

## ENTOMOLOGY

# Brewery's waste streams as a valuable substrate for Black Soldier Fly *Hermetia illucens* (Diptera: Stratiomyidae)

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## Abstract

The growing population and, consequently, the demand for food, has led to an increase in the agro-food activities, causing irreversible exploitation of natural resources. The use of insects for the management and recycling of by-products of the agro-food industry and for the production of proteins is growing. The Diptera *Hermetia illucens* is of particular interest as larvae are able to grow rapidly on numerous organic matters and are rich in proteins and fats. The identification of a low-cost and low-impact substrate suitable for the mass rearing of *H. illucens* is conse-

quently of great interest. In this study, we evaluated the suitability of brewery wastes, spent grain and trub (used separately or mixed), as rearing substrate for the larvae. We evaluated the effect of these diets on preimaginal performance (survival, developmental time, and weight reached) and adult traits (sex-ratio, dimension, and female fertility). As a result, the different substrates significantly impacted different biological traits. Best results, both for larvae and adults, were reported when the brewery wastes were provided together, leading to similar results obtained in a control diet and comparable to other previous studies. We conclude that brewery by-products can be valuable substrate for larval growth and in this way can be efficiently recycled and valorised.

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Key words: brewers' spent grain, trub, biological traits, performances, protein production

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## Introduction

The increase in the world population and the progressive raising of living standards (United Nations, 2013), determine a continuous growing of agro and food industry activities, that induces in turn an excessive and irreversible exploitation of natural resources and at the same time of the areas dedicated to agricultural production and therefore of food goods (Gibbs *et al.*, 2010). Areas of research even distant by type, found strong points of contact in the solution of contingent problems. One of these is the identification of new and less environmental impacting strategies for food production and the recycling of food waste. In this context rises the worldwide interest in the rearing of insects on wastes and by-products of the agro and food industry, due to the ability of these animals to convert low-value organic substrates into macromolecules of high nutritional value, in particular proteins and fats (Cappellozza *et al.*, 2019; Fowles & Nansen, 2019; Varelas, 2019). *Hermetia illucens* L. (Diptera Stratiomyidae), also known as the black soldier fly, is considered among the insects of particular interest for its nutritional value and its ability to grow on organic substrates of different nature with an efficient reduction, up to 75%, of the waste masses (Newton *et al.* 2005; Diener *et al.* 2011; Barragan-Fonseca *et al.*, 2017; Jucker *et al.* 2017).

Assuming in the areas of high anthropic density many agro-food industries are present and huge quantities of food waste are produced, the use of *H. illucens* to manage and valorise these wastes through their conversion into food, feed or fuel represents a current challenge (Diener *et al.*, 2011; Bava *et al.*, 2019; Lalander *et al.*, 2019). The growing insect industrial production still relies on the use of artificial diets greatly impacting the final cost of the product and increasing the environmental impact (Ooninx & De Boer, 2012; Bava *et al.*, 2019). Thus, the identifi-

cation of low-cost and low-impact substrates suitable for the mass rearing of insects is of great interest and can reduce the future competition for grains.

Among numerous agro-food by-products, in this research we considered the waste from the beer production, one of the most consumed alcoholic drinks in the world whose consumption has increased over the past ten years (Filladeau *et al.*, 2006). Despite Italy is one of the countries with the lowest per capita consumption in Europe, beer consumption and production in the country are continuously increasing. In particular, small and micro-breweries increased by 345% in the last ten years (AssoBirra, 2018). As a result, Italy actually represents the ninth European producer by production volumes and ranks fifth for the number of breweries.

Beer production yields abundant amount of low-cost organic wastes available throughout the year, largely underutilized despite their potential nutritional value, and their disposal entails costs for the brewery (Tang *et al.*, 2009; Robertson *et al.*, 2010). The main waste products of beer brewing are brewers' spent grain, which represents 85% of the total dry waste and hot trub, characterized by high humidity levels, up to 90% (Rocha dos Santos *et al.*, 2014; De Marco *et al.*, 2016). The research of alternative utilisation of brewery by-products is still ongoing (Aliyu & Bala, 2011; Kerby & Vriesekoop, 2017).

