
		hypothetical protein DAPPUDRAFT_37179, partial	DN18534_c0_g5_i1	17.0	$7.51e^{-4}$
		hypothetical protein g.47064	DN18473_c2_g1_i1	11.7	$8.68e^{-3}$
		hypothetical protein g.7198	DN16128_c4_g1_i1	11.1	$3.86e^{-3}$
		large neutral amino acids transporter small subunit 1	DN20157_c2_g3_i2	66.6	$1.50e^{-4}$
		nocturnin isoform X1	DN15927_c10_g1_i6	15.1	$2.82e^{-4}$
		predicted protein	DN21032_c17_g1_i2	27.0	$2.19e^{-3}$
		predicted protein	DN21032_c17_g1_i1	23.9	$6.41e^{-3}$
		uncharacterized protein LOC105736982	DN21288_c9_g7_i2	28.5	$8.64e^{-4}$
		uncharacterized protein LOC103515486	DN16253_c6_g2_i4	12.6	0.04
		profilin	DN19422_c2_g2_i1	162.4	$7.00e^{-9}$
		protein hu-li tai shao	DN19744_c0_g2_i2	12.2	0.01
		putative senescence-associated protein	DN18133_c2_g1_i3	28.0	$1.41e^{-3}$
		Regulator of rDNA transcription protein 15	DN16244_c2_g3_i1	24.8	$1.62e^{-3}$
		Regulator of rDNA transcription protein 15	DN21288_c9_g7_i1	16.4	$6.43e^{-3}$
		Regulator of rDNA transcription protein 15	DN16244_c2_g3_i2	15.6	$7.58e^{-4}$
		Regulator of rDNA transcription protein 15	DN16244_c2_g3_i3	13.0	$5.46e^{-3}$
		reverse transcriptase-like protein	DN21162_c2_g1_i4	12.4	0.02
		sodium/potassium-transporting ATPase subunit alpha-like	DN14485_c0_g1_i1	17.9	$5.33e^{-4}$
		UNC93-like protein MFSD11	DN16539_c2_g6_i1	48.5	0.04
		Uncharacterized protein ART2	DN19721_c0_g1_i3	9.8	0.02
		uncharacterized protein LOC105206152	DN18133_c2_g1_i1	22.8	$1.45e^{-3}$
		uncharacterized protein LOC105693632	DN19721_c0_g4_i1	7.9	$8.56e^{-3}$

		uncharacterized protein LOC113219380	DN19326_c1_g1_i2	35.7	$6.21e^{-4}$
		uncharacterized protein LOC113469268	DN20635_c0_g1_i1	37.4	0.02
	Down-regulated	leptin receptor overlapping transcript-like 1	DN20058_c2_g3_i4	-26.7	0.02
		methylglutaconyl-CoA hydratase, mitochondrial	DN18628_c4_g1_i2	-11.3	$1.27e^{-3}$
		putative juvenile hormone binding protein	DN21423_c1_g12_i2	-12.3	0.04
		Soluble NSF attachment protein	DN21196_c2_g1_i1	-19.8	$5.64e^{-7}$
		translation elongation factor 2	DN21188_c1_g7_i2	-11.9	0.02
		tubulin-folding cofactor B	DN17504_c3_g2_i6	-4.5	$1.82e^{-3}$
		uncharacterized protein LOC103513992	DN16335_c5_g1_i1	-16.7	$5.18e^{-3}$
		zinc transporter ZIP10 isoform X1	DN19132_c8_g1_i5	-43.4	0.02
***A3 - *CLas*$^{+}$ x *CLas*$^{-}$	**Up-regulated**	2-oxoglutarate dehydrogenase, mitochondrial-like	DN14833_c0_g1_i1	8.6	0.03
		Ac1147-like protein	DN21090_c8_g2_i1	50.3	0.04
		BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	DN20168_c2_g3_i4	21.0	0.04
		beta-1,3-galactosyltransferase 5-like	DN18243_c2_g2_i2	20.2	$7.31e^{-3}$
		cleavage and polyadenylation specificity factor subunit CG7185-like	DN15201_c0_g1_i1	15.6	$6.05e^{-3}$
		cytochrome P450-like TBP	DN19721_c0_g1_i4	35.7	0.05
		dynein heavy chain 7, axonemal-like	DN35915_c0_g1_i1	17.3	0.05
		gamma-glutamyl hydrolase-like	DN18300_c5_g1_i2	16.7	$3.96e^{-5}$
		GATA zinc finger domain-containing protein 14-like	DN16387_c3_g2_i1	12.2	$6.66e^{-3}$
		glycerophosphocholine phosphodiesterase GPCPD1-like isoform X1	DN15741_c0_g2_i2	41.6	$1.10e^{-4}$
		hydrocephalus-inducing protein homolog	DN14616_c0_g1_i2	26.6	0.03
		hydrocephalus-inducing protein homolog	DN14616_c0_g2_i2	16.6	0.02
		hypothetical protein ALC57_18598	DN17391_c0_g1_i2	230.6	$1.56e^{-4}$
		hypothetical protein FF38_03795	DN18516_c0_g3_i1	67.0	0.02
		hypothetical protein LOTGIDRAFT_202939	DN17968_c0_g2_i3	101.2	0.03
		hypothetical protein Phum_PHUM590900	DN17430_c2_g2_i1	24.2	0.02
		keratin-associated protein 5-3-like	DN15230_c0_g1_i2	14.2	$6.76e^{-4}$
		keratinocyte proline-rich protein-like	DN20284_c2_g1_i3	11.5	0.02
		mucin-5AC-like, partial	DN16207_c6_g5_i1	7.7	0.04
		uncharacterized protein LOC103511862	DN17183_c5_g2_i3	9.4	0.04

uncharacterized protein LOC103513455	DN17270_c3_g2_i1	46.8	0.04
uncharacterized protein LOC103514633	DN18467_c1_g5_i1	31.7	$3.61e^{-5}$
uncharacterized protein LOC103515486	DN16253_c6_g2_i4	12.6	0.04
uncharacterized protein LOC103518002	DN18156_c6_g2_i2	10.0	0.03
uncharacterized protein LOC103521603	DN17270_c3_g2_i2	63.7	0.01
uncharacterized protein LOC103521674	DN15182_c0_g1_i1	9.3	0.03
protein AMN1 homolog	DN15340_c0_g1_i1	8.9	0.04
protein FAM133-like isoform X1	DN19474_c1_g3_i11	15.4	$8.40e^{-3}$
protein PRRC2C-like	DN15390_c0_g1_i1	21.0	$3.47e^{-4}$
protein suppressor of forked	DN20501_c1_g1_i2	50.7	0.04
putative juvenile hormone binding protein	DN21423_c1_g12_i1	33.0	$5.4e^{-5}$
putative juvenile hormone binding protein	DN21423_c1_g12_i2	9.4	$8.34e^{-3}$
RNA-directed DNA polymerase from mobile element jockey-like	DN19725_c3_g1_i3	14.1	$6.95e^{-3}$
senescence-associated protein	DN16041_c1_g1_i2	198.7	$1.67e^{-4}$
SWI/SNF chromatin-remodeling complex subunit SNF5-like	DN18520_c6_g1_i1	52.5	$1.18e^{-5}$
uncharacterized protein Dyak_GE27401	DN16348_c2_g1_i1	18.0	0.02
uncharacterized protein LOC103507494 isoform X2	DN20548_c5_g2_i7	13.1	0.01
uncharacterized protein LOC103507496	DN20260_c2_g2_i2	17.0	$6.6e^{-4}$
uncharacterized protein LOC103507787	DN20585_c3_g1_i2	60.0	$8.58e^{-3}$
uncharacterized protein LOC103512709	DN20458_c5_g2_i2	29.7	0.03
uncharacterized protein LOC103514651	DN18692_c1_g1_i7	41.1	$9.13e^{-4}$
uncharacterized protein LOC103516995	DN14754_c0_g3_i1	39.3	$2.89e^{-3}$
uncharacterized protein LOC103518490	DN15191_c0_g1_i2	105.3	0.05
uncharacterized protein LOC103518490	DN15191_c0_g1_i4	85.7	$7.47e^{-3}$
uncharacterized protein LOC103519237	DN15191_c0_g1_i7	36.9	0.01
uncharacterized protein LOC103520317	DN17514_c8_g2_i1	10.7	0.01
uncharacterized protein LOC103524424	DN14888_c0_g2_i1	7.8	0.04
uncharacterized protein LOC108252545 isoform X1	DN16387_c3_g1_i9	7.6	0.03
uncharacterized protein LOC113219351	DN17193_c2_g1_i6	74.5	$8.74e^{-4}$
uncharacterized protein LOC113219380	DN18715_c0_g1_i6	305.6	$9.53e^{-5}$
uncharacterized protein LOC113219380	DN21090_c7_g10_i1	82.0	0.04
uncharacterized protein LOC113469268	DN20635_c0_g3_i2	73.8	0.03
uncharacterized protein LOC113469268	DN20635_c0_g1_i1	37.4	0.02

	uncharacterized protein LOC113469268	DN20635_c0_g3_i1	36.9	0.02
	uncharacterized protein LOC113469388	DN14497_c0_g1_i2	8.9	0.04
	uncharacterized protein YMR317W-like	DN15237_c0_g1_i1	60.2	$8.52e^{-4}$
	Zgc:165536 protein	DN19721_c0_g2_i1	186.9	$8.52e^{-4}$
Down-regulated	annexin B9-like isoform X1	DN19784_c8_g1_i2	-11.0	0.03
	cytosolic purine 5'-nucleotidase isoform X4	DN16811_c2_g1_i5	-37.4	0.02
	probable 4-coumarate--CoA ligase 1	DN19422_c2_g3_i12	-7.4	0.05
	UDP-glucuronosyltransferase 2C1-like	DN20095_c3_g1_i5	-12.4	$1.92e^{-3}$

* A1- *CLas*+ x *CLas*-: Differential gene expression of adults with 1 to 2 days of feeding; ** A2- *CLas*+ x *CLas*-: Differential gene expression of adults with 3 to 4 days of feeding; *** A3- *CLas*+ x *CLas*-: Differential gene expression of adults with 5 to 6 days of feeding

From the total number of differentially expressed transcripts of $CLas^{+}$ when compared to their respective $CLas^{-}$ psyllids, only one transcript was differentially expressed in $CLas^{+}$ psyllids regardless the time they were allowed to feed on CLas-infected citrus plants as compared to psyllids fed on CLas-uninfected citrus plants (Fig 2). Most of the DE transcripts detected in $CLas^{+}$ psyllids differed only at the specific stage they were compared. Seventeen (71%) of the DE transcripts detected in the gut of adult psyllids after 1-2 d, 31 (88%) after 3-4 d and 60 (88%) after 5-6 d of feeding in CLas-infected plants differed from controls specifically at each particular stage (Fig 2).

3. Discussion

The gut differential gene expression in adult psyllids differed depending on the duration of feeding on CLas-infected citrus plants. Longer was the duration of feeding on CLas-infected citrus plants, higher were the number of DE transcripts. Most interesting, the majority of DE transcripts detected were specific to each one of the periods of feeding psyllids remained exposed to CLas-infected plants.

