THE QUALITY OF NEW SUSTAINABLE ALTERNATIVE FOOD SOURCES: EDIBLE INSECTS AND TOMATOES IRRIGATED WITH WASTEWATER

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Doctoral Dissertation
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KAKOVOST NOVIH TRAJNOSTNIH ALTERNATIVNIH VIROV HRANE: UŽITNE ŽUŽELKE IN PARADIŽNIKI NAMAKANI Z ODPADNO VODO

Doktorska disertacija

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To my family

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Abstract

Globally, there is increasing awareness of the need for sustainable food systems, driven by projected population growth, rising demand for protein, and tightening constraints on land and water resources. Despite high agricultural productivity, the European Union remains highly dependent on imports of protein-rich feed, with approximately 66% of high-protein feed used in livestock production sourced externally. Simultaneously, water scarcity is emerging as a critical issue in southern Europe. Even in a relatively water-rich, Alpine-influenced country such as Slovenia, regional and seasonal water deficits are increasingly common due to increased drought frequency and variability in water availability, which can threaten agricultural productivity. Both of these challenges, although distinct, have driven interest in circular food production systems that reduce waste, optimise resource use, and close resource loops.

Addressing those challenges, this doctoral thesis investigates two sustainable food sources: farmed edible insects and tomatoes irrigated with treated wastewater (TWW), with a focus on their quality. The research covers four main areas: (i) nutritional characterisation of edible insects, (ii) the impact of fermentation on insect quality, (iii) the effects of TWW and the presence of contaminants of emerging concern (CECs) on tomato quality in hydroponic and soil cultivation systems, and (iv) the application of explainable machine learning for identifying the compounds that contribute most to the differentiation of tomatoes across treatments.

This thesis's findings confirmed that farmed edible insects (*Schistocerca gregaria, Tenebrio molitor, Acheta domesticus, Locusta migratoria, Gryllus assimilis, Alphitobius diaperinus, and Blaberus discoidalis*) are a rich source of essential fatty acids, amino acids, and minerals, with species, developmental stage, and sex significantly influencing fatty acid and polyphenol composition, while amino acid profiles remained consistent between sexes. Fermentation effects were found to be species- and culture-dependent and generally less beneficial than expected, with limited impact on proximate and fatty acid composition but resulting in reductions in essential amino acids (e.g., lysine, methionine, threonine) and oxidation stability (*Tenebrio molitor* and *Locusta migratoria*).

The impact of CECs on tomato quality was influenced by their uptake, the cultivation system, and the growth media. In a hydroponic system, higher concentrations of bisphenol S, 2,4-bisphenol F, and naproxen correlated with increased levels of Mn, Cu, and Zn, suggesting modifications in elemental composition. In contrast, lower uptake in soil-grown tomatoes led to minimal differences among TWW treatments, although TWW irrigation resulted in higher B and Na concentrations than potable water. In a hydroponic system, CEC exposure increased yield without affecting sugars, organic acids, carotenoids, polyphenols, or fatty acids, but it significantly decreased specific amino acids and altered volatile organic compound profiles, likely due to stress, endocrine-disrupting or other toxic effects.

The application of an explainable machine learning approach successfully differentiated between tomatoes grown across five treatments (HPW, HSPW, LPW, LWW, and LSWW), achieving high classification accuracy (75–88%) despite a limited sample size. Furthermore,

SHAP (SHapley Additive exPlanations) analysis identified key metabolites and elements at both global and local levels and provided insight into feature contributions, including misclassifications.

Overall, this thesis demonstrates that farmed edible insects and tomatoes irrigated with treated wastewater are promising sustainable food sources. The results contribute to the optimisation of insect rearing and processing and reveal the impact of biological factors and fermentation on their composition and quality. The work is also informative for producers, stakeholders, consumers and regulators regarding the potential effects of TWW reuse on product quality. Finally, it demonstrates the value of explainable machine learning as an effective approach for analysing complex datasets and underscores its broader potential in food and agricultural research.

Povzetek

Na svetovni ravni se vse bolj zavedamo nujnosti trajnostnih prehranskih sistemov, kar je posledica predvidene rasti prebivalstva, naraščajoče potrebe po beljakovinah ter vse večjih omejitev zemljišč in vodnih virov. Kljub visoki kmetijski produktivnosti Evropska unija ostaja odvisna od uvoza beljakovinsko bogate krme, saj 66 % visoko beljakovinske krme, uporabljene v živinoreji, uvozimo iz tujine. Hkrati postaja pomanjkanje vode vse resnejši problem, tudi v južni Evropi. Celo v relativno vodnati, alpsko obarvani državi, kot je Slovenija, so regionalni in sezonski primanjkljaji vode vse pogostejši zaradi povečane pogostosti suš in spremenljivosti razpoložljivosti vode, kar lahko ogrozi kmetijsko produktivnost. Ti izzivi spodbujajo zanimanje za razvoj krožnih sistemov proizvodnje hrane, ki zmanjšujejo količino odpadkov, optimizirajo rabo virov in zapirajo snovne tokove.

Kot odgovor na te izzive se doktorska disertacija osredotoča na dva trajnostna vira hrane: gojene užitne žuželke in paradižnik, namakan s prečiščeno odpadno vodo (angl. Treated WasteWater, TWW), s poudarkom na njuni kakovosti. Raziskava zajema štiri ključna področja: (i) prehransko karakterizacijo užitnih žuželk, (ii) vpliv fermentacije na kakovost žuželk, (iii) vpliv TWW in onesnaževal, ki vzbujajo zaskrbljenost (angl. Contaminants of Emerging Concern, CECs), na kakovost paradižnika v hidroponskih in talnih sistemih ter (iv) uporabo razložljivega strojnega učenja za identifikacijo spojin, ki najbolj vplivajo na ločbo paradižnika iz različnih načinov gojenja.

Izsledki te disertacije so pokazali, da so gojene užitne žuželke (*Schistocerca gregaria, Tenebrio molitor, Acheta domesticus, Locusta migratoria, Gryllus assimilis, Alphitobius diaperinus, and Blaberus discoidalis*) bogat vir esencialnih maščobnih kislin, aminokislin in mineralov. Vrsta, razvojna stopnja in spol so pomembno vplivali na sestavo maščobnih kislin in polifenolov, medtem ko se profili aminokislin med spoloma niso bistveno razlikovali. Učinki fermentacije so bili odvisni od vrste in uporabljene mikrobne kulture ter so bili na splošno manj ugodni od pričakovanj, saj so imeli omejen vpliv na osnovno kemijsko sestavo in maščobne kisline ter so zmanjšali vsebnosti esencialnih aminokislin (lizin, metionin, treonin) in oksidacijsko stabilnost (*Tenebrio molitor* and *Locusta migratoria*).

Vpliv CECs na kakovost paradižnika je bil odvisen od njihovega vnosa, sistema gojenja in substrata. V hidroponskih sistemih so bile višje koncentracije bisfenola S, 2,4-bisfenola F in naproksena v paradižniku povezane s povečano vsebnostjo Mn, Cu in Zn, kar kaže na spremembe v elementni sestavi. Nasprotno je bil vnos teh spojin pri paradižnikih, gojenih v tleh, nižji, zato med obdelavami s TWW niso bile ugotovljene pomembne razlike, čeprav je namakanje s TWW v paradižnikih povečalo vsebnost B in Na v primerjavi s pitno vodo. Izpostavljenost paradižnikov onesnaževalom v hidroponiki je povečala pridelek, ne da bi vplivala na vsebnost sladkorjev, organskih kislin, karotenoidov, polifenolov ali maščobnih kislin, vendar je zmanjšala vsebnost nekaterih aminokislin in spremenila profile hlapnih organskih spojin, verjetno zaradi stresnih, endokrinih ali drugih strupenostnih učinkov.

