

Allergenicity assessment of the edible cricket *Acheta domesticus* in terms of thermal and gastrointestinal processing and IgE cross-reactivity with shrimp

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ABSTRACT

The allergenic potency of the cricket *Acheta domesticus*, a promising edible insect, has never been assessed. This work aims to study the immunoreactivity of *Acheta domesticus*, and its cross-reactivity with the shrimp *Litopenaeus vannamei*, assessing the effect of cooking and gastrointestinal digestion on their allergenic properties. Different cricket proteins were detected by immunoblotting with shrimp-allergic patients' sera. Tropomyosin was identified as the most relevant IgE-binding protein, and its cross-reactivity with shrimp tropomyosin was demonstrated by ELISA. While shrimp tropomyosin showed scarce stability to gastric digestion, cricket tropomyosin withstood the whole digestion process. The sarcoplasmic calcium-binding protein, specifically detected in shrimp, showed exceptional stability to gastrointestinal digestion. IgE-binding proteins in a model of enriched baked products were partially protected from proteolysis. In conclusion, the ingestion of *A. domesticus* proteins poses serious concerns to the Crustacean-allergic population. The high stability of tropomyosin may represent a risk of primary sensitization and clinical cross-reactivity.

1. Introduction

The Food and Agriculture Organization of the United Nations estimated that the world's population would increase by 34% and reach about 9.3 billion people by 2050 (FAO, 2009; Kourimská & Adámková, 2016), representing a food security challenge since conventional food sources will be insufficient to nourish the world's population. For this reason, alternative food sources, such as edible insects, will be required.

Entomophagy (i.e., the practice of eating insects) is common in many countries, even though people from western countries consider this practice disgusting. Up to now, about 2000 species of insects are currently eaten by over 2 billion people with an unequal distribution (Barennes, Phimmasane, Rajaonarivo, & Schooling, 2015). Promoting entomophagy might have various positive implications for the

environment, e.g. the emission of fewer greenhouse gases and water pollution than livestock, the reduced land-dependency, the requirement of small spaces for growing, and the possibility to exploit a wide range of plants and organic waste streams as feed (Akhtar & Isman, 2018; Fowles & Nansen, 2020; van Huis et al., 2013).

Insects could be considered a source of polyunsaturated fatty acids (linoleic and α -linolenic acids) and essential amino acids; moreover, they provide as much energy as meat (Rumpold & Schlüter, 2013; Zielinska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015).

However, eating insects could expose consumers to certain risks, like the accumulation of toxic chemicals, such as heavy metals or hazardous substances like cyanogenic glycosides (Kourimská & Adámková, 2016). Insects may also represent a potential source of microbiological hazards due to their high total bacterial load; thus, it is strongly suggested to

Abbreviations: TM, tropomyosin; SCP, sarcoplasmic calcium-binding protein; MW, molecular weight; %T, percentage of acrylamide.

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consume insects only after a sufficient heat treatment to inactivate or at least reduce the total microbial load (Fernandez-Cassi, Supeanu, Jansson, Boqvist, & Vagsholm, 2018). A further health risk is associated with their suggested allergenic potential. Many insects have been reported to cause allergic reactions through inhalation, direct contact and also by ingestion (Rumpold & Schlüter, 2013; van Huis et al., 2013; Verhoeckx et al., 2014; Akhtar et al., 2017; Belluco, Mantovani, & Ricci, 2018). The adverse reactions (including anaphylactic shock) described after the consumption of insects, can be attributed to a mechanism of primary allergic sensitization or more likely of IgE cross-reactivity with other taxonomically-related allergenic species belonging to arthropods (Broekman et al., 2017; de Gier & Verhoeckx, 2018; Downs, Johnson, & Zeece, 2016; Kamemura et al., 2019). In line with this, common allergens from arthropods, such as tropomyosin, arginine kinase and glyceraldehyde 3-phosphate dehydrogenase have been described (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2015; Verhoeckx et al., 2014).

One of the most promising edible insects is the house cricket *Acheta domesticus* (Fernandez-Cassi et al., 2018). A recent specific risk profile evaluation on reared *A. domesticus* recognized homologue proteins shared between different species as a source of potential pan-allergenic reactions (Fernandez-Cassi et al., 2018). Tropomyosin (TM), an actin-binding protein, is the major pan-allergen responsible for the cross-reactivity among different invertebrates (Reese, Ayuso, & Lehrer, 1999). However, although cross-reactivity with other species has been strongly suggested, no cricket (or orthoptera in general) protein has been confirmed as allergenic yet, and therefore, to the best of our knowledge, no orthopteran allergens have been included in the International Union of Immunological Societies (IUIS) database. Only the cross-reactivity of cricket *Gryllus bimaculatus* with crustaceans has been studied so far (Kamemura et al., 2019; Srinroch et al., 2015), while specific studies on *A. domesticus* are missing. Despite the potential allergenicity of crickets, in Europe, and in general in Western society, insects are increasingly used to fortify food products in order to improve their nutritional properties and, at the same time, to hide the unappealing nature of eating whole insects. With the growing production of insect-derived foods, it must be considered that the different food processing methods can lead to diverse effects on food protein and thus on allergenic epitopes (Maleki, Chung, Champagne, & Raufman, 2000; Sathe & Sharma, 2009). In general, class I (also termed genuine or true) food allergens are stable to digestion and reach the intestinal mucosa, in an immunologically active form (Astwood, Leach, & Fuchs, 1996). For this reason, resistance to simulated digestion is one of the most used criteria for the assessment of the risk of reaction to food allergens and novel foods (Moreno, 2007). This work aims to investigate the allergenic relevance of *A. domesticus* in terms of cross-reactivity with the pacific white shrimp *L. vannamei*, one of the main consumed shrimp worldwide, and to evaluate the effect of the baking process and simulated gastroenteric digestion on the allergenic potency of the identified cricket proteins.

2. Materials and methods

2.1. Human sera and antibodies

Eight sera (# 1–8) of patients with confirmed IgE antibody to shrimp were obtained from PlasmaLab (Everett, MA, USA), and 12 sera of patients with adverse reactions upon ingestion of shrimp were supplied by the Allergy Unit, Dept of Dermatology University Hospital Erlangen (Erlangen, Germany). Written informed consent was obtained from all patients, and the study was approved by the local ethics committee. The clinical characteristics of patients and the results of the diagnostic analysis are summarized in Table 1.

Specific IgE for recombinant *Peneus aztecus* tropomyosin (rPen a 1) and shrimp were measured by ImmunoCAPTM (Thermo Fisher Scientific, Waltham, MA, USA). Values ≥ 0.35 kUA/L were considered as positive.

Table 1

Clinical data of the allergic subjects.

