Antibacterial activity of fat extracts from black soldier fly (*Hermetia illucens*) fat larvae against antibiotic-resistant *Campylobacter* strains

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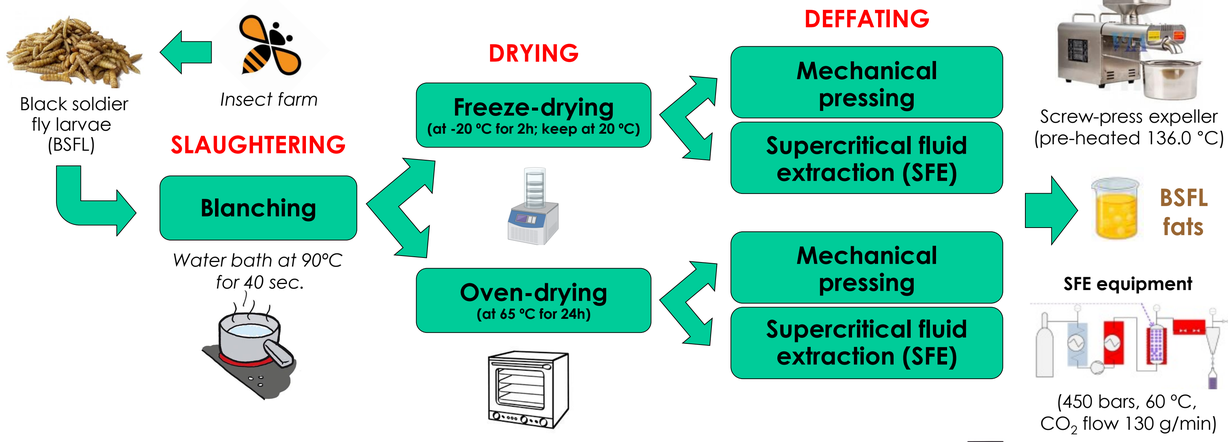
INTRODUCTION

Campylobacter is considered the main food-borne pathogen causing gastrointestinal illnesses worldwide, with chicken being the main source of infection in humans. Strategies for controlling *Campylobacter* in the food chain are gaining attention due to rising antibiotic resistance (1). The use of natural antibacterial compounds can help minimize *Campylobacter* contamination. Insects ingredients are emerging as a source of antibacterial compounds which offers an innovative and sustainable approach to enhancing food safety and addressing the growing concern of antibiotic resistance (2). Black soldier fly larvae (BSFL) (*Hermetia illucens*) have a great potential as an alternative protein source for food and feed, and from the antibacterial point of view of BSFL fat have recently described a relevant antibacterial activity (3). The objective of the present work was to explore the antibacterial activity of fat extracts obtained from *H. illucens* by different process against antibiotic-resistant *C. jejuni* and *C. coli* strains.

MATERIALS & METHODS

▪ Production of black soldier larvae (BSFL) fats



▪ Characterization of free fatty acids (FFAs) of BSFL fats

GC-MS-FID

Analysis of FFAs was performed by GC-MS-FID (3):

- Derivatization by N,O-Bis(trimethylsilyl)trifluoroacetamide.
- Analytical separation: column HP-5MS (30m x 0.25mm x 0.25µm); carrier: helium at 2 mL/min.
- Parameters: splitless mode; injector at 260 °C, MS ion source at 230 °C, interface at 280 °C.
- Analysis: started at 50 °C for 3 min, increasing at 15 °C/min up to 310 °C, and held for 25 min.
- Mass spectra: electronic impact at 70 eV; scan rate 1.6 scans/s at a mass range of 30-700 amu.
- Commercial standards of FFAs (lauric, myristic, palmitic, oleic, and linoleic acids).