Pristop razložljivega strojnega učenja je omogočil uspešno razlikovanje paradižnikov med petimi obdelavami (HPW, HSPW, LPW, LWW, and LSWW) in dosegel visoko natančnost razvrščanja (75–88 %) kljub omejenemu številu vzorcev. Analiza SHAP

(Shapleyeve aditivne razlage, angl. SHapley Additive exPlanations) je identificirala ključne metabolite in elemente na globalni in lokalni ravni ter omogočila boljše razumevanje vpliva posameznih značilnosti, vključno z napačnimi razvrstitvami.

Disertacija dokazuje, da so gojene užitne žuželke in paradižnik, namakan s prečiščeno odpadno vodo, obetavni trajnostni viri hrane. Rezultati prispevajo k optimizaciji reje in predelave žuželk ter razkrivajo vpliv bioloških dejavnikov in fermentacije na njihovo sestavo in kakovost. Ta študija je zanimiva tudi za pridelovalce, druge deležnike, potrošnike in regulativne organe glede morebitnih vplivov ponovne uporabe TWW na kakovost izdelkov. Hkrati prikazuje, da je razložljivo strojno učenje učinkovito orodje za analizo kompleksnih podatkov in poudarja njegov velik potencial za nadaljnje raziskave na področju prehrane in kmetijstva.

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Abbreviations

ANOVA	...	Analysis of Variance
AOAC	...	Association of Official Agricultural Chemists
ARSO	...	Slovenian Environment Agency
BDE-47	...	2,2',4,4'-tetrabromodiphenyl ether
BW	...	Body Weight
CAPP	...	Cold Atmospheric Pressure Plasma
CB	...	Cubist Regression
CEC	...	Contaminant of Emerging Concern
DM	...	Dry Matter
EC	...	European Commission
EFSA	...	European Food Safety Authority
EU	...	European Union
FCR	...	Feed Conversion Ratio
GAE	...	Gallic Acid Equivalents
GC-FID	...	Gas Chromatography-Flame Ionisation Detector
GC-MS	...	Gas Chromatography-Mass Spectrometry
GDPR	...	General Data Protection Regulation
HHP	...	High Hydrostatic Pressure
HPLC-DAD	...	High-Performance Liquid Chromatography with Diode Array Detection
HPLC-DAD-MS/MS	...	High-Performance Liquid Chromatography with Diode Array Detection and Tandem Mass Spectrometry
HPLC-RI	...	High-Performance Liquid Chromatography with a Refractive Index Detector
HPLC-UV	...	High-Performance Liquid Chromatography with Ultraviolet-Visible detection
HS-SPME-GC-MS	...	Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry
INPROFF	...	Quality, Safety, and Authenticity of Insect Protein-based Food and Feed
IPIFF	...	International Platform of Insects for Food and Feed
LC-MS	...	Liquid Chromatography-Mass Spectrometry
LightGBM	...	Light Gradient-Boosting Machine
LIME	...	Local Interpretable Model-agnostic Explanations
MLR	...	Multiple Linear Regression
MUFA	...	Monounsaturated Fatty Acid
NN	...	Neural Network
OH/CNS pyrolizer/EA-TCD	...	Oxygen Hydrogen/ Carbon Nitrogen Sulphur Pyrolizer/Elemental Analyser with Thermal Conductivity Detector

PAH	...	Polycyclic Aromatic Hydrocarbon
PCA	...	Principal Component Analysis
PCB	...	Polychlorinated Biphenyls
PBDEs	...	Polybrominated diphenyl ether
PEF	...	Pulsed Electric Field
PFAS	...	Per- and polyfluoroalkyl substance
PLS	...	Partial Least Squares Regression
PTE	...	Potentially Toxic Element
PUFA	...	Polyunsaturated Fatty Acid
RF	...	Random Forest
SFA	...	Saturated Fatty Acid
SHAP	...	SHapley Additive exPlanations
SVR	...	Support Vector Machine Regression
TSS	...	Total Suspended Solids
TWW	...	Treated Wastewater
UNESCO	...	United Nations Educational, Scientific, and Cultural Organisation
US	...	Ultrasound
VOC	...	Volatile Organic Compound
WWTP	...	Wastewater Treatment Plant
XAI	...	Explainable Artificial Intelligence
XGBoost	...	eXtreme Gradient Boosting

Chapter 1

Introduction

The global population is expected to continue growing over the next fifty to sixty years, reaching a peak of around 10.3 billion people in the mid-2080s, up from 8.3 billion in 2024 (United Nations Department of Economic and Social Affairs, Population Division, 2024). With population growth comes the challenge of ensuring everyone has access to sufficient, safe, and nutritious food. In its latest report, "State of Food Security and Nutrition in the World 2025", the FAO estimates that between 638 and 720 million people, corresponding to 7.8 and 8.8% of the global population, respectively, faced hunger in 2024, indicating a slight decrease compared to 2022 (FAO et al., 2025). About 2.3 billion people worldwide are estimated to have been moderately or severely food-insecure in 2024. It is projected that 512 million people may be chronically undernourished in 2030, depending on conflicts, climate extremes, economic slowdowns and downturns, and growing inequality (FAO et al., 2025), which underscores the need for quality, alternative, sustainable food sources.

In Europe, the situation is different and is more about food security, since, despite high overall agricultural productivity, the European Union remains strongly dependent on imports of protein-rich feed commodities. Approximately 66% of high-protein feed used in the EU livestock sector is imported, with soybean meal representing the most critical dependency, as up to 96% of it is imported from outside Europe (European Commission, 2024). Overall, the EU compensates for a structural plant-protein deficit through imports amounting to approximately 19 million tonnes of crude protein annually (European Commission, 2024). In addition to soybean products, imports of feed grains and protein-rich oilseed meals are essential to sustaining European animal production systems. This reliance on external resources creates economic vulnerability, environmental impacts, and strategic concerns, thereby driving interest in alternative protein sources produced within circular and regional food systems (European Commission, 2024).

At the same time, water scarcity is a critical concern in an increasing number of regions, including the south and south-western USA, north-eastern Brazil, central Argentina, Northern and Eastern Africa, the Middle East, the Caucasus, northern India, northern China, and southern Europe (García-Ruiz et al., 2011; Huggins et al., 2022). Due to climate change scenarios, severe water imbalances are anticipated to intensify worldwide, potentially causing substantial infrastructure damage and economic impacts (Schewe et al., 2019). Extreme weather events such as extended heatwaves and floods all around the world are occurring at increased frequency and severity, causing further disturbances to the hydrological cycle, and emphasising that urgent actions need to be undertaken (Mueller & Gasteyer, 2021; Naddaf, 2023; Rahman et al., 2023; Schumacher et al., 2019). It is estimated that by 2030, 1.6 billion people will lack access to safely managed drinking water, and 2.8 billion people will lack access to safely managed sanitation (The Sustainable Development Goals Report 2022, 2022).

In response to these challenges, circular food production approaches are seen as a way to reduce waste, optimise resource use, and promote sustainability by closing the loop within food systems (Cities and Circular Economy for Food, 2019). These approaches focus on reducing environmental impact by reusing organic materials, minimising waste, and improving resource efficiency. Key principles include waste valorisation, in which agricultural residues, forestry residues, horticultural waste, animal waste, industrial food waste, and urban and municipal food waste are transformed into valuable inputs, such as animal feed, biofuels, biopolymers, fertilisers, bioactive compounds, and enzymes (Pant et al., 2026). In addition, the reuse of treated wastewater (TWW) in agriculture is a key element that allows for the recycling of wastewater nutrients and supports crop irrigation, thereby conserving freshwater resources and helping manage water scarcity (Jaramillo & Restrepo, 2017). By reducing dependence on external inputs and enhancing resource recovery across the food chain, circular approaches can contribute to more sustainable and resilient food systems, while also supporting food security, lowering greenhouse gas emissions, and strengthening environmental resilience (Rizwan et al., 2025).

