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Assessing the impact of insect protein sources on intestinal health and disease: insights from human *ex vivo* and rat *in vivo* models

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The exploration of edible insects, specifically *Alphitobius diaperinus* and *Tenebrio molitor*, as sustainable sources of protein for human consumption is an emerging field. However, research into their effects on intestinal health, especially in relation to inflammation and permeability, remains limited. Using *ex vivo* and *in vivo* models of intestinal health and disease, in this study we assess the impact of the above insects on intestinal function by focusing on inflammation, barrier dysfunction and morphological changes. Initially, human intestinal explants were exposed to *in vitro*-digested extracts of these insects, almond and beef. Immune secretome analysis showed that the inflammatory response to insect-treated samples was comparatively lower than it was for samples exposed to almond and beef. Animal studies using yellow mealworm (*Tenebrio molitor*) and buffalo (*Alphitobius diaperinus*) flours were then used to evaluate their safety in healthy rats and LPS-induced intestinal dysfunction rats. Chronic administration of these insect-derived flours showed no adverse effects on behavior, metabolism, intestinal morphology or immune response (such as inflammation or allergy markers) in healthy Wistar rats. Notably, in rats subjected to proinflammatory LPS-induced intestinal dysfunction, *T. molitor* consumption did not exacerbate symptoms, nor did it increase allergic responses. These findings validate the safety of these edible insects under healthy conditions, demonstrate their innocuity in a model of intestinal dysfunction, and underscore their promise as sustainable and nutritionally valuable dietary protein sources.

Introduction

There is an urgent need nowadays to feed an exponentially growing human population. As a result, the demand for protein is expected to increase by 70% in the next 30 years.[1] Current food research therefore focuses on incorporating foods derived from novel and sustainable protein sources such as insects.[2,3] Although insects have long been a common component of the human diet, in Western countries entomophagy is still in its early stages.[4] However, several reasons support incorporating insects into our diets as a valuable source of protein.

Firstly, insects contain a large amount of protein, accounting for over 50 percent of their crude weight. This abundant protein has high biological quality thanks to the presence of all essential amino acids.[5–7] Insects also contain large amounts of unsaturated fatty acids, minerals, and vitamins.[8] In addition, insects have been identified as a source of bioactive compounds with anti-hypertensive, antidiabetic, antioxidant, and anti-inflammatory activity.[9]

Also in support of insect consumption is the fact that insect production is more environmentally sustainable than the production of conventional livestock.[8] Indeed, insect-based protein is emerging as a cost-effective alternative to meat protein since its production employs minimal resources, requires less water and less land, and is responsible for a much lower carbon footprint than the production of meat protein.[4,10] Moreover, since insects can feed on bio-waste (such as mushroom waste and fruit peels), they can mitigate the environmental impact by turning waste into compost.[11]

For all these reasons, in the not-too-distant future insect protein will probably become a component of the total protein consumed by humans.[5] To enhance social acceptance, insect protein can also be processed into food ingredients such as flour, which can then be incorporated into food products.[12]

The yellow mealworm *Tenebrio molitor*, and the lesser mealworm *Alphitobius diaperinus* (Coleoptera: family Tenebrionidae),

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are both larvae of the beetles that grow in flour and grain cereals. These edible larvae possess significant nutritional value as they encompass a full spectrum of amino acids that are rich in essential fatty acids and vitamins as well as a higher mineral content (including calcium, copper, magnesium, iron and zinc) than conventional meats and eggs.[10,13–15] *Tenebrio molitor* commonly breeds in Europe and, together with *Alphitobius diaperinus*, is the main species used for farm-animal feed and considered for human consumption.[10]

Recent reports from the European Food Safety Authority (EFSA) positively evaluated both these mealworms as novel foods suitable for human consumption.[16,17] The safety profiles of *T. molitor* and *A. diaperinus* larvae as novel foods are based on nutritional studies that assessed the microbiological risk of zoonosis, heavy metal contamination and allergy. However, the EFSA recommends that further research should be conducted in this area.[14,17]

Recent studies have suggested beneficial effects of edible insects in terms of inflammation and intestinal health. For instance, a study with *Tenebrio molitor* larvae powder described its ability to attenuate pathologic changes in colon tissue and down-regulate the expression of inflammatory cytokine genes in mice (DSS)-induced colitis.[18] Moreover, an *in silico* analysis indicated that *Tenebrio molitor* could be a source of peptides with anti-inflammatory activity.[19] Further, *in vitro*-digested proteins from three different insects have been proposed to exhibit antioxidant and anti-inflammatory effects.[20] Additionally, in a human intervention study, cricket consumption was found to improve gut health and reduce systemic TNF-α levels;[21] while other studies also indicated improvements in microbiota and metabolic benefits in animals after the consumption of mealworms.[22,23] Collectively, this evidence suggests potential health benefits associated with insect consumption, particularly in the context of intestinal tract and inflammatory response.[24]

However, a current concern about insect consumption is the potential allergic effects on sensitized individuals or those allergic to other inhalant or food allergen sources such as house dust mites or crustaceans.[24,25] The yellow mealworm and lesser mealworm belong to the Insecta class, one of the four subphyla of Arthropoda. In relation to arthropods, several allergens have been reported, including tropomyosin,[26] arginine kinase,[27] chitinases[28] and glutathione *S*-transferase,[29] that affect humans through cross-reactivity. However, studies in this context suggest that the allergenicity of edible insects is species-specific and that thermal processing may partially reduce cross-allergenicity.[30] In the context of insect mealworm protein, individuals allergic to shrimps exhibited cross-reactivity due to high protein homology across different arthropod subphyla,[31] while one case report that documented food-induced anaphylaxis to *T. molitor* in a patient allergic to dust mites attributed the allergic response to α-amylase, tubulin, and larval cuticle proteins.[32]