▪ Bacterial strains, growth media and culture conditions

- Six *Campylobacter* spp. strains isolated from different points of the chicken food chain were used:

Species	Strain references
C. jejuni (3)	CJ1, CJ2, CJ3
C. coli (3)	CC1, CC2, CC3

Campylobacter

VAIN workstation

- Selective growth media: Brucella Broth (BB) and Mueller-Hinton agar supplemented with 5% sheep blood (MHB)
- Growth conditions: strains reactivation into MHB and incubation for 48 h at 40ºC, in microaerophilic atmosphere in VAIN workstation (85% N_2, 10% CO_2, 5% O_2)

▪ Determination of antibiotic susceptibility of *Campylobacter* strains

- Antibiotic susceptibility was performed by the *E-test* (4).
- Six antibacterial agents representing five different antibiotic families were used:
  - Erythromycin (ERY)
  - Tetracycline (TET)
  - Gentamicin (GEN)
  - Amoxicillin (AMX)
  - Amoxicillin-clavulanic acid (AMX-CLA)
  - Ciprofloxacin (CIP)

▪ Antibacterial activity

- Procedure:
  - BSFL fats were solubilized in 10% (v/v) Tween-80
  - 1 mL of BFSL fats (14% final concentration) disolved in BB
  - 4 mL BB (or 5 mL BB for control growth)
  - 100 µL bacterial inoculum (~1x10^8 CFU/mL)
  - Incubation for 24 h at 40ºC, 150 rpm, in microaerophilic atmosphere (VAIN)
  - Serial dilutions of mixtures were plated onto fresh MHB agar
  - Incubation microaerobically for 72 h at 40ºC in microaerophilic atmosphere (VAIN)
  - Antibacterial activity determination by CFU counting

Bacterial culture

RESULTS

➢ Identification of free fatty acids (FFAs) of BSFL fats

Table 1. Free fatty acids composition of BSFL fats obtained by different processing methods. Results are expressed as g FFAs/100 g of total lipids (average ± SD).

	Freeze-drying		Oven-drying	
FFAs	**Pressing**	**SFE**	**Pressing**	**SFE**
Lauric acid (C12:0)	2.19 ± 1.95 ^{ab}	5.12 ± 2.17 ^{b}	0.04 ± 0.00 ^{a}	0.09 ± 0.03 ^{a}
Myristic acid (C14:0)	0.39 ± 0.32 ^{ab}	1.08 ± 0.39 ^{b}	0.01 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}
Palmitic acid (C16:0)	0.70 ± 0.49 ^{a}	2.45 ± 0.38 ^{b}	0.12 ± 0.01 ^{a}	0.26 ± 0.04 ^{a}
Oleic acid (C18:1)	2.40 ± 1.09 ^{ab}	4.16 ± 0.09 ^{b}	0.22 ± 0.02 ^{a}	0.41 ± 0.08 ^{a}
Linoleic acid (C18:2)	1.96 ± 0.85 ^{a}	3.23 ± 0.11 ^{b}	0.10 ± 0.01 ^{a}	0.21 ± 0.04 ^{a}
∑ FFAs	7.64 ± 4.70 ^{ab}	16.04 ± 2.92 ^{b}	0.49 ± 0.05 ^{a}	1.00 ± 0.21 ^{a}

^{a,b} Different letters between the treatments mean significant differences in the content of each FFAs.
SFE: supercritical fluid extraction

BSFL fats

GC-MS-FID

➢ Antibiotic susceptibility of *Campylobacter* strains

Table 2. Antibiotic susceptibility profile and minimal inhibitory concentration (MIC) values for the *Campylobacter* spp. strains.

Specie	Strain	CIP	TET	ERY	GEN	AMC	AMX	Strain resistance rate
C. jejuni	CJ1	R (>32)	S (0.032)	S (0.25)	S (0.5)	S (0.19)	S (3)	1/6
	CJ2	R (>32)	R (32)	S (0.5)	S (0.25)	S (0.5)	R (16)	3/6
	CJ3	R (>32)	R (32)	S (0.5)	S (0.38)	S (0.5)	R (32)	3/6
C. coli	CC1	R (>32)	S (0.032)	S (0.125)	S (0.75)	S (2)	S (8)	1/6
	CC2	R (>32)	R (>256)	S (1)	S (0.5)	S (1)	S (6)	2/6
	CC3	R (>32)	R (>256)	S (2)	S (0.5)	S (0.75)	S (8)	2/6
Antibiotic resistance rate		6/6	4/6	0/6	0/6	0/6	2/6	

S: susceptible; R: resistant. MIC values are given between brackets.
CIP: ciprofloxacin; TET: tetracycline; ERY: erythromycin; GEN: gentamycin; AMC: amoxicillin-clavulanic acid; AMX: amoxicillin.
The breakpoints were defined following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (v11.0) and French Society of Microbiology.