Gene expression of CLas in the gut of adult psyllids after different times of feeding on CLas-infected citrus plants were quite different. CLas transcription in the gut of adult psyllids was highly active soon after adult feeding started, but expression of a large set of genes was significantly increased at later stages of feeding (*A3CLas*$^{+}$).

Immune attack and immune defense responses

Bacteria that gain access to the hemocoel of insects find an easy way through the gut epithelium of the midgut, once the epithelium in this region does not have the cuticle lining protecting the fore- and the hindgut. Psyllids, as other sap-feeding insects do not carry the peritrophic membrane that protects the midgut epithelium of several other groups of insects (Lehane & Billingsley 1996, Erlandson et al. 2019). But regardless the presence of such physical barriers, the front line of defense of the gut immune system involves the activation of the epithelial innate immune system for the production of reactive oxygen species (ROS). Bacteria that survive in the gut of insects are either resistant to or are able to metabolize ROS (Buchon et al. 2013, Vallet-Gely et al. 2008).

Analysis of the CLas gene expression in the gut of adult psyllids demonstrated that CLas expressed the cytoprotective antioxidant enzyme peroxiredoxin (DN17546_c4_g1_i1), capable of reducing ROS and reactive nitrogen species (RNS) produced in the process of gut infection (Perkins et al., 2015; Knoops et al., 2016). The significant increase in the expression of peroxiredoxin from *A1CLas*$^{+}$ to *A3CLas*$^{+}$ (FC = 13.48) indicates this enzyme provides an increased contribution in the infection process as CLas cells also established an intracellular interaction with the gut epithelium of psyllids. CLas expression of peroxiredoxin occurred despite the expected reduction of ROS in the gut epithelium of adult psyllids, as the major source for ROS production in

the gut, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3 (DN17549_c4_g1_i2), was down-regulated in *CLas*$^+$ psyllids.

These results suggest that CLas can inhibit hydrogen peroxide production in the host insect, and that peroxiredoxin is certainly serving roles other than its antioxidative contribution. The adipokinetic hormone (ADK) is involved in the regulation of the oxidative stress response in insects (Kodrík et al. 2015). Unfortunately, the expression levels of ADK-related genes by CLas infection could not be verified as ADK is produced and stored in neurosecretory cells of the *corpora cardiaca*. Nevertheless, regulation of this neuropeptide hormone in the gut epithelium of *CLas*$^+$ psyllids could occur by the up-regulation of the metallo-endopeptidase-like 1 isoform X2 (DN16798_c0_g1_i1) neprilysin. Neprilysins are metalloproteases better known for their neuropeptide degrading activity, while also carrying peptide-degrading activity in other tissues, including the gut (Turner et al. 2001).

CLas peroxiredoxin could also be acting in the regulation of H_2O_2-mediated cell signaling processes, such as those involved in growth and immune responses (Shears & Hayakawa 2019). The oxidative response produced by gut epithelia due to microbial infection leads to cell proliferation and modulation of the innate immune response (Neish 2013), and ROS is required for inducing nitric oxide production in the gut. Nitric oxide production will in turn trigger the synthesis of antimicrobial peptides (AMP) and activate organ-to-organ communication (Wu et al. 2012). Therefore, the down-regulation of ROS production in the gut of *CLas*$^+$ psyllids could explain the lack of differential expression of genes belonging to the AMP production pathways. AMP synthesis can also be elicited by the recognition of peptidoglycans released by bacteria by membrane-bound peptidoglycan recognition proteins if peptidoglycans survive to amidase degradation (Vallet-Gely et al. 2008).

There is very little information on insect gut phagocytosis, although proteomic analysis of anal droplets of the beetle *Cryptorhynchus lapathi* larvae led to the identification of several proteins to support an immune cellular response at the gut level (Jing et al. 2018). Moreover, phagocytic receptors identified in the gut epithelium of *Drosophila* were proven to play crucial role in the phagocytosis of both Gram bacteria, and in controlling hemocele infection by bacteria invasion through the gut epithelium (Melcarne et al. 2019). We could not detect any changes at the molecular level in *CLas*$^{+}$ psyllids that would demonstrate a phagocytic response in the gut, as well as we did not observe adult psyllids to build an immune response to CLas infection by activating ROS and AMP pathways.

Nevertheless, differential gene expression analysis of the gut of *A1CLas*$^{+}$ psyllids demonstrated the mounting of a defensive response of the gut epithelium based on the increased expression of a CLIP domain-containing serine protease 2-like (DN16851_c1_g2_i2). CLIP domain-containing serine protease 2-like has been recently reported to be up-regulated in the midgut of CLas-infected psyllids (Yu et al., 2020). Serine proteases containing a CLIP domain are involved in the regulation of humoral responses through the activation of prophenoloxidases (PPO) and the Toll immune signaling pathway. Activation of Toll pathway leads to antimicrobial peptides production, while the activation of PPO results in melanogenesis (Anderson 2000; Nakhleh et al. 2017). Toll activation requires a multi-step proteolytic cascade (Stokes et al. 2015), and the lack of additional differentially expressed serine proteases and the antimicrobial peptides transcripts in the psyllid transcriptome support the argument that the up-regulation of CLIP-domain serine protease in the gut of ACP feeding on CLas-infected plants leads to the activation of melanization as an immune response against CLas infection. We also believe the activation of melanization at this stage of psyllid-

CLas interaction was triggered by a local response caused by cell injury and invasion of the gut epithelium by CLas cells, resulting in the production of wound clots in order to avoid infected, damaged cells to suffer further damage, die and be replaced by *nidi* cells. This hypothesis is also supported by the down-regulation of pangolin (*Pan*) (DN15424_c0_g3_i2), a key regulator of the Wnt/Wg pathway. Wnt proteins are highly conserved and participate in the control of growth, patterning, tissue and energy homeostasis. Pangolin was reported to bind to the β-catenin homologue Armadillo, acting directly in the regulation of gene expression in response to Wnt signaling (Brunner et al. 1997; Franz et al. 2017). The Wnt signaling in *Drosophila* is an important process for the homeostasis of the gut tissue as it is involved in the regeneration of adult gut epithelial cells (Strand & Micchelli 2011, Cordero et al. 2012). Thus, the down-regulation of pangolin interferes with the activation of the Wnt signaling to regulate gene expression involved in the replacement of CLas-infected cells by the activation of the nidi cells to differentiate into new, active cells of the gut epithelium.

Studies on the role of genotype-genotype interactions of insects and parasites demonstrated activation and differential gene expression of the host immune machinery depending on the interacting parasite genotype. Alternative splicing was also reported as a required element in the specificity of the immune response of insects to specific parasite genotypes (Riddell et al. 2014). The nuclear speckles carry pre-mRNA splicing machinery composed by nuclear speckle-related proteins that mediate alternative splice site selection in targeted pre-mRNAs (Girard et al. 2012; Galganski et al., 2017). The detected up-regulation of proteins that mediate alternative splice site selection in targeted mRNAs, the nuclear speckle splicing regulatory protein 1-like

(DN17904_c2_g1_i3) in *CLas*$^{+}$ psyllids, suggests CLas acts on the regulation of gene expression of infected psyllids at the molecular level.

The psyllid initial defense response against CLas infection also included the up-regulation of arylsulfatase B (DN19237_c6_g3_i2), an enzyme stored in lysosomes that acts on large glycosaminoglycan molecules by removing attached sulfate groups (Bhattacharyya et al., 2014). Glycosaminoglycans are components of proteoglycans commonly exploited by pathogens as receptors for their adherence to different tissues (García et al. 2016). Glycosaminoglycans are produced by some pathogenic bacteria as extracellular capsule and used in the process of host infection and colonization to facilitate pathogen attachment, invasion and/or evasion of host defensive mechanisms (Roberts, 1996). Arylsulfatases B can also participate in processes of biotransformation, mediating the sulfonation of xenobiotics by other enzymes to facilitate their excretion (Zhao et al., 2016). In this case, increase in the arylsulfatase could be related to the psyllid physiological needs to metabolize the high levels of flavonoids (hesperidin, narirutin and dydimin) produced in citrus plants infected by *Ca.* Liberibacter (Massenti et al., 2016; Dala-Paula et al., 2018; Kiefl et al., 2018). Flavanoids are important metabolites produced by plants in response to abiotic and biotic stressors and play a relevant contribution in plant resistance against microbes and herbivores (Treutter 2006), including citrus plants in response to CLas infection (Hijaz et al. 2013, 2020).

The overexpression of immune related genes and the required increased cell metabolism early in the process of interaction of the psyllid gut epithelium with CLas can explain the up-regulation of the mitochondrial cytochrome c oxidase subunit IV (DN18774_c0_g1_i7) in *CLas*$^{+}$ insects. Mitochondrial cytochrome c oxidase subunit IV is the major regulation site for oxidative phosphorylation by catalyzing the final step of electron transfer in mitochondria (Li et al. 2006b).

374

375 **Cross-talk in the colonization of the gut lumen**

376 Bacteria that survive the harsh chemical environment and the immune barriers
377 available in the gut will have to use strategies to avoid their rapid elimination from the
378 gut with faeces (Vallet-Gely et al. 2008, Benguettat et al. 2018). Insects are active
379 feeders and food fastly transits through the gut. The fast food transit in the gut has been
380 argued as one condition to explain the controversial lack of a resident microbiome in
381 lepidopteran larvae, for example (Hammer et al. 2017).

382 CLas seems to employ different strategies to colonize the gut lumen by avoiding
383 its elimination with faeces: regulation of gut peristalsis, synthesis of adherence proteins,
384 and biofilm formation.

385 CLas regulation of the psyllid gut peristalsis is thought to occur through the up-
386 regulation of the host neprilysin as earlier discussed. Neprilysins are reported to degrade
387 peptide hormones, and the gut of insects contains several hormone-producing cells.
388 Peptide hormones produced in the gut act as signaling molecules that are involved in the
389 regulation of a range of processes, including gut peristalsis (Wegener & Veenstra 2015,
390 Caccia et al. 2019, Wu et al. 2020). The hypothesis that CLas modulates the gut
391 peristalsis of psyllids is supported by the down-regulation of titin (DN21409_c5_g2_i1)
392 transcription in *CLas*$^{+}$ psyllids. Muscle degeneration is marked by a reduction in titin
393 proteins, and gut-associated muscles are in charge of producing the peristaltic
394 movement observed in the gut. Additionaly, the intracellular non-catalytic domain of
395 neprilysins in excess is shown to induce muscle degeneration (Panz et al. 2012). Thus,
396 the degeneration of muscles associated with the gut would certainly impair gut
397 peristalsis and consequently reduce the elimination of bacteria with faeces (Vallet-Gely
398 et al. 2008, Benguettat et al. 2018).

399 Regulation of signaling in the gut is also evidenced by the up-regulation of
400 arylalkylamine N-acetyltransferase (DN20634_c1_g1_i13) transcription in *A2CLas*$^{+}$
401 psyllids. Arylalkylamine N-acetyltransferases are involved in the N-acetylation of
402 arylalkylamines, playing an important role in the synthesis of melatonin in vertebrates
403 and invertebrates (Klein 2007; Hiragaki et al. 2015). But in insects, arylalkylamine N-
404 acetyltransferases also inactivate arylalkymines that play important roles as
405 neuromodulators, such as octopamine, dopamine and serotonin (Brodbeck et al. 1998;
406 Amherd et al. 2000). These neuromodulators are also implicated in the double brain-gut
407 communication circuitry (Solari et al. 2017; Zhang et al. 2018).