The origins of allergic diseases have traditionally been explained by immunoglobulin E-mediated immune responses to account for asthma, atopic dermatitis, atopic rhinitis, and food allergy. Research insights into disease origins support a broader array of factors that predispose, initiate, or exacerbate altered immunity in allergic diseases, such as (1) inherent epithelial barrier dysfunction; (2) loss of immune tolerance; (3) disturbances in the gut; and (4) organ-specific microbiomes, diet, and age.[33] Here, we focus on the gut barrier function as a key factor in preventing or facilitating food allergy. In fact, Galli *et al.* previously reported that epithelial barrier deficiencies often lead to a state of constant inflammation that makes tissue repair difficult. Deficient barrier integrity facilitates allergen entry and lowers the threshold for sensitization to innocuous substances because of the inflammatory environment, and therefore likely precipitates allergic sensitization at distal organs.[34]

Although the consumption of *Alphitobius diaperinus* and *Tenebrio molitor* has raised concerns about their potential to induce food allergic reactions in sensitized or allergic individuals, research into the specific health impacts of these insects, including both their detrimental and beneficial effects, is still limited and further studies are needed to understand their role in inflammation and intestinal dysfunction.

Thus, this paper analyses how insect flours interacted with the gastrointestinal tract in the human intestine *ex vivo* and how this interaction might influence the response of the whole organism in rats, focussing on the inmunologic response. From a more holistic view of allergy development, we evaluated how the effects of low-dose insect supplementation depend on disease status by comparing the effects on a healthy animal *vs.* a model of LPS-induced inflammation and intestinal barrier dysfunction.

Experimental methods

Ex vivo study with human colonic explants exposed to *in vitro* digested insect, beef and almond

Buffalo (*A. diaperinus*) powder and insect protein concentrate powder (IPC) from *A. diaperinus* (Protifarm, Belgium), a lean portion of beef (*Bos primogenitors*) from the Central Market in Tarragona, Spain, and almond (*Prunus dulcis*) flour from Borges Agricultural & Industrial Nuts (BAIN) were used for human colon treatments. Samples were stored in the dark at −20 °C until use.

Buffalo, IPC, beef and almond food powders were then *in vitro* digested according to the INFOGEST harmonized protocol.[35] This digestion involved simulating *in vivo* digestion in oral, gastric and intestinal stages *in vitro* using commercial enzymes such as amylase, pancreatin and trypsin and inactivating the enzymes. The detailed procedures and characterization of the digestion products have been described previously.[36]

To check the effectiveness of enzymatic digestion, SDS-PAGE was performed with the samples obtained.[37]

For the explant culture, healthy viable colon tissues were obtained from 10 patients (average age 65 years) who had undergone colon surgery. All donor patients met the study criteria and gave their informed consent. The exclusion criteria

were anti-inflammatory drug use, alcohol abuse, and intestinal disease. The study was approved by the Clinical Research Ethics Committee (CEIC) of the Hospital Universitari Joan XXIII in Tarragona (CEIm 101/2017).

Tissues obtained from the proximal and distal colon were transferred to the culture laboratory within 30 min in cold oxygenated Krebs–Ringer buffer (pH = 7.4) with D-mannitol 10 mM.[38]

The excised samples were cut up to generate six explants from each human donor. The 5 mm diameter explants were placed in a 48-well plate pre-filled with 400 µL of KRB buffer with D-mannitol, and divided into five treatment groups: control, beef, almond, insect, and IPC. The control group was treated with the same KRB buffer with glucose. The digested samples were adjusted to a dose of 5 mg protein per mL. The medium was collected after 30 minutes of incubation and stored at −80 C.

The secretion of human inflammatory cytokines and intestinal immunoglobulins TNF-α, IL-10, IL-8, IgE and sIgA was quantified using Enzyme-linked Immunosorbent Assay (ELISA) with colorimetric detection. The overall experimental procedure is shown in Fig. 1. For immunosecretome analysis in human colon explants, intestinal secretion of IL-10, IL-1β, IgE and sIgA was measured with the Elabscience ELISA kit (Texas, United States). The human TNF-α ultrasensitive ELISA kit was obtained from Thermofisher (Invitrogen, Barcelona) (Cat. no: KHC3014). Colon IL-8 secretion was measured with Millipore (Sigma Aldrich, Madrid) (Cat. no: RAB0319). All immunomarkers were measured according to manufacturers' instructions.

In vivo study of the consumption of *A. diaperinus* and *T. molitor* flour by Wistar rats

Buffalo (*A. diaperinus*) flour was obtained from Protifarm NV (Ermelo, Gelderland, The Netherlands) with a caloric content of 6.1 kcal g^{-1} and a macronutrient composition of 56.31% protein, 18.82% fat, 7.44% fibre, and 6.3% carbohydrates (1.30% starch).

T. molitor flour from larvae was prepared from insects purchased from a local supplier (Iberinsect, S.L; Reus, Spain). Insect flour was prepared and processed by the FoodIE Research Group and the Mobiofood Research Group at

Universitat Rovira i Virgili (URV), Spain. The composition of *T. molitor* flour was 56.10% protein, 26.31% lipids and 7.78% carbohydrates (3.34% starch), as measured by AGROLAB, S.A. The caloric content was determined to be 6.23 kcal g^{-1} by the Institute of Agrifood Research and Technology (IRTA, Catalonia, Spain).

The microbial content of *A. diaperinus* flour was determined at Protifarm (Ermelo, Gelderland, The Netherlands). The microorganism content of *T. molitor* flour was analysed at AGROLAB S.A (Tarragona, Spain). Both microbiological analyses showed levels of bacteria, moulds and yeasts absent or below the toxicity levels for both insects.

Forty, six-week-old, Wistar female rats (Janvier, Castellar del Vallès, Spain) were included in the *in vivo* study. The animals spent an adaptation period of 14 days at the Universitat Rovira Virgili animal facility under standard conditions. They were caged in pairs at 22 °C with a standard 12-hour light–dark cycle, ventilation, *ad libitum* access to tap water, and a standard Teklad diet (Cat no: Teklad 2014, Envigo++, Barcelona, Spain) consisting of 20% protein, 13% fat and 63% carbohydrates. The rats' standard diet was a plant-based protein maintenance diet with 4.073 kcal g^{-1}, as measured by IRTA, Catalonia, Spain.