➢ Antibacterial activity of BSFL fats against *Campylobacter* strains

Table 3. Antibacterial activity of BSFL fats (14% final concentration) against antibiotic-resistant *Campylobacter* strains. Results are expressed as log CFU/mL ± SD (n=3).

Campylobacter strains	Control growth	Freeze-drying		Oven-drying	
		Pressing	SFE	Pressing	SFE
CJ1	9.47 ± 0.17^{d}	8.34 ± 0.23^{c} (1.1)	4.65 ± 0.12^{a} (4.8)	8.43 ± 0.10^{c} (1.0)	7.75 ± 0.09^{b} (1.7)
CJ2	8.89 ± 0.04^{c}	6.28 ± 0.05^{b} (2.6)	4.82 ± 0.08^{a} (4.1)	9.10 ± 0.04^{d} (0.0)	8.55 ± 0.09^{c} (0.3)
CJ3	8.95 ± 0.09^{c}	6.58 ± 0.03^{b} (2.4)	3.37 ± 0.05^{a} (5.6)	9.10 ± 0.09^{c} (0.0)	8.77 ± 0.09^{c} (0.2)
CC1	7.53 ± 0.12^{c}	6.94 ± 0.06^{b} (0.6)	< 1.48^{a} (>6.0)	8.53 ± 0.16^{c} (0.0)	8.12 ± 0.03^{c} (0.0)
CC2	9.77 ± 0.01^{e}	7.04 ± 0.08^{b} (2.7)	< 1.48^{a} (>8.3)	9.24 ± 0.04^{d} (0.5)	8.25 ± 0.07^{c} (1.5)
CC3	9.58 ± 0.13^{e}	4.98 ± 0.05^{b} (4.6)	< 1.48^{a} (>8.1)	9.08 ± 0.11^{d} (0.5)	7.26 ± 0.04^{c} (2.3)

Bactericidal effect is expressed as "< 1.48". CFU detection limit was 1.48 log CFU/mL (30 CFU per plate).
$^{a-e}$ Log CFU/mL values in the same row marked with different letters indicate significant differences according to ANOVA post hoc Tukey test ($p \leq 0.05$). Values shown in parentheses indicate the log reduction CFU/mL compared to the control growth.
SFE: supercritical fluid extraction.

CONCLUSIONS

- ❑ BSFL fats with varying FFAs contents were obtained depending on the larvae processing conditions. Considering both drying and the defatting methods, the freeze-dried fats followed by SFE nearly doubled the total FFAs content compared to the freeze-dried fats subjected to mechanical pressing (close to 8%), while the FFAs content in the oven-dried fats was negligible (≤ 1%). Lauric acid was the predominant FFA, accounting for nearly 30% of the total, followed by oleic and linoleic acids, with lower concentrations of palmitic and myristic acids.
- ❑ All *Campylobacter* strains were resistant to at least one of the six commonly antibiotics used for campilobacteriosis treatment. In addition two of the *C. jejuni* strains (CJ2 and CJ3) were classified as multidrug resistant (MDR) due to their lack of response against drugs belonging to three different antibiotic families. All strains exhibited resistance to ciprofloxacin, however no erythromycin-, gentamicin-, and amoxicillin/clavulanic-resistant strains were detected, confirming as ones of the most effective antibiotics against *Campylobacter*.
- ❑ The antibacterial effect was directly correlated with the FFAs content in the BSFL fats. Accordingly, the antibacterial activity of the oven-dried fats was lower than that of the freeze-dried fats, which is consistent with the lower concentration of FFAs in the oven-dried fats. Fats obtained by freeze-drying and defatting by SFE were the most effective as antibacterial against *Campylobacter* spp., significantly reducing bacterial growth ($p \leq 0.05$) by 4.1 to 5.6 log CFU/mL in *C. jejuni* and being bactericidal (<1.48 log CFU/mL) for all *C. coli* strains.
- ❑ The results obtained in this study constitute a potential promise for the utilization of BSFL fat in poultry feed for the control of *Campylobacter* spp.

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