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Antibiotic treatment reduces fecundity and nutrient content in females of *Anastrepha fraterculus* (Diptera: Tephritidae) in a diet dependent way

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ABSTRACT

Insect microbiota, particularly, gut bacteria has recently gained especial attention in Tephritidae fruit flies, being Enterobacteriaceae the predominant bacterial group. This bacterial group has been postulated to contribute to the fitness of fruit flies through several life-history traits. Particularly in *Anastrepha fraterculus*, removal of Enterobacteria from male gut via antibiotic treatment impaired their mating behavior. Because the impact of gut bacteria on female reproduction was not yet addressed, we here analysed the effect of antibiotic treatment on female fecundity and nutritional status, and further explored the role of bacteria under different dietary regimes. The removal of culturable Enterobacteria from the gut of females was associated to a reduction in fecundity as well as in the protein and lipid reserves. However, fecundity reduction depended on the dietary regime; being more pronounced when females fed a poor diet. Our results suggest that nutrient reserves of females are determined, at least to some extent, by intestinal bacteria (particularly Enterobacteria). The effect of antibiotics on fecundity could be explained, thus, as a consequence of a poorer nutritional status in antibiotic-treated females compared to control females. Our results contribute to understand the interaction between gut bacteria and Tephritidae fruit flies. Considering the relevance of this insect as fruit pest and the widespread use of the sterile insect technique to control them, these findings may lead to practical applications, such as development of efficient mass rearing protocols of *A. fraterculus* that supplement the adult diet with probiotics.

1. Introduction

Insect gut bacterial community has been shown to contribute to host nutrition, immune response and other relevant biological functions, including reproduction (reviewed by Dillon and Dillon, 2004; Lee et al., 2017). Enterobacteriaceae is the dominant group in the gut of various insect species (Douglas, 2015 and references herein; Raza et al., 2020) and this group has been associated, at least, to nutrient bioavailability, nitrogen fixation, degradation and nutrient biosynthesis; mainly proteins and lipids (Bar-Shmuel et al., 2020; Jing et al., 2020: Salem et al., 2014; Stathopoulou et al., 2021). This has led to propose the addition of bacteria to the diet of artificially-reared insects as to increase both female reproduction and male sexual competitiveness (Augustinos et al. 2015; Stathopoulou et al., 2021 and references herein). Thus, understanding the role of bacteria on insect nutrition and metabolism as well as evaluating the use of gut bacteria as probiotics became an emerging field of research particularly due to its potential use for pest management in association with the sterile insect technique (SIT) (Noman et al., 2020). The SIT requires millions of highly-competitive sterile insects to be released into the field to compete with wild fertile males to access and mate with wild females reducing reproduction of wild populations (Knipling, 1955). During the last decades, the SIT has been widely used in different insect species (Dyck et al., 2021) and particularly in Tephritidae fruit flies (Hendrichs et al., 2021).

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In most Tephritidae species analyzed so far, Enterobacteriaceae is the predominant bacterial group in gut community (Bourtzis et al., 2003; Juárez et al., 2019; Ventura et al., 2018; Augustinos et al., 2019; Noman et al., 2020; Salgueiro et al., 2020). Enterobacteriaceae has been suggested to be involved in biochemical pathways related to membrane transport and the metabolism of carbohydrates, amino acids, cofactors and lipids (see Ventura et al., 2018 and references herein); including fixation of nitrogen in the gut of *Ceratitis capitata* (Behar et al., 2005) and urea metabolization in *Bactrocera oleae* (Ben-Yosef et al., 2014). By suppressing bacteria in the gut of *B. oleae* with the addition of antibiotics, Ben-Yosef et al. (2014) indirectly evidenced the contribution of bacteria to the nitrogen availability of the host, which in turn increases egg production (Dimou et al., 2010; Ben-Yosef et al., 2014). Similar studies showed the contribution of the bacterial communities to reproduction of other tephritids, such as *Bactrocera tryoni* (Nguyen et al., 2021) and *Rhagoletis completa* (Tsiropoulos, 1981). It was also reported that changes in the bacterial associations, due to antibiotic treatment, during the larval stage affected ovarian development of *Zeugodacus tau* (Noman et al., 2021). Furthermore, it was postulated that Enterobacteria could serve as proteinaceous food for their host (Drew et al., 1983; Lemos and Terra, 1991; Deutscher et al., 2019 and references herein). For example, *Bactrocera minax* fecundity is increased when the diet contains members of the Enterobacteriaceae family (Andongma et al., 2018; Rashid et al., 2018). This beneficial effect of gut bacteria on Tephritidae fruit fly reproduction, mediated by nutrition, seem to be more relevant when flies consume diets of low nutritional quality (Ben-Yosef et al., 2010; 2014). In such cases, it has been proposed that symbiotic bacteria allow flies to improve their fitness when feeding on food sources rather marginal in terms of their nutrient composition or nutrient bioavailability (Ben-Yosef et al., 2008; Juárez et al., 2019). Although the mechanisms are not fully understood, evidence suggests that bacteria would be involved in specific physiological functions related to the metabolism of proteins (Tsiropoulos, 1981; Lauzon et al., 2000; Andongma et al., 2018) and lipids (Ben-Yosef et al., 2008).