However, an important yet still insufficiently explored dimension of sustainable food production concerns the effects of such alternative production systems on food quality. While existing research has largely focused on their environmental and resource-efficiency benefits, considerably less attention has been paid to how these systems influence the nutritional and chemical composition of food products, despite the fact that these characteristics are essential for consumer acceptance, food safety, and long-term sustainability.

To help address this gap, the present PhD thesis focuses on two complementary circular food production strategies: farmed edible insects and the reuse of treated wastewater in tomato cultivation. In the context of Europe's reliance on imported high-protein plant feed, increasing water scarcity, and the need to promote circular food production, these two systems were selected as case studies through which nutritional and chemical food quality could be examined. The primary aim of this doctoral research was to analyse and gain insights into the characteristics of farmed insects and the effects of processing, specifically fermentation (a traditional technique to improve the nutritional, shelf-life, and sensory properties of food), on their quality. For tomatoes, the research focused on understanding how exposure to TWW and potentially to contaminants of emerging concern (CECs) influences product quality. Such a multidisciplinary approach results in large and heterogeneous datasets that require appropriate analytical handling. Accordingly, this PhD thesis applies novel machine learning-based methods for data processing and analysis.

1.1 Insect Farming

According to recent studies, approximately 2,205 insect species are consumed worldwide (Omuse et al., 2024). Among continents, Asia hosts the highest diversity of edible insect species, followed by North America (mainly Mexico) and Africa (Omuse et al., 2024). The majority of edible species, however, are harvested from the wild, with only 8% of edible insect species having been successfully farmed (Yen, 2015). Despite this, edible insects can serve both as human food and as animal feed, with animal feed being far more widespread.

According to Van Huis (2024), the global insect-for-feed market is projected to expand from USD 5 billion in 2022 to USD 97 billion by 2031, while the insect-for-food market is expected to grow from USD 654 million in 2023 to USD 1,093 million by 2033 (Figure 1.1). In 2022–2023, insects as feed accounted for approximately 88% of the total market,

compared to 12% for food. By 2031–2033, it is projected that feed's proportion of the market will increase to 99%, leaving food at 1%.

In Europe, production forecasts (Figure 1.1) indicate strong sectoral growth: insect-based food output is expected to rise from 803 tonnes in 2023 to 43,044 tonnes by 2030, while feed production is projected to increase from 21,619 tonnes to 870,370 tonnes (IPIFF, 2023a, 2023b). While in 2023, 4% of production was allocated to food and 96% to feed, by 2030, the shares are forecast to shift marginally to 5% and 95%, respectively, suggesting that insects for food remain a niche segment relative to the rapidly expanding feed market.

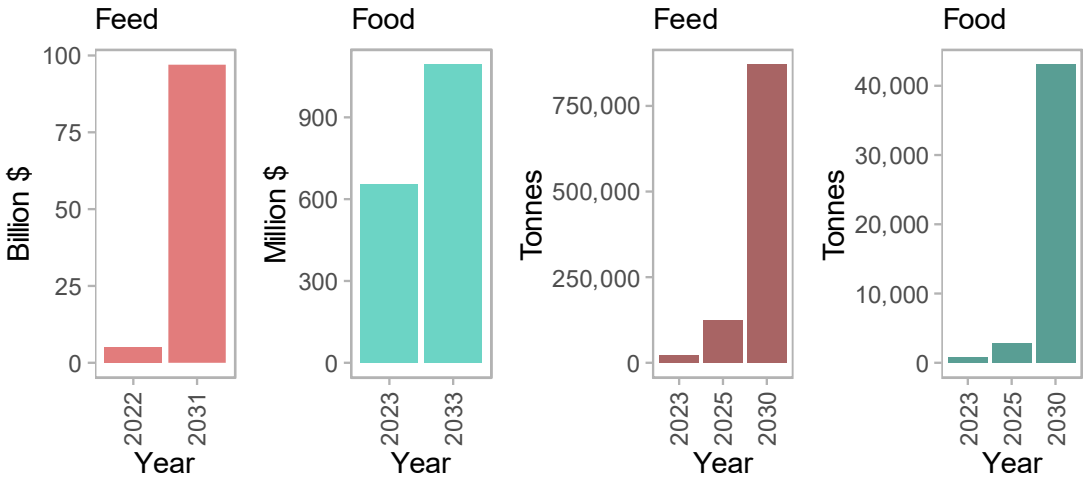


Figure 1.1: Global insect market development forecast for feed and food ($) (Van Huis, 2024) and expected production of insect-based feed and food in Europe (tonnes) (IPIFF, 2023a, 2023b).

The insect farming market in Slovenia is still in its early stages. Currently, two companies are involved in insect farming: Mokar, Tilen Zajec s.p., which breeds yellow mealworms and produces organic fertilisers from their frass, and Čričkoteka, which produces house crickets for food consumption. Additionally, ŽIPO Lenart d.o.o. focuses on breeding yellow mealworms for research purposes, utilising agricultural by-products from their farm.

1.1.1 EU regulations

The potential of insect farming has been recognised by the European Commission, which has approved several insect species as novel foods and feed. So far, four insect species have been authorised for marketing as novel foods, including:

- frozen, dried, powder and UV-treated powder forms of yellow mealworm larvae – *Tenebrio molitor* (*Commission Regulation (EU) 2022/169*, 2022; *Commission Regulation (EU) 2025/89*, 2025),
- frozen, dried, powdered, and defatted powdered forms of house cricket – *Acheta domesticus* (*Commission Regulation (EU) 2022/188*, 2022, *Commission Regulation (EU) 2023/5*, 2023),
- frozen, dried, and powder forms of migratory locust – *Locusta migratoria* (*Commission Regulation (EU) 2021/1975*, 2021),
- frozen, paste, dried, and powdered forms of lesser mealworm larvae – *Alphitobius diaperinus* (*Commission Regulation (EU) 2023/58*, 2023).

Commission Regulations (EU) 2017/893 (2017) and *(EU) 2021/1925* (2021) authorised eight insect species for the production of processed animal proteins intended as feed for

farmed animals (other than fur animals). These are tropical house cricket (*Gryllodes sigillatus*), Jamaican field cricket (*Gryllus assimilis*), house cricket (*Acheta domesticus*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), black soldier fly (*Hermetia illucens*), house fly (*Musca domestica*), and silkworm (*Bombyx mori*).

Insect farmers and insect product producers must comply with many regulations, among others, regulations regarding general principles and standards governing food and feed safety (*Regulation (EC) 178/2002*, 2002); hygiene rules (*Regulation (EC) 852/2004*, 2004); animal health (*Regulation (EU) 2016/429*, 2016); proper labelling with regard to allergen information, nutrition labelling, and country of origin labelling (*Regulation (EU) 1169/2011*, 2011); importation (*Regulation (EU) 2021/404*, 2021); and use of animal by-products and derived products not intended for human consumption (*Regulation (EU) 142/2011*, 2011).

Strict regulations also exist regarding animal feed, including placing feed on the market concerning safety, labelling, and traceability requirements (*Regulation (EC) 767/2009*, 2009) and a catalogue of authorised feed materials, which includes insects and their products, that can be used in animal feed formulations (*Commission Regulation (EU) 68/2013*, 2013). Moreover, in the EU, insect protein is classified as processed animal protein, and its use, production, and labelling are strictly controlled by specific rules (*Commission Regulation (EU) 2017/893*, 2017).

A detailed review of all EU regulations is published by the International Platform of Insects for Food and Feed (IPIFF) in the Guide on Good Hygiene Practices (IPIFF Guide on Good Hygiene and Practices, 2024) and is also available on their website (IPIFF, 2025).

