UNIVERSITÀ DEGLI STUDI DI UDINE
hic sunt futura

www.uniud.it

Corso di dottorato di ricerca in:

“Scienze e Biotecnologie Agrarie”

Ciclo XXXIII

Molecular based identification of insect ingredients in Feed and Food

Dottorando
Dott. Enrico Daniso

Supervisore
Prof.ssa Francesca Tulli

Anno 2021

Abstract

The use of insects as ingredients for animal feeds production or as processed products for human consumption has increased significantly in recent years. At the same time, the lack of methods for the detection of these compounds requires the adaptation and improvement of some technologies for their detection. My PhD thesis was mainly aimed to give a contribution towards this objective.

Indeed, with suitably designed primers, it was possible to detect the presence of target DNA in complex matrices such as feeds, bars and other mixtures by RT-PCR technology. Specifically, it was possible to accurately discriminate the presence of *Gryllodes sigillatus* within detecting up to 0.02 ng / µl of target DNA in compounds subjected to different technological processing treatments and with the possible presence of inhibitors. Therefore, the combination of a specific pair of primers is able to certify the unique identification of the target species. However, if the method has to be applied for relative quantification, specific calibration curves need to be developed and validated for the different technological processes as they affect DNA integrity. Subsequently based on the results of PCR, an innovative OLED-based biosensor based on target DNA recognition was developed. After several investigations on the more appropriate chemical deposition reaction of primers PCR-derived DNA probes, the presence of *Hermetia illucens* has been detected in several commercial compound feeds. The technology was able to detect target DNA with a detection limit of 0.75 ng / µl; without false positive results and avoiding the need for amplification as occurs in PCR approach. Data obtained by the biosensor system were validated by RT-PCR analyzes, which confirmed the reliability of the new system for future applications in the food and feed control area. The positive results support the implementation needed to make this system a Point of Care.

Another subject considered in my thesis work was the evaluation of the effect of the dietary inclusion of *Hermetia illucens* (HI) meal on the activation of specific metabolic pathways in the gastrointestinal tract of gilthead sea bream within the EU AQUAEXCEL project carried out at the HCMR (GR) laboratories. Results underlined that it is possible to partially replace the traditional ingredients with HI meal resulting in positive effects on the gastrointestinal tract. In particular, the presence of this new ingredient resulted in a mitigation of the tissue damage of the gastric mucosa due to the use of vegetable-based diets. In addition, animal growth performance was not hampered by the dietary composition and the overexpression of hepatic SOD suggests an increase in antioxidative capacity. In any case, a dose response effect of the enzymes for the hydrolysis of chitin was not detected although the expression of these genes was analysed.

Content

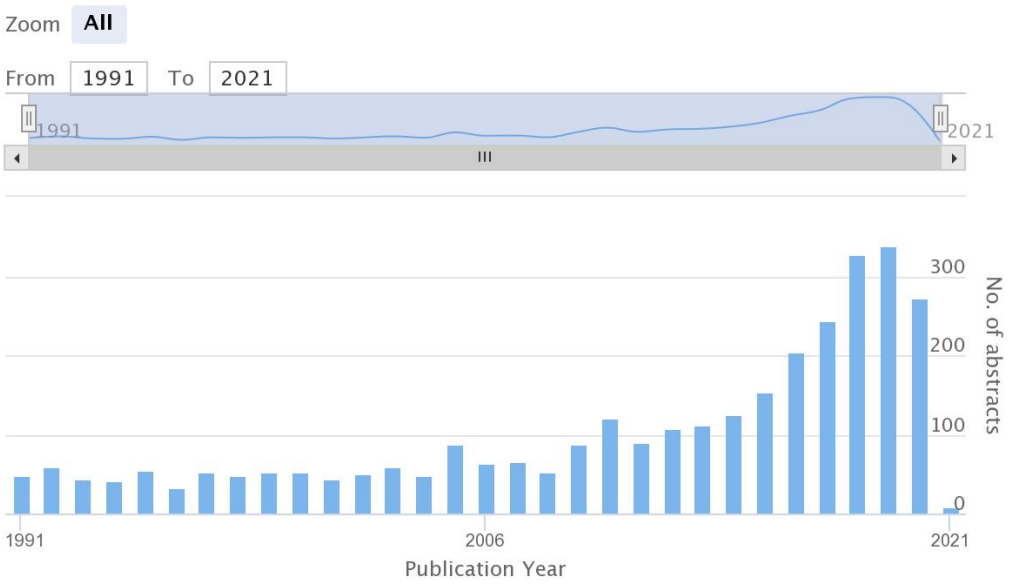
INTRODUCTION

1 Insects new perspectives

Entomophagy, or the act of eating insects, has been a common practice that began with early hominids (Barennes et al., 2015), for this reason, insects have been part of the human diet for centuries (Bukkens, 1997). In tropical countries, edible insects are often used for home consumption or they provide a source of income when marketed. They are traditionally harvested from nature and they are a seasonal product as most species depend on host plants; anyway because of the enlarge deforestation, agricultural intensification, uncontrolled use of pesticides, habitat changes and environmental pollution, this natural resource can be threatened (Ramos-Elorduy, 2006; van Huis and Oonincx, 2017).

However, if entomophagy is already a common practice and an important food resource in the eastern countries, recently the interest towards edible insects started to be relevant for food and feed purposes in western cultures and insects are being proposed as an alternative protein source for livestock, fish and humans. This new worldwide trend follows the document entitled "Edible Insects: Future Perspectives of Food and Nutrition Security" from the Food and Agriculture Organization of the United Nations (Fao, 2013), where insects have been promoted as human food and animal feed. After this publication, the increased interest towards insect and insect derivatives is illustrated by the rise of scientific publications on the topics (Figure 1) and is also registered in the food market (Jantzen da Silva Lucas et al., 2020) following the exponential growth in companies that establish standardized pipelines to mass production of insects for commercial use (Francuski and Beukeboom, 2020). With a growing world population, increasingly demanding consumers, and the restricted availability of agricultural land, there is an urgent need to increase food supply and to find alternatives to conventional protein sources (van

Figure 1 Number of research article concerning edible insects in the period 1971-2021. Data from Cab direct 11.10.2020)



Huis and Oonincx, 2017). As the world population rises, the global food system faces an impending crisis and a major component of this crisis is the forecast that the livestock sector is growing at a rate that is deemed unsustainable and is an important contributor to climate change. According to several authors, current livestock production contributes greatly to several environmental problems such as acidification due to leaching of ammonia, climate change due to greenhouse gas emissions, loss of plant

biodiversity, desertification, deforestation, soil erosion, and water pollution (Foley et al., 2011; Gerber and Hui, 2013; Herrero et al., 2016; Herrero and Stuckey, 2015; Steinfeld et al., 2006).
For those reasons, insects have been proposed as one of the most promising alternative sources of proteins and have been evaluated to solve the global issue of protein shortage and enlarge the panel of new and safe food and feed available on the market. Edible insects are currently celebrated as a sustainable solution to food insecurity and environmental pollution when compared to the meat industry across the globe (Fao, 2013; Gahukar, 2016; Tomberlin and van Huis, 2020) .
The insect-based food and feed industry already produces tons of insects daily (Van Huis, 2016) and their use as a feed ingredient is showing an increased interest too (van Huis and Oonincx, 2017). In most European countries so far, insects have been generally used for pet feed (such as birds, reptiles, and amphibians) rather than for human or farm animal consumption. However, this trend seems to be changing mainly due to research efforts in this field and they started to be included in feeds not only for pets but also for farmed animals like pigs, poultry and fish (Biasato et al., 2019).

1.1 Sustainability and Environmental impact of insect production

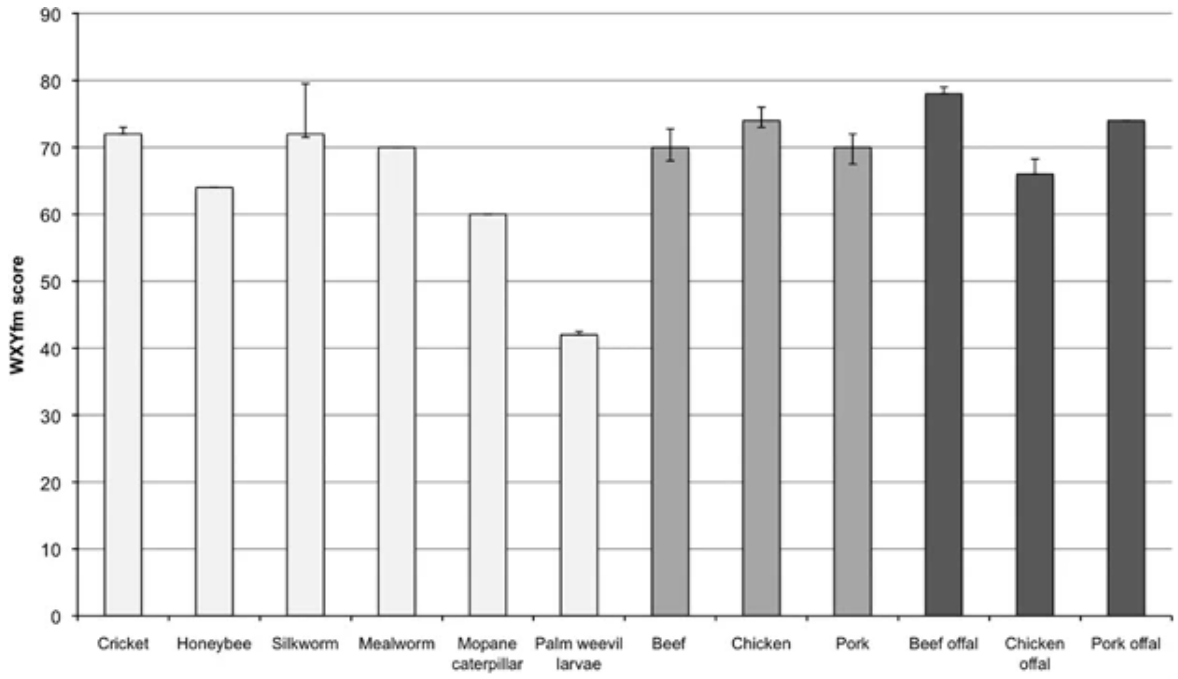
Sustainability of the livestock production systems is increasingly important in the world we live in (Gerber and Hui, 2013). Therefore, alternative food sources must be found in order to replace traditional ones, less sustainable, because livestock contribute to decrease food supply since a third of the world's cereal production is fed to animals, but it could be used for human consumption and they contribute approximately to 14.5% of all anthropogenic greenhouse gas (GHG) emissions (Gerber and Hui, 2013; Mottet and Tempio, 2017). The increase in global demand for meat and the restricted availability of land prompt the search for alternative protein sources (FAO (Food and Agriculture Organization), 2011; Jongema, 2017). On all, the above-mentioned negative effects, insects perform better. Insects face several challenges approaching in the near future: population growth and increasing demand for animal protein, food and feed insecurity and environmental pressures (Tomberlin and van Huis, 2020). Insect farming is one of the most efficient protein production systems and could challenge the increasing demand for protein products as well as the limited land area available for agriculture (Sosa and Fogliano, 2017; Veldkamp et al., 2012). The impact of rearing insects on the environment has several implications. Principally at the food chain level, as in an ideal case, insects could be directly used as food, which would maximise the gain because they are thus not an extra trophic level in the food web, but replace another trophic level. When insects are used as feed material an extra trophic level in the food production chain or web is plugged in. In addition, insects can be reared on organic side streams, contributing to a circular economy, now pursued by most governments as about one-third of our food and agricultural production is wasted (Food and Agriculture Organization, 2013).The mass production of insect presents environmental advantages from an environmental perspective because of the low land and water utilization/competition for their production (Hoekstra and Mekonnen, 2012), low greenhouse gas emissions (Gerber and Hui, 2013), high feed conversion efficiencies (Makkar et al., 2014; van Huis and Tomberlin, 2017), possible breeding on bio-waste, enabling the transformation of bio-waste biomass into high-quality products (Meneguz et al., 2018) and possible use of certain insect species in pig and poultry diet or in aquafeeds, for the substitution of traditional ingredients, which are becoming increasingly scarce or expensive on the market. Insects do not produce methane, they have a high reproduction rate and a short maturation period; furthermore, their feed conversion ratio is similar to values reported for chicken (2.3), higher than the figure reported for the most of finfish species, but lower than that for pigs (4.0) and beef cattle (2.7−8.8) (Oonincx and de Boer, 2012). While the poikilothermic nature of insect species improves their ability to efficiently convert food into body mass, this alone cannot be the sole characteristic that denotes edible insects as an environmentally sustainable food option. Husbandry contributions to GHG emissions are much lower for insects (2−122 g/kg mass gain) than for beef cattle (2850 g/kg mass gain), and in the lower range when compared to pigs (80−1130 g/kg mass gain). While insects for food and feed show great potential as an environmentally friendly choice, there is still very limited information to enable the complete assessment of the sustainability of the whole production systems to be undertaken. So further investigations and researches are requested to provide a more complete picture of the LCA of the insect mass production systems (Halloran et al., 2016; van Huis and Oonincx, 2017).

1.2 Insects and nutritional aspects

It is difficult to make general statements about the nutritional value of insects and data reveal that their nutritional value varies widely between species and according to environmental rearing conditions (temperature, humidity and light conditions).
Insects have received increased attention as an important source of sustainable raw materials for animal feeds, especially in fish, poultry and swine and it has been assumed that these animals are evolutionarily adapted to eating them as a part of their regular diet because insects are consumed naturally. In particular, the most promising species in western countries are represented by the black soldier fly (*Hermetia illucens*, HI), the yellow mealworm (*Tenebrio molitor*, TM), and the common house fly (*Musca domestica*, MD).

The nutritional value of some insect species for human diet reveals that insect products are better than meat, as can be seen in the first systematic comparison of the nutritional composition of insects and meat and their relative healthiness according to contemporary nutrient profiling models for human diet utilizing the 'Nutrient Value Score' (NVS) developed by the United Nations World Food Programme to inform the composition of food baskets and commodity vouchers (Figure 2). It classifies individual foods on a continuous scale based on their nutrient composition, with an emphasis on micronutrient content (Payne et al., 2016)

Figure 2 – Bar graph showing the median values and the interquartile range of Nutrient Value Score for insects, meat and offal. Higher score indicate healthier foods (from Payne et al., 2016)



As a starting point, a large part of the literature focused on the nutritional quality and the benefit related to the whole insect consumption. Currently, insects are commercialized as whole insects, paste, in-ground, de-fatted meal, but also as oil. The attention focused on proteins and the most important parameter is their total concentration.
When measured on a fresh weight basis, the insect protein concentration is within the range of other animals, being between 10 and 30%. This makes the insect proteins particularly interesting from a nutritional point of view, not only for their high concentration and the adequate amino acid composition, but also for their potential to meet all the criteria of a protein source of the future, being sustainable, healthy, affordable, reliable and, palatable according to the principles depicted in the recent EU-

SUSFANS project (Rutten et al., 2018).

Insects are rich in lipids. This component is either obtained from the diet or *de novo* synthesized and its content ranges from 10 up to 50% dry matter basis. The amount of lipids that can be extracted from insects depends on the species and the range of extraction capacity could be between 40 and 82% of the total lipid content. Insect lipids are composed mainly of triacylglycerols and in minor amounts, they include cholesterol, partial glycerides, free fatty acids (FFA), phospholipids and wax esters. This derived by-product is usually obtained by an aqueous oil extraction method, which is a green alternative to conventional oil extraction with n-hexane (Kumar et al., 2017)and also provides oils that do not require further oil refinements (Latif and Anwar, 2011; Mat Yusoff et al., 2015; Preece et al., 2017). The oil that is usually obtained from the insects presents a wide range of melting peaks, that go from -30.7 up to 22.7 °C that make them liquid-like at room temperature as insect oils are rich in unsaturated fatty acids (>60% of total FA) being C18:1n9 and C18:2n6 the most abundant. In certain species, like black soldier fly, the lipid composition is characterized by high content in saturated FA being lauric (C12:0 ≈45% of total FA), myristic (C14:0 ≈9% of total FA) and palmitic (C16:0 ≈12% of total FA) acids the most abundant (Sosa and Fogliano, 2017). This insect fat is especially interesting for its food and feed applications with a concentration in C12:0 similar to that of coconut oil and for the antimicrobial properties that make it of interest as a functional ingredient. The food-grade oils thus obtained could also be used as an ingredient in foods as it has the chemical and physical characteristics for food applications, such as the parameters of thermal behavior, color and aromatic compounds, that are key characteristics in edible oils and fats (Cecchi and Alfei, 2013; Tzompa-Sosa et al., 2019). Besides, most oils had compounds related to pleasant aromas, such as yellow mealworm oil, lesser mealworm oil and cricket oil that present organoleptic characteristics even desirable for table oils and oils use as food ingredients (Sosa and Fogliano, 2017)

To promote the insect derivatives as food and feed alternative, increase them acceptances and create a global trend, it requires a large-scale production of insects with high health standards and accessible commercial price; besides, innovative and promising bio-products derivatives, as bioactive peptides, could represent an additional promising biotech business that has also to be promoted. Therefore, to achieve all these objectives, a multidisciplinary approach will be necessary (de Castro et al., 2018). In the last few years, the research in this field has innovated and sought alternative solutions to improve processing and to increase the shelf life of insect and insect by-products, in order to increase availability and consumer acceptance. One of the main proposed solutions for them acceptance would be the isolation of proteins and lipids from insects to be used as food ingredients for other industrial preparations (Sosa and Fogliano, 2017); this objective requires an in-depth knowledge of the physicochemical characteristics of these proteins and lipids, their functionality and an elaborating consumers' perceptions and motivations. Further studies show a recent advance in the use of insect peptides as antihypertensive, antimicrobial and antioxidant agents, demonstrating the wide applicability of these proteins but additional investigations are requested (de Castro et al., 2018; Hall et al., 2018).

Insect species have the largest unexplored reservoir of antimicrobial peptides. They can be exploited as alternatives to conventional antibiotics to counter the threat of multidrug-resistant pathogens. Like all Arthropods, insects are characterized by high chitin levels that it is present in their exoskeleton. From chitin, a long-chain polymer of β-(1→4)-N-acetyl-D-glucosamine, the chitosan is produced commercially by deacetylation. The latter is a recognized valuable compound used as a feed additive in several animals feeds as fat-reducing factor in poultry (Lokman et al., 2019) and as a modulator of rumen fermentation (Haryati et al., 2019). Its dietary effects in aquafeeds are under investigation in terms of antimicrobial and immune-stimulation properties, growth promotion and antioxidant activity (Abdel-Ghany et al., 2019). In humans, chitosan has been used as supporting material for pharmaceutical applications, gene delivery, cell culture, tissue engineering and for its several pharmacological activities such as anti-hypertensive, anti-ulcer, wound healing, anti-cancer, blood coagulant/anti-coagulant, anti-microbial, anti-viral, hypolipidemic and hypocholesterolemic effects (Tripathi et al., 2017). Even for plants, chitosan has been proposed to improve health: it activates plant defence including elicitation of phytoalexins and pathogenesis-related protein (Pichyangkura and Chadchawan, 2015). Anyway, more research on side effects, such as possible cytotoxicity, immunogenicity and allergenicity that may pose a risk to human and animal health upon the use of

insects for feed or food, are required (van der Fels-Klerx et al., 2018).

After the publications made by the FAO, where the insects are considered as one of the solutions for future protein scarcity and support their use for human consumption, more than 300 insect producing companies have been created or they move from the traditional way to mass-scale production in controlled environments. About half of those companies are placed in Europe and produce black soldier fly (*Hermetia illucens*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*) and banded house cricket (*Gryllodes sigillatus*) (Derrien and Boccuni, 2018)and they can be used to bring new, safe and innovative byproducts to the EU market (Tzompa-Sosa et al., 2019).

1.3 European legislation

In the last few years, the European Union started to define novel foods and ingredients and to regulate their production and utilization. EU law regulates the conditions for food and feed business operators, such as insect producers, to produce and commercialize their products in the European Union. In addition to the 'general food hygiene requirements', the production and marketing of insects as food in Europe is governed by the so-called 'Novel Foods' legislation. Notably, EU policy makers have adopted – in the early 2000' – a package of legislative texts which define general principles and standards in the area of food and feed safety.

Beginning with the REGULATION (EC) No 258/97 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 January 1991 defining that the term novel foods and novel food ingredients " *shall apply to the placing on the market within the Community of foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the Community and which fall under the following categories:*

(a) foods and food ingredients containing or consisting of genetically modified organisms within the meaning of Directive 90/220/EEC;

(b) foods and food ingredients produced from, but not containing, genetically modified organisms;

(c) foods and food ingredients with a new or intentionally modified primary molecular structure;

(d) foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae;

(e) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use;

(f) foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances" (European Parliament and EU Council, 1997). In this regulation the European Union tried to discriminate what can be consider novel food using general parameters not easy to be applied.

Subsequently, the REGULATION (EU) 2015/2283 of 25 November 2015 actually in force on novel foods, precises and clarifies the different categories of novel foods, thus amending previous Regulations (Reg. EU No 1169/2011; Reg. EC No 258/97; Reg. EC No 1852/2001): "*The scope of this Regulation should, in principle, remain the same as the scope of Regulation (EC) No 258/97. However, on the basis of scientific and technological developments that have occurred since 1997, it is appropriate to review, clarify and update the categories of food which constitute novel foods.*" In particular the new regulation define a new category that " *cover whole insects and their parts*" (EU 2015/2283, 2015; Ue et al., 2015). With this new regulation insects started to be part of the possible commercialized novel food for human consumption. Consequently, producers – like any other food or feed business operator, will be subjected to hygiene standards according the General Food Law Regulation (European Parliament and Council, 2002) and the Regulations contained in the 'Hygiene Package' (Regulation (852/2004, 2004) and Regulation (183/2005/EC, 2005)).

Anyway only with the REG. (EU) No 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011, the European Commission regulates and defines the possible processed animal protein that can be used in livestock production. "*The amendment to Regulation (EC) No 999/2001 with a view to authorise processed animal protein derived from insects for feeding aquaculture animals is likely to open the opportunity for bigger production of processed animal protein derived from insects in the Union. Whereas the current small scale rearing of insects for pet-food can adequately be addressed by existing national control schemes, Union provisions addressing animal health, public health, plant health or environmental risks are appropriate to ensure*

that insect rearing within the Union on a larger scale is safe. With respect to the insect species reared in the Union, these should not be pathogenic or have other adverse effects on plant, animal or human health; they should not be recognised as vectors of human, animal or plant pathogens and they should not be protected or defined as invasive alien species. Taking into account these national risk assessments, as well as the EFSA opinion of 8 October 2015, the following insect species can be identified as those insect species currently reared in the Union which fullfill the above mentioned safety conditions for insect production for feed use: Black Soldier Fly (Hermetia illucens), Common Housefly (Musca domestica), Yellow Mealworm (Tenebrio molitor), Lesser Mealworm (Alphitobius diaperinus), House cricket (Acheta domesticus), Banded cricket (Gryllodes sigillatus) and Field Cricket (Gryllus assimilis)" (Commission, 2017).

Anyhow, after the approval of insects for food and feed applications, methods for the detection and identification of these ingredients in complex matrices were needed. Following the guidelines of the EU Commission Regulation No. 2009/152, where is defined that the " *Deoxyribonucleic acid (DNA) fragments of animal origin which may be present in feed materials and compound feed are detected by a genetic amplification technique through PCR, targeting species-specific DNA sequences*" (EC, 2009),several new detection protocols based on PCR and innovative biosensors are going to be proposed.

1.4 Quality and safety of insect production

The production, marketing, and use of edible insects as food and feed pervades a wide range of regulatory institutions, which should ensure aspects such as the quality and safety of the products obtained and the environmental impact of insect breeding. A crucial point is that consumer acceptance is probably associated with the development of an appropriate processing strategy, such as the extraction, purification and use of insect proteins as a food additive (Huis, A., Iterbeeck, J., Klunder,H., Mertens, E., Halloran, A. Muir, G. and Vantomme, 2013). Insects production for human or animal consumption must fulfill all legal requirements like hygiene and chemical residues like pesticides and antibiotics and must not transmit diseases. An upcoming problem may be the already established production of insects for pet food (van Huis and Oonincx, 2017). Adulteration of high-quality insect products for human consumption with lower quality pet food insects may take place. In addition, the use of not recognizable insects, such as insect flour, may be more acceptable to consumers than entire insects. In any case, food control laboratory must be capable to detect insects in food products. However, to a certain degree and as inevitable ingredient, insect material is expected to be found in many food products already. It may be a result of contamination during harvest, storage and production process as insects are omnipresent. Therefore, quantitative analysis methods are desirable to fix a threshold value to distinguish usual contamination from intentional insect ingredients (Köppel et al., 2019).

Thesis outline

This thesis is composed of several research activities performed in collaboration with different research groups in different laboratories

- University of Udine − Dept. of Agriculture, Food, Environment and Animal Science,
- Hellenic Centre of Marine Resources laboratory in Crete (Greece), within the EU-AQUAEXCEL Programme of access to Excellence Infrastructures (Contract N. 652831)
- Or-El d.o.o. Startup in Nova Gorica (Slovenia)
- Polymer Competence Center Leoben GmbH (PCCL) Leoben (Austria).

The first chapter of the thesis presents a state of art for the detection of insects using the PCR (polymerase chain reaction) technique and the experimental work carried out to quantitative detection of insects in feeds and food with a special emphasis to *Gryllodes sigillatus* conducted at the Department of Agricultural, Food, Environmental and Animal Sciences of Udine University.

In the second chapter the state of the art of biosensors and the experimental works that were performed at the University of Udine in collaboration with Or-El d.o.o. (Slovenia) and the PCCL (Austria) is presented. The aim of the study was to evaluate the most suitable functionalization of PE substrate and to develop a DNA biosensor based on O-LED technology for insect DNA quantitative detection.

The experiment discussed in the third chapter was performed in collaboration with the HCMR of Crete (Greece) and founded by the European AquaExcel project. The effects of dietary addition of *Hermetia illucense* in aquafeeds were evaluated on gut morphology and target gene expression. The results have been submitted to A research paper will been submitted to the Animals Journal.

The outcomes of the research activity carried out during the three years PhD period are here listed:

- Daniso E., ·Tulli F., · Cardinaletti G., · Cerri R.,· Tibaldi E. (2020) Molecular approach for insect detection in feed and food: the case of *Gryllodes sigillatus*. European Research and Technology Journal (DOI 10.1007/s00217-020-03573-1)
- Daniso E., Patrizia Melpignano P., Tulli F. (2020) An OLED-based genosensor for the detection of *Hermetia illucens* in feeds. Food Control 113 107179 (DOI 10.1016/j.foodcont.2020.107179)
- Daniso E., Maroh B., Feldbacher S., Mühlbacherb I., Schlöglb S., Melpignano P..Tailoring the chemical functionalization of a transparent polyethylene foil for its application in an OLED-based DNA biosensor. Biosensor and Bioelectronic. (Submitted)
- Daniso E., Sarropoulou E., Beraldo P., Tibaldi E., Cerri E., Cardinaletti G.. Graded level of Hermetial illucens pupae meal in plant protein-based diet lacking fishmeal for gilthead sea bream (Sparus aurata, L.): liver and gut gene expression and histological evaluation. Acquaculture. (under submission)
- Daniso E., Messina M., Tulli F. (2019) Molecular based identification of insect ingredients in animal feeds. Proceedings of 23rd National Congress of the Animal Science and Production Association ″New challenges in Animal Science″ Sorrento (Naples), 11-14 June 2019 (oral presentation).
- Daniso E., Tulli F. (2019) Molecular tools for insect detection in animal feeds. Proceedings of 70° EAAP Meeting, Ghent (Belgium) 26-30 August 2019. EAAP Book of Abstracts, Volume 25. Editor Scientific Committee. 713pp. eISBN: 978-90-8686-890-2 (oral presentation)

2 Detection methods of species-specific DNA use for insect detection

The nucleic acid-based approaches like PCR or sequencing are the most frequently applied methods for reliable detection of species-specific DNA. An Other innovative method that is also available is metabarcoding, this method allows large-scale taxonomic identification of complex environmental samples via analysis of DNA sequences for short regions of one or a few genes (called DNA barcodes) using universal primers; this approaches powered by Next Generation Sequencing (NGS) technology that relies on high-throughput DNA sequencing, it yields millions of DNA sequences in parallel and allow large-scale analysis of environmental samples., but the success of these methods strictly depend on the choice of appropriate universal primers as well as on the ability to detect the possible small presence of the target DNA sequence in a complex matrix do to the no specificity of the primers utilised. Moreover, this technique still requires cost-intensive, high-end machines, qualify operators and access to comprehensive sequence libraries (Elbrecht et al., 2016). On the contrary, PCR allows precise quantification of specific nucleic acids sequence in a complex mixture, also this method is capable to detect the presence of the target sequence even if the quantity present in the analyzed samples is in a very low concentration (fg).

The PCR process generally consists of a series of temperature cycle changes that are repeated 25−50 times during which the specific primers bind the target sequence and the polymerization occurs, carried out by the DNA polymerase enzyme. In this method, appropriated targeted primers are fundamental for specific and univocal detection of the target. In the real-time PCR, the amplification of the target sequence of DNA is monitored every cycle, and not as in the Endpoint PCR where just the final product is detected. For the in real time monitoring, two are the common methods utilized for the detection of PCR products; the first is a non-specific fluorescent dyes that intercalate with any dsDNA, ex. SybrGreen , and second is a sequence-specific DNA probes that are labelled with a fluorescent reporter (Shah, 2019), ex. TaqMan probes that are constituted of a fluorophore covalently attached to the 5′-end of the probe and a quencher at the 3′-end., this system permits detection only after hybridization of the probe with its complementary sequence and his cleavage (Thermo Fisher Scientific, 2011), other techniques such as Scorpion, where the probe is physically coupled to the primer which means that the reaction leading to signal generation is a unimolecular one, are also available but not frequently applied.

Real-time PCR has revolutionized the 21st century biological science due to its capability of detection and expression analysis of genes in real-time applied in quantitative genotyping, genetic variation of inter and intra organisms, early diagnosis of disease, forensic, detection and quantification of a specific target in different matrixes etc., and the obtained results were also validated through ring trials with the help of the National Reference Laboratories or sequencing the genomic fragment resulted from the PCR amplification and blasting the resulted sequence with the deposited ones . For that reasons, PCR technology was identified as one of the methods capable to fulfill all legal requirements of traceability and food control required from the law (EC, 2009). Due to the lack of or expensive alternatives for the detection of insects, PCR may help to fill the existing analytical gaps for the species of interest most rapidly and cost-efficient in comparison with other technologies.

Base on this considerations, recently fully validated protocols applied to the PCR technology were published for the detection of different insects such as *Tenebrio molitor* (Debode et al., 2017), where using Taqman technology a limit of detection of 20 gene copy number, approximately 10.4 pg of the target DNA, was reached without having cross reactivity with the DNA of the other analyzed species; *Hermetia illucens*, where the taget DNA was identify in commercial fish feed with a concentration of 0.01% of *Hermetia* DNA (Zagon et al., 2018); but also multiplex real-time PCR for the simultaneous detection of Tenebrio molitor, Locusta migratoria and Achaeta domestica was performed but with a

limit of detection of 0.1% (w/w) ten times less in comparison with the previous (Köppel et al., 2019).

Taking in consideration this background, during the first period of the PhD at the University of Udine, a detection method, based on real-time PCR system, was performed for the detection of Gryllodes sigillatus; one of the seven allowed species for human consumption an animal feed. The detection of the target was based on SybrGreen intercalator and a specific pair of primer designed on the Gryllodes sigillatus mitochondrial cytochrome b (CYB) gene, the Taqman technology utilized in the citated articles was not used because it needs two primers and an internal probe, this make the technique more specific but for the subsequent creation of a biosensor based on the recognition of DNA target, it was necessary to have a protocol with only two very specific primers.. The usual parameters for the accuracy of the analysis, like sensitivity and specificity, were validated. The method showed no cross reaction to vertebrates and plants samples that were used, and the system was capable to detect low concentration of the target DNA in complex matrix. These results allow the use of the designed primers for other applications such as the construction of a DNA biosensor.

2.1 **Molecular approach for insect detection in feed and food: the case of *Gryllodes sigillatus***

European Food Research and Technology
https://doi.org/10.1007/s00217-020-03573-1

ORIGINAL PAPER

Molecular approach for insect detection in feed and food: the case of *Gryllodes sigillatus*

Enrico Daniso[1] · Francesca Tulli[1] · Gloriana Cardinaletti[1] · Roberto Cerri[1] · Emilio Tibaldi[1]

Received: 1 April 2020 / Revised: 16 July 2020 / Accepted: 18 July 2020

Abstract
The production of insects on an industrial scale has attracted the attention of the research and agricultural industry as novel protein sources. To detect the presence of *Gryllodes sigillatus* (GS) in feed and food, a real-time PCR method based on the mitochondrial cytochrome b (CYB) gene is proposed by this study. Forty DNA samples of animal and plant origin were used to confirm the specificity of the qPCR system. The detection method's performance was evaluated on different processed GS matrices including native GS (UnGS) and different commercial products: crunchy roasted samples (RoGS), insect meal mixtures (ACGS) and energetic snacks containing GS (GSS). Data on sequencing were aligned with the reference gene to confirm the PCR products. The regression curve ($y = -3.394\ x + 42.521$; $R^2 = 0.994$, d.f. 14) between C_t values and Log DNA concentrations of *Gryllodes sigillatus* resulted in an efficiency of 96.4%. The severity of the technological processing treatments and the matrix structure affected the intensity of the PCR signal with the same amount of insect DNA as observed by different y-intercepts of the three-regression lines for RoGS, ACGS, and GSS. The real-time PCR method resulted in robust and sensitive outcomes able to detect low amounts of GS DNA (5 g/100 g) in a complex matrix, making it suitable for detecting the presence or absence of labeled Gryllodes sigillatus material both in feed and food.

Keywords *Gryllodes sigillatus* · Detection · Edible insects · Real-time PCR · Processed food · Feed

Highlights

- The method allows for the identification of *Gryllodes sigillatus* in a complex matrix.
- Primers exhibited high sensitivity and accuracy.
- PCR discrimination among insect species.
- The qPCR system was successfully tested in complex matrices.

Introduction

Global welfare standards and the increasing world population [1] require/necessitate/demand/call for the consideration of protein sources derived from sustainable production systems capable of efficiently converting biomass [2–5]. The novel proteins from unconventional sources should be safe, nutritious, flexible and reliable as well as accepted by consumers. Insects may represent a more efficient way of producing animal protein and seem to meet this goal due to the use of cost-effective raw materials and environmental sustainability along with high nutritional value [6, 7]. Recently, the production of insects on an industrial scale has attracted the attention of the research and agricultural industry [8], because they are a good source of protein, vitamins, and energy [9] and are widely used for human consumption in many countries in Asia, Africa and South America [10]. Whole insects can be used raw, dried, crushed, textured, pulverized, ground, eaten after technological food processing or preserved by freeze-drying with or without the exoskeleton of chitin [11]. They can be consumed as snacks, used as ingredients of some typical preparations or undergo

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00217-020-03573-1) contains supplementary material, which is available to authorized users.

✉ Francesca Tulli
francesca.tulli@uniud.it

[1] Department of Agricultural, Food, Environment and Animal Science, University of Udine, Via Sondrio 2 A, 33100 Udine, Italy

Published online: 31 August 2020

Molecular approach for insect detection in feed and food: the case of *Gryllodes sigillatus*

Enrico Daniso[a], Francesca Tulli[a]*, Gloriana Cardinaletti[a], Roberto Cerri[a], Emilio Tibaldi[a]

[a] Department of Agricultural, Food, Environment and Animal Science, University of Udine, Udine, Italy

* Corresponding author: francesca.tulli@uniud.it

European Food Research and Technology 2020, doi.org/10.1007/s00217-020-03573-1

Abstract

The production of insects on an industrial scale has attracted the attention of the research and agricultural industry as novel protein sources. To detect the presence of *Gryllodes sigillatus* (GS) in feed and food, a real-time PCR method based on the mitochondrial cytochrome b (CYB) gene is proposed by this study. Forty DNA samples of animal and plant origin were used to confirm the specificity of the qPCR system. The detection method′s performance was evaluated on different processed GS matrices including native GS (UnGS) and different commercial products: crunchy roasted samples (RoGS), insect meal mixtures (ACGS) and energetic snacks containing GS (GSS). Data on sequencing were aligned with the reference gene to confirm the PCR products. The regression curve (y=-3.394 x +42.521; R^2 =0.994, d.f. 14) between Ct values and Log DNA concentrations of Gryllodes sigillatus resulted in an efficiency of 96.4%. The severity of the technological processing treatments and the matrix structure affected the intensity of the PCR signal with the same amount of insect DNA as observed by different y-intercepts of the three-regression lines for RoGS, ACGS, and GSS. The real-time PCR method resulted in robust and sensitive outcomes able to detect low amounts of GS DNA (5g/100g) in a complex matrix, making it suitable for detecting the presence or absence of labeled *Gryllodes sigillatus* material both in feed and food.

Highlights

- The method allows for the identification of *Gryllodes sigillatus* in a complex matrix.
- Primers exhibited high sensitivity and accuracy.
- PCR discrimination among insect species.
- The qPCR system was successfully tested in complex matrices.

Keywords: *Gryllodes sigillatus*, detection, edible insects, real-time PCR, processed food, feed

1. Introduction

Global welfare standards and the increasing world population [1] require/necessitate/demand/call for the consideration of protein sources derived from sustainable production systems capable of efficiently converting biomass [2−5]. The novel proteins from unconventional sources should be safe, nutritious, flexible and reliable as well as accepted by consumers. Insects may represent a more efficient way of producing animal protein and seem to meet this goal due to the use of cost-effective raw materials and environmental sustainability along with high nutritional value [6, 7]. Recently, the production of insects on an industrial scale has attracted the attention of the research and agricultural industry [8] because they are a good source of protein, vitamins, and energy[9] and are widely used for human consumption in many countries in Asia, Africa and South America [10]. Whole insects can be used raw, dried, crushed, textured, pulverized, ground, eaten after technological food processing or preserved by freeze-drying with or without the exoskeleton of chitin [11]. They can be consumed as snacks, used as ingredients of some typical preparations or undergo other technological treatments, depending on cultural habits [12]. In contrast, people in Western countries are not familiar with this kind of food even if some compounds derived from insects are already exploited by the food industry as coloring agents [11] that can be found in Smarties, yogurt, Campari, etc. [13]. Nevertheless, interest in including insects in the Western diet is increasing along with the number of restaurants serving insects as a delicacy and the number of recipe books dedicated to insect preparation [14, 15]. The expectation is that in the coming decades this novel protein source will represent 40% of the traditional meat consumption [16, 17], but estimated entry time into the market [18, 19] will vary depending on cultures and traditions. Recently, several studies have been carried out on the functionality, processing and industrial applications of this novel ingredient, trying to exploit byproducts and waste streams obtained in other

production processes. Moreover, research studies indicate that insects possess several features that make them wellsuited for use in animal feed. The European Union identified seven insect species as suitable for use in aquafeed: *Gryllodes sigillatus* (GS) (tropical house cricket), *Gryllus assimilis* (Jamaican field cricket), *Acheta domesticus* (AC) (house cricket), *Alphitobius diaperinus* (lesser mealworm), *Musca domestica* (common housefly), *Tenebrio molitor* (yellow mealworm) and *Hermetia illucens* (black soldier fly)[20].

The production and marketing of insects as food in Europe is regulated by the so-called 'Novel Foods' legislation − i.e. Regulation [14]. This Regulation applies to all categories of foods that were not used for human consumption to a significant degree within the European Union before May 15, 1997, which is the case of insects. Since January 1, 2018, the Novel Food regulation has been in force; therefore an authorization form must be submitted for each insect species intended to be considered as food and sold on the market. So far, Kreca ProtiFarm (NL), Micronutris (FR) and BiiF (BE) have applied for the inclusion of the following species in the European Union's list: *Alphitobius diaperinus*, *Gryllodes sigillatus, Tenebrio molitor, Locusta migratoria* and *Acheta domesticus*. Among these, A. domesticus and *G. sigillatus* have increased their presence in the food market as a promising protein source for the human diet. Today, GS is commonly marketed as an alternative to AC, due to its ability to withstand cricket paralysis virus. GS, like other Gryllidae species, is susceptible to *Acheta domesticus* densovirus (AdDNV) with a variable degree, but without cases of severe mortality which instead occurs in AC, where AdDNV provokes high mortality and serious and irreparable damages to the production. For this reason, interest in rearing GS against AC increased after the bankruptcy of AC facilities [21].