To evaluate the possible use of brewery's wastes in the rearing system of *H. illucens*, in the present study we assessed the suitability of this waste for the development of the insect. In detail, different biological parameters were used as instrument to evaluate the feasibility of the rearing system on the brewers' spent grain and hot trub produced by a craft-brewery.

## Materials and Methods

Larvae of *Hermetia illucens* used in the experimental trials came from a laboratory stock culture at the University of Milan. Larvae were reared on hen feed mixed with water (500 g/800 mL water) until pupation. Pupae were then placed in containers with wood sawdust; adults emerged were transferred in a cage (100×80×60 cm) where they mated and laid eggs on corrugated cardboard, positioned on a plastic container (20×10×5 cm) and filled with hen diet mixed with water as attractant for females (Jucker *et al.*, 2017). Neonate larvae (four-day old), collected from the container, were used for the trials.

### Growing substrates and experimental trials

The substrates (brewer's spent grain and trub) were provided by a craft brewery (Birrificio Lambrate s.r.l, Milan, Italy). Substrates were directly collected from the production process in the brewery. Once collected, the two substrates were separately placed in buckets with lids, then placed in freezer bags (500 cc), and stored at -18°C. Before insect feeding, each substrate was placed at room temperature for 24h.

The following substrates were tested: 1) brewers' spent grain; 2) chopped brewers' spent grain; 3) trub; 4) brewers' spent grain added with trub; 5) standard diet. Brewers' spent grain was tested as it was or chopped with a mixer to test if homogenization was more appreciated by the larvae. Moreover, a mixture in equal parts (1:1) of the two brewer by-products was tested. A standard diet, composed by 50% wheat germ, 30% alfalfa, and 20% corn flour added with water was used as a control (Jucker *et al.*, accepted). Different diets were added with water, when necessary, to obtain a moisture of 50-60%.

Three replicates per substrate/control were prepared. Replicates consisted of 200 four-days-larvae, placed on the differ-

ent substrates in a 500 mL container with a perforated lid covered with a mesh netting and fed *ad libitum*. Containers were maintained in a climate chamber at T 25±0.5°C; RH 60±0.5%; light cycle 12:12 L:D.

### Biological traits

To evaluate the growth rate on the different diets, larval weight was measured every three days on a subsample of ten larvae and then returned to their respective container. Feed supply was stopped when 40% of the larvae reached the prepupal stage in each replicate. Prepupae were removed daily from the trial, counted and put in a separate container with wood shaving waiting for adult emergence. Twenty pupae per replicate were weighted. When adults emerged, they were counted and sexed based on genitalia dimorphism. A sample of 20 males and 20 females for each replicate (a total of 60 for diet) was measured with a caliper, dried at 105°C for 48 h, and weighed. All weights were conducted using an analytical balance (Sartorius CP64, accuracy 0.1 mg).

In order to acquire data on adult performance in terms of fertility, a separate set of 500 larvae was reared on the experimental growing substrates under the same climatic condition. Adults (50 males and 50 females) from the different diets were placed in separate boxes (one for each treatment, 60 x 40 x 40 cm) at room temperature (25±0.5°C and 30±0.5% RH) and egg-laying cardboards were provided in each box in presence of hen diet as female attractant. Cardboard strips (2 mm in width; three flute openings per cm) were checked every two days for egg masses (Booth and Sheppard, 1984). Ten egg clutches for each treatment were collected. After weighted, the clutches with the analytical balance, eggs were counted with the help of a counter under the stereomicroscope (Leica MZ 12, Germany).

### Statistical analysis

Experimental data on larval development, final larval and pupal weight, adult weight and length, survival of different stages, weight of egg clutches and number of eggs were compared by one-way analysis of variance (ANOVA). Prior to analyses, all data were examined with Levene's test for homogeneity of variance and with the Shapiro-Wilk test for normal distribution. Where significant differences occurred, the Tukey-Kramer honestly significant difference (HSD) multiple comparisons test was performed for mean separation (P<0.05). Moreover, the two-tailed Student's t-test was used to assess significant differences between female and male body weight and length (P<0.05). The SPSS for Windows statistical package, Version 25.0 (SPSS Inc., Chicago) was used for the statistical analysis.