After this adaptation period, the animals were individualized and randomly divided into five experimental groups, with identical initial body weight, each of which was given a different treatment. Lipopolysaccharide (LPS) treatment was given, based on the body weight (BW) of the rats, to induce intestinal immune disruption. LPS was obtained from *E. coli* serotype O111.B5 (Merck Lifesciences, Madrid, Spain; Cat no: 4357765). As detailed in Miguéns-Gómez *et al.*,[39] the treatment lasted 26 days (also shown in Fig. 2) and involved five groups as follows:

Group 1 comprised animals that received a standard (STD) diet *ad libitum* (C group);

Group 2 comprised animals that received an STD diet plus five intraperitoneal (i.p.) doses of LPS at 0.5 mg kg^{-1} of BW on the last five days (C + LPS group);

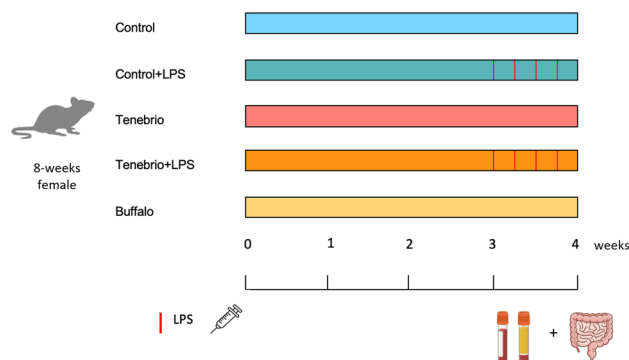**Fig. 1** Graphical representation of the experimental design of the explant's study.

Fig. 2 Experimental design of Wistar rat study.

Group 3 comprised animals that received an STD diet plus a daily oral dose of *T. molitor* flour (300 mg of protein per kg BW per day) (T group);

Group 4 comprised animals that received STD diet plus a daily oral dose of *T. molitor* flour (300 mg of protein per kg BW per day) and an i.p. dose of LPS 0.5 mg per kg of BW on the last five days (T + LPS group); and

Group 5 comprised animals that received STD diet plus a daily oral dose with *A. diaperinus* flour (300 mg of protein per kg BW per day) (Buffalo group).

The insect flours were administered by controlled voluntary oral intake with a syringe, at a dose of 300 mg of protein per kilogram of body weight dissolved in water, at 6 pm. The treatment thus consisted only of raw insect meal mixed with tap water, while the control groups received the equivalent volume of tap water.

During the experiment, the health status of the animals was regularly monitored for cleanliness, general physical appearance, faecal consistency, and stress symptoms (hair loss, lack of appetite, *etc.*).

After 21 days, the animals were euthanized *via* exsanguination under anaesthesia, administered at a dose of 100 mg per kg BW of pentobarbital. Some of the rats' main organs (the duodenum, jejunum and ileum from the small intestine; the distal and proximal large intestine; and the thymus, spleen, kidney and liver) were excised, weighed and frozen immediately in liquid nitrogen for future analysis. White abdominal adipose tissue (WAT) from mesenteric, retroperitoneal and epididymal locations was excised and weighed to calculate the percentage of adiposity. Blood was collected with ethylenediaminetetraacetic acid (Deltalab, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation at $1500g$ for 15 min at 4 °C and frozen immediately at −80 °C for future parameter quantification. All procedures were approved by the GENCAT Animal Experimentation Committee (number 11701).

Biochemical analysis

Colorimetric kits from QCA, (Tarragona, Spain), Materlab (Madrid, Spain) and Wako (Kyoto, Japan) were used to determine the following plasma biochemical parameters: cholesterol (QCA, Ref. 995282), glucose (QCA, Ref. 998282), triacylglycerols (QCA, Ref. 992330), urea (QCA, Ref. 993648), creatinine (QCA, Ref. 990310) and ketone bodies (β-hydroxybutyrate), (Materlab, Ref. HB8855).

Intestinal barrier, immunological and allergenic analyses

Two days before euthanasia, the ovalbumin (OVA) test was run to determine intestinal permeability.[40] These analyses were carried out using Ovalbumin ELISA kits (Cat. no. MBS2000240) from MyBioSource (San Diego, CA, USA).

Plasma levels of total IgE and histamine were measured as biomarkers of allergy at the end of the experimental period. IgE (Cat. no. CN: E-EL-R0517) and histamine (Cat. no. CN: E-EL-0032) kits were purchased from Elabscience (Texas, United States) and performed according to the manufacturer's instructions.

Plasma inflammatory cytokine markers (IL-1β, IL-10, and TNF-α) were determined by ELISA. The ELISA kit for IL-10 (Cat. no. CN: 88-50629) was purchased from Thermofisher (Invitrogen) (Life Technologies, Madrid, Spain). ELISA for TNF-α (Cat. no. E-EL-R2856) and IL-1β (Cat. no. E-ELL-R0012) were purchased from Elabscience.

After the intestine was removed and before the intestinal parts were excised, the intestinal lumen contents were subjected to two lavages with 4 mL of PBS. The intestinal lumen contents of the small and large intestine were obtained separately and frozen immediately at −80 °C for future analysis. Secretory IgA (sIgA) levels were measured in small and large intestinal lavage fluids using the ELISA kit from MyBioSource (Cat. no. MBS9711882).

Total RNA and cDNA were obtained as previously reported.[41] Quantitative PCR amplification was performed using specific TaqMan® probes for the sIgA inducing protein (Rn01406210_s1) and IL-1β (Rn00580432_m1) genes, and PPIA (cyclophilin) (Rn00690933_m1) as reference gene. The relative expression of each gene was compared with the control group using the 2-ΔΔCt method and with the cyclophilin gene as reference.

Histological analysis of the intestinal sections

One centimetre of each part of the intestine was fixed for 24 h in 4% formaldehyde solution and transferred to 70% ethanol solution for preservation until it was embedded in paraffin blocks. The samples analysed (at the Laboratory of Biology and Pathology of the Endocrine Pancreas of the Unité de Biologie Fonctionnelle et Adaptative, CNRS at Université Paris Cité, F-75013 Paris, France) were the duodenum, jejunum, ileum and ascending colon from five animals from each experimental group.