408 CLas adherence to the psyllid gut was achieved by the expression of three genes
409 of a subtype of the type IVb pilins, the tight adherence pili (Tad = Flp). Flp pilins are
410 common to several Gram^{+} and Gram^{-} bacteria. Type IV pili are highly diverse and
411 involved in a number of protein-protein interactions, but flp pili are better known for
412 their role in adherence to living and nonliving surfaces. Pili are also involved in cell
413 motility, secretion of exoproteins, and in host cell manipulation under extreme
414 conditions (Giltner et al. 2012, Kazmierczak et al. 2015).

415 The expression of Type IVc pili of *Ca.* L. asiaticus was reported to be higher in
416 psyllids than in plants, but only one (*flp3 pilin*) out of five *flp* genes was reported to be
417 up-regulated in psyllids (Andrade & Wang 2019). The *flp3 pilin* gene was demonstrated
418 to be under the regulation of VisN and VisR, demonstrating these proteins are important
419 in the colonization of the host vector (Andrade & Wang 2019). In our analysis of the
420 transcriptional profile of CLas in the gut of adult psyllids, we detected the expression of
421 several isoforms of two *flp* genes (DN14425_c0_g1; DN18703_c0_g1), but both
422 belonging to the family Type IVb pilin. Three isoforms of gene DN14425_c0_g1
423 (DN14425_c0_g1_i2; DN14425_c0_g1_i3; DN14425_c0_g1_i4) and two of gene

DN18703_c0_g1 (DN18703_c0_g1_i21 and DN18703_c0_g1_i23) were highly and consistently expressed in the gut of psyllids in all sampling periods. Three LuxR family transcriptional regulators (DN1177_c0_g1_i1, DN14940_c0_g2_i1, and DN38633_c0_g1_i1) were also observed in the gut of *CLas*$^{+}$ psyllids; nevertheless, none of them was identified as the VisR or VisN regulators of *flp3 pilin* gene reported by Andrade & Wang (2019).

The expression of CLas flagellins (*FlgA*) (DN3624_c0_g2_i1 and DN40834_c0_g1_i1) and several genes involved in the assembling and functioning of the flagellar machinery (*FliE, FliF*, *FliG*, *FliK, FliN, FliR, FliP, FlgB, FlgD, FlgE, FlgF, FlgG, FlgH, FlgI, FlgK, FlhA, FlhB, MotB,* and the *flagellar C-ring protein*) would indicate CLas assembles flagella for colonizing the gut of adult psyllids, although FlgF and FlgI were detected exclusively in *A3CLas*$^{+}$. The expression of genes involved in the flagellar machinery corroborates recent data on the higher expression of genes encoding the flagellum apparatus of CLas in the gut of psyllids than in plants (Andrade et al. 2020a). Although we detected a much higher number of transcripts of the flagellar system of the CLas isolate we worked with than those reported to be expressed in the gut of psyllids by Andrade et al. (2020a), we did not detect the expression of three genes they evaluated (*fliQ, fliL*, and *flgA*). Differences in the overall number of genes of the flagellar system among lineages are expected to occur, but not among isolates of the same species (Bardy et al. 2003).

The production of flagellum in the gut by infecting CLas is also supported by transmission electron microscopy images, although most of the CLas cells observed lacked a flagellum (Andrade et al. 2020a). Analysis of images of the flagellum of CLas cells clearly shows CLas carries the secondary flagellar system, producing a lateral flagellum. The secondary flagellar systems arose twice in the evolution of bacteria, once

449 in alpha-proteobacteria and once in the common ancestor of beta/gamma-proteobacteria
450 (Liu & Ochman 2007). The primary flagellar system produces a polar flagellum and
451 contributes with cell motility in liquid media, while the secondary flagellum system
452 produces a lateral flagellum that is involved in adhesion and cell swarming on surfaces.
453 Bacterial flagella serve bacteria not only as a motor apparatus, but also as a protein
454 export/assembly apparatus. The motor apparatus of flagella can provide bacteria the
455 movement required to remain in the gut, as demonstrated in the trypanosomatid
456 *Vickermania* (Kostygov et al. 2020). Moreover, the flagellum pattern can be altered by
457 bacteria depending on the environmental conditions faced, and bacterial flagella are
458 recognized as important virulence factors (Moens & Vanderleyden 1996, Macnab 2003,
459 Duan et al. 2013). Chemotaxis is an important contribution of bacterial flagellum to
460 virulence (Matilla & Krell 2018), and the detection of the expression of four chemotaxis
461 protein genes (DN15199, DN31800, DN46197 and DN50630) suggests the flagellum
462 participates in cell adhesion to and swarming on the psyllid gut epithelium. Yet, we also
463 propose the flagellum aids CLas to become chemically oriented for the localization of
464 suitable host cells for invasion.

465 Biofilm formation requires bacteria to synthesize an extracellular matrix.
466 Biofilms include extracellular proteins, cell surface adhesins and subunits of flagella
467 and pili proteins (Fong & Yildiz 2015). We did not identify the expression of adhesins
468 in the transcriptome of CLas in the gut of adult psyllids. Nevertheless, several
469 chaperones were highly expressed, and many chaperones can act as adhesins in bacteria.
470 Additionally, we believe the cold shock protein (DN10086_c0_g2_i1) of CLas is also
471 playing key roles in biofilm formation. Cold shock proteins were demonstrated not only
472 to participate in biofilm formation but also to support cell adhesion and motility, and to

stimulate cell aggregation, interfering thus with virulence (Eshwar et al. 2017, Ray et al. 2020).

Cross-talk for infecting the gut epithelium

Several gut-associated bacterial symbionts of hemipterans enter in close contact with epithelial cells to establish an intracellular phase through endocytosis (Nardi et al. 2019). CLas also enters the gut epithelium of psyllids through endocytosis, remaining inside vacuoles formed from or surrounded by endoplasmic reticulum membrane (Ghanim et al. 2017). The differential gene expression analysis of the gut of *CLas*$^{+}$ psyllids led to the identification of transcripts involved with ultrastructural alterations of the gut epithelial cells, allowing the identification of the molecular intermediates that participate in the route CLas follows to invade the gut epithelium.

In addition to the regulation of the immune related responses earlier described in the gut epithelium of *CLas*$^{+}$ psyllids, epithelial cells of the gut were altered early in the infection process (*A1CLas*$^{+}$ adults). Alterations are perceived by the transcriptional down-regulation of zonadhesin-like (DN14644_c0_g1_i1), a transcriptional response already reported by Ramsey et al. (2015). Zonadhesin-like proteins represent an expansion of the zonadhesin multi-domain proteins that are implicated in the binding of sperm and egg in a species-specific manner. In the gut, zonadhesin-like proteins are thought to function as mucins, and two zonadhesin-like proteins of *D. citri* were previously predicted to be part of the extracellular matrix and to have a role in cell-cell adhesion. Mucins protect the gut epithelium from microbial infections and inflammation (Hansson 2012). Mucins are components of the perimicrovillar layer that in psyllids and other paraneopterans (Silva et al. 2004) replaces the chitin-based matrix (peritrophic

membrane) that provides a protective barrier to the gut epithelial cells from pathogenic bacteria (Kuraishi et al. 2011).

In vertebrates the mucin composition of the mucous layer can vary among gut regions, with diet composition and gut microbial infection (Paone & Cani 2020). Mucins can also be exploited as a nutritional resource by bacteria, and degradation of mucins by a commensal microbe was demonstrated to facilitate the penetration of the epithelium by viruses (Schroeder 2019, Wu et al. 2019). The role different mucins can have on the host-pathogen interaction can explain the up-regulation of the psyllid gene coding for mucin-5AC (DN16207_c6_g5_i1) in *A3CLas*$^{+}$ psyllids. The higher abundance of this protein in *CLas*$^{+}$ psyllids has been demonstrated in previous proteomic analysis (Ramsey et al. 2017). Regulation of the host mucin-5AC has been reported in vertebrate hosts infected by Gram^{-} and Gram^{+} bacteria, and in one system modulation of mucin-5AC has been linked with increased adhesion of bacteria to the gut tissue (Dohrman et al. 1998, Quintana-Hayashi et al. 2015).

The differential expression of genes coding for cytoskeletal proteins involved in the regulation of the submembranous actin-spectrin network in cells of the gut epithelium of *CLas*$^{+}$ psyllids demonstrates CLas interferes with the remodeling of gut epithelial junctions in adult psyllids, leading to endocytosis of intercellular junctions with cellular organelles. Adducins (protein Hu-li tai shao - DN19744_c0_g2_i2) form connections with membranes, promote spectrin-actin interactions and regulate actin filaments (Matsuoka et al. 2000). Internalization of apical junction and/or tight junction proteins may disrupt the epithelial barriers and favor the movement of bacterial toxins into cells (Hopkins et al. 2003).

The intracellular events in preparation for endocytosis can also be detected as indicated by the up-regulation of the psyllid phosphatase 1 regulatory subunit 21

(DN19112_c0_g1_i1) in the gut of *A1CLas*$^{+}$. This protein participates in early (sorting process) or late (maturation) endosome pathway, which leads to the endocytosis of several types of materials, including pathogenic agents. Our hypothesis is that early endosomes will also internalize the leptin receptor overlapping protein-like 1. The leptin receptor overlapping transcript-like 1 (DN20058_c2_g3_i4) was down-regulated in the gut epithelium of *A2CLas*$^{+}$ psyllids. Internalization of such receptors in early endosomes also affects cell signaling, and in this particular case can severely interferes with the gut epithelium resistance to microbial infection. Down-regulation of leptin/leptin receptor was reported to drastically affect cell resistance to amoeba and bacterial infection in several species (Faggioni et al. 2001, Guo et al. 2011, Mackey-Lawrence & Petri Jr 2012).

The endocytic vesicles detach and become free endocytic carrier vesicles transporting their cargoes to late endosomes to finally fuse with endoplasmic reticulum, where CLas cells aggregate in associated vacuoles as indicated by ultrastructural analysis (Ghanim et al. 2017). Fusion of lysosomes to cell aggregates vacuoles is inhibited by the down-regulation of soluble NSF attachment protein (SNAPs) (DN21196_c2_g1_i1) in *A2CLas*$^{+}$. SNAPs are highly conserved proteins that participate in intracellular membrane fusion and vesicular trafficking (Stenbeck, 1998). Intracellular membrane fusion requires both SNAPs and NSF to act in concert, and inhibition of one of them will lead to the failure of membrane fusion and the accumulation of vesicles one cannot fuse (Rothman, 1994).

SNAPs are characterized by the presence of a tetratricopeptide repeat (TPR) domain (Lakhssassi et al., 2017). Tetratricopeptide repeat proteins (TRP) are directly involved in virulence, particularly due the translocation of virulence factors into host cells and in the blockage of phagolysosomal maturation, among others (Cerveny et al.