The impact of marginal diets on Tephritidae reproduction has been well documented (Tsitsipis, 1989; Drew and Yuval, 2000; Yuval et al., 2007; Chen et al., 2017; Goane et al., 2019). In females, fecundity is drastically reduced when flies are fed a diet with a low-quality protein source, such as non-hydrolyzed brewer's yeast, or high-quality hydrolyzed yeast protein source but at low proportions (Fanson & Taylor, 2012, Jácome et al., 1999; Chang, 2009, Morelli et al., 2012, Goane et al., 2019). In males, lack of access to high-quality protein sources affects their sexual competitiveness, pheromone release rate and courtship behavior (Liendo et al., 2013; Pereira et al., 2013 and references herein; Taylor et al., 2013; Juárez et al., 2019). Studies on the effect of diet on the fly metabolism are scarce, but it has been shown that a poor diet induces a reproductive halt causing flies to enter into a "waiting mode", in which nutrients are derived to tissue maintenance until external cues (i.e., adequate nutrient source found) indicate that reproduction can be resumed (Romanyukha et al., 2004; Yap et al., 2015). These physiological states (waiting vs. reproductive) are accompanied with anabolism-catabolism cycles in which flies keep nutritional reserves approximately constant (Aluja et al., 2011). Likewise, the potential role of gut bacteria on nutrient content associated with the quality of diets and its consequences on reproduction has been seldom addressed (Ben-Yosef et al., 2008, Juárez et al., 2019), but studies concur in showing that removal of bacteria has a detrimental effect, both on reproduction and nutrient content.

Anastrepha fraterculus is one of the most important Tephritidae fruit fly species in South America (Malavasi et al., 2000, Cladera et al., 2014) due to its wide distribution and host range, which includes several commercial fruit crops (Norrbom, 2004). The SIT has been proposed as a key component in area-wide action programs against this pest and therefore mass-rearing should be optimized. Because the diet provided to the flies in the mass rearing is relevant for SIT feasibility and success (Pérez-Staples et al., 2021), previous studies analyzed the role of nutrients, mainly different yeast derivatives and wheat germ, on female fecundity. Goane et al. (2019) showed that the type of yeast provided to the females conditions their fecundity, probably because of the lack of specific amino acids, vitamins or sterols in low quality yeast derivatives, such as brewer's yeast or also due to its low assimilation. Yet, no studies explored the impact of bacteria nor their interaction with the type of yeast derivative provided on *A. fraterculus* female reproduction. Our current knowledge on *A. fraterculus* bacterial community suggests a dominance of Proteobacteria in the gut of larvae and adults (Müller, 2013; Augustinos et al., 2019; Juárez et al., 2019; Salgueiro et al., 2020). Removal of Enterobacteria from *A. fraterculus* male gut via antibiotic treatments (Juárez et al., 2019), impaired host nutrition, specifically protein content, and male mating success in a diet dependent way. Antibiotic treatment reduced the ratio of sexual displays (wing fanning and exposure of salivary glands), the amount of pheromone emitted by nutritionally stressed males (fed only sugar) and the sexual success of protein (hydrolyzed yeast) fed males. Unveiling the role of bacteria in the reproductive biology of *A. fraterculus* females would contribute to the development of mass rearing protocols (Cáceres et al. 2019) through the implementation of different approaches (Ben-Ami et al., 2010; Deutscher et al., 2019; Raza et al., 2020) such as supplementing the adult diet with probiotics or maintaining a healthy gut bacterial community of reared flies.

Here, we analysed the impact of a combination of antibiotics, known to affect gut bacterial load of *A. fraterculus* males and more specifically the abundance of Enterobacteriaceae, on the fecundity and nutritional status of females fed two different yeast derivatives (hydrolysed yeast and brewer's yeast) with proven contrasting effect on *A. fraterculus* fecundity. The experimental design aimed at unveiling whether gut bacteria contributed, at least indirectly, to female fecundity and nutritional status, and if this contribution depends on the quality of the adult diet.

2. Materials and methods

2.1. Insects and general procedures

Anastrepha fraterculus pupae were obtained from the artificial colony corresponding to the *A. fraterculus* sp. 1 or Brazilian 1 morphotype (Selivon et al., 2005; Goday et al., 2006; Hernández-Ortiz et al., 2012) kept at Instituto de Genética "Ewald A. Favret" (IGEAF). Flies were reared on a larval diet based on sugar, wheat germ and brewer's yeast in the same proportion (Salles, 1995) and sorted by sex upon emergence. Newly-emerged females were transferred in groups of 15 to 15-L plastic container and provided one of the diets described below. Five containers were set per type of diet (see below). Males were housed in 12-L plastic containers (400 individuals per container) and fed on a solid diet containing hydrolyzed yeast (MP Biomedicals®, Ohio, USA) and sucrose in a 1:3 ratio. Both sexes were kept at room temperature (25 ± 4 °C) and 12 h-12 h light: dark photoperiod. Water was supplied in plastic containers with a moistened cloth. After 10 days, 15 sexually mature males were placed into the female containers to allow the flies to freely copulate. Five mated females of the same diet were then placed in 1-L plastic containers with the corresponding diet and water, covered with a lid of voile cloth to evaluate fecundity (see 2.5).

2.2. Diets and antibiotics

Two yeast derivatives with proven contrasting effect on *A. fraterculus* fecundity (Goane et al., 2019) were evaluated: hydrolyzed yeast (MP Biomedicals®, Ohio, USA) abbreviated as "HY" which is a concentrated source of soluble proteins, carbohydrates and vitamins; and brewer's yeast (Calsa®, Tucumán, Argentina) abbreviated as "BY", which is widely used in the larval diets and has been shown to allow egg production of *A. fraterculus* females but at a low rate (Goane et al., 2019). Both yeast derivatives were offered in combination with sucrose (1:3 w/

w proportion) and were either supplied with antibiotics ("AB") or kept antibiotics-free. Ciprofloxacin (10 μg mL^{-1}) and Piperacillin (200 μg mL^{-1}) were used as these antibiotics showed to severely affect the gut bacteria community (undetectable by PCR) of *A. fraterculus* adults, particularly those of the Enterobacteriaceae family (Juárez et al., 2019). This combination of the yeast derivative and the presence or not of antibiotics resulted in four diets (treatments) which were supplied to the flies since their emergence.