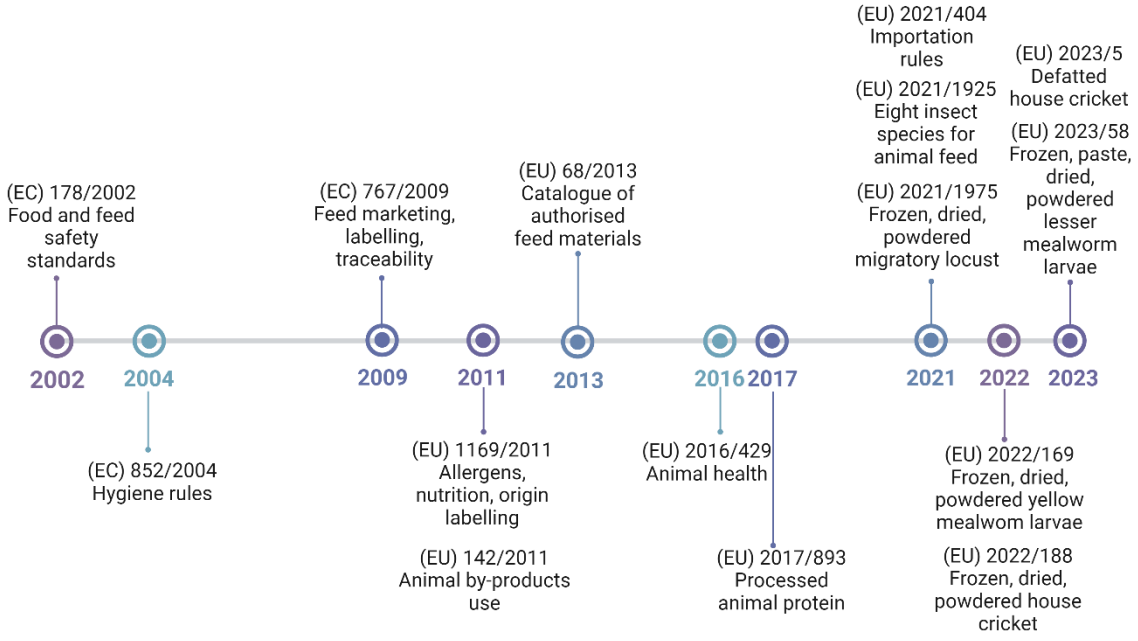


Figure 1.2: Timeline of EU Regulations for insects as food and feed.

1.1.2 Green perspective of farming insects

Insect farming is increasingly recognised for its environmental benefits, particularly its relatively high feed conversion efficiency. The feed conversion ratio (FCR) of insects is strongly dependent on the nutritional quality and composition of the rearing substrate. Under optimised feeding conditions, insects generally convert plant-derived nutrients into biomass more efficiently than most conventional livestock. Insects can achieve efficiencies

comparable to those of poultry (2.5) and pork production systems (5, Table 1.1). However, when substrates do not adequately meet species-specific nutritional requirements, such as when unbalanced agricultural by-products are used, FCR values can increase substantially.

For example, Oonincx et al. (2015) showed that yellow mealworms fed a balanced control diet (commercial diet) exhibited FCR values comparable to or lower than those reported for pork (FCR=5). In contrast, diets characterised by low protein content combined with either high or low-fat levels resulted in markedly higher FCR values (FCR=10.9-19.1), comparable to or exceeding those associated with beef production (FCR=10). These results demonstrate that substrate formulation is a key determinant of production efficiency in insect farming and that optimising feed composition is essential for achieving favourable conversion efficiency and environmental performance. The edible portion of insects is also relatively high, reaching up to 80% for house crickets, and 100% for yellow mealworm, house fly, and black soldier fly larvae, whereas pigs and chickens provide approximately 55%, and cattle yield only about 40% of their body mass as consumable meat (Nakagaki et al., 1987).

Although comparisons of water consumption must be interpreted with caution due to substantial methodological variability across assessment approaches, insect farming generally requires less freshwater than conventional livestock production systems (Table 1.1). Reported values depend strongly on system boundaries, allocation methods, and assumptions about feed production and processing (Smetana et al., 2015). Nevertheless, several studies indicate that certain insect species, particularly black soldier fly larvae (285-478 m^3/edible tonne) and house crickets (420-3,300 m^3/edible tonne), require considerably less water than traditional livestock (4,325-15,415 m^3/edible tonne) (Bava et al., 2019; Halloran et al., 2017; Mekkonnen & Hoekstra, 2010; Tapanen, 2018). Yellow mealworms (4,341 m^3/edible tonne) have a comparable water demand to that of chicken meat production (4,325 m^3/edible tonne), while house fly larvae (9,072 m^3/edible tonne) may even exhibit higher water consumption than chicken and pork systems (4,325-5,988 m^3/edible tonne) under specific production scenarios (Mekkonnen & Hoekstra, 2010; Miglietta et al., 2015; Van Zanten et al., 2015). Compared to fishmeal (0.161-1.57 m^3/edible tonne), insects require more water, while compared to soybean (1,800-2,500 m^3/edible tonne), only black soldier fly larvae (285-478 m^3/edible tonne) require less water (Barrera et al., 2026; Bava et al., 2019; Fréon et al., 2017). These findings highlight both the potential water-use advantages of insect farming and the importance of standardised assessment methodologies when comparing environmental footprints across protein sources.

Insect rearing is also not restricted to prime agricultural land and can be integrated into vertical production systems, resulting in substantially lower land use compared with conventional livestock production. Land requirements for farmed insects range from approximately 0.01 to 18 m^2 per kg of edible protein (Table 1.1), which is considerably lower than values reported for chicken (42–52 m^2/kg edible protein), pork (47–64 m^2/kg edible protein), and beef (144–258 m^2/kg edible protein). Land use associated with insect production can be comparable to or higher than that of certain feed ingredients such as fishmeal (0.0 m^2/kg edible protein) and soybean meal (3.4–8.7 m^2/kg edible protein) (Bosch et al., 2019; De Vries & De Boer, 2010; Halloran et al., 2016; Oonincx & De Boer, 2012; Roffeis et al., 2015; Salomone et al., 2017; Smetana et al., 2015, 2016; Thévenot et al., 2018; Van Zanten et al., 2015; Williams et al., 2006).

Land requirements, however, are strongly influenced by the type of feed substrate used for insect rearing. Van Zanten et al. (2015) demonstrated that the highest land demand was associated with black soldier fly larvae reared on feed materials suitable for direct human consumption (67 m^2/kg edible protein), followed by co-products typically used in livestock feeding (3 m^2/kg edible protein), whereas substrates not suitable either as food or feed resulted in negligible land use requirements (0.0 m^2/kg edible protein). These

findings emphasise that the environmental performance of insect production systems depends not only on the species reared but also on feed sourcing and resource-allocation strategies.

Energy use in insect production includes energy required for resource production and processing, as well as operational inputs such as heating, climate control, and insect separation, being strongly influenced by the type and quality of the rearing substrate. Bosch et al. (2019) showed that energy demand is highest when black soldier fly larvae are reared on substrates suitable for human consumption, intermediate when fed co-products or former foodstuffs used in animal feed, and lowest when organic residual streams such as food waste or manure are utilised. In contrast, Roffeis et al. (2015) reported relatively high energy requirements for house fly larvae reared on pig manure (209–345 MJ/kg edible protein), largely driven by climate control, particularly heating and air conditioning, while drying and illumination contributed only marginally to total energy demand.