2013). Three TRPs (DN11192_c0_g1_i1; DN11192_c0_g1_i2; DN14826_c0_g1_i2) were expressed in the gut of *CLas*$^{+}$ psyllids, but only DN14826_c0_g1_i2 was differentially expressed. The expression of different TRPs in all sampling periods and their time-specific differential expression indicates TRPs participates in different processes of CLas interactions with the host cells, from CLas establishment in the gut lumen to epithelial cell invasion and access to the hemocoel.

The down-regulation of the psyllids annexin B9-like isoform X1 (DN19784_c8_g1_i2) in the gut of *A3CLas*$^{+}$ would interferes with the development of multivesicular bodies, once annexin B9 is involved in endosomal trafficking to multivesicular bodies (Tjota et al. 2011). This change will affect the transfer of the contents of CLas-containing endosomal vesicles to lysosomes for proteolytic degradation.

We propose that the establishment of the intracellular cycle and survival of CLas within host cells was putatively aided by the expression of CLas peptidyl-prolyl isomerase (PPI) (DN14386_c0_g1_i1), a protein that has been proved to participate in intracellular infection and virulence of other Gram^{-} bacteria (Norville et al. 2011, Pandey et al. 2017, Rasch et al. 2018). The Mpi PPI from *Legionella pneumophila* was demonstrated to require an active enzymatic site to enhance a proper host cell invasion (Helbig et al. 2003), although the preservation of the active site was not a requirement for the PPI of *Burkholderia pseudomallei* (Norville et al. 2011). Ghanim et al. (2017) suggested earlier that CLas cells do not enter the ER, but instead CLas cells recruit ER to transform the phagosome into a host-immune free space suitable for CLas survival and multiplication. These same ER-derived structures were already observed in *Legionella* and *Brucella* (Celli & Gorvel 2004, Robinson & Roy 2006).

Early endosomes and endoplasmic reticulum share contact sites that are used to bind to microtubules at or close to their contact sites, and both organelles remain bounded as endosomes traffic and mature (Friedman et al. 2013). Endosome trafficking within the cell involves the membrane binding to motor proteins and its transport along the actin and microtubule cytoskeleton (Granger et al. 2014). Trafficking of endosomes in *CLas*$^{+}$ psyllids is provided by the up-regulation of kinesin-like protein KIF3B isoform X4 (DN10161_c0_g1_i1) in the gut of psyllids. Kinesins are motor proteins involved in the movement of multiple cytoplasmic organelles (Granger et al. 2014).

Profilin (DN19422_c2_g2_i1), another protein involved in intracellular movement of organelles, was also up-regulated in the gut of *A2CLas*$^{+}$ psyllids. Profilins are actin-binding proteins capable of regulating actin polymerization and the availability of the actin cytoskeleton for binding to the endosomes; but new roles for profilins are being identified in vertebrates (Witke 2004). Additionally, profilin 1 play an important role in host–pathogen interactions. Intracellular pathogens such as *Listeria monocytogenes* and *Shigella flexneri* use the host-cell actin cytoskeleton to propel themselves through the cytoplasm and to spread to neighboring cells without entering the extracellular space (Kocks, 1994; Witke, 2004). Dynein (DN35915_c0_g1_i1), another motor protein, was up-regulated in the gut of *A3CLas*$^{+}$ psyllids. Dyneins also contribute to microtubule-based transport in eukaryotic cells (reviewed in Holzbaur & Vallee, 1994; Porter, 1996; Hirokawa, 1998).

Cross-talk for moving to the hemocoel

After CLas inhibits the early immune response and invades the intracellular space of the gut epithelium by regulating a clathrin-independent endocytosis mechanism, hiding from the host's immune activators within vacuoles surrounded by

endoplasmic reticulum membrane, where CLas multiplies, the molecular mechanism behind the release of CLas bacteria from epithelial cells in the hemocoel may require additional data.

The higher expression in *AD3CLas*$^{+}$ of seven (DN15630_c0_g2_i3 = CLIBASIA_00255; DN15330_c0_g1_i2 = CLIBASIA_00880; DN14809_c0_g1_i = CLIBASIA_03170; DN14146_c0_g1_i1 = CLIBASIA = 04410; DN10141_c0_g1_i1 = CLIBASIA_00995; DN15141_c0_g1_i1 = CLIBASIA_01620; DN15414_c0_g1_i4 = CLIBASIA_01605) out of the 67 Sec-dependent proteins common to Las species (Thapa et al. 2020) indicates these candidate effector proteins play an important role in the dynamics of CLas infection of the psyllid gut epithelium at this stage. In fact, genomic analysis of Liberibacter species predicted a total of 166 proteins containing Sec-dependent signal peptides in the CLas strain psy62, from which 86 have been already experimentally validated (Prasad et al. 2016). These are potential effector proteins, and 106 of them share common homologues with Las_Ishi-1 and Las_gxpsy. But only 45 Sec-dependent proteins were shared among HLB-associated *Ca*. Liberibacter species (CLas, CLam and CLaf) (Wang et al. 2017; Andrade et al. 2020b). The detection of transcripts of the general secretion system provides further support for the participation of the detected Sec-dependent proteins in the late process of infection of the gut epithelium of *D. citri*. The Sec pathway secretion system transports proteins involved in bacterial cell functions and survival (Thapa et al. 2020). The Sec pathway is represented by two independent pathways, both detected in the gut of *CLas*$^{+}$ psyllids: the post-translational pathway represented by the expression of SecA (DN14962_c0_g1_i1) and SecY (DN15568_c0_g3_i3); and the co-translational pathway represented by the signal recognition particle receptor FtsY (DN1686_c0_g1_i1) (Green & Mecsas 2016). We did not detect SecB expression in the

gut of adult psyllids, indicating SecA can also act as a chaperone complementing the required activity of SecB as reported in *Escherichia coli* (McFarland et al. 1993).

Combined ultrastructure, proteomics and transcriptomics analysis of the gut epithelium of CLas-infected psyllids suggested CLas acquisition into the hemocoel would rely on the programmed cell death of CLas-infected epithelial gut cells, although CLas infection has little impact on the fitness of adult psyllids (Kruse et al. 2017). The low fitness impact of CLas infection to adult *D. citri* indicates CLas acquisition into the hemocoel would occur through an exocytosis process, as suggested for the *Ca.* Liberibacter solanacearum vectored by potato psyllids (Cicero et al. 2017).

By following the proposed subroutines based on mechanistic and essential aspects of cell death proposed by the Nomenclature Committee on Cell Death (Galluzzi et al 2018), our transcriptomic analysis could not support the proposition that CLas-infected epithelial gut cells of psyllids would initiate the process of programmed cell death (PCD) as proposed by Kruse et al. (2017). We did not observed transcription of caspases, a very common protein to several subroutines of cell death, as well as other aspects that would allow this characterization (Galluzzi et al 2018). Yet, we were unable to confidently characterize one of the subroutines of cell death described when analyzing the transcriptional profile of *CLas*$^{+}$ psyllids, once the molecular mechanisms involved were not all represented. However, we can confidently report the activation/inhibition of the expression of genes involved in processes of regulated cell death. The high expression of the lysosomal cathepsin L1 (DN15698_c4_g11_i2) in *A2CLas*$^{+}$ psyllids would support the activation of lysosome-dependent cell death, which requires intracellular perturbations to permeabilize the lysosomal membrane to result in the release of cytosolic cathepsins. Mitochondrial outer membrane permeabilization and caspases are not necessarily required for this process of cell death, and lysosome-

dependent cell death is an important response to pathophysiological conditions induced by intracellular pathogens (Galluzzi et al 2018).

The overexpression of the psyllid BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) (DN_20168_c2_g3_i4) in *A3CLas*$^{+}$ also supports the gut epithelium of *CLas*$^{+}$ psyllids go into lysosome-dependent cell death. BNIP3 are pro-apoptotic proteins that open the pores of mitochondrial outer membrane, resulting in mitochondria dysregulation due the loss of membrane potential and ROS production in mitochondria (Vande Velde et al 2000, Moy & Cherry 2013). But BNIP3 oligomerization with BCL2 has been demonstarted not to be required for cell death (Vande Velde et al. 2000). Besides, the down-regulation of the psyllid senescence-associated protein transcript (DN16041_c1_g1_i2) in the gut of *A3CLas*$^{+}$ psyllids demonstrates CLas suppresses cell senescence and the further dysfunctional growth of the epithelial cells due the activation of senescence-associated secretory phenotype (Münch et al 2008, Ito & Igaki 2016). Cellular senescence is not considered a form of regulated cell death (Galluzzi et al 2018).

The differential expression of a putative juvenile hormone binding protein (JHBP) (DN21423_c1_g12_i2; DN21423_c1_g12_i1) in *CLas*$^{+}$ psyllids (down-regulation in the gut of *A2CLas*$^{+}$; up-regulation in *A3CLas*$^{+}$) demonstrates the epithelial cells count with different levels of hormonal stimulation. JHBPs are proteins that act as shuttles for juvenile hormone (JH) in the hemolymph, avoiding JH degradation by JH-esterases and JH-epoxide hydrolases (Zalewska et al., 2009). JH is produced and released primarily by neurosecretory cells of the *corpora allata* of the central nervous system, and the availability of JHBP in the gut, if its activity in the gut is the same played in the hemolymph, indicates JH is alo available in the gut epithelial cells. JH has recently been shown to be produced by intestinal stem cells and enteroblasts of the gut

671 epithelium of *D. melanogaster,* and to regulate cell growth and survival. The local JH
672 activity was also shown relevant for damage response by gut cells, playing important
673 roles in gut homeostasis (Rahman et al 2017). If the pattern of expression of JHBP
674 correlates with the availability of JH we can argue that at the same time CLas acts on
675 the regulation of the gut epithelium by suppressing cell senescence, it can also induce
676 the proliferation of stem cells in order to replace cells that were damaged due the release
677 of CLas cell into the hemocoel and/or entered the lysosome-mediated cell death as
678 discussed earlier.

679 The observed up-regulation of the transcriptional levels of nocturnin
680 (DN19927_c10_g1_i6) in the gut of *A2CLas*$^{+}$ psyllids suggests that this circadian
681 rhythm effector protein may be acting together with JH in regulating gut genes, just as
682 the joint action of the circadian genes and JH in the regulation of genes acting on the
683 transitional states of *Pyrrhocoris apterus* adults to reproductive diapause or not (Bajgar
684 et al. 2013). Nocturnin is classified as a deadenylase acting on the catalysis of poly(A)
685 tail of target mRNAs and/or targeting noncoding RNAs, and has been implicated in
686 metabolic regulation, development and differentiation (Hughes et al. 2018). But recent
687 studies with *curled,* the nocturnin ortholog in *Drosophila* proved nocturnin is an
688 NADP(H)2'-phosphatase acting on the conversion of the dinucleotide $NADP^{+}$ into
689 NAD^{+} and NADPH into NADH, regulating mitochondrial activity and cellular
690 metabolism in response to circadian clock (Estrella et al. 2019). NADP(H)2'-
691 phosphatase activity of nocturnin was later reported in vertebrates, with the
692 demonstration of the colocalization of nocturnin in mitochondria, cytosol and
693 endoplasmic reticulum-bound pools depending on the isoform (Laothamatas et al.
694 2020). Thus, nocturnin acts as a regulator of the intracellular levels of NADP(H) and
695 the oxidative stress response. The role of nocturnin in the regulation of cell metabolism

and the oxidative stress response supports the required energy supply to sustain the increased gene expression activity in *CLas*$^{+}$ gut epithelium cells.