2.3. Diet preparation and dispensing

To prepare the diets, sucrose and the corresponding yeast derivative were blended at the fixed proportions and stored in a refrigerator. Daily, 3 g of diet were mixed in 30 cc of water (with or without antibiotics) and vigorously shaked to obtain a homogeneous mixture. Diets were imbibed in small pieces of cotton and then offered *ad libitum* to the flies. The cotton with food was renewed daily.

2.4. Dissections and bacterial load estimation

Bacterial load of females was estimated at the end of the oviposition period (24 days after emergence) to confirm bacterial removal. Confirmation of bacterial removal was done on homogenates. From each replicate, three mature females (24 days-old) were randomly selected at the end of the oviposition period and sterilized/disinfected by immersing them, individually and sequentially, in i) sterile water; ii) 0.05% sodium hypochlorite; iii) sterile water; iv) 70% ethanol; v) sterile water; vi) sterile PBS 1X and then dissected in PBS 1X. Dissection was also performed in sterility under stereoscopic microscope (Olympus SZ30, 40 × zoom) inside the flow cabinet, with sterile forceps and needles on previously autoclaved slides. The dissection and the anatomical recognition of the intestine was performed following Caetano et al. (2006). Subsequently, a pool of three guts was homogenized in 500 μl sterile PBS 1X.

2.4.1. DAPI stain

In order to proceed with the comparison of total bacterial count between treatments, each homogenate was diluted by half, and 3 μl were transferred to a sterile slide which was then stained (10 min on ice, complete darkness) with 10 μl of DAPI (49,69-diamidino-2-phenylindole 0.050 μg/ml) previously prepared using 2x SSC as solvent. Excess DAPI was removed with cold distilled water. Slides were then air-dried in the dark and subsequently treated with antifade. The staining was developed using Olympus BX51 epifluorescence (Olympus, Tokyo, Japan) microscope with 1600X objective and pictures of 10 random fields were captured (Robarts and Sephton, 1981; Walker et al., 1988). Percentage of field area covered by total microbial cells, including culturable and no culturable bacteria, was estimated with Image J software (Rueden et al., 2017).

2.4.2. Colony forming units (CFU) evaluation

To estimate the culturable bacteria in the homogenates, serial dilutions of the homogenates, from 0 to −9, were performed on solid LB plates (37 °C overnight) in order to find an appropriate concentration in which defined colonies could be counted. Afterwards, the homogenate was diluted to the concentration selected (C1) for CFU counting, registering CFU/μl values for each replicate.

2.4.3. MacConkey test

In order to estimate the proportion of culturable Enterobacteria, 100 μl of C1 were taken and homogeneously distributed on LB plates. All the colonies obtained were isolated, numbered and replicated to a plate with MacConkey medium and to a second LB plate (LB control). This medium allowed to estimate the percentage of CFU corresponding to Enterobacteriaceae. The number of colonies capable of degrading lactose was then counted and standardized over the total number of CFU evaluated for each replicate.

2.5. Effect of the addition of antibiotics in the diet with different yeast derivatives on fecundity

Fecundity was evaluated on the mated females placed in 1-L plastic containers (five females of the same diet per container) with the corresponding diet and water, covered with a lid of voile cloth (described in 2.1). A green colored agar disc was placed on top of the voile lid as oviposition substrate and replaced every 48 h throughout a period of 14 days. The number of eggs laid on the agar disk was counted under a stereoscopic microscope (10X). Female fecundity was estimated by dividing the total number of eggs in the oviposition substrate by the number of females still alive in the container when replacing the oviposition substrate. This experiment was repeated 3 times with 5 replicates (containers) each, totalizing 75 females evaluated for each combination of diet and antibiotic treatment.

2.6. Effect of the addition of antibiotics in the diet with different yeast derivatives on nutrient content

Nutrient content of females was determined at the end of the oviposition period (24 days after emergence) following standard biochemical techniques. Protein content was determined with the Bradford method (Bradford, 1976) using Coomassie brillant blue G-250 reagent (Biopack®, Buenos Aires, Argentina). Lipid and carbohydrate contents were determined with a method adapted by Kaufmann (2015). According to the protocol, 2% sodium sulfate was added to precipitate the glycogen and separate it from the rest of carbohydrates by centrifugation. Lipid content was measured with vanillin reagent (Sigma-Aldrich, St. Louis, USA) whereas total sugar and glycogen contents were measured with anthrone reagent (Cicarelli, Santa Fe, Argentina). Optical density was measured on a ZL-5100 Zeltee Spectrophotometer. Ten randomly selected females from each combination of diet and antibiotic were evaluated. Determinations were carried out individually and the dry weight of each female was recorded (Precision scale 0.0001g, Ohaus corporation, USA) to standardize nutrient content by body weight for each fly. Neither antibiotic treatment ($F_{1,36} = 0.09$, $P = 0.763$) nor yeast derivative ($F_{1,36} = 2.97$, $P = 0.094$) affected dry weight of post-oviposition females, with non-significant interaction between factors ($F_{1,36} = 3.17$, $P = 0.084$). Weight of non-treated females varied from 5.65 ± 0.42 when fed BY to 6.86 ± 0.23 when fed HY. Weight of antibiotic- treated females varied from 6.16 ± 0.4 when fed BY to 6.14 ± 0.27 when fed HY.