Samples were cut at 5 mm and placed on glass slides. Haematoxylin and eosin (H&E) staining was then performed following standard procedures.

Using an OLYMPUS BX60 microscope equipped with Histolab 10.5.1 (Microvision Instruments, Evry, France) software for histology and morphometrics counting, the intestinal epithelium was analysed by measuring the villus height, villus width, crypt depth, epithelium height and villus-to-crypt ratio, as described in our previous study.[42] The percentage of goblet cells was also analysed by counting the number of goblet cells and the number of epithelial cells.

Statistical analysis

Results are presented as means ± SEM. Data analysis was conducted with the XLSTAT 2023 statistical software (Addinsoft, USA). In the *ex vivo* human experiment, group differences were assessed through one-way ANOVA, followed by Tukey HSD multiple comparisons test.

For the rat experiment, statistical differences were analysed using Student's *t* test, comparing each experimental group with the corresponding control group. Specifically, Tenebrio, Buffalo and Control + LPS were compared with Control, while Tenebrio + LPS was compared to Control + LPS.

Statistical significance was considered for mean differences if $p < 0.05$.

Paper

Results

Insect-derived flours produced a healthier inflammatory secretome in human colonic explants

To assess how the human colonic immune system responds to various protein sources, we exposed healthy human colon explants to digested flours for 30 minutes at a concentration protein that stimulated enterohormone secretions in paired colonic samples.[37]

The levels of proinflammatory protein IL-8 and TNF-α were determined in the media of colonic explants after insect, almond and beef exposure (Fig. 3). IL-8 levels from human explants treated with insect and IPC showed lower inflammatory profiles than those treated with beef or almond (Fig. 3A). Beef also had a proinflammatory effect by inducing an increase in TNF-α levels, whereas neither insect, IPC nor almond treatment led to elevated TNF-α levels (Fig. 3B).

Also assessed was the anti-inflammatory cytokine IL-10, which was significantly higher in explants treated with almond flour but remained unchanged after insect, IPC or beef treatment (Fig. 3C).

The levels of sIgA showed the extent of protection from intestinal pathogens in the intestinal lumen. No treatment showed changes in sIgA levels with respect to the control (Fig. 3D).
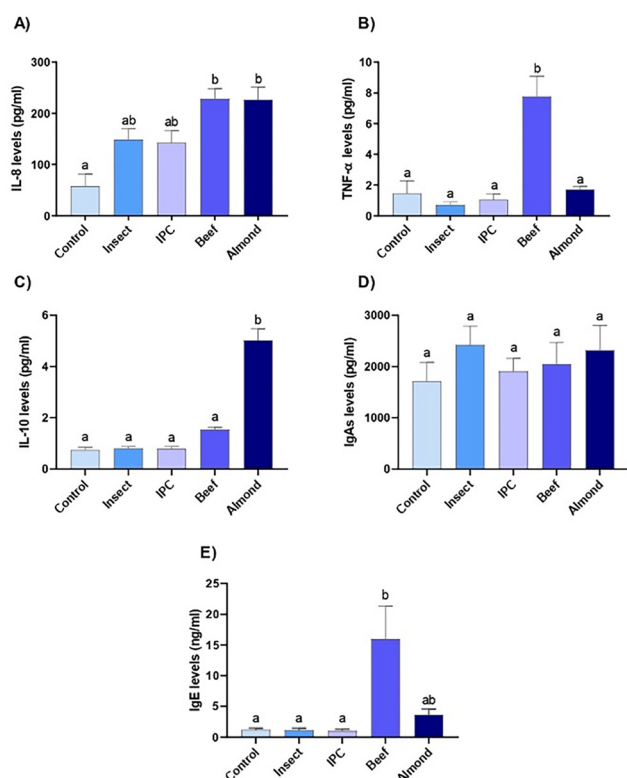
Also determined was the level of allergy-related IgE immunoglobulin (Fig. 3E). Neither whole insect nor IPC from *A. diaperinus* showed a similar allergenic profile to that of almond extract, whereas beef induced a significant increase in IgE levels.

Whole-health status of insect-fed rats

To analyse the effects on an *in vivo* system, we worked with two animal models, one of which was a healthy rat and the other was a model of LPS-induced mild inflammation and intestinal dysfunction.[43] This LPS-induced disease model wants to mimic all pathologies that presented an altered physical barrier and a proinflammatory intestinal environment in the small intestine which would be the best candidates to be sensitive to this atypical food component.[44,45] Using these animal models, we tested two species of insects to detect potential species-specific effects.

In the healthy rat model, after 21 days of insect administration no animal showed any physical sign of stress (gastrointestinal, respiratory, or skin alteration) during the nutritional intervention. Treatment with buffalo flour did not change the weight of any organs in comparison with those of the control. Supplementation with *T. molitor* insect flour produced a trend towards an increase in % body weight gain but not in adiposity and did not change the weight of the thymus, liver, stomach, spleen, or kidney. There were no differences in total intestinal length between the groups (Table 1). In healthy rats, the ingestion of insect flour did not modify glucose, triglycerides (TAGs) or total cholesterol (Table 2). The levels of ketone bodies were undetectable and urea and creatinine in plasma were unchanged by insect consumption.

In line with the results in humans, in this healthy model the administration of insect protein (Buffalo or Tenebrio flour) did not alter the levels of the proinflammatory cytokines IL-1β and TNF-α. Similarly, no changes were observed in IL-10.

In the intestinal dysfunction model, the weights of the liver and spleen in the LPS group clearly increased while that of the thymus decreased (Table 1). *Tenebrio molitor* ingestion in LPS-

Fig. 3 Effect of different digested proteins (5 mg protein per mL) on levels of IL-8 (A), TNF-α (B), IL-10 (C), sIgA (D) and IgE (E) in human colon explants. Results are represented as mean ± SEM. *n* = 10 humans per group. We used ANOVA and Tukey multiple comparisons test. *P* values < 0.01. Different letters indicate significant differences.