The expression profile of CLas infecting the gut epithelium of *A3CLas*$^{+}$ psyllids demonstrates CLas has an increased protein synthesis activity as expected by the overexpression of DEAD/DEAH box helicase (DN4052_c0_g1_i1), a protein involved in ribosome biogenesis, RNA turnover and translation initiation (Redder et al., 2015). Increased protein activity is also supported by the increased expression of molecular chaperones, such as the trigger factor (DN13945_c0_g1_i1), GroEL (DN14757_c0_g1_i1) and DnaK (DN15220_c0_g1_i1), which prevent protein misfolding and aggregation (Agashe et al 2004, Merz et al. 2006).

CLas multiprotein complexes

The protein HlyD family efflux transporter periplasmic adaptor subunit (DN11766_c0_g1_i1) and the outer membrane factor translocation protein TolB (DN15087_c0_g1_i3) are essential components for functioning the pump of tripartite efflux systems. HlyD connects primary and secondary inner membrane transporters to the outer membrane factor TolB. CLas expressed several inner membrane transporters belonging to the MSF and ABC families. TolB has been shown to interact with a range of proteins including cell-killing proteins (Carr et al. 2000, Loftus et al. 2006). In *Xylella fastidiosa* TolB was shown to be involved in biofilm development (Santos et al. 2015).

Additional CLas transcriptional regulators

The detection of several other uncharacterized response regulators (DN10585_c0_g1_i1; DN10585_c0_g2_i1; DN12200_c0_g1_i1; DN58285_c0_g1_i1)

and transcriptional regulators (DN11066_c0_g1_i1; DN15145_c0_g1_i2; DN15145_c0_g1_i5) indicates other CLas two-component systems are also being activated by environmental stimuli, such as the two-component system sensor histidine kinase AtoS (DN14305_c0_g2_i1; DN15701_c0_g1_i1). The two-component system sensor histidine kinase AtoS is a member of the AtoS/AtoC regulatory system, but we did not identify the AtoC response regulator in the CLas transcriptome. This regulatory system is better known by the induction of AtoS by acetoacetate. AtoS then phosphorylates AtoC that will in turn stimulate the expression of the atoDAEB operon for the catabolism of short chain fatty acids. We could not reliably identify transcripts belonging to the atoDAEB operon to demonstrate CLas requirements for short chain fatty acids. We propose that the activation of the AtoS/AtoC regulatory system is instead acting on the regulation of the flagellar regulon, regulating the expression of CLas genes involved in cell motility and chemotaxis, as reported for *Escherichia coli* in response to acetoacetate or spermidine (Theodorou et al. 2012).

Cross-talk on nutrient deficiency

The high expression of sulfonate ABC transporter permease (DN15690_c0_g1_i2) in all *CLas*$^{+}$ psyllids suggests CLas signals the host to supply its requirements for sulfur. Sulfur is a ubiquitous element involved in a number of different processes in organisms (Beinert 2000). The sulfonate ABC transporter permease is involved in sulfur/sulfonate uptake, and the higher expression observed in *A3CLas*$^{+}$ as compared to *A1CLas*$^{+}$ points for an increased demand late in the infection process.

Sulfur is also used in the biogenesis of Fe-S clusters, which are produced in conditions of oxidative stress and iron deprivation. The biogenesis of Fe-S clusters was observed by the expression of two unclassified cysteine sulfurases (DN12917_c0_g1_i1

and DN35541_c0_g1_i1), the cysteine desulfuration protein SufE (DN1144_c0_g1_i1), the Fe-S cluster assembly protein SulfB (DN14252_c0_g1_i1), and the iron-sulfur cluster carrier protein ApbC (DN17762_c4_g2_i3) (Layer et al. 2007, Roche et al. 2013). All four genes had an increased expression in *A3CLas*$^{+}$ as compared to *A1CLas*$^{+}$. Sulfur requirements by CLas could also be used for protection against oxidative stress as demonstrated by the expression of the thioredoxin-dependent thiol peroxidase (DN10526_c0_g1_i1) (Lu & Holmgren 2014; Wang et al. 2020).

The high expression of CLas phosphate ABC transporter permease subunit PstC (DN40256_c0_g1_i1; DN67283_c0_g1_i1), putative two-component sensor histidine kinase transcriptional regulatory protein (DN14305_c0_g2_i1), two-component sensor histidine kinase (PhoR) (DN28075_c0_g1_i1; DN67626_c0_g1_i1), two component response regulator protein (PhoB) (DN14869_c0_g1_i1), alkaline phosphatase (DN12828_c0_g1_i2; DN39297_c0_g1_i2) and NTP pyrophosphohydrolase (DN2128_c0_g1_i1) demonstrates CLas cells are exposed to phosphate restriction while infecting the gut epithelium of adult psyllids. Phosphate is generally the major source of phosphorus to bacteria, a vital nutrient for living organisms. Phosphorus is important in several processes (energy metabolism, intracellular signaling, among others). Misregulation of phosphorus availability in bacteria is sensed by the PhoB/PhoR two-component regulatory system, leading to the activation of the Pho regulon. The Pho regulon regulates the expression of other genes, producing phenotypes that can differ in morphology and virulence in response to phosphate deprivation (Lamarche at al. 2008, Santos-Beneit 2015). Thus, the expression of both members of the PhoB/PhoR two component regulatory system and the phosphate ABC transporter permease subunit PstC, that is in charge to transport extracellular phosphate to the cytosol, proves CLas was exposed to phosphate concentrations below 4 μM, a threshold that generally turns

771 on the expression of the response regulator PhoB. Furthermore, the expression of
772 alkaline phosphatase and NTP pyrophosphohydrolase, which both act on phosphorus-
773 containing substrates (Lamarche et al. 2008) demonstrates CLas increased the
774 catabolism of substrates capable of releasing phosphorus nutrient.

775 The requirement of CLas for phosphate is also demonstrated by the up-
776 regulation of the psyllid glycerophosphocholine phosphodiesterase GPCPD1 gene, as
777 observed by the increased abundance of its transcript (DN15741_c0_g2_i2) in
778 *A3CLas*$^{+}$. The contribution of GPCPD1 in phosphate production results from the
779 downstream processing of GPCPD1 hydrolysis products of glycerophosphocholine,
780 choline and glycerolphosphate. At the same time glycerophosphocholine hydrolysis can
781 provide phosphates for cell metabolism, the choline can serve as as substrate for
782 phosphatidylcholine synthesis. Phosphatidylcholine is a major lipid component of
783 membranes of eukaryotes, although several bacterial symbionts and pathogens carry
784 phosphatidylcholine synthases, showing their requirement for this lipid component of
785 cell membranes, including those belonging to *Rhizobiaceae* as *Ca*. Liberibacter. Choline
786 serves as substrate for the osmoprotectant glycine betaine synthesis and as an energy
787 substrate to support cell growth in *Rhizobiaceae* (Sohlenkamp et al., 2003; Dupont et
788 al., 2004).

789 The role of host-produced choline in CLas metabolism is supported by the up-
790 regulation of CLas glycine/betaine ABC transporter substrate-binding protein
791 (DN15178_c0_g1_i1) in the gut of *A3CLas*$^{+}$. ABC transporters were reported to have
792 several important roles in *Ca.* Liberibacter asiaticus, from the importation of nutrients
793 like choline to the exportation of virulence factors (Li et al., 2012). Thus, we believe the
794 increased activity of GPCPD1 in *A3CLas*$^{+}$ is a result of the recycling of
795 glycerophosphocholine for choline utilization in the recovery of membranes of the gut

796 epithelial cells of the host psyllid as a response to cell infection and/or in the
797 supplementation of choline to CLas metabolism.

798 CLas is known to induce profound physiological changes in citrus plants,
799 affecting the nutritional content of CLas-infected plants to psyllids, which have in turn
800 altered transcriptional profiles and protein abundance when feeding on CLas-infected
801 and health citrus plants (Fu et al. 2016, Ramsey et al. 2017). Therefore, it is impossible
802 to distinguish if the indication of nutritional restriction CLas encounters when infecting
803 the gut epithelium of psyllids is an indirect effect of the host plant on the psyllids or if
804 this is a direct response of psyllids to avoid CLas infection.

805

806 **4. Material and Methods**

807 **4.1. *Diaphorina citri* rearing and plants**

808 A colony of CLas-free Asian Citrus Psyllid (ACP) was initiated with insects
809 collected from *Murraya paniculata* (L.) Jack, syn. *Murraya exotica* L. (Sapindales:
810 Rutaceae) in the state of São Paulo, Brazil in 2009.

811

812 **4.2. *Diaphorina citri* gut collection and RNA extraction**

813 Adults of *D. citri* (7 to 10 days after emergence) were transferred to uninfected
814 and CLas-infected citrus plants [*Citrus* x *sinensis* (L.) Osbeck, grafted in 'Rangpur'
815 lime (*C.* x *limonia* Osbeck)] for an exposure period (EP) of 1, 2, 3, 4, 5 and 6 days. For
816 each exposure period, three biological replicates were collected (1 replicate = 100
817 individuals). Third-instars of *D. citri* were also transferred to CLas-infected and
818 uninfected (control) citrus plants for collection of RNA and differential transcriptional
819 analysis. Nymphs were allowed to feed for 4 days on CLas-infected and control plants.

Afterwards, three biological replicates/treatment (1 replicate = 200 nymphs) were collected and stored in RNALater for further processing and analysis.

After each exposure time, adults were collected and the gut dissected under aseptic conditions. A similar procedure was used for nymphs, but in this case insects we opted by extracting RNA from whole nymphs, as initial attempts to dissect the suitable gut samples for downstream analysis were very time-consuming and had a low success rate.

The obtained guts/nymphs were stored in RNALater (Invitrogen/ThermoFisher Scientific, Waltham, MA, EUA) at -80°C until RNA extraction. Total RNA extraction was performed using the SV RNA Isolation System Kit (Promega), following the manufacturers' recommendations. Samples were lysed in 175 µL of RNA lysis buffer added with β-mercaptoethanol and 350 µL of RNA dilution buffer for tissue disruption using a TissueLyser II LT™ (Qiagen). Samples were incubated at 70°C for 3 min, centrifuged (12,000 *g* × 10 min × 4°C), trapped in a column and washed with 95% ethanol by centrifugation (12,000 *g* × 10 min × 4°C). Total RNA was recovered in 350 µL of RNA wash solution following centrifugation (12,000 *g* × 1 min × 4°C). Afterwards, samples were treated with 50 µL of DNAse mix (40 µL buffer 'yellow core'+ 5 µL 0.09 M $MnCl_2$ + 5 µL DNAse I) for 15 min at room temperature. Samples were added with 200 µL of DNAse stop solution and subjected to centrifugation (13,000 *g* × 1 min × 4°C). Samples were washed in 600 µL cleaning solution followed by a second wash in 250 µL following centrifugation (12,000 *g* × 1 min × 4°C). The pelleted RNA was recovered in 30 µL of nuclease-free water, and RNA concentration verified using NanoDrop V 3.8.1 (ThermoFischer).