2.7. Data analysis

Three models were built to analyze the impact of antibiotic treatment on gut bacterial load, one for each technique used. Percentage of area covered by bacteria in DAPI stained photographs was analyzed by Kruskal-Wallis Rank Sum test. A generalized linear model with negative binomial distribution was performed to analyze the number of CFU, with type of yeast derivative and antibiotics as fixed factors. Guts from females fed diets with antibiotics had no detectable levels of Enterobacteria; therefore, Enterobacteriaceae load (detected with MacConkey test) was analyzed by a generalized linear model with quasibinomial family given the overdispersion detected when fitting to a binomial family to assess the impact of the yeast derivative on Enterobacteriaceae load. Enterobacteria number was considered the response variable, yeast derivative the fixed factor and total bacteria the covariable. Analyses were performed with Infostat and R (Di Rienzo et al., 2019; R Development Core Team, 2015).

In order to address the effect of antibiotic treatment and the type of yeast derivative on fecundity, we fitted a zero-inflated negative binomial model (Hilbe, 2014; Beaujean and Grant, 2016) since we found high frequency of count observations consisting in zeros (Supplementary

Fig. 1). These models use a mixture of distributions: a binomial distribution with logit link is used to model the non-occurrence of the event (here, lack of eggs) and a negative binomial distribution with a log link is used to model the occurrence of the event (here, the number of eggs laid). Repeated experiments were considered as a blocking factor in the model and time tendency was modelled by means of a quadratic polynomial (the cubic component was not significant). Considering the significant interaction between antibiotic treatment and yeast derivative in a previous analysis (Supplementary Table1), the model for occurrence was parameterized according to the contrasts of interest with yeast derivative, the antibiotic treatment inside each yeast derivative (HY and BY) and the time as a quadratic polynomial function. For the non-occurrence, we considered yeast derivative and antibiotic treatment effects. Contrasts were built with dummy variables. Model fitting was performed by maximum likelihood. Analyses were performed with R (R Development Core Team, 2015) using the package pscl (Jackman et al., 2015).

The effect of antibiotics and the type of yeast derivative on nutrient content of post-oviposition females was analyzed by means of separate general linear models built for each nutrient (protein, lipid, carbohydrates and glycogen). Yeast derivative and antibiotic treatment were included in each model as fixed factors. To eliminate the effect of fly weight variation, we standardize nutrient content by dividing this value of each fly to its corresponding weight (Yuval et al., 1998; Foray et al., 2012; Wong et al., 2014; Goane et al., 2019; Juárez et al., 2019) resulting in a value expressed in μg per mg of fly weight (μg/mg) which was used in the statistical analysis. Normality (Q-Q plot) and homogeneity of standardized residuals (residuals' plot) were verified in all cases (Crawley, 2007). Heteroscedasticity were corrected by the power variance function (weights = varPower(.), reml R package) when required (Pinheiro and Bates, 2000).

3. Results

3.1. Antibiotic treatment on gut bacteria load.

3.1.1. DAPI stain

The percentage of the area covered by total microbial cells, including culturable and no culturable bacteria in DAPI images was significantly lower when antibiotics were included in the adult diet ($H = 142.83$, $P < 0.001$) with the lowest values for females fed BY with AB diet followed for females fed HY with AB diets (Fig. 1a). No differences were detected between diets with no AB.

3.1.2. CFU evaluation

A drastic reduction of culturable bacteria was detected in females fed diets with antibiotic treatment ($Z = 7.551$, residual deviance: 32.546 on 20 d.f., $P < 0.001$), independently of the yeast derivative in the adult diet ($Z = -0.047$, residual deviance: 32.546 on 20 d.f., $P = 0.962$) with no significant interaction between factors ($Z = 0.305$, residual deviance: 32.546 on 20 d.f., $P = 0.761$) (Fig. 1b).

3.1.3. MacConkey test

No Enterobacteria were found in AB-treated females. About 40% of the culturable bacteria found in the guts of non-AB treated females were Enterobacteria (Fig. 1c) with no significant differences attributable to the yeast derivative present in the adult diet ($T = 1.14$, d.f. = 10, $P = 0.282$). Average percentage of Enterobacteria was 36.32 ± 12.23 for BY and 35.61 ± 4.59 for HY.

3.2. Effect of the addition of antibiotics in the diet with different yeast derivatives on fecundity

Both, the addition of antibiotics and the yeast derivative in the diet affected female fecundity (Table 1). Antibiotic-treated females laid significantly fewer eggs than non-treated females irrespective of the yeast derivative provided to the females (Fig. 2). Females fed a diet with HY laid more eggs than females fed a diet with BY (Fig. 2). Moreover, the estimated model parameters showed that the addition of antibiotics to the diet affected female fecundity in a diet-dependent way (Table 1). The presence of antibiotics in the diet decreased to 11% the probability of laying eggs when females fed a diet with HY and to 41% when females fed a diet with BY.