	ID	Shrimp CAP (kU/L)	rPen a 1 CAP (kU/L)
1	20197-BH	54.1	45.5
2	9735-RE	14.8	9.58
3	15742-JT	5.01	0.06
4	17694-SS	3.31	0.79
5	20770-MH	13.5	1.86
6	2496-LB	4.65	0.04
7	20318-SN	8.22	0.65
8	20775-AW	2.51	0.18
9	VM 74	–	0.37
10	VM 75	>100	96.5
11	VM 76	–	1.16
12	VM 78	–	23.8
13	VM 79	0.65	0.00
14	VM 80	15.3	0.04
15	VM 81	1.62	0.01
16	VM 82	3.87	0.28
17	VM 83	0.77	0.02
18	VM 84	2.05	0.01
19	VM 85	1.26	0.21
20	VM 88	2.76	2.83

–: not performed

A polyclonal antibody (named ANTI-TM) was raised against recombinant *Crangon crangon* tropomyosin (rCra c 1, Paul-Ehrlich-Institut) into two Balb/c mice as described previously (Zoccatelli et al., 2012). Experiments with mice were carried out according to the ethical regulations established by the European Directive 86/609/EEC (permission of Ministero della Salute No. 106/2011-B).

2.2. Samples preparation

2.2.1. Shrimp and cricket extraction

Cricket (*Acheta domesticus*) flour was purchased by Thailand Unique (JR Unique Foods Ltd., Thailand). This product contained finely ground thermally-treated crickets. Raw white leg shrimps (*Litopenaeus vannamei*) were purchased at the local market. Peeled shrimp tails were frozen in liquid nitrogen and finely ground with mortar and pestle to a powder. Samples were washed with 10 volumes of cold acetone for 90 min, centrifuged at 3500g for 10 min, and pellets were filtered through Whatman paper, dried overnight and stored at -20°C . Alternatively, shrimps' tails were dipped into boiling water for about 2 min, drained, cut into small and thin slices and lyophilized for 46 h. The material was finely ground to a powder and stored at -20°C . This last sample was used for the digestion experiments and the enrichment of biscuits (see below).

Protein was extracted from 50 mg of shrimp powder or cricket flour with 2 mL of 7 M Urea, 2 M Thiourea, and 4% CHAPS (IPG buffer). Samples were left at room temperature in continuous stirring for 1 h and centrifuged at 21,000g for 5 min, and supernatants were stored at -20°C .

2.2.2. Enriched biscuits

The biscuits were made following a classic recipe that included: 180 g of wheat flour, 130 g of margarine, 75 g of sugar, 2 g of baking powder and required amount of water. In the enriched biscuits wheat flour was replaced with 10% (w/w) of shrimp powder or cricket flour. Biscuits were cooked in a static oven at a temperature of 180°C for about 15 min until they turned brown.

Biscuit proteins were extracted with 0.2 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 5% β -mercaptoethanol (SDS-PAGE buffer). Samples were left at room temperature in continuous stirring for 1 h and centrifuged at 21,000g for 5 min, and supernatants were stored at -20°C .

2.3. Protein quantification

The protein concentration of the extracts was measured by RC DC™ Protein Assay Kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions with bovine serum albumin (BSA) as a standard. The linearity of the standard curve was in the range 0.125–2 mg/mL of BSA. Absorbance was measured by a PowerWave microtiter plate reader (Bio-Tek Instruments, Winooski, VT). All analyses were carried out in triplicate.

2.4. Nitrogen quantification and protein content determination in the samples

Shrimp powder, cricket flour and enriched biscuits were mineralized according to the method of (Hach, Brayton, & Kopelove, 1985) and nitrogen was quantified by the AOAC method 33.056 (Williams, 1984).

2.5. SDS-PAGE

Extracts and samples from the *in vitro* digestion (paragraph 2.13) were mixed with a 2 × loading buffer (62.5 mM Tris-HCl at pH 6.8, 1.3% (w/v) SDS, 10% (w/v) glycerol, and 4% β-mercaptoethanol) and heated at 100 °C for 5 min. Proteins (50 µg/cm of lane width) were separated in 12% or 16% acrylamide gels at 20 mA current using a Mini Protean III apparatus (Bio-Rad, Hercules, CA) in 25 mM Tris–192 mM glycine and 0.1% SDS (pH 8.4). The gels were stained by EZ Blue™ Coomassie Brilliant Blue G-250 reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) or subjected to electroblotting onto PVDF membrane as described below.

2.6. 2-D Electrophoresis

The first dimension of the 2-DE was performed with ReadyStrip™ IPG, 11 cm, pH 3–10 or pH 4–7 (Bio-Rad). Strips were passively rehydrated with the extracts and eventually diluted in IPG buffer containing 1% dithiothreitol (DTT). The strips were subjected to isoelectric focusing using a Protean IEF Cell (Bio-Rad) at 20 °C until 32,000 Vh was reached. After focusing, the strips were equilibrated for 15 min in SDS-PAGE buffer with 1% DTT and then for 15 min in the same buffer containing 2% iodoacetamide at room temperature.

The second dimension was performed by SDS-PAGE as described above, and gels were stained with EZ Blue™ Coomassie Brilliant Blue G-250 or subjected to electroblotting onto PVDF membrane.

2.7. Immunoblotting

Proteins separated by SDS-PAGE and 2-DE were electroblotted onto a 0.45 µm pore-size PVDF membrane (Millipore, Merck KGaA, Darmstadt, Germany) at 80 V for 150 min. The membrane was blocked with 3% skimmed milk in PBS containing 0.05% Tween-20 (PT buffer) and incubated for 90 min with a mouse anti-tropomyosin polyclonal antibody (dilution 1:20000) or overnight with the patients' sera at 1:10 or 1:50 dilution. Mouse IgG was detected by an HRP-conjugated goat anti-mouse IgG antibody (dilution 1:20000, Sigma-Aldrich), whereas human IgE was detected by a mouse HRP-conjugated anti-human IgE antibody (dilution 1:1500, Southern Biotech, Birmingham, AL, USA). Chemiluminescence reaction was carried out with Immobilon™ Western HRP Substrate (Millipore) and the image acquired by a Chemidoc XRS apparatus (Bio-Rad).

2.8. In situ digestion and peptide extraction

Protein spots of interest were excised with a razor blade and in-gel digested by trypsin (Promega, Mannheim, Germany) following the protocol described by (Shevchenko, Tomas, Havliš, Olsen, and Mann (2007).

2.9. MALDI-TOF-MS

The extracted peptides were spotted onto the target plate using dried droplet method, and the mass spectra were obtained on a Bruker UltrafleXtreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in reflectron mode. Instrument calibration was performed in a mass range from 500 Da to 4000 Da, using a peptide standard mixture. Database searches were conducted using the ion search of Mascot (Matrix Science, London, England) against SwissProt and non-redundant NCBI. The query was restricted to a database for the taxa of Metazoa (Animalia) with the following parameters: specific trypsin digestion, up to one missed cleavage, fixed global modification: carbamidomethyl (Cys), mass tolerances: ±0.8 Da. The protein spots were considered "significant hit" ($P < 0.05$) based on individual peptide ion score, directly calculated by Mascot programme as $10 \times \log(P)$, where P is the probability that an observed match is a random event. The identity of the spot is established as the protein that produced the highest score and consequently, the best match with peptide sequences.

2.10. nanoHPLC-ESI-MS/MS

Protein identification was performed by Proteome Factory (Proteome Factory AG, Berlin, Germany). The LCMS system consisted of an Agilent 1100 nanoHPLC system (Agilent, Waldbronn, Germany), Pico-Tip electrospray emitter (New Objective, Woburn, MA) and an Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany). Peptides were first trapped and desalted on the enrichment column (Zorbax 300SB-C18, 0.3 × 5 mm, Agilent) for 5 min (solvent: 2.5% acetonitrile/0.5% formic acid), then separated on a Zorbax 300SB-C18, 75 µm × 150 mm column (Agilent) using a linear gradient from 10% to 32% B (solvent A: 5% acetonitrile in water, solvent B: acetonitrile, both with 0.1% formic acid). Ions of interest were data-dependently subjected to MS/MS according to the expected charge state distribution of peptide ions. Proteins were identified by database search against the Swissprot protein database 01/2020 using MS/MS ion search of the Mascot search engine. Peptide mass tolerance was ±3 ppm, and fragment mass tolerance was ±0.6 Da. Carbamidomethylation of cysteine was assigned as fixed while Deamidation (NQ) and Oxidation (M) were selected as variable modifications.

2.11. Densitometry

Tropomyosin in the shrimp and cricket extracts was quantified by densitometry performed on Coomassie-stained SDS-PAGE gels, as described by Vincenzi et al. (2005). Different dilutions of the extracts and different amounts of rCr c 1 (from 0.25 to 4 µg) were separated by 10% SDS-PAGE as described above. After the staining, the gel pattern was acquired with ChemiDoc XRS apparatus (Bio-Rad), and analyzed by Quantity One software (Bio-Rad). The intensity of the bands relative to shrimp and cricket TM were quantified and compared to the rCr c 1 standard curve to infer the amount of loaded protein.

2.12. Competitive ELISA

Competitive ELISA was performed on Nunc MaxiSorp™ flat-bottom 96 wells-microplates (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). rCr c 1 protein (0.5 µg per well) was coated in PBS for 1 h at 37 °C. After washing with PBS buffer containing 0.5% Tween 20 (PTB buffer) wells were blocked with 1% BSA in PTB buffer for 30 min at 37 °C. Patients' sera were diluted at 1:10 in PTB and pre-incubated for 1 h with serial dilutions of cricket or shrimp extracts in the range of 0.016–0.001 µg/ml of tropomyosin equivalent. Then sera were placed in the wells and incubated for 3 h at 37 °C. After washing, wells were further incubated with an unlabelled mouse monoclonal anti-human IgE antibody (dilution 1:500, Southern Biotech) for 1 h at 37 °C and probed with a goat anti-mouse IgG HRP-conjugated antibody (dilution 1:1000,

Sigma-Aldrich) for 1 h at 37 °C both diluted in PTB.

Tetramethylbenzidine (Sigma-Aldrich) was used as the chromogenic substrate, and optical density (OD) was monitored at 450 nm by a Microplate Reader (BioTek Instruments, Inc.).

The percentage of inhibition was calculated as follows:

$$\%inhibition = \frac{Ab - Ax}{Ab} \times 100$$

where Ax is the absorbance of the inhibited sample and Ab the absorbance of the non-inhibited control. All analyses were carried out in triplicate.

2.13. Simulated gastrointestinal digestion

Simulated gastrointestinal digestion of the powders (shrimp and cricket) and the enriched biscuits was performed following the Infogest protocol described by Minekus et al. (2014) for simulated *in vitro* digestion with some modifications. The enzymes utilized for the digestion were: a) α -amylase from porcine pancreas (catalogue number: A3176, Sigma-Aldrich); b) pepsin from porcine gastric mucosa (catalogue number: P6887, Sigma-Aldrich); c) pancreatin from porcine pancreas (catalogue number: P1750, Sigma-Aldrich). The stock of simulated digestion fluids was produced as suggested by the protocol, whereas the final enzyme concentration for each digestion sample was 65 units of α -amylase/mL, 2250 units of pepsin/mL, and 99 TAME units for pancreatin/mL. All the samples were digested using a fixed enzyme: protein ratio (unit of enzyme: mg of protein) of 10:1 for pepsin and 2:1 for pancreatin.

Simulated digestion fluids and samples were mixed in a 1:1 ratio,

except for the oral phase in which the ratio was set to obtain the consistency of a paste, as indicated by the protocol. The reaction was performed at 37 °C in a rotating shaker, and aliquots of 100 μ L were collected after 2 min of oral digestion, 1 h of gastric digestion, and after 1 h and 2 h of intestinal digestion. The digestion was stopped by adding 2 volumes of SDS-PAGE buffer and heating the samples at 100 °C for 5 min. The samples were centrifuged at 21,000g at room temperature, and the supernatants were collected and stored at -20 °C.

3. Results

3.1. Immunoreactivity of shrimp and cricket proteins

The immunoreactivity of both shrimp and cricket extracts was assayed by immunoblotting using 20 sera from shrimp-allergic/sensitized patients (Fig. 1A and B). The binding profile varied substantially according to the serum used. Sixteen out of 20 patients (80%) showed positive signals towards shrimp proteins in the range 20–200 kDa (Fig. 1A), the negative control was represented by the serum of a non-sensitized donor who did not show any reaction to shrimp proteins (lane N). Lanes 1, 7, 10, 11, 12 presented a particular intense IgE reactivity, serum 20197-BH (lane 1) and sera VM75, VM76 and VM78 (lanes 10, 11, and 12 respectively) produced a strong signal, in accordance to their moderate to high CAP values, for proteins with a molecular weight range comprised between 30 and 66 kDa.

However, not all the tested sera produced a reaction for the shrimp proteins, despite their positive CAP value. For instance, sera 15742-JT (lane 3) and 2496-LB (lane 6) showed positive CAP values to shrimp proteins, but no IgE-binding protein was detected, probably due to