RNA samples containing residual DNA contaminants were further treated with 2 µL of buffer (10×) and 2 µL of Turbo™ DNAse (2 U/µl) (Ambion®). Samples were

incubated at 37°C for 30 min followed by DNAse inactivation by adding 2 µL of DNAse Inactivation Reagent (Ambion®). After 5 min at room temperature, samples were centrifuged (13,000 g × 1.5 min × 4°C) and the supernatant collected and stored at -80°C. DNA elimination was confirmed by testing the amplification of the wingless gene (*wg*) of ACP (Manjunath et al., 2008).

All plants and insects were subjected to quantitative PCR analysis for verification of CLas infection using the TaqMan qPCR Master Mix Kit (Ambion®) following Li et al. (2006a).

In insects, the average Ct value found for the *wg* gene in the libraries was 30.7, while the average Ct value in plants for the 16S rRNA gene from CLas was 29.6. Samples with a Ct value under 35 were considered positive for CLas.

After confirmation of the *Ca.* L. asiaticus infection status of each sample, RNA obtained from the gut samples of adults were pooled in equimolar concentrations to yield three exposure periods: *i) A1CLas*$^{-}$: adults that fed on healthy citrus plant (CLas^{-}) for 1-2 days; *ii) A2CLas*$^{-}$: for 3-4 days; *iii) A3CLas*$^{-}$: for 5-6 days; *iv) A1CLas*$^{+}$: adults that fed on infected citrus plant (CLas^{+}) for 1-2 days; *v) A2CLas*$^{+}$: for 3-4 days; and *vi) A3CLas*$^{+}$: for 5-6 days. In the case of the whole nymphs, two samples were produced: *N1CLas*$^{+}$: for nymphs after 4 days of feeding on CLas-infected citrus plants, and *N1CLas*$^{-}$: for nymphs after 4 days of feeding on control plants.

Samples were subjected to mRNA enrichment through eukaryote and prokaryote rRNA removal using the Ribo-Zero rRNA Removal Epidemiology Kit (Illumina®), following the manufacturers' instructions. RNA integrity was confirmed using the Agilent Bioanalyzer 1000 (Agilent Technologies).

4.4. Library preparation and sequencing

cDNA libraries were prepared for sequencing using the cDNA TruSeq RNA Library Prep Kit (Illumina®) following a paired-end (2 × 100 bp) strategy. The cDNA produced was end-repaired and adenosine was added at the 3′ end of each cDNA fragment to guide the ligation of specific adapters. Adapters consisted of primers for transcription and a specific index to code each sample. Samples were enriched with limited-cycle PCR and analyzed to confirm the success of sample preparation before sequencing using the Illumina© HiScanSQ platform available at the Multiusers Center of Agricultural Biotechnology at the Department of Animal Sciences, ESALQ/USP.

4.5. *De novo* transcriptome assembly

Reads quality were visualized in FastQC software (Andrews, 2010) before adapters removal and quality filtering by trimming the leading (LEADING:3) and trailing (TRAILING:3) nucleotides until the quality was higher than 3, and then using a sliding window of 4 nucleotides and trimming when scores were lower than 22 (SLIDINGWINDOW 4:22). Quality filtering was done using Trimmomatic-0.36 (Bolger et al., 2014).

All reads obtained were used to assemble a *de novo* transcriptome using the pipeline available in the Trinity-v.2.4.0 software (Haas et al., 2013). Both paired and unpaired trimmed and quality-filtered reads were used to assemble the *de novo* transcriptome, which was further used as the reference transcriptome for the RNA-Seq experiments. Assemblage was obtained using normalization of the reads coverage (<50) and the minimum contig size selected was 200 nucleotides.

The transcripts obtained were functionally annotated using the BlastX algorithm for putative identification of homologous sequences, with an *e*-value cut-off < 10^{-3}. Annotated sequences were curated and grouped into categories according to their

function using Blast2Go® (Conesa et al., 2005) with an *e*-value cut-off < 10^{-6} and EggNOG-mapper 4.5.1 (Huerta-Cepas et al., 2017). Transcripts putatively identified as belonging to insects and *Ca.* L. asiaticus were checked against the KEGG database (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa & Goto, 2000) to verify the metabolic pathways represented in the obtained *de novo* transcriptome. Transcripts of *Diaphorina citri*, *Ca.* Liberibacter spp. and *Wolbachia* spp. were filtered using Blast2go version Pro (Götz et al. 2008).

4.6. Differential gene expression analyses

Changes in the pattern of gene expression were evaluated separately in gut of ACP adults infected or not by *Ca*. L. asiaticus using the CLC Genomics Workbench 20.0 software (QIAGEN, Aarhus, Denmark). Reads from each library were counted against the *de novo* transcriptome, and counts were normalized as transcripts per million reads (TPM). TPM values for each sample were used to calculate fold-change ratios for comparative analyses of the gene expression of control ($CLas^-$) versus CLas-infected insects ($CLas^+$) within each feeding interval (1-2 d, 3-4 d, 5-6 d). Only the transcript that were counted in two of the replicates of a particular treatment were further taken for comparative analysis. Data were analyzed using multifactorial statistics based on a negative binomial Generalized Linear Model (GLM). The values of fold change obtained were corrected with False Discovery Rate (FDR) method and only transcripts that showed *p*-value ≤0.05 and log fold change > |2| between treatments were considered differentially expressed.

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Author's contribution

FLC and NAW designed the experiments; FLC, NAW and LP secured the funds; JCD collected insect samples and extracted RNA; BLM processed the sequencing data and assembled the *de novo* transcriptome; FMMB analyzed the data; FMMB and FLC wrote the paper; all authors commented the initial draft and approved the final version of this manuscript.

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S1 Table. Differentially expressed CLas genes in the gut of *Diaphorina citri* adults after 1-2 d (*A1CLas*$^+$), 3-4 d (*A2CLas*$^+$) and 5-6 d (*A3CLas*$^+$) of infection by feeding on CLas-infected citrus plants.

Comparison	Annotation	Transcript ID	FC	FDR
*A1CLas+ x ***A3CLas+	2-octaprenyl-6-methoxyphenyl hydroxylase	DN14876_c0_g1_i1	13.9	0.02
	5-(carboxyamino)imidazole ribonucleotide synthase	DN14087_c0_g1_i2	12.6	0.03
	50S ribosomal protein L2	DN11710_c0_g1_i2	16.9	0.04
	AAA family ATPase	DN1679_c0_g1_i1	13.8	0.05
	alanine--tRNA ligase	DN14954_c0_g1_i1	17.2	0.01
	alpha/beta hydrolase	DN39212_c0_g1_i1	14.0	0.01
	amino acid ABC transporter permease	DN15630_c0_g2_i3	14.1	0.02
	argininosuccinate lyase	DN13884_c0_g1_i1	13.4	0.01
	aspartate aminotransferase	DN14989_c0_g1_i4	12.0	0.02
	aspartate aminotransferase family protein	DN11157_c0_g1_i1	13.9	0.03
	ATP-dependent protease ATPase subunit HslU	DN20210_c0_g3_i1	12.0	0.03
	beta-ketoacyl-ACP synthase I	DN14910_c0_g1_i1	13.8	0.04
	bifunctional glutamate N-acetyltransferase/amino-acid acetyltransferase ArgJ	DN10638_c0_g1_i1	14.6	0.03
	carbamoyl phosphate synthase small subunit	DN13724_c0_g1_i1	19.0	$9.3e^{-3}$
	Chloramphenicol-sensitive protein rarD	DN13473_c0_g1_i1	17.2	0.05
	chromosomal replication initiation protein	DN15038_c0_g1_i2	16.8	$7.41e^{-3}$
	cold-shock protein	DN10086_c0_g2_i1	20.0	0.02
	DEAD/DEAH box helicase	DN4052_c0_g1_i1	24.2	0.02
	DNA polymerase III subunit chi	DN12825_c0_g1_i1	11.2	0.04
	DNA topoisomerase I	DN14080_c0_g1_i1	11.9	0.04
	DNA topoisomerase IV subunit B	DN10717_c0_g1_i1	37.8	$8.87e^{-4}$
	DNA translocase FtsK	DN14450_c0_g1_i1	22.7	$2.49e^{-3}$
	DUF1874 domain-containing protein	DN14305_c0_g1_i1	31.4	$9.46e^{-4}$
	elongation factor G	DN17043_c0_g1_i1	14.2	0.05
	elongation factor P	DN12683_c0_g1_i1	31.9	0.01
	elongation factor Ts	DN14637_c0_g1_i1	11.1	0.04
	excinuclease ABC subunit C	DN45886_c0_g1_i1	14.8	0.03
	exodeoxyribonuclease VII large subunit	DN17192_c0_g4_i3	14.0	$9.3e^{-3}$
	exonuclease I	DN15585_c7_g1_i1	11.2	0.02
	fumarate hydratase	DN14954_c0_g1_i2	23.9	$6.8e^{-3}$
	glutaminase	DN14270_c0_g1_i1	13.0	0.03
	glutathione S-transferase	DN4544_c0_g1_i1	17.5	0.04
	glycerol kinase	DN16960_c2_g1_i14	34.4	0.01
	glycine/betaine ABC transporter substrate-binding protein	DN15178_c0_g1_i1	33.1	$2.07e^{-3}$

hypothetical protein	DN14113_c1_g1_i1	16.2	0.02
hypothetical protein	DN17456_c0_g1_i2	23.7	0.01
hypothetical protein	DN17456_c0_g1_i3	14.2	0.03
hypothetical protein	DN17456_c0_g1_i4	22.0	$3.66e^{-3}$
hypothetical protein	DN17456_c0_g2_i1	17.3	0.04
hypothetical protein CGUJ_01660	DN14113_c0_g2_i1	16.5	0.05
hypothetical protein DJ66_0664	DN16265_c0_g2_i2	30.5	$1.15e^{-3}$
IMP dehydrogenase	DN6964_c0_g2_i1	13.6	0.03
isocitrate dehydrogenase	DN15328_c0_g1_i1	13.5	0.06
leucyl aminopeptidase	DN13997_c0_g1_i1	34.9	0.02
LuxR family transcriptional regulator	DN14940_c0_g2_i1	11.9	0.05
molecular chaperone DnaK	DN15220_c0_g1_i1	28.2	0.01
molecular chaperone GroEL	DN14757_c0_g1_i1	20.2	0.03
N-acetyl-gamma-glutamyl-phosphate reductase	DN15330_c0_g1_i2	14.7	0.05
NADH dehydrogenase	DN15210_c0_g1_i2	14.4	0.03
NifU family protein	DN5715_c0_g1_i1	11.8	0.05
nitrate ABC transporter ATP-binding protein	DN14488_c0_g2_i1	12.8	0.03
orotidine 5'-phosphate decarboxylase/BAX inhibitor (BI)-1/YccA family protein	DN14873_c0_g1_i1	13.5	0.04
PAS domain-containing sensor histidine kinase	DN14346_c0_g1_i1	13.9	0.02
penicillin-binding protein	DN14783_c0_g1_i1	11.9	0.03
periplasmic solute binding protein	DN15099_c0_g1_i1	9.7	0.04
permease	DN13997_c0_g2_i1	14.8	0.03
peroxiredoxin	DN17546_c4_g1_i1	13.5	0.03
phage repressor protein	DN15458_c0_g2_i5	20.6	0.03
phosphopyruvate hydratase	DN14337_c0_g1_i1	9.4	0.03
phosphoribosylformylglycinamidine cyclo-ligase	DN27217_c0_g1_i1	17.8	$4.73e^{-3}$
pilus assembly protein	DN13708_c0_g1_i2	10.8	0.03
poly(A) polymerase	DN12871_c0_g1_i1	11.9	0.05
porin	DN10141_c0_g1_i1	86.2	$5.55e^{-4}$
porin family protein	DN15690_c0_g1_i2	19.8	0.03
putative protease IV transmembrane protein	DN14809_c0_g1_i2	12.7	0.05
pyridoxine 5'-phosphate synthase	DN15414_c0_g1_i4	19.1	0.01
pyruvate kinase/carbamoyl phosphate synthase large subunit	DN15141_c0_g1_i1	15.8	0.04
replicative DNA helicase	DN15002_c0_g1_i2	20.9	0.03
response regulator	DN12200_c0_g1_i1	16.8	0.02
ribonuclease D	DN10298_c0_g1_i1	16.4	0.03
signal recognition particle protein	DN15243_c0_g1_i2	38.6	$1.17e^{-3}$
succinyl-diaminopimelate desuccinylase	DN14376_c0_g1_i1	12.7	0.03
sulfonate ABC transporter permease	DN15690_c0_g1_i1	10.2	0.03