Due to substantial variability among studies (Table 1.1), direct comparisons across insect production systems remain difficult. Overall, energy use for insect-derived protein (yellow mealworm: 80–173 MJ/kg edible protein; house fly: 19.5–345 MJ/kg edible protein; black soldier fly: 15.1–174 MJ/kg edible protein) generally exceeds that of soybean meal (4.1–9 MJ/kg edible protein), whereas comparisons with fishmeal (44 MJ/kg edible protein), chicken (80–152 MJ/kg edible protein), pork (95–236 MJ/kg edible protein), and beef (177–273 MJ/kg edible protein) vary widely, reflecting strong dependence on substrate selection and production conditions (Bosch et al., 2019; De Vries & De Boer, 2010; Oonincx & De Boer, 2012; Roffeis et al., 2015; Salomone et al., 2017; Smetana et al., 2015, p. 201, 2016; Van Zanten et al., 2015; Williams et al., 2006).

Consequently, greenhouse gas emissions associated with edible insect production (Table 1.1) exhibit substantial variability across species, feeding substrates, and production conditions. At the lower end, emissions reported for house fly (0.77 kg CO_2-eq/kg edible protein), black soldier fly (1.36–3 kg CO_2-eq/kg edible protein) and house cricket (3.87 CO_2-eq/kg edible protein) are comparable to those of fishmeal (2.8 kg CO_2-eq/kg edible protein) and soybean meal (1.1–1.7 kg CO_2-eq/kg edible protein) (Bosch et al., 2019; Halloran et al., 2017; Salomone et al., 2017; Smetana et al., 2016; Van Zanten et al., 2015). In contrast, higher emission estimates, particularly for black soldier fly (15.1-19 kg CO_2-eq/kg edible protein) and house cricket production (15.1-49 kg CO_2-eq/kg edible protein), approach values observed for chicken (18–36 kg CO_2-eq/kg edible protein) and pork (21–53 kg CO_2-eq/kg edible protein), while generally remaining lower than those associated with beef production (75–170 kg CO_2-eq/kg edible protein) (Bosch et al., 2019; De Vries & De Boer, 2010; Salomone et al., 2017; Smetana et al., 2016; Tapanen, 2018; Williams et al., 2006).

Table 1.1: Feed conversion, edible portion, and environmental footprint of farmed insect species (yellow mealworm larvae, house fly larvae, black soldier fly larvae, house cricket) in comparison with conventional protein sources, including fishmeal, soybean meal, and livestock meat (chicken, pork, and beef).

	Feed conversion rate (kg/kg BW)	Edible portion (%)	Water consumption (m^3/edible tonne)	Land requirements (m^2/kg edible protein)	Energy use (MJ/kg edible protein)	Global warming potential (kg CO_2-eq/kg edible protein)
Yellow mealworm larvae	2.2-19.1[e,u]	100[e]	4,341[a]	3.75-18[c,e,o]	80-173[e,o]	7.1-14[e,o]
House fly larvae	4[d]	100[j]	9,072[d]	0.032-5.32[d,i,j,k]	19.5-345[d,i]	0.77[d]
Black soldier fly larvae	1.4-4.22[r,u]	100[k]	285-478[r]	0.01-67[j,k,l]	15.1-174[k,j,l]	1.36-19[k,j,l]
House cricket	1.08-10.5[f,u]	80[n]	420-3,300[p,q]	2.63-3.97[f]		3.87-49[p,q]
Fishmeal			0.161-1.57[t]	0.0[j]	44[j]	2.8[j]
Soybean meal			1,800-2,500[s]	3.4-8.7[j,k]	4.1-9[j,k]	1.1-1.7[j,k]
Chicken	2.5[m]	55[n]	4,325[b]	42-52[g,h]	80-152[g,h]	18-36[g,h]
Pork	5[m]	55[n]	5,988[b]	47-64[g,h]	95-236[g,h]	21-53[g,h]
Beef	10[m]	40[n]	15,415[b]	144-258[g,h]	177-273[g,h]	75-170[g,h]

Sources: [a]Miglietta et al., 2015; [b]Mekkonnen & Hoekstra, 2010; [c]Thévenot et al., 2018; [d]Van Zanten et al., 2015; [e]Oonincx & De Boer, 2012; [f]Halloran et al., 2016; [g]De Vries & De Boer, 2010; [h]Williams et al., 2006; [i]Roffeis et al., 2015; [j]Bosch et al., 2019; [k]Salomone et al., 2017; [l]Smetana et al., 2016; [m]Smil, 2002; [n]Nakagaki et al., 1987; [o]Smetana et al., 2015; [p]Halloran et al., 2017; [q]Tapanen, 2018; [r]Bava et al., 2019; [s]Barrera et al., 2026; [t]Fréon et al., 2017; [u]Oonincx et al., 2015

Moreover, insect farming is essentially waste-free, since leftover substrate and frass can be used as a fertiliser. Due to its nutrient and microbial profile, this leftover product has the potential to be utilised as a biofertiliser for high-value crop production. Studies using the frass of various insects (e.g., black soldier flies, houseflies, and mealworms) have shown results comparable to or better than those of inorganic fertilisers (Antoniadis et al., 2023; Chavez & Uchanski, 2021).

Feeding edible insects on agricultural by-products and food waste represents one of the most sustainable and circular strategies for alternative protein production, further minimising their already low environmental footprint (Heath et al., 2024). This approach offers substantial environmental and economic advantages but is also accompanied by several technical and regulatory challenges (Van Huis & Oonincx, 2017).

Insects such as the black soldier fly, mealworms, and crickets can efficiently convert agricultural residues and food waste into high-quality protein, thereby reducing the amount of biodegradable waste sent to landfills and lowering associated methane and CO_2 emissions (Barbi et al., 2020; Bava et al., 2019; Kuo & Fisher, 2022; Oonincx et al., 2015). Moreover, this practice supports zero-waste principles and contributes to the development of circular food systems that transform waste into valuable resources (Smetana et al., 2016). Moreover, due to their flexibility, insect farms can be located near farms or food processing facilities, reducing transportation costs and emissions while strengthening local food systems (Bava et al., 2019).

Rearing insects on agricultural by-products and food waste also presents risks. Such substrates may contain pesticide residues, heavy metals, or microbial contaminants that can accumulate in insects and pose potential health hazards to animals and humans (EFSA Scientific Committee, 2015). The European Union strictly regulates the types of waste that may be used for insect rearing, prohibiting the use of manure, catering waste, or meat-containing by-products (*Commission Regulation (EU) 2017/893*, 2017). Additionally, nutrient variability in agricultural residues requires diet balancing or supplementation to ensure optimal insect growth (Bava et al., 2019; Kuo & Fisher, 2022; Oonincx et al., 2015; Smetana et al., 2016).

Overall, insect farming could represent a sustainable, resource-efficient alternative to conventional livestock, offering high feed conversion efficiency, low emissions, and reduced land and water use. The use of agricultural by-products and food waste supports a circular economy and produces valuable organic fertiliser.

1.1.3 Nutritional value of farmed edible insects

Edible insects are a nutrient-dense and sustainable food source with significant potential to address global nutritional and food security challenges (Gautam et al., 2025). Their nutritional value can vary based on several factors, including their diet, developmental stage, sex, species, rearing environment, and processing methods (Meyer-Rochow et al., 2021). There is a broad consensus among researchers that insects are rich in protein, fat, vitamins, and other essential nutrients, offering a unique combination of macronutrients, micronutrients, and bioactive compounds, making them a viable alternative to conventional animal- and plant-based food sources (Gautam et al., 2025; Rumpold & Schlüter, 2013b).

1.1.3.1 Protein

Protein is a key macronutrient in edible insects, with concentrations ranging from 40% to 75% on a dry-matter (DM) basis (Table 1.2). Depending on the species, the protein content of insects can be comparable or even outperform other sources such as chicken (54.7%

DM), eggs (52.7% DM), beef (40.5 % DM), pork (27.7% DM), and soybean (35.8 % DM) (Ghosh et al., 2017; Rumpold & Schlüter, 2015). The quality of protein is determined by both the digestibility of the protein and the amino acid composition. Digestibility stands for the amount of protein available for absorption after passing through the upper part of the gastrointestinal tract (Lampová et al., 2024).