Table 1 Organ weights of healthy Wistar female rats and LPS–Wistar inflamed rats after daily ingestion of *T. molitor* or *A. diaperinus* for 21 days

Organ (g)	Control	Tenebrio	Buffalo	LPS	LPS + Tenebrio
Liver	7.46 ± 0.35	7.86 ± 0.19	8.06 ± 0.25	8.91 ± 0.37*	9.41 ± 0.28
Stomach	1.38 ± 0.07	1.42 ± 0.04	1.41 ± 0.03	1.33 ± 0.03	1.37 ± 0.05
Kidney	0.34 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.34 ± 0.01
Thymus	0.49 ± 0.04	0.57 ± 0.09	0.52 ± 0.10	0.33 ± 0.03*	0.49 ± 0.06$
Small Intestine length (cm)	97.6 ± 0.57	100.1 ± 0.17	98.0 ± 0.44	96.2 ± 0.68	100.6 ± 0.52
Spleen	0.84 ± 0.05	0.84 ± 0.03	0.86 ± 0.04	1.53 ± 0.10*	1.49 ± 0.13
% BW gain	9.42 ± 1.03	13.98 ± 2.83#	9.80 ± 1.04	7.01 ± 1.40	6.64 ± 1.26
% Adiposity	4.38 ± 0.25	5.12 ± 0.59	4.26 ± 0.26	4.12 ± 0.21	4.37 ± 0.49

n = 7–8 animals per group. Data are mean ± SEM. Student's *t* test was performed. * indicates *p* < 0.05 *vs.* control group; # indicates *p* < 0.1 *vs.* control group; $ *p* < 0.05 *vs.* LPS.

Table 2 Biochemical markers of metabolic status in healthy and LPS-inflamed female Wistar rats with a daily insect intake of *Tenebrio molitor* or *Alphitobius diaperinus* for 21 days

Plasma	Control	Tenebrio	Buffalo	LPS	LPS + Tenebrio
Glucose (mM)	7.18 ± 0.15	7.21 ± 0.38	7.35 ± 0.14	6.44 ± 0.24*	7.09 ± 0.16$
TAGs (mM)	0.18 ± 0.02	0.22 ± 0.05	0.17 ± 0.02	0.18 ± 0.03	0.20 ± 0.01
Cholesterol(mM)	0.45 ± 0.01	0.46 ± 0.03	0.48 ± 0.03	0.35 ± 0.02*	0.37 ± 0.02
Urea (mM)	3.20 ± 0.21	2.93 ± 0.13	3.28 ± 0.13	1.70 ± 0.18*	2.11 ± 0.13$
Creatinine (μM)	2.68 ± 0.49	2.59 ± 0.28	3.18 ± 0.24	2.64 ± 0.56	3.43 ± 0.34

Plasma levels of glucose, triglycerides, cholesterol, urea and creatinine at the end of the study. n = 7–8 animals per group. Data are mean ± SEM. Student's t test was performed. * indicates $p < 0.05$ *vs.* control group; $ 0.05 > p < 0.1$ *vs.* LPS.

treated animals slightly prevented the decrease in thymus weight compared to the LPS group. The disease model presented mild alterations in some biochemical parameters (Table 2). LPS treatment reduced the plasma levels of total cholesterol, glucose and the urea. However, TAGs and creatinine remained unchanged. Interestingly, ingestion of *T. molitor* seemed to prevent the decrease in blood glycemia and uraemia.

Effects of insect consumption on intestinal permeability, immune barrier and allergenicity

Both LPS-treated groups showed an increase in intestinal permeability compared to controls, though insect supplementation did not exert any significant effect with this time and dose (Fig. 4a). In healthy rats, insect supplementation showed no change in intestinal permeability compared to controls (Fig. 4a).

To evaluate the effect of insect protein consumption in a pro-inflammatory environment, we measured the serum levels of TNF-α, IL-1β and IL-10. Fig. 4c shows that treatment with LPS significantly increased the levels of TNF-α and increased Il-10 and IL-1β almost three-fold (Fig. 4b and d) in comparison with the control group. Treatment with *T. molitor*, on the other hand, did not modify the secretion of these cytokines.

To assess the health and functionality of the mucosal immune system, we quantified the secretion of secretory immunoglobulin A (sIgA) at the intestinal level. In the healthy model, the results showed that more sIgA was secreted in the small intestine than in the colon (Table 3). Ingestion of the Buffalo and Tenebrio flours alone had no effect on sIgA levels in either the small intestine or the colon.

In the disease model, when comparing the LPS group with the Control group, the statistical analysis indicates a tendency to increase. However, animals that received both LPS injection and consumed Tenebrio did not show significant differences or a trend, neither with the Tenebrio group nor the LPS group. Intraperitoneal LPS administration had local immunology effects in the small intestine but not in the colon. We also analyzed relative expression in the ileum of sIgA activation protein (Table 3) but observed no significant changes in gene expression in either the healthy or the LPS-induced inflammatory group.

To evaluate potential *in vivo* sensitization to the consumption of *A. diaperinus* or *T. molitor* insect flour for 21 days, IgE and histamine plasma biomarkers were tested. In the healthy
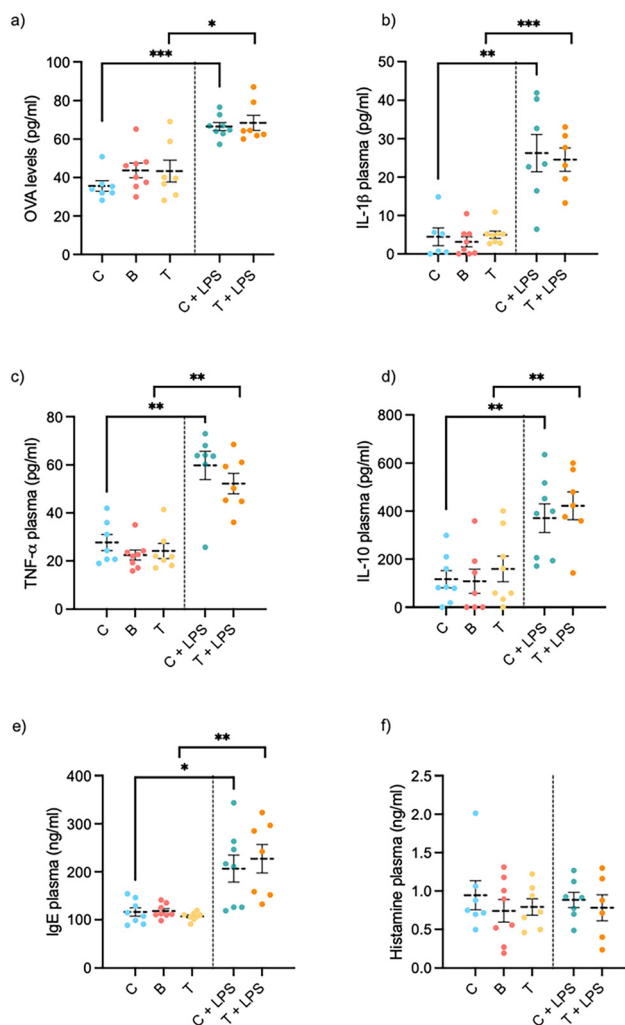

Fig. 4 Changes in intestinal permeability ((a) OVA test), plasma systemic inflammation ((b) IL-1β, (c)TNF-α and (d) IL-10), allergenic response ((e) IgE and (f) histamine) after chronic doses of insect protein flour in in health and in LPS-induced inflammation female rats. Animals (n = 7–8 per treatment) were treated for 21 days. Experimental groups are (C) standard diet; (C + LPS) Control diet plus i.p. LPS (0.5 mg per kg BW) for the last five days; (T) 300 mg protein per kg BW daily supplement of *Tenebrio molitor* flour; (T + LPS); 300 mg protein per kg BW daily supplement of *Tenebrio molitor* flour diet plus i.p. LPS (0.5 mg per kg BW) for the last five days; (B) 300 mg protein per kg BW daily supplement of *Alphitobius diaperinus* flour. *, ** or *** correlation's p-value <0.05, 0.01 or 0.001, respectively, denotes statistical significance by Student's t test.

Table 3 Secretory immunoglobulin A (sIgA) at the intestinal level

	Control	Tenebrio	Buffalo	LPS	LPS + Tenebrio
Small intestine sIgA (μg ml⁻¹)	9.94 ± 1.25	8.00 ± 0.63	8.65 ± 1.37	17.2 ± 3.49#	13.6 ± 1.95
Ileal IgA i.p. gene expression	0.91 ± 0.14	1.19 ± 0.30	0.87 ± 0.07	1.04 ± 0.19	0.76 ± 0.20
Colonic sIgA (μg ml⁻¹)	1.27 ± 0.09	1.19 ± 0.08	1.17 ± 0.05	1.13 ± 0.05	1.23 ± 0.08

n = 7–8 animals per group. Data are mean ± SEM. Student's t test was performed. # indicates $0.05 > p < 0.1$ *vs.* control.

Table 4 Morphometric intestinal variables affected by insect consumption in healthy and LPS-Wistar rats

Small intestine	Control	Tenebrio	Buffalo	LPS	LPS + Tenebrio
Duodenum (µm)					
Villus length	548.13 ± 31.24	616.33 ± 29.62	597.29 ± 23.19	558.27 ± 25.37	549.71 ± 34.04
Villus width	78.48 ± 3.27	79.57 ± 2.43	76.21 ± 3.03	83.32 ± 2.88	79.80 ± 2.43
Crypt depth	168.94 ± 8.63	196.92 ± 17.41	187.20 ± 10.81	191.89 ± 17.18	181.79 ± 9.71
Crypt width	71.25 ± 4.42	71.05 ± 2.77	74.86 ± 4.77	78.86 ± 5.83	73.85 ± 2.31
Epithelium w	777.34 ± 22.76	848.24 ± 54.28	854.31 ± 48.23	803.30 ± 40.45	791.76 ± 45.40
Villus/crypt ratio	3.34 ± 0.45	3.50 ± 0.67	3.20 ± 0.08	3.01 ± 0.32	3.06 ± 0.24
M (surface ratio)	8.4 ± 0.12	9.4 ± 0.52	8.79 ± 0.46	7.93 ± 0.60	8.17 ± 0.52
Jejunum (µm)					
Villus length	404.72 ± 14.96	407.33 ± 14.02	414.78 ± 12.81	393.50 ± 26.82	366.32 ± 17.30
Villus width	55.28 ± 2.74	55.86 ± 5.69	53.77 ± 5.20	58.14 ± 2.68	52.73 ± 5.39
Crypt depth	168.94 ± 8.63	196.92 ± 17.41	187.20 ± 10.81	191.89 ± 17.18	181.79 ± 9.71
Crypt width	58.34 ± 4.42	58.44 ± 5.54	51.92 ± 3.79	56.76 ± 3.00	54.76 ± 2.49
Epithelium w.	586.74 ± 24.38	576.25 ± 20.85	588.07 ± 18.35	576.25 ± 20.85	533.05 ± 22.47
Villus/crypt ratio	2.90 ± 0.03	3.37 ± 0.12*	3.22 ± 0.15&	3.28 ± 0.33	3.00 ± 0.05
M (surface ratio)	7.8 ± 0.43	7.96 ± 0.67	8.8 ± 0.51	7.7 ± 0.57	7.45 ± 0.39
Ileum (µm)					
Villus length	269.50 ± 21.89	282.90 ± 5.14	248.96 ± 16.71	244.04 ± 10.28	261.89 ± 11.79
Villus width	55.92 ± 6.38	46.60 ± 2.03	47.93 ± 7.06	53.98 ± 5.08	47.16 ± 1.33
Crypt depth	102.55 ± 7.06	100.05 ± 5.51	103.73 ± 3.39	105.62 ± 3.13	104.95 ± 3.64
Crypt width	58.14 ± 1.98	54.08 ± 2.60	55.74 ± 1.43	53.10 ± 2.79	51.49 ± 1.67
Epithelium w	408.46 ± 24.50	429.35 ± 12.06	381.82 ± 20.50	374.73 ± 16.02	397.30 ± 15.42
Villus/crypt ratio	2.61 ± 0.15	2.85 ± 0.13	2.41 ± 0.20	2.21 ± 0.01*	2.49 ± 0.05[$]
M (surface ratio)	5.41 ± 0.52	6.02 ± 0.29	5.20 ± 0.20	5.28 ± 0.05	5.87 ± 1.21
Large intestine	**Control**	**Tenebrio**	**Buffalo**	**LPS**	**LPS + Tenebrio**
Colon (µm)					
Crypt depth	113.76 ± 15.40	103.56 ± 8.61	106.84 ± 7.06	106.13 ± 6.58	95.22 ± 6.27
Crypt width	40.95 ± 1.35	40.75 ± 1.50	38.55 ± 2.27	39.46 ± 1.53	39.47 ± 1.69