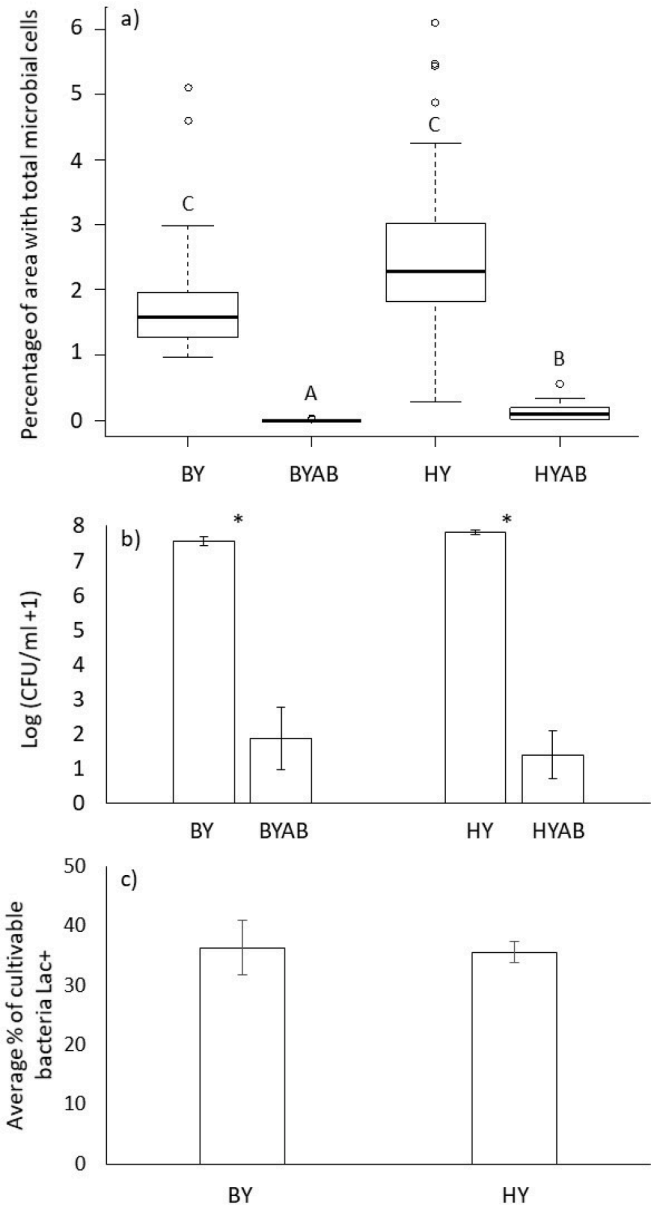

Fig. 1. Effect of antibiotic treatment offered in the adult liquid diet on gut bacteria load of *Anastrepha fraterculus* females analyzing percentage of the area covered by total microbial cells (culturable and no culturable bacteria) in DAPI images (a); colony forming units of culturable bacteria (b); and average percentage of culturable bacteria which resulted positive to the lactose degradation test by McConkey and therefore identified as Enterobacteria (c).

3.3. Effect of the addition of antibiotics in the diet with different yeast derivatives on nutrient content

3.3.1. Proteins

Antibiotic treatment negatively affected protein content of females ($F_{1,37} = 103.92$, $P < 0.001$), irrespective of the yeast derivative evaluated ($F_{1,37} = 0.16$, $P = 0.745$) (Fig. 3). No significant interaction was

Table 1
Estimates of the model parameters (mixture of two distributions) to analyze the effect of antibiotic treatment on *Anastrepha fraterculus* fecundity according to the yeast derivative considered in the adult diet.

	Estimate	Std. Error	Z-value	Pr(>|z|)
Occurrence (non-zeros)				
Experiment II	0.5418	0.0764	7.096	< 0.001
Experiment III	0.5750	0.0730	7.878	< 0.001
Days	0.3920	0.0905	4.330	< 0.001
Days^2	−0.0114	0.0026	−4.442	< 0.001
Yeast derivative (HY vs BY)	−1.0719	0.0340	−31.525	< 0.001
Antibiotics within HY	−0.1094	0.0360	−3.042	0.0024
Antibiotics within BY	−0.5288	0.0532	−9.946	< 0.001
Log (Theta)	1.1325	0.0934	12.131	< 0.001
Non-occurrence (zeros)				
Yeast derivative (HY vs BY)	1.9522	0.2641	7.393	< 0.001
Antibiotics	0.7338	0.1360	5.396	< 0.001

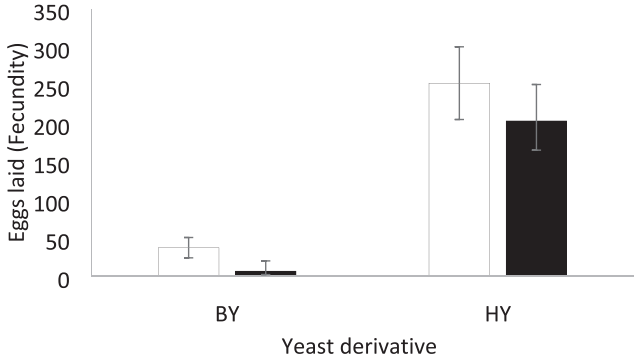

Fig. 2. Average number of eggs laid by *Anastrepha fraterculus* mature females fed on brewer's yeast or hydrolyzed yeast diet with (black bars) or without (white bars) antibiotics. Females were 10 d-old at the beginning of the readings and data at 48 h interval period (considering alive females at each interval period) were averaged.

recorded between the yeast derivative and antibiotic treatment ($F_{1,37}$ = 0.61, P = 0.440).

3.3.2. Lipids

Lipid content of females was also negatively affected by antibiotic treatment ($F_{1,37}$ = 6.71, P = 0.014) irrespective of the yeast derivative used for the diet ($F_{1,37}$ = 0.29, P = 0.595) with no statistically significant interaction between factors ($F_{1,37}$ = 1.17, P = 0.287) (Fig. 3).

3.3.3. Carbohydrates

Yeast derivative ($F_{1,36}$ = 7.59, P = 0.009) but not antibiotic treatment ($F_{1,36}$ = 2.27, P = 0.141) affected carbohydrate content of females; with no statistically significant interaction between factors ($F_{1,36}$ = 3.26, P = 0.079). Carbohydrate content was lower for females fed HY diet than for females fed BY diet (Fig. 3).

3.3.4. Glycogen

Yeast derivative ($F_{1,36}$ = 17.15, P < 0.01) and antibiotic treatment ($F_{1,36}$ = 4.60, P = 0.039) affected glycogen content of females with statistically significant interaction ($F_{1,36}$ = 4.90, P = 0.033). Non-antibiotic treated females showed the lowest glycogen contents when fed HY diet relative to the other females (Fig. 3).