## Results and Discussion

### Preimaginal stages

*H. illucens* was able to feed, survive and complete its life cycle on all the tested diets. Nevertheless, growing substrates significantly impacted its biological traits and the success of the development.

Larval developmental time varied considerably in relation to the diet provided ( $F_{4,10}=27.96$ ;  $P<0.05$ ) (Table 1; Figure 1): only the brewers' spent grain mixed with trub was statistically similar to the standard diet, with a mean of 18.7±0.3 days to reach the prepupal stage; larvae reared on all the other experimental substrates took longer. Furthermore, when brewers' spent grain was supplied finely chopped larval development period was even greater (mean of 33.0±2.3 days). These results are not comparable with the shorter development time reported in other researches where waste streams

from brewing industry were tested (Chia *et al.*, 2018; Liu *et al.*, 2018; Meneguz *et al.*, 2018). On the contrary, the results are in line with the ones observed when *H. illucens* was reared on other vegetables substrates, such as sorghum, cowpea, a mix of vegetables or fruits (Nguyen *et al.*, 2013; Jucker *et al.*, 2017; Tinder *et al.*, 2017; Barragan-Fonseca *et al.*, 2018). It is well known that the quality of the feeding substrates strongly influences the preimaginal and adult traits and performance in different insect species (Gobbi *et al.*, 2013; Cammack & Tomberlin, 2017; Favaro *et al.*, 2017; Tinder *et al.*, 2017; Jucker *et al.*, accepted). In general, slower larval development is observed on low-protein and low-energy diets (e.g. fruits and vegetables), while balanced diets allow an optimal growth. In addition, other authors (Nguyen *et al.*, 2013; Jucker *et al.*, accepted), observed that a high-fat-diet could be detrimental for *H. illucens* larval growth. The longer larval development time observed in brewers' spent grain could be attributed to the high content of cellulose, lignocellulose and lignine (Aliyu and Bala, 2011; Mathias *et al.*, 2015; Liu *et al.*, 2018) which affects the time required to complete the larval cycle, the survival rate and the final larval

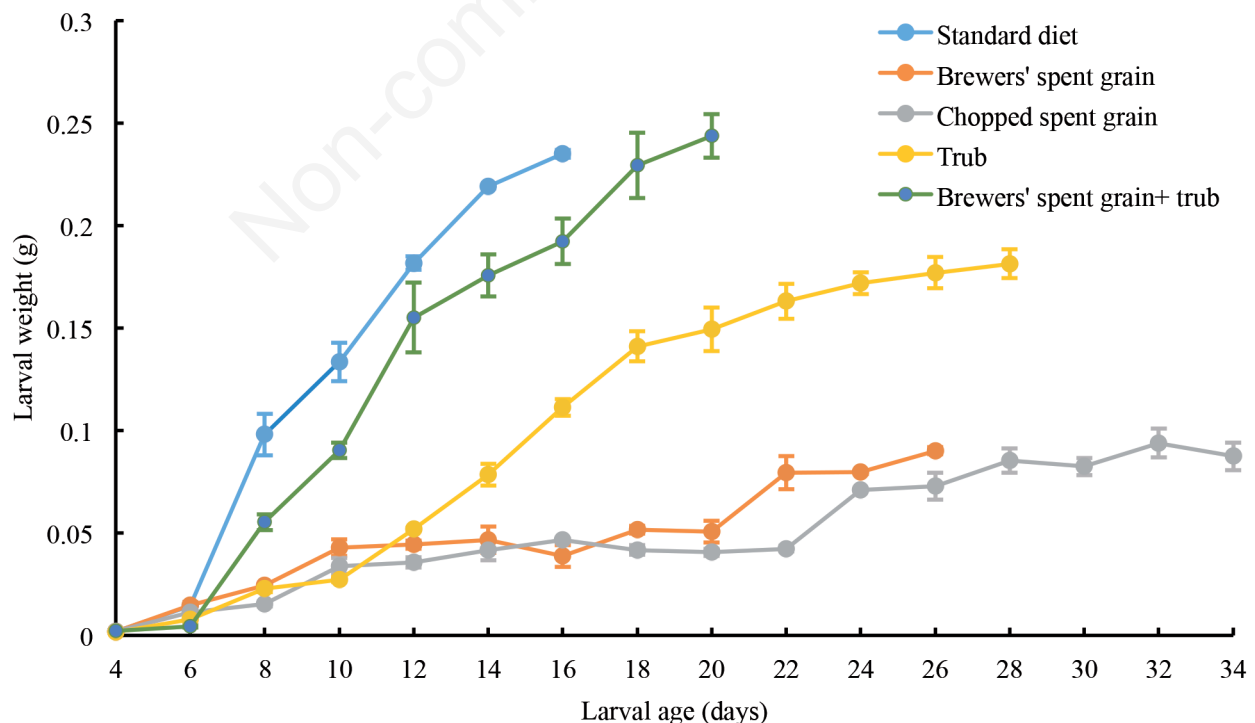
weight (ur Rehman *et al.*, 2017). Instead, the extreme moisture of the trub (nearly 90%, Mathias *et al.*, 2015) could have negatively influenced the duration of the larval development. Holmes *et al.* (2012) reported that an increase in humidity in the growing substrates up to 70% promotes larval growth, while the presence of an excessive liquid fraction, as observed in trub, negatively affects larval development (Kalová & Borkovcová, 2013). On the contrary, mixing the two by-products has led to an optimal larval growth, due to the reduction of the liquid fraction thanks to its absorption by brewers' spent grain, and also to a more balanced diet, as the trub protein content is reported in Mathias *et al.* (2015) as 49%.