In this context, controlling the composition of both feed and food is an important issue according to European rules, especially after outbreaks such as BSE (Bovine Spongiform Encephalopathy) or minced beef contaminated with horse meat; in any case, the safety and the control systems for insect components in food are still being developed [18, 22]. Today, ingredients of animal origin, used as raw materials, are identified based on typical and microscopically identifiable characteristics or by using polymerase chain reaction (PCR) following the provisions laid down in Annex VI of EU Commission Regulation No. 2019/152 [23]. Molecular biology techniques provide robust and valuable tools for the identification of both plant or animal species in feedstuffs [24]. DNA detection and identification of animal materials employing PCR, because of its high sensitivity, is considered the official analytical method to: (i) determine the species origin of processed animal proteins (PAPs); (ii) detect the presence of prohibited ingredients; (iii) confirm the presence of ingredients in animal feeds. Among the detection/identification of PAPs, insect species identification may represent a new challenge and the PCR methods may provide a possible solution. In particular, end-point PCR was developed to detect the presence of GS directly in foods [25], but a quantification was not possible with this technique. For this reason, an RT-PCR method is proposed in the present study.

Several methods have been applied for insect species identification, such as Enzyme-Linked Immunosorbent Assays (ELISA) for TM and nucleic acid-based approach applied to reliably detect the black soldier fly specific DNA [26]. In more detail, DNA barcoding sequencing has been widely used in species identification and biodiversity research [27]. It has been shown that in many groups, including insects, interspecific variation in DNA sequences of some genes are higher than intraspecific variation thus providing an opportunity for DNA-based species identification. In any case, this technique is time consuming in comparison with qPCR and it requires a pure sample and DNA from complex matrices can give unclear results [28]. An improved tool supporting molecular methods is Next Generation Sequencing (NGS), which enables highthroughput and parallel taxonomic identification of multiple species, but a specialized working team and different statistical parameters should be employed to describe and evaluate the quality of the final genome assembly derived from NGS data output [26, 29]. In any case, the PCR method applied on specific DNA fragments is being advocated for species identification because it is fast, cheap and has high sensitivity and specificity and has been employed to confirm morphological fragment identification [23, 30, 31].

The present research aims to develop a PCR based method to detect *Gryllodes sigillatus* as a raw material in different processed feed and food preparations. The species specific PCR primers and their accuracy are presented.

2. Material and methods

2.1. Samples

The samples presented in the following list were used for method development and GS1 primer specificity test. Untreated insect samples of *Gryllodes sigillatus* (UnGS), *Hermetia illucens*, *Acheta domesticus* (AC), *Gryllus assimilis*, *Musca domestica*, *Alphitobius diaperinus*, *Tenebrio molitor* were purchased alive from Agripetgarden S.r.l. (PD) − Italy. Untreated insect samples of *Bombus terrestris*, *Nezara viridula, Oecanthus pellucens, Sitophilus granaries, Sitophilus oryzae, Rhyzopertha dominica, Halyomorpha halys, Apis mellifera, Bombyx mori* were kindly provided by the entomology division of the University of Udine, Italy. Species identification was confirmed by the examination of morphological features of adult individuals according to scientific standard literature [32, 33]. Muscle samples from selected animal species such as *Sparus aurata* (gilthead sea bream), *Dicentrarchus labrax* (European sea bass), *Oncorhynchus mykiss* (rainbow trout), *Bos taurus* (cattle), *Ovis aries* (sheep), *Sepia officinalis* (cuttlefish), *Loligo vulgaris* (squid), *Mytilus edulis* (mussel), *Parapenaeus longirostris* (pink shrimp), *Penaeus kerathurus* (striped prawn), *Chamelea gallina* (clam), *Pleoticus muelleri (*Argentine red Shrimp), *Atherina boyeri*, *Pecten jacobaeus* (scallop) and *Aequipecten opercularis* were purchased from GDO commerce, Italy.

Acheta domesticus (mAC) and *Locusta migratoria* flour were obtained from Crunchy Critters (Ilkeston, United Kingdom).

Gryllodes sigillatus meal (mGS), Cruncky Roasted Gryllodes sigillatus (RoGS) and energetic bars for human consumption (GSS) with different GS inclusion level (0, 5.2, 6.3, 10.6 g/100g in GSS0, GSS1, GSS2 and GSS3, respectively) were purchased from Eat Grub Ltd, London, United Kingdom.

Finally, a serial dilution of commercial GS meal (mGS) from Eat Grub, and AC meal (mAC) from Crunchy Critters were also prepared as mixtures (approximately 100g) at the laboratories of the University of Udine (Table 1) to be used as calibration material.

Table 1 Mixtures of GS meal (mGS) and AC meal (mAC) prepared to obtain different percentages of target species inclusion

Sample name	*Gryllodes sigillatus (mGS)*	*Acheta domesticus (mAC)*	*Gryllodes sigillatus inclusion*
ACGS10	10 g	90 g	10%
ACGS20	20 g	80 g	20%
ACGS40	40 g	60 g	40%
ACGS60	60 g	40 g	60%
ACGS80	80 g	20 g	80%
ACGS100	100 g	0 g	100%

2.2. Sample preparation and DNA extraction

All the samples were stored at -20 °C; if needed tissues were cut using a scalpel, put into a Lysing matrix A tube (MP biomedicals, Santa Ana, California, USA) and disrupted by a TissueLyser II bead mill (Qiagen, Hilden, Germany).

DNA was extracted from 20-25 mg of whole insect/pupae and samples of animals and feeds and food with the DNeasy Blood & Tissue ® (Qiagen, Hilden, Germany) according to the manufacturer′s instruction. The DNA concentration and purity were evaluated by measuring the absorbance at 260 nm and the 260/280 nm ratio, respectively, using a NanoDrop One Micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.). DNA integrity was also tested by denaturing 1.2 % agarose gel electrophoresis in 1X TBE buffer and ethidium bromide staining.

2.3 DNA primers

All samples were analyzed using Unspecific insect primers, Ins3.2F (ATAATTGGTGGATTT-GGAAATTGA) and Ins3.2R (GGTGGATAA ACAGTT CATCCTGT), designed according to Koppel et al., (2019) [31]. A specific pair of primers for Gryllodes sigillatus was designed by retrieving the sequence of the mitochondrial cytochrome b (CYB) gene (accession no. KR903358.1) from GenBank. The pair of primers GS1Fw (GATCAAACAATCCCCTAGGTGTC) and GS1re (CTGGGTCTCCAAGTATTAAGGATTAG) were designed using the online software Primer3 (http://primer3.ut.ee) with Lucia algorithm. The expected GS1 amplified sequence was blasted in BLASTn to evaluate the specificity of the sequence against the deposited sequences in GenBank. The end-point PCR technique was performed to screen the primers specific amplification by using the GoTaq DNA polymerase Kit (Promega, Milano, Italy). Each PCR reaction was conducted in a final volume of 20 µl, using 4 µl of PCR Green or colorless GoTaq Reaction Buffer (1.5 mMol MgCl2), 13.5 µl of distilled water, 0.4 µl of PCR nucleotide Mix at 10 mM, 0.1 µl of GoTaq DNA polymerase (5 u/µl), 0.5 µl of 10 µM of each primer and 1 µl of DNA template (<0.5 µg/50 µl) were used. The PCR thermal protocol consisted of one cycle of 3 min at 98 °C, then 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C for 35 cycles and a final cycle of 5 min at 72 °C. Each PCR product was subsequently gel verified for the expected product length (bp).

The pair of primers amplified the target sequence and was tested for amplification efficiency and sensitivity in qPCR. These parameters were determined by six serial tenfold dilutions of pure GS DNA in three replicates. Real-time PCR was performed in a total volume of 20 µl, 10 µl of EvaGreen Buffer mix, 8.2 µl of molecular biology grade water, 0.4 µl of each primer, Forward and Reverse at a final concentration of 200 nM, and 1 µl of DNA. qPCR was carried out on a CX-96 (Biorad Srl, Milano, Italy) with the following standard program: initial denaturation steps of 3 min at 98 °C, followed by 45 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. Besides, a melting curve was determined using the following parameters: starting at 65°C for 90 seconds temperature increment 1°C, 5 seconds each step, 95 °C was reached. In all runs a No Template Control (NTC) with pure water instead of DNA was added. All tested samples were diluted to obtain a concentration of 10 ng/µl and evaluated using a NanoDrop One Micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.). When results of different runs were compared (e.g. in specificity testing) the threshold was always set manually to the same level. The baseline setting was done automatically.

The specificity of the primer pairs was estimated using 10 ng of DNA extracted from 40 samples including animals and ingredients. The GS1 primers sensitivity was evaluated by six serial 10-fold dilutions in the concentration range from 10 ng to 10 fg, of DNA isolated from the target insect species (UnGS) and the processed products (RoGS, ACGS).

2.4 Sequencing

The samples, that in PCR have been amplified for both Ins3.2 and GS1 were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer′s instructions. The purified products from GS1 PCR were subsequently sequenced (Eurofins Genomics, Milano, Italy). The forward and reversed sequences were clustered and aligned with the reference gene sequence (accession no. KR903358.1) using the multiple sequence alignment program CLUSTAL OMEGA (https://www.ebi.ac.uk/) (data shown in supplementary material).

2.5 Data analysis

The limit of detection (LOD) for GS1 was defined as the lowest dilution level for which all replicates still gave a positive result. When appropriate, data were subjected to the linear regression model. The efficiency [E= (10 -1/s -1) *100] of each qPCR system was calculated based on the slope(s) of the regression curve obtained by plotting Ct-values against log DNA concentrations. The regression lines were compared and tested for parallelism. The following model was used:

$$y = \beta 0 + \beta 1x + \beta 2z + \beta 3xz + \varepsilon$$

where ε is the error associated with the model and z is an ″independent″ dummy variable as described by Andrade & Estévez-Pérez (2014). The Student′s t-test was used to evaluate the null hypothesis H0: $\beta 3 = 0$.

3. Results and discussion

3.1. In silico analysis

The GS PCR system was developed based on the mitochondrial cytochrome b (CYB). The sequence from GenBank Accession No. KR903358.1 (346 bp) was chosen as *Gryllodes sigillatus* reference for method development and alignments with all other species. The GS1Fw and GS1re primers amplified a sequence of 149 bp included in this region. The specificity of the whole amplicon and the primer binding sites checked in silico by BLASTn database revealed 85% of identity with *Lepidogryllus comparatus* and 83% of identity with *Gryllus bimaculatus* for the whole amplicon. A comparison between the *Gryllodes* specific primers and the sequences from other Arthropods demonstrated an encouraging number of mismatches.

The genus Lepidogryllus sp. is of minor interest regarding the intended scope of the system developed primarily for feed and food on the European market, where those species are not allowed. In the data set, only *Acheta domesticus* revealed close identities (82%) with the targeted *Gryllodes sigillatus* sequence; in any case, the AC samples gave negative results with the GS primers.

To achieve 100% specificity for an insect targeted PCR system seems to be unrealistic taking into consideration the biodiversity of insects [35]. The in silico analysis confirmed the application of the reported primers in the qPCR test and the results obtained in the present study revealed good specificity for GS against the species allowed for feed and the ones proposed for human consumption.

3.2. Specificity testing with biological samples

The primers specificity was tested in triplicate on forty different DNA samples of animal and plant origin with the starting concentration of DNA at 10 ng/µl for each pair of primers. Data are reported in Table 2. The Ins3.2 primers amplified only the samples belonging to an insect family, as previously observed in Koppel et al., (2019). On the contrary, the GS1 primers gave clear positive signals in qPCR (Ct=21.5) only for the target species, Gryllodes sigillatus., thus showing the specificity of the GS1 primers compared to the unspecific insect detection of Ins3.2. These results allow the use of GS1 primers to discriminate GS DNA in a complex matrix without the risk of unspecific amplification sequences.

Table 2 : animal species and ingredients used to evaluate the cross-reaction

Species / product	*Insect unspecific Ins3.2*	*Gryllodes sigillatus GS1*
Insecta		
Gryllodes sigillatus (UnGS)	*Pos*	*Pos*
Hermetia illucens	*Pos*	*Neg*
Gryllus assimilis	*Pos*	*Neg*
Acheta domesticus	*Pos*	*Neg*
Musca domestica	*Pos*	*Neg*
Alphitobius diaperinus	*Pos*	*Neg*
Tenebrio molitor	*Pos*	*Neg*
Bombus terrestris	*Pos*	*Neg*
Nezara viridula	*Pos*	*Neg*
Oecanthus pellucens	*Pos*	*Neg*
Sitophilus granarius	*Pos*	*Neg*
Sitophilus oryzae	*Pos*	*Neg*
Rhyzopertha dominica	*Pos*	*Neg*
Halyomorpha halys	*Pos*	*Neg*
Apis mellifera	*Pos*	*Neg*
Bombyx mori	*Pos*	*Neg*
Locusta migratoria	*Pos*	*Neg*
Crustacea		
Parapenaeus longirostris	*Neg*	*Neg*
Pleoticus muelleri	*Neg*	*Neg*
Penaeus kerathurus	*Neg*	*Neg*
Mollusca		
Pecten jacobaeus	*Neg*	*Neg*
Mytilus edulis	*Neg*	*Neg*
Chamelea gallina	*Neg*	*Neg*
Aequipecten opercularis	*Neg*	*Neg*
Sepia officinalis	*Neg*	*Neg*
Loligo vulgaris	*Neg*	*Neg*
Vertebrates		
Bos taurus	*Neg*	*Neg*
Ovis aries	*Neg*	*Neg*
Oncorhynchus mykiss	*Neg*	*Neg*
Dicentrarchus labrax	*Neg*	*Neg*
Sparus aurata	*Neg*	*Neg*
Atherina boyeri	*Neg*	*Neg*
Ingredients		
Pea protein concentrate	*Neg*	*Neg*
Soybean meal	*Neg*	*Neg*
Dry brewer's yeast	*Neg*	*Neg*
Fish meal (anchovy)	*Neg*	*Neg*
Poultry & turkey by-product	*Neg*	*Neg*
Poultry by-product	*Neg*	*Neg*
Corn gluten	*Neg*	*Neg*
Wheat gluten	*Neg*	*Neg*

3.3. Efficiency and limit of detection (LOD)

The efficiency of the GS1 qPCR system evaluated by serial ten-fold dilutions of UnGS, RoGS, and ACGS100 DNA is presented in Figure 1. Positive results were observed until 10-5 dilutions. Such values reflect the higher presence of mitochondrial target genomes relative to genomic DNA copies in the cell. Linear regression relationship was observed within the dilution range between Ct values and Log DNA concentrations. An efficiency of 96.4% was calculated from the regression curve of UnGS (y=-3.394 x +42.521; R^2 =0.994, d.f. 14) and RoGS (y=-3.412 x +43.023; R^2 =0.999, d.f. 14) while an efficiency of 95.4% resulted from the regression curve of ACGS100 (y=-3.436 x +47.010; R^2 =0.998, d.f. 8). The slope value, the amplification efficiency and the determination co-efficient of the linear relationships resulted within the range of the requested parameters reported in the guidelines of the European Network of GMO laboratories for the verification of a quantitative real-time PCR method [36]. The values of the slope of the standard curves were in the range of -3.6 ≤ slope ≤ -3.1 and the amplification efficiencies were in the range 90 - 110 %. Those results show the specificity of the primers designed for the target DNA and the melting curve underlines the amplification of a single fragment, confirmed by gel electrophoresis run.

3.4. qPCR validation

All the samples and complex matrices were tested in qPCR using the Ins3.2 primers; the samples that gave a positive result were subsequently tested in qPCR with GS1 primers. Only the samples UnGS, RoGS, ACGS meal mix and GSS (1,2,3) resulted in a positive signal and the amplified products were afterward sequenced to verify the amplified sequences that were blasted against the reference gene (CYB accession no. KR903358.1). The alignment (supplementary materials) shows no differences between the UnGS (reference DNA) and the other five sequenced samples, thus confirming that the qPCR analyses amplified selectively only the target G. sigillatus DNA; on the contrary, the six sequenced sequences of the qPCR amplification resulted in four different bases when compared with the reference CYB sequence (accession no. KR903358.1). Such discrepancy could be due to low accuracy in the deposited sequence in GenBank or punctiform intraspecies variation of the selected gene; anyway, these variations did not prevent the specific identification of the species.

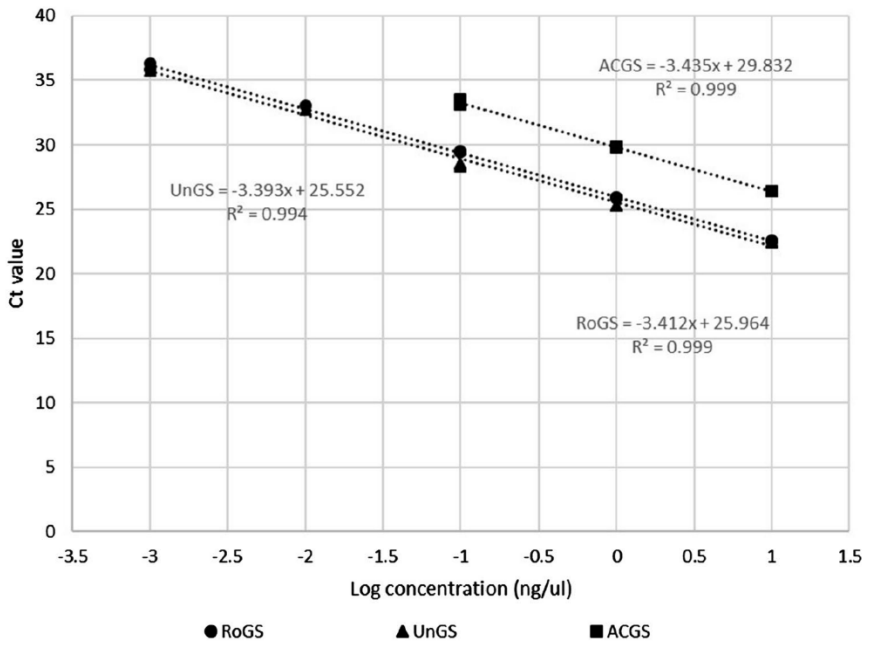


Fig. 1 PCR Linear regression relationship from five tenfold dilutions of Untreated GS (UnGS), roasted GS (RoGS) and ACGS mix (ACGS) DNA

3.5. Effect of technological treatment

The effect of GS matrix processing was evaluated on the detection method performance by utilizing the native *G. sigillatus* (UnGS) and the different commercial products: crunchy roasted samples (RoGS), the mixture of AC and GS commercial meals (ACGS) and energetic bars containing GS (GSS).

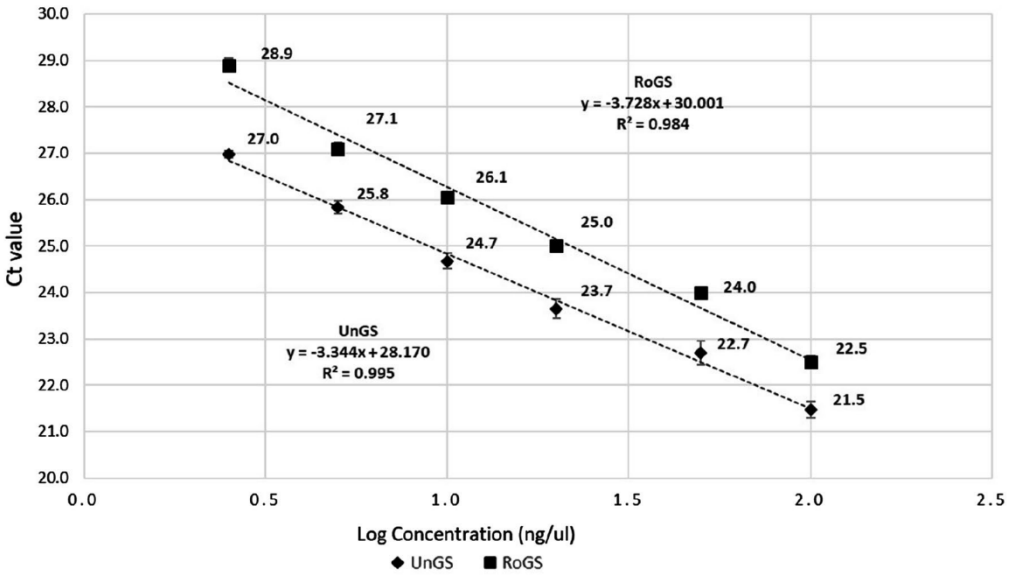


Fig. 2 GS PCR regression curve resulting from six-fold dilutions of Untreated GS (UnGS) and Roasted GS (RoGS) DNA. Data expressed as mean value ± standard deviation of triplicate measurements

The linear regression of six ½ dilutions of 10 ng of DNA extract from UnGS and RoGS samples are shown in Figure 2. The 10 ng/µl of UnGS DNA sample resulted in a Ct value 21.5 and the normalized curve of its six ½ dilutions is expressed by the relationship y =-3.344 (± 0.090) x + 28.170 (± 0.118) (R^2 = 0.995, d.f. = 17). The 10 ng/µl of RoGS DNA sample resulted in a figure of Ct value of 22.5 and the normalized curve of six ½ dilution is expressed by the relationship: y =-3.728 (± 0.157) x + 30.001 (±0.205) (R^2 = 0.984, d.f. = 17). Testing the two regression lines for parallelism, the fol-lowing relationship was obtained for the overall data y = 42.521 (±0.288) -3.394 (±0.064) x − 0.018 (± 0.090) z + 0.502 (± 0.408) xz where the ± indicate the standard error associated to each coeffi-cient. The Student′s t-test for the coefficient of the interaction x x z (t=1.231) was not significant (P= 0.234) so the lines are parallel with no significant difference between slope values (-3.344 and -3.727 for UnGS and RoGS, respectively). It should be noted that the dilutions from RoGS DNA samples resulted in approximately two additional cycles to be detected. The PCR results on the DNA extracted from the snacks for human consumption characterized by different GS inclusion (GSS) and from the mixture of AC and GS commercial meal samples (ACGS) are presented in Figure 3. A clear positive signal was detected for the 10 ng DNA/PCR samples. No template control (NTC) and negative controls were tested and resulted in negative or weak signals. The samples with different levels of GS inclusion in the complex matrix of the snacks samples (GSS) resulted in a normalized relationship expressed by y = -10.867 (± 1.094) x + 39.201 (± 1.021) (R^2 = 0.936, d.f. = 8). The linear relationship obtained with the ACGS mixture is expressed by y = -4.5572 (± 0.244) x + 35.67 (± 0.395) (R^2 = 0.965, d.f. = 20).

When the regression lines of UnGS and ACGS were tested for parallelism, the following relation-ship was obtained for the overall data y = 34.834 (±0.450) -3.908 (±0.314) x − 6.665 (± 0.475) z + 0.564 (± 0.335) xz. The Student′s t-test for the coefficient of the interaction x x z (t=1.681) was not significant (P= 0.105) so the lines resulted parallel with no significant difference between slopes (-3.344 and -4.557 for UnGS and ACGS, respectively).

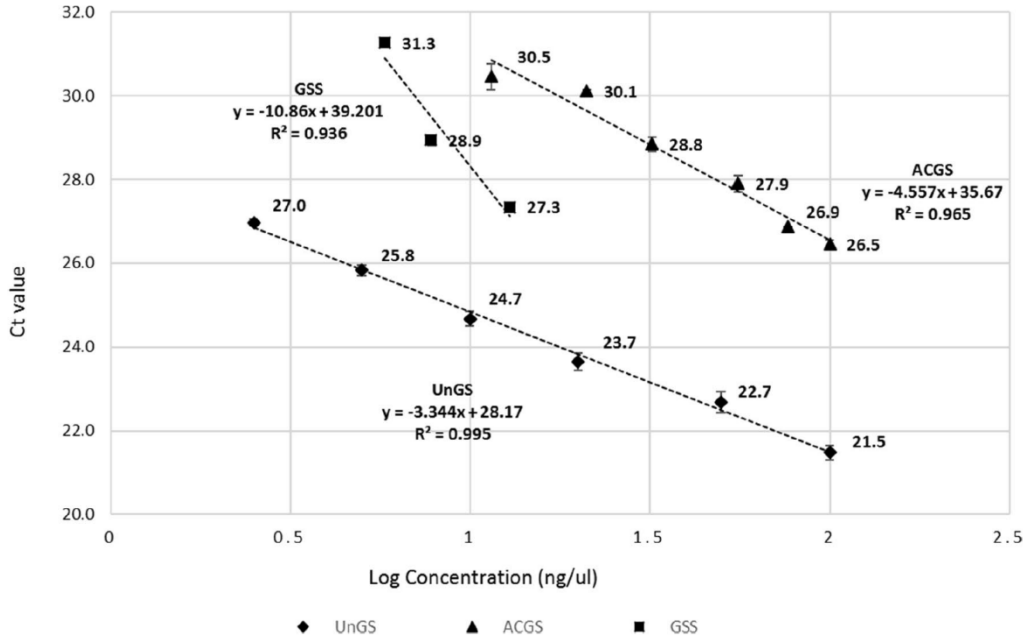


Fig. 3 GS PCR regression curve of different percentages of GS derivatives inclusion in the snack (GSS) and the mixture of commercial AC and GS meals (ACGS) compared with the UnGS regression curve. Data expressed as mean value ± standard deviation of triplicate measurements

It was worthwhile to note that the comparison between the UnGS regression line and the ACGS regression line reveals that additional PCR cycles are needed to detect the samples from the mixtures (ACGS) in comparison with the samples not subjected to technological treatments (UnGS).

In contrast, the GSS regression line presents a significantly different slope if compared to the UnGS linear relationship (P= 0.001). Testing the regression lines of UnGS and GSS for parallelism, the following relationship was obtained for the overall data y = 28.170 (±0.175) -3.344 (±0.134) x + 11.030 (± 0.703) z − 7.523 (± 0.742) xz. The Student′s t-test for the coefficient of the interaction x x z (t=-10.133) indicates that the lines are not parallel. The relationships presented in Figure 3 underline the different characteristics of the matrices considered in the test. As observed by Hrnčírová, Bergerová, & Siekel (2008) the technological processing could be one of the factors affecting the integrity of the DNA extracted from food matrices that influences the CT needed for the qPCR de-tection. In fact, the nature/intensity of the processing treatments determined weak or not negligi-ble degrading effect, as also suggested by the different y-intercepts of the regression lines for UnGS, RoGS, and ACGS. In addition, the possible presence of inhibitors, that could not be eliminated during the DNA extraction process, could affect the CT response of a complex matrix.

Moreover, in the case of GSS, the different particle size of the bars ingredients represents another factor that should be taken into account; this might have made the DNA extraction from fine com-ponents, like insect meal, easier with respect to the other ingredients. Consequently, such factor could have affected the sample expected ingredients ratio.

All these factors should be taken into account and specific calibration curves based on the sample characteristics should be considered to quantify the target DNA in different processed samples.

4. Conclusions

The real-time PCR method described in the present study resulted in robust and sensitive outcomes making it suitable for the purpose for which it was designed.

The use of the two pairs of primers enables a clear insect and GS discrimination and the sequencing of the GS1 qPCR products confirms the accuracy of the *Gryllodes sigillatus* detection and the robustness of the technique.

In addition, the results of the present study indicate that with the same initial amount of insect DNA, different signals are obtained depending on the severity of the technological processing treatment which the matrix is subjected to and the possible presence of inhibitors.

In conclusion, the complexity of the matrix, possible inhibitors, and the integrity of the extracted DNA can affect the quantification of GS in the sample, but the method is always capable of detecting the GS target DNA and the sequencing of the PCR product confirms the specificity of the amplified product. Moreover the combination of the two pairs of primers is able to certify the univocal identification. However, if the method must be applied for relative quantification, specific calibration curves should be developed and validated for different technological processes.

For these reasons, the method is suitable for detecting the presence/absence of *Gryllodes sigillatus* DNA both in feed and food and GS1 primers will be used to implement a genosensor biochip [38].

Acknowledgments

This work was supported by project Ager SUSHIN cod. 2016-0112. The authors wish to thank prof. P. Zandigiacomo for kindly confirming insect species identification and Dr. Dianne Cannizzo for accurate language revision.

References

ADDIN Mendeley Bibliography CSL_BIBLIOGRAPHY 1. FAO (Food and Agriculture Organization) (2011) World Livestock 2011 Livestock in food security World

2. Halloran A, Flore R, Vantomme P, Roos N (2018) Edible Insects in Sustainable Food Systems

3. Boland MJ, Rae AN, Vereijken JM, et al (2013) The future supply of animal-derived protein for human consumption. Trends Food Sci. Technol.

4. Fao (2013) Edible insects. Future prospects for food and feed security

5. Remans R (2020) The changing nature of our food systems. Nat Food 1:21–21. https://doi.org/10.1038/s43016-019-0015-z

6. Smetana SM, Mathys A, Heinz V (2016) Life Cycle Assessment Perspectives of Insect Proteins for Feed and Food. In: Paper presented to Putting LCA into Practice - 10th International Conference on LCA of Food 2016 (Dublin, Ireland: 19-21 October 2016)

7. Dobermann D, Swift JA, Field LM (2017) Opportunities and hurdles of edible insects for food and feed. Nutr. Bull.

8. van Huis A, Tomberlin JK (2017) Insects as food and feed: from production to consumption (free online e-module). Insects as food Feed from Prod to Consum (free online e-module)

9. Ramos-Elorduy J, Moreno JMP, Prado EE, et al (1997) Nutritional value of edible insects from the state of Oaxaca, Mexico. J Food Compos Anal. https://doi.org/10.1006/jfca.1997.0530

10. Gahukar RT (2016) Edible Insects Farming: Efficiency and Impact on Family Livelihood, Food Security, and Environment Compared With Livestock and Crops. In: Insects as Sustainable Food Ingredients

11. Schabel HG (2010) Forest insects as food: a global review. In: Forest insects as food: humans bite back

12. DeFoliart GR (1992) Insects as human food. Gene DeFoliart discusses some nutritional and economic aspects. Crop Prot. https://doi.org/10.1016/0261-2194(92)90020-6

13. DeFoliart GR (1999) INSECTS AS FOOD: Why the Western Attitude Is Important. Annu Rev Entomol. https://doi.org/10.1146/annurev.ento.44.1.21

14. EU 2015/2283 (2015) Regulation (Eu) 2015/2283 of the European Parliament. Off J Eur Union

15. La Barbera F, Verneau F, Videbæk PN, et al (2020) A self-report measure of attitudes toward the eating of insects: construction and validation of the Entomophagy Attitude Questionnaire. Food Qual Prefer 79:103757. https://doi.org/10.1016/j.foodqual.2019.103757

16. de Kuijer O, Wielenga DK (1999) Een vergelijking van de milieubelasting van vlees en vleesalternatieven en de aantrekkelijkheid van de alternatieven voor consumenten

17. Sogari G, Amato M, Biasato I, et al (2019) The potential role of insects as feed: A multi-perspective review. Animals

18. van der Spiegel M, Noordam MY, van der Fels-Klerx HJ (2013) Safety of novel protein sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application in food and feed production. Compr Rev Food Sci Food Saf. https://doi.org/10.1111/1541-4337.12032

19. Alexandratos N, Bruinsma J (2012) World agriculture towards 2015/2030: the 2012 revision. ESA Working paper (Vol. 12 No. 3)

20. European Commission (2017) Commision Regulation 2017/893 … as regards to the provisions on processed animal proteins. Off. J. Eur. Union

21. Weissman DB, Gray DA, Thi Pham H, Tijssen P (2012) Billions and billions sold: Pet-feeder crickets (Orthoptera: Gryllidae), commercial cricket farms, an epizootic densovirus, and government regulations make for a potential disaster. Zootaxa. https://doi.org/10.11646/zootaxa.3504.1.3

22. Vandeweyer D, Wynants E, Crauwels S, et al (2018) Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (Gryllodes sigillatus) for Human Consumption . Appl Environ Microbiol. https://doi.org/10.1128/aem.00255-18

23. EC (2009) Commission regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Off J Eur Union 36:1–130

24. Peres B, Barlet N, Loiseau G, Montet D (2007) Review of the current methods of analytical traceability allowing determination of the origin of foodstuffs. Food Control. https://doi.org/10.1016/j.foodcont.2005.09.018

25. Tramuta C, Gallina S, Bellio A, et al (2018) A set of multiplex polymerase chain reactions for genomic detection of nine edible insect species in foods. J Insect Sci 18:.

https://doi.org/10.1093/jisesa/iey087

26. Zagon J, di Rienzo V, Potkura J, et al (2018) A real-time PCR method for the detection of black soldier fly (*Hermetia illucens*) in feedstuff. Food Control 91:440–448. https://doi.org/10.1016/j.foodcont.2018.04.032

27. Liu ZF, Ci XQ, Li L, et al (2017) DNA barcoding evaluation and implications for phylogenetic relationships in Lauraceae from China. PLoS One. https://doi.org/10.1371/journal.pone.0175788

28. Kim D-W, Yoo WG, Park HC, et al (2012) DNA Barcoding of Fish, Insects, and Shellfish in Korea. Genomics Inform. https://doi.org/10.5808/gi.2012.10.3.206

29. Chun J, Oren A, Ventosa A, et al (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int J Syst Evol Microbiol. https://doi.org/10.1099/ijsem.0.002516

30. Calvo JH, Zaragoza P, Osta R (2001) Technical note: A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. J Anim Sci

31. Köppel R, Schum R, Habermacher M, et al (2019) Multiplex real-time PCR for the detection of insect DNA and determination of contents of *Tenebrio molitor*, *Locusta migratoria* and *Achaeta domestica* in food. Eur Food Res Technol 245:559–567. https://doi.org/10.1007/s00217-018-03225-5

32. Capinera JL (2008) Encyclopedia of Entomology, Volume 4

33. Klausnitzer B (2011) Exkursionsfauna von Deutschland

34. Andrade JM, Estévez-Pérez MG (2014) Statistical comparison of the slopes of two regression lines: A tutorial. Anal. Chim. Acta

35. Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc B Biol Sci 270:313–321. https://doi.org/10.1098/rspb.2002.2218

36. Goerlich O, Grohmann L, Mazzara M, et al (2017) Verification of Analytical Methods for GMO Testing When Implementing Interlaboratory Validated Methods. Version 2. Test. Anal. GMO-containing Foods Feed

37. Hrnčírová Z, Bergerová E, Siekel P (2008) Effects of technological treatment on DNA degradation in selected food matrices of plant origin. J Food Nutr Res

38. Daniso E, Melpignano P, Tulli F (2020) An OLED-based genosensor for the detection of *Hermetia illucens* in feeds. Food Control 113:. https://doi.org/10.1016/j.foodcont.2020.107179

Supplementary materials

PCR amplification sequencing

```
KR903358.1_Gryllodes_sigillatus_(CYB)      -CAGGATCAAACAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATTCCATTCCATCCA 59
ACGS_Fw                                    ---GGATCAAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 57
GSS3_Fw                                    --AAGGGACAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 58
GSS1_Fw                                    ACAGGAATCAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 60
GSS2_Fw                                    --AGGAATCAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 58
UnGS_Fw                                    -----GATCAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 55
RoGS_Fw                                    ---CGGATCAATAATCCCCTAGGTGTCAACAGAAATCTAGATAAAATCCCATTCCATCCA 57
                                                   ** ************************************ ************

KR903358.1_Gryllodes_sigillatus_(CYB)      TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 119
ACGS_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 117
GSS3_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 118
GSS1_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 120
GSS2_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 118
UnGS_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 115
RoGS_Fw                                    TACTTCACATTTAAGGATATTACTGGATTCGTTATCATAATTATATTTTTAACTATTCTT 117
                                           ************************************************************

KR903358.1_Gryllodes_sigillatus_(CYB)      TCATTAACTAATCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 179
ACGS_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 177
GSS3_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 178
GSS1_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 180
GSS2_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 178
UnGS_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 175
RoGS_Fw                                    TCACTAACTAACCCTTATATACTTGGAGACCCAGATAATTTTACACCTGCAAATCCATTA 177
                                           *** ******* **************************************************

KR903358.1_Gryllodes_sigillatus_(CYB)      GTTACCCCCGTCCACATCCA 199
ACGS_Fw                                    GTTACCCCCATTCCC----- 192
GSS3_Fw                                    GT-ACCCC------------ 185
GSS1_Fw                                    GTTACCCCCCGTCCACTA-- 198
GSS2_Fw                                    GTTACCCCC-GTCCACAA-- 195
UnGS_Fw                                    GTTACCCCC-GTCCACAGA- 193
RoGS_Fw                                    GTTACCCCC-GTCCAC---- 192
                                           ** *****
```

```
KR903358.1_Gryllodes_sigillatus_(CYB)      GTAAAATTATCTGGGTCTCCAAGTATATAAGGATTAGTTAATGAAAGAATAGTTAAAAAT 60
GSS1_re                                    -GGAATTATCTGGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 59
UnGS_re                                    --GGAATATCTGGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 58
GSS2_re                                    TGGGAATATCTGGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 60
GSS3_re                                    --GGAATTTCTGGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 58
ACGS_re                                    -GGGAATTTCTGGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 59
RoGS_re                                    ----------CTGGGTCTCCAAGTATATAAGGGTTAGTTAGTGAAAGAATAGTTAAAAAT 50
                                                    ******************** ******* ********************

KR903358.1_Gryllodes_sigillatus_(CYB)      ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGAATT 120
GSS1_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 119
UnGS_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 118
GSS2_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 120
GSS3_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 118
ACGS_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 119
RoGS_re                                    ATAATTATGATAACGAATCCAGTAATATCCTTAAATGTGAAGTATGGATGGAATGGGATT 110
                                           ************************************************************ ***

KR903358.1_Gryllodes_sigillatus_(CYB)      TTATCTAGATTTCTGTTGACACCTAGGGGATTGTTTGATCCTGTTTGATGAAGGAATAAT 180
GSS1_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 179
UnGS_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 178
GSS2_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 180
GSS3_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 178
ACGS_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 179
RoGS_re                                    TTATCTAGATTTCTGTTGACACCTAGGGGATTATTTGATCCTGTTTGATGAAGGAATAAT 170
                                           ********************************* ****************************

KR903358.1_Gryllodes_sigillatus_(CYB)      AAATGAATTATAACGAAGGCGGCAACAA 208
GSS1_re                                    AAATGAATTATAACGAAGGCGGCA---- 203
UnGS_re                                    AAATGAATTATAACGAAGGCGGCA---- 202
GSS2_re                                    AAATGAATTATAACGAAGGCGGCA---- 204
GSS3_re                                    AAATGAATTATAACGAAGGCGGCA---- 202
ACGS_re                                    AAATGAATTATAACGAAGGCGGCA---- 203
RoGS_re                                    AAATGAATTATAACGAAGGCGGCACAAC 198
                                           ************************

                ** *****
```

3 Biosensors overview

In recent decades, the interest in developing a innovative fast analytical methods for the detection of pathogens and /or metabolites and biomolecules has been growing exponentially mainly due to the implementation of human health and food safety regulations. With the ongoing miniaturization in electronics, emerging technologies can enable portable instruments and minimize the need for bulky laboratory infrastructures and well-trained operators for the benefit of point of care (POC) analysis (Khalilpour et al., 2015; Liu et al., 2019). These innovative systems for rapid detection are so-called biosensors. Several technologies can be applied for the development of the biosensors and they take advantage of different strategies of detection; anyway all these biosensor technologies try to fulfil the current desirable features and characteristics, such as low-cost equipment, accuracy, portability, simplicity, rapid response and automation (Martín-Fernández et al., 2017). According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is defined as "a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is in direct spatial contact with a transduction element" (Thévenot et al., 2001).

In general, a biosensor system is capable to translate a biological variation in the sample into a signal detectable by the specific detection system; according to the detected signal, biosensors can be classified into four categories: electrical (Metkar and Girigoswami, 2019), optical (Bañuls et al., 2013; Homola et al., 1999; Melpignano et al., 2016), chemical (De Mora et al., 2011) and mechanical (Kaewphinit et al., 2012).

These innovative technologies are simple in the protocol analyses, have a low-cost price for sample unite analyzed, provide results in a short time and use fewer reagents in comparison to the traditional ones (Samanta and Sarkar, 2011); beyond that, they require a reduced number of operators/hours and not specifically trained Additionally, the specificity provided by biorecognition components, utilized in those innovative biosensors, can relieve the cross-reactivity and sample preparation problems associated with samples in complex matrices (Farka et al., 2017).

Other conventional biological methods such as enzyme-linked immunosorbent assay (ELISA), culturing or polymerase chain reaction (PCR) have been widely used to detect and quantify pathogens and other biomolecules with high sensitivity and specificity and are considered as gold standard methods for the validation of innovative protocols (Jordan and Durso, 2005). Unfortunately, these methods are not easy to be adapted for a point-of-care (POC) analyses, since they require a long protocol time, adequate environment and often complex, cumbersome or expensive tools or evaluate the results (Khalilpour et al., 2015). However, the knowledge acquired from the use of antibodies, aptamers and DNA / RNA in these methodologies can be used for the development of new innovative systems. Besides, these molecules can be used for bio-recognition in point-of-care (POC) diagnostic technologies allowing rapid screening of metabolites and biomolecules outside traditional laboratory settings thanks to new methods of detecting the biological signal generated (Liu et al., 2019; Zhan et al., 2020).