	TerC family protein	DN11390_c0_g1_i1	10.8	0.05
	tetratricopeptide repeat protein	DN14826_c0_g1_i2	16.4	$8.26e^{-3}$
	thioredoxin-dependent thiol peroxidase	DN10526_c0_g1_i1	10.8	0.05
	threonine--tRNA ligase	DN10111_c0_g1_i1	11.8	0.04
	thymidylate kinase	DN14879_c0_g1_i1	13.2	0.03
	TIGR02300 family protein	DN20462_c0_g8_i1	13.9	0.03
	translation initiation factor IF-3	DN14949_c0_g2_i1	14.6	0.03
	trigger factor	DN13945_c0_g1_i1	11.5	0.04
	tRNA(5-methylaminomethyl-2-thiouridylate)- methyltransferase	DN53941_c0_g1_i1	17.1	0.05
	tRNA-dihydrouridine synthase A	DN14326_c0_g1_i1	14.2	0.02
	two-component system sensor histidine kinase AtoS	DN14305_c0_g2_i1	20.4	0.04
	type I methionyl aminopeptidase	DN13945_c0_g2_i1	16.4	$7.51e^{-3}$
	ubiquinone biosynthesis protein UbiB	DN13521_c0_g1_i1	9.8	0.03
	3-oxoacyl-ACP synthase	DN17762_c4_g2_i2	36.8	$1.85e^{-3}$
	bordetella phage Bbp38 like protein	DN15458_c0_g2_i1	9.6	0.03
	cell wall-associated hydrolase	DN19816_c1_g6_i1	42.2	0.02
	Chloramphenicol-sensitive protein rarD	DN13473_c0_g1_i1	7.5	0.05
	DEAD/DEAH box helicase	DN4052_c0_g1_i1	9.7	0.02
	dicarboxylate/amino acid:cation symporter	DN14605_c0_g1_i1	7.0	0.04
	DNA topoisomerase IV subunit B	DN10717_c0_g1_i1	8.2	0.05
	ferrochelatase	DN15257_c0_g1_i1	7.0	0.01
	glycerol kinase	DN16085_c2_g1_i12	38.8	$2.50e^{-4}$
	glycerol kinase	DN16960_c2_g1_i14	24.0	$3.44e^{-3}$
	glycine/betaine ABC transporter substrate-binding protein	DN15178_c0_g1_i1	135.0	$3.57e^{-4}$
	helix-turn-helix transcriptional regulator	DN15227_c0_g1_i1	36.9	0.05
	HsdR family type I site-specific deoxyribonuclease	DN14127_c0_g1_i1	11.3	$5.14e^{-3}$
	hypothetical protein	DN10205_c0_g1_i1	13.1	0.02
	hypothetical protein	DN14146_c0_g1_i1	7.3	0.06
	hypothetical protein	DN17456_c0_g1_i4	7.1	0.02
	phage repressor protein/Prophage antirepressor	DN15458_c0_g2_i5	16.5	$5.14e^{-3}$
	phenylalanine--tRNA ligase subunit beta	DN14949_c0_g1_i2	19.9	0.02
	SAM-dependent methyltransferase	DN13350_c0_g1_i2	38.0	$2.35e^{-4}$
	threonine--tRNA ligase	DN10111_c0_g1_i1	8.9	0.01

****A2*CLas*+ x A3*CLas*+**

*A1*CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 1-2 days;
**A2*CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 3-4 days; and
***A3*CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 5-6 days.

Fig S1. The analysis of the 248,850 contigs was conducted by Blast2Go, using Blastx to recover annotations with significant homology from the NCBI. All terms 'Biological Processes', 'Molecular Function' and 'Cellular Component' at level 2 are represented as percent over total number of sequences.

Fig S2. Distribution of alignments and transcripts of *de novo* assembly of *Diaphorina citri* gut that fed on health and CLas-infected citrus plant, after search for similarity in NCBI databank.

Fig 1. Venn diagram of *Candidatus* Liberibacter asiaticus transcripts differentially expressed in gut of adults at different periods of feeding on CLas-infected citrus plant. *A1CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 1-2 days; *A2CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 3-4 days; and *A3CLas*$^{+}$: adults that fed on CLas-infected citrus plant for 5-6 days.

Fig 2. Venn diagram of *Diaphorina citri* transcripts differentially expressed in gut of adults at different periods of feeding on CLas-infected citrus plant compared to insects that fed on healthy citrus plant. A1 - *CLas*$^{+}$ x *CLas*$^{-}$: adults that fed on CLas-infected versus uninfected citrus plant for 1-2 days; A2 - *CLas*$^{+}$x *CLas*$^{-}$: adults that fed on CLas-infected versus uninfected for 3-4 days; and A3 - *CLas*$^{+}$x *CLas*$^{-}$: adults that fed on CLas-infected versus uninfected for 5-6 days.

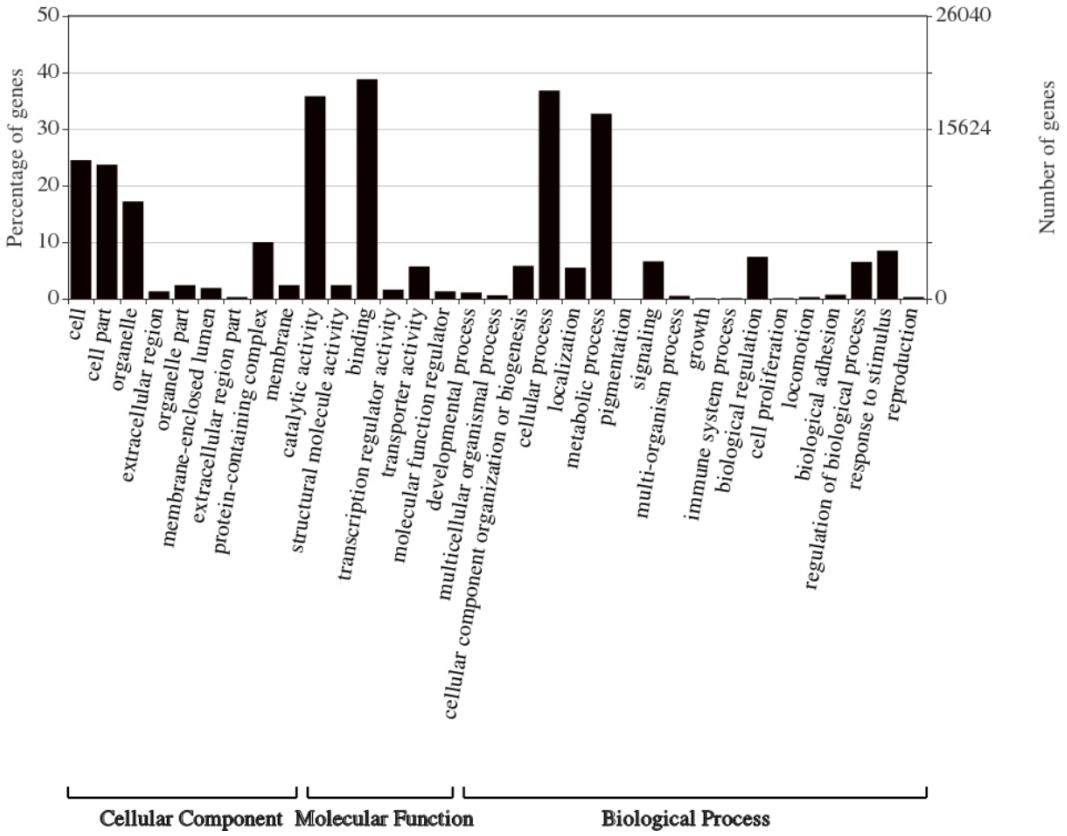

Fig S1.

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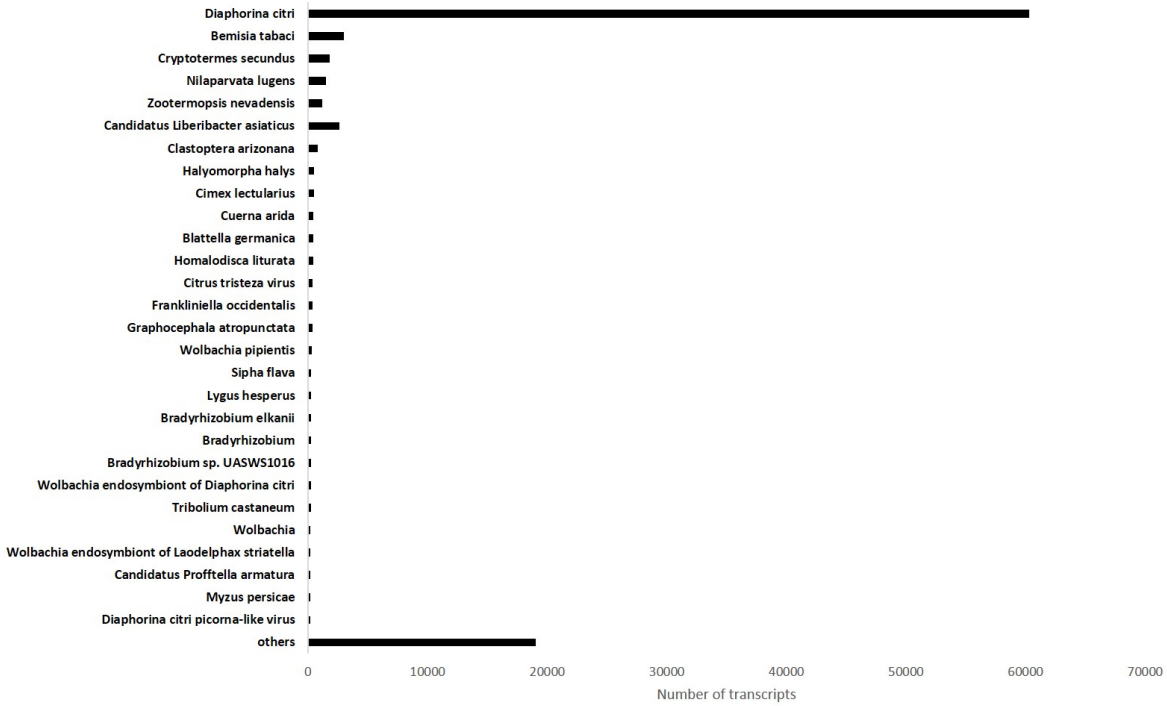