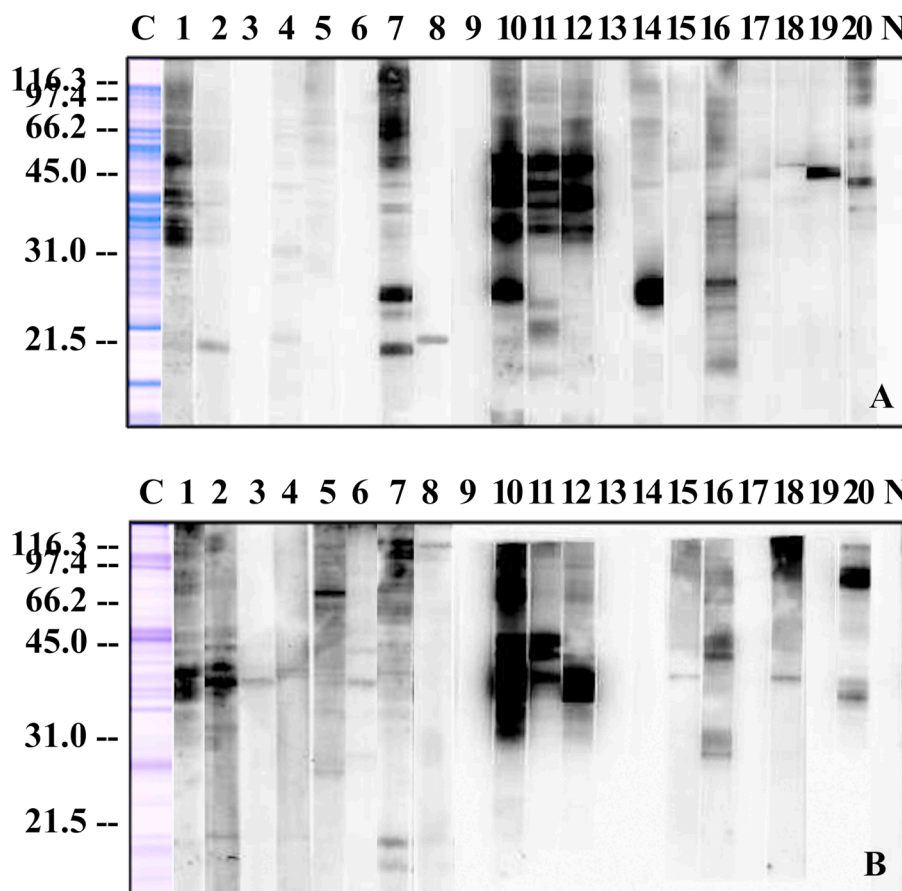


Fig. 1. SDS-PAGE (12%T) and immunoblotting of shrimp (A) or cricket (B) protein extract. Lane C, Coomassie staining; lane 1–20 patient's sera: 1) 20197-BH; 2) 9735-RE; 3) 15742-JT; 4) 17694-SS; 5) 20770-MH; 6) 2496-LB; 7) 20318-SN; 8) 20775-AW; 9) VM74; 10) VM75; 11) VM76; 12) VM78; 13) VM79; 14) VM80; 15) VM81; 16) VM82; 17) VM83; 18) VM84; 19) VM85; 20) VM88; Lane N: non-sensitized control.

different extraction protocols and/or to the denaturing action of SDS that might have affected IgE epitopes.

Similarly to what was observed for shrimp, the percentage of sera that showed IgE reactivity to cricket proteins accounted for 75%, but the IgE binding pattern of each serum was quite different (Fig. 1B). Serum VM75 (lane 10) was the most reactive also towards cricket proteins, producing a strong and extended signal. Patients VM80, VM83 and VM85 (lanes 14, 17, and 19, respectively), despite showing IgE binding to shrimp proteins, did not recognize any cricket proteins.

3.2. 2-DE analysis and protein identification

To further characterize and identify the cricket and shrimp immunoreactive proteins, the two protein extracts were analyzed by 2-D electrophoresis (Fig. 2). Fig. 2A shows the Coomassie-stained 2-DE gel profile of the shrimp extract, in which several protein spots were separated between 14.4 kDa and 200 kDa in the pI range 4–7. 2-DE spots were assayed by immunoblotting using four selected patients' sera i.e., VM75, VM76, VM78 and VM80 (Fig. 2, panels B, C, D, and E). Fig. 2F shows the detection by the mouse anti-TM polyclonal antibody: this recognized a spot around 36 kDa, consistent with tropomyosin (the spot "a" in Fig. 2A) and a protein doublet at a slightly lower molecular weight, at 31 and 33 kDa. The same IgE-binding pattern was observed for sera VM75, VM76 and VM78. A different profile was shown by serum VM80, that produced a signal at ~ 22 kDa (Fig. 2, panel D), in agreement

with the immunoblotting presented in Fig. 1A. The case of serum VM76 is peculiar since, as shown in Fig. 1A, it recognized proteins around 36–38 kDa, compatible with TM, and at 45 kDa, but once used to probe 2D-separated proteins the 45 kDa protein was not detectable (Fig. 2C).

Among the proteins recognized by tested patients' sera, MS analysis allowed to identify only the spots "a" at 36 kDa and "e" at 22 kDa (Fig. 2, panel A), as TM and sarcoplasmic calcium-binding protein (SCP) respectively. For spots "b", "c" and "d" the results obtained by MS analysis were not statistically relevant ($p > 0.05$). The spots at 31 and 33 kDa, recognized by both anti-TM antibody and by patients' sera, might represent tropomyosin isoforms as already described by Itoh and Fujinoki (2008), who characterized 3 TMs in the mussel *Mytilus galloprovincialis* by a 2-DE approach.

Fig. 2G shows the 2-DE profile of the cricket flour extract. IEF has been performed using a wider pH range to obtain an optimal separation of the cricket protein on the base of their pI.

The same patients' sera and the anti-tropomyosin polyclonal antibody were used to identify putative allergenic cricket proteins, (Fig. 2, panels H–L). Different binding patterns were obtained: sera VM75 and VM78 recognized a highly reactive 38 kDa protein spot and other faint spots in the range between 70 and 100 kDa (Fig. 2H and J), whereas serum VM76 detected several spots at 48–50 kDa and pIs ranging from 3.5 to 7.0 (Fig. 2I). A weak signal was also detected a 38 kDa. As expected, serum VM80 tested negative (Fig. 2K) according to Fig. 1B (lane 14), while the binding pattern obtained with the polyclonal antibody