3.1 Optical biosensors

Among the class of biosensors, optical biosensors are the most reported ones. An optical biosensor is usually a compact analytical device containing a biorecognition sensing element that is integrated with an optical transducer system capable to detect the presence and/or the concentration of a target analyte (Damborský et al., 2016). For its construction various biological materials can be used as biorecognition elements, like enzymes, antibodies, antigens, receptors, nucleic acids, whole cells and tissues. Besides, optical biosensors can be divided into two main categories: a label-free category, when the detected signal is generated directly by the interaction of the target analyte and the transducer and a label-based sensing category, when for the detection of the target a label is used and an optical signal is then generated by a colorimetric, fluorescent or luminescent method. However, in label-based sensing, the labelling can alter the binding properties of one of the bio reactants and therefore could introduce systematic error to the biosensor analysis; this aspect should be taken into account during the development of new assays (*Handb. Surf. Plasmon Reson.*, 2008). Among the different optical biosensors available on the market, the surface plasmon resonance (SPR) is the most widely used as the phenomenon used for the development of those biosensors is well known. The SPR occurs on the surface of conducting materials at the interface of two media (usually glass and liquid) when it is illuminated by polarized light at a specific angle. The principle of the assay is that when the target binds to the bio-recognition molecule immobilized on the metal sensor surface, it generates surface plasmons and consequently a reduction in the intensity of the reflected light at a specific angle known as the resonance angle. This effect is proportional to the mass that is bonded to the surface. In all configurations, the SPR phenomenon enables direct, label-free and real-time changes of refractive index at the sensor surface, which is proportionate to the biomolecule concentration (Masson, 2017).

Exploiting the same physical principle of SPR, other methodologies were developed such as SPR imaging (SPRi) that merges the sensitivity of SPR and spatial imaging in a microarray format, allowing the simultaneous study of multiple different interactions, or Localized SPR (LSPR) based on metallic nanostructures (MNPs) that induced plasmons oscillate locally on the nanostructure rather than along the metal/dielectric interface.

Other method are also available such as evanescent wave fluorescence biosensors; in this case the biological recognition of the target is due to the binding event occurred within the boundaries of an evanescent wave (the evanescent wave arises from the manner in which light behaves when confined in an optical waveguide or fiber), or bioluminescent optical fibre biosensors where engineered microorganisms emit detectable a bioluminescent signal in the presence of the target molecule, this signal is subsequently transferred from the analyte by an optical fibre to the detector.

However, many other techniques are utilised for the construction of a optical biosensor, in the following articles a fluorescence biosensor base on the excitation of the label by a OLED souce will be proposed for the detection of target DNA in complex matrix as fish feed

3.2 Biosensors development

There are three essential components that should be investigated when a new biosensor is to be developed: biological recognition elements (like enzyme, antibody, DNA, etc.), a signal transducer that transforms the detected signal into a quantified, readable output and a signal processor to convert the transformed signal in an easy and clear output. In particular, during the design of a new biosensor, a fundamental aspect that must be carefully studied is the immobilization procedure of the recognition or reaction biomolecules with the selected biosensor surface (Bhalla et al., 2016; Lakshmipriya and Gopinath, 2018)The objective of the direct hooking of the biomolecules on the surface can be achieved by using several methods, based on the mechanism of attachment: the non-covalent interactions or the covalent bonds. In the case of immobilization through non-covalent interactions, the immobilization of the target molecule on the surface can be achieved through electrostatic, hydrophobic, or polar interactions; while in the second immobilization strategy, the target biomolecules can be anchored on the suitable functionalized substrate by the reaction between the exposed functional groups of the biomolecules with the functional groups present on the surface of the substrate for the biosensor construction. The advantage of the covalent bond formation is that the molecules that are covalently bounded are not removed by buffers during assays, and this is a fundamental aspect to take into consideration during the protocol analysis design (Samanta and Sarkar, 2011).
In the proposed article, five different surface treatments were evaluated for their capacity to bind on the polyethylene (PE) biosensor surface the DNA probes for the construction of a DNA biosensor base on OLED light excitation.

3.2.1 Tailoring the chemical functionalization of a transparent polyethylene foil for its application in an OLED-based DNA biosensor

Elsevier Editorial System(tm) for Biosensors and Bioelectronics

Manuscript Draft

Manuscript Number:

Title: TAILORING THE CHEMICAL FUNCTIONALIZATION OF A TRANSPARENT POLYETHYLENE FOIL FOR ITS APPLICATION IN AN OLED-BASED DNA BIOSENSOR

Article Type: Full Length Article

Section/Category: Affinity (nucleic acids)

Keywords: Polymeric surface modification, oligonucleotide bio-probe, fluorescence detection, genosensor, Hermetia illucens

Corresponding Author: Dr. enrico daniso, Ph.D.

Corresponding Author's Institution: University of Udine

First Author: enrico daniso, Ph.D.

Order of Authors: enrico daniso, Ph.D.; Boris Maroh; Sonja Feldbacher; Sandra Schlögl; Inge Mühlbacher; Patrizia Melpignano

TAILORING THE CHEMICAL FUNCTIONALIZATION OF A TRANSPARENT POLYETHYLENE FOIL FOR ITS APPLICATION IN AN OLED-BASED DNA BIOSENSOR

Enrico Daniso[a*1], Boris Maroh [b1], Sonja Feldbacher [b], Inge Mühlbacher[b], Sandra Schlögl[b], Patrizia Melpignano[c]

[a] Department of Agricultural, Food, Environment and Animal Science, University of Udine, Udine, Italy

[b] Polymer Competence Center Leoben GmbH, Roseggerstrasse 12, AT-8700 Leoben, Austria

[c] OR-EL d.o.o., Volariceva Ulica 6, 5222 Kobarid, Slovenia

[*]Corresponding Author: Enrico Daniso, enrico.daniso@uniud.it, Via Sondrio 2°, Udine (UD), 33100, Italy.

[1] These authors contributed equally to the present work

Declarations of interest: none

Keywords : Polymeric surface modification, oligonucleotide bio-probe, fluorescence detection, genosensor.

Abstract

For the development of a bio-sensor based on fluorescence excitation in transmission, a plastic substrate with excellent optical characteristics, such as high optical transmission in the visible range and low self-fluorescence, is required. Polyethylene (PE) films have been found to meet these optical characteristics. However, the adhesion of short sequences of oligonucleotides used as bio-probes, which are needed to develop a genosensor, is not ideal on this substrate, due to its olefinic composition. In order to overcome this challenge, a physico-chemical surface modification of PE films has been performed using a corona treatment followed by the attachment of functional organic compounds. The surface modified substrates have been characterized for their wettability, surface energy, zeta potential and surface topography. The bio-probes′ adhesion on the treated surfaces has then been tested by monitoring the fluorescence of dye-conjugated complementary sequences of the oligonucleotides deposited on the modified PE substrates. Finally, two modified PE substrates, comprising the best adhesion of the bio-probes, have been used to prepare a genosensor for the detection of the DNA of the insect *Hermetia illucens.* As expected*,* the better adhesion of bio-probes on the functionalized PE substrate has allowed a significant improvement of the bio-sensor limit of detection (LOD).

Introduction

In the last decades, the interest to develop innovative and fast analytical methods for the detection of pathogens and other important biomolecules is exponential increasing. These innovative devices are so-called biosensors and they are capable to convert a biological response into an electrical (Metkar and Girigoswami, 2019), optical (Bañuls et al., 2013; Daniso et al., 2020b; Homola et al., 1999; Melpignano et al., 2016) , chemical (De Mora et al., 2011) and/or mechanical (Kaewphinit et al., 2012) signal. They usually offer a simple low-cost, fast analysis with a significant reagent reduction (Samanta and Sarkar, 2011) and they do not require specialized personal to operate them. Additionally, the specificity provided by biorecognition components, utilized in some of those innovative biosensors, can relieve the cross-reactivity and sample preparation problems associated with the presence of analytes in a complicated matrix (Farka et al., 2017). At contrary, traditional analysis tools such as mass spectrometry, gas chromatography, fluorescence spectrometry, etc. require more expensive and bulky instruments and often need time-consuming handling from well-trained operators and a well-equipped laboratory (Liu et al., 2019).

Conventional biological diagnostic methods such as enzyme linked immunosorbent assay (ELISA), culturing or polymerase chain reaction (PCR) have been widely used to detect and quantify pathogens and other biomolecules with high sensitivity and specificity (Jordan and Durso, 2005) but are not adaptable to be used such as point-of-care (POC) analysis because they require long assay times, and complex and expensive

instruments to run the analysis or evaluate the results (Khalilpour et al., 2015); but the same bio-probes used in these traditional diagnostic systems, such as antibodies, aptamers and DNA/RNA can also be utilized for biorecognition in point-of-care diagnostic technologies to enable rapid screening of metabolites and biomolecules outside of traditional laboratory settings (Zhan et al., 2020). The development of such innovative platforms, that are affordable and rapid, for the control of infectious diseases is one of the top priorities for improving human health allowing a point-of-care detection in unusual settings as the COVID-19 pandemic has demonstrated (Green et al., 2020; Metkar and Girigoswami, 2019), but they can be also used for the screening of environmental pollutants, pesticides or other molecules such as 2,4,6-trinitrotoluene (TNT) that dissipate in soil and groundwater and can be absorbed by direct contact of skin and result in the malfunction of liver and anemia (Liu et al., 2019).

During the development of a new POC biosensor, the immobilization of recognition biomolecules (bio-probes) on the biosensor′s active surface is an integral part of the research (Bhalla et al., 2016; Lakshmipriya and Gopinath, 2018). Direct attachment of biomolecules on the surface can be reached taking advantage of the non-covalent interactions or covalent bond of the bio-probes with the substrate. In non-covalent interactions, immobilization on the surface can be achieved through electrostatic, hydrophobic or polar interactions; while in covalent bound the biomolecules can be anchored on a suitably functionalized substrate via covalent bond formation by reaction with exposed functional groups; the advantage of the second methodology is that molecules covalently bound are not removed by buffer washing during assays (Samanta and Sarkar, 2011).

In the present study the optimization of the bio-probes′ adhesion on the active surface of our optical biosensor is described. In particular, the detection of the fluorescence excited by an organic light emitting diode (OLED), detected using an image sensor (Daniso et al., 2020a; Manzano et al., 2015; Melpignano et al., 2016), in a point of care system is possible due to the deposition of the bio-probes on a transparent polymer film . The specific bio-probes envisaged for our diagnostic test are short sequences of oligonucleotides (short ssDNA sequences), which have to be bonded on the polymeric substrate to allow the DNA recognition of selected insect species in different matrices. However, the transparent polymer film used as surface for our biosensor must satisfy specific requirements. In terms of carrier material, certain polymers are considered here due to physical/mechanical (e.g. cutable, light weight, unbreakable), chemical and optical properties (e.g. transparency, haze, colour, clarity). Promising types of polymers are unfilled amorphous thermoplastics (crystalline part < 5%) such as polystyrene (PS), polycarbonate (PC), polyvinylchloride (PVC), polymethylmethacrylate (PMMA) or polyethylene (PE). However, it has to be considered that not all of them have necessarily a high transmission in the fluorescence excitation region. A high content of low deep-blue/UV light absorbing parts (e.g. from C-H groups in the polymer backbone) are preferred here (Oreski et al., 2010). In previous work, a PS surface has been used as transparent substrate to bind the bio-probes in a low-density matrix due to the surface chemistry compatible to further procedure. However, the optical transmission of PS is not ideal for the targeted fluorescence excitation. Compared to PS, fluorinated polymers (e.g. ETFE) and polyolefines (e.g. PE) show very low absorbance in the region over

400 nm (Seymour et al., 1984). Thus, to improve our biosensor sensitivity, a thin film of PE has been selected as substrate in the current study, due to its optical characteristics comparable to that of a thin glass sheet. The chemical nature of PE involving alkane groups results in a hydrophobic surface with low surface energy. Thus, wettability and covalent adsorption of the targeted bio-probes are therefore very limited. In a new approach, we tailored the polarity and chemical surface composition of the PE substrate by applying selected physical and chemical surface treatments to enhance the attachment of the respective bio-probes.

Polymers with a lower surface energy tend to form intrinsically poor adhesion bonds without some type of surface treatment, which does only affect the region near the surface and does not alter the bulk properties. Today there are several treatment methods available to tailor polymer surface properties. Chemical modification techniques include wet etching, grafting, acid-induced oxidation, and plasma polymerization (Manhart et al., 2016, 2015; Sahin et al., 2018). Besides chemical treatments there are also several physical surface treatment methods described that include corona discharge, ion or electron beam, photon beams, plasma discharge, and flame oxidation (Ebnesajjad, 2011; Johansson, 2017; Ramkumar et al., 2018). Physical modification techniques can be also applied as activation procedures for further modification steps and has been known in this manner already for decades. To prepare a highly oxidized surface which can be tailored with different chemical modifications, corona discharge at atmospheric pressure has been used(Iwata et al., 1988; Levitin et al., 2012). In this paper, we report the application of such surface modification routes on PE substrates to significantly improve the adhesion properties of oligonucleotides used as bio-probes to detect the DNA of the insect *Hermetia Illucens* in the final biosensor.

Material and methods

Materials and chemicals

Commercially available polyethylene (PE) foils, 180 µm thick, were obtained from Goodfellow. (3-glycidyloxypropyl)triethoxysilane (GPTES), (3-aminopropyl)triethoxysilane (APTES), [3-(2-aminoethylamino)propyl] trimethoxysilane (AEAPTMS) and all other chemicals were supplied by Sigma-Aldrich and used without further purification.

Corona treatment of PE substrates

PE substrates were treated with a corona discharge using a benchtop corona instrument from Ahlbrandt GmbH (Germany). to increase the surface energy and to create active groups, such as −COOH, COH, -OH and −OOH. The exposure dose amounted to 1 J/cm^2 using a 10 seconds exposure time.

Surface modification with 1,6-hexanediamine (HMDA)

After the corona treatment, the PE substrates were immediately incubated with a solution of 10 wt% 1,6-hexanediamine in 100 mM borate buffer at pH 9.5 for 2 h. The substrates were then rinsed two times with MilliQ water for 10 min each. Finally, the PE substrates were dried overnight at 30 °C (Fixe et al., 2004).

Surface modification with organo-functional silanes

The corona treated PE substrates were immediately immersed into an anhydrous ethanol solution containing 1 wt% of a selected organosilane (GPTES, APTES or AEAPTMS). After heating the reaction formulation at 60 °C, the PE substrates removed after 4 hours and rinsed three times with anhydrous ethanol. Drying of the samples was carried out at 120 °C for 60 min in an air-circulating drying oven.

Contact angle measurements

The surface energy and the wettability of the four chemically modified substrates and the corona treated PE foil were measured using a contact angle technique. Contact angle measurements were carried out by using a DSA 100 Drop Shape Analysis System (Krüss, Germany). Deionized water and diiodomethane were used as test liquids. The droplet′s volume of both test liquids amounted to 2 µL. For the experiments, a drop was metered onto a solid sample (sessile drop) and an image of the drop was recorded with the help of a camera and transferred to the drop shape analysis software. A contour recognition was initially carried out based on a grey-scale analysis of the image. In the second step, a geometrical model describing the drop shape was applied to fit the contour of the drop. Thus, as the basic method the Young-Laplace-Fit, which is appropriate for ideal sessile drop oblate by its own weight and contact angles in the range of 10 ° to 180 °, was chosen. The surface energy was then calculated according to Owens, Wendt, Rabel and Kaelble (Kaelble, 1970; Owens and Wendt, 1969; Rabel, 1971). For each test liquid at least 10 measurements were taken, and reproducibility was assured by a maximum standard deviation of ± 3 °. A non-modified PE substrate served as reference.

Zeta potential and AFM surface topography measurements

Zeta potential analysis was carried out with the ″SurPASS″ electrokinetic analyzer (Anton Paar GmbH, Austria). In general, the streaming potential method was chosen to measure the zeta potential of modified PE substrates in 1mM KCl, whereby the analysis started from the natural pH level to lower acidic values by increasing the titration media up to 50 mM HCl or to higher pH values by increasing up to 50 mM NaOH with an autotitration unit (RTU, Anton Paar KG, Austria).

AFM measurements were carried out on an atomic force microscope Tosca 400 (Anton Paar GmbH, Austria) in a tapping mode with a resonance frequency of 274 Hz. For all substrates the scan size was 1x1 μm^2 at a speed of 1 line/s.

Design and Labelling of the detection probe

Three oligonucleotides probes were designed by retrieving the *Hermetia illucense's* sequence of mitochondrial cytochrome oxidase subunit I (COI) gene (accession no. GQ465783.1) from GenBank. A complementary pare of probe, forward (HI5Fw) and complementary reverse (HI5FwSpec), that showed low self-complementary and no hairpin formation, have been used for evaluating the binding capacity of the ssDNA on the different treated surfaces. The third probe (HI5reSpec) has been designed on the same gene sequence used for HI5Fw probe but in a different region of the considered sequence for the binding evaluation of real Hermetia DNA. For the attachment on the different surfaces, the probe HI5Fw (CTACCCTTCATGGAACACAAATATC) was ordered with and without 5′ amino modification. The HI5FwSpec (GATATTTGTGTTCCATGAAGGGTAG) HI5reSpec (CATAGGGGGATTTGTACACTGATAC) were ordered with the addition of an amino group in 5′ for the conjugation with a customized fluorophore. For the dye probe conjugation, 150 µg of Atto 430LS has been mixed with 17.5 µL of DMSO (dimethylsulfoxide) (Sigma, Milan, Italy), then 102.5 µL of sodium tetraborohydrate 0.1M (pH8.5) and 4 µL of the selected probe at a concentration of 25 µg/µL, were added. The tube has been incubated overnight at room temperature under agitation. After incubation one-tenth volume of 3M NaCl and two and a half volume of cold absolute ethanol were mixed in the tube and incubated at -20 °C for 30 min. Then the tube has been centrifuged at 12,000 x g for 30 min. The supernatant has been removed, the pellet let dry and subsequently dissolved in 100 µl of triethylammonium acetate (TEAA). The labelled oligonucleotide probe was then purified by reverse-phase HPLC using a standard analytical C18 column. The dissolved pellet has been loaded onto the column with 0.1 M (TEAA) to run a linear 5-95% acetonitrile gradient over 30 minutes (Daniso et al., 2020).

Hermetia DNA extraction

For the test of the genosensor, DNA from *Hermetia illucens* (HI) pupae were used. The pupae were frozen to −20 °C; then they were cut using a scalpel, put into a Lysing matrix A tube (MP biomedicals, USA) and finally disrupted by a TissueLyser II bead mill (Qiagen, Hilden, Germany). DNA has been extracted from 20 to 25 ng of whole disrupted pupae using Dneasy Blood & Tissue ® (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

The concentration and the purity of the extracted DNA have been evaluated measuring the absorbance at 260 nm and the 260/280 nm ratio, respectively, using a NanoDrop One Micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Also, DNA integrity has been tested by denaturing 1.2% agarose gel electrophoresis in 1X TBE buffer and ethidium bromide staining.

Support and probe adhesion

A black plastic cartridge with a reaction chamber of 200 µL volume has been used (Melpignano et al., 2016) for the genosensor preparation. The bottom of the reaction chamber was the transparent PE substrate previously prepared as described in paragraph 2.1, where the HI5Fw probe has been deposited as a 2 µL drop in triplicate, using the four concentrations and the four reaction mix showed in table 1. Mix 1 has a final concentration of 33.3 µM and mix 2 of 16.6 µM of the HI5Fw probe (which correspond to a dilution of 1:3 and 1:6 starting from an initial concentration of 100 µM). In all the different mix prepared, the cross-linking activator EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), coupled with different amount of 0.1M Imidazole, has been used in different amount, as described in Table 1. After the coating, the slides were incubated overnight at room temperature.

Table 1: Protocols of the different surface treatments used to improve the oligonucleotide probes (forward HI5Fw) binding to the PE surface.

Experimental Protocol	Sample n°	PE treatment	Reaction mix for attaching the HI5Fw primer on the PE substrate
P1-co	1-3	CORONA treatment	2 µl drop of HI5Fw primer at 100µM
P2-co	4-6	CORONA treatment	2 µl drop of HI5Fw amilate primer at 100µM
P3-co	7-9	CORONA treatment	2 µl drop of HI5Fw amilate primer at 50µM
P4-co	10-12	CORONA treatment	2 µl drop of HI5Fw amilate primer at 25µM
P5-co	13-15	CORONA treatment	2 µl drop of HI5Fw amilate primer at 12.5µM
P6-co	16-18	CORONA treatment	2 µl drop of Mix 1 (40 µl HI5Fw primer at 100µM + 80 µl Imidazole 0.1M + 0.5 mg EDC)
P7-co	19-21	CORONA treatment	2 µl drop of Mix 2 (40 µl HI5Fw primer at 100µM+ 160 µl Imidazole 0.1M + 1 mg EDC)
P8-hm	22-24	HMDA treatment	2 µl drop of Mix 1 (40 µl HI5Fw primer at 100µM + 80 µl Imidazole 0.1M + 0.5 mg EDC)

P9-hm	25-27	HMDA treatment	2 µl drop of Mix 2 (40 µl HI5Fw primer at 100µM + 160 µl Imidazole 0.1M + 1 mg EDC)
P10-gp	28-30	GPTES treatment	2 µl drop of HI5Fw amilate primer at 100µM
P11-gp	31-33	GPTES treatment	2 µl drop of HI5Fw amilate primer at 50µM
P12-gp	34-36	GPTES treatment	2 µl drop of HI5Fw amilate primer at 25µM
P13-gp	37-39	GPTES treatment	2 µl drop of Mix 1 (40 µl HI5Fw primer at 100µM + 80 µl Imidazole 0.1M + 0.5 mg EDC)
P14-gp	40-42	GPTES treatment	2 µl drop of Mix 2 (40 µl HI5Fw primer at100µM + 160 µl Imidazole 0.1M + 1 mg EDC)
P15-ap	43-45	APTES treatment	2 µl drop of Mix 1 (40 µl HI5Fw primer at 100µM + 80 µl Imidazole 0.1M + 0.5mg EDC)
P16-ap	46-48	APTES treatment	2 µl drop of Mix 2 (40 µl HI5Fw primer 100µM + 160 µl Imidazole 0.1M + 1 mg EDC)
P17-ae	49-51	AEAPTMS treatment	2 µl drop of Mix 1 (40 µl HI5Fw primer at 100µM + 80 µl Imidazole 0.1M + 0.5mg EDC)
P18-ae	52-54	AEAPTMS treatment	2 µl drop of Mix 2 (40 µl HI5Fw primer 100µM + 160 µl Imidazole 0.1M + 1 mg EDC)

Annealing of the fluorescence complementary probe

The prepared cartridges have been washed twice in deionized water before the utilization. Then, 2 µL of the RE labelled probe at the 5′ end with the Atto430LS dye (25 ng/ µL) and 198 µL of milliQ water have been pipetted on each slide. Then, the slides have been incubated at 62 °C for 1 h in a sterile petri dish to prevent evaporation; this incubation time is needed for the FW primer and RE primer to hybridize; subsequently, the slides have been washed twice with milliQ water to eliminate unbound labelled primer.

DNA annealing reaction

The plastic cartridge, with the different treated substrates and capture probe coating with a concentration of 33.3 µM, as described in paragraph 2.6, has been used for the genosensor construction and validation of the analysis with HI extracted DNA. Before the utilization, the cartridges have been washed twice in deionized water. Subsequently, 1 µL of the DNA serial dilutions samples (25 ng/µL, 50 ng/µL and 100 ng/µL), 4 µL of the HI DNA-detect-probe (HI5reSpec labelled at the 5′ end with the Atto430LS dye) and 195 µL of milliQ water have been pipetted on each slide after 7 min of denaturation at 95 °C. Then, the slides have been incubated at 62 °C for 1 h in a sterile petri dish to prevent evaporation; during this incubation time the capture and detection probes hybridize with the target HI DNA; subsequently, the slides have been washed twice with milliQ water to eliminate unbound DNA and unbound HI5reSpec probe. In each run, a No Template Control (NTC) with pure water substituting DNA was tested.

Fluorescence optical image acquisition and processing

For all tests, the fluorescence signals have been acquired using a high-sensitive CCD camera (Hamamatsu Orca C8484-03G02) integrated with a microscope objective. The image digitalization was set at 12 bits with a greyscale ranging from 0 to 4095. Image processing was performed with the shareware software ImageJ (Rasband, W.S., USA National Institutes of Health, Bethesda Maryland, USA).

A bottom-emission, small molecule-based, OLED, developed according to the OR-EL Patent (US 8,647,578 B2) optimized to obtain a deep blue colour emission with a peak wavelength of 434 nm (DB-OLED-Marcello), has been used to excite the fluorescence of the commercial dye Atto 430LS (Attotech, Germany), with the absorption peak located at 436 nm and the emission peak located at 545 nm. An in-depth physical description and characterization of the patented OLED, adopted in this point-of-care device, using the fluorescent molecule, a-NPD [*N*, *N*′-diphenyl-*N*, *N*′-bis (1-naphthylphenyl)-1, 1′-biphenyl-4, 4′-diamine] as an emitter, has been reported by (Marcello et al., 2013). After the reaction with the DNA target, the plastic cartridges have been put in contact with the exciting OLED source. A high-pass optical emission filter with high transmission in the fluorophore emission spectral region and a high extinction coefficient at the wavelength corresponding to the OLED emission (transmission (T<10^{-5}) has been used before the signal capture camera. The DB-OLED was used at 8.0 V with a total optical energy density of 155 W/cm^2. For image acquisition, no gain was used on the CCD and the integration time has been set to 20 s. For the analysis, the background of each image has been subtracted, and the images obtained have been analysed by applying a threshold, to automatically identify and measure the circular fluorescence spots. To obtain the sensitivity curve, the fluorescence intensity has been calculated by averaging the pixel values inside the image bright spots area.

Data analysis

The normality and homogeneity of the fluorescence data were tested using Shapiro−Wilk and Levene′s tests, respectively. Data were subjected to ANOVA test considering quantity of deposited probe and PE treatment as fixed factors and the interaction between PE treatment and deposited probe was also considered in the model; the Tukey′s test was used to evaluate significant differences among mean values (P<0.05). The statistical analyses were performed using R software, version 3.4.0 (Team, 2016). In the manuscript, values are reported as mean ± St.Dev.

Results and Discussion

Contact angle measurements

Contact angle measurements were carried out to determine the change of wettability and surface energy of the PE films after different modification steps (Table 2). PE films are not wettable with water-based

solutions due to their nonpolar surface characteristics involving a low polar part of the surface energy (0.3 mN/m) and a water contact angle of 97.9°. After the corona treatment of the PE surface, we observed a significant increase in the polar part from 0.3 to 29.6 mN/m, which is mainly attributed to formation of oxygen containing polar species such as −OH, -COOH, -COC or -COH groups. The formation of various polar groups on the surface improved the wettability of the PE films and the water contact angle decreased from 97.9° to 27.8°. Those polar groups were then used as reactive anchor groups to immobilize organosilanes by a condensation reaction. With the two-step modification, we were able to tailor surface polarity and surface reactivity over a broad range. Depending on the applied organosilane the polar part was varied between 3.2 mN/m (attachment of GPTES) and 7.8 mN/m (attachment of APTES). Along with organosilanes, the corona activated PE substrate was also modified with HMDA. The HMDA modified PE surface was characterized by a high wettability and a polar part of the surface energy of 15.3 mN/m.

Table 2: Surface energy and contact angle of PE substrates as a function of the applied treatment

Treatment of PE substrate	Surface energy (mN/m)	Polar part (mN/m)	Disperse part (mN/m)	Theta H_2O (°)	Theta CH_2I_2 (°)
untreated	35.5	0.3	35.3	97.9 ± 0.7	48.2 ± 1.6
Corona treated	70.2	29.6	40.7	27.8 ± 2.5	37.8 ± 2.9
GPTES modified	42.8	3.2	39.6	80.9 ± 2.3	40.1 ± 2.8
AEAPTMS modified	46.4	6.0	40.4	72.8 ± 2.4	38.5 ± 2.7
APTES modified	49.8	7.8	41.9	67.7 ± 3.0	35.2 ± 2.8
HMDA modified	54.8	15.3	39.5	55.0 ± 3.1	40.3 ± 2.5

Zeta potential and AFM measurements

In addition to contact angle measurements, zeta potential measurements were carried out to determine the success of the chemical modification of the PE substrates (Fig. 1). It can be observed that all three modifications with amino-functional compounds (AEAPTMS, APTES and HMDA) shift the isoelectric point (IEP) more into the basic region compared to the untreated PE substrate, which had an IEP of 2.9.

That confirms the presence of basic amino groups on the surface. It should be noted that the shift was more pronounced for organosilanes compared to HMDA. Organofunctional silanes are known to build oligomers and a siloxane network with higher reaction times (Matisons, 2012). The higher number of amino groups that are inside a siloxane network are able to shift the IEP to higher values. The formation of siloxane networks was also confirmed by AFM images (Fig. 1), where we observed significant changes in the surface topography compared to the untreated PE foil and PE foil modified with HMDA where the surface topography stayed nearly the same. The highest change in the topography was observed when APTES was applied to modify the surface. In comparison to AEAPTMS with two amino groups in the structure to APTES with only one, AFM images also explain the higher IEP shift from 2.9 to 7.1 for APTES and to 6.5 for AEAPTMS. Having a more pronounced change in the topography reveals that we have more APTES groups on the surface compared to AEAPTMS, which leads in the end to more amino groups in the network and therefore a higher IEP. In contrast, the immobilization of GPTES lead to a IEP shift in the more acidic region due to the presence of polar −OH groups after the hydrolyzation of epoxy groups.

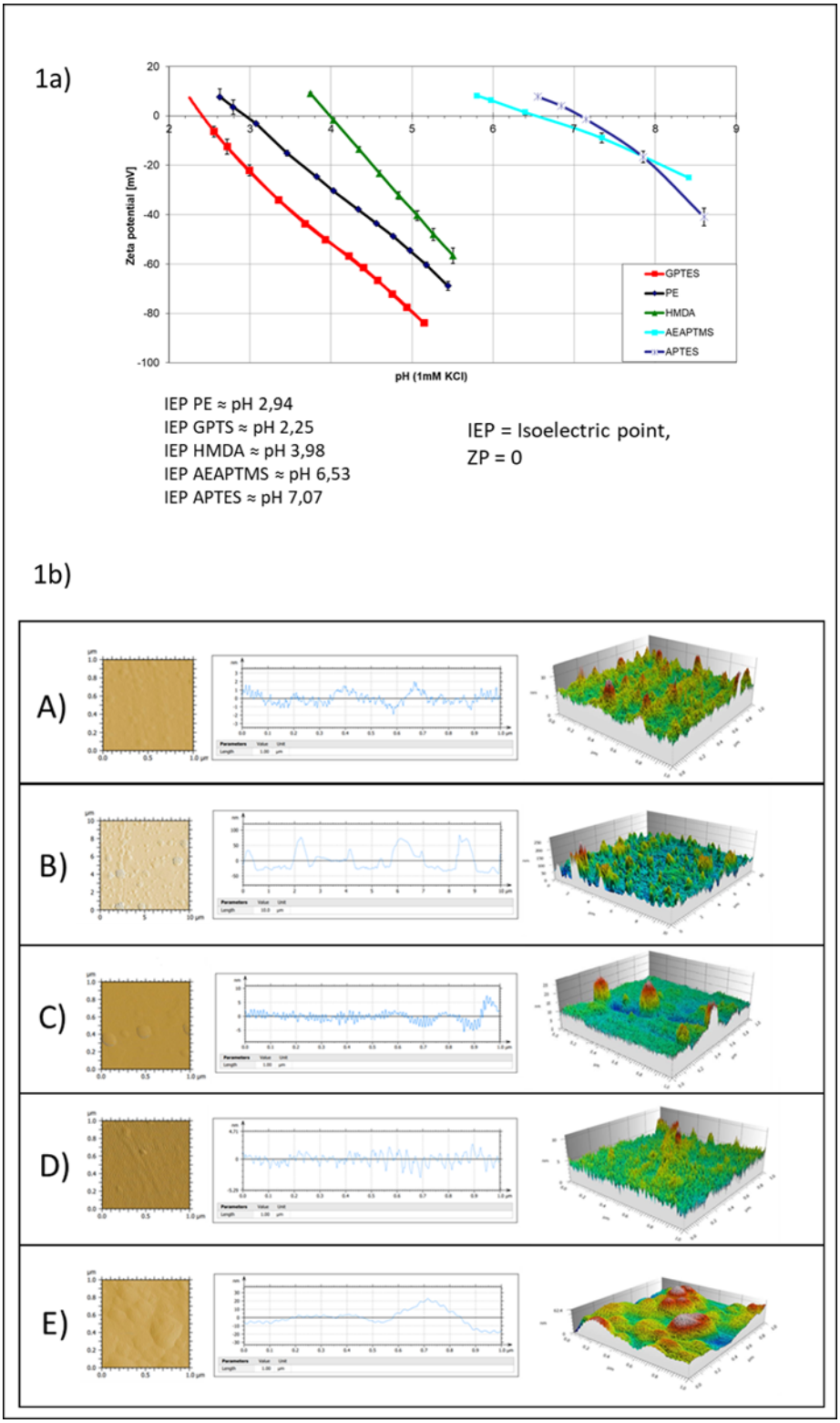


Fig. 1: (a) Zeta potential curves of PE substrates prior to and after the modification with selected organosilanes and HMDA, (b) AFM micrographs (1x1 µm²) from (A) untreated PE surfaces (Ra = 0.86 +/- 0.22 nm, Rq = 1.2 +/- 0.34 nm, Rt 5.89 +/- 1.62 nm) and after the functionalization with (B) APTES (Ra = 17.56 +/- 7.17 nm, Rq = 24.63 +/- 9.62 nm, Rt = 113.3 +/- 40.54 nm) , (C) GPTES (Ra = 1.98 +/- 1.09 nm, Rq = 2.99 +/- 1.74 nm, Rt = 12.01 +/- 5.75 nm), (D) HMDA (Ra = 0.73 +/- 0.14 nm, Rq = 0.95 +/- 0.21 nm, Rt = 5.22 +/- 1.2 nm) and (E) AEAPTMS (Ra = 9.13 +/- 1.77 nm, Rq = 11.38 +/- 1.9, Rt = 42.5 +/- 5.93 nm)

Evaluation of oligonucleotides adhesion on the surface modified PE substrates

The effective oligonucleotide adhesion on the different modified PE surfaces has been studied by measuring the fluorescence of the complementary probes conjugated with the Atto 430 LS fluorophore. The annealing procedure used for all the different treated substrates is described in paragraph 2.6. After the annealing, the resultant fluorescence has been measured to verify the effective adhesion of the DNA probes to the various substrates with the procedure described in paragraph 2.8.

The images of the fluorescence spots obtained using the different treated substrates are shown in Fig. 2, where the fluorescence 3D intensity distribution, in terms of count per pixel is also reported. The numerical results of the fluorescence spots are reported in Fig. 3. In this figure, the average value of the results obtained measuring the fluorescence of the samples produced in triplicate is reported with the standard deviation (StDev) for each experimental protocol used to modify the surface. As can be seen from Fig.3, no probe adhesion has been observed, independently on the surface treatment, if EDC is not used in the reaction mix where the oligonucleotide probes are dissolved to be deposited on the surfaces. EDC is generally utilized as a carboxyl activating agent for amide bonding with primary amines. Moreover, it can also be applied in a carbodiimide-mediate reaction with phosphate groups. EDC activates the phosphate group to form an intermediate phosphate ester, which reacts with amino groups to form a stable phosphoramidate bond (Hermanson, 2013a). For each surface treatment, two different dilutions of oligonucleotide probes (1:3 and 1:6) have been placed on the different substrates, in order to verify the proportionality of the fluorescent signal as a function of the probe concentrations. As can be seen from Fig. 3, using the same amount of complementary dye conjugated probe (50 ng) it is effectively possible to observe a fluorescent signal proportional to the amount of oligonucleotide probes deposited on the modified PE surfaces with the exception of the signal measured on the PE surface functionalized with AEAPTMS, as confirmed by the two ways ANOVA statistical analysis. The fluorescence signals obtained with the higher probes′ dilution on all the treated surfaces are statistically similar, using a Tukye test, except the result obtained for the surface functionalized with GPTES that shows a significantly higher value (see Greek letters in Fig. 3). Moreover, the results obtained with the lower probes′ dilution (33 µM deposited probes) are statistically similar for the substrates treated with corona activation only and with corona activation plus the successive functionalization with APTES and AEAPTMS. In contrast, the substrates functionalized with HMDA and GPTES give a higher fluorescence signal proportional to the degree of probe adhesion (see Latin letters in Fig. 3). From the images and the statistical analysis, it is clear that the substrate functionalized with the GPTES shows a significant better probe adhesion than all the other treated surface. The lower reactivity of amino-functional PE substrates can be explained by the steric hindrance of the crosslinked silane layers (see also AFM micrographs in Fig. 2), which reduces the mobility and the number of free $-NH_2$ groups available for the coupling reaction. In contrast, steric hindrance on GPTES modified PE surfaces is less pronounced, whilst under the applied reaction conditions the epoxy group of GPTES will hydrolyse yielding free −OH groups, which enhance the wettability and are able to react with the EDC activated phosphate functions under the formation of phosphoester links (Hermanson, 2013b). Furthermore, samples treated with HMDA with a

lower zeta potential shift compared to APTES and AEAPTMS have more available −NH2 groups on the surface since no stearic hindrance is present.

In addition, the PE surface modified with only the corona treatment presents a probe adhesion similar to that obtained with the successive chemical functionalization with amino-group based compounds. This is due to the treatment-induced creation of active groups, such as −COOH that in the presence of the zero-length cross-linker EDC, are able to form covalent bonding with phosphate groups present in the oligonucleotide probes. In this case no modification with an amino group is required to achieve the surface bonding, unlike the oligonucleotide probes used for the bonding on the chemically modified surfaces. However, while the corona treatment itself could be used to improve the oligonucleotide adhesion on PE surface, the treatment effect on the PE surface is time limited due to the relatively fast hydrophobic recovery. Thus, the interaction with the oligonucleotide probes must be done shortly after the treatment to be effective. In contrast, the chemical modification with HDMA, GPTES, APTES and AEAPTMS is permanent, allowing the oligonucleotide probes deposition also after weeks from the surface functionalization.

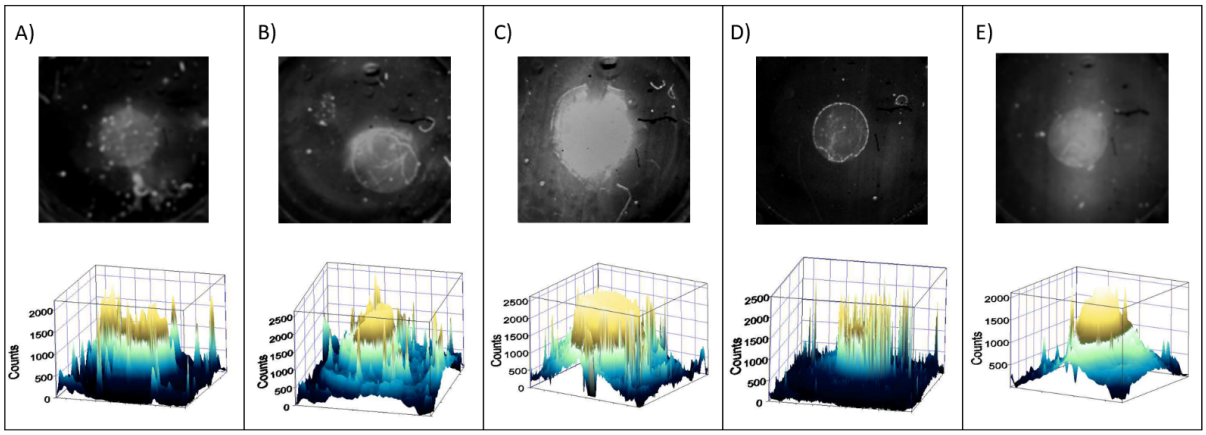


Fig. 2: Top: fluorescence image of 2 µL at [33.3 µM] of HI5Fw binding probe hybridized with its complementary dye conjugated probe HI5FwSpec, acquired with an integration time of 20 s, Bottom: 3D image of the same spot as in top 2D image. The images are showing that the number of counts in the central part of the spot for PE substrates modified with (A) HMDA, (B) Corona activation, (C) GPTES, (D) APTES and (E) AEAPTMS, are between 1500 and 2500 (background has been subtracted).