Despite variations in digestion methodologies, the digestion of edible insects is considered high, ranging from 80% to 90% (Rodríguez-Rodríguez et al., 2022). Moreover, insects are rich in amino acids, provide all essential amino acids, and generally fulfil the World Health Organisation's (WHO) requirements for essential amino acids (Gautam et al., 2025; Zhou et al., 2022). The content of essential amino acids represents 37-63% of the total amino acids (Table 1.3).

1.1.3.2 Lipids

Edible insects contain 6.2 to 43.1% of fat in dry matter, depending on the species and rearing conditions (Table 1.2). Fat content is usually higher in the larval and pupal stages than in adults (Gautam et al., 2025). Insect fat is present in several forms. Triacylglycerols represent around 80% of fat. They serve as an energy reserve during periods when insects need high energy, such as during long flights. Another large group is phospholipids, which play a role in the structure of cell membranes and are usually below 20% (Kouřimská & Adámková, 2016).

In general, edible insects have high content of C18 fatty acids, including stearic, oleic, and linoleic acid, and relatively high levels of palmitic acid (Table 1.4). Some fatty acid contents are species-specific; for example, black soldier fly contains very high levels of lauric acid, while migratory locust contains high levels of α-linolenic acid. Also, their saturated fatty acid (SFA), monounsaturated (MUFA), and polyunsaturated fatty acid (PUFA) contents depend on the species, while they, in general, contain a high proportion of PUFA, particularly omega-6 and omega-3 (Kouřimská & Adámková, 2016; Zhou et al., 2022). These fatty acids reduce inflammation, improve heart and brain function, and lower disease risk through their antioxidant and lipid-regulating effects (Djuricic & Calder, 2021).

Table 1.2: Proximate composition of farmed edible insects.

	Tropical house cricket	Jamaican field cricket	House cricket	Migratory locust	Yellow mealworm	Lesser mealworm	Black soldier fly	House fly	Silkworm
Protein (%)	51.5-70.0	55.6-71.0	62.41–72.0	50.8-75.3	46.4-60.3	44.2-64.8	41.1-56.1	42.3-62.5	51.6-70.6
Fat (%)	7.44-19.1	7.00-34.0	9.80-29.6	6.41-34.9	26.8-43.1	22.2-26.9	12.8-34.8	9.00-26.0	6.20-37.1
Fibre (%)	3.36-10.3	7.00-8.28	10.2-10.4		4.58-15.0		7	1.60-8.60	2.50-6.4
Carbohydrates (%)	0.1-14.9	8.60-12.5	9.77	6.72-15.5	7.10-13.4	19.5			25.4
Ash (%)	4.43-15.4	4.08-6.40	3.60–9.10	2.42-40.0	1.00-4.50	3.14-4.10	12.6-28.4	5.60-17.3	2.59-10.6
Energy (kcal/100g)	338.8-452.0	397.0-537.5	454.88-455.19	377.44-568.8	539.6-652.5		528.2	480.4-583.2	389.6-616.6
Source	Magara et al., 2025; Zielińska et al., 2015	Magara et al., 2021	Bosch et al., 2014; Brogan et al., 2021; Finke, 2002; Kittibunchakul et al., 2023; Magara et al., 2021	Brogan et al., 2021; Clarkson et al., 2018b; Kleden et al., 2023; Oonincx & Van Der Poel, 2011	Bosch et al., 2014; Finke, 2002; Kröncke et al., 2019; Makkar et al., 2014; Ravzanaadii et al., 2012	Bosch et al., 2014; Purkiewicz et al., 2025	Bosch et al., 2014; Makkar et al., 2014	Bosch et al., 2014; Makkar et al., 2014	Brogan et al., 2021; Finke, 2002; Makkar et al., 2014

Table 1.3: Amino acids (g/100 g DM) in farmed edible insects.

	Tropical house cricket	Jamaican field cricket	House cricket	Migratory locust	Yellow mealworm	Lesser mealworm	Black soldier fly	House fly	Silkworm moth
Alanine	4.06	2.92-4.02	5.74-5.89	7.56	1.71-4.04	2.25	2.45-2.55	3.42	2.43-2.46
Arginine	3.26	3.20-8.64	3.63-4.06	3.84	1.13-2.55	1.76-3.11	1.78-2.19	2.63-2.87	2.25-2.76
Aspartic acid	5.10	2.87-3.02	5.52-5.66	4.74	1.67-3.99		4.09-4.14	5.02	3.53-5.22
Cystine	0.77	0.73-0.74	0.15-0.55	0.47	0.24-0.42			0.46	0.46-0.72
Glutamic acid	7.46	3.64-4.30	6.48-7.09	6.2	2.64-5.54	65.54	4.17-4.42	7.27	4.99-5.38
Glycine	2.85	2.41-2.63	3.36-3.50	3.94	1.12-2.73	0.73	1.72-1.80	2.49	2.35-3.24
Histidine	1.20	1.23-1.32	1.52-2.40	1.56	0.71-2.65	3.18	0.76-2.47	2.12-3.00	1.39-1.69
Isoleucine	1.86	1.52-2.12	2.52-3.05	2.92	1.65-2.47	2.98	1.83-2.24	2.21-2.50	1.73-2.35
Leucine	4.05	3.04-4.96	4.62-6.66	5.04	1.58-5.22	3.08-4.34	2.66-3.42	3.53-3.81	2.83-3.7
Lysine	2.69	2.06-7.91	3.57-4.09	3.64	1.35-2.86	4.21-4.96	2.05-3.03	3.87-3.88	2.54-3.68
Methionine	1.11	0.63-1.03	0.82-1.13	0.9	0.31-0.73	0.84	0.77-0.89	1.48-1.63	0.75-1.49
Phenylalanine	1.54	0.72-1.93	1.69-2.34	2.03	0.82-1.77	2.53	1.72-1.83	3.09-3.25	1.56-2.68
Proline	3.79	1.26-2.54	3.54-3.73	4.31	1.40-3.41	0.86		2.24	1.85-1.96
Serine	2.83	0.61-1.95	2.87-3.36	2.22	0.97-2.52	1.36	1.37-1.42	2.31	1.97-1.98
Threonine	2.58	1.76-3.55	2.34-2.54	2.33	0.84-2.02	2.59	1.58-2.02	2.37-2.38	1.68-2.27
Tryptophan		0.95	0.20-0.68	0.52	0.39	4.30		0.85	0.4-0.9
Tyrosine	2.23	3.29-5.44	3.18-3.70	3.63	1.61-3.60	4.25	2.22-2.57	3.47	1.68-3.19
Valine	3.29	2.26-4.62	3.47-4.02	4.18	1.13-3.28	3.31-3.82	2.97-3.09	3.13-2.90	2.13-2.2
Total EAA	21.32	14.82-26.78	21.56-24.22	23.12	8.39-19.58	24.49	14.47-21.15	22.42-23.56	15.09-21.77
Total AA	51.30	39.24-56.56	57.86-60.65	60.91	20.88-48.37		32.78-33.56	51.97	37.86-46.61
Source	Zielińska et al., 2015	Bednářová et al., 2013; Oliveira et al., 2024	Bosch et al., 2014; Brogan et al., 2021; Finke, 2002; Kittibunchakul et al., 2023	Brogan et al., 2021	Bosch et al., 2014; Finke, 2002; Ravzanaadii et al., 2012	Bosch et al., 2014; Purkiewicz et al., 2025	Bosch et al., 2014; Sealey et al., 2011	Bosch et al., 2014; Zuidhof et al., 2003	Brogan et al., 2021; Finke, 2002

Table 1.4: Fatty acids (%) in farmed insects.