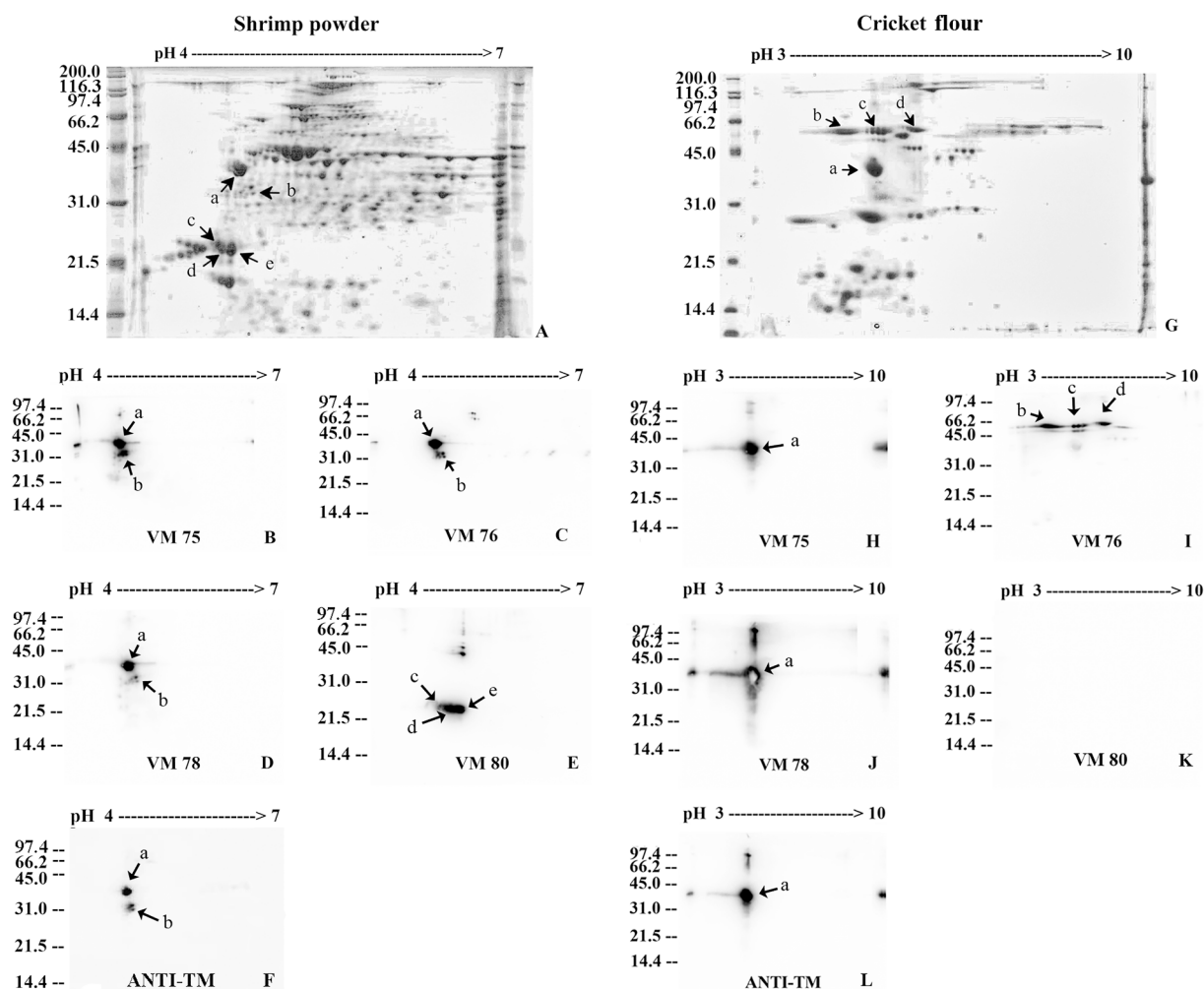


Fig. 2. 2-DE (12%T in 2nd dimension) separation of shrimp powder (A–F) and cricket flour (G–L) protein extracts. Coomassie staining (A, G); immunoblotting developed with patient's serum VM75 (B, H), VM76 (C, I), VM78 (D, J), VM80 (E, K), and polyclonal anti-TM antibody (F, L). The protein spots indicated by the letters were analyzed by MS.

(Fig. 2L) presented the same 38 kDa recognized by patients' sera. This protein was finally identified by HPLC-MS/MS as tropomyosin. The anti-TM tropomyosin antibody also recognized higher MW spots with a binding pattern very similar to that obtained with sera VM75 and VM78. We can speculate that these spots might represent tropomyosin oligomers due to the innate tendency of this protein to dimerize (Hooper & Thuma, 2005).

3.3. IgE-crossreactivity to TM

The potential cross-reactivity between shrimp and cricket tropomyosins was studied by competitive ELISA on rCra c 1 coated plates. The binding of IgE to the immobilized protein was inhibited by either shrimp or cricket tropomyosins pre-incubating the sera of selected rPen a 1-sensitized allergic patients, i.e., VM75, VM76, VM78, and VM88 with shrimp or cricket extracts at different dilutions. To this purpose, the concentration of the respective TMs in the extracts was measured by densitometry (data not shown). Serum VM80 was not employed since, as mentioned before, it did not react to TMs.

In the case of serum VM75 (Fig. 3A), shrimp and cricket extracts produced very similar inhibition profiles, at the highest concentration of inhibitor used the percentage inhibition of the IgE-binding to the rCra c 1 was 74% and 72% respectively. Similar results were obtained with sera VM76 and VM88 (Fig. 3, panels B and D) and indicate a high level of epitopes homology. On the contrary, for serum VM78 (Fig. 3C), the inhibition profile given by the cricket extract differed from the self-inhibition achieved with the shrimp extract.

3.4. Stability to simulated gastrointestinal digestion

Resistance to simulated gastrointestinal digestion could be a marker

for potential allergenicity of a given protein, which can also be affected by the food processing. To evaluate the digestion profile of shrimp and cricket proteins and whether the baking process might modify their IgE-binding capacity and their susceptibility to digestion, selected patients' sera (i.e., VM75, VM76, VM78, and VM80) were used to assay the IgE-binding properties of the digests.

Fig. 4 shows the digestion pattern of shrimp powder and shrimp-enriched biscuits, whereas Fig. 5 shows the digestion pattern of cricket flour and cricket-enriched biscuits. In order to render the results between each ingredient (shrimp powder or cricket flour) and the related baked product comparable, in all electrophoretic lanes the equivalent of 80 µg of either shrimp or cricket proteins was separated, regardless of wheat protein amount.

3.4.1. Shrimp powder

The immunoblotting carried out with sera VM75, VM78, together with the polyclonal antibody, recognized the 36 kDa band (lane 1 of Fig. 4, panels B, C, and E), i.e. shrimp TM. The protein was digested during the gastric phase, with the consequent appearance of lower MW bands deriving from the fragmentation of the protein in peptides. The fragments could be immunodetected up to 1 h during the intestinal phase. The detection with serum VM76 (Fig. 4, panel D), showed a major immunodetected band at 45 kDa only in the undigested sample with a lower signal around 36 kDa. Noteworthy, the major band was observed in Fig. 1A but not in Fig. 2C, suggesting that this protein might be precipitated during the first separating dimension, i.e., isoelectric focusing, or that the IgE epitopes are sensitive to alkylation as already shown in a previous work of our team (Sega et al., 2012).

As already observed in Fig. 2, patients' sera VM75 and VM80 (Fig. 4, panels C and F) showed reactivity to a 22 kDa protein identified as SCP. SCP remained visible through all the digestion process, indicating that

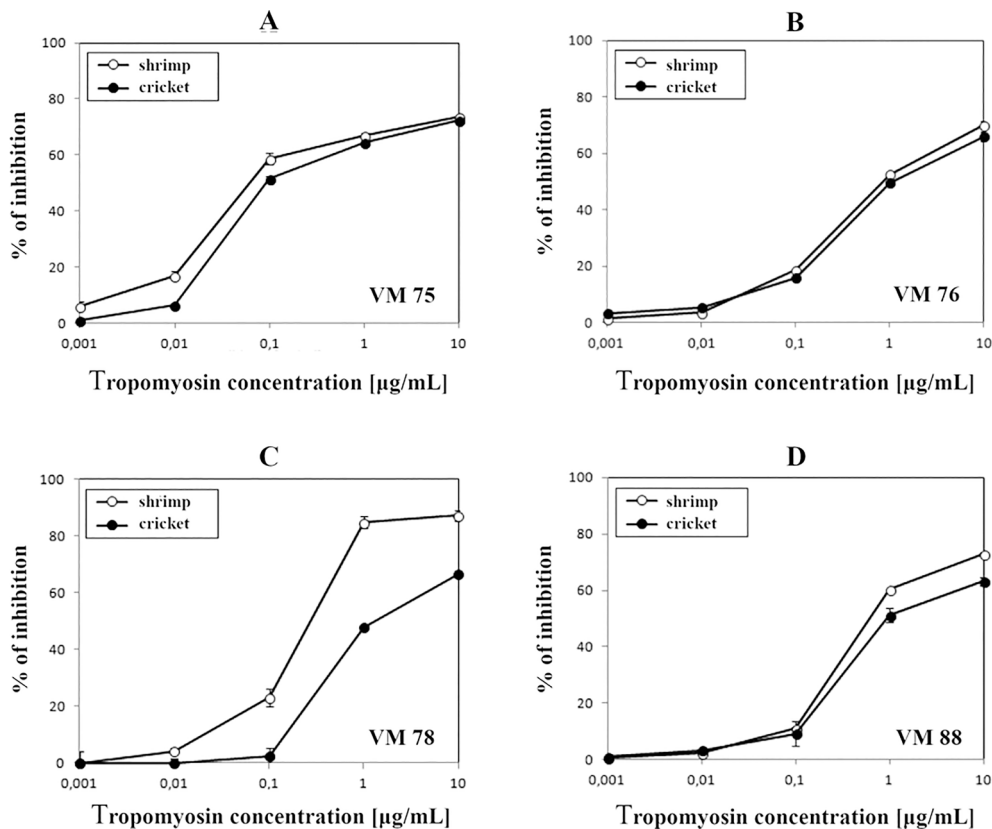


Fig. 3. Competitive ELISA performed with selected patients' sera. Sera were pre-incubated with the correspondent arthropod extract at different dilutions, ranging from 0.001 to 10 µg/mL of tropomyosin equivalents. White and black dots represent the inhibition of the IgE-binding given by shrimp and cricket extracts respectively. The results are presented as mean $\pm$ standard deviation of three independent experiments.

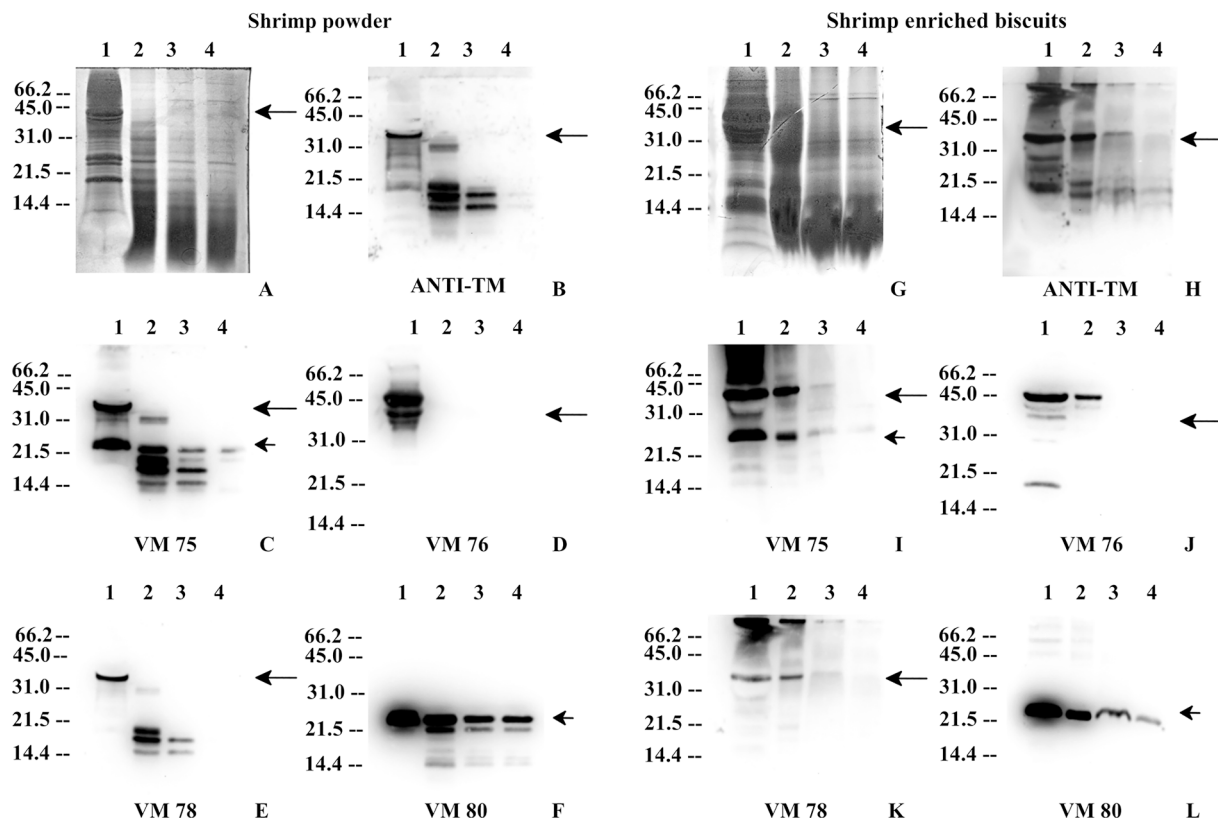


Fig. 4. SDS-PAGE (16%T) of the digestion products of shrimp powder (A-F) and shrimp-enriched biscuit (right G-L). Coomassie staining (A, G), immunoblotting incubated with: polyclonal anti-TM antibody (B, H), serum VM75 (C, I), serum VM76 (D, J), serum VM78 (E, K), serum VM80 (F, L). 1: undigested sample; 2: sample after 1 h of gastric digestion; 3: sample after 1 h of intestinal digestion; 4: sample after 2 h of intestinal digestion. Long arrows indicate TM, short arrows indicate SCP. In each lane 80 μ g of shrimp protein was loaded.

the IgE-binding capacity of this protein is minimally affected by proteases.

3.4.2. Shrimp-enriched biscuits

The polyclonal anti-TM antibody (Fig. 4, panel H) and the sera VM75 and VM78 (Fig. 4, panels I and K) recognized TM also in the biscuits extract, confirming its stability to the baking process, but in this case, the protein was detectable also after the gastric phase. A weak signal recognized by the polyclonal antibody and serum VM75 is observable also after 1 h of intestinal digestion. The binding pattern of serum VM76 (Fig. 4J) allowed detecting the 45 kDa protein band observed for shrimp powder (Fig. 4D) that remained partially detectable after the gastric digestion. As observed for the shrimp powder, the IgE of sera VM75 and VM80 reacted to SCP (Fig. 4, panels I and L), with a signal detectable through all the digestion process, even though the small peptides (<20 kDa), well visible in Fig. 4C and F, here were not recognized by IgE. Anyway, the results confirmed that the baking process did not much alter the immunoreactivity of SCP nor its susceptibility to proteolysis. Serum VM76 recognized only a smaller MW protein band (18 kDa) (Fig. 4J) that was proteolyzed during the gastric phase.

Notably, in comparison with the results obtained for shrimp powder, the immunodetection of biscuit samples gave rise to several “apparent” aspecific signals using the polyclonal antibody and the patients’ sera, especially in the case of VM75, VM78, and VM80. The possibility that the signal observed in biscuit extract, but not in shrimp powder, might be originated by sensitization to wheat proteins was excluded since the immunodetection of wheat protein with the same sera gave negative results (data not shown).

3.4.3. Cricket

With respect to the digestion of cricket flour (Fig. 5), tropomyosin at

38 kDa seems to be only partially digested by pepsin, and in the samples collected upon intestinal digestion, a protein band with a slightly lower MW (36 kDa) was immunodetected (Fig. 5B, C and E), without evidence of lower MW fragments. This would indicate that the cricket tropomyosin is more stable to digestion than the shrimp homologue. In the blotting incubated with serum VM76 (Fig. 5D), the detection of a doublet at 48–50 kDa (in accordance with Fig. 2I) was visible only in the undigested sample, while a very faint signal at 38 kDa was detectable at the following time point.

3.4.4. Cricket-enriched biscuits

Concerning the digestion of cricket-enriched biscuits, the incubation with the anti-TM antibody (Fig. 5G) and the sera VM75 and VM78 (Fig. 5, panels H and J, respectively) showed a similar pattern of digestion, with TM almost unaffected during the gastric phase. Even if during the intestinal phase the intensity of the tropomyosin signal decreased, this was still visible up to 1 h, whereas the fragments at lower MW were detectable up to 2 h of digestion.

On the contrary, serum VM76 (Fig. 5I) showed strong reactivity to the cricket proteins, producing an extended and intense signal in a range of molecular weight from 30 kDa to 50 kDa, partially detectable also after the gastric phase. Like in the case of shrimp-enriched biscuit, also, in this case, IgE binding proteins at very high MW were detected.

4. Discussion

Edible insects represent a potential nutrients source that might contribute to solving the great food security challenge that will arise in the next years due to the progressive growth of the world population.

A. domesticus is considered a potential nutrient source due to its attractive nutritional profile and lower feed conversion ratio compared

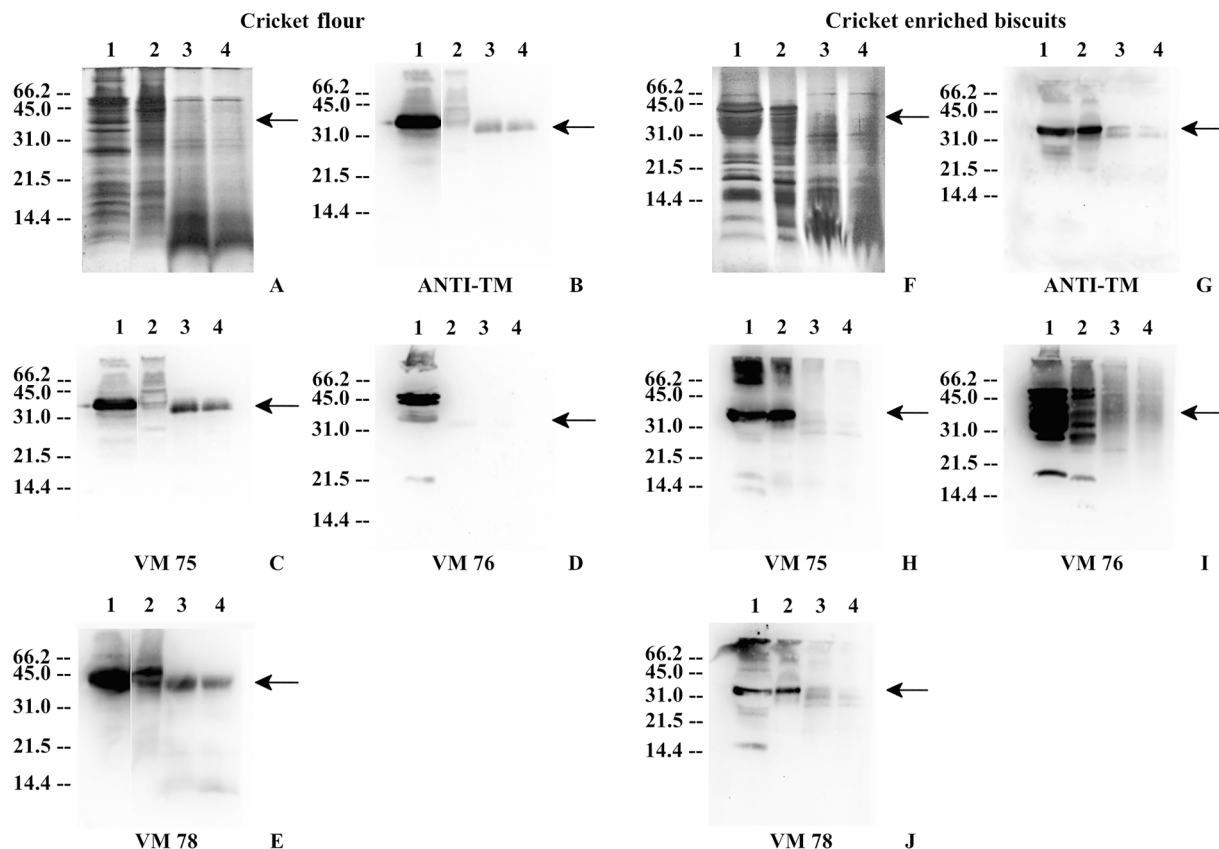


Fig. 5. SDS-PAGE (16%T) of the digestion products of cricket flour (A-E) and cricket-enriched biscuit (right F-K). Coomassie staining (A, F), immunoblotting incubated with: polyclonal anti-TM antibody (B, G), serum VM75 (C, H), serum VM76 (D, I), serum VM78 (E, J). 1: undigested sample; 2: sample after 1 h of gastric digestion; 3: sample after 1 h of intestinal digestion; 4: sample after 2 h of intestinal digestion. Long arrows indicate TM. In each lane 80 μ g of cricket protein was loaded.

to other animals. However, an evaluation of its allergenic potency at the molecular level is still missing. This is necessary since edible insects are considered “novel foods” according to EU regulation 2015/2283 and because the primary sensitization to other invertebrates like crustaceans might lead to clinical manifestations upon ingestion of the insect due to clinical cross-reactivity mechanism.

The results of the immunodetection experiments carried out with shrimp allergic patients' sera showed that in most of the cases, cricket proteins present epitopes recognized by shrimp sensitized patients. Different IgE-binding patterns depending on the subject were detected, thus highlighting a strong inter-individual variability, probably as a consequence of the sensitization to different proteins. Indeed, it is possible that the co-sensitization to other allergens, such as house-dut mites, might contribute to the variability of the IgE-binding profiles (Van Broekhoven, Bastiaan-Net, De Jong, & Wichers, 2016). Negative results observed in some cases might be due to the lack of cross-reacting epitopes, but also to a low concentration of specific IgE, like in the case of serum VM74, incapable of reacting even towards shrimp proteins. However, we cannot exclude that denaturing agents like SDS or urea may have altered the nature of structural epitopes or that the thermal treatment operated by the producer to stabilize crickets from a microbiological point of view might have affected some epitopes.

To gain information on the nature of the IgE binding proteins, 2-DE and immunoblotting with selected patients' sera were carried out. The analysis of the spots excised from the 2-DE gels performed by MALDI-TOF showed positive hits for shrimp, leading to the identification of tropomyosin and SCP, but in the case of cricket, no statistically significant identifications were obtained (data not shown). For this reason, the cricket putative TM spot (spot “a” in Fig. 2G) was also subjected to HPLC-MS/MS that allowed us to identify the IgE-binding protein as

tropomyosin.

The cross-reactivity between shrimp TM and cricket TM was confirmed by competitive ELISA. Indeed, the results indicate that the cricket and shrimp tropomyosins share high epitope homology since, in the majority of the cases (VM75, VM76, and VM88), almost superimposable IgE-binding inhibition profiles were obtained. The serum VM78 presented a different pattern in which cricket extract inhibited the IgE binding to rCr a 1 only at higher concentrations in comparison to shrimp extract. This would suggest that the serum either contains a lower titer of IgE or that this is characterized by less affinity for cricket tropomyosin compared to the other sera.

The assessment of the stability upon food processing and gastrointestinal digestion of cricket proteins, when used as ingredients to enrich food products, is crucial to infer essential data about the risk associated with their ingestion. In our work, we decided to employ a simple but realistic model of a bakery product such as biscuits, enriched either with 10% shrimp powder or cricket flour, and to investigate the stability of the epitopes by immunoblotting (Fig. 4 and Fig. 5). In our hands, shrimp TM showed scarce stability to gastric digestion even if IgE epitopes harbored on the generated fragments were recognized up to 1 h of intestinal digestion. These results are coherent with the work of Gámez et al. (2015), who observed that tropomyosin from the shrimp *Solenocera melanthero*, either purified or present in the shrimp extract, did not resist peptic digestion, even if the latter was more stable. On the contrary, Liu et al. (2019) observed a complete degradation of *Litopenaeus vannamei* TM by simulated gastric fluid only when the shrimp was previously boiled for 15 min. In our case, shrimps were boiled for two minutes to simulate a microbiological stabilization process, so it is possible that this short time did not bring to a significant alteration of the structure of TM.

The different results obtained in our study compared to the literature may also be attributed to different settings of gastrointestinal digestion parameters, in particular to the enzyme-to-protein ratio. We employed a largely accepted consensus protocol (Minekus et al., 2014) which is supposed to simulate to the best the human physiologic conditions of digestion. However, a specific enzyme: protein ratio has not been considered by the authors, and for this reason, we decided to introduce a fixed ratio in order to normalize the digestion conditions and to allow the comparison of the proteolysis of the different samples as they possess different protein concentrations. Another point that might explain the differences between our results and those described in the literature is the choice to treat the entire food matrix rather than an extract. In our opinion, the digestion of the whole matrix is the best choice since it is more representative of the real digestion process and takes into account the differences among the food constituents. In the present study, even though TM is degraded, the generated epitopes are still immunologically active, being these recognized by the patients' sera and by the polyclonal anti-TM antibody. This contributes to explain why TM are considered allergens capable of inducing the primary sensitization of a subject (Reese et al., 1999). The results concerning SCP strongly indicate that this protein is highly resistant to digestion, in agreement with the results of Chen et al. (2013) on crayfish SCP.

The analysis of shrimp-enriched biscuits highlighted a sort of protective effect exerted by the complex food matrix towards IgE-binding proteins. Rather than becoming more susceptible to digestion as a consequence of the thermal treatment, TM was recognized by patients' sera IgE after the gastric digestion and also up to 1 h of intestinal digestion. Also, the 45 kDa band, when extracted from biscuits, was recognized by serum VM76 after gastric digestion. This finding is of great importance since it indicates that the clinical outcome of its ingestion might vary, depending on the physiochemical modifications of TM and the interactions with other food matrix components occurred during processing (Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005) and on the interactions established (Mouécoucou, Villaume, Sanchez, & Méjean, 2004; Schulten, Lauer, Scheurer, Thalhammer, & Bohle, 2011). Although Liu et al. (2019) observed that similarly to tropomyosin, also SCP stability to digestion is reduced by previous thermal treatment, in our study, SCP showed to be highly stable to both gastric and intestinal environments even if it underwent the baking process.

The simulated digestion of cricket flour showed a different proteolysis pattern for TM. Indeed, even if this was partially digested during the gastric phase, the action of proteases seems to be limited to a region close to either the N or C terminus of the protein since the MW of the generated fragment was similar to that of the undigested protein, and no lower MW fragments were visible.

Also, in the case of cricket-enriched biscuits, the immunoblotting results indicated an improvement of the stability of TM in the gastric phase with the persistence of the intact protein during the first part of the intestinal digestion (1 h), followed by the generation of lower MW fragments immunodetectable until the last phase of the digestion process, even if with apparently less intensity. The lack of reactivity of IgE from shrimp allergic patients to cricket SCP was expected. In fact, while crustacean's SCPs share a high percentage of sequence homology (80–98%), this is not the case among crustaceans and molluscs (15–21%) (Mita, Koketsu, Ishizaki, & Shiomi, 2013). Inhibition experiments also confirmed the lack of cross-reactivity between crustaceans SCPs and those belonging to arthropods like mites and cockroach (Ayuso et al., 2009), suggesting that SCP is involved only in cases of cross-reactivity between crustaceans.

In both kinds of enriched biscuits we also observed high molecular weight signals that were not detectable in shrimp and cricket. The signals are probably due to aggregation phenomena between arthropod and wheat flour proteins catalyzed by the high temperatures typical of baking processes. In this way, IgE could to higher MW protein aggregates. Similar results were observed previously for wheat allergens (Simonato et al., 2001).

In conclusion, our results demonstrate that there is a high risk that individuals sensitized to crustacean's like shrimp might experience allergic symptoms upon ingestion of *A. domesticus* proteins, especially when tropomyosin is involved since a high degree of IgE cross-reactivity was clearly shown. This has been recently reported also for the orthopteran *Gryllus bimaculatus* (Kamemura et al., 2019). The particular resistance of *A. domesticus* tropomyosin to gastrointestinal digestion and its enhanced stability after the baking process suggests that increasing the consumption of this protein among populations might also lead to a primary sensitization of subjects, opening a new scenario in which the cross-reactivity with other invertebrates (e.g., cockroach, dust mites, mealworm, etc.) might bring to new clinical outcomes upon their contact or ingestion. However, more information, especially on the IgE epitopes of *A. domesticus* tropomyosin, needs to be addressed.

CRediT authorship contribution statement

Laura De Marchi: Investigation, Writing - original draft, Data curation. **Federica Mainente:** Investigation, Writing - original draft, Data curation. **Massimo Leonardi:** Investigation. **Stephan Scheurer:** Methodology, Resources. **Andrea Wangorsch:** Investigation. **Vera Mahler:** Resources. **Rosa Pilolli:** Data curation. **Daniela Sorio:** Investigation. **Gianni Zoccatelli:** Conceptualization, Supervision, Writing - original draft.

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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