4. Discussion

Our study indicates that antibiotic treatment affected fecundity of *A. fraterculus* females, being this effect more pronounced in poor diets. Changes in gut bacterial load caused by the addition of antibiotics in the adult diet were confirmed by three techniques; with no culturable Enterobacteria recorded in AB-treated females. Nutrient content was also affected by the treatments. Protein content was markedly low in antibiotic treated females. Lipid content was also low but to a lesser extent. Carbohydrate content was affected by the yeast derivative and a significant interaction between factors (yeast derivative and antibiotic treatment) was recorded for glycogen content. Our results provide the first, yet indirect, evidence of a potential beneficial role of gut bacteria on the fitness of *A. fraterculus* females.

A low gut bacterial load, specifically Enterobacteria, was associated with a low fecundity of *A. fraterculus* females in a diet-dependent way. Fecundity reduction in antibiotic treated females was more pronounced when they were fed BY than HY. The contribution of bacteria to fecundity has previously been demonstrated for *B. oleae* (Ben-Yosef et al., 2010, 2014; Dimou et al., 2010); and particularly, Ben-Yosef et al. (2010) showed that the effect was diet dependent. Females fed on a diet without essential amino acids and treated with antibiotics laid fewer eggs than control females (same diet, no antibiotics), whereas the fecundity of females fed a diet with the complete set of amino acids was not affected by antibiotic treatment (Ben-Yosef et al., 2010). These results lead the authors to conclude that the microbial community allows the hosts to subsist on food sources considered marginal in terms of their nitrogenous composition; something that is thought to occur in nature (Mattson, 1980, Drew and Yuval, 2000). A similar experiment with *B. minax* (Andongma et al., 2018) showed that no eggs were laid by females fed only sucrose; and the addition of antibiotics decreased fecundity even when the flies were fed a diet that fulfills the amino acids requirements. Here we evaluated two yeasts with different output in female reproduction (Goane et al., 2019): one highly hydrolyzed yeast (HY) and one non-hydrolyzed yeast (BY). The consistent diet-dependent effect of antibiotics on fecundity and the fact that fecundity of Tephritidae fruit flies, including *A. fraterculus* (Goane et al., 2019), depends on adult female nutrition (Meats and Leighton, 2004), strongly evidence the role of gut bacteria, particularly Enterobacteria on nutrition. This impact on host nutrition could be either because bacteria provide metabolites required for reproduction such as essential amino acids, sterols or vitamins, or because they contribute to their incorporation and utilization in a more efficient way.

The effect of gut bacteria on biological parameters within Tephritidae fruit flies was also explored by adding Enterobacteria to the adult diet, with positive results in terms of male sexual performance and female fecundity (Andongma et al., 2018; Rashid et al., 2018). These authors ruled out the possibility that bacteria could be used directly by the flies as the only proteinaceous food source because no egg production was obtained in flies fed only sucrose supplemented with bacteria. In contrast, other studies propose that the contribution of gut bacteria is related to a nutritive role of bacteria in which fruit flies, including *A. fraterculus* (Lemos and Terra, 1991), acquire short-chain protein, amino acids or other nutrients by simply digesting the bacteria growing in their guts (Drew et al., 1983; Lemos and Terra, 1991; Deutscher et al., 2019 and references herein). Drew et al., (1983) showed that a diet containing sugar and *Klebsiella oxytoca* isolated and cultivated on agar increased female fecundity of flies demonstrating that ingestion of bacteria satisfied the female requirements for egg maturation. However, for the same species, females feeding on sugar and *K. oxytoca* or *Klebsiella pneumoniae* laid fewer eggs than females fed a complete diet (mix of autolyzed yeast and sucrose) (Meats et al., 2009); reinforcing the idea of a functional contribution of microbial community to the host. Studies in which dead (autoclaved) and alive bacteria are supplemented to the fly diet might shed light on the mechanisms by which bacteria contribute to reproduction in *A. fraterculus*. Neither antibiotic treatment nor yeast derivative affected dry weight of post-oviposition females (see material and methods section). As a result, antibiotic-treated females showed similar dry weight when fed BY or HY diet than non-treated females, reinforcing the idea of normal feeding of females on all diets which has been confirmed for males in previous studies (Juárez et al.