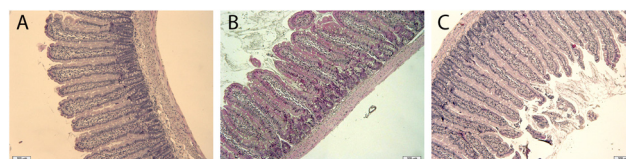n = 7–8 animals per group. Data are mean ± SEM. Student's t test was performed. * indicates $p < 0.05$ $vs.$ control group; [$] $p < 0.05$ $vs.$ LPS; & $0.05 > p < 0.1$ $vs.$ control.

model, neither the histamine nor the IgE levels showed any significant change between groups when the animals received insect flour (see Fig. 4f). In the disease model, IgE plasmatic levels increased significantly compared to controls, while histamine levels remained unchanged after insect consumption.

Intestinal morphometry in healthy and LPS-Wistar rat models under insect supplementation

To evaluate any putative micro-gut morphological alterations caused by the consumption of insect flour from mealworm and buffalo, we histochemically analysed the morphometric size of the villus and crypt and determined the total epithelium and surface amplification ratio of all intestinal sections (duodenum, jejunum, ileum and proximal colon) (see Table 4) as well as their Goblet cell percentage. Scheme 1 shows examples of the images used for the analysis.

In healthy animals, our results showed that insect flour consumption did not modify villus width, villus height, crypt depth, villus-to-crypt ratio, epithelium width or the M-surface amplification ratio from any intestinal location. This is an indicator of the absence of a deleterious effect. Only Tenebrio increased the villus-to-crypt ration in the jejunum. No changes in Goblet cell number were observed in any intestinal section .

Scheme 1 Examples of images obtained of jejunal sections of (A) Control group, (B) Tenebrio group and (C) LPS group. Magnification 10×.

The same parameters were also measured in the inflammatory rat model with and without insect flour supplementation (Table 4). In the inflammatory model, a slight proinflammatory state influenced some parameters, including the ileum villus-to-crypt ratio, which decreased, thus indicating a morphological deleterious effect. Interestingly, *T. molitor* supplementation prevented this effect. No changes in Goblet cell number were observed.

Discussion

We have investigated the effect of a sustainable source of protein on human explants and Wistar rats, focusing on intestinal immunological effects. In a previous complementary work[39] we analysed their effects in food intake and enteroho-

mone modulation. Specifically, two edible insect species approved for human consumption by EFSA were analysed: *Alphitobius diaperinus* and *Tenebrio molitor*.[47] Here our results showed healthy responses with regard to systemic and intestinal inflammation, allergenic response, and intestinal morphology in rats after chronic insect supplementation in both healthy and intestinal disfunction models. This is the first description of the non-allergenic effect of insect ingestion in a disrupted permeability and intestinal inflammation animal model.

The gastrointestinal tract – the primary barrier against food components – plays a crucial role in host defence and immune response.[46] Some studies have reported that increased permeability of the intestinal epithelium can facilitate the entry of potential food allergens, thus heightening sensitization and allergy risks.[33,47] Notably, the intraperitoneal injection of lipopolysaccharides (LPS) leads to systematic and local alterations, including increased intestinal permeability and exacerbated inflammation.[43,48]

In the context of insect consumption, the potential allergenic response is a significant concern. While most existing research focuses on pre-sensitized individuals or those with allergies to crustaceans and inhalants,[49] our study aimed to advance this field by assessing allergenic responses in the context of disrupted intestinal permeability and inflammation. Our results from the LPS model rats revealed an impaired inflammatory and allergic response, as has previously been described.[43] However, in the group that received insect supplementation, no aggravation of this situation was observed, which suggests that the insect did not exert any allergenic response in this rat model of intestinal dysfunction.

Moreover, by evaluating the response to insect consumption in both healthy and non-sensitized human and rat models, our study offers a comprehensive overview of the allergenic potential of low-dose insect consumption. In this sense, the healthy rats that were chronically supplemented by yellow mealworm or lesser mealworm had a normal profile of secreted inflammatory and anti-inflammatory cytokines, while histamine and total IgE were not altered. Previous studies conducted with Sprague-Dawley rats, with longer oral exposures to insects and maximum doses (300–3000 mg kg^{-1} day^{-1}) of mealworm, also found no statistically significant increases in serum histamine or IgE concentrations.[50] Our data with doses of 300 mg kg^{-1} day^{-1} for 21 days in Wistar females corroborate those results.

We also directly compared the human colonic immune response to insect extracts to the response to almond and beef protein sources. Our results indicated that exposure to insect extracts led to a lower secretion of inflammatory cytokines and allergenic immunoglobulin compared to almond or beef extracts. Beef especially appeared to be more proinflammatory, as was evidenced by higher cytokines and IgE levels. On the other hand, the response to almond appeared to be counterbalanced by an anti-inflammatory effect. These findings align with previous *in vivo* studies that suggest that beef consumption can alter gut microbiota and activate inflammatory pathways,[51,52] while almond consumption is known to exert a protective effect against the development of gastrointestinal inflammation.[53] Moreover, while explants treated with beef exhibited higher IgE secretion, these levels are still within the low and normal range for intestinal tissue,[54] which suggests that this is not indicative of an allergic response. All these results support the notion of a healthier inflammatory secretome for insect-derived flours compared to those from almond and beef.