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1684 **Fig S2.**

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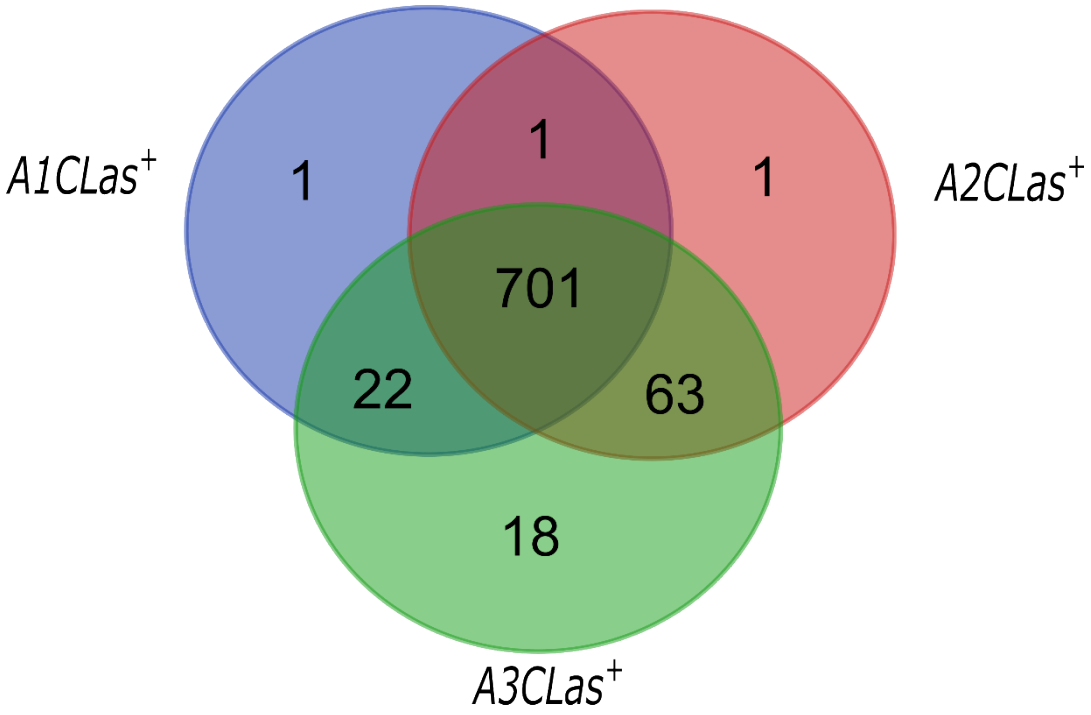

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1689 **Fig 1.**

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1692 **Fig 2.**

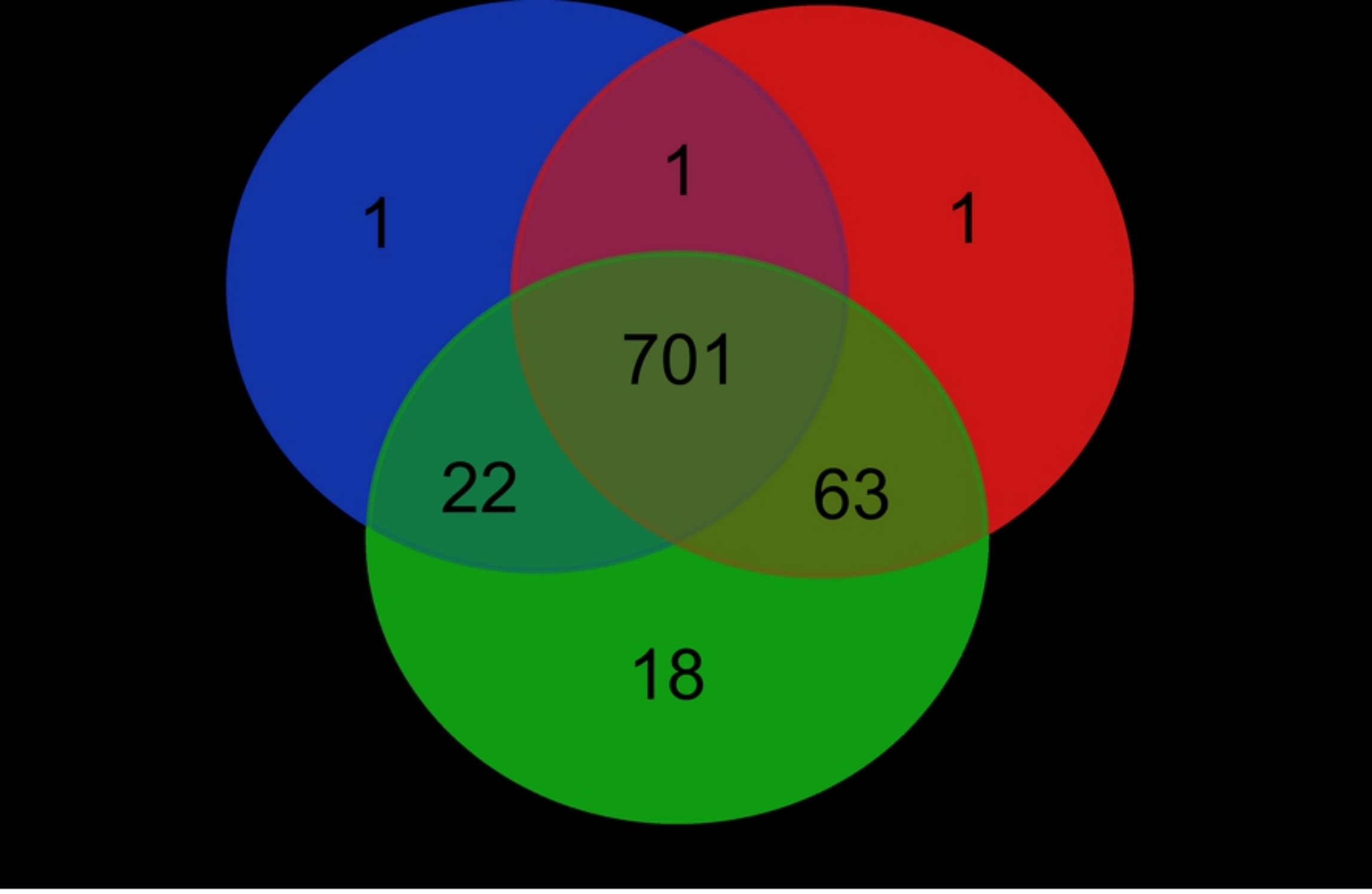

Figure 1

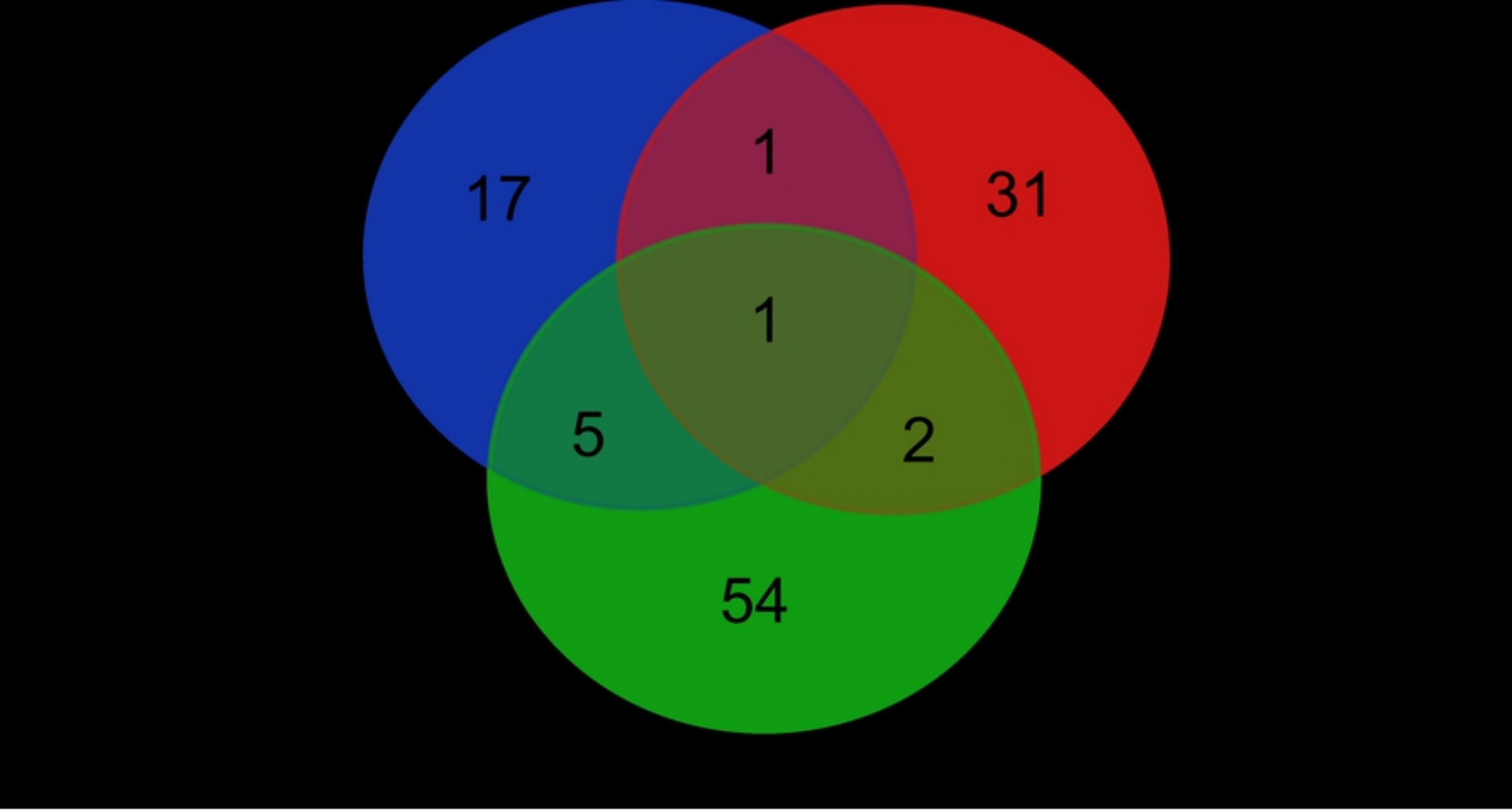

Figure 2