Larval mortality ( $F_{4,10}=24.66$ ;  $P<0.05$ ) and pupal mortality ( $F_{4,10}=20.64$ ;  $P<0.05$ ) were also significantly affected by the growing substrates (Figure 2). In detail, the feeding on trub alone resulted in the highest larval mortality, while feeding on all other diets produced a high survival comparable with larvae grown on the standard diet. Pupal mortality on the contrary was higher than larval mortality in all the tested diets. Pupae from brewers' spent grain (as it or finely chopped) reached a mortality higher than 46%, leading

**Table 1. Duration of larval period (days), final larval weight and pupal weight (g), adult sex ratio on the different growing substrates (mean±ES).**

	Larval development (days)	Final larval weight (g)	Pupal weight (g)	Adult sex ratio (M:F)
Brewers' spent grain	24.7±0.3 <sup>bc</sup>	0.860±0.003 <sup>a</sup>	0.054±0.002 <sup>a</sup>	0.64±0.05 <sup>a</sup>
Chopped spent grain	33.0±2.3 <sup>d</sup>	0.873±0.007 <sup>a</sup>	0.064±0.002 <sup>a</sup>	0.66±0.02 <sup>ab</sup>
Trub	28.3±1.9 <sup>cd</sup>	0.180±0.008 <sup>b</sup>	0.108±0.003 <sup>b</sup>	0.96±0.09 <sup>bc</sup>
Brewers' spent grain+ trub	18.7±0.3 <sup>ab</sup>	0.244±0.011 <sup>c</sup>	0.160±0.014 <sup>c</sup>	0.95±0.09 <sup>ab</sup>
Standard diet	15.3±0.3 <sup>a</sup>	0.235±0.002 <sup>c</sup>	0.142±0.002 <sup>c</sup>	1.02±0.06 <sup>c</sup>

Different letters indicate significant differences across diets ( $P<0.05$ ).



**Figure 1. Larval growth on the different experimental substrates. Larvae were weighted until 40% reached the prepupal stage.**

to a total mortality (from larvae to adult emergence) around 50%. Pupal mortality was lower when trub was added to the diet.