An important effector function of gut-associated lymphoid tissue involves the production and secretion of immunoglobulin A (IgA). This immunoglobulin plays a crucial role in protecting the intestine from pathogens, mainly by limiting their interactions with the epithelial cell monolayer.[55] The transcytosis of dimeric IgA antibodies through epithelial cells is mediated by the polymeric Ig receptor, which results in the release of secretory IgA (sIgA).[56] In this context, local antigen exposure could lead to increased sIgA levels in both the exposed area and the enteric mucosa. Measuring IgA in intestinal lavage fluids is therefore a common method for assessing mucosal immune responses.[55] Our animal study showed a tendency only for increased sIgA secretion in rats administered with LPS. In contrast, neither human explants nor rats treated solely with protein sources showed significant alterations in this intestinal immune marker. This suggests that the consumption of these protein sources, including insects, does not cause major immune disturbances, thereby aligning with findings reported by Stull *et al.*[21]

To continue with the intestinal analysis, our findings also showed that insect supplementation did not affect normal intestinal morphology. The typical gradient of morphometric indices from the duodenum to the ileum was maintained, which corroborates the preservation of physiological gut development and absorption processes.[57] Similar to these results obtained by Biasato *et al.* in chickens, chronic insect consumption did not alter the intestinal morphology. Main modification was in the jejunum, where animals supplemented with insects exhibited a higher villus-height to crypt-depth ratio. This effect has been suggested as adaptative response to expand the surface area for nutrient absorption.[58] Our study does not allow support this hypothesis, because there was not a clear effect on food intake, we saw an increased food intake at the first week, but it was lost afterwards.[39] And the other challenging aspect regarding insects-derived products is their digestibility. In this respect, we have previously shown a similar digestibility of *A. diaperinus* to beef samples.[59] Moreover, a lower ileal villus-to-crypt ratio was observed in rats administered LPS, which confirms the finding from studies on mice that the intraperitoneal injection of LPS affects the integrity of the small intestine.[60] Interestingly, the consumption of *T. molitor* appeared to counteract this reduction in the ileal ratio.

Moreover, our findings reveal a reduction in thymus weight following LPS injections, consistent with other studies that have previously reported the potential for LPS-induced thymic atrophy.[61,62] The thymus is a vital organ of the immune system, and its atrophy could compromise its ability to effec-

tively respond to other infections.[63] Interestingly, our observations suggest that the consumption of *T. molitor* may confer protection to the thymus against changes induced by LPS. Despite not observing other improvements in terms of inflammatory status, this result aligns with the potential anti-inflammatory effects associated with insect consumption, as indicated by existing literature.[24] Furthermore, some authors have proposed that thymus gland dysfunction can be ameliorated through nutritional intervention, involving a balance of macronutrients, micronutrients, and the incorporation of probiotics, indicating thymus-gut connections.[64,65] Thus, the potential of insect supplementation to modulate gut microbiota with prebiotic effects,[66] may be linked to thymus healthy. Further research exploring both the inflammatory protection and the prebiotic effects of insect consumption could contribute to a comprehensive understanding of its impact on inflammation.

Although this study primarily focused on the immune system's response, we also considered whole-health parameters. With regard to the plasma parameters, our study indicates good metabolic tolerance to insect administration, with *T. molitor* intake notably ameliorating LPS-altered glucose and urea levels. Our study does not allow to explain these effects, but we can clearly state that they cannot relate it with improvement of TNF-α or IL-1β, which have been demonstrated to induce hypoglycaemia.[45] We cannot discard results from other studies that have reported improved circulating triglycerides, glucose and cholesterol levels in humans and animals following insect consumption.[1] Our initial screening of plasmatic cytokine levels, known to participate in systemic inflammation and exhibiting increased levels in the LPS situation,[67] revealed that LPS administration alone increased the immunological parameters, but the ingestion of insect flour did not exacerbate this effect. Notably, the LPS-induced increase in IL-10 plasma levels is a typical counter-regulatory response to intraperitoneal insult mediated by T-regulatory cells to resolve inflammation.[68]

Note that the EFSA recently approved the insects used in this study as novel food for human consumption and encouraged further research in this area.[16,17] There is growing consensus on the need for further studies to demonstrate the health benefits of insect consumption and enhance its social acceptance, especially in Western diets, which urgently need to change from a conventional to a more sustainable source of protein.[69] The present study describes, for the first time, the chronic effects of consuming two species of insects on both general and intestinal health in rat and human samples and provides novel insights into the immune response in a healthy and a disrupted intestinal model.

In view of all the evidence in support of adding insects to diets and the encouraging results of this study, to validate these findings further research should involve human participants without known allergies. Long-term studies are needed to fully understand the impact of edible insects on human health. As the food industry explores alternative sources of protein, edible insects present a unique opportunity to improve environmental sustainability while also providing substantial nutritional advantages.

Conclusions

The human *ex vivo* studies have shown that *A. diaperinus* digested *in vitro* induced a less inflammatory profile than either almond or beef extract in colon samples. Moreover, neither *A. diaperinus* nor *T. molitor* administered chronically in moderate doses to healthy Wistar rats compromised gut morphology, plasma biochemistry profile or immune response. In the LPS-induced intestinal dysfunction model, insect consumption ameliorated glycaemia, uraemia and gut villus-to-crypt ratio. These data reinforce the healthy immunological profile of both species of insect assayed. More insights are needed on the bioactive effects of insect flour, especially with regard to the less studied *Alphitobius diaperinus*.

Author contributions

HS, FJ and MSC: data curation, formal analysis, investigation; FF: investigation; ERG, JM and XT: methodology; AA, MTB, XT and MP: conceptualization, supervision. AA, MTB, HS: project administration, original draft; MP, XT, JM and AA: resources. All the authors participated in funding acquisition and revised and edited the final document before submission.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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