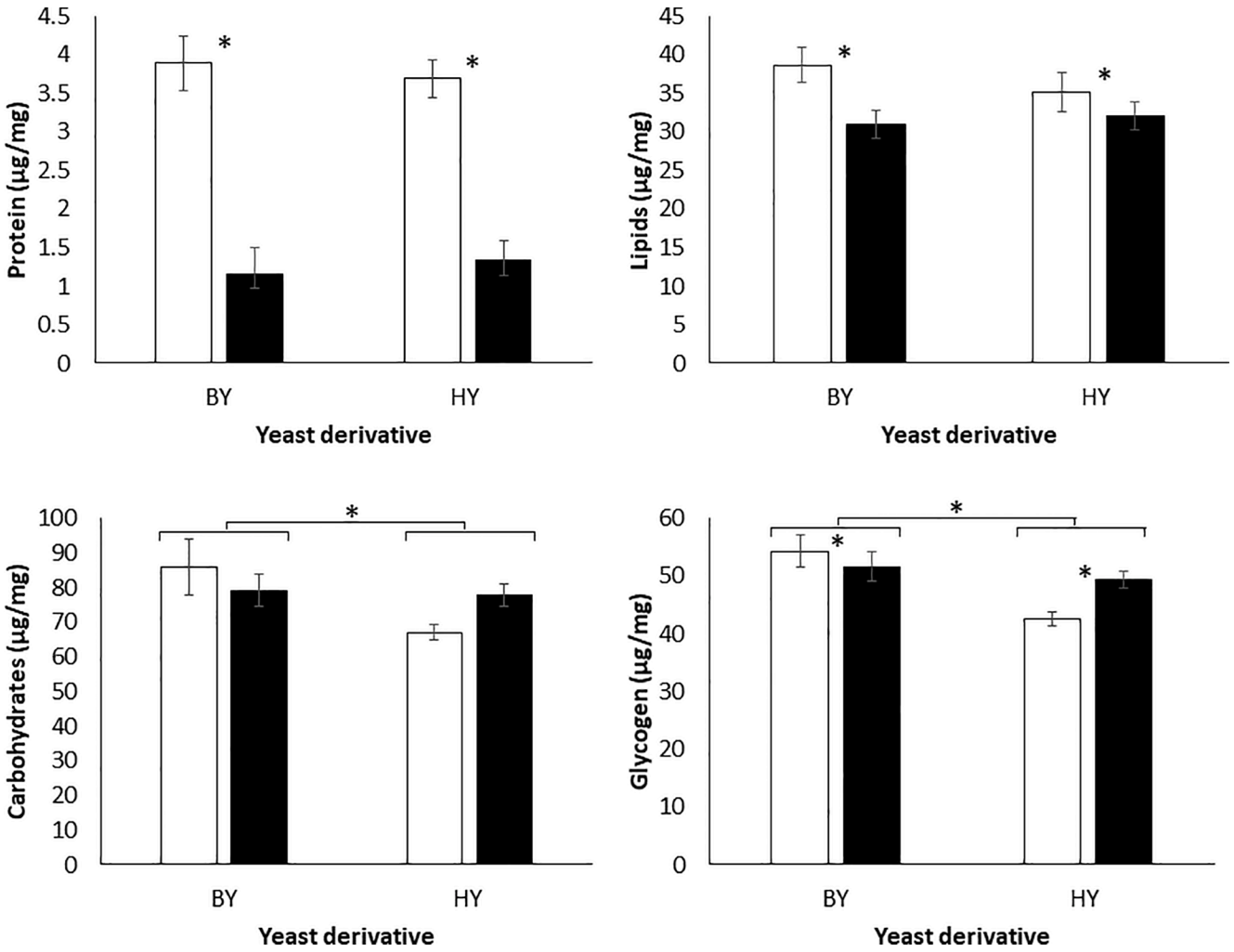

Fig. 3. Average nutrient content per fly (µg/mg) of *Anastrepha fraterculus* females fed adult liquid diet with (black bars) or without (white bars) antibiotic treatment and different yeast derivative: hydrolyzed yeast (MP Biomedicals®, HY) and brewer's yeast (CALSA®, BY). Analysis was separately performed for each female condition.

2019). In addition, the number of living females was recorded throughout the experiment for fecundity estimations. In this context, it should be noted that all antibiotic-treated flies were alive if fed HY and 90% if fed BY. Alive females without antibiotics were 100% and 93% for HY and BY, respectively. These values show that antibiotic treatments did not cause damage to the flies, at least during the time of the experiment.

Removal of bacteria affected protein and lipid content of *A. fraterculus* females. The decrease of protein content in flies treated with antibiotics was also documented for *A. fraterculus* males (Juárez et al., 2019) and suggests that bacteria would contribute to the degradation of long protein chains followed by assimilation or to the synthesis of certain amino acids (Tsiropoulos, 1981; Lauzon et al., 2000; Andongma et al., 2018; Jing et al., 2020). In addition, Enterobacteriaceae has been reported to fix atmospheric nitrogen in the guts of *C. capitata* (Behar et al., 2005). Furthermore, Enterobacteria isolated from the gut of *A. fraterculus* has been documented to fix atmospheric nitrogen when cultivated under ambient conditions (Salgueiro, pers. obs.). Low lipid levels in females feeding on a diet with antibiotics have also been reported for *C. capitata* females (Ben-Yosef et al., 2008). Bacteria have no capacity to synthetize cholesterol (Douglas, 2015) but they are involved in lipolysis (Ventura et al., 2018), probably making lipids available to the host (Madigan et al., 2004; Huang and London, 2016). Thus, antibiotic-treated flies could have reduced its ability to degrade lipids affecting both lipid availability and reserves. Bacteria also contribute to the synthesis of sterols and most of the non-saponifiable lipid vitamins. Also, different peptide hormones are involved in lipid metabolism (Toprak, 2020). Therefore, it can be expected that a drop in protein content would translate in a drop of lipids content. In addition, we observed that even when the interaction between factors (yeast derivative and antibiotic) was not statistically significant, lipid reduction caused by antibiotic treatment was more evident for females fed BY diet (from 38.67 µg to 30.89 µg/mg) than HY diet (from 35.24 µg to 32.04 µg/mg) confirming our expectation.

Interestingly, the yeast derivative had no effect on protein and lipid contents, even though there was a marked difference on female fecundity according to the yeast they were fed. This could suggest that a "poor food" produces "high-quality" flies in terms of their protein and lipid content (see in Fig. 3 that females fed on BY had the same protein and lipid content than females fed on HY) but does not produce flies with high fecundity. This apparent uncoupling between fecundity and nutritional status may be a consequence of metabolism regulation and resource allocation (Boggs, 2009) which would allow the insect to resist nutritional restrictions (Aluja et al., 2011; Nestel et al., 2016) by entering into a reproductive "wainting mode" (Zur et al., 2009). Individuals keep nutrients at an optimal level but require a minimum level of specific nutrient for body maintenance, survival and reproduction. Proteins have several functions and total content may not necessarily correlate with each particular function (such as reproduction, movement, structure and regulation). Under the resource allocation lens

(Boggs, 2009), flies would allocate their resource to reach the optimal level for body maintenance and after this is met, they would start allocating resources to reproduction (Romanyukha et al., 2004; Yap et al., 2015). In our study, HY-fed females with bacteria fulfill body maintenance and reproduction requirements whereas, BY-fed females with bacteria fulfill body maintenance but not reproductive requirements. When bacteria are removed, flies are faced to an unexpected nutritional scenario, which seems to have a big impact on protein content, regardless of the food eaten. In this scenario, protein content decrease and flies apparently reach a level of protein that has no impact on survival but limits reproduction. Considering our results, it is possible that antibiotic-treated flies (fed on both foods) are around this minimum protein level. So, to avoid losing more protein than the minimum required for their bodies, flies reduced fecundity rates. The reduction of fecundity reached minimum values in BY flies but not in HY flies. In this sense, egg production is a complex process (Attardo et al., 2005) and depends not only on the total amount of protein ingested in the diet, but also on the bioavailability of some very specific nutrients (Kambysellis et al., 1989). HY flies would have more available metabolites (because they fed a highly hydrolyzed yeast) than BY flies fed this non-hydrolyzed yeast. Alternatively, flies may have lost muscle mass. In our experiment, flies were confined in 1-L containers so flight was rather restricted and therefore we did not detect an impact on this trait. Future studies in nutrition and resource allocation will surely contribute to better understand the metabolism of *A. fraterculus* and its microbiome.

Previous studies already shown phenotypic plasticity associated with reproduction in response to dietary restriction in another genus of Tephritidae, *Bactrocera dorsalis* (Chen et al., 2017). Analyzing trade-offs and gene expression changes by RNA-Seq technology, these authors showed that diet restriction is related with down-regulation of genes associated with digestion and vitellogenesis (Chen et al., 2017). Although our analysis of protein, lipid and carbohydrate content does not reveal the specific chemical component affected by the poor diet, it may reflect the metabolic plasticity of the flies; and fecundity results may be related to the diet effect on vitellogenesis pathways. Future studies with RNA-seq technology in *A. fraterculus* may reveal specific gene down-regulations in response to different diets with or without antibiotic. Further, some bacterial families detected in Tephritidae (Noman et al., 2020) such as Burkholderiaceae may establish metabolic cross-talks with vitellogenesis pathways of the host. This group of bacteria, particularly those from the Burkholderia genus, modulate host gene expression of hexamerin and vitellogenin in a hemipteran species, *Riptortus pedestris* (Lee et al., 2017) and has been detected in the gut of *A. fraterculus* sp.1 female adults fed HY diet (Salgueiro *et. al.*, 2020). Besides affecting Enterobacteria, antibiotic treatment may have affected *Burkholderia* genus hosted in *A. fraterculus*, giving a major effect on vitellogenesis. Future studies are needed to analyze potential cross-talks between gut bacteria and the host vitellogenesis.

Yeast derivative, but not antibiotics, affected post-oviposition carbohydrate content of females; being lower for females fed HY diet than those fed BY diet. Both yeast derivatives were offered in a mixture with sucrose at the same ratio (1:3). In previous studies (Oviedo et al., 2011), we showed that *A. fraterculus* females stop consuming a nutrient source when they reach a certain level of protein, referred as the intake target (Simpson and Raubenheimer, 1995; Chown and Nicolson, 2004), because high amounts of protein could be detrimental (Kaspi and Yuval, 2000; Prabhu et al., 2008; van Schoor et al., 2020). Here, females probably attained their protein intake target with lower amounts of the HY diet compared to the required for the BY diet, resulting in different consumption rates and therefore a low carbohydrate content for females fed the HY diet. Additionally, the lowest glycogen content recorded for females fed HY diet without antibiotics was accompanied by the highest fecundity. Due to their high egg production level, these females probably required more energy than the other females. Therefore, these females had low glycogen content as they consumed most of their ingested energy to produce eggs, having no capacity to build up energetic reserves.

In all, this study represents an advance in the knowledge of specific functions of the intestinal bacterial community of *A. fraterculus*. Our findings add to the evidence that tephritid gut microbiota contributes to the fitness of the host, particularly to a rather unexplored life history trait such as female fecundity. Not surprisingly, our results suggest that this contribution is affected by the quality of the diet female feeds on, which affects their potential fecundity. In particular, females fed marginal diets depend most on the contribution of bacteria to produce offspring than females fed complete diets. Our findings suggest that nutrient content of females is also mediated by intestinal bacteria (particularly Enterobacteria) which in turn explains, at least to some extent, the differences in female fecundity between antibiotic treated and non-treated females. In this respect, it should be noted that proteins, lipids and carbohydrates were determined in the complete insect which included fat body, ovaries (involved in female fecundity) as well as structural components and bioavailability of these biomolecules. Although our data may not support exclusively the bioavailability of the nutrients, our approaches allow us to go deeper into the physiology of *A. fraterculus* and lead to plan further hemolymph analysis for future studies. Yet, our experimental approach does not allow us to dismiss possible contributions of other symbionts (e.g., *Wolbachia*) (Conte et al., 2019) not measured in this study, which could have been indirectly affected by the addition of antibiotics. Further research is still needed to better understand the specific role of the different members of the microbiota on nutrients content, particularly proteins and lipids, and more specifically on fecundity. This will surely contribute not only to better understand the role of gut bacteria on Tephritidae physiology but also to improve mass-rearing protocols in support of SIT application (Cáceres et al., 2019) and other pest-specific control methods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2022.104396.

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