Data on larval survival are in agreement with what reported by Chia *et al.* (2018) on brewery wastes, and with other authors testing different vegetable or animal wastes (Jucker *et al.*, 2017; Barragan-Fonseca, 2018; Miranda *et al.*, 2019; Jucker *et al.*, accepted), and even higher than what reported by Nguyen *et al.* (2013) on a wide type of organic resources. Low larval mortality indicates that our tested substrates can efficiently sustain *H. illucens* population development. The high mortality of pupae (in this study reported only on the brewers' spent grain), is not in contrast with this affirmation as similar levels of mortality in pupae were already observed by different authors on *H. illucens* larvae reared on vegetables, fruits but also on kitchen waste, liver or fish waste (Nguyen *et al.*, 2013; Cammack & Tomberlin, 2017; Jucker *et al.*, 2017). Chia *et al.* (2018) registered a pupal mortality up to 35% on different brewery waste streams.

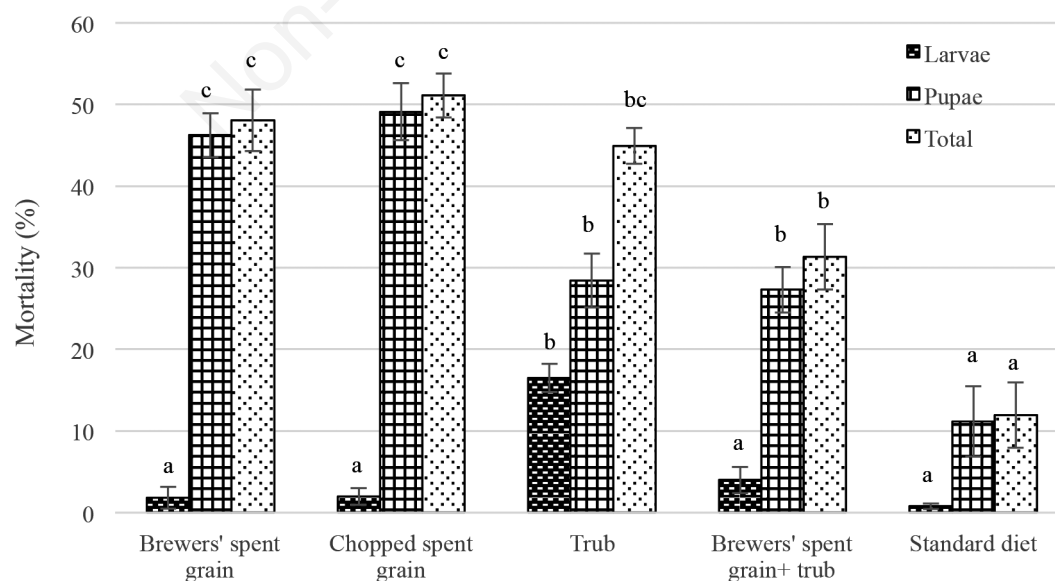
Final larval weight was significantly different ( $F_{4,10}=126.02$ ;  $P<0.05$ ) among larval diets (Table 1). Individuals fed only with brewer's spent grain were the lightest, with a weight 65% lower than those on the mixture of the two waste streams. The addition of the trub to the brewers' spent grain greatly increased the final larval weight and no statistical differences were noticed with the standard diet. Trub alone gave intermediate results. Statistical differences were detected also on pupal weight, following the larval trend ( $F_{4,295}=47.45$ ;  $P<0.05$ ) (Table 1). Pupae coming from the mixture were similar to the standard diet, while when the by-products were provided alone, pupae were lighter, in particular on the brewer's spent grain (as it or chopped). Weight obtained with prepupae reared on trub or on the mixture of trub and brewery are comparable or higher with previous investigations on a similar substrate, which report a prepupal weight of 0.08-0.17 g (Chia *et al.*, 2018; Nyakeri *et al.*, 2017; Meneguz *et al.*, 2018; Bava *et al.*, 2019) and a pupal weight of 0.11-0.15 g (Chia *et al.*, 2018). Also other vegetable wastes resulted in similar larval weight (Barragan-Fonseca *et al.*, 2018; Jucker *et al.*, 2017; Lalander *et al.*, 2019), while substrates nutritionally richer allow best performance resulting also in heavier larvae (Nguyen *et al.*, 2013; Lalander *et al.*,