	Tropical house cricket	Jamaican field cricket	House cricket	Migratory locust	Yellow mealworm	Lesser mealworm	Black soldier fly	House fly	Silkworm moth
Lauric acid C12:0	0.01-0.25	0.12	0.10			0.04-0.34	23.6-37.1		
Myristic acid 14:0	1.22-3.96	1.28	0.44-0.63	1.67-2.69	2.20-7.86	0.56-1.57	5.1-6.3	5.5	2.4
Palmitic acid 16:0	15.73-37.06	25.85	22.65-24.68	27.3-28.44	16.72-21.89	25.34-26.68	17.3-19.8	31.1	13.5
Palmitoleic acid 16:1	3.78	1.92	0.34-1.42	1.17	1.42-2.81	0.27-1.06	6.3-7.6	13.4	0.79
Stearic acid 18:0	7.35-18.48	14.07	8.54-9.18	7.23-8.27	2.49-4.27	8.89-10.57	2.0-6.5	3.4	9.5
Oleic acid 18:1	5.97-60.32	25.03	20.18-24.37	31.60-37.02	32.93-45.88	31.23-31.41	18.8-22.7	24.8	25.4
Linoleic acid 18:2	9.23-30.11	26.13	36.23-41.39	8.94-16.96	16.63-37.66	20.27-29.1	5.9-6.8	19.8	27.8
α-linolenic acid 18:3	0.46-2.13		0.95-1.11	10.47-15.64	0.21-1.66	0.38-1.9	0.0-0.5	2	11.1
SFA	21.88-65.49	43.72	32.22-36.08	37.22-39.21	21.5-32.06	35.74-39.16	55-62.7	40	32.5
MUFA	5.97-60.32	27.49	21.31-25.95	33.02-38.49	34.50-50.37	32.98-33.21	29-26.4	38.2	27.8
PUFA	9.69-31.91	28.8	37.18-42.63	24.57-27.77	17.33-39.32	21.21-31.00	6.9-12.5	21.8	38.9
Source	Magara et al., 2025; Zielińska et al., 2015	Mlček et al., 2018	Finke, 2002; Paul et al., 2017	Clarkson et al., 2018b; Perez-Santaescolastica et al., 2023	Dreassi et al., 2017; Finke, 2002; Kröncke et al., 2019; Ravzanaadii et al., 2012	Mattioli et al., 2024; Purkiewicz et al., 2025	Sealey et al., 2011	Makkar et al., 2014	Finke, 2002

1.1.3.3 Minerals and vitamins

Insects are also a significant source of many minerals, such as iron, zinc, potassium, sodium, calcium, phosphorus, magnesium, manganese, selenium, and copper (Kouřimská & Adámková, 2016; Rumpold & Schlüter, 2013a; C. Tang, 2019), often surpassing the levels found in conventional meat (Gautam et al., 2025). Especially iron and zinc may be of considerable nutritional importance (Mwangi et al., 2018). Mineral composition and content vary considerably, depending on the species, the environment in which the insect is grown, and the composition of its diet (Zhou et al., 2022). Insects have already demonstrated potential as an effective dietary source of essential minerals for animals, and their inclusion in the human diet could be promoted as a strategy to combat mineral deficiencies (Gautam et al., 2025; Köhler et al., 2019).

Vitamins A, D2, D3, C, E, K, thiamine, riboflavin, pantothenic acid, niacin, pyridoxine, folic acid, D-biotin, and B12 are also found in insects (Finke, 2013), which are typically rich in riboflavin, pantothenic acid, and biotin. Insects of the order Orthoptera (grasshoppers, crickets, locusts) and Coleoptera (beetles) are also rich in folic acid. On the other hand, they are not an efficient source of vitamin A, C, niacin, and, in most cases, thiamine (Rumpold & Schlüter, 2013a). Whereas the content of vitamins and minerals in wild edible insects is seasonal in the case of farmed species, it can be controlled via feed (Kouřimská & Adámková, 2016).

1.1.3.4 Chitin

Insects contain a significant amount of fibre, ranging from 0.12% to 29%, depending on the species. The content of nitrogen-free extract, which represents carbohydrates other than fibre, ranges from 4% to 23% (Rumpold & Schlüter, 2013a). Carbohydrates in insects are mainly found in the exoskeleton and glycogen in the body (Kim et al., 2019). Around 90% of the external skeleton of insects is composed of a structural polysaccharide called chitin, which is mainly composed of the polymer N-acetyl-D-glucosamine, and is also the main source of cellulose and chitosan (Qian et al., 2022). The chitin content of edible insects varies by species and developmental stage, typically ranging from 5% to 15% (Gautam et al., 2025). Large amounts of chitin can be used to produce new substances such as bioactive chitosan (Qian et al., 2022). Interest in chitin and chitosan stems from their wide range of biological and technological properties, including mucoadhesive, anti-inflammatory, antioxidant, antimicrobial, antifungal, antihyperglycemic, antitumor, and wound-healing activities (Aranaz et al., 2021).

1.1.3.5 Polyphenols

Although polyphenols are typically associated with plants, insects also contain these compounds, which exhibit antioxidant, anti-inflammatory, anticancer, antimicrobial, and antibacterial effects, as well as their roles in enzyme activity and metabolic regulation (David-Birman et al., 2018; Escobar-Ortiz et al., 2022; Hayakawa et al., 2022; Issaoui et al., 2020; Niedzwiecki et al., 2016; Sadat et al., 2022; Sajadimajd et al., 2020). Insects use polyphenols as pigments and chemical defences and can synthesise or store non-dietary phenolic compounds through sclerotisation (Nino et al., 2021). They can also absorb and accumulate flavonoids from their host plants during the larval stage, making diet a key factor influencing the type and concentration of polyphenols present (Aiello et al., 2023). To date, 225 polyphenols have been identified across various insect species, although research on their composition and bioactive properties remains ongoing (Aiello et al., 2023).

Total phenolic content ranges from 260 to 5,000 mg gallic acid equivalents (GAE) per 100 g of extract, depending on the species and method used (Table 1.5).

Table 1.5: Total phenolic content (mg GAE/100 g) in farmed insect species.

Insect species	Total phenolic content (mg GAE/100 g)	Source
Tropical house cricket	515.76	Zielińska et al., 2021
House cricket	300-5,000	Navarro del Hierro et al., 2020; Nino et al., 2021
Yellow mealworm	260-3,545	Navarro del Hierro et al., 2020; Pyo et al., 2020; Zielińska et al., 2021

1.1.4 Risks associated with eating insects

Eating insects could pose certain risks that must be considered. In 2015, EFSA assessed the risk profile of insect production and consumption, concluding that it is comparable to other conventional protein sources (e.g., meat, poultry, and fish), provided insects are reared under controlled conditions and fed with safe, authorised feed (EFSA Scientific Committee, 2015).

Insects are known to cause allergic reactions such as eczema, conjunctivitis, angioedema, and bronchial asthma. The most common allergic reactions can be caused by insect bites or stings, by inhalation or contact (Van Huis, 2013). The most common insect allergens are glycoproteins, for example, tropomyosin, arginine kinase, triose phosphate isomerase, tubulin, and glyceraldehyde 3-phosphate dehydrogenase (Srinroch et al., 2015; Verhoeckx et al., 2014). Chitin and its derivative, chitosan, are not directly allergenic but may exhibit immunomodulatory properties depending on the route of administration and particle size. These properties could influence allergic reactions to other allergens (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2010; Lee et al., 2008).