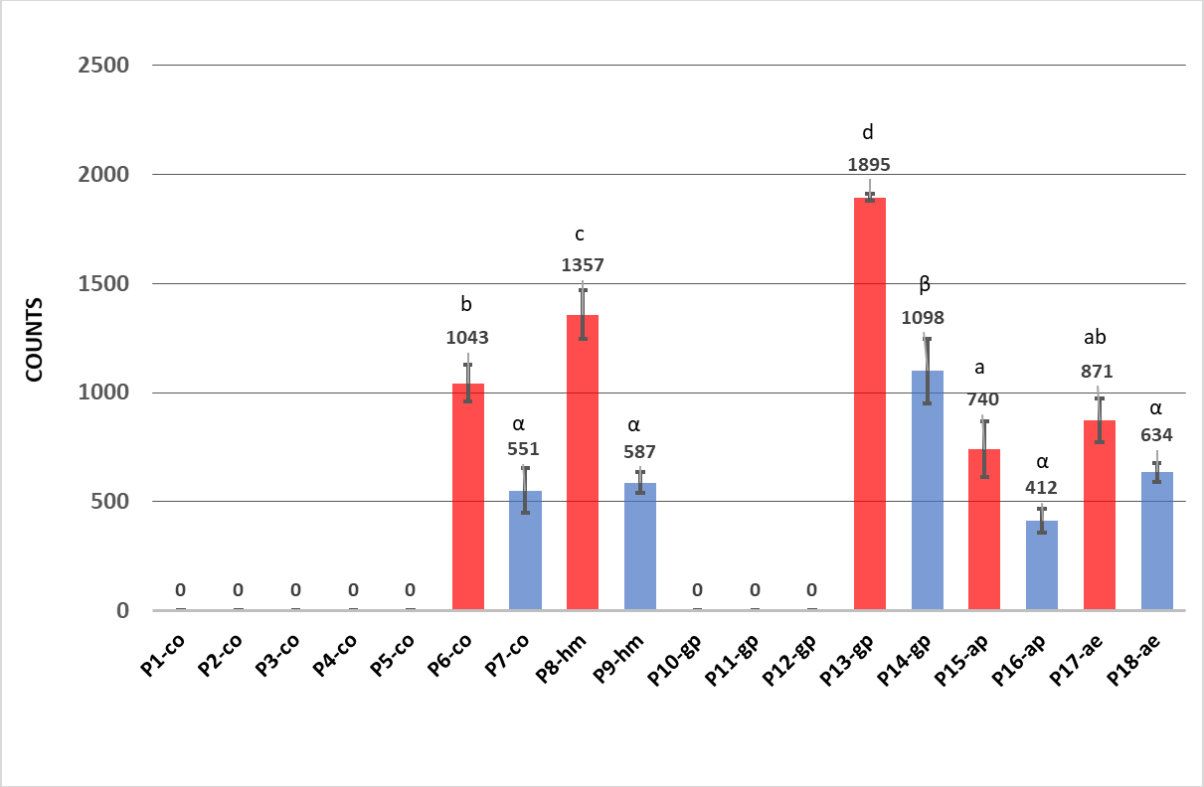


Fig. 3: Number of counts obtained using the OLED-based genosensor with an integration time of 20 sec to measure the fluorescence intensity of the annealed probe on the different proposed substrate treatments. Values are mean ± StDev of three replicate tests for each experimental protocol. The Latin letters represent the Tukye test results for the 1:3 HI5Fw probe dilution and the Greek letters for the 1:6 dilution.

Final experiment with DNA

To assess the effectiveness of the surface treatments in a real DNA biosensor, the two best surface treatments, HMDA and GPTES, have been applied to prepare a genosensor prototype. In this case, the same oligonucleotide probe of the first experiment, has been used at a concentration of 33.3 µM to detect the presence of the DNA of the insect *Hermetia Illucens.* This probe was used to bind a single strain of a DNA, previously denaturated, that have reacted with a secondary oligonucleotide dye-conjugated probe (complementary to a different region of the ssDNA target). The presence of the DNA is then detected by measuring the fluorescence. To verify the linear response of the analysis, an experiment with three different DNA concentrations has been performed in triplicate on the two selected substrates. The results of the experiments are reported in Fig.4

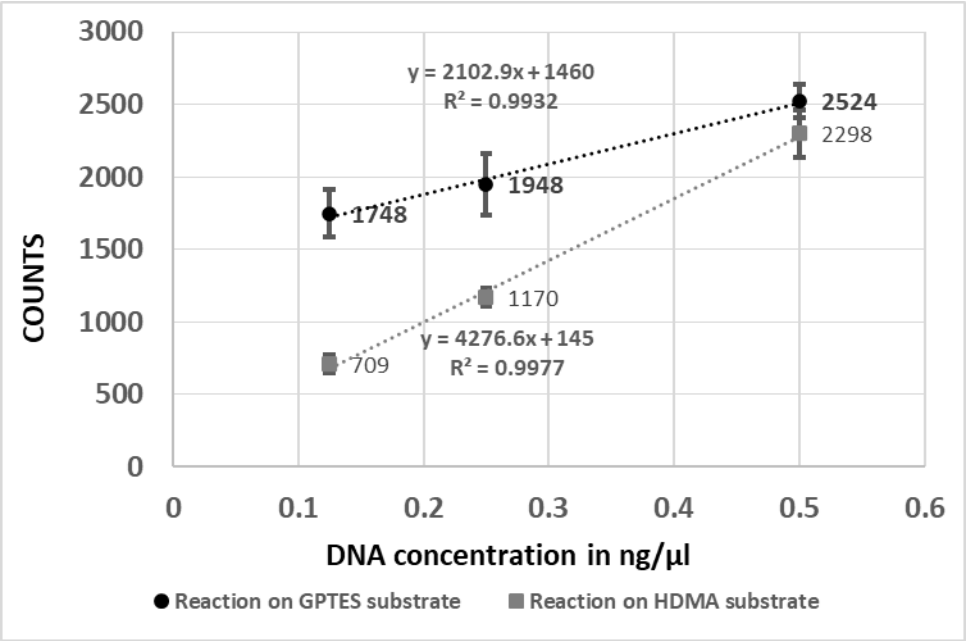


Fig. 4: Linear regression of the fluorescence signal (measured in counts) dependent on DNA concentration, obtained using the two PE substrates modified with GPTES and HDMA. For each DNA concentration the samples have been measured in triplicate.

Before to start the experiment with the DNA, the cut-off counts, obtained using a solution of HI5reSpec labelled at the 5′ end with the Atto430LS dye but without insect DNA and the two substrates coated with a drop of 2 L of 33.3 µM of the HI5Fw probe plus EDC, have been measured. 500 counts and 750 counts have been obtained for the substrate functionalized with HDMA and GPTES, respectively. The fluorescence measure was then been performed using various DNA concentrations (0.5, 0.25 and 0.125 ng/ L) in a total volume of 200 L of HI5reSpec labelled solution, without any amplification procedure. As can be seen from the graph both genosensors show a linear dependence of the fluorescence intensity vs. the DNA concentration in the sample. However, the genosensor built using the GPTES modified PE substrate shows a much higher counting rate that will allow, extrapolating the linear regression, to have a high fluorescence signal (> 1500 counts) also for a DNA concentration of 0.1 ng/ L. However, at the same DNA concentration the genosensor prepared using the HMDA modified PE substrate will give a fluorescence signal in the range of 500 counts, which is very close to its cut-off limit. Thus, in this case, it has reached its limit of detection at 0.125 ng/ L. In contrast, the genosensor built using the GPTES modified PE substrate has a signal of more than 1600 counts for the same DNA concentration, well beyond its cut-off limit of 750 counts, allowing to obtain a better LOD than that obtained with the HMDA functionalized PE substrate.

Conclusion

To improve the surface adhesion of oligonucleotides DNA probes on a transparent PE substrate, suitable for its excellent optical characteristics in a fluorescence-based biosensor, different physical and chemical surface treatments have been applied. A corona treatment was first used, followed by chemical functionalization with three different organosilanes: APTES, AEAPTMS and GPTES. A fourth functionalization was performed using the molecule HMDA, to obtain an amilated surface. The successful bonding of the functional groups was confirmed by contact angle, zeta potential and AFM measurements. Their ability to bound DNA oligonucleotide probes was then tested by measuring the fluorescence emitted after the reaction with a specular dye-conjugated DNA oligonucleotide. The results of this test revealed that the higher fluorescence was obtained with the PE substrate modified with GPTES, followed by that modified with HMDA. The other substrate modifications show very similar results in terms of emitted fluorescence but were lower than the first two. Finally, the two best substrates were used to prepare a final genosensor, using three different concentration of insect target DNA. The result of this final experiment shows that using the transparent PE foil functionalized with GPTES it is possible to obtain the best probe adhesion allowing to improve the limit of detection of the final genosensor to a LOD value better than 0.1 ng/ L without any DNA amplification procedure.

Acknowledgments

The research has been carried out within the project RETINA, which is being implemented and co-financed from the European Union - European Regional Development Fund in the frame of the Cooperation Programme Interreg V-A Slovenia-Austria in the programme period 2014-2020. The authors like to thank Institute for Chemistry and Technology of Materials as well as the HRSM-Soft Matter Application Lab at the Graz University of Technology for the possibility to use their AFM device.

References

ADDIN Mendeley Bibliography CSL_BIBLIOGRAPHY Bañuls, M.J., Puchades, R., Maquieira, Á., 2013. Chemical surface modifications for the development of silicon-based label-free integrated optical (IO) biosensors: A review. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2013.01.025

Bhalla, N., Jolly, P., Formisano, N., Estrela, P., 2016. Introduction to biosensors. Essays Biochem. 60, 1–8. https://doi.org/10.1042/EBC20150001

Daniso, E., Melpignano, P., Tulli, F., 2020a. An OLED-based genosensor for the detection of *Hermetia illucens in feed*s. Food Control 113. https://doi.org/10.1016/j.foodcont.2020.107179

Daniso, E., Tulli, F., Cardinaletti, G., Cerri, R., Tibaldi, E., 2020b. Molecular approach for insect detection in feed and food : the case of *Gryllodes sigillatus*. Eur. Food Res. Technol. https://doi.org/10.1007/s00217-020-03573-1

De Mora, K., Joshi, N., Balint, B.L., Ward, F.B., Elfick, A., French, C.E., 2011. A pH-based biosensor for detection of arsenic in drinking water. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-011-4815-8

Ebnesajjad, S., 2011. Material surface preparation techniques, in: Handbook of Adhesives and Surface Preparation. https://doi.org/10.1016/B978-1-4377-4461-3.10005-7

Farka, Z., Juřík, T., Kovář, D., Trnková, L., Skládal, P., 2017. Nanoparticle-Based Immunochemical Biosensors and Assays: Recent Advances and Challenges. Chem. Rev. https://doi.org/10.1021/acs.chemrev.7b00037

Fixe, F., Dufva, M., Telleman, P., Christensen, C.B., 2004. Functionalization of poly(methyl methacrylate) (PMMA) as a substrate for DNA microarrays. Nucleic Acids Res. https://doi.org/10.1093/nar/gng157

Green, K., Graziadio, S., Turner, P., Fanshawe, T., Allen, J., 2020. Molecular and antibody point-of-care tests to support the screening, diagnosis and monitoring of COVID-19. Oxford COVID-19 Evid. Serv.

Hermanson, G.T., 2013a. Chapter 2 - Functional Targets for Bioconjugation BT - Bioconjugate Techniques (Third edition), in: Bioconjugate Techniques (Third Edition).

Hermanson, G.T., 2013b. Chapter 3 - The Reactions of Bioconjugation BT - Bioconjugate Techniques (Third edition), in: Bioconjugate Techniques (Third Edition).

Homola, J., Yee, S.S., Gauglitz, G., 1999. Surface plasmon resonance sensors: review. Sensors Actuators, B Chem. https://doi.org/10.1016/S0925-4005(98)00321-9

Iwata, H., Kishida, A., Suzuki, M., Hata, Y., Ikada, Y., 1988. Oxidation of polyethylene surface by corona discharge and the subsequent graft polymerization. J. Polym. Sci. Part A Polym. Chem. https://doi.org/10.1002/pola.1988.080261216

Johansson, K.S., 2017. Surface Modification of Plastics, in: Applied Plastics Engineering Handbook: Processing, Materials, and Applications: Second Edition. https://doi.org/10.1016/B978-0-323-39040-8.00020-1

Jordan, J.A., Durso, M.B., 2005. Real-time polymerase chain reaction for detecting bacterial DNA directly from blood of neonates being evaluated for sepsis. J. Mol. Diagnostics. https://doi.org/10.1016/S1525-1578(10)60590-9

Kaelble, D.H., 1970. Dispersion-Polar Surface Tension Properties of Organic Solids. J. Adhes. https://doi.org/10.1080/0021846708544582

Kaewphinit, T., Santiwatanakul, S., Chansiri, K., 2012. Quartz crystal microbalance DNA based biosensor for diagnosis: A review. Sensors and Transducers.

Khalilpour, A., Kilic, T., Khalilpour, S., Álvarez, M.M., Yazdi, I.K., Liu, Y., Yang, D., Yu, T., Jiang, X., Tokel, O., Yildiz, U.H., Inci, F., Durmus, N.G., Ekiz, O.O., Turker, B., Cetin, C., Rao, S., Sridhar, K., Natarajan, N., Shafiee, H., Dana, A., Demirci, U., Samanta, D., Sarkar, A., 2015. Portable Microfluidic Integrated Plasmonic Platform for Pathogen Detection. Sci. Rep. 40, 475–491. https://doi.org/10.1039/c0cs00056f

Lakshmipriya, T., Gopinath, S.C.B., 2018. An introduction to biosensors and biomolecules, in: Nanobiosensors for Biomolecular Targeting. https://doi.org/10.1016/B978-0-12-813900-4.00001-4

Levitin, G., Reinhardt, K., Hess, D.W., 2012. Plasma Cleaning for Electronic, Photonic, Biological, and Archeological Applications, in: Developments in Surface Contamination and Cleaning: Contaminant Removal and Monitoring. https://doi.org/10.1016/B978-1-4377-7881-6.00002-8

Liu, R., Li, Z., Huang, Z., Li, K., Lv, Y., 2019. Trends in Analytical Chemistry Biosensors for explosives : State of art and future trends 118. https://doi.org/10.1016/j.trac.2019.05.034

Manhart, J., Kramer, R., Schaller, R., Holzner, A., Kern, W., Schlögl, S., 2016. Surface Functionalization of Natural Rubber by UV-Induced Thiol-ene Chemistry. Macromol. Symp. https://doi.org/10.1002/masy.201650016

Manhart, J., Lenko, D., Mühlbacher, I., Hausberger, A., Schaller, R., Holzner, A., Kern, W., Schlögl, S., 2015. Photo-patterned natural rubber surfaces with tunable tribological properties. Eur. Polym. J. https://doi.org/10.1016/j.eurpolymj.2015.02.024

Manzano, M., Cecchini, F., Fontanot, M., Iacumin, L., Comi, G., Melpignano, P., 2015. OLED-based DNA biochip for *Campylobacter spp.* detection in poultry meat samples. Biosens. Bioelectron. 66, 271–276. https://doi.org/10.1016/j.bios.2014.11.042

Marcello, A., Sblattero, D., Cioarec, C., Maiuri, P., Melpignano, P., 2013. A deep-blue OLED-based biochip for protein microarray fluorescence detection. Biosens. Bioelectron. https://doi.org/10.1016/j.bios.2013.02.017

Matisons, J.G., 2012. Silanes and Siloxanes as Coupling Agents to Glass: A Perspective. https://doi.org/10.1007/978-94-007-3876-8_10

Melpignano, P., Daniso, E., Vidergar, N., 2016. Multiparametric oled-based biosensor for rapid dengue serotype recognition with a new pointof-care serological test. Inf. MIDEM 46.

Metkar, S.K., Girigoswami, K., 2019. Diagnostic biosensors in medicine – A review. Biocatal. Agric. Biotechnol. 17, 271–283. https://doi.org/10.1016/j.bcab.2018.11.029

Oreski, G., Tscharnuter, D., Wallner, G.M., 2010. Determination of solar optical properties of transparent polymer films using UV/vis spectroscopy. Sol. Energy Mater. Sol. Cells. https://doi.org/10.1016/j.solmat.2010.01.012

Owens, D.K., Wendt, R.C., 1969. Estimation of the surface free energy of polymers. J. Appl. Polym. Sci. https://doi.org/10.1002/app.1969.070130815

Rabel, W., 1971. Einige Aspekte der Benetzungstheorie und ihre Anwendung auf die Untersuchung und Veränderung der Oberflächeneigenschaften von Polymeren. Farbe und Lack.

Ramkumar, M.C., Cools, P., Arunkumar, A., De Geyter, N., Morent, R., Kumar, V., Udaykumar, S., Gopinath, P., Jaganathan, S.K., Pandiyaraj, K.N., 2018. Polymer coatings for biocompatibility and reduced nonspecific adsorption, in: Functionalised Cardiovascular Stents. https://doi.org/10.1016/b978-0-08-100496-8.00009-3

Sahin, M., Schlögl, S., Kalinka, G., Wang, J., Kaynak, B., Mühlbacher, I., Ziegler, W., Kern, W., Grützmacher, H., 2018. Tailoring the interfaces in glass fiber-reinforced photopolymer composites. Polymer (Guildf). https://doi.org/10.1016/j.polymer.2018.03.020

Samanta, D., Sarkar, A., 2011. Immobilization of bio-macromolecules on self-assembled monolayers: Methods and sensor applications. Chem. Soc. Rev. https://doi.org/10.1039/c0cs00056f

Seymour, R.B., Carraher, C.E., Seymour, R.B., Carraher, C.E., 1984. Properties of Polyolefins, in: Structure—Property Relationships in Polymers. https://doi.org/10.1007/978-1-4684-4748-4_12

Team, R.C., 2016. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput.

Zhan, L., Granade, T., Liu, Y., Wei, X., Youngpairoj, A., Sullivan, V., Johnson, J., Bischof, J., 2020. Development and optimization of thermal contrast ampli fi cation lateral fl ow immunoassays for ultrasensitive HIV p24 protein detection. Microsystems Nanoeng. https://doi.org/10.1038/s41378-020-0168-9

3.3 USE of DNA for Biosensor construction

Among the different strategies for the construction of a biosensor, the natural capacity of single strand DNA (ssDNA) to hybridize with his complementary DNA sequence and form the double-strand DNA (dsDNA) was the base of the DNA-biosensor device′s development. The DNA hybridize capacity was chosen in my thesis work for the creation of an innovative biosensor system based on insect DNA target recognition which exploits the excitation of a fluorescent DNA probe by an Oled light source.In DNA-biosensors, ssDNA molecules, that are covalently bond on the biosensor reaction chamber surface to form a layer, serve as the target recognition element and are known as the probe ss-DNA. The functional principle of the DNA biosensor is based on the DNA hybridization event between the probe ss-DNA with complementary target ss-DNA in the analysed sample; the recognition from the probe of its complementary DNA target and the formation of a DNA double helix will lead to changes in the mass transport, light absorption, emission, or proton concentration, which results in the generation of a signal (Saidur et al., 2017). This hybridization event can be converted into a quantifiable signal by means of an appropriate transducer element such as an optical, electrochemical or thermal element; the transducer is capable of transforming and quantifying the biorecognition event that has occurred in the analyzed sample into an output element which it′s translated into an electrical, optical or mass-sensitive signal (Sassolas et al., 2008).

Recently, this strategy has also been applied for the development of new assay such as gold nanorod-based optical DNA biosensor for the diagnosis of pathogens or for the analysis of bioactive constituents based on plasmon resonance (Minunni et al., 2005; Parab et al., 2010) , or for the detection of anti-gliadin antibodie based on optical fibers(Corres et al., 2008), or the accurate molecular measurements directly in undiluted whole blood measuring electrochemical variation, etc. (Barrias et al., 2019; Qiu et al., 2020; Rashid and Yusof, 2017).

3.3.1 An OLED-based genosensor for the detection of *Hermetia illucens* in feeds

Food Control 113 (2020) 107179

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

An OLED-based genosensor for the detection of *Hermetia illucens* in feeds

Enrico Daniso[a,1,*], Patrizia Melpignano[b,1], Francesca Tulli[a]

[a] Department of Agricultural, Food, Environmental and Animal Science, University of Udine, Udine, Italy
[b] OR-EL d.o.o., Volaričeva Ulica 6, 5222, Kobarid, Slovenia

ARTICLE INFO

Keywords:
Food control
OLED based geno-sensor
Hermetia illucens
qPCR
DNA bio-chip

ABSTRACT

Recently, the insects meal has been released by the European Union as an ingredient in aquafeeds and only seven insect species have been allowed to be used for these preparations. To avoid misleading results, a suitable method for the detection of these insect species in food/feed, would be highly desirable. In this paper, we present an innovative, sensitive, specific and low-cost Point-of-Care apparatus, based on DNA probes for the detection of one of the most popular insects used in aquafeed preparation, *Hermetia illucens (HI)*, commonly known as black soldier fly. The DNA probes adopted for this genosensor have been designed to enable a very specific insect recognition which were derived from DNA primers. The DNA primers were first tested with qPCR. Forty different samples (animal species and common aquafeed ingredients) have been tested to check the possible cross-reactivity, confirming the adequate primers specificity. The primers have been used in a practical application, like the detection of HI in complex matrices of practical aquafeeds. The DNA probes derived by these primers have been then applied to implement an Organic Light Emitting Diode (OLED) based genosensor. The developed genosensor has been tested with HI DNA directly extracted from the insect, without any DNA amplification; a good test sensitivity has been obtained, being 0.75 ng/μl its detection (LOD) limit. Then, the genosensor has been tested with the DNA extracted from the same aquafeeds used in the qPCR experiments. The results obtained by these two methods have then been compared giving very good consistency and both allowed to detect HI meal up to a minimum threshold of 5 g/100gr feed.

1. Introduction

Many different insect species are worldwide part of the traditional human diet (Bukkens, 1997; Ramos-Elorduy et al., 1997), some of them are also used in animal feeding. Recently, the adoption of insects as a source of protein for feed and food purposes has been claimed (Fao, 2013) and the advantage of insect-based products on the market is justified by their low environmental footprint and nutritional properties (Premalatha, Abbasi, Abbasi, & Abbasi, 2011; Sogari, Amato, Biasato, Chiesa, & Gasco, 2019). Recently, the European Union has adopted this approach allowing the use of seven insect species for feed production and in particular, for aquafeeds. The insect species selection has been based on the following criteria: not to be pathogenic or have other adverse effects on plant, animal or human health, not recognized as vectors of human, animal or plant pathogens and not to be invasive alien species. The seven selected insect species are: *Hermetia illucens* (HI) (black soldier fly), *Gryllodes sigillatus* (GS) (tropical house cricket), *Gryllus assimilis* (GA) (Jamaican field cricket), *Acheta domesticus* (AC) (house cricket), *Alphitobius diaperinus* (AD) (small mealworm), *Tenebrio molitor* (TM) (yellow mealworm) and *Musca domestica* (MD) (common housefly) (Commission, 2017). Among these species, HI, MD and TM, due to their short development cycle, high survival of immature, high oviposition rate and very high potential to convert organic waste in biomass, are receiving particular attention, as they could collectively convert 1.3 billion tons of bio-waste per year into edible food and feed (Veldkamp et al., 2012). In particular, HI exhibits a consolidated rearing method and shows great ability to utilize cheap and less restrictive substrate than TM. For this reason, black soldier fly prepupae meal has preferentially been selected for inclusion as fish meal substitute in aquafeed preparations ("The historical spread of the Black Soldier Fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada," 2015). Its nutritional and physical properties have been proven a promising component of a complete fish diet for rainbow trout (Cardinaletti et al., 2019; St-Hilaire et al., 2007), catfish, tilapia (Cummins et al., 2017; Józefiak et al., 2019; Kumlawan, Arief, Agustono, & Lamid, 2018), juvenile Japanese seabass (Wang et al., 2019), European seabass (Magalhães et al., 2017) and Atlantic salmon (Belghit, Waagbø, Lock, & Liland, 2019). However, aquafeeds

* Corresponding author. Department of Agricultural, Food, Environmental and Animal Science, University of Udine, via Sondrio 2A, Udine, 33100, UD, Italy.
E-mail address: enrico.daniso@uniud.it (E. Daniso).
[1] These authors contributed equally to the present work.

https://doi.org/10.1016/j.foodcont.2020.107179
Received 28 November 2019; Received in revised form 18 February 2020; Accepted 19 February 2020
Available online 22 February 2020

AN OLED-BASED GENOSENSOR for the DETECTION OF *HERMETIA ILLUCENS* IN FEEDS

Enrico Daniso[a*1], Patrizia Melpignano[b1], Francesca Tulli[a]

[a] Department of Agricultural, Food, Environment and Animal Science, University of Udine, Udine, Italy

[b] OR-EL d.o.o., Volariceva Ulica 6, 5222 Kobarid, Slovenia

[*]Corresponding Autor: enrico.daniso@uniud.it

[1] These authors contributed equally to the present work

Food control 2020, doi.org/10.1016/j.foodcont.2020.107179

Abstract

Recently, the use of insects has been released by the European Union as an ingredient in aquafeeds and only seven insect species have been allowed to be used for these preparations. To avoid misleading results, a suitable method for the detection of these insect species in food/feed, would be highly desirable. In this paper, we present an innovative, sensitive, specific and low-cost Point-of-Care apparatus, based on DNA probes for the detection of one of the most popular insects used in aquafeed preparation, *Hermetia illucens (HI),* commonly known as black soldier fly. The DNA probes adopted for this genosensor have been designed to enable a very specific insect recognition which were derived from DNA primers. The DNA primers were first tested with qPCR. Forty different samples (insects and other aquafeed ingredients) have been tested to check the possible cross-reactivity, confirming the adequate primers specificity. The primers have been used in a practical application, like the detection of *HI* in complex matrices of practical aquafeeds. The DNA probes derived by these primers were then applied to implement an Organic Light Emitting Diode (OLED) based genosensor. The developed genosensor has been tested with *HI* DNA directly extracted from the insect, without any DNA amplification; a good test sensitivity has been obtained, being 0,75 ng/µl its detection (LOD) limit. Then, the genosensor has been tested with the DNA extracted from the same aqua-feeds used in the qPCR experiments. The results obtained by these two methods have then been compared giving very good consistency and both allowed to detect HI meal up to a minimum threshold of 5g/100gr feed.

Highlights:

- A new DNA method for the identification of the black soldier fly *(Hermetia illucens)* in feeds.
- Sensitivity and accuracy of OLED-based Genosensor compared to qPCR.
- Discrimination among insect species using a new DNA based Genosensor.
- *Hermetia illucens* DNA detection in complex matrices.

Introduction

Many different insect species are worldwide part of the traditional human diet (Bukkens, 1997; Ramos-Elorduy et al., 1997), some of them are also used in animal feeding. Recently, the adoption of insects as a source of protein for feed and food purposes has been claimed (Fao, 2013), the advantage of insect-based products on the market are justified for their low environmental footprint and nutritional properties (Premalatha et al., 2011; Sogari et al., 2019). Recently, the European Union has adopted this approach allowing the use of seven insect species for feed production and in particular, for aquafeeds. The insect species selection has been based on the following criteria: not to be pathogenic or have other adverse effects on plant, animal or human health, not recognized as vectors of human, animal or plant pathogens and not to be invasive alien species. The seven selected insect species are: *Hermetia illucens* (HI) (black soldier fly), *Gryllodus sigillatus* (GS) (tropical house cricket), *Gryllus assimilis* (GA) (Jamaican field cricket), *Acheta*

domesticus (AC) (house cricket), *Alphitobius diaperinus* (AD) (small mealworm), *Tenebrio molitor* (TM) (yellow mealworm), and *Musca domestica* (MD) (common housefly) (Commission, 2017). Among these species, *HI, MD* and *TM*, due to their short developmental cycle, high survival of immaturity, high oviposition rate and very high potential to convert organic waste in biomass, are receiving more attention, as they could collectively convert 1.3 billion tons of bio-waste per year into edible food and feed (Veldkamp et al., 2012). In particular, HI exhibits a consolidated rearing method and shows great ability to utilize cheap and less restrictive substrate than TM. For this reason, black soldier fly prepupae meal has preferentially been selected for inclusion as fish meal substitute in aqua-feed preparations ("The historical spread of the Black Soldier Fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada," 2015). Its nutritional and physical properties have been proven a promising component of a complete fish diet for rainbow trout (Cardinaletti et al., 2019; St-Hilaire et al., 2007), catfish, tilapia (Cummins et al., 2017; Józefiak et al., 2019; Kurniawan et al., 2018), juvenile Japanese seabass (Wang et al., 2019), European seabass (Magalhães et al., 2017) and Atlantic salmon (Belghit et al., 2019). However, aqua-feeds including HI have higher costs than the ones including other fish meal protein substitutes, like plant proteins or processed animal proteins (PAPs), and the price is justified by the high protein quality and limited market availability of HI meal. Therefore, it is important to certify the effective presence of the declared feed components to ensure that the farmers pay a correct price for the provided quality.

Moreover, the importance of authentication of products used in feed and food is a priority in the EU. This issue is even more pronounced for novel ingredients in a worldwide food and feed supply chain. In the last few years, food fraud and adulterations are causing serious economic consequences in the global market. Furthermore, undeclared raw material components, used in the food and feed production, could introduce additional and unpredictable food safety risks (Hong et al., 2017). To contrast such fraud and adulterations, guidelines to identify ingredients of animal origin have been set by the European Union Commission. The techniques for their identification are based on typical and microscopically identifiable characteristics or molecular biology techniques, such as polymerase chain reaction (PCR) (Annex VI (EC, 2009)). In particular, this latter technique, due to its good detection and accuracy, is worldwide used to determine the species of raw materials of animal origin and to evidence the absence of prohibited ingredients. For its high sensitivity and specificity, the molecular method is often preferred as identification system (Valentin et al., 2016). Currently, the detection in complex feed matrix of novel food is a newly emerging issue (Debode et al., 2017; Marien et al., 2018; Zagon et al., 2018). In particular, the TaqMan PCR technology has been proposed to detect one target species (Debode et al., 2017; Zagon, di Rienzo, Potkura, Lampen, & Braeuning, 2018) or more insect DNA targets simultaneously, in a multiplex real-time PCR. However, this latter technique, using multiple probes simultaneously, presents some disadvantages like kit costs and the possibility that, during the polymerase chain reaction, many secondary structures can be produced. This could generate a positive signal in NTC (no template control) at 24 thermal cycles, making the discrimination between a positive or negative signal difficult without an accurate evaluation of the qPCR melting curve (Köppel et al., 2019).

The present study aim has been the development of a low-cost and efficient qualitative and quantitative genosensor for the detection of *Hermetia illucens,* as an innovative portable molecular system. The present point-of-care system is based on the detection of an organic light emitting diode (OLED) excited fluorescence by an image sensor (Manzano et al., 2015; Marcello et al., 2013), to verify the presence of the investigated target insect species.

The developed genosensor has been first tested using HI DNA extracted from the insect and, then, using the DNA extracted from commercial aquafeeds including different HI meal levels.

The genosensor results have then been validated using the qPCR technique on the same samples and with the same DNA probes sequence used in the genosensor. The validation of this method for the detection of HI will be the first step to a more complex test, where different insect species will be measured simultaneously in a multi-parametric test, to eventually detect the presence of illegal insect in feeds.

2. Material and methods

2.1. Samples

Different insect and animal species, as well as other ingredients commonly used in aquafeeds, have been used to verify the potential cross-reactivity of the primers designed for the detection of *Hermetia illucens*. Insect samples of *Hermetia illucens* (HI), *Gryllodes sigillatus* (GS), *Gryllus assimilis* (GA), *Acheta domesticus* (AC), *Musca domestica* (MD), *Alphitobius diaperinus* (AD) and *Tenebrio molitor* (TM) were purchased live from Agripetgarden S.r.l. (PD) − Italy. Insect samples of *Bombus terrestris* (BT), *Nezara viridula* (NV), *Oecanthus pellucens* (OP), *Sitophilus granaries* (SG), *Sitophilus oryzae* (SO), *Rhyzopertha dominica* (RD), *Halyomorpha halys* (HH), *Apis mellifera* (AM), *Locusta migratoria* (LM) and *Bombyx mori* (BM) were kindly provided by the Entomology Division of the University of Udine, Italy. Species identification has been confirmed by the examination of morphological features of adult individuals according to scientific standard literature (Capinera, 2008; Klausnitzer, 2011). Muscle samples from selected animal species such as *Oncorhynchus mykiss* (rainbow trout) (RT), *Dicentrarchus labrax* (European sea bass) (ESB), *Sparus aurata* (gilthead sea bream) (GSB), *Bos taurus* (cattle) (BT), *Ovis aries* (sheep) (OA), *Loligo vulgaris* (squid) (LV), *Sepia officinalis* (cuttlefish) (SO), *Mytilus edulis* (blue mussel) (ME), *Penaeus kerathurus* (striped prawn) (PK), *Parapenaeus longirostris* (pink shrimp) (PL), *Pleoticus muelleri* (Argentine red Shrimp) (PM), *Chamelea gallina* (clam) (CG), *Aequipecten opercularis* (AO), *Pecten jacobaeus* (scallop) (PJ) and *Atherina boyeri* (AB) have been purchased from Mass Market Retailers, Italy.

Other raw materials, commonly used as aquafeed ingredients, such as Concentrate peameal (CPM), Soybean meal (SBM), Dry brewer′s yeast (DBY), Chile Prime Fish meal (FM) and Mays gluten meal (MG) were provided by Skretting Italia S.p.A. and Wheat gluten meal (WG) was supplied by Sacchetto S.p.A. (Torino, Italy); different poultry by-product meals (PBM) from Italian rendering industry were also used: chicken meal (PBM100) provided by ECB company (Bergamo Italy), 65% chicken and 35% turkey meal (PBM65)

provided by Agricola tre valli società cooperative (Verona, Italy). Practical aquafeeds with different percentages of HI inclusion were purchased from Cycle Farms, Beaufort en Vallée, France (Table 1).

2.2. Sample preparation and DNA extraction

Insects were freezed to -20°C; then the samples were cut using a scalpel and put into a Lysing matrix A tube (MP biomedicals, USA) finally disrupted by a TissueLyser II bead mill (Qiagen, Hilden, Germany).

DNA has been extracted from 20 to 25 ng of whole insect/pupae, animal tissue, and raw materials or feeds using Dneasy Blood & Tissue ® (Qiagen,Hilden, Germany) according to the manufacturer′s instruction. The DNA concentration and the purity of the extracts have been evaluated measuring the absorbance at 260 nm and the 260/280 nm ratio, respectively, using a NanoDrop One Micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA integrity has been tested by denaturing 1.2 % agarose gel electrophoresis in 1X TBE buffer and ethidium bromide staining.

Table 1
Complete aquafeed samples, with different HI inclusion tested in the present research.

Sample code	Hermetia inclusion g/100g	Product Treatment	Main Ingredients
NC (Negative control)	0	Extruded feed	Fish meal and oil, corn meal and gluten, feather meal
CR5	5	Cuticle removed, cold pelleted	Fish meal and oil, corn meal and gluten, feather meal
CR5F	5	Cuticle removed, cold pelleted, fine grain size	Fish meal and oil, corn meal and gluten, feather meal
CR5ext	5	Cuticle removed, extruded feed	Fish meal and oil, corn meal and gluten, feather meal
CR10ext	10	Cuticle removed, extruded feed	Fish meal and oil, corn meal and gluten, feather meal
Cut50F	50	Fine grain size, with cuticle	Wheat gluten, wheat meal, toasted soybean meal, sardine oil, tilapia trimmings
Cut50C	50	Coarse grain size, with cuticle	Wheat gluten, wheat meal, toasted soybean meal, tilapia trimmings and corn meal

2.3 DNA primers design and test

Specific primers for *HI* have been designed by retrieving the sequence of mitochondrial cytochrome oxidase subunit I (COI) gene (accession no. GQ465783.1) from GenBank; COI gene is usually used for barcoding analysis due to its high copy number in the genome and its low mutation rate. The pair of primers have been designed using the online software Primer3 (http://primer3.ut.ee) with Lucia algorithm obtaining the following forward and reverse primers:

Forward: HI5Fw (CTACCCTTCATGGAACACAAATATC)

Reverse: HI5re (GTATCAGTGTACAAATCCCCCTATG).

The end-point PCR technique has been performed to screen the target primer fragment amplification of 217bp using the GoTaq DNA polymerase Kit (Promega, USA). PCR reaction has been carried out in a final volume of 20 µl, using 4 µl of PCR Green or colorless GoTaq Reaction Buffer (1.5 mMol MgCl2), 13.5 µl distilled water, 0.4 µl PCR nucleotide Mix, 10 mM each (final concentration 0.2 mM of each dNTP), 0.1 µl GoTaq DNA polymerase (5 U/µl), 0.5 µl 10 µM of each primer and 1µl DNA template (<0.5 µg/50 µl) were used. The PCR thermal regime consisted of one cycle of 3 min at 98 °C, then 1 min at 95 °C, 1 min at 60 °C

and 1 min at 72 °C for 35 cycles and a final cycle of 5 min at 72 °C. Each PCR product has been subsequently gel verified for the expected product length (bp).

Real-time PCR has been performed in a total volume of 20 µl, 10 µl of EvaGreen Buffer mix, 8.2 µl of molecular biology grade water, 0.4 µl of each primer, Forward and Reverse at a final concentration of 200 nM, and 1 µl of DNA. qPCR has been carried out on a CX-96 (Biorad Srl, Milano, Italy) with the following program: initial denaturation steps of 3 min at 98 °C, followed by 40 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. Besides, a melting curve was determined starting at 65°C for 90 seconds, with a temperature increment step of 1°C/ 5 sec, till 95 °C. A No Template Control (NTC) with pure water substituting DNA was included in each run. All tested samples were diluted to obtain a concentration of 10 ng/µl and evaluated using a NanoDrop One Micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). When results of different runs were compared (e.g. in specificity testing), the threshold was always set manually to the same level. The baseline setting automatically carried out.

2.4 Design and Labelling of the detection probe

The pair of primers HI5, forward and reverse, that showed no cross-reactivity with the other samples in the PCR tests, have been used for the development of the genosensor with some modifications. The first modification has been the design of the reverse primer HI5re as its complementary probe, HI5reSpec (CATAGGGGGATTTGTACACTGATAC) with the addition of an amino group in 5′ for the conjugation with a customized fluorophore (Fig.1). For the primer conjugation, 150 µg of Atto 430LS has been mixed with 17.5 µl of DMSO (Dimethylsulfoxide) (Sigma, Milan, Italy), then 102.5 µl of sodium tetraborohydrate 0.1M (pH8.5) and 4 µl of the HI5reSpec at a concentration of 25 µg/µl, were added. The tube has been incubated overnight at room temperature under agitation. After incubation one-tenth volume of 3M NaCl and two and a half volume of cold absolute ethanol were mixed in the tube and incubated at -20 °C for 30 min. Then the tube has been centrifuged at 12,000 x g for 30min. The supernatant has been removed, the pellet let dry and subsequently dissolved in 100 µl of triethylammonium acetate (TEAA). The labeled oligonucleotides were then purified by reverse-phase HPLC using a standard analytical C18 column. The dissolved pellet has been loaded onto the column with 0.1 M (TEAA) to run a linear 5-95% acetonitrile gradient over 30 minutes.

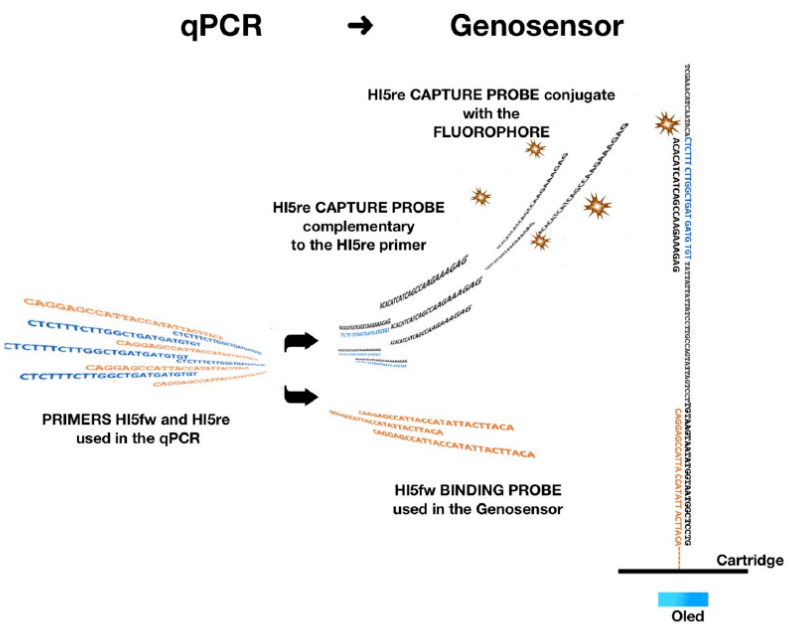


Fig. 1. HI5 Forward (fw) and Revers (re) primers were tested in qPCR, subsequently they were modified, and the fw primer became the binding probe attached to the polystyrene substrate (cartridge) and the re complementary primer conjugated with the fluorophore became the capture probe.

2.5 Support and probe adhesion

A black plastic cartridge with a reaction chamber of 150 µl volume has been used (Melpignano et al., 2016) for the genosensor development. The bottom of the reaction chamber is a polystyrene transparent substrate where the HI-capture-probe, forward HI primer (HI5Fw), has been deposited as a 1 µl drop. To improve the DNA probe adhesion on the polystyrene substrate a drop of 5 µl of Poly-L-lysin 0.005 (% w/v) has been deposited till dried. After a washing step with milliQ water, 1 µl of HI-capture-probe (HI5Fw primer) at 100 ng/µl has been deposited on the Poly-L-lysin in each cartridge and incubated at room temperature for 3 hrs to allow the support binding before washing with milliQ water. A 3 µl of DNA Blocking-probe at 1 µg/ µl has been deposited on the previous drop in each slide and incubated at room temperature overnight to block all the Poly-L-lysine sites still available.

2.6. Genosensor run protocol and sensitivity curve

The plastic cartridge, treated as described in paragraph 2.5, has been used for the genosensor construction and the measure of the sensitivity curve with the HI probes.

The prepared cartridges have been washed twice in deionized water before the utilization. Then, 1 µl of the DNA samples (25 ng/ µl), 4 µl of the HI-detect-probe (HI5reSpec labeled at the 5′ end with the Atto430LS dye) and 95 µl of milliQ water have been pipetted on each slide in a ratio 1:4 after 7 min of denaturation at 95 °C. Then, the slides have been incubated at 62 °C for 1 h in a sterile petri dish to prevent evaporation; this incubation time is needed for the capture and detection probes to hybridize with the target HI DNA; subsequently, the slides have been washed twice with milliQ water to eliminate unbound DNA and unbound HI-detect-probe. In each run, a No Template Control (NTC) with pure water substituting DNA and a positive

control HI DNA 25 ng/ µl have been included. The sensitivity curve has been determined using DNA serial dilutions (25, 12.5, 6.25, 3.12, 1.50, 0.75 ng/ µl) from HI and the same amount of the HI-detect-probe (100 ng for each well) and control tests have been performed to prevent false-negative results of the genosensor on (i) no capture probe (omission of the Fw5HI probe); (ii) no DNA (omission of the DNA sample); and (iii) no fluorophore (omission of the dye conjugated Re5HI probe).

2.7. Fluorescence optical image acquisition and processing

For all tests, the fluorescence signals have been acquired using a high-sensitive CCD camera (Hamamatsu Orca C8484-03G02) integrated with a microscope objective. The image digitalization was set at 12 bits with a grey scale ranging from 0 to 4095. The image processing was performed with the shareware software ImageJ (Rasband, W.S., USA National Institutes of Health, Bethesda Maryland, USA).

A bottom-emission, small molecule-based, OLED, developed according to the OR-EL Patent (US 8,647,578 B2) optimized to obtain a deep blue color emission with a peak wavelength of 434 nm (DB-OLED-Marcello), has been used to excite the fluorescence of the commercial dye Atto 430LS (Attotech, Germany), with the absorption peak located at 436 nm and the emission peak located at 545 nm. An in-depth physical description and characterization of the patented OLED, adopted in this point-of-care device, using the fluorescent molecule, a-NPD [N, N′-diphenyl-N, N′-bis (1-naphthylphenyl)-1, 1′-biphenyl-4, 4′-diamine] as an emitter, has been been reported by (Marcello et al., 2013). After the reaction with the DNA target, the plastic cartridges have been put in contact with the exciting OLED source. A high-pass optical emission filter with high transmission in the fluorophore emission spectral region and a high extinction coefficient at the wavelength corresponding to the OLED emission (transmission (T<10^{-5}) has been used before the signal capture camera. The DB-OLED was used at 8.0 V with a total optical energy density of 155 W/cm^2. For image acquisition, no gain was used on the CCD and the integration time has been set to 10 s. For the analysis, the background of each image has been subtracted, and the images obtained have been analysed by applying a threshold, to automatically identify and measure the circular fluorescence spots. To obtain the sensitivity curve, the fluorescence intensity has been calculated by averaging the pixel values inside the image bright spots area.

2.8 Determination of Primers LOD and efficiency

The primer amplification efficiency and sensitivity have been determined by six serial tenfold dilutions of HI DNA in triplicate. The PCR amplification efficiency, defined as the ratio of the final target gene molecules number divided by the initial number of target molecules at the beginning of the PCR cycle, has been calculated using the following formula:

Primer amplification Eff. (%) =

where *Slope* is the angular coefficient of the PCR linear regression obtained by plotting Ct-values against the log DNA concentrations. The limit of detection (LOD) was defined as the lowest dilution level for which all

three replicates still gave a positive result.

2.9 Data analysis

Data have been subjected to one-way ANOVA and, if adequate, means have been compared using Tukey's test, set for $P < 0.05$. Data analysis has been performed by using the SPSS package (Release 17.0, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Sensitivity and specificity of the DNA primers

When the efficiency of the DNA primers designed to detect the HI mitochondrial DNA has been evaluated, a linear relationship ($y = -3.360x + 24.42$, $R^2 = 0.998$; d.f. 14) between thermal cycles Ct values and Log DNA concentrations has been obtained within the dilution range (from 10 ng/µl to 100 fg/µl), as shown in Fig.2. Considering that the angular coefficient of the measured standard curve was -3.360, a primers efficiency of 99.8 % has been obtained.

According to the guidelines of the European Network of GMO laboratories for the validation of a quantitative real-time PCR method (Goerlich et al., 2017), the correct values for the qPCR standard curve angular coefficient should be within the range of $-3.6 \leq$ slope ≤ -3.1 and the amplification efficiency should be within the range 90% - 110%. The primers tested in the present study exhibited high efficiency (99.8%) and specificity (-3.360) according to the above-mentioned guidelines. Moreover, PCR positive signals have been observed until a dilution of 10^{-5} of the initial DNA solution, corresponding to a limit of detection (LOD) of 100 fg/ l. Such values reflect the higher presence of mitochondrial target genomes relatively to genomic DNA copies.

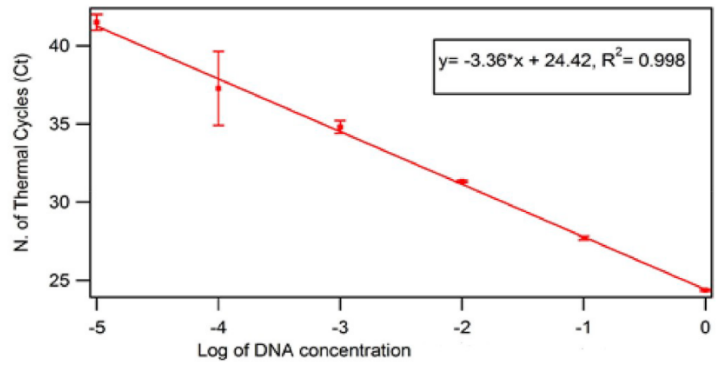


Fig. 2. HI primers efficiency from six tenfold dilution of HI starting from an initial concentration of 10 ng/µl measured using a NanoDrop One Micro-UV/ Vis spectrophotometer.

The specificity of the primers has been experimentally tested with DNA isolated from 46 different samples of animal and plant origin. All the samples have been evaluated in triplicate at an initial DNA concentration of 10 ng/ l and the number of amplification cycles needed to detect a fluorescence signal in qPCR was determined. As shown in Table 2, only the target species, *Hermetia illucens,* resulted in clear positive signals in qPCR after 24.3 thermal amplification cycles. Data indicate no cross-reaction between *HI* DNA and other DNA samples at a concentration of 10 ng/µl and these results underline the specificity of the designed primers. So, the use of the HI5 primers in a complex matrix is allowed, giving the negative signal obtained

when the ingredients have been tested, avoiding the risk of potential false positive.

Table 2
Samples of different species and ingredients tested with qPCR method and the associated Ct value reported as mean value and standard deviation of triplicate measurements.

Sample code	Species/product	Ct Detection
Insecta		
HI	*Hermetia illucens*	*24.30 ± 0.18*
GS	*Gryllodes sigillatus*	*N/A*
GA	*Gryllus assimilis*	*N/A*
AC	*Acheta domesticus*	*N/A*
MD	*Musca domestica*	*N/A*
AD	*Alphitobius diaperinus*	*N/A*
TM	*Tenebrio molitor*	*N/A*
BT	*Bombus terrestris*	*N/A*
NV	*Nezara viridula*	*N/A*
OP	*Oecanthus pellucens*	*N/A*
SG	*Sitophilus granarius*	*N/A*
SO	*Sitophilus oryzae*	*N/A*
RD	*Rhyzopertha dominica*	*N/A*
HH	*Halyomorpha halys*	*N/A*
AM	*Apis mellifera*	*N/A*
BM	*Bombyx mori*	*N/A*
LM	*Locusta migratoria*	*N/A*
Crustacea		
PL	*Parapenaeus longirostris*	*N/A*
PM	*Pleoticus muelleri*	*N/A*
PK	*Penaeus kerathurus*	*N/A*
Mollusca		
PJ	*Pecten jacobaeus*	*N/A*
ME	*Mytilus edulis*	*N/A*
CG	*Chamelea gallina*	*N/A*
AO	*Aequipecten opercularis*	*N/A*
SO	*Sepia officinalis*	*N/A*
LV	*Loligo vulgaris*	*N/A*
Vertebrates		
BT	*Bos taurus*	*N/A*
OA	*Ovis aries*	*N/A*
RT	*Oncorhynchus mykiss*	*N/A*
ESB	*Dicentrarchus labrax*	*N/A*
GSB	*Sparus aurata*	*N/A*
AB	*Atherina boyeri*	*N/A*
Ingredients		
CPM	*Pea protein concentrate*	*N/A*
SBM	*Soybean meal*	*N/A*
DBY	*Dry brewer's yeast*	*N/A*
FM	*Fish meal (anchovy)*	*N/A*
PBM65	*Poultry & turkey by-product*	*N/A*
PBM100	*Poultry by-product*	*N/A*
CG	*Corn gluten*	*N/A*
WG	*Wheat gluten*	*N/A*

3.2 PCR performance with practical samples

The PCR performance of the designed primers has been tested with practical commercial aquafeed samples as representative of practical complete mixtures and processing methods. The DNA extracted from 10 ng/µl sample of the six diets containing different HI inclusion levels resulted in a positive signal (31-36 Ct) while the negative control, without HI, resulted in no signal. Significant statistical differences have been observed among the different samples ($P<0.001$), as shown in Fig.3. Diets CR5, CR5F, CR5ext including 5 g/100g HI meal resulted in similar Ct values (36.40 ± 0.19 Ct), while, as expected, feed containing 10 g/100g HI meal resulted in significantly lower Ct value (CR10ext: 35.31± 0.61).

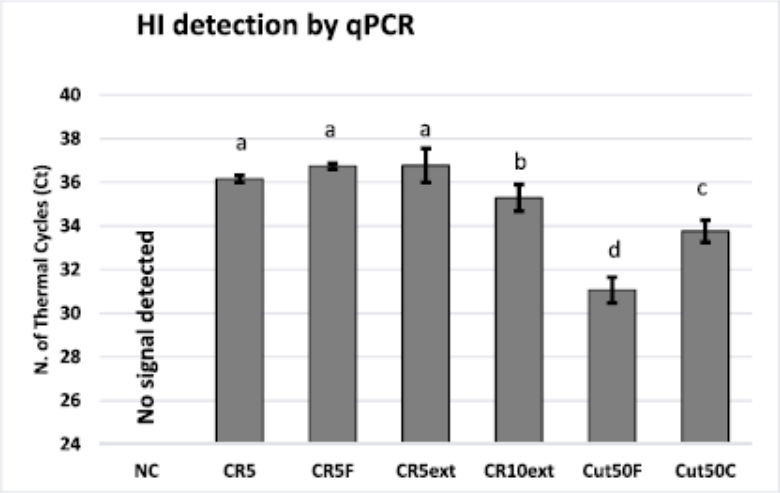


Fig. 3. Number of Thermal Cycles observed in qPCR for aquafeeds containing different level of *Hermetia illucens* meal. Values are mean ± StDev of six replicate tests per each sample. Different letters indicate significant differences among mean values ($P < 0.001$).

When considering 50 g/100g HI meal inclusion feeds, Cut50F resulted in a Ct value of 31.10 ± 0.59 as expected according to the sensitivity curve, while Cut50C resulted in a Ct value of 33.76 ± 0.51. The physical difference between the two aquafeeds samples could be implied in this behaviour. As declared by the producer, HI meal with different mesh size has been used thus consequently affecting the cuticle mesh size and the related chitin content of the sample subjected to the determination. Even if the size and percentage of chitin have been not measured, optical microscope observations confirmed that the cuticle fragments in Cut50C were larger and more abundant than in Cut50F confirming what reported by the producer. It could also be possible that the DNA extraction procedure from a coarse powder could have been less performant than in the case of fine powder. Such technological feature could have resulted in sample differences in HI DNA content between Cut50C and Cut50F samples, as reflected in qPCR measurements.

It should be noted that the Ct numbers required to detect a positive signal for all the practical samples have been higher than expected from the calibration curve when considering aquafeeds within a range of HI meal from 5-50 g/100g of the total ingredients. These data support the observation of (Hrnčírová et al., 2008) on

the partial DNA degradation of the raw material including the insect target species due to the mechanical and physical process adopted for feed production. The DNA degradation can be different depending on the specific treatment, allowing to detect only a fraction of the amount of insect effectively present in the complex matrix.

The presence of the HI insect meal in the different commercial tested aquafeeds, supplied by Cycle Farms, has been always clearly identified by qPCR technique but it was only roughly possible to discriminate among samples differing in target insect meal inclusion level. Anyway, a precise quantification of the insect inclusion was not possible due to the different DNA degradation, depending on the particular technique used to produce the feeds and on the different amount and size of cuticle present in the samples, that can reduce the effective DNA contents.

These results confirm the accuracy of the method for HI meal detection in complex matrices, but, due to the random degradation of the insect meal during the production process of the feed, this method could be semi-quantitative, only.

3.3 Sensitivity and specificity of the OLED-based genosensor

The DNA probes derived by the same primers used for the PCR measurements have been then used to build the disposable cartridge previously described (paragraphs 2.3/4/5/6) that takes advantage of the HI5reSpec probe, dye conjugated in 5′, fluorescence emission, excited by a specific OLED, to detect and quantify HI DNA. Two series of two-fold DNA dilution (statistical repetition), starting from an initial concentration of 25 ng/ l without DNA amplification step, have been plotted against the average number of counts measured in the luminous spots (count range between 0 and 4095) for the sensitivity curve using the genosensor (Fig. 4). Three samples for each serial dilution have been measured with an acquisition time set to 10 sec. The two series, used to evaluate the method reproducibility, resulted in a linear relationship (R^2: 0.989-0.992) with a limit of detection of 0.75 ng/ l. The angular coefficient values reflect differences in the initial DNA concentration of the two-fold independent DNA serial dilution.

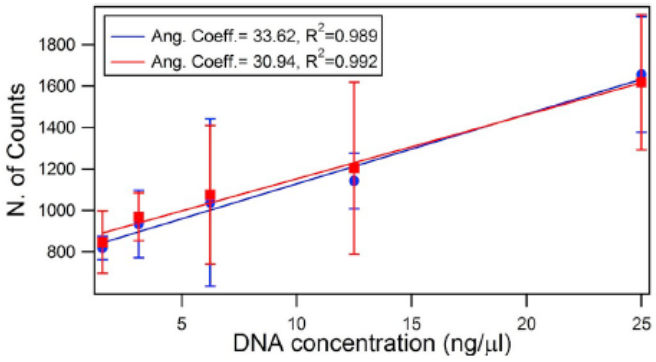


Fig. 4. Sensitivity curves obtained using different DNA concentrations (measured in triplicate for each sensitivity curve). The mean number of counts (range 0 - 4095) of the fluorescent signals, recorded with a 12bit digitalization, is reported as a function of DNA concentration. A linear relationship between the measured fluorescence signals and DNA concentration has been estimated for each serial dilution.

In Fig. 5 two fluorescence images of the HI DNA sample at 25 ng/µl (a) and 6.25 ng/ µl (b), acquired at an integration time of 10 seconds, are plotted together with their 3D graph. Control tests have been performed to prevent false-negative results of the genosensor on (i) no capture probe (omission of the Fw5HI probe); (ii) no DNA (omission of the DNA sample- no HI in the sample); and (iii) no fluorophore (omission of the dye conjugated Re5HI probe) all the tests confirmed the specificity of the test (images not shown). Moreover, all the samples showed in Table 2 were tested in the genosensor for cross-reactivity and negative results were obtained.

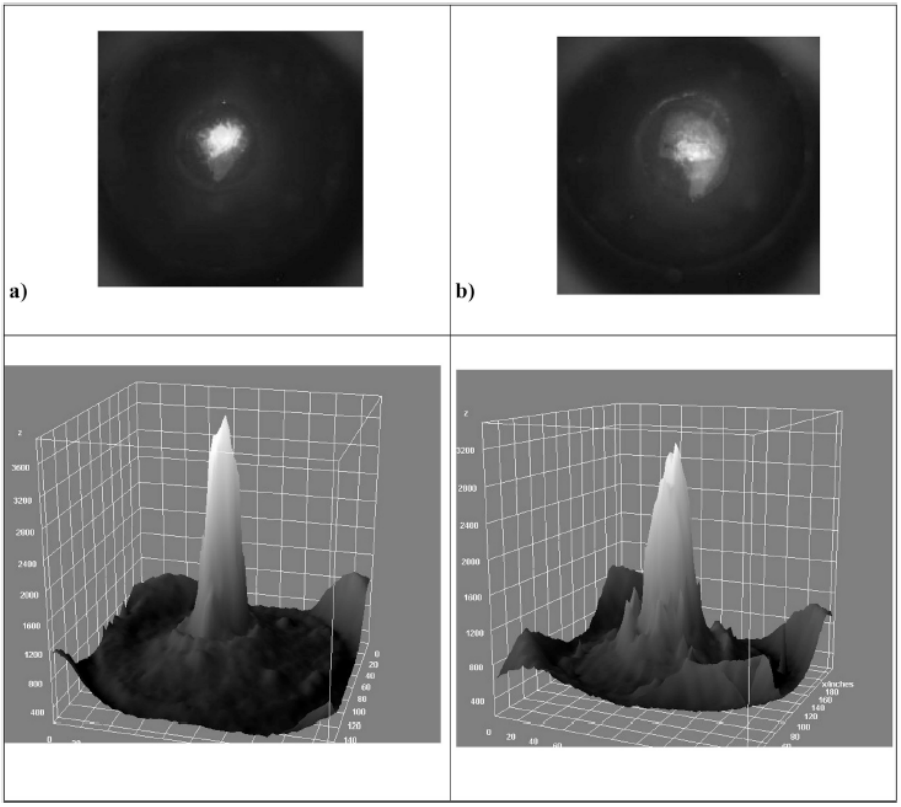


Fig. 5. a) Top: fluorescence image of 25 ng/µl of HI DNA acquired with an integration time of 10 s, Bottom: 3D image of the same spot as in image a) showing that the number of counts in the central part of the spot is between 3200 and 3600. b) Top: fluorescence image of 6.25 ng/µl of HI DNA acquired with an integration time of 10 s, Bottom: 3D image of the same spot as in image b) showing that the number of counts in the central part of the spot is between 2800 and 3200.

3.5 Genosensor test with practical samples

The OLED-based genosensor detected the presence of HI in feeds. Also, in this case, no DNA amplification has been performed on feed extracted DNA. In detail, 50 ng/µl of feed extracted DNA have been used in each test and an integration time of 20 sec was set for the image acquisition. The results of the feeds tested are presented in Fig. 6.

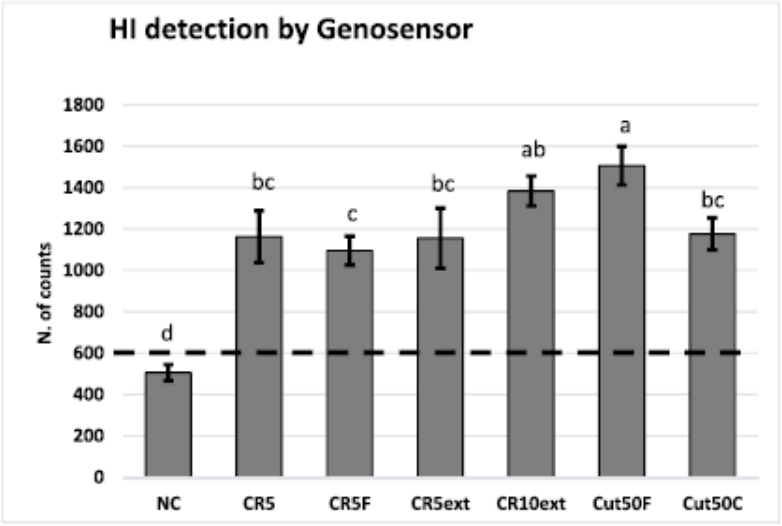


Fig. 6. Number of counts obtained using the OLED-based genosensor with an integration time of 20 s to measure the feed samples characterized by different inclusion levels of *Hermetia illucens* meal. The dotted line indicates the cut-off measured on negative samples at the same integration time. Values are mean ± StDev of three replicate tests per each sample. Different letters indicate significant differences among mean values ($P < 0.001$).

All HI meal containing samples resulted in count numbers significantly higher than the value obtained with the negative control sample, where no HI was included in the feed, (506±39) and a cut-off value of 600 counts has been set for the analysis (P>0.001). No significant differences have been observed in the signal values registered for the samples CR5, CR5F, and CR5ext. The sample CR10ext, containing 10 g/100g of HI meal, showed a higher signal (1383±73) as expected, while the average signal obtained with the Cut50F sample (1506±93) was higher by more than 300 counts than that obtained for the Cut50C sample (1176±77), despite their declared equivalence in terms of HI content. As already observed with qPCR, also in the genosensor analysis the Cut50C sample showed a lower number of counts than the sample Cut50F.

The results obtained by the two detection methods, qPCR and OLED-based genosensor, are presented in (Table 3). As in qPCR detection, where a higher thermal cycle number is needed for the detection of practical samples, also for the genosensor, a higher integration time (20 sec) has been used to obtain significant signal respect to the sensitivity curve.

In general, the genosensor results are supported by the qPCR data except for the ratio between the samples CR10ext and Cut50C, even if the qPCR still results in higher sensitivity than the genosensor. Anyway, both diagnostic methods allow to determine with good sensitivity (5% of inclusion) the presence of *Hermetia illucens* meal in complex matrices. Nevertheless, a correct quantification of the amount of such ingredient in the complex matrix is not really possible as both methods are affected by the uncertainty of DNA degradation during the processing method.

Conclusion

A new genosensor, based on the detection of the fluorescence emitted by a DNA probe conjugated with an emitting dye and excited by an OLED, has been designed for the detection of *Hermetia illucens,* recently approved by the European Community as a feed ingredient. The DNA probes used in the genosensor were derived from the DNA primers validated in qPCR experiments for HI detection. The genosensor was tested to detect the presence of HI in complex matrices, like HI meal based aquafeeds and the results compared with those obtained using the golden standard technique qPCR. Both methods demonstrated a good sensitivity, being able to resolve a percentage as low as 5 g/100g of HI meal inclusion in a complex matrix. The obtained results suggest the possibility of using this new sensor for the detection of HI presence in aquafeeds; still, as in the qPCR method, the precise quantification of insect contents in the matrix was not reliable. The OLED-based genosensor technique is an extremely promising one, being suitable for a Point-of-Care system. It is insensitive to contaminants, it does not require DNA amplification, resulting in a sensitive, accurate and low-cost diagnostic device that could be used in field to check the presence, as well as the absence, of HI in complex matrices.

Acknowledgments

This study was supported by RIC_LIB Tulli. The authors wish to thank prof. P. Zandigiacomo for kindly confirming insect species identification and Mr. G. Le Reste from Halieutica (F) for kindly providing aquafeed samples.

References

Belghit, I., Waagbø, R., Lock, E. J., & Liland, N. S. (2019). Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. *Aquaculture Nutrition*. https://doi.org/10.1111/anu.12860

Bukkens, S. G. F. (1997). The nutritional value of edible insects. *Ecology of Food Nutrition*. https://doi.org/10.1080/03670244.1997.9991521

Capinera, J. L. (2008). Encyclopedia of Entomology, Volume 4. In *Encyclopedia of Entomology*. https://doi.org/10.1007/978-1-4020-6359-6

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., … Tulli, F. (2019). Effects of graded dietary inclusion level of full-fat hermetia illucens prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). *Animals*. https://doi.org/10.3390/ani9050251

Commission, T. H. E. E. (2017). *COMMISSION REGULATION (EU) 2017/ 893 - of 24 May 2017 - amending Annexes I and IV to Regulation (EC) No 999 / 2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) . 2017*(May). Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R0893&from=EN

Cummins, V. C., Rawles, S. D., Thompson, K. R., Velasquez, A., Kobayashi, Y., Hager, J., & Webster, C. D. (2017). Evaluation of black soldier fly (*Hermetia illucens*) larvae meal as partial or total replacement of marine fish meal in practical diets for Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2017.02.022

Debode, F., Marien, A., Gérard, A., Francis, F., Fumière, O., & Berben, G. (2017). Development of real-time PCR tests for the detection of *Tenebrio molitor* in food and feed. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *34*(8), 1421–1426. https://doi.org/10.1080/19440049.2017.1320811

EC. (2009). Commission regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Official Journal of the European Union*, *36* (152), 1–130.

Fao. (2013). Edible insects. Future prospects for food and feed security. In *Future prospects for food and feed security*. https://doi.org/10.1017/CBO9781107415324.004

Goerlich, O., Grohmann, L., Mazzara, M., Gatto, F., Zel, J., Lieske, K., … Ovesna, J. (2017). Verification of Analytical Methods for GMO Testing When Implementing Interlaboratory Validated Methods. Version 2. *Testing and Analysis of GMO-Containing Foods and Feed*. https://doi.org/10.1201/9781315178592-8

Hong, E., Lee, S. Y., Jeong, J. Y., Park, J. M., Kim, B. H., Kwon, K., & Chun, H. S. (2017). Modern analytical methods for the detection of food fraud and adulteration by food category. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.8364

Hrnčírová, Z., Bergerová, E., & Siekel, P. (2008). Effects of technological treatment on DNA degradation in selected food matrices of plant origin. *Journal of Food and Nutrition Research*.

Józefiak, A., Nogales-Mérida, S., Mikołajczak, Z., Rawski, M., Kierończyk, B., & Mazurkiewicz, J. (2019). The utilization of full-fat insect meal in rainbow trout (*Oncorhynchus mykiss*) nutrition: The effects on growth performance, intestinal microbiota and gastro-intestinal tract histomorphology. *Annals of Animal Science*. https://doi.org/10.2478/aoas-2019-0020

Klausnitzer, B. (2011). Exkursionsfauna von Deutschland. In *Exkursionsfauna von Deutschland*. https://doi.org/10.1007/978-3-8274-2452-5

Köppel, R., Schum, R., Habermacher, M., Sester, C., Piller, L. E., Meissner, S., & Pietsch, K. (2019). Multiplex real-time PCR for the detection of insect DNA and determination of contents of *Tenebrio molitor*, *Locusta migratoria* and *Achaeta domestica* in food. *European Food Research and Technology*, *245*(3), 559–567. https://doi.org/10.1007/s00217-018-03225-5

Kurniawan, D. R., Arief, M., Agustono, & Lamid, M. (2018). Effect of maggot (*Hermetia illucens)* flour in commercial feed on protein retention, energy retention, protein content and fat content in tilapia (*Oreochromis niloticus*). *IOP Conference Series: Earth and Environmental Science*. https://doi.org/10.1088/1755-1315/137/1/012072

Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., & Peres, H. (2017). Black soldier fly (Hermetia illucens) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2017.04.021

Manzano, M., Cecchini, F., Fontanot, M., Iacumin, L., Comi, G., & Melpignano, P. (2015). OLED-based DNA biochip for *Campylobacter spp*. detection in poultry meat samples. *Biosensors and Bioelectronics*, *66*, 271–276. https://doi.org/10.1016/j.bios.2014.11.042

Marcello, A., Sblattero, D., Cioarec, C., Maiuri, P., & Melpignano, P. (2013). A deep-blue OLED-based biochip for protein microarray fluorescence detection. *Biosensors and Bioelectronics*. https://doi.org/10.1016/j.bios.2013.02.017

Marien, A., Debode, F., Aerts, C., Ancion, C., Francis, F., & Berben, G. (2018). Detection of *Hermetia illucens* by real-time PCR. *Journal of Insects as Food and Feed*. https://doi.org/10.3920/JIFF2017.0069

Melpignano, P., Daniso, E., & Vidergar, N. (2016). Multiparametric oled-based biosensor for rapid dengue serotype recognition with a new pointof-care serological test. *Informacije MIDEM*, *46*(4).

Premalatha, M., Abbasi, T., Abbasi, T., & Abbasi, S. A. (2011). Energy-efficient food production to reduce global warming and ecodegradation: The use of edible insects. *Renewable and Sustainable Energy Reviews*. https://doi.org/10.1016/j.rser.2011.07.115

Ramos-Elorduy, J., Moreno, J. M. P., Prado, E. E., Perez, M. A., Otero, J. L., & De Guevara, O. L. (1997). Nutritional value of edible insects from the state of Oaxaca, Mexico. *Journal of Food Composition*

and Analysis. https://doi.org/10.1006/jfca.1997.0530

Sogari, G., Amato, M., Biasato, I., Chiesa, S., & Gasco, L. (2019). The potential role of insects as feed: A multi-perspective review. *Animals*. https://doi.org/10.3390/ani9040119

St-Hilaire, S., Sheppard, C., Tomberlin, J. K., Irving, S., Newton, L., McGuire, M. A., … Sealey, W. (2007). Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society*. https://doi.org/10.1111/j.1749-7345.2006.00073.x

The historical spread of the Black Soldier Fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada. (2015). *The Journal of the Entomological Society of Ontario*.

Valentin, R. E., Maslo, B., Lockwood, J. L., Pote, J., & Fonseca, D. M. (2016). Real-time PCR assay to detect brown marmorated stink bug, Halyomorpha halys (Stål), in environmental DNA. *Pest Management Science*. https://doi.org/10.1002/ps.4217

Veldkamp, T., van Duinkerken, G., van Huis, A., Ottevanger, E., Bosch, G., & van Boekel, T. (2012). Insects as a sustainable feed ingredient in pig and poultry diets : a feasibility study = Insecten als duurzame diervoedergrondstof in varkens- en pluimveevoeders : een haalbaarheidsstudie. *Food Chemistry*, *50*(October), 192–195.

Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., & Huang, Y. (2019). Evaluation of defatted black soldier fly *(Hermetia illucens L.)* larvae meal as an alternative protein ingredient for juvenile Japanese seabass (*Lateolabrax japonicus)* diets. *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2019.04.023

4 Alternative protein source for healthy and sustainable aqua-feeds: the case of insect

Intensive farming of carnivorous fish species, relies on the use of complete feeds where fish meal (FM) and fish oil (FO) have historically represented ideal sources of energy and nutrients able to ensure optimal growth, health and nutritional quality of cultured fish. However, due to the gap between the high demand for these commodities by the worldwide growing aquaculture industry, as well as the unsustainability for any further exploitation of the marine resources to be converted into FM and FO, their inclusion levels in commercial aquafeeds have been drastically cut down and are expected to be further reduced (Tacon and Metian, 2015). Consequently, the search for alternatives to FM and FO in aquafeeds has represented and still represents a major challenge for the further sustainable development of the aquaculture production.

For most of the EU farmed fish species (Atlantic salmon, rainbow trout, European sea bass and gilthead sea bream), research studies on dietary alternatives to FM and FO have been mostly directed towards the use of terrestrial plant protein-rich derivatives and vegetable oils as the most ready available and cost-effective commodities on the feed market (Gatlin et al., 2007). The studies performed so far, have shown that fish responses to highly substituted diets is often species-specific, depending on life stage, nutritional/anti-nutritional characteristics and dietary inclusion levels of the vegetable ingredients. Adverse effects are mainly observed on feed intake, nutrient-energy digestibility, retention efficiency which in turn affect the growth performance through physiological and molecular mechanisms involving digestive-absorptive functions, metabolism and factors regulating somatic growth (Bakke-McKellep et al., 2000; Benedito-Palos et al., 2008; Caballero et al., 2003; Conceição et al., 2012; Dias et al., 2005; Glencross et al., 2007; Gómez-Requeni et al., 2004; Kaushik et al., 2004; Krogdahl et al., 2010; Laporte and Trushenski, 2012; Merrifield et al., 2009; Montero and Izquierdo, 2010; Oliva-Teles, 2012; Oliva-Teles et al., 2015; Tacchi et al., 2012; Tibaldi et al., 2006). High levels of dietary vegetable proteins and oils in fish diets were also shown to impair stress tolerance, gut and liver integrity, as well as immune response, by physiological and molecular mechanisms which are not still fully understood. Short or long term harmful effects on immune defences deserve particular attention as they could directly affect fish disease susceptibility and resistance. Impaired immune response has been ascribed to changes in the immune-cells populations, humoral response, and on direct effects on the intestinal mucosa such as composition and morphological alterations, changes in microbiota biodiversity, alterations on membrane permeability or in the gut associated immune system (GALT) function (Montero and Izquierdo, 2010). Hence, ensuring fish health and welfare standards is a major criteria to be met in order to select and validate feed ingredients in fish diets (Waagbø et al., 2013). In addition, the adverse side effects of certain vegetable-based diets on feed intake, growth, health and quality in fish, have been attributed to the lack of feeding stimulants, limiting or unbalance levels of indispensable amino acids, to the absence of (LC)n-3PUFA and disproportion of the n-3 to n-6 PUFA ratios (which characterise most vegetable oils), to the high level of indigestible non starch polysaccharides that affect nutrient and energy bioavailability, to the action of specific antinutritional factors (ANFs) supplied by certain plant-protein rich derivatives (Krogdahl et al., 2010). Despite, the aforementioned drawbacks of plant-based diets, the great deal of research carried out in the last two decades has set limits of plant-materials inclusion in fish diets which are currently broadly used in commercial aquafeeds and will maintain a significant application in the near future (Tacon and Metian,

2015). However, it should be also considered that, although cost-effective and beneficial at reducing reliance from ocean resources, the use of vegetable ingredients in aquafeeds could give rise to a growing conflict with other livestock and human consumption as feed/foods. This makes their inclusion in fish diets not easily sustainable on a global scale, in particular considering the demand for livestock feeds and the human demographic scenario expected for the year 2050 are taken into account (FAO, 2018). In this contest, it would be worth considering and evaluating, novel underexploited feed sources, as protein and lipid ingredients for aquafeeds, which could be less impacting, conflicting, and able to mitigate or prevent the adverse physiological effects exhibited by farmed fish fed "veggy" diets.

An increasing number of studies have been carried out to demonstrate the feasibility of the use of insect meal in aquafeeds for different fish species (Hua, 2021). In most of the studies performed so far, insect meals were used to partially or totally replace fish meal (Hua, 2021; Li et al., 2020). Since the gastrointestinal tract is the primary target of dietary changes and challenges, the integrity of liver and intestinal epithelium is fundamental to secure nutrient catabolism and absorption, so optimal growth, as well as defence against pathogenic factors, lets say in "healthy fish". In fish nutrition studies, attention has been given on liver welfare since liver plays a key role in many metabolic pathways and its morphological structure and macromolecular composition are deeply influenced by the diet (Vargas et al., 2018; 2019; Bruni et al. 2020; Cardinaletti et al., 2019). Moreover, emphasis should also be payed on gut integrity since any alteration may modify nutrient absorption and thus fish welfare and growth (Krogdahl et al., 2010; Santigosa et al., 2011; Voorhees et al., 2019).

The positive effect of insect on animal immune system is linked to the presence of the chitin (Gasco et al., 2018). For example, in sea bream it has been shown that chitin increased the activity in the innate immune system (Ramos-Elorduy et al., 1997; Esteban et al., 2000); however, the studies used non-insect chitin or chitin derivatives and the administration was via injection. In rainbow trout, chitin administration stimulated macrophage activity (Sakai et al., 1992) and also stimulated respiratory burst, phagocytic activity, and cytotoxic activity in sea bream (Esteban et al., 2000). Furthermore, some evidences suggest that chitin confers protection against infections. For instance, rainbow trout and Catfishindividuals injected with chitin exhibited increased resistance to *Vibrio anguillarum* (Sakai et al., 1992) and *Pasteurella piscicida* (Kawakami et al., 1998), respectively. A few studies have added chitin to diet, and results indicated an improvement of *Ephinephelus bruneus* bloch immune response (Harikrisnan et al. 2012). In addition, the observed non-specific modulation of hemolytic complement activity, leucocyte respiratory burst activity and cytotoxicity indicated the enhancement of sea bream immune activity (Esteban et al., 2001). It is important to note that these studies added pure chitin to the diet. Nevertheless, the inclusion of insect support the results obtained with pure chitin. For instance, Ming et al. (2013) found that feeding Black carp (*Mylopharyngodon piceus*) with a diet supplemented at 2.5 % with house fly maggot meal (*Musca domestica*) increased the lysozyme, serum alkaline phosphatase, and glutathione peroxidase in serum. Moreover, hepatic superoxide dismutase and catalase activities increased, and it is also reduced the malondialdehyde (MDA) levels in the serum and liver. Furthermore, *Aeromonas hydrophila* infestation resulted in lower mortality in fish fed housefly diet (Ming et al., 2013). This result clearly indicated that chitin from insects played a role in the immune system, similarly to the results observed when pure chitin was administered via injection. In European seabass juveniles fed a diet including insect meal from *Tenebrio molitor* (TM), showed an enhancement in serum lysozyme antibacterial activity and serum trypsin inhibition (Henry et al., 2018b). In yellow catfish (*Pelteobagrus fulvidraco*), fish fed diet including 18% TM meal resulted in an improved immune resistance against *Edwardsiella ictaluri* without any negative effect on fish growth and gastrointestinal tract function. Rainbow trout (*Oncorhynchus mykiss*) fed diets including black soldier fly (BSF) (*Hermetia illucens*) larval meal resulted in a positive effect on the gut microbiota, coupled

with an increased incidence of *Carnobacterium genus* bacteria, known to act as probiotics, thus indirectly influencing performance and health status in salmonids (Karlsen et al., 2017). The oxidative stress is considered a welfare indicator and previous study demonstrated that insect might affect this metabolic pathway. A dietary inclusion (4.2%-5%) of maggot meal (*Musca domestica*) can increase the antioxidative capability and non-specific immunity of black carp (*Mylopharyngodon piceus*) (Dong et al., 2013). On the contrary, silkworm pupae meal dietary inclusion above 50% in common carp caused oxidative stress, which significantly reduced fish growth, decreased superoxide dismutase (SOD) and intestinal protease activities, and finally increased the amount of heat shock protein (Ji et al., 2015). These contradictory results may be ascribable to the possible bioaccumulation of contaminants in insects, so great attention should be attaining on the substrate used to rear insect (Ravi et al., 2020). Additional studies have been performed to investigate antioxidant response at intestinal level. Results obtained on rainbow trout fed yellow mealworm supplemented diets showed an increased in SOD, catalase (CAT) and glucose-6-phosphate dehydrogenase (G6DP) activity and a reduction in lipid peroxidation (MDA) in the proximal and distal intestine (Henry et al., 2018a) . Yellow catfish fed TM-containing diets increase plasma SOD activity and decrease the plasma MDA (Su et al., 2017); on the other hand in the Adriatic salmon fed with BSF larvae meal SOD was unaffected by dietary treatment, thus suggesting that TM is probably more effective in enhancing the fish antioxidant defence system than BSF (Henry et al., 2018a). Studies at gene expression level of specific markers involved in welfare metabolic pathway are considered useful tools to investigate the effects of dietary insect meal on fish physiological response. One of the markers that has been taken into account in different fish species is the HSP70 (heat-shock protein-70) protein complex that it is known to be markedly induced in response both to physical-chemical environmental stressors (Yamashita et al., 2010) and dietary changes (Li et al., 2019). This gene resulted up-regulated in the liver of Jian carp fed diets including defatted BSF larvae meal, suggesting an activation of the tissue stress response (Li et al., 2017); similar results were also observed in trout (Cardinaletti et al., 2019). On the other hand, Atlantic salmon and zebrafish fed diets including 15% and 50% of HI meal (Belghit et al., 2019; Vargas-Chacoff et al., 2018; Zarantoniello et al., 2018) hsp70 was unaffected. Recently, total replacement of fish meal with black soldier fly larvae meal did not compromise the gut health of Atlantic salmon. Dietary insect meal inclusion resulted in a reduction of excessive lipid deposition within the enterocytes (steatosis) in the proximal intestine and few differentially expressed genes were identified in the proximal or distal intestine with a down-regulation of the matrix metalloproteinase 13 gene involved in tissue reorganization, and choline kinase involved in *de novo* synthesis of phosphatidilcoline (Li et al., 2020).

Nowadays, several laboratory techniques are available for better understanding fish responses to new ingredients. Histology is the traditional technique that has been widely used over a long time, to detect, among others, signs of morphological alterations in digestive system organs (mainly gut and liver) induced by inappropriate diet composition (Cardinaletti et al., 2019; Robaina et al., 1995). Recently, the use of molecular tools also represents a valid choice of investigation that allow precocious detection of inflammation even in absence of clear histopathological evidences (Sahlmann et al., 2013).

In the following article, histology and molecular methods were applied to evaluate the effect of high plant protein based diet lacking fish meal and including increasing amount of commercial insect (*Hermetia illucens*) meal on the gastrointestinal tract of gilthead sea bream (*Sparus aurata*), one of the most reared species among the Mediterranean country.

Graded level of *Hermetia illucens* pupae meal in plant protein-based diet lacking fishmeal for gilthead sea bream (*Sparus aurata*, L.): liver and gut gene expression and histological evaluation.

Enrico Daniso [1$], Elena Sarropoulou [2$], Paola Beraldo [1], Emilio Tibaldi [1], Roberto Cerri [1], Gloriana Cardinaletti [1,*]

[1] Department of Agri-food, Environment and Animal Science, University of Udine, via Sondrio, 2, 33100 Udine, Italy; enrico.daniso@spes.uniud.it (E.D.); paola.beraldo@uniud.it (P.B.); emilio.tibaldi@uniud.it (E.T.); roberto.cerri@spes.uniud.it (E.D.); gloriana.cardinaletti@uniud.it (G.C.)

[2] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, P.O. Box 2214, 71003, Iraklion, Crete, Greece; sarris@hcmr.gr

[*] Correspondence: gloriana.cardinaletti@uniud.it; Tel.: +39 0432 558179 (G.C.)

[$] These two authors equally contributed to the study

Received: date; Accepted: date; Published: date

Abstract: The present study evaluated liver and gut response in juveniles gilthead sea bream, *Sparus aurata*, fed zero-fish meal plant protein-based diets including graded level of defatted *Hermetia illucens* pupae meal (Hi), by using gene expression and histology. Three iso-nitrogenous (Nx6.25, 450 g kg^{-1} diet) and iso-lipidic (total fat, 200 g kg^{-1} diet) extruded diets were formulated. A basal diet (CV), rich in vegetable plant protein derivatives including soybean meal was prepared to have a 10:90 and 20:80 fish to vegetable protein and lipid ratios, respectively. Two other test diets were obtained by replacing graded levels of the CV protein (20 and 40%) by using Hi meal and maintaining the same vegetable to fish lipid ratio as in the CV diet. Diets were tested in triplicate for 45 days in juveniles fish of initial average weight of 49g and fish were hand-fed to apparent satiation. Final body weight and feed efficiency were unaffected by dietary treatments. In all dietary treatments, the foregut, midgut and hindgut showed a general structural integrity in intestinal folds, enterocytes and no signs of inflammation were; however, occasionally mild lymphocytic infiltration of the epithelium and *lamina propria* was observed in the pyloric caeca of CV and Hi20 diets. Histological examination of the stomach showed that CV diet induced moderate severe lymphocytic gastritis accompanied by a down-regulation of *Hsp70* ($p < 0.05$). Moderate lymphocytic infiltrates were observed in liver of fish fed the CV diet, which correspond a down-regulation of the *Sod1*. In the hindgut, an up-regulation of *Sod1*, *Sod2* and *iap* and a down-regulation of *Hsp70* were detected in insect meal including diet relative to CV, respectively. In summary zero-fish meal diet including insect meal help in ameliorate histological distortion of tissue, by activating specific correlated genes.

Keywords: *Hermetia illucens*; *Sparus aurata*; plant-protein; chitinase; oxidative stress; Hsp70; intestinal alkaline phosphatase

1. Introduction

In the last twenty years, the increasing aquaculture production to satisfy the high fish demand for human consumption has led to increase from 8 to 48 million tonnes the manufacture industrial aquaculture feeds (FAO, 2018). This trend has put pressure on fish nutritionist and feed companies to develop more sustainable and cost-effective aqua-feed formulations, resulting in an increase conventional marine resources replacement by plant counterparts (Hardy, 2010; Naylor et al., 2009; Tacon, 2020), since these commodities are easily available in the market in high quantity (Gatlin et al., 2007). Currently, commercial manufactured diets for carnivorous fish include a wide range of vegetable sources alternative to fishmeal and oil (Gatlin et al., 2007; Glencross et al., 2020), such as oilseed meals (soybean, canola, and sunflower), grains (wheat and corn), and legumes (lupine, bean, and peas) (Gatlin et al, 2007). However, plant protein meals and fishmeal differ in their nutrient composition and because the former contains varying levels of anti-nutritional factors (ANFs) (Francis et al., 2001), it is pertinent to study their effect on the gastrointestinal tract (Krogdahl et al., 2010; Omnes et al., 2015). In particular, when considering the soybean products, histo-patological effects are associated with the enteritis developed in the distal intestinal mucosa of Atlantic salmon (Krogdahl et al., 2010), and several other teleost species react to soy in a similar manner (Burrells et al., 1999; Hedrera et al., 2013; Matulić et al., 2020; Urán et al., 2008). Nevertheless, exceptions to this generalization exist, since different taxa respond differently to soy derivatives and to the ANFs. For example the distal intestine of cobia (*Rachycentron canadum*) (Romarheim et al., 2008), Egyptian sole (*Solea aegyptiaca*) (Bonaldo et al., 2006) and European seabass (*Dicentrarchus labrax*) (Bonaldo et al., 2008) are completely unaffected by feeding soybean meal. On the contrary, gilthead sea bream (*Sparus aurata*) fed diets including 30 or 60% soybean meal, exhibited distal intestinal abnormalities, as infiltration of leukocytes into the lamina propria or dilated structure of the submucosa (Bonaldo et al., 2008; Kokou et al., 2015, 2012). Immune status disorders and impairment on oxidative stress resistance (Kokou et al., 2015, 2012; Sitjà-Bobadilla et al., 2005) were also observed in sea bream fed high fish meal replacement diets, in combinations with physiological and/or histo-morphological alterations in liver tissue (Baeza-Ariño et al., 2016; Bonaldo et al., 2008; Sitjà-Bobadilla et al.,

2005). Research effort in implementing processing technology to improve the nutritional value of plant feedstuff (Drew et al., 2007) has contributed to reach zero-fish meal diet formulation. In this contest, Estruch et al. (Estruch et al., 2018a, 2015) reported the successful use of a 100% plant-protein mixture based diet for sea bream without hampering gut histology. However a decrease in different pro-inflammatory mediators (*il-1b*, *il-6* and *cox2*) and immune-related molecules (*IgM* and *alp*) as well as gut microbial imbalances was observed (Estruch et al., 2018a, 2015). This effects were mitigated by supplemented the vegetable diet with 10 and 5% marine by-product such as squid and krill meals, respectively.

If in the past the discussions on aquaculture feeds focused on alternatives to marine resources; nowadays, the sustainability of the sector is linked to new and more sustainable raw materials than vegetable feedstuff, such as proteins from insect meal (Laura Gasco et al., 2018; Nogales-Mérida et al., 2019), recently authorized by the European Union Commission (2017/893 -24/05/2017).

Among the seven insect species allowanced, the black soldier fly (*Hermetia illucens*) (Hi) is the most studied in different fish species, since it is commercially available in the EU market due to the development of its mass rearing method. Increasing number of studies have been carried out to test the effectiveness of including insect as fish meal substitute in feeds of various fish species (Hua, 2021). For example, in the false percula clownfish, *Amphiprion ocellaris,* (Vargas-Abúndez et al., 2019) 60% dietary inclusion of Hi meal did not affect intestinal mucosa and liver parenchyma or stress related gene (*gr* and *Hsp70*). On the contrary, in rainbow trout (Cardinaletti et al., 2019; Elia et al., 2018) 50% fish meal substitution by full-fat Hi meal resulted in an up-regulation of *Hsp70* in the liver and activation of the inflammatory response (*il-10*, *tnf-α*, and *tlr-5*) in medium intestine together with histological changes related to a reduction in villi length. Histopathological examination of liver, spleen and gut in the same fish species was performed by Elia (Elia et al., 2018) and results revealed no structural adverse effects of insect meal; but an unbalance oxidative homeostasis in liver and kidney was noted. Similar results on oxidative stress markers were also observed in Siberian sturgeon (*Acipanser baerii*) by Caimi et al. (Caimi et al., 2020). Finally, studies performed on zebrafish (*Danio rerio*) have shown that over 75% dietary inclusion levels of Hi affect larval liver histology and induce a general increase in liver lipid accumulation and an up-regulation in the immune and stress-related genes (Vargas et al., 2018; Zarantoniello et al., 2020).

If at the beginning insects have attracted interest by the scientific community for its high nutritious properties (protein, mineral and amino acidic profile content) (Nogales-Mérida et al., 2019); recently the attention has been focused on their potential nutraceutical effects. In fact, the interesting thing of this novel alternative ingredient is the presence of biologically active compounds, such as chitin and short-medium FAs, able to improve fish innate immune response (promoting immunomodulatory effects) (Esteban et al., 2001; L. Gasco et al., 2018). Chitin has also been shown to possess antimicrobial and bacteriostatic properties against several harmful Gram-negative bacteria (Benhabiles et al., 2012); while some short-medium-chain fatty acids, such as lauric acid (C12), are known to have antimicrobial activity on Gram-positive bacteria (Skřivanová et al., 2005) and anti-inflammatory properties at intestinal level (Spranghers et al., 2018).

Chitin is the most abundant polysaccharide in nature functioning as the major structural polymer in many organisms (Plantae, Fungi and Invertebrate) (Nogales-Mérida et al., 2019). The ability of animals to hydrolyze chitin-fibres depend on a specific set of chitinolytic enzymes, such as chitinases and chitobiase, whose gene expression and enzymatic activity seem to be governed by the feeding habits (Ikeda et al., 2017; Tabata et al., 2018). Available data on sea bream feeding habits in the wild highlight that this species is an opportunistic feeder and its diet mainly consists of gastropods, bivalves and some crustacea (Pita et al., 2002). These natural prays, suggest that sea bream, as other Sparidae (Karasuda et al., 2004), can have active chitinolytic enzymes in the stomach or along the gastrointestinal tracts able to digest chitin, making it able to accept feeds including insect. The chitinase nucleotide sequence has been identified from stomach and other tissues of several Osteichthyes, Chondrichthyes, Crustaceans, Amphibians, Avians and

Mammalians (Ikeda et al., 2017; Tabata et al., 2019, 2018), but to date no information are available in gilthead sea bream. In addition when looking at literature on the use of insect meal in fish nutrition (Hua, 2021) very few data concern sea bream (Haro et al., 2016; Iaconisi et al., 2019; Piccolo et al., 2017) and only two have considered black soldier fly (*Hermetia illucens*) (Fabrikov et al., 2020; Karapanagiotidis et al., 2014). Moreover, in most of the study performed so far, insect meal were use in partial or even total substitution of FM (Hua, 2021; Li et al., 2020) and until now, no study has been addressed on using *Hermetia illucens* to replace plant-protein based diet-lacking fishmeal.

In fish nutrition studies, when alternative ingredients are evaluated to replace conventional marine raw materials, the stomach represents the most underexplored organ. Few information are available on the physiological and histological effect of alternative ingredient on fish stomach (Amin et al., 2019; Ogueji et al., 2020; Refstie et al., 2006) including those concerning sea bream (Estruch et al., 2015; Omnes et al., 2015; Pérez-Sánchez et al., 2013), thus stimulating investigation of this aspect in relation to the insects use. Stomach is involved in important physico -chemical processes for the subsequent macronutrient transit and processing at intestinal level, and the gastric surface mucous cells contribute to not only efficient digestion but also represent the first line of defense against insults derived from ingested food.

Based on this and taking into account the future trend of aquafeed formulations with zero-fish meal inclusion, the aim of the present study was to evaluate, in gilthead sea bream (*Sparus aurata*), the effect of plant-protein based diet lacking of fishmeal and including increasing amount of alternative protein source from commercial defatted *Hermetia illucens* meal. To this end, gene expression study of a set of selected genes related to chitin hydrolysis, some oxidative stress markers, cellular homeostasis, protection of the mucosal epithelial surface, protein degradation and processing, maintenance of gut homeostasis were investigated along the gastrointestinal tract. The gene study was supplemented with histological evaluation of different gastrointestinal target organs considering, for the first time in such kind of study, the stomach.

2. Materials and Methods

2.1 Ethical statement

The fish feeding trial was conducted at the Experimental Facility of the Di4A (Pagnacco, Udine, Italy, code 068UD047). All procedures for animal handling and care were accomplished according to the guidelines of the European Union (Directive 2010/63/EU, 2010) on the protection of animals used for scientific purposes and Italian law (D.L. 26/2014). The experimental protocol was approved by the Ethical Committee of the University of Udine (Prot. N. 1/2018) and it has been authorized by the Italian Ministry of Health (n.°290/2019-PR).

2.2 Experimental diets

Three test diets were formulated to be grossly iso-proteic and iso-lipidic. A plant-based control diet (CV) was designed in order to obtain a 90:10 weight ratio between vegetable and fish protein and a 67:33 weight ratio between vegetable and fish lipid, calculated from the crude protein and crude lipid contribution to the whole diet of all marine and plant-based dietary ingredients. Two other test diets, coined Hi20 and Hi40, were prepared by replacing 20 and 40% of crude protein from the CV diet by the crude protein from a commercial *Hermetia illucens* pupae meal (Hi) (ProteinX™, Protix, Dongen, The Netherlands) by maintaining constant the 67:33 weight ratio between vegetable and fish lipid as in the CV diet.

Where necessary the diets were supplemented with essential amino acids to meet the nutrient requirement of *Sparus aurata* (Council, 2011). All diets were manufactured at SPAROS Lda. (Portugal) by extrusion in two pellet size (3 and 5 mm) and stored in a cold room (+4°C) until used. The ingredient composition and proximate analysis of the test diets are shown in Table 1.

Feed samples were analyzed in duplicate for dry matter, DM (AOAC #950.46), crude protein, CP Nx6.25 (AOAC #976.05) and ash (AOAC #920.153) contents according to AOAC International ("AOAC International - AOAC," n.d.) and total lipid content according to Burja et al. (Burja et al., 2007).

Table 1. Ingredient composition (g $100g^{-1}$), proximate composition (% as fed) of the test diets.

Title 1	Test diets		
	CV	**Hi20**	**Hi40**
Ingredient composition			
SBM [1]	26.5	20.3	14.2
Protein-rich veg. mix [2]	42.5	32.3	22.4
Hermetia meal [3]	-	16.2	32.4
CPSP [4]	3.5	3.5	3.5
Squid meal [*]	2.0	2.0	2.0
Wheat meal [*]	0.4	1.6	4.5
Whole pea [*]	3.0	5.8	6.0
Fish oil [5]	6.2	6.2	6.2
Veg. oil mix [6]	11.4	8.4	5.4
Vit. & Min. Premix [7]	3.5	3.1	2.9
L-Lysine [8]	0.5	0.2	0.2
DL-Methionine [9]	0.5	0.4	0.3
Proximate composition			
Moisture	6.5	6.0	4.5
Protein (Nx6.25)	45.1	45.3	45.2
Total lipid	20.4	20.2	20.4
Ash	5.8	6.6	6.6
Chitin [#]	0.02	0.76	1.51
NFE [$]	22.2	21.2	21.9

[1] Dehulled solvent-extracted soybean meal: 470 g/kg CP, 26 g/kg CF; CARGILL

[2] Protein-rich veg. mix % composition: soy protein concentrate-Soycomil, 20; corn gluten, 18; wheat gluten, 15, rapeseed meal, 8

[3] ProteinX™, Protix, Dongen, The Netherlands

[4] Fish protein concentrate, Sopropeche, Boulogne sur mer, France

[5] Fish oil: Savinor UTS

[6] Vegetable oil mixture % composition: rapeseed oil, 56; linseed oil, 26; palm oil, 18

[7] Supplying per kg of vitamin supplement: Vit. A, 4,000,000 IU; Vit D3, 850,000 IU; Vit. K3, 5,000 mg; Vit.B1, 4,000 mg; Vit. B2, 10,000 mg: Vit B3, 15,000 mg; Vit. B5, 35,000 mg; Vit B6, 5,000 mg, Vit. B9, 3,000 mg; Vit. B12, 50 mg, Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg. Supplying per kg of mineral supplement: Ca, 77,000 mg; Cu, 2,500 mg; Fe, 30,000 mg; I, 750 mg; Se, 10,000 mg; Zn, 25 mg.

[8] Biolys: l-lysine sulphate, 546 g/kg lysine; EVONIK Nutrition & Care GmbH

[9] DL-Methionine: 990 g/kg; EVONIK Nutrition & Care GmbH

[*] Wherever not specified the ingredients composing the diets were obtained from local providers by Sparos Lda.

[§] Estimated as 100-(Moisture + Protein + Total lipid + Ash + Chitin).

[#] Estimated based on chitin content of the ingredients used (squid meal, 0.9% and *Hermetia illucens* meal 4.69%).

2.3 Fish rearing conditions and tissue sampling

Fifty-four fish (mean body weight 49.1 ± 9.1 g) were randomly divided among 9 cylindrical fiberglass tanks with a capacity of 250-L. Tanks were connected to a marine recirculating aquaculture system (daily replacement rate, 3% in volume) equipped with sand-mechanical and biological filters, protein skimmer, ozonator and UV lamp (Scubla, Italia). During the feeding trial fish were kept under constant day length and intensity (12 hours per day at 400 lux) provided by fluorescent light tubes. Water temperature, salinity, dissolved oxygen, pH, TAN and N-NO2 were as follows: 23.4 ± 0.75 °C; 31 ± 0.7 g/L; 6.6 ± 0.4 mg/L; 8.0 ± 0.1; < 0.02 mg/L and < 1.0 mg/L. After stocking, fish were fed two weeks with a commercial diet (Protec, Skretting) and adapted to the experimental conditions. After that, dietary treatments were randomly assigned in triplicate to the 9 groups. Fish were hand-fed the experimental diets over 45 das days, six days a week in two daily meals (8:00 am and 4:00 pm), until the first feed item was refused. Feed distributed was daily recorded.

At the end of the feeding trial, after 24 h fasting period, all fish were subjected to stage 3 anaesthesia with 80 ppm of MS-222 (PHARMAQ Ltd., Fordingbridge, UK) and biometry measurements (total length and body weight) were recorded. Subsequently, three fish per tank (9 fish per dietary treatment) were sacrificed with an overdose (200 ppm) of the same anaesthetic and liver (L) and different gastrointestinal organs (GITs) were immediately excised put in individual cryovial, frozen in liquid nitrogen and stored at -80 °C for gene expression analyses (see Section 2.4). The GITs were sectioned into stomach (ST, the fundus portion); pyloric caeca (PC); foregut (F, defined as the intestine from the most proximal to the most distal pyloric caeca); midgut (M, defined as the intestine between the most distal pyloric caeca and the appearance of transverse luminal folds) and hindgut (H, defined as the region characterised by the transverse luminal folds and increased intestinal diameter to the anus). Subsamples of L, S, PC and H were also properly processed for the subsequent histological analysis (See Section 2.5) performed by light microscopy.

Moreover, at the end of sampling, Fulton's condition factor (K) and feed efficiency (FE) were calculated as follow:

$K = 100 \times (\text{body weight} / \text{total length}^3)$

FE = (final body weight – initial body weight)/feed supply

2.4 Gene expression analyses

2.4.1 RNA extraction and cDNA synthesis

Total and small RNA was extracted from L, S, PC, F, M and H samples (approximatively 30 mg) using Nucleospin miRNA extraction kit, (Machinery Nagel, Germany) and following the manufacturer's instructions. RNA concentration were analysed by NanoDrop® (Thermofisher, Germany) while its integrity by Ethidium Bromide staining of 18S and 5S ribosomal RNA bands on 1% agarose gel. After extraction, complementary DNA (cDNA) was synthesised from 1 µg of total RNA by using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio), following the manufacturer's instruction. At the end of the procedure, the RNA was diluted 1:50 in RNase-DNase free water (Sigma, Aldrich) and stored at −80 °C until quantitative real-time PCR (qPCR). An aliquot of cDNA was used to check primer pair specificity.

2.4.2 Real time qPCR and data acquisition

chitin hydrolysis, protein degradation and processing, intestinal mucins, some oxidative stress biomarkers including those involved in the intestinal homeostasis maintenance were investigated along the gastrointestinal tract.

Changes in the expression levels of several target gene related to chitin hydrolysis, some oxidative stress markers, cellular homeostasis, and protection of the mucosal epithelial surface, protein degradation and processing, maintenance of gut homeostasis were differentially evaluated according to the tissue samples: L, S, PC, F, M and H. Those transcript included gastric-chitinase (*CHIA*) and chitobiase (*CTBS*), copper–zinc superoxide dismutase (*Sod1*) and manganese superoxide dismutase (*Sod2*), glutathione S-transferase 3 (*GST3*), heat shock protein 70 (*Hsp70*), cysteine cathepsins (*Cat B* and *Cat Z*), membrane-bound mucin (intestinal mucin, *I-Muc* and mucin13, *Muc13*) and intestinal alkaline phosphatase (*IAP*).

In silico studies on the aforementioned target genes were performed using Primer3 (Kõressaar et al., 2018; Untergasser et al., 2012) (http://frodo.wi.mit.edu/primer3) and Beacon Designer 8.0 software (PREMIER Biosoft International, USA). The objective was to: (i) design new forward and reverse primers based on available sequences on NCBI (http://ncbi.nlm.nih.gov) or check correct thermal parameters of other primers retrieved by scientific literature; (ii) avoid hairpin and oligo-dimer formation, and/or self-complementarity; (iii) set-up correct melting temperature and/or annealing temperature to use in real-time RT-PCR. Primers were synthesized by Merck KGaA (Darmstadt, Germany) and are listed in Table 2.

Table 2. Primer pair sequences, amplicon size (bp), annealing temperature (°C) and Gene accession number (n°) for genes used for real-time PCR. Gray shaded rows correspond to reference genes.

Acronym	5'-3' primer sequence		bp	Ta (°C)	n°	Ref.
	Forward	Reverse				
Hsp70	AAGGTTGGGTCTGAAAGGAACG	CTCTGCGATGAAGTGGTTGACCG	170	57	EU805481	Present study
GST3	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC	98	57	JQ308828.1	(Teles et al., 2019)
Sod1	AGAATCATGGCGGTCCTAC	ACTGAGAGTGAGCATCTTGTC	116	57	JQ308832.1	(Estensoro et al., 2016)
Sod2	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATATTTCTCCTCTG	134	57	JQ308833.1	(Teles et al., 2019)
Cat B	GGACTTCTGGACCAAAGACGG	CATTCACATGGTGCTCGGAG	101	57	KJ524457.1	Present study
Cat Z	GCAAACCCTTCAACGAATGTG	CTCCGCCATCATCTTCTCC	125	57	XM_030420146.1	Present study
IAP	CCGCTATGAGTTGGACCGTGAT	GCTTTCTCCACCATCTCAGTAAGGG	63	59	KF857309	Present study
I-Muc	GTGTGACCTCTTCCGTTA	GCAATGACAGCAATGACA	102	57	JQ277712.1	(Pérez-Sánchez et al., 2013)
Muc13	GCAATCCGCACATCAAAGC	GTAGTCGCCGCATCACAGG	184	57	JQ277713.1	(Pérez-Sánchez et al., 2013)
CHIA	ATCTTGTCTATGCCTTTGCTG	GGTTGCTGTTCTCGTTCTTC	111	57	XM_030419000	Present study
CSTB	ACAGGGAGTTACCAGGTTCAC	ACAGCAGGTCGCAGGATTC	111	59	XM_030433419	Present study
18S	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	164	57	AM490061.1	Present study
L13	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTAAGAGCAG	148	57	CB177089.1	(Kokou et al., 2015)
FAU	GACACCCAAGGTTGACAAGCAG	GGCATAGAAGCACTTAGGAGTTG	148	57	AM951436.1	(Kokou et al., 2015)
β-actin	CTGGGATGACATGGAGAAGA	CTTGATGTCACGCACGATTT	206	57	X89920.1	Present study

Real-time qPCR was performed using the mic qPCR cycler detection system (Bio Molecular Systems, Australia). Briefly, reactions were set on 8-well strips tube by mixing for each sample 2.5 µL cDNA diluted 1:50; 4 µL of SYBR® FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA) as the fluorescent intercalating agent and 0.2 µL of specific forward and reverse primer of initial concentration of 10 µM. The thermal profile for all reactions was 3 min at 95 °C, followed by 40 cycles of 5 s at 95°C; 25 s at 57°C and 5 s at 72°C (annealing and extension step). After each cycle a plate read for fluorescent signal assessment was performed and a dissociation curve analysis with a gradient of 50°C to 95°C was performed to evaluate the specificity of the amplification and to verify the absence of primer dimers. The efficiency of PCR reactions were always higher than 90%, negative controls without samples templates were routinely used for each primer set and no primer-dimer formation was found in control templates. Ct-values were calculated based on the exported raw fluorescence data applying the Miner software (Zhao and Fernald, 2005). The

Miner software is a method for quantifying qRT-PCR results using calculations based on the kinetics of individual PCR reactions without the need of standard curves (Zhao and Fernald, 2005). The raw fluorescence data fit as a function of PCR cycles to identify the exponential amplification phase of the reaction using a four-parameter logistic model. This algorithm is an objective and noise-resistant method to quantify qRT-PCR results. Four candidate reference genes (*β-actin*, *FAU*, *L13* and *18S*) were tested as housekeeping gene (HKG) in the gene expression assay. NormFinder and geNorm were used to identify the most stable HKG among the selected; the stability values of the individual HKG in each tissue are presented in table 3. The best combination of two HKGs for each tissue sample was used for normalization. Normalized gene expression values were subsequent evaluated by the GenEX 7.0 software program (bioMCC, Freising-Weihenstephan, Germany).

Table 3. Housekeeping gene stability among tissue evaluated with NormFinder (SD value) and geNORM (M-value). Within each type of tissue gray shaded cell indicate the most stable gene used to normalized expression data.

	Housekeeping gene							
	NormFinder				***geNORM***			
Tissue	***18S***	***L13***	***FAU***	***β-actin***	***18S***	***L13***	***FAU***	***β-actin***
Stomach	0.312	0.6428	0.7273	**0.2882**	**0.025904**	0.044074	0.05284	0.025904
Liver	**1.3455**	1.4049	1.4036	1.8055	0.132678	0.127954	**0.127954**	0.140401
Pyloric caeca	**0.0368**	0.2785	0.3719	0.3182	0.02084	**0.02084**	0.02565	0.02830
Foregut	**0.6503**	1.1083	0.9325	1.2163	0.0831	0.0932	**0.0831**	0.1026
Midgut	1.3996	**0.3565**	1.3167	0.8652	0.1084	0.0736	0.0951	**0.0736**
Hindgut	**0.1101**	0.1242	0.1431	0.4352	0.0108	**0.0108**	0.0122	0.0222

2.5 Histology

At the end of the experimental period, portion of liver, stomach, pyloric caeca, and hindgut were collected, fixed in 4% neutral buffered formaldehyde (Bio-Optica, Milan, Italy) for 48h. The samples were routinely processed for histology, cut into 3µm-thick sections (microtome LEICA RM 2255, Nusslock, Germany) and stained with Haematoxylin and Eosin (HE) (Sigma-Aldrich, Milan, Italy). To detect neutral and acid mucins, Alcian blue/periodic acid-Schiff (AB/PAS) straining were also achieved.

The specimens were blindly examined under DMRB light microscope (Leica, Germany) and images were captured using a digital camera (Nikon Fi3), connected to a workstation computer with imaging software (NIS-Elements Br, Nikon).

A semiquantitative scoring system was set up in order to compare the diet effects, paying attention most of all to inflammatory morphological changes. Furthermore, each sampled organs was evaluated for its general architecture, parameter in which any circulatory disturbances, regressive and progressive alterations were taken into account. A score from 1 to 5 is semi-

quantitatively attributed to both each parameters and the architecture on the basis of the phenomenon extent. Details of the semiquantitative scoring system utilised is given in Table 4. Inflammatory cell infiltrates were carried out at the level of all layers of the stomach wall, pyloric and distal intestine, distinguishing the type and distribution (diffuse, focal and multifocal). The score is defined by the mean number of cells on 3 fields at 400 magnifications (1 = absent; 2 = 2 - 5 cells / field; 3 = 5 - 20 cells / field; 4 = 20 - 50 cells / field; 5 = 50 -> 150 cells / field).

Total number of AB/PAS-positive goblet cells (GC) was counted on 3 fields at 100 magnifications of pyloric caeca and distal intestine sections by automated counting (at binary level combining intensity thresholding and feature restriction).

Table 4. Semi-quantitative scoring system adopted for the histopatological traits.

DESCRIPTORS	SCORING				
	1	**2**	**3**	**4**	**5**
ARCHITECTURE*	*Normal*	*Mild alterations*	*Moderate alt.*	*Severe alt.*	*Unrecognasible*
LIVER					
Hepatocyte fat accumulation	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
STOMACH					
Mucosal integrity	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
PYLORIC CAECA					
Globet cells density	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
DISTAL INTESTINE					
Globet cells density	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Very abundant*

*This parameter was evaluated for each organic district.

2.6 Statistical analyses

Data of growth parameters were subjected to one-way ANOVA using diet as an independent variable. Data on gene expression and histology evaluation were tested for normality and homogeneity of variances by using Shapiro-Wilk's and Levene's tests, respectively. When both conditions were satisfied, a one-way ANOVA (*p-value* < 0.05) followed by a Tukey's post hoc test was performed to assess the effects of the diets. If the criteria (normality and homogeneity) were not met, a non-parametric test using Kruskal-Wallis ANOVA on ranks followed by Mann-Whitney Rank Sum, were used for the analysis. All analyses were conducted using R software (version 2.14.0) and the Stats package. Gene expression data are presented as the mean value ± standard error of the mean of nine biological replicates.

3. Results

3.1. Fish growth

The fish readily accepted the test diets and all feeds were consumed without rejection or loss. No morality was recorded during the 45 days of feeding and the final body weight, condition factor and feed conversion ratio resulted similar (p > 0.05) among the different dietary treatments as reported in table 3.

Table 3. Growth parameters of sea bream fed the test diets over 45 days. Data are reported as mean ± SD.

	CV	**Hi20**	**Hi40**
FBW (g) [1]	90.7±9.3	91.7±9.8	93.5±8.5
K [2]	1.9±0.14	1.9±0.17	1.9±0.15

FE [3]	1.17±0.51	1.07±0.13	1.03±0.67

[1] Final body weight, [2] Condition factor, [3] Feed Efficiency.

3.2. Gene expression

All data for tissue gene expression are shown in Figure 1. Note that as expected *CHIA* was expressed only in the stomach (Fig. 1a). The expression of mucin genes *(I-Muc* and *Muc13*), *Sod2* and *IAP* were undetectable or absent in the liver (Fig. 2b). On the other hand, the remaining genes (10) were expressed at detectable levels in all six analysed tissues of the gastrointestinal tract with the exception for *I-Muc* in the midgut (Fig. 1e).

Gene expression results observed in the fish liver tissue (Fig. 1a) highlighted a significant (p < 0.05) highest *Sod1* expression in the Hi40 dietary treatments; while the *Cat Z* expression was significantly lower in fish fed the CV diet than those fed Hi40 diet and an intermediate values was observed in the Hi20 treatment. Considering the stomach (Fig. 1b), no differences (p > 0.05) where observed among the fish fed the different diets for all set of genes investigated, with the exception of *Hsp70* that was significantly upregulated in the Hi20 diet compared to the CV test diet (p < 0.05), while an intermediate value was recorded in fish fed the Hi40. As regards the gene expression observed in the pyloric caeca (Fig. 1c) segment, a significant downregulation was observed in the *GST3* in fish fed the Hi40 diet compared to those fed the CV diet. An opposite pattern was recorded in the *Hsp70* expression as the Hi40 treatment showed the higher values than Hi20 and CV diets (p < 0.05). Concerning the *IAP* expression fish fed the CV diet exhibit the lowest value (p < 0.05). Considering the foregut (Fig. 1d) and midgut (Fig 1e), no changes (p > 0.05) in the gene expression levels were detected among all the set of genes considered. Finally, in the hindgut, 40% of the genes studied were affected by dietary treatment (Fig. 1f). In particular, *Sod1* and *Sod2* showed the same expression pattern with the highest values recorded in fish fed the Hi20 diet (p < 0.05) and the lowest one in the CV treatment (p < 0.05). As regards *Hsp70* expression a significant higher values were detected in fish fed the CV diet than those fed Hi20 and Hi40 diets (p < 0.05). On the contrary, a significant *IAP* upregulation was exhibited in fish fed the Hi40 with respect to both Hi20 and CV diets (p < 0.05).

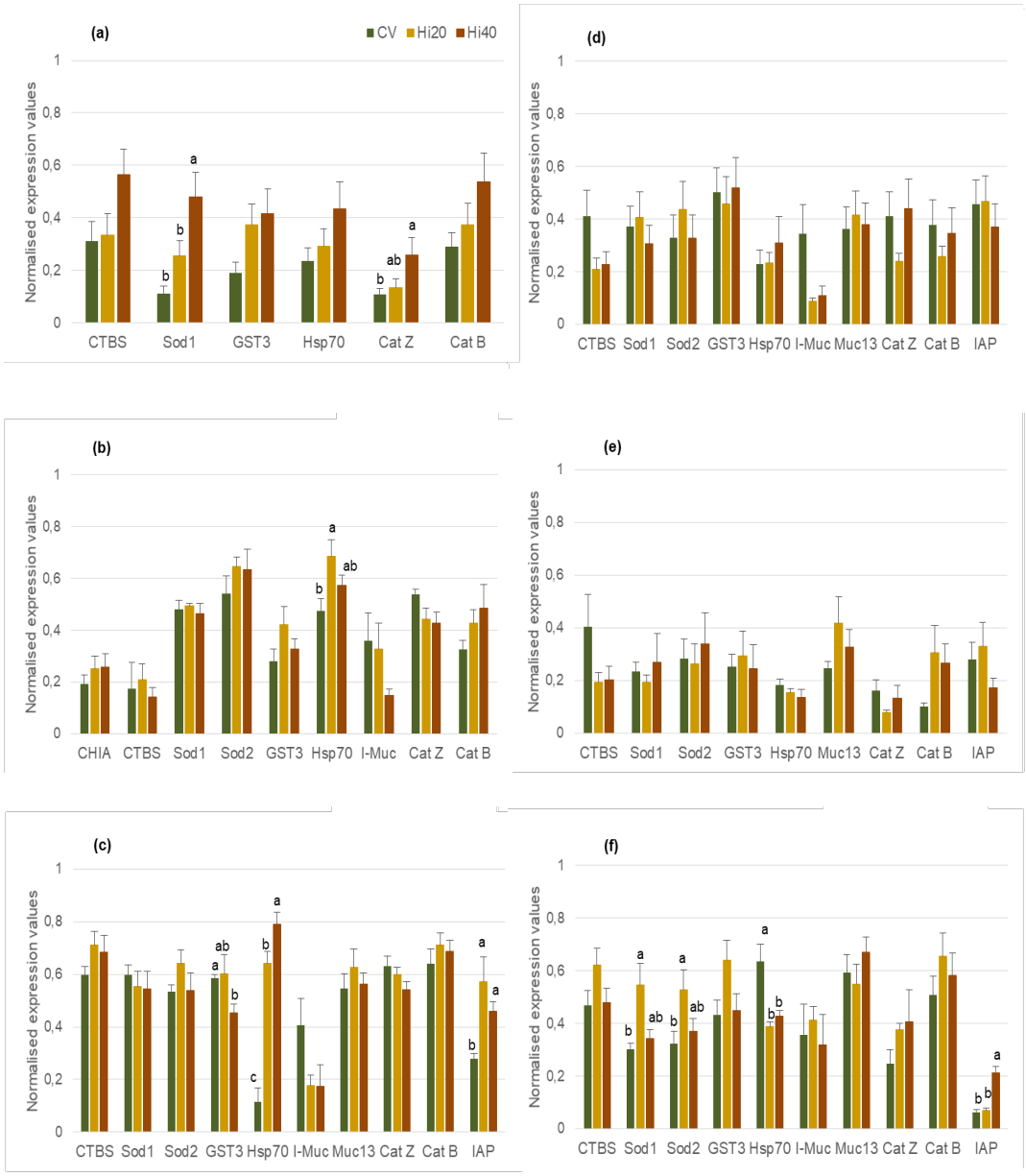


Figure 1. Differential gene expression values along the gastrointestinal tract of sea bream fed over 45 days with the different test diets. Green bar, CV diet; Ocher bar, Hi20 diet; Sienna color, Hi40. (a) liver, (b) stomach, (c) pyloric caeca, (d) foregut, (e) midgut and (f) hindgut. For each gastrointestinal tract considered and within each target gene, different letters indicate statistically significant differences among the test diets ($P < 0.05$). Values are presented as mean ± sem (n=9).

3.3. Histology

Generally, the histological assessment of the different digestive organs no differences between diets were found in the parameters analysed except for the stomach (table 5).

The liver structure was similar in CV, Hi20 and Hi40 fish with low lipid accumulation, but in CV-fish multifocal lymphocytic infiltrates (occasionally associated to hepatocytes necrosis) and aggregates of macrophages surrounding pancreatic acini were observable (figure 2a), causing mild hepatic alterations.

At the gastric level, CV-fish exhibit a histopathological pattern of moderate severity significantly different from that observed in Hi20 and Hi40 fish. In particular, a chronic aspecific gastritis (lymphocytic gastritis) was observed, characterized by a marked inflammatory infiltrate consisting mainly of lymphocytes (dispersed or organized in nodular structures with central necrosis) and mastocytes (syn. eosinophilic granular cells) within the lamina propria and glandular epithelium (figure 2b). In some cases, the inflammatory process interested all the gastric layers and small erosions in the epithelium of gastric mucosa are observed.

In all the fish, the villi and the brush border of pyloric caeca and distal intestine appear normal and intact, without mucosa desquamation into the gut lumen. Nevertheless, in fish fed the CV and Hi20 diets there is a mild lymphocytic infiltration of the epithelium and lamina propria of pyloric caeca, even if no significant statistical differences are evident.

Table. 5. Scores and goblet cell count (GCc) of the different parameters of the digestive apparatus.

	LIVER			STOMACH			PYLORIC CAECA				DISTAL INTESTINE			
	ARC	HFA	IIF	ARC	GMI	IIF	ARC	IIF	GCD	GCC	ARC	IIF	GCD	GCC
VEG	1,7	2,3	2,3	3,0a	3,0a	2,8a	1,3	2,7	3,3	403,8	1,0	1,3	3,0	473,4
H20	1,0	2,7	2,0	1,7b	1,3b	3,0a	1,7	2,3	3,3	320,7	1,0	1,0	1,7	445,1
H40	1,0	1,3	1,3	1,0b	1,3b	1,8b	1,0	1,0	3,3	382,1	1,0	1,0	2,0	334,9

ARC, architecture; HFA, hepatocyte fat accumulation; IIF Inflammatory infiltrate; GMI, gastric mucosal integrity; GCD, goblet cell density; GCC, goblet count.

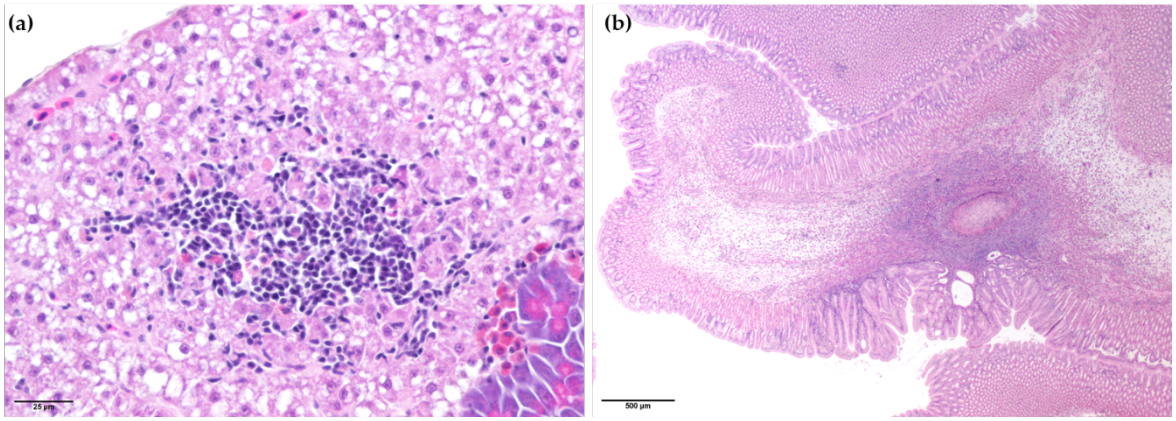


Figure 2. a) Focal lymphocytic inflammatory infiltrate in liver of CV fish, HE; (b) severe lymphocytic infiltrate organized in nodular structures with central necrosis in *lamina propria* of gastric mucosa of CV fish, HE.

4. Discussion

Finding novel protein-rich ingredients able to partial or total substitute conventional raw material is a key challenge for the future aquaculture development and nowadays insect meal represents the most promising candidate (Hua, 2021; Williams et al., 2016) due to its nutritious and nutraceutical properties (Finke, 2013; L. Gasco et al., 2018) as well its mass rearing production that meet the circular economy principle (Ravi et al., 2020).

The relevant subject of this study was to establish in gilthead sea bream the effect (45 days) of plant-protein based diet lacking fishmeal and replaced by increasing level of protein from insect meal (*Hermetia illucens*). The diet formulation was inspired by previous results obtained in Sparidae as gilthead sea bream (Estruch et al., 2018a) and red sea bream (Kader et al., 2012) were zero-fish meal diets were successful used by supplement the test diet with complementary marine by-products ingredients or fish soluble in a range of 15-20%. In our study, all the test diets were included with lower amount of marine by-products (squid meal and fish soluble) known to

act in high vegetable diet as an attractant (Kader et al., 2010). In fact fish feeding monitoring confirmed this property since all the test diets were promptly accepted; after 45 days, as expected due to the short term feeding period, no differences in the final growth parameter were detected, suggesting that 100% fishmeal can be successfully replaced by vegetable counterparts or by insect meal in juvenile sea bream diets. Results obtained are not easily comparable to other studies since to date Hi were mostly evaluated as fish meal substitutes in diets containing only low levels of vegetable proteins (Elia et al., 2018; Iaconisi et al., 2019) or different types and composition of Hi used (Cardinaletti et al., 2019; Zarantoniello et al., 2018). However, albeit not significant ($p>0.05$) differences among dietary treatment in growth parameters', a tendency in improving final body weight and feed efficiency with the increasing dietary insect protein inclusion was observed, suggesting no detrimental effect of insect meal in this specie (Fabrikov et al., 2020). Although, this assumption need to be further confirmed in a longer feeding trial, our preliminary data seems to confirm what has been previously observed by in sea bream fed high plant-protein based diet supplemented with a blend of marine by-products (Estruch et al., 2018a, 2018b, Kader et al., 2012, Monge-Ortiz et al., 2016) or in other fish species were insect meal do not affect final growth performance (Hua, 2021).

When using high plant protein based diet and when testing new ingredients, it is important to consider the impacts on the gastrointestinal as by its integrity depends both the digestive functions and the protection barrier against pathogens (Merrifield et al., 2011). In the present study gene expression analysis and histological evaluations of the digestive system were considered as good indicators of fish health status (Caballero et al., 2003; Couto et al., 2015, 2014; Messina et al., 2019). For the first time the use of insect meal in gilthead sea bream was investigated at molecular and histological level to better understand the fish physiological response.

In the CV diet, fish showed a moderate/severe lymphocytic gastritis that may impaired the digestive functions. To our knowledge, very few studies are available on the effects of alternative protein sources on fish gastric mucosa. Omnes et al. (Omnes et al., 2015) reported no sign of structural pathological effects in the gilthead seabream stomach fed during one month with diets including 200 g kg^{-1} of white lupin or canola/rapeseed. In a recent study 100% fish meal replacement by discarded cashew nut reveal in *Clarias gariepinus* moderate to severe histological alterations, which include degeneration of columnar epithelium, degeneration/shrinkage of gastric glands/mucosa and serosa vacuolization (Ogueji et al., 2020). In the present study, the most serious gastric damages were observed in the CV fish and based on the type of inflammatory pattern, the potential presence of anti-nutritional factors coming from the plan protein sources utilized, could be the trigger, since the absence of such inflammatory lesions in fish prior to the start of this trial. The type of histological changes observed was somewhat similar to that observed in soy dependent enteritis of salmonids (Krogdahl et al., 2010). In the stomach, it was also observed an up-regulation of the *Hsp70* gene expression in the Hi20 and Hi40 diets, compared to the CV. Heat shock proteins are crucial for the maintenance of cell integrity during normal cell growth as well as during pathophysiological conditions. In the rat stomach, *Hsp70* has been shown to have an important role in the defense of the gastric mucosa by its over-expression, which suppresses exfoliation and necrosis of gastric mucosal cells inhibiting the apoptosis phenomena. In the present study, the upregulation of *Hsp70* was interpreted as a selective induction of the chaperone which aims to limit gastric harmful events (Rokutan, 2000). However, the observed effect of CV diet and Hi20 and Hi40 counterparts on gastric morphology in gilthead sea bream deserve further investigated.

Another important finding delivered by the present study concern the endogenous expression of the gene related to chitin hydrolysis both in the stomach, liver and along intestinal tract. Despite, the unaffected effected of insect meal dietary inclusion ($p>0.05$) on both gastric-chitinase (*CHIA*) and chitobiase (*CTBS*) expression, due to the high variability among the biological replicates, the expression pattern reflect what previously reported in other insectivorous mammals by Tabata et al. (Tabata et al., 2019, 2018). However, our finding are not consistent with previous one on zebrafish where fish fed a 50% fish meal diet replacement by full-

fat *Hermetia illucens* prepupae meal, up-regulate the *Chia1* expression after 7 days post-hatching (Vargas et al., 2018; Zarantoniello et al., 2018). The chitinase genes have been sequenced in few marine teleosts including the Japanese flounder (*Paralichthys olivaceus*) (Kurokawa et al., 2004), turbot (*Scophthalmus maximus*) (Gao et al., 2017) and the striped bass (*Morone saxatilis*) (GenBank accession no. EU048546) and chitinase enzymes related with chitin hydrolysis have been measured in the gastric and intestinal mucosa, pyloric caeca and pancreas of marine fish (Ikeda et al., 2017; Jeuniaux, 1993) confirming that Teleosts are capable of endogenous production of chitinase and supporting our preliminary findings in sea bream. However, there is a need to further investigate the chitin digestion by fish fed insect meal including diets in order to better understand and clarify the debate among the contribution of host endogenous production and the chitinoclastic bacteria present in the digestive tracts (Lindsay et al., 1984; Ramesh and Venugopalan, 1989).

One way of improving the animal's oxidative status is by incorporating in feeds specific components that stimulate the antioxidant defense and chitin has been recognized as an antioxidant molecule, preventing deleterious effects in various diseases (Ngo and Kim, 2014). The superoxide dismutase considered in the present study, the copper-zinc superoxide dismutase (*Sod1*) and manganese superoxide dismutase (*Sod2*), contributes in the cyto-protection by catalyzing the breakdown of ROS-generating O_2^-. They are structurally different and present differ cellular localization: *Sod1* is found in cytosolic and extracellular fractions, whereas *Sod2* is mostly mitochondrial (Zelko et al., 2002). In the liver, the highest insect meal inclusion level (Hi40) increased the expression of *Sod1* compared to CV diet whereas in the hindgut an up regulation of both *Sod1* and *Sod2* was observed in fish fed intermediate insect meal inclusion (Hi20). At gene expression level *Sod1* mRNA, can be up-regulated in response to a wide array of chemical, and biological messengers such as heat shock (Hae Yong Yoo et al., 1999a), heavy metal (H. Y. Yoo et al., 1999) and arachidonic acid (Hae Yong Yoo et al., 1999b); while cytokines, lipopolysaccharide (LPS) and IFN-γ are potent activators at transcriptional level of *Sod2* in different tissues and cell types (Visner et al., 1990; Zelko et al., 2002). Despite the absence of histological alterations in gut and the lack of inflammatory markers investigation (Fehrmann-Cartes et al., 2019), it is only possible to hypothesize an interplay between *Sod2* and inflammatory response; on the other hand, it cannot also be ruled out that upregulation of *Sod1* may be related to some contaminant related to insect quality (Fels-Klerx et al., 2018). However the effect of insect meal in the oxidative stress response have been previously investigated in freshwater fish species where it was observed an increase in the antioxidant enzymatic activity due to the chitin content present in the insect diet (Caimi et al., 2020; Elia et al., 2018) and chitin dietary content has exerted a boost in the SOD activity in orange-spotted grouper (*Epinephelus coioides*) (Zhang et al., 2012). Although it can not be ruled out the pro-oxidant role of chitin when optimal threshold is encompassed (Caimi et al., 2020) it is important to take into account that in Mammals *Sod1* and *Sod2* activity is affected by dietary Cu, Zn and or Mn content (Hidalgo et al., 2002; Lin et al., 2008). Similarly, in seabream the activity of SOD was related to the bioavailability of the manganese in the diet (Pérez-Jiménez et al., 2012). Finally, it is reported that, in the presence of oxidative stress, the transcription of GPx and GST increase in order to control the high level of ROS produced (Wilhelm Filho, 1996). In agreement with this and in the absence of histological tissue damage the the decrease in GST transcription level in the pyloric caeca of fish fed the highest insect meal (Hi40) suggests that both oxidative stress damage decreased and that feed components did not contain toxicant chemicals that may adversely affect fish performance.

Finally another interesting outcome of the present study is the up-regulation recorded in the pyloric caeca and hindgut of fish fed insect meal diet in the intestinal alkaline phosphatase, that have an important role in maintaining the intestinal homeostasis (Lallès, 2010). Intestinal alkaline phosphatase can be subjective to nutritional modulation by protein content and composition or by fat source and type of dietary fatty acid at both gene and enzymatic level (Lallès, 2020) but it is difficult to make a comparison of our results and those previous published since the differences in trials source and composition of protein and fat, treatment duration, fish species and stage of development. However, the up-regulation observed in seabream fed insect

meal may be considered consistent and similar to what has been previously recorded at gene level by using probiotic (Reyes-Becerril et al., 2013) or bioactive compounds from olive oil (Gisbert et al., 2017) in the diet. In addition by considering the role of alkaline phosphatase as an enhancer gut mucosal defense factor (Chen et al., 2011) it is clear its potential beneficial effect in fish welfare (Rimoldi et al., 2019; Terova et al., 2019).

5. Conclusions

In conclusion, the present study unveiled a major adverse effect in the stomach of fish fed the vegetable diet even during a short-term feeding period, verified mostly by a modulation of the *Hsp70* that acts as hallmarks of fundamental gastric mucosal defense, as supported by the histological evaluation. Whereas no clear dose dependent response was detected in the gene expression induction of chitin hydrolysis related genes, for the first time we have demonstrated, that sea bream has and express its own sets of chitinolitic genes, and that these are distributed along the entire gastrointestinal tract. The modulation of the response of some biomarkers involved in oxidative stress deserved to be further investigated moreover, understanding the potential nutraceutical role of insect meal on fish intestinal alkaline phosphatase at gastrointestinal level will allow to better optimize fish performance and health in aquaculture programmes (Lallès, 2019).

Author Contributions: G.C., E.D. and E.S. conceived and designed the experiment; G.C. and E.T. formulated the diets; R.C. support in fish husbandry; G.C. and E.D. contributed to collect the samples. E.D. carried out the nucleotides extraction and the real time PCR analyses; E.S and G.C. contributed to qPCR data analysis and interpretation of the results; P.B. carried out histological evaluation; R.C performed the statistical histological data analyses. G.C., E.S., P.B. and E.T. contributed to write the first draft of the manuscript; E.D., E.S., P.B., E.T. and G.C. contributed in reviewing and editing the final version of article.

Funding: This research was funded by AQUAEXCEL2020 (grant agreement No. 652831) under TNA project AE120006, and partially supported by Ager (AGER2-SUSHIN Cod 2016-0112) under grant-funded project SUSHIN (SUstainable fiSH feeds INnovative ingredients).

Acknowledgments: The authors gratefully acknowledge Dr. Fabio Mina for the diets analyses; Carla Calligaro histological tissue samples preparation and staining; Dr. Maria Papadaki for technical support at the HCMR laboratory during E.D. staying; Dr. Costas Tsigenopoulos for administrative assistance within AQUAEXCEL2020 program. We also apologize to those colleagues whose works we were unable to cite due to space limitation.

Conflicts of Interest: "The authors declare no conflict of interest."

References

ADDIN ZOTERO_BIBL {"uncited":[],"omitted":[],"custom":[]} CSL_BIBLIOGRAPHY Amin, A., El Asely, A., Abd El-Naby, A.S., Samir, F., El-Ashram, A., Sudhakaran, R., Dawood, M.A.O., 2019. Growth performance, intestinal histomorphology and growth-related gene expression in response to dietary Ziziphus mauritiana in Nile tilapia (*Oreochromis niloticus*). Aquaculture 512, 734301. https://doi.org/10.1016/j.aquaculture.2019.734301

AOAC International - AOAC [WWW Document], n.d. . AOAC International. URL https://www.aoac.org/ (accessed 10.14.20).

Baeza-Ariño, R., Martínez-Llorens, S., Nogales-Mérida, S., Jover-Cerda, M., Tomás-Vidal, A., 2016. Study of liver and gut alterations in sea bream, *Sparus aurata L.*, fed a mixture of vegetable protein concentrates. Aquaculture Research 47, 460–471.

https://doi.org/10.1111/are.12507
Benhabiles, M.S., Salah, R., Lounici, H., Drouiche, N., Goosen, M.F.A., Mameri, N., 2012. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocolloids 29, 48−56. https://doi.org/10.1016/j.foodhyd.2012.02.013
Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I., Gatta, P.P., 2008. Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream *(Sparus aurata L.*) and European sea bass (Dicentrarchus labrax L.). Aquaculture Research 39, 970−978. https://doi.org/10.1111/j.1365-2109.2008.01958.x
Bonaldo, A., Roem, A.J., Pecchini, A., Grilli, E., Gatta, P.P., 2006. Influence of dietary soybean meal levels on growth, feed utilization and gut histology of Egyptian sole (Solea aegyptiaca) juveniles. Aquaculture 261, 580−586. https://doi.org/10.1016/j.aquaculture.2006.08.013
Burja, A.M., Armenta, R.E., Radianingtyas, H., Barrow, C.J., 2007. Evaluation of Fatty Acid Extraction Methods for Thraustochytrium sp. ONC-T18. J. Agric. Food Chem. 55, 4795−4801. https://doi.org/10.1021/jf070412s
Burrells, C., Williams, P.D., Southgate, P.J., Crampton, V.O., 1999. Immunological, physiological and pathological responses of rainbow trout (*Oncorhynchus mykiss*) to increasing dietary concentrations of soybean proteins. Vet Immunol Immunopathol 72, 277−288. https://doi.org/10.1016/s0165-2427(99)00143-9
Caballero, M.J., Izquierdo, M.S., Kjørsvik, E., Montero, D., Socorro, J., Fernández, A.J., Rosenlund, G., 2003. Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225, 325−340. https://doi.org/10.1016/S0044-8486(03)00299-0
Caimi, C., Gasco, L., Biasato, I., Malfatto, V., Varello, K., Prearo, M., Pastorino, P., Bona, M.C., Francese, D.R., Schiavone, A., Elia, A.C., Dörr, A.J.M., Gai, F., 2020. Could Dietary Black Soldier Fly Meal Inclusion Affect the Liver and Intestinal Histological Traits and the Oxidative Stress Biomarkers of Siberian Sturgeon (*Acipenser baerii*) Juveniles? Animals (Basel) 10. https://doi.org/10.3390/ani10010155
Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., Bruni, L., Parisi, G., Olivotto, I., Tulli, F., 2019. Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals 9, 251. https://doi.org/10.3390/ani9050251
Chen, K.T., Malo, M.S., Beasley-Topliffe, L.K., Poelstra, K., Millan, J.L., Mostafa, G., Alam, S.N., Ramasamy, S., Warren, H.S., Hohmann, E.L., Hodin, R.A., 2011. A Role for Intestinal Alkaline Phosphatase in the Maintenance of Local Gut Immunity. Dig Dis Sci 56, 1020−1027. https://doi.org/10.1007/s10620-010-1396-x
Council, N.R., 2011. Nutrient Requirements of Fish and Shrimp. https://doi.org/10.17226/13039
Couto, A., Kortner, T.M., Penn, M., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2014. Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 432, 295−303. https://doi.org/10.1016/j.aquaculture.2014.05.009
Couto, A., Kortner, T.M., Penn, M., Østby, G., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2015. Saponins and phytosterols in diets for European sea bass (*Dicentrarchus labrax*) juveniles: effects on growth, intestinal morphology and physiology. Aquaculture Nutrition 21, 180−193. https://doi.org/10.1111/anu.12146
Drew, M.D., Borgeson, T.L., Thiessen, D.L., 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science and Technology, Nutrition Technologies in Animal Feed Science and Technology 138, 118−136. https://doi.org/10.1016/j.anifeedsci.2007.06.019

Elia, A.C., Capucchio, M.T., Caldaroni, B., Magara, G., Dörr, A.J.M., Biasato, I., Biasibetti, E., Righetti, M., Pastorino, P., Prearo, M., Gai, F., Schiavone, A., Gasco, L., 2018. Influence of Hermetia illucens meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 496, 50−57. https://doi.org/10.1016/j.aquaculture.2018.07.009

Esteban, M.A., Cuesta, A., Ortuño, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata L.*) innate immune system. Fish & Shellfish Immunology 11, 303−315. https://doi.org/10.1006/fsim.2000.0315

Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Grammes, F., Martos-Sitcha, J.A., Mydland, L.-T., Calduch-Giner, J.A., Fuentes, J., Karalazos, V., Ortiz, Á., Øverland, M., Sitjà -Bobadilla, A., Pérez-Sánchez, J., 2016. Dietary Butyrate Helps to Restore the Intestinal Status of a Marine Teleost (*Sparus aurata*) Fed Extreme Diets Low in Fish Meal and Fish Oil. PLOS ONE 11, e0166564. https://doi.org/10.1371/journal.pone.0166564

Estruch, G., Collado, M.C., Monge-Ortiz, R., Tomás-Vidal, A., Jover-Cerdá, M., Peñaranda, D.S., Pérez Martínez, G., Martínez-Llorens, S., 2018a. Long-term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata, L.*) leads to changes in the inflammatory and immune related gene expression at intestinal level. BMC Vet Res 14, 302. https://doi.org/10.1186/s12917-018-1626-6

Estruch, G., Collado, M.C., Peñaranda, D.S., Vidal, A.T., Cerdá, M.J., Martínez, G.P., Martinez-Llorens, S., 2015. Impact of Fishmeal Replacement in Diets for Gilthead Sea Bream (*Sparus aurata*) on the Gastrointestinal Microbiota Determined by Pyrosequencing the 16S rRNA Gene. PLOS ONE 10, e0136389. https://doi.org/10.1371/journal.pone.0136389

Estruch, G., Tomás-Vidal, A., Nokrashy, A.M.E., Monge-Ortiz, R., Godoy-Olmos, S., Cerdá, M.J., Martínez-Llorens, S., 2018b. Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead sea bream (*Sparus aurata, L.*): effects on digestibility, amino acid retention, ammonia excretion and enzyme activity. Archives of Animal Nutrition 72, 321−339. https://doi.org/10.1080/1745039X.2018.1472408

Fabrikov, D., Sánchez-Muros, M.J., Barroso, F.G., Tomás-Almenar, C., Melenchón, F., Hidalgo, M.C., Morales, A.E., Rodriguez-Rodriguez, M., Montes-Lopez, J., 2020. Comparative study of growth performance and amino acid catabolism in *Oncorhynchus mykiss, Tinca tinca* and *Sparus aurata* and the catabolic changes in response to insect meal inclusion in the diet. Aquaculture 529, 735731. https://doi.org/10.1016/j.aquaculture.2020.735731

FAO (Ed.), 2018. Meeting the sustainable development goals, The state of world fisheries and aquaculture. Rome.

Fehrmann-Cartes, K., Coronado, M., Hernández, A.J., Allende, M.L., Feijoo, C.G., 2019. Anti -inflammatory effects of aloe vera on soy meal-induced intestinal inflammation in zebrafish. Fish & Shellfish Immunology 95, 564−573. https://doi.org/10.1016/j.fsi.2019.10.075

Fels-Klerx, H.J. van der, Camenzuli, L., Belluco, S., Meijer, N., Ricci, A., 2018. Food Safety Issues Related to Uses of Insects for Feeds and Foods. Comprehensive Reviews in Food Science and Food Safety 17, 1172−1183. https://doi.org/10.1111/1541-4337.12385

Finke, M.D., 2013. Complete Nutrient Content of Four Species of Feeder Insects. Zoo Biology 32, 27−36. https://doi.org/10.1002/zoo.21012

Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199, 197–227. https://doi.org/10.1016/S0044-8486(01)00526-9

Galkanda-Arachchige, H.S.C., Wilson, A.E., Davis, D.A., 2020. Success of fishmeal replacement through poultry by-product meal in aquaculture feed formulations: a meta-analysis. Reviews in Aquaculture 12, 1624–1636. https://doi.org/10.1111/raq.12401

Gao, C., Cai, X., Zhang, Y., Su, B., Song, H., Wenqi, W., Li, C., 2017. Characterization and expression analysis of chitinase genes (CHIT1, CHIT2 and CHIT3) in turbot (Scophthalmus maximus L.) following bacterial challenge. Fish & Shellfish Immunology 64, 357–366. https://doi.org/10.1016/j.fsi.2017.03.019

Gasco, L., Finke, M., van Huis, A., 2018. Can diets containing insects promote animal health? Journal of Insects as Food and Feed 4, 1–4. https://doi.org/10.3920/JIFF2018.x001

Gasco, Laura, Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., Caruso, G., 2018. Fishmeal Alternative Protein Sources for Aquaculture Feeds, in: Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., Caruso, G. (Eds.), Feeds for the Aquaculture Sector: Current Situation and Alternative Sources, SpringerBriefs in Molecular Science. Springer International Publishing, Cham, pp. 1–28. https://doi.org/10.1007/978-3-319-77941-6_1

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research 38, 551–579. https://doi.org/10.1111/j.1365-2109.2007.01704.x

Gisbert, E., Andree, K.B., Quintela, J.C., Calduch-Giner, J.A., Ipharraguerre, I.R., Pérez-Sánchez, J., 2017. Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*). British Journal of Nutrition 117, 351–363. https://doi.org/10.1017/S0007114517000228

Glencross, B.D., Baily, J., Berntssen, M.H.G., Hardy, R., MacKenzie, S., Tocher, D.R., 2020. Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. Reviews in Aquaculture 12, 703–758. https://doi.org/10.1111/raq.12347

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research 41, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x

Haro, C. de, Bueno, R.P.R., Barroso, F.G., Muros, M.J.S., Cervera, M.Á.R., Guil-Guerrero, J.L., 2016. Insect larvae as feed ingredient selectively increase arachidonic acid content in farmed gilthead sea bream (*Sparus aurata L.)*. Aquaculture Research 47, 2881–2887. https://doi.org/10.1111/are.12738

Hedrera, M.I., Galdames, J.A., Jimenez-Reyes, M.F., Reyes, A.E., Avendaño-Herrera, R., Romero, J., Feijóo, C.G., 2013. Soybean Meal Induces Intestinal Inflammation in Zebrafish Larvae. PLOS ONE 8, e69983. https://doi.org/10.1371/journal.pone.0069983

Hidalgo, M.C., Expósito, A., Palma, J.M., Higuera, M. de la, 2002. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). The International Journal of Biochemistry & Cell Biology 34, 183–193. https://doi.org/10.1016/S1357-2725(01)00105-4

Hua, K., 2021. A meta-analysis of the effects of replacing fish meals with insect meals on growth performance of fish. Aquaculture 530, 735732. https://doi.org/10.1016/j.aquaculture.2020.735732

Iaconisi, V., Secci, G., Sabatino, G., Piccolo, G., Gasco, L., Papini, A.M., Parisi, G., 2019. Effect of mealworm (*Tenebrio molitor L.*) larvae meal on amino acid composition of gilthead sea bream (Sparus aurata L.) and rainbow trout (*Oncorhynchus mykiss W.*) fillets. Aquaculture 513, 734403. https://doi.org/10.1016/j.aquaculture.2019.734403

Ikeda, M., Kakizaki, H., Matsumiya, M., 2017. Biochemistry of fish stomach chitinase. International Journal of Biological Macromolecules, 11th APCCS-2016-Chemistry, Environmental, Biotechnology and Biomedical Aspects of Chitin and Chitosan 104, 1672−1681. https://doi.org/10.1016/j.ijbiomac.2017.03.118

Jeuniaux, C., 1993. Chitinolytic systems in the digestive tract of vertebrates : a review. Chitin Enzymology 1, 233−244.

Kader, Md.A., Bulbul, M., Koshio, S., Ishikawa, M., Yokoyama, S., Nguyen, B.T., Komilus, C.F., 2012. Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, Pagrus major. Aquaculture 350−353, 109−116. https://doi.org/10.1016/j.aquaculture.2012.04.009

Kader, Md.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., 2010. Supplemental effects of some crude ingredients in improving nutritive values of low fishmeal diets for red sea bream, Pagrus major. Aquaculture 308, 136−144. https://doi.org/10.1016/j.aquaculture.2010.07.037

Karapanagiotidis, Daskalopoulou E., Vogiatzis I., Rumbos C., Mente E., Athanassiou C.G., 2014. Substitution of fishmeal by fly Hermetia illucens prepupae meal in the diet of gilthead seabream (Sparus aurata) [WWW Document]. URL http://hydromedit2014.apae.uth.gr/index.php?lang=en&Itemid=185 (accessed 10.29.20).

Karasuda, S., Yamamoto, K., Kono, M., Sakuda, S., Koga, D., 2004. Kinetic Analysis of a Chitinase from Red Sea Bream, <I>Pagrus major</I>. Bioscience, Biotechnology, and Biochemistry 68, 1338−1344. https://doi.org/10.1271/bbb.68.1338

Kokou, F., Rigos, G., Henry, M., Kentouri, M., Alexis, M., 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata L.*) fed graded levels of a bioprocessed soybean meal. Aquaculture 364−365, 74−81. https://doi.org/10.1016/j.aquaculture.2012.08.009

Kokou, F., Sarropoulou, E., Cotou, E., Rigos, G., Henry, M., Alexis, M., Kentouri, M., 2015. Effects of Fish Meal Replacement by a Soybean Protein on Growth, Histology, Selected Immune and Oxidative Status Markers of Gilthead Sea Bream, Sparus aurata. Journal of the World Aquaculture Society 46, 115−128. https://doi.org/10.1111/jwas.12181

Kõressaar, T., Lepamets, M., Kaplinski, L., Raime, K., Andreson, R., Remm, M., 2018. Primer3_masker: integrating masking of template sequence with primer design software. Bioinformatics 34, 1937−1938. https://doi.org/10.1093/bioinformatics/bty036

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquaculture Research 41, 333−344. https://doi.org/10.1111/j.1365-2109.2009.02426.x

Kurokawa, T., Uji, S., Suzuki, T., 2004. Molecular cloning of multiple chitinase genes in Japanese flounder, Paralichthys olivaceus. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 138, 255−264. https://doi.org/10.1016/j.cbpc.2004.03.015

Lallès, J.-P., 2020. Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. Reviews in Aquaculture 12, 555−581. https://doi.org/10.1111/raq.12340

Lallès, J.-P., 2019. Recent advances in intestinal alkaline phosphatase, inflammation, and nutrition. Nutr Rev 77, 710−724. https://doi.org/10.1093/nutrit/nuz015
Lallès, J.-P., 2010. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutr Rev 68, 323−332. https://doi.org/10.1111/j.1753-4887.2010.00292.x
Li, Y., Kortner, T.M., Chikwati, E.M., Belghit, I., Lock, E.-J., Krogdahl, Å., 2020. Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar)*. Aquaculture 520, 734967. https://doi.org/10.1016/j.aquaculture.2020.734967
Lin, Y.-H., Lin, S.-M., Shiau, S.-Y., 2008. Dietary manganese requirements of juvenile tilapia, Oreochromis niloticus × O. aureus. Aquaculture 284, 207−210. https://doi.org/10.1016/j.aquaculture.2008.07.049
Lindsay, G.J.H., Walton, M.J., Adron, J.W., Fletcher, T.C., Cho, C.Y., Cowey, C.B., 1984. The growth of rainbow trout (*Salmo gairdneri)* given diets containing chitin and its relationship to chitinolytic enzymes and chitin digestibility. Aquaculture 37, 315−334. https://doi.org/10.1016/0044-8486(84)90297-7
Matulić, D., Barišić, J., Aničić, I., Tomljanović, T., Safner, R., Treer, T., Gao, J., Glojnarić, I., Čož-Rakovac, R., 2020. Growth, health aspects and histopathology of brown bullhead (*Ameiurus nebulosus L.*): replacing fishmeal with soybean meal and brewer′s yeast. Scientific Reports 10, 1104. https://doi.org/10.1038/s41598-020-57722-3
Merrifield, D.L., Olsen, R.E., Myklebust, R., Ringø, E., 2011. Dietary Effect of Soybean (Glycine max) Products on Gut Histology and Microbiota of Fish. Soybean and Nutrition. https://doi.org/10.5772/20101
Messina, M., Bulfon, C., Beraldo, P., Tibaldi, E., Cardinaletti, G., 2019. Intestinal morpho-physiology and innate immune status of European sea bass (*Dicentrarchus labrax*) in response to diets including a blend of two marine microalgae, Tisochrysis lutea and Tetraselmis suecica. Aquaculture 500, 660−669. https://doi.org/10.1016/j.aquaculture.2018.09.054
Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. PNAS 106, 15103−15110. https://doi.org/10.1073/pnas.0905235106
Ngo, D.-H., Kim, S.-K., 2014. Chapter Two - Antioxidant Effects of Chitin, Chitosan, and Their Derivatives, in: Kim, S.-K. (Ed.), Advances in Food and Nutrition Research, Marine Carbohydrates: Fundamentals and Applications, Part B. Academic Press, pp. 15−31. https://doi.org/10.1016/B978-0-12-800268-1.00002-0
Nogales-Mérida, S., Gobbi, P., Józefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., Kierończyk, B., Józefiak, A., 2019. Insect meals in fish nutrition. Reviews in Aquaculture 11, 1080−1103. https://doi.org/10.1111/raq.12281
Ogueji, E.O., Iheanacho, S.C., Mbah, C.E., Yaji, A.J., Ezemagu, U., 2020. Effect of partial and complete replacement of soybean with discarded cashew nut (*Anacardium occidentale L*) on liver and stomach histology of Clarias gariepinus (Burchell, 1822). Aquaculture and Fisheries 5, 86−91. https://doi.org/10.1016/j.aaf.2019.10.005
Omnes, M.H., Silva, F.C.P., Moriceau, J., Aguirre, P., Kaushik, S., Gatesoupe, F.J., 2015. Influence of lupin and rapeseed meals on the integrity of digestive tract and organs in gilthead seabream (*Sparus aurata L.*) and goldfish (*Carassius auratus L.*) juveniles. Aquaculture Nutrition 21, 223−233. https://doi.org/10.1111/anu.12162
Panserat, S., Hortopan, G.A., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., Esquerré, D., Geurden, I., Médale, F., Kaushik, S., Corraze, G., 2009. Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture 294, 123−131.

https://doi.org/10.1016/j.aquaculture.2009.05.013
Pérez-Jiménez, A., Peres, H., Cruz Rubio, V., Oliva-Teles, A., 2012. The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (*Sparus aurata*). Br J Nutr 108, 1202−1209. https://doi.org/10.1017/S0007114511006556
Pérez-Sánchez, J., Estensoro, I., Redondo, M.J., Calduch-Giner, J.A., Kaushik, S., Sitjà-Bobadilla, A., 2013. Mucins as Diagnostic and Prognostic Biomarkers in a Fish-Parasite Model: Transcriptional and Functional Analysis. PLoS ONE 8, e65457. https://doi.org/10.1371/journal.pone.0065457
Piccolo, G., Iaconisi, V., Marono, S., Gasco, L., Loponte, R., Nizza, S., Bovera, F., Parisi, G., 2017. Effect of Tenebrio molitor larvae meal on growth performance, in vivo nutrients digestibility, somatic and marketable indexes of gilthead sea bream (*Sparus aurata*). Animal Feed Science and Technology 226, 12−20. https://doi.org/10.1016/j.anifeedsci.2017.02.007
Pita, C., Gamito, S., Erzini, K., 2002. Feeding habits of the gilthead seabream (*Sparus aurata*) from the Ria Formosa (southern Portugal) as compared to the black seabream (*Spondyliosoma cantharus*) and the annular seabream (*Diplodus annularis*). Journal of Applied Ichthyology 18, 81−86. https://doi.org/10.1046/j.1439-0426.2002.00336.x
Ramesh, A., Venugopalan, V.K., 1989. Role of luminous bacteria in chitin degradation in the intestine of fish. World J Microbiol Biotechnol 5, 55−59. https://doi.org/10.1007/BF01724959
Ravi, H.K., Degrou, A., Costil, J., Trespeuch, C., Chemat, F., Vian, M.A., 2020. Larvae Mediated Valorization of Industrial, Agriculture and Food Wastes: Biorefinery Concept through Bioconversion, Processes, Procedures, and Products. Processes 8, 857. https://doi.org/10.3390/pr8070857
Refstie, S., Glencross, B., Landsverk, T., Sørensen, M., Lilleeng, E., Hawkins, W., Krogdahl, Å., 2006. Digestive function and intestinal integrity in Atlantic salmon (*Salmo salar*) fed kernel meals and protein concentrates made from yellow or narrow-leafed lupins. Aquaculture 261, 1382−1395. https://doi.org/10.1016/j.aquaculture.2006.07.046
Reyes-Becerril, M., Guardiola, F., Rojas, M., Ascencio-Valle, F., Esteban, M.Á., 2013. Dietary administration of microalgae Navicula sp. affects immune status and gene expression of gilthead seabream (*Sparus aurata*). Fish & Shellfish Immunology 35, 883−889. https://doi.org/10.1016/j.fsi.2013.06.026
Rimoldi, S., Gini, E., Iannini, F., Gasco, L., Terova, G., 2019. The effects of dietary insect meal from Hermetia illucens Prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). Animals 9, 143. https://doi.org/10.3390/ani9040143
Rokutan, K., 2000. Role of heat shock proteins in gastric mucosal protection. J Gastroenterol Hepatol 15 Suppl, D12-19. https://doi.org/10.1046/j.1440-1746.2000.02144.x
Romarheim, O.H., Zhang, C., Penn, M., Liu, Y.-J., Tian, L.-X., Skrede, A., Krogdahl, Å., Storebakken, T., 2008. Growth and intestinal morphology in cobia (*Rachycentron canadum*) fed extruded diets with two types of soybean meal partly replacing fish meal. Aquaculture Nutrition 14, 174−180. https://doi.org/10.1111/j.1365-2095.2007.00517.x
Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249, 387−400. https://doi.org/10.1016/j.aquaculture.2005.03.031
Skřivanová, E., Marounek, M., Dlouhá, G., Kaňka, J., 2005. Susceptibility of Clostridium perfringens to C2−C18 fatty acids. Letters in Applied Microbiology 41, 77−81. https://doi.org/10.1111/j.1472-765X.2005.01709.x
Spranghers, T., Michiels, J., Vrancx, J., Ovyn, A., Eeckhout, M., De Clercq, P., De Smet, S., 2018. Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia*

illucens L.) prepupae for weaned piglets. Animal Feed Science and Technology 235, 33−42. https://doi.org/10.1016/j.anifeedsci.2017.08.012

Tabata, E., Kashimura, A., Kikuchi, A., Masuda, H., Miyahara, R., Hiruma, Y., Wakita, S., Ohno, M., Sakaguchi, M., Sugahara, Y., Matoska, V., Bauer, P.O., Oyama, F., 2018. Chitin digestibility is dependent on feeding behaviors, which determine acidic chitinase mRNA levels in mammalian and poultry stomachs. Scientific Reports 8, 1461. https://doi.org/10.1038/s41598-018-19940-8

Tabata, E., Kashimura, A., Uehara, M., Wakita, S., Sakaguchi, M., Sugahara, Y., Yurimoto, T., Sasaki, E., Matoska, V., Bauer, P.O., Oyama, F., 2019. High expression of acidic chitinase and chitin digestibility in the stomach of common marmoset (*Callithrix jacchus*), an insectivorous nonhuman primate. Scientific Reports 9, 159. https://doi.org/10.1038/s41598-018-36477-y

Tacon, A.G.J., 2020. Trends in Global Aquaculture and Aquafeed Production: 2000−2017. Reviews in Fisheries Science & Aquaculture 28, 43−56. https://doi.org/10.1080/23308249.2019.1649634

Teles, M., Oliveira, M., Jerez-Cepa, I., Franco-Martínez, L., Tvarijonaviciute, A., Tort, L., Mancera, J.M., 2019. Transport and Recovery of Gilthead Sea Bream (*Sparus aurata L.*) Sedated With Clove Oil and MS222: Effects on Oxidative Stress Status. Front. Physiol. 10. https://doi.org/10.3389/fphys.2019.00523

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., Gasco, L., 2019. Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from Hermetia illucens prepupae in the diet. Reviews in Fish Biology and Fisheries 29, 465−486. https://doi.org/10.1007/s11160-019-09558-y

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res 40, e115. https://doi.org/10.1093/nar/gks596

Urán, P.A., Gonçalves, A.A., Taverne-Thiele, J.J., Schrama, J.W., Verreth, J.A.J., Rombout, J.H.W.M., 2008. Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio L.*). Fish & Shellfish Immunology 25, 751−760. https://doi.org/10.1016/j.fsi.2008.02.013

Vargas, A., Randazzo, B., Riolo, P., Truzzi, C., Gioacchini, G., Giorgini, E., Loreto, N., Ruschioni, S., Zarantoniello, M., Antonucci, M., Polverini, S., Cardinaletti, G., Sabbatini, S., Tulli, F., Olivotto, I., 2018. Rearing Zebrafish on Black Soldier Fly (*Hermetia illucens*): Biometric, Histological, Spectroscopic, Biochemical, and Molecular Implications. Zebrafish 15, 404−419. https://doi.org/10.1089/zeb.2017.1559

Vargas-Abúndez, A.J., Randazzo, B., Foddai, M., Sanchini, L., Truzzi, C., Giorgini, E., Gasco, L., Olivotto, I., 2019. Insect meal based diets for clownfish: Biometric, histological, spectroscopic, biochemical and molecular implications. Aquaculture 498, 1−11. https://doi.org/10.1016/j.aquaculture.2018.08.018

Visner, G.A., Dougall, W.C., Wilson, J.M., Burr, I.A., Nick, H.S., 1990. Regulation of manganese superoxide dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor. Role in the acute inflammatory response. J. Biol. Chem. 265, 2856−2864.

Wilhelm Filho, D., 1996. Fish antioxidant defenses--a comparative approach. Braz J Med Biol Res 29, 1735−1742.

Williams, J.P., Williams, J.R., Kirabo, A., Chester, D., Peterson, M., 2016. Chapter 3 - Nutrient Content and Health Benefits of Insects, in: Dossey, A.T., Morales-Ramos, J.A., Rojas, M.G. (Eds.), Insects as Sustainable Food Ingredients. Academic Press, San Diego, pp. 61−84. https://doi.org/10.1016/B978-0-12-802856-8.00003-X

Yoo, Hae Yong, Chang, M.S., Rho, H.M., 1999a. The Activation of the Rat Copper/Zinc Superoxide Dismutase Gene by Hydrogen Peroxide through the Hydrogen Peroxide-responsive Element and by Paraquat and Heat Shock through the Same Heat Shock Element. J. Biol. Chem. 274, 23887−23892. https://doi.org/10.1074/jbc.274.34.23887

Yoo, H. Y., Chang, M.S., Rho, H.M., 1999. Heavy metal-mediated activation of the rat Cu/Zn superoxide dismutase gene via a metal-responsive element. Mol Gen Genet 262, 310−313. https://doi.org/10.1007/s004380051088

Yoo, Hae Yong, Chang, M.S., Rho, H.M., 1999b. Induction of the rat Cu/Zn superoxide dismutase gene through the peroxisome proliferator-responsive element by arachidonic acid. Gene 234, 87−91. https://doi.org/10.1016/S0378-1119(99)00176-6

Zarantoniello, M., Bruni, L., Randazzo, B., Vargas, A., Gioacchini, G., Truzzi, C., Annibaldi, A., Riolo, P., Parisi, G., Cardinaletti, G., Tulli, F., Olivotto, I., 2018. Partial Dietary Inclusion of *Hermetia illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications. Zebrafish 15, 519−532. https://doi.org/10.1089/zeb.2018.1596

Zarantoniello, M., Zimbelli, A., Randazzo, B., Compagni, M.D., Truzzi, C., Antonucci, M., Riolo, P., Loreto, N., Osimani, A., Milanović, V., Giorgini, E., Cardinaletti, G., Tulli, F., Cipriani, R., Gioacchini, G., Olivotto, I., 2020. Black Soldier Fly (*Hermetia illucens*) reared on roasted coffee by-product and Schizochytrium sp. as a sustainable terrestrial ingredient for aquafeeds production. Aquaculture 518, 734659. https://doi.org/10.1016/j.aquaculture.2019.734659

Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology and Medicine 33, 337−349. https://doi.org/10.1016/S0891-5849(02)00905-X

Zhang, Y., Feng, S., Chen, J., Qin, C., Lin, H., Li, W., 2012. Stimulatory effects of chitinase on growth and immune defense of orange-spotted grouper (*Epinephelus coioides*). Fish & Shellfish Immunology 32, 844−854. https://doi.org/10.1016/j.fsi.2012.02.009

Zhao, S., Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12, 1047−1064. https://doi.org/10.1089/cmb.2005.12.1047

5 Conclusion

Insects are part of the natural diet of both freshwater and marine fish and thanks to the fact that they are rich in protein, lipids, vitamins and minerals and have a low ecological footprint, they have been considered as potential candidate ingredients and alternatives to fish meal and fish oil, in diets for reared fish. Their ability to renewal waste and their potential usage as feed in livestock and aquaculture production has recently attracted the attention of the European Council. An official EU document (EU Regulation 2017/893) has defined seven breeding insect species that are at present allowed in aquafeeds: *Hermetia illucens, Musca domestica, Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus, Gryllodes sigillatus* and *Gryllus assimilis*. Their use has resulted in the need to find innovative and practical methods for identifying these new ingredients in complex matrices such as feeds and food.

To achieve this goal, the possible ability of species-specific primers to selectively amplify the target DNA segment in complex matrices by RT-PCR technology has been evaluated. Primers have been designed for the identification of *Gryllodes sigillatus* (GS), their efficiency was subsequently evaluated and it was tested whether the pair of primers amplified any non-target DNA segments using other insect species and raw materials commonly used in the formulation of common feeds. Subsequently, the primers were used to verify the presence of GS DNA in various complex matrices different commercial products: crunchy roasted sam-ples (RoGS), insect meal mixtures (ACGS) and energetic snacks containing GS (GSS). Data on sequencing were aligned with the reference gene to confirm the PCR products. The regression curve ($y=-3.394 x +42.521$; $R^2 =0.994$, d.f. 14) between Ct values and Log DNA concentrations of *Gryllodes sigillatus* resulted in an efficiency of 96.4%. The severity of the technological processing treatments and the matrix structure affected the intensity of the PCR signal with the same amount of insect DNA as observed by different y-intercepts of the three-regression lines for RoGS, ACGS, and GSS. Results indicate that the real-time PCR method produced robust and sensitive results capable of detecting small amounts of GS DNA (5 g / 100 g) in complex matrices,

making it suitable to detect the presence or absence of GS material labeled in both feeds and food.

With the same strategy a pair of primers specific for *Hermetia illicens* (HI) mitochondrial DNA were identified and their efficiency was evaluated. A linear relationship ($y = -3.360x + 24.42$, $R^2 = 0.998$; d.f. 14) between thermal cycles Ct values and Log DNA concentrations was obtained within the dilution range (from 10 ng/µl to 100 fg/µl) and considering that the angular coefficient of the measured standard curve was -3.360, a primers efficiency of 99.8 % was obtained. The PCR performance of the designed primers was tested with commercial aquafeed samples as representative of practical complete mixtures and processing methods. In general the Ct numbers required to detect a positive signal for all the practical samples was higher than expected from the calibration curve when considering aquafeeds within a range of HI meal from 5-50 g/100g of the total ingredients due to the partial DNA degradation of the raw material including the insect target species as a consequence of the mechanical and physical process adopted for feed production. Subsequently, the primers were used to design probes utilised for the construction of a DNA-based biosensor based on the detection of a fluorescent signal, excited by an organic light emitting diode (OLED) source coupled with a high sensitivity image sensor (CMOS). The results obtained by the biosensor were validated with the ″golden standard″ technology of RT-PCR. Both the RT-PCR and the biosensor were able to detect the presence of HI DNA in all the samples that contained the target DNA by clearly discriminating them from the negative samples. The biosensor and of the RT-PCR specificity was evaluated using DNA of different species of arthropods, mammals and ingredients used in aquaculture and no cross reactivity was detected. Subsequently, the DNA extracted from seven commercial feeds that had a variable inclusion of HI flour and a negative control were analyzed using the innovative method and the data obtained had matched with the ones obtained from RT-PCR. The innovative biosensor was capable to detect the presence of the target with a concentration of 5g / 100g without amplification.

Another topic studied during the PhD period was the physiological response of fish fed HI meal including diets using histological and molecular investigations. Specifically, the expression of genes involved in both digestive processes and animal welfare was evaluated *in vivo* along the gastrointestinal tract of gilthead seabream (*Sparus aurata*) as animal model. In particular were considered: genes involved in the hydrolysis of the exoskeletal components of arthropods (chitinase and chitobiase); genes involved in the synthesis of some carboxypeptidases that regulate numerous physiological processes (cathepsin B and Z); biomarkers involved in oxidative stress (superoxide dismutase (Sod1 and Sod2) and glutathione S transferase (GST3)) as well as those involved in protein synthesis (heat shock proteins (Hsp70) crucial for maintaining cell integrity); genes involved in the synthesis of mucins that play a protective role in the gastrointestinal mucosa (I-Muc and Muc13) and finally a marker involved in the synthesis of intestinal alkaline phosphatase involved in maintaining intestinal homeostasis. Fish were fed a control diet, consisting of vegetable ingredients, free from fish meal (CV), from which two other formulations (Hi20 and Hi40) were prepared including 20 and 40% of partially defatted larvae of the black soldier fly (HI), respectively, to replace the protein plant content of the CV. At the end of the feeding test, liver, pyloric blind stomach, upper, middle and posterior portions of the intestine were analysed. The results of the gene expression revealed that although both dietary formulations guaranteed similar growth performance of the animals, the presence of insect meal attenuated the pathological picture in the intestine observed in fish fed the plant-based diet (CV). For the first time, the expression of genes involved in the hydrolysis of chitin was demonstrated along the gastrointestinal tract of sea bream juveniles, although this was not influenced by the dose of insect provided. Furthermore, the expression of the genes involved in oxidative stress, with a dose-response effect at the hepatic level and the expression of the alkaline phosphatase involved in the maintenance of intestinal homeostasis was significantly higher in animals fed the insect-based diets ($P<0.05$). So feeding diets including (16%) HI meal stimulated genes related to oxidative stress and mitigated the negative effects of plant-based diets in the stomach of gilthead seabream.

Acknowledgments

This thesis was possible thanks to the fruitful collaboration with Patrizia Melpignano from Or.El.d.o.o. (Slovenia) , Elena Sarropoulou from HCMR (Greece) and Boris Maroh and Sonja Feldbacher from PCCL (Austria).

6 References

(References have been listed according to the guidelines of Elsevier – Aquaculture)

ADDIN Mendeley Bibliography CSL_BIBLIOGRAPHY 183/2005/EC, 2005. Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. Off. Jounal Eur. Union L 35, 1–22.

852/2004, R. (EC) N., 2004. REGULATION (EC) No 852/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 on the hygiene of foodstuffs. Off. J. Eur. Union.

Abdel-Ghany, H.M., El-Sayed, A.F.M., Ezzat, A.A., Essa, M.A., Helal, A.M., 2019. Dietary lipid sources affect cold tolerance of Nile tilapia (*Oreochromis niloticus*). J. Therm. Biol. https://doi.org/10.1016/j.jtherbio.2018.11.009

Bakke-McKellep, A.M., McL Press, C., Baeverfjord, G., Krogdahl, Å., Landsverk, T., 2000. Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, Salmo salar L., with soybean meal-induced enteritis. J. Fish Dis. https://doi.org/10.1046/j.1365-2761.2000.00218.x

Bañuls, M.J., Puchades, R., Maquieira, Á., 2013. Chemical surface modifications for the development of silicon-based label-free integrated optical (IO) biosensors: A review. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2013.01.025

Barennes, H., Phimmasane, M., Rajaonarivo, C., 2015. Insect consumption to address undernutrition, a national survey on the prevalence of insect consumption among adults and vendors in Laos. PLoS One. https://doi.org/10.1371/journal.pone.0136458

Barrias, S., Fernandes, J.R., Eiras-Dias, J.E., Brazão, J., Martins-Lopes, P., 2019. Label free DNA-based optical biosensor as a potential system for wine authenticity. Food Chem. https://doi.org/10.1016/j.foodchem.2018.07.058

Belghit, I., Waagbø, R., Lock, E.J., Liland, N.S., 2019. Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. Aquac. Nutr. https://doi.org/10.1111/anu.12860

Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Gordon Bell, J., Kaushik, S., Pérez-Sánchez, J., 2008. High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata L.*): Growth performance, muscle fatty acid profiles and histological alterations of target tissues. Br. J. Nutr. https://doi.org/10.1017/S0007114508966071

Bhalla, N., Jolly, P., Formisano, N., Estrela, P., 2016. Introduction to biosensors. Essays Biochem. 60, 1–8. https://doi.org/10.1042/EBC20150001

Biasato, I., Renna, M., Gai, F., Dabbou, S., Meneguz, M., Perona, G., Martinez, S., Lajusticia, A.C.B., Bergagna, S., Sardi, L., Capucchio, M.T., Bressan, E., Dama, A., Schiavone, A., Gasco, L., 2019. Partially defatted black soldier fly larva meal inclusion in piglet diets: Effects on the growth performance, nutrient digestibility, blood profile, gut morphology and histological features. J. Anim. Sci. Biotechnol. https://doi.org/10.1186/s40104-019-0325-x

Bukkens, S.G.F., 1997. The nutritional value of edible insects. Ecol. Food Nutr. https://doi.org/10.1080/03670244.1997.9991521

Caballero, M.J., Izquierdo, M.S., Kjørsvik, E., Montero, D., Socorro, J., Fernández, A.J., Rosenlund, G., 2003. Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources, in: Aquaculture. https://doi.org/10.1016/S0044-8486(03)00299-0

Capinera, J.L., 2008. Encyclopedia of Entomology, Volume 4, Encyclopedia of Entomology. https://doi.org/10.1007/978-1-4020-6359-6

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., Bruni, L., Parisi, G., Olivotto, I., Tulli, F., 2019. Effects of graded dietary inclusion level of full-fat hermetia illucens prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals. https://doi.org/10.3390/ani9050251

Cecchi, T., Alfei, B., 2013. Volatile profiles of Italian monovarietal extra virgin olive oils via HS-SPME-GC-MS: Newly identified compounds, flavors molecular markers, and terpenic profile. Food Chem. https://doi.org/10.1016/j.foodchem.2013.05.090

Commission, T.H.E.E., 2017. COMMISSION REGULATION (EU) 2017/ 893 - of 24 May 2017 - amending Annexes I and IV to Regulation (EC) No 999 / 2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) 2017.

Conceição, L.E.C., Aragão, C., Dias, J., Costas, B., Terova, G., Martins, C., Tort, L., 2012. Dietary nitrogen and fish welfare. Fish Physiol. Biochem. https://doi.org/10.1007/s10695-011-9592-y

Corres, J.M., Matias, I.R., Bravo, J., Arregui, F.J., 2008. Tapered optical fiber biosensor for the detection of anti-gliadin antibodies. Sensors Actuators, B Chem. https://doi.org/10.1016/j.snb.2008.08.008

Cummins, V.C., Rawles, S.D., Thompson, K.R., Velasquez, A., Kobayashi, Y., Hager, J., Webster, C.D., 2017. Evaluation of black soldier fly (Hermetia illucens) larvae meal as partial or total replacement of marine fish meal in practical diets for Pacific white shrimp (Litopenaeus vannamei). Aquaculture. https://doi.org/10.1016/j.aquaculture.2017.02.022

Damborský, P., Švitel, J., Katrlík, J., 2016. Optical biosensors. Essays Biochem. https://doi.org/10.1042/EBC20150010

Daniso, E., Melpignano, P., Tulli, F., 2020. An OLED-based genosensor for the detection of *Hermetia illucens* in feeds. Food Control 113. https://doi.org/10.1016/j.foodcont.2020.107179

de Castro, R.J.S., Ohara, A., Aguilar, J.G. dos S., Domingues, M.A.F., 2018. Nutritional, functional and biological properties of insect proteins: Processes for obtaining, consumption and future challenges. Trends Food Sci. Technol. https://doi.org/10.1016/j.tifs.2018.04.006

De Mora, K., Joshi, N., Balint, B.L., Ward, F.B., Elfick, A., French, C.E., 2011. A pH-based biosensor for detection of arsenic in drinking water. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-011-4815-8

Debode, F., Marien, A., Gérard, A., Francis, F., Fumière, O., Berben, G., 2017. Development of real-time PCR tests for the detection of *Tenebrio molitor* in food and feed. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 34, 1421–1426. https://doi.org/10.1080/19440049.2017.1320811

Derrien, C., Boccuni, A., 2018. Current status of the insect producing industry in Europe, in: Edible Insects in Sustainable Food Systems. https://doi.org/10.1007/978-3-319-74011-9_30

Dias, J., Alvarez, M.J., Arzel, J., Corraze, G., Diez, A., Bautista, J.M., Kaushik, S.J., 2005. Dietary protein source affects lipid metabolism in the European seabass (Dicentrarchus labrax). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. https://doi.org/10.1016/j.cbpb.2005.07.005

Dong, G.F., Yang, Y.O., Song, X.M., Yu, L., Zhao, T.T., Huang, G.L., Hu, Z.J., Zhang, J.L., 2013. Comparative effects of dietary supplementation with maggot meal and soybean meal in gibel carp (*Carassius auratus gibelio*) and darkbarbel catfish (*Pelteobagrus vachelli*): Growth performance and antioxidant responses. Aquac. Nutr. https://doi.org/10.1111/anu.12006

EC, 2009. Commission regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Off. J. Eur. Union 36, 1–130.

Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, P., Beisel, J.N., Coissac, E., Boyer, F., Leese, F., 2016. Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. PeerJ. https://doi.org/10.7717/peerj.1966

EU 2015/2283, 2015. Regulation (Eu) 2015/2283 of the European Parliament. Off. J. Eur. Union.

European Parliament and Council, 2002. Regulation (EC) N^{o} 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Off. J. Eur. Communities L31, 1–24.

Fao, 2013. Edible insects. Future prospects for food and feed security, Future prospects for food and feed security. https://doi.org/10.1017/CBO9781107415324.004

FAO (Food and Agriculture Organization), 2011. World Livestock 2011 Livestock in food security World, World. https://doi.org/10.1080/00036841003742587

Farka, Z., Juřík, T., Kovář, D., Trnková, L., Skládal, P., 2017. Nanoparticle-Based Immunochemical Biosensors and Assays: Recent Advances and Challenges. Chem. Rev. https://doi.org/10.1021/acs.chemrev.7b00037

Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M., Mueller, N.D., O'Connell, C., Ray, D.K., West, P.C., Balzer, C., Bennett, E.M., Carpenter, S.R., Hill, J., Monfreda, C., Polasky, S., Rockström, J., Sheehan, J., Siebert, S., Tilman, D., Zaks, D.P.M., 2011. Solutions for a cultivated planet. Nature. https://doi.org/10.1038/nature10452

Food and Agriculture Organization, 2013. Food wastage footprint, Food and Agriculture Organization of the United Nations (FAO).

Francuski, L., Beukeboom, L.W., 2020. Insects in production – an introduction. Entomol. Exp. Appl. https://doi.org/10.1111/eea.12935

Gahukar, R.T., 2016. Edible Insects Farming: Efficiency and Impact on Family Livelihood, Food Security, and Environment Compared With Livestock and Crops, in: Insects as Sustainable Food Ingredients. https://doi.org/10.1016/b978-0-12-802856-8.00004-1

Gasco, L., Finke, M., van Huis, A., 2018. Can diets containing insects promote animal health? J. Insects as Food Feed. https://doi.org/10.3920/JIFF2018.x001

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: A review. Aquac. Res. https://doi.org/10.1111/j.1365-2109.2007.01704.x

Gerber, E.M., Hui, J., 2013. Crowdfunding: Motivations and deterrents for participation. ACM Trans. Comput. Interact. https://doi.org/10.1145/2530540

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients - A review of ingredient evaluation strategies for aquaculture feeds. Aquac. Nutr. https://doi.org/10.1111/j.1365-2095.2007.00450.x

Goerlich, O., Grohmann, L., Mazzara, M., Gatto, F., Zel, J., Lieske, K., Hougs, L., Scholtens, I.M.J., Papazova, N., Narendja, F., Ovesna, J., 2017. Verification of Analytical Methods for GMO Testing When Implementing Interlaboratory Validated Methods. Version 2. Test. Anal. GMO-containing Foods Feed. https://doi.org/10.1201/9781315178592-8

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. https://doi.org/10.1016/S0044-8486(03)00532-5

Hall, F., Johnson, P.E., Liceaga, A., 2018. Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (*Gryllodes sigillatus*) protein. Food Chem. https://doi.org/10.1016/j.foodchem.2018.04.058

Halloran, A., Roos, N., Eilenberg, J., Cerutti, A., Bruun, S., 2016. Life cycle assessment of edible insects for food protein: A review. Agron. Sustain. Dev. https://doi.org/10.1007/s13593-016-0392-8

Handbook of Surface Plasmon Resonance, 2008. , Handbook of Surface Plasmon Resonance. https://doi.org/10.1039/9781847558220

Haryati, R.P., Jayanegara, A., Laconi, E.B., Ridla, M., Suptijah, P., 2019. Evaluation of chitin and chitosan from insect as feed additives to mitigate ruminal methane emission, in: AIP Conference Proceedings. https://doi.org/10.1063/1.5115646

Henry, M.A., Gai, F., Enes, P., Peréz-Jiménez, A., Gasco, L., 2018a. Effect of partial dietary replacement of fishmeal by yellow mealworm (*Tenebrio molitor*) larvae meal on the innate immune response and intestinal antioxidant enzymes of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. https://doi.org/10.1016/j.fsi.2018.09.040

Henry, M.A., Gasco, L., Chatzifotis, S., Piccolo, G., 2018b. Does dietary insect meal affect the fish immune system? The case of mealworm, *Tenebrio molitor* on European sea bass, *Dicentrarchus labrax*. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2017.12.002

Herrero, M., Henderson, B., Havlík, P., Thornton, P.K., Conant, R.T., Smith, P., Wirsenius, S., Hristov, A.N., Gerber, P., Gill, M., Butterbach-Bahl, K., Valin, H., Garnett, T., Stehfest, E., 2016. Greenhouse gas mitigation potentials in the livestock sector. Nat. Clim. Chang. https://doi.org/10.1038/nclimate2925

Herrero, M., Stuckey, D.C., 2015. Bioaugmentation and its application in wastewater treatment: A review. Chemosphere. https://doi.org/10.1016/j.chemosphere.2014.10.033

Hoekstra, A.Y., Mekonnen, M.M., 2012. The water footprint of humanity. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.1109936109

Homola, J., Yee, S.S., Gauglitz, G., 1999. Surface plasmon resonance sensors: review. Sensors Actuators, B Chem. https://doi.org/10.1016/S0925-4005(98)00321-9

Hong, E., Lee, S.Y., Jeong, J.Y., Park, J.M., Kim, B.H., Kwon, K., Chun, H.S., 2017. Modern analytical methods for the detection of food fraud and adulteration by food category. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.8364

Hrnčírová, Z., Bergerová, E., Siekel, P., 2008. Effects of technological treatment on DNA degradation in selected food matrices of plant origin. J. Food Nutr. Res.

Hua, K., 2021. A meta-analysis of the effects of replacing fish meals with insect meals on growth performance of fish. Aquaculture. https://doi.org/10.1016/j.aquaculture.2020.735732

Huis, A., Iterbeeck, J., Klunder,H., Mertens, E., Halloran, A. Muir, G. and Vantomme, P., 2013. Edible insects Future prospects for food and feed security Edible insects Future prospects for food and feed security. FAO.

Jantzen da Silva Lucas, A., Menegon de Oliveira, L., da Rocha, M., Prentice, C., 2020. Edible insects: An alternative of nutritional, functional and bioactive compounds. Food Chem. 311, 126022. https://doi.org/10.1016/j.foodchem.2019.126022

Ji, H., Zhang, J.L., Huang, J.Q., Cheng, X.F., Liu, C., 2015. Effect of replacement of dietary fish meal with silkworm pupae meal on growth performance, body composition, intestinal protease activity and health status in juvenile Jian carp (*Cyprinus carpio var. Jian*). Aquac. Res. 46, 1209–1221. https://doi.org/10.1111/are.12276

Jongema, Y., 2017. World list of edible insects [WWW Document]. Wageningen Univ.

Jordan, J.A., Durso, M.B., 2005. Real-time polymerase chain reaction for detecting bacterial DNA directly from blood of neonates being evaluated for sepsis. J. Mol. Diagnostics. https://doi.org/10.1016/S1525-1578(10)60590-9

Józefiak, A., Nogales-Mérida, S., Mikołajczak, Z., Rawski, M., Kierończyk, B., Mazurkiewicz, J., 2019. The utilization of full-fat insect meal in rainbow trout (*Oncorhynchus mykiss*) nutrition: The effects on growth performance, intestinal microbiota and gastro-intestinal tract histomorphology. Ann. Anim. Sci. https://doi.org/10.2478/aoas-2019-0020

Kaewphinit, T., Santiwatanakul, S., Chansiri, K., 2012. Quartz crystal microbalance DNA based biosensor for diagnosis: A review. Sensors and Transducers.

Karlsen, Ø., Amlund, H., Berg, A., Olsen, R.E., 2017. The effect of dietary chitin on growth and nutrient digestibility in farmed Atlantic cod, Atlantic salmon and Atlantic halibut. Aquac. Res. https://doi.org/10.1111/are.12867

Kaushik, S.J., Covès, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture. https://doi.org/10.1016/S0044-8486(03)00422-8

Khalilpour, A., Kilic, T., Khalilpour, S., Álvarez, M.M., Yazdi, I.K., Liu, Y., Yang, D., Yu, T., Jiang, X., Tokel, O., Yildiz, U.H., Inci, F., Durmus, N.G., Ekiz, O.O., Turker, B., Cetin, C., Rao, S., Sridhar, K., Natarajan, N., Shafiee, H., Dana, A., Demirci, U., Samanta, D., Sarkar, A., 2015. Portable Microfluidic Integrated Plasmonic Platform for Pathogen Detection. Sci. Rep. 40, 475–491. https://doi.org/10.1039/c0cs00056f

Klausnitzer, B., 2011. Exkursionsfauna von Deutschland, Exkursionsfauna von Deutschland. https://doi.org/10.1007/978-3-8274-2452-5

Köppel, R., Schum, R., Habermacher, M., Sester, C., Piller, L.E., Meissner, S., Pietsch, K., 2019. Multiplex real-time PCR for the detection of insect DNA and determination of contents of *Tenebrio molitor*, *Locusta migratoria* and *Achaeta domestica* in food. Eur. Food Res. Technol. 245, 559–567. https://doi.org/10.1007/s00217-018-03225-5

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquac. Res. https://doi.org/10.1111/j.1365-2109.2009.02426.x

Kumar, S.P.J., Prasad, S.R., Banerjee, R., Agarwal, D.K., Kulkarni, K.S., Ramesh, K. V., 2017. Green solvents and technologies for oil extraction from oilseeds. Chem. Cent. J. https://doi.org/10.1186/s13065-017-0238-8

Kurniawan, D.R., Arief, M., Agustono, Lamid, M., 2018. Effect of maggot (Hermetia illucens) flour in commercial feed on protein retention, energy retention, protein content and fat content in tilapia (*Oreochromis niloticus*), in: IOP Conference Series: Earth and Environmental Science. https://doi.org/10.1088/1755-1315/137/1/012072

Lakshmipriya, T., Gopinath, S.C.B., 2018. An introduction to biosensors and biomolecules, in: Nanobiosensors for Biomolecular Targeting. https://doi.org/10.1016/B978-0-12-813900-4.00001-4

Laporte, J., Trushenski, J., 2012. Production performance, stress tolerance and intestinal integrity of sunshine bass fed increasing levels of soybean meal. J. Anim. Physiol. Anim. Nutr. (Berl). https://doi.org/10.1111/j.1439-0396.2011.01174.x

Latif, S., Anwar, F., 2011. Aqueous enzymatic sesame oil and protein extraction. Food Chem. https://doi.org/10.1016/j.foodchem.2010.09.064

Li, S., Ji, H., Zhang, B., Zhou, J., Yu, H., 2017. Defatted black soldier fly (*Hermetia illucens*) larvae meal in diets for juvenile Jian carp (*Cyprinus carpio var. Jian*): Growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. Aquaculture. https://doi.org/10.1016/j.aquaculture.2017.04.015

Li, Y., Kortner, T.M., Chikwati, E.M., Belghit, I., Lock, E.J., Krogdahl, Å., 2020. Total replacement of fish meal with black soldier fly (Hermetia illucens) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*). Aquaculture. https://doi.org/10.1016/j.aquaculture.2020.734967

Li, Y., Kortner, T.M., Chikwati, E.M., Munang'andu, H.M., Lock, E.J., Krogdahl, Å., 2019. Gut health and vaccination response in pre-smolt Atlantic salmon (Salmo salar) fed black soldier fly (*Hermetia illucens)* larvae meal. Fish Shellfish Immunol. https://doi.org/10.1016/j.fsi.2018.12.057

Liu, R., Li, Z., Huang, Z., Li, K., Lv, Y., 2019. Trends in Analytical Chemistry Biosensors for explosives : State of art and future trends 118. https://doi.org/10.1016/j.trac.2019.05.034

Lokman, I.H., Ibitoye, E.B., Hezmee, M.N.M., Goh, Y.M., Zuki, A.B.Z., Jimoh, A.A., 2019. Effects of chitin and chitosan from cricket and shrimp on growth and carcass performance of broiler chickens. Trop. Anim. Health Prod. https://doi.org/10.1007/s11250-019-01936-9

Magalhães, R., Sánchez-López, A., Leal, R.S., Martínez-Llorens, S., Oliva-Teles, A., Peres, H., 2017. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). Aquaculture. https://doi.org/10.1016/j.aquaculture.2017.04.021

Makkar, H.P.S., Tran, G., Heuzé, V., Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197, 1–33. https://doi.org/10.1016/j.anifeedsci.2014.07.008

Manzano, M., Cecchini, F., Fontanot, M., Iacumin, L., Comi, G., Melpignano, P., 2015. OLED-based DNA biochip for *Campylobacter spp.* detection in poultry meat samples. Biosens. Bioelectron. 66, 271–276. https://doi.org/10.1016/j.bios.2014.11.042

Marcello, A., Sblattero, D., Cioarec, C., Maiuri, P., Melpignano, P., 2013. A deep-blue OLED-based biochip for protein microarray fluorescence detection. Biosens. Bioelectron. https://doi.org/10.1016/j.bios.2013.02.017

Marien, A., Debode, F., Aerts, C., Ancion, C., Francis, F., Berben, G., 2018. Detection of *Hermetia illucens* by real-time PCR. J. Insects as Food Feed. https://doi.org/10.3920/JIFF2017.0069

Martín-Fernández, B., Manzanares-Palenzuela, C.L., Sánchez-Paniagua López, M., de-los-Santos-Álvarez, N., López-Ruiz, B., 2017. Electrochemical genosensors in food safety assessment. Crit. Rev. Food Sci. Nutr. https://doi.org/10.1080/10408398.2015.1067597

Masson, J.F., 2017. Surface Plasmon Resonance Clinical Biosensors for Medical Diagnostics. ACS Sensors. https://doi.org/10.1021/acssensors.6b00763

Mat Yusoff, M., Gordon, M.H., Niranjan, K., 2015. Aqueous enzyme assisted oil extraction from oilseeds andemulsion de-emulsifying methods: A review. Trends Food Sci. Technol. https://doi.org/10.1016/j.tifs.2014.09.003

Melpignano, P., Daniso, E., Vidergar, N., 2016. Multiparametric oled-based biosensor for rapid dengue serotype recognition with a new pointof-care serological test. Inf. MIDEM 46.

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M., Gasco, L., 2018. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.9127

Merrifield, D.L., Dimitroglou, A., Bradley, G., Baker, R.T.M., Davies, S.J., 2009. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, oncorhynchus mykiss (Walbaum). J. Fish Dis. https://doi.org/10.1111/j.1365-2761.2009.01052.x

Metkar, S.K., Girigoswami, K., 2019. Diagnostic biosensors in medicine – A review. Biocatal. Agric. Biotechnol. 17, 271–283. https://doi.org/10.1016/j.bcab.2018.11.029

Minunni, M., Tombelli, S., Mascini, M., Bilia, A.R., Bergonzi, M.C., Vincieri, F.F., 2005. An optical DNA-based biosensor for the analysis of bioactive constituents with application in drug and herbal drug screening. Talanta. https://doi.org/10.1016/j.talanta.2004.07.020

Montero, D., Izquierdo, M., 2010. Welfare and health of fish fed vegetable oils as alternative lipid sources to fish oil, in: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. https://doi.org/10.1201/9781439808634

Mottet, A., Tempio, G., 2017. Global poultry production: Current state and future outlook and challenges. Worlds. Poult. Sci. J. https://doi.org/10.1017/S0043933917000071

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. https://doi.org/10.1111/j.1365-2761.2011.01333.x

Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish, in: Feed and Feeding Practices in Aquaculture. https://doi.org/10.1016/b978-0-08-100506-4.00008-8

Oonincx, D.G.A.B., de Boer, I.J.M., 2012. Environmental Impact of the Production of Mealworms as a Protein Source for Humans - A Life Cycle Assessment. PLoS One. https://doi.org/10.1371/journal.pone.0051145

Parab, H.J., Jung, C., Lee, J.H., Park, H.G., 2010. A gold nanorod-based optical DNA biosensor for the diagnosis of pathogens. Biosens. Bioelectron. https://doi.org/10.1016/j.bios.2010.06.067

Payne, C.L.R., Scarborough, P., Rayner, M., Nonaka, K., 2016. Are edible insects more or less "healthy" than commonly consumed meats? A comparison using two nutrient profiling models developed to combat over- and undernutrition. Eur. J. Clin. Nutr. https://doi.org/10.1038/ejcn.2015.149

Pichyangkura, R., Chadchawan, S., 2015. Biostimulant activity of chitosan in horticulture. Sci. Hortic. (Amsterdam). https://doi.org/10.1016/j.scienta.2015.09.031

Preece, K.E., Hooshyar, N., Zuidam, N.J., 2017. Whole soybean protein extraction processes: A review. Innov. Food Sci. Emerg. Technol. https://doi.org/10.1016/j.ifset.2017.07.024

Premalatha, M., Abbasi, Tasneem, Abbasi, Tabassum, Abbasi, S.A., 2011. Energy-efficient food production to reduce global warming and ecodegradation: The use of edible insects. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2011.07.115

Qiu, G., Gai, Z., Tao, Y., Schmitt, J., Kullak-Ublick, G.A., Wang, J., 2020. Dual-Functional Plasmonic Photothermal Biosensors for Highly Accurate Severe Acute Respiratory Syndrome Coronavirus 2 Detection. ACS Nano. https://doi.org/10.1021/acsnano.0c02439

Ramos-Elorduy, J., 2006. Threatened edible insects in Hidalgo, Mexico and some measures to preserve them. J. Ethnobiol. Ethnomed. https://doi.org/10.1186/1746-4269-2-51

Ramos-Elorduy, J., Moreno, J.M.P., Prado, E.E., Perez, M.A., Otero, J.L., De Guevara, O.L., 1997. Nutritional value of edible insects from the state of Oaxaca, Mexico. J. Food Compos. Anal. https://doi.org/10.1006/jfca.1997.0530

Rashid, J.I.A., Yusof, N.A., 2017. The strategies of DNA immobilization and hybridization detection mechanism in the construction of electrochemical DNA sensor: A review. Sens. Bio-Sensing Res. 16, 19–31. https://doi.org/10.1016/j.sbsr.2017.09.001

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D., Fernández-Palacios, H., 1995. Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. Aquaculture. https://doi.org/10.1016/0044-8486(94)00225-D

Rutten, M., Achterbosch, T.J., de Boer, I.J.M., Cuaresma, J.C., Geleijnse, J.M., Havlík, P., Heckelei, T., Ingram, J., Leip, A., Marette, S., van Meijl, H., Soler, L.G., Swinnen, J., van't Veer, P., Vervoort, J., Zimmermann, A., Zimmermann, K.L., Zurek, M., 2018. Metrics, models and foresight for European sustainable food and nutrition security: The vision of the SUSFANS project. Agric. Syst. https://doi.org/10.1016/j.agsy.2016.10.014

Sahlmann, C., Sutherland, B.J.G., Kortner, T.M., Koop, B.F., Krogdahl, Å., Bakke, A.M., 2013. Early response of gene expression in the distal intestine of Atlantic salmon (*Salmo salar L.*) during the development of soybean meal induced enteritis. Fish Shellfish Immunol. https://doi.org/10.1016/j.fsi.2012.11.031

Saidur, M.R., Aziz, A.R.A., Basirun, W.J., 2017. Recent advances in DNA-based electrochemical biosensors for heavy metal ion detection: A review. Biosens. Bioelectron. https://doi.org/10.1016/j.bios.2016.11.039

Samanta, D., Sarkar, A., 2011. Immobilization of bio-macromolecules on self-assembled monolayers: Methods and sensor applications. Chem. Soc. Rev. https://doi.org/10.1039/c0cs00056f

Santigosa, E., García-Meilán, I., Valentín, J.M., Navarro, I., Pérez-Sánchez, J., Gallardo, M.Á., 2011. Plant oils' inclusion in high fish meal-substituted diets: Effect on digestion and nutrient absorption in gilthead sea bream (*Sparus aurata L.*). Aquac. Res. https://doi.org/10.1111/j.1365-2109.2010.02679.x

Sassolas, A., Leca-Bouvier, B.D., Blum, L.J., 2008. DNA biosensors and microarrays. Chem. Rev. https://doi.org/10.1021/cr0684467

Shah, N.J., 2019. Polymerase chain reaction, in: Introduction to Basics of Pharmacology and Toxicology: Volume 1: General and Molecular Pharmacology: Principles of Drug Action. https://doi.org/10.1007/978-981-32-9779-1_31

Sogari, G., Amato, M., Biasato, I., Chiesa, S., Gasco, L., 2019. The potential role of insects as feed: A multi-perspective review. Animals. https://doi.org/10.3390/ani9040119

Sosa, D.A.T., Fogliano, V., 2017. Potential of Insect-Derived Ingredients for Food Applications, in: Insect Physiology and Ecology. https://doi.org/10.5772/67318

St-Hilaire, S., Sheppard, C., Tomberlin, J.K., Irving, S., Newton, L., McGuire, M.A., Mosley, E.E., Hardy, R.W., Sealey, W., 2007. Fly prepupae as a feedstuff for rainbow trout, Oncorhynchus mykiss. J. World Aquac. Soc. https://doi.org/10.1111/j.1749-7345.2006.00073.x

Steinfeld, H., Wassenaar, T., Jutzi, S., 2006. Livestock production systems in developing countries: Status, drivers, trends. OIE Rev. Sci. Tech. https://doi.org/10.20506/rst.25.2.1677

Su, J., Gong, Y., Cao, S., Lu, F., Han, D., Liu, H., Jin, J., Yang, Y., Zhu, X., Xie, S., 2017. Effects of dietary *Tenebrio molitor* meal on the growth performance, immune response and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*). Fish Shellfish Immunol. https://doi.org/10.1016/j.fsi.2017.08.008

Tacchi, L., Secombes, C.J., Bickerdike, R., Adler, M.A., Venegas, C., Takle, H., Martin, S.A.M., 2012. Transcriptomic and physiological responses to fishmeal substitution with plant proteins in formulated feed in farmed Atlantic salmon (*Salmo salar*). BMC Genomics. https://doi.org/10.1186/1471-2164-13-363

Tacon, A.G.J., Metian, M., 2015. Feed matters: Satisfying the feed demand of aquaculture. Rev. Fish. Sci. Aquac. https://doi.org/10.1080/23308249.2014.987209

The European Parliament and the Council of the European Union., 1997. REGULATION (EC) No 258/97 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 January 1991 concerning novel foods and novel food ingredients. Off. J. Eur. Union L 43, 1–6.

The historical spread of the Black Soldier Fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada, 2015. . J. Entomol. Soc. Ontario.

Thermo Fisher Scientific, 2011. Application note: Using Taqman ® endogenous Control Assays to select an endogenous control for experimental studies. White Pap.

Thévenot, D.R., Toth, K., Durst, R.A., Wilson, G.S., 2001. Electrochemical biosensors: Recommended definitions and classification. Biosens. Bioelectron. https://doi.org/10.1016/S0956-5663(01)00115-4

Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M., Luzzana, U., Harpaz, S., 2006. Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). Aquaculture. https://doi.org/10.1016/j.aquaculture.2006.06.026

Tomberlin, J.K., van Huis, A., 2020. Black soldier fly from pest to "crown jewel" of the insects as feed industry: An historical perspective. J. Insects as Food Feed. https://doi.org/10.3920/JIFF2020.0003

Tripathi, N., Choppala, G., Singh, R.S., 2017. Evaluation of modified chitosan for remediation of zinc contaminated soils. J. Geochemical Explor. https://doi.org/10.1016/j.gexplo.2016.08.011

Tzompa-Sosa, D.A., Yi, L., van Valenberg, H.J.F., Lakemond, C.M.M., 2019. Four insect oils as food ingredient: Physical and chemical characterisation of insect oils obtained by an aqueous oil extraction. J. Insects as Food Feed 5, 279–292. https://doi.org/10.3920/JIFF2018.0020

Ue, R., Parlamento, D.E.L., Del, E.E., 2015. Regolamento (UE) 2015/2283 del Parlamento Europeo e del Consiglio del 25 novembre 2015 relativo ai nuovi alimento e che modifica il regolamento (UE) n. 1169/2011 del Parlamento europeo e del Consiglio e abroga il regolamento (CE) n. 258/97 del Parlamento . Gazz. Uff. dell'Unione Eur. 2015, 2003–2005. https://doi.org/10.2903/j.efsa.2007.555.

Valentin, R.E., Maslo, B., Lockwood, J.L., Pote, J., Fonseca, D.M., 2016. Real-time PCR assay to detect brown marmorated stink bug, Halyomorpha halys (Stål), in environmental DNA. Pest Manag. Sci. https://doi.org/10.1002/ps.4217

van der Fels-Klerx, H.J., Camenzuli, L., Belluco, S., Meijer, N., Ricci, A., 2018. Food Safety Issues Related to Uses of Insects for Feeds and Foods. Compr. Rev. Food Sci. Food Saf. https://doi.org/10.1111/1541-4337.12385

Van Huis, A., 2016. Edible insects are the future?, in: Proceedings of the Nutrition Society. https://doi.org/10.1017/S0029665116000069

van Huis, A., Oonincx, D.G.A.B., 2017. The environmental sustainability of insects as food and feed. A review. Agron. Sustain. Dev. 37. https://doi.org/10.1007/s13593-017-0452-8

van Huis, A., Tomberlin, J.K., 2017. Insects as food and feed: from production to consumption (free online e-module). Insects as food Feed from Prod. to Consum. (free online e-module).

Vargas-Chacoff, L., Regish, A.M., Weinstock, A., McCormick, S.D., 2018. Effects of elevated temperature on osmoregulation and stress responses in Atlantic salmon *Salmo salar* smolts in fresh water and seawater. J. Fish Biol. https://doi.org/10.1111/jfb.13683

Veldkamp, T., van Duinkerken, G., van Huis, A., Ottevanger, E., Bosch, G., van Boekel, T., 2012. Insects as a sustainable feed ingredient in pig and poultry diets : a feasibility study = Insecten als duurzame diervoedergrondstof in varkens- en pluimveevoeders : een haalbaarheidsstudie. Food Chem. 50, 192–195.

Voorhees, J.M., Barnes, M.E., Chipps, S.R., Brown, M.L., 2019. Bioprocessed soybean meal replacement of fish meal in rainbow trout (*Oncorhynchus mykiss*) diets. Cogent Food Agric. https://doi.org/10.1080/23311932.2019.1579482

Waagbø, R., Berntssen, M.H.G., Danielsen, T., Helberg, H., Kleppa, A.L., Berg Lea, T., Rosenlund, G., Tvenning, L., Susort, S., Vikeså, V., Breck, O., 2013. Feeding Atlantic salmon diets with plant ingredients during the seawater phase - a full-scale net production of marine protein with focus on biological performance, welfare, product quality and safety. Aquac. Nutr.

https://doi.org/10.1111/anu.12010

Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., Huang, Y., 2019. Evaluation of defatted black soldier fly (Hermetia illucens L.) larvae meal as an alternative protein ingredient for juvenile Japanese seabass (*Lateolabrax japonicus*) diets. Aquaculture. https://doi.org/10.1016/j.aquaculture.2019.04.023

Yamashita, M., Yabu, T., Ojima, N., 2010. Stress Protein HSP70 in Fish. Aqua-BioScience Monogr. https://doi.org/10.5047/absm.2010.00304.0111

Zagon, J., di Rienzo, V., Potkura, J., Lampen, A., Braeuning, A., 2018. A real-time PCR method for the detection of black soldier fly (*Hermetia illucens*) in feedstuff. Food Control 91, 440–448. https://doi.org/10.1016/j.foodcont.2018.04.032

Zarantoniello, M., Bruni, L., Randazzo, B., Vargas, A., Gioacchini, G., Truzzi, C., Annibaldi, A., Riolo, P., Parisi, G., Cardinaletti, G., Tulli, F., Olivotto, I., 2018. Partial Dietary Inclusion of *Hermetia illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications. Zebrafish. https://doi.org/10.1089/zeb.2018.1596

Zhan, L., Granade, T., Liu, Y., Wei, X., Youngpairoj, A., Sullivan, V., Johnson, J., Bischof, J., 2020. Development and optimization of thermal contrast ampli fi cation lateral fl ow immunoassays for ultrasensitive HIV p24 protein detection. Microsystems Nanoeng. https://doi.org/10.1038/s41378-020-0168-9