2019; Jucker *et al.*, accepted). In this study, the mixture of the two brewery by-products produced results similar to the standard diet, thanks to its chemical composition, as already stated. The prepupal weight is an important parameter as represents the insect stage usually collected in *H. illucens* mass rearing for protein production and it shows an optimal nutritional profile (Sheppard *et al.*, 1994; Spranghers *et al.*, 2017; Tomberlin & Cammack, 2017). Moreover, as prepupae stop feeding, their weight (and that of pupae) is important as it affects, with the nutrients stored by the larval stage, adult weight and performances.

## Adults

Sex ratio (males: females) of the emerged adults was assessed for all experimental treatments and was influenced by the growing diets ( $F_{4,10}=7.26$ ;  $P<0.05$ ) (Table 1). Sex-ratio was female-biased on brewer's spent grain, provided both as it or chopped. The ratio between males and female was more balanced in all other tested substrates. Barragan-Fonseca *et al.* (2018) found a sex ratio of 0.93 and 1.22 in trials using different vegetables ingredients for larval growth; Jucker *et al.* (2017) determined a sex ratio of 0.99 when larvae were fed only on fruits diet, while a male-biased sex-ratio was found on vegetables diet. The proportion between males and females has to be taken in consideration in artificial mass rearing as it has been reported that fertility is influenced by sex ratio. In male-based population, each female lays more eggs than in female-dominant populations due to the lower density of males that delays mating, reducing the size of female oviposition (Hoc *et al.*, 2019). Nevertheless, the total egg production is higher for female-dominant population due to the highest number of females. Thus, considering a *H. illucens* mass production, a slightly female-biased population is strictly recommended (Hoc *et al.*, 2019).

Adult weight (Females:  $F_{4,295}=4033.18$ ;  $P<0.05$ ; Males  $F_{4,295}=2344.69$ ;  $P<0.05$ ) and length (Females:  $F_{4,295}=148.48$ ;  $P<0.05$ ; Males  $F_{4,295}=125.54$ ;  $P<0.05$ ) were impacted by larval feeding (Figures 3 and 4). Individuals emerged from the brewers' spent grain were lighter and smaller, both for females and males. The mixture of brewers' spent grain and trub provided the highest weight and length for females, even more than the standard diet.



**Figure 2.** Mortality of *H. illucens* larvae, pupae and total mortality (mean $\pm$ SE). Significant differences among means of the different growing substrates are indicated by different lower case letters (Tukey's test,  $P<0.05$ ).

Males emerged from the mixture significantly differed from other tested wastes with higher parameters. Trub alone supplied intermediate values. The adult weights strongly reflected the trend already observed in preimaginal stages. As known, larvae of *H. illucens* are very voracious and accumulate fat body only during the larval stage, while imaginal feeding is less fundamental and mostly influences adult longevity (Nakamura *et al.*, 2016; Lupi *et al.*, 2019).

Comparing the weight of the two sexes, females were heavier than males in all experimental substrates (Figures 3 and 4) ( $P < 0.05$ ). Females were longer than males except for those emerged from trub and standard diet. This difference in the weight between females and males was already observed by previous authors (Tomberlin *et al.*, 2009; Jucker *et al.*, 2017; Chia *et al.*, 2019; Jones and Tomberlin, 2019; Miranda *et al.*, 2019;). In particular, Tomberlin *et*

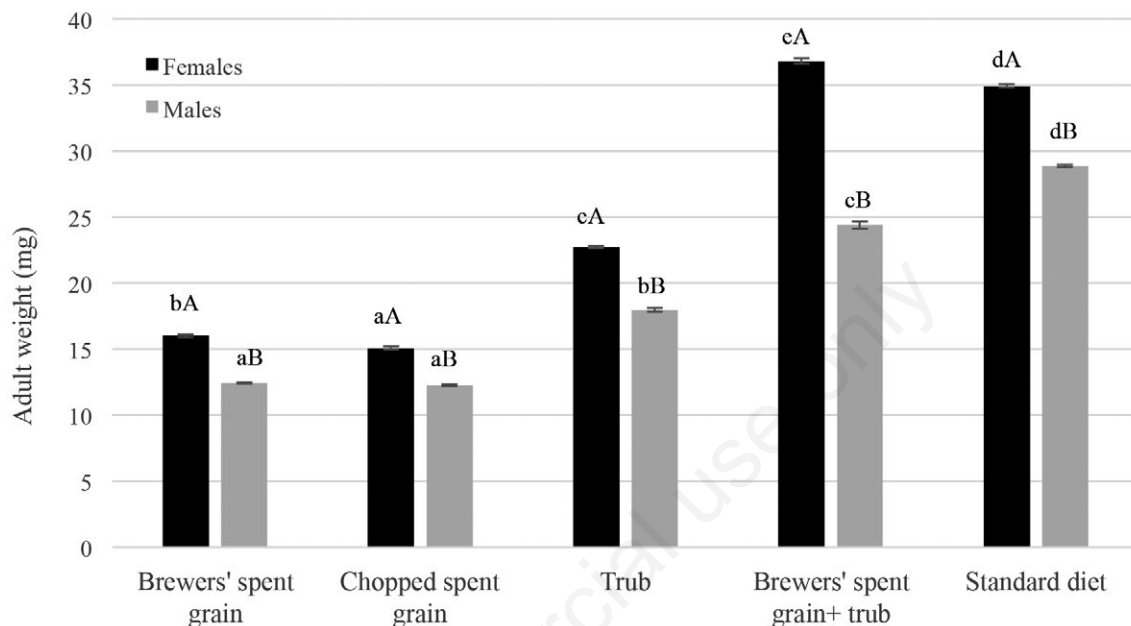


Figure 3. Mean ( $\pm$ SE) of female and male weight. Significant differences among means of the different growing substrates are indicated by different lower-case letters (Tukey's test,  $P < 0.05$ ), while capital letters indicate differences among sexes (t-test,  $P < 0.05$ ).

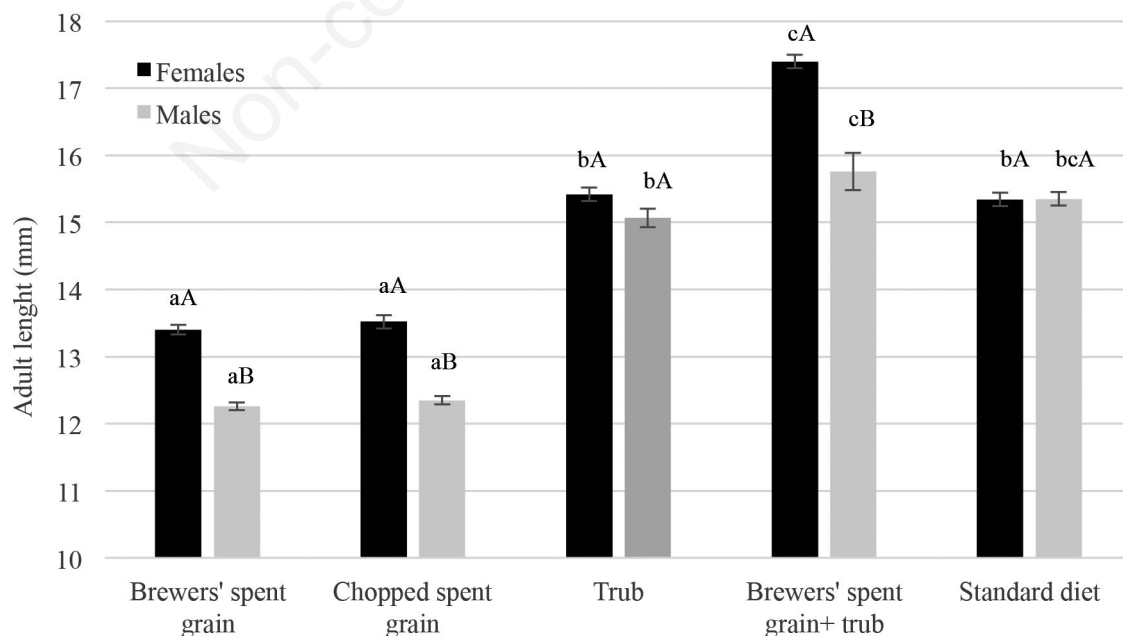


Figure 4. Mean ( $\pm$ SE) of female and male length. Significant differences among means of the different growing substrates are indicated by different lower-case letters (Tukey's test,  $P < 0.05$ ), while capital letters indicate differences among sexes (t-test,  $P < 0.05$ ).

**Table 2. Mean ( $\pm$ ES) weight of egg clutches and mean number of eggs per clutch deriving from females emerged from the different growing substrates.**

	Egg clutch weight (mg)		Egg number/clutch	
	Mean $\pm$ ES	Mean $\pm$ ES	Min	Max
Brewers' spent grain	8.19 $\pm$ 0.83 <sup>a</sup>	395 $\pm$ 47	209	721
Chopped spent grain	11.18 $\pm$ 1.32 <sup>ab</sup>	492 $\pm$ 49	207	686
Trub	13.17 $\pm$ 1.49 <sup>ab</sup>	581 $\pm$ 44	341	791
Brewers' spent grain+ trub	10.05 $\pm$ 1.30 <sup>ab</sup>	423 $\pm$ 53	206	685
Standard diet	15.29 $\pm$ 1.72 <sup>b</sup>	562 $\pm$ 47	383	868

Different letters indicate significant differences across diets ( $P < 0.05$ ).

al. (2009) underlined that females take more time to develop as they need to accumulate more energy reserves essential for their egg production. Also other insect species show differences in the body dimension between sexes (Lewandowski *et al.*, 2004).

Egg clutches deposit by females emerged from the different diets showed significant differences only in their weight ( $F_{4,45}=4.073$ ;  $p < 0.05$ ), but not on the egg number ( $F_{4,45}=2.945$ ;  $P > 0.05$ ) (Table 2). Statistical differences were recorded between the weight of egg clutches of standard diet and brewer's spent grain. Mean number of eggs per clutch varied between 395 (brewers' spent grain) and 581 (trub), with a minimum value of 206 in the mixture (brewer's spent and trub) and a maximum of 868 in the standard diet (Table 2). Data are in accordance with available literature reporting a mean number of eggs per mass between 120 and 1000 (Booth & Sheppard, 1984; Tomberlin *et al.*, 2002; Jucker *et al.*, 2017; Barragan-Fonseca *et al.*, 2019; Bertinetti *et al.*, 2019). Adult nutrition influences the longevity, but does not have a direct effect on fertility and egg clutches size and weight. Thus, the difference observed in the egg number could be related to larval dietary content (Barragan-Fonseca *et al.*, 2019; Bertinetti *et al.*, 2019; Lupi *et al.*, 2019).

## Conclusions

This study contributes to investigate the potential applications of the brewery's wastes and adds additional information on the recent interest for brewery by-products as growing substrate for the larvae of *H. illucens* (Chia *et al.*, 2018; Liu *et al.*, 2018; Bava *et al.*, 2019). The tested substrates sustained the larval development and adult reproduction, showing similar or better performance than other vegetable substrates. In particular, the combined use of the two wastes (brewer's spent grain and trub) provided the best results for all the considered biological traits of *H. illucens*, proving to be a promising rearing substrate for the insect mass production.

In this study we did not consider the nutritional values of the larvae, that is known to be influenced by the diet composition (Barragan-Fonseca *et al.*, 2017), but previous studies on similar substrates referred a protein content of about 50-54%, in line or even higher than the protein content of larvae grown on other vegetable matters (Liu *et al.*, 2018; Bava *et al.*, 2019). Other aspects to be deepened in view of a circular economy application are the waste reduction and the bioconversion efficiency of the brewery's waste, by *H. illucens* larvae, already partially studied by Bava *et al.* (2019). Overall, the use of the studied side-streams as larval feeding contributes to create a low-cost growing media and allows to recapture the nutrients that can be recycle in the market as insect proteins for animal feed.

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