Biological risk is an important factor in assuring the safety and shelf-life stability of edible insects. Insects can carry pathogens, with contamination levels varying by species, collection method (wild or farmed), and processing hygiene practices (Rumpold & Schlüter, 2013a). Pathogenic bacteria and fungi, which typically reside in the insect (farmed and wild) gut or on the surface, include *Alternaria*, *Aspergillus*, *Bacillus*, *Campylobacter*, *Candida*, *Clostridium*, *Enterococcus*, *Enterobacteriaceae*, *Micrococcus*, *Neisseria*, *Penicillium*, *Pseudomonas*, *Salmonella*, *Shigella, Staphylococcus*, *Streptococcus and Vibrio* (Kim et al., 2019; Lange & Nakamura, 2021). Effective control measures and hygiene practices must be implemented throughout the edible insect food chain, including production, processing, preservation, and handling, to eliminate microbial infection risks (Lange & Nakamura, 2021). Additionally, proper fasting, thermal treatment, and storage conditions are essential for decontaminating insects and extending their shelf life (Kouřimská & Adámková, 2016).

Insects may also face risks from viruses, parasites, and prions. Viruses infecting insects (such as Iridoviridae, Parvoviridae, Iflaviridae, Dicistroviridae, and Reoviridae) pose no direct risk to humans or vertebrate animals, but they can affect insect farming operations (EFSA Scientific Committee, 2015). A potential issue is the presence of parasites, which can be pathogenic for insects only (*Nosema* spp, *Gregarine* spp., *Nyctotherus* spp., *Steinernema* spp., *Gordiidae*, *H. diesigni*, Thelastomidae, and *Thelastoma* spp.) or parasitic for animals and potentially pathogenic for humans (*Cryptosporidium* spp., *Isospora* spp., *Balantidium* spp., *Entamoeba* spp., Cestoda, *Pharyngodon* spp., *Physaloptera* spp.,

Spiruroidea, Acanthocephal, Pentastomida)(Gałęcki & Sokół, 2019). In response, Maciel-Vergara et al. (2021) proposed a list of general recommendations for hygiene and good practices to be followed. Additionally, prions may pose a risk if introduced through contaminated feed, so edible insects intended for food and feed should be provided with non-human, non-ruminant substrates to minimise these risks (EFSA Scientific Committee, 2015).

Concentrations of potentially toxic elements in insects depend on the characteristics of the elements, their concentrations in the substrate, and the insect species and developmental stage (EFSA Scientific Committee, 2015). Bioaccumulation of potentially toxic elements (As, Cd, Pb, Hg) has already been reported in numerous studies (Meyer et al., 2021, and references therein). For this reason, it is essential to prevent pollution by using uncontaminated feed and by controlling insect production.

Insects can also contain antinutrients, substances that can inhibit the intake, digestion, absorption, and utilisation of macro- and micronutrients and may produce adverse health effects in consumers (Lange & Nakamura, 2021). The main anti-nutrient compounds detected in edible insects are oxalates, phytic acid, cyanogenic glycosides, and saponins (Sánchez-Estrada et al., 2024).

Organic contaminants represent a significant area of concern in edible insect safety, with ongoing research focusing on biotransfer, bioaccumulation, and biotransformation processes (Labu et al., 2022; Poma et al., 2017, 2019, 2021). Studies show that contaminant uptake varies across species, developmental stages, substrates, and rearing conditions, underscoring the need for comprehensive monitoring and risk assessment (Meyer et al., 2021). The organic contaminants include toxins produced by or accumulated in insects, veterinary drugs and hormones, pesticide residues, polycyclic aromatic hydrocarbons, dioxins, and dioxin-like polychlorinated biphenyls and packaging migration contaminants (Amorello et al., 2024; EFSA Scientific Committee, 2015). Their presence in insects can be controlled by regulating contaminant levels in the substrate (Meyer et al., 2021).

1.1.5 Insect processing and its impact on the quality of edible insect products

Insect processing is crucial for enhancing safety, shelf life, sensory attributes, and consumer acceptability. The first step, pre-processing, involves harvesting, separating insects from substrate residuals, inactivating/killing insects, removing wings/legs, and washing (Rumpold & Schlüter, 2013b). Harvesting can be manual or automatic, including sieving (Ojha et al., 2021). A starvation period before harvesting helps empty insect intestines, improving taste, cleanliness, and reducing microbial load (Garofalo et al., 2019; Wynants et al., 2017).

If not sold as live, insects are killed by freezing, drowning in hot or boiling water, or steaming (Ojha et al., 2021), which can affect product quality, including microbial load, proximate composition, colour, and taste (Adámková et al., 2017; Farina, 2017; Larouche et al., 2019). Freeze-killing is considered the most humane technique (Adámková et al., 2017; Pali-Schöll et al., 2019).

1.1.5.1 Processing of whole insects

Insect pre-processing alone is insufficient to ensure safety; further processing is required to produce a microbiologically stable, safe food product. Processing methods vary depending on whether insects are used whole or powdered. For whole insects, decontamination methods include blanching, boiling, steaming, marinating, smoking, toasting, frying, and their combinations (Melgar-Lalanne et al., 2019; Ojha et al., 2021). These insects can then

be marketed chilled, frozen, or dried (Ojha et al., 2021). Drying, the most common method for extending shelf life, includes techniques such as fluidised-bed drying, freeze-drying, hot-air oven-drying, microwave drying, smoke drying, roasting, sun-drying, and vacuum drying (Hernández-Álvarez et al., 2021). Each method impacts the chemical, microbiological, and sensory properties differently.

Thermal treatments like boiling, toasting, and blanching are most effective for microbial reduction, while drying is less effective unless combined with heat (Nyangena et al., 2020; Ojha et al., 2021). High temperatures can also affect nutritional composition, reducing protein solubility, vitamin B12 levels, and mineral bioaccessibility (Egan et al., 2014; Kinyuru et al., 2010; Lenaerts et al., 2018; Madibela et al., 2007; Manditsera et al., 2019; Womeni et al., 2012). Blanching inactivates browning enzymes but may cause non-enzymatic browning (Azzollini et al., 2016; Tonneijck-Srpová et al., 2019), while toasting and roasting result in a crunchy texture and meat-like aroma (Melgar-Lalanne et al., 2019).

Marination, a non-thermal treatment, is still under-explored but could improve shelf life and sensory properties. Borremans et al. (2018) found that marinating yellow mealworm larvae in red wine or soy sauce for 6 days inhibited microbial growth, extending shelf life by at least 7 days. However, marination may promote bacterial endospore germination, posing challenges for longer storage. Further research is needed on the nutritional and sensory benefits of marinated insect products.

1.1.5.1.1 Drying techniques

Drying is a critical process for extending the shelf life of edible insects by reducing water content, thereby limiting enzymatic and microbial degradation (Melgar-Lalanne et al., 2019). This method also helps mitigate rancidity, improve colour, and enhance texture (Hernández-Álvarez et al., 2021). Sun-drying, often used at the household level due to its low energy consumption, has limited antimicrobial efficacy unless combined with heat treatment (Azzollini et al., 2016; Nyangena et al., 2020). It may also lead to increased peroxide values, oxidative degradation (Tiencheu et al., 2013), and a reduction in vitamin content and protein digestibility (Kinyuru et al., 2010). Inconsistent drying results, driven by the reliance on appearance alone, can also lead to contamination risks through inadequate hygiene during processing and contact with soil and air (Melgar-Lalanne et al., 2019).

Smoking, a traditional preservation technique, enhances lipid stability and extends shelf life due to dehydration and the antimicrobial and antioxidant properties of smoke constituents such as formaldehyde, carboxylic acids, and phenolics (Hernández-Álvarez et al., 2021; Tiencheu et al., 2013). It also imparts a desirable smoky aroma, which is generally well accepted by consumers (Mmari et al., 2017). However, the presence of polycyclic aromatic hydrocarbons, potential carcinogens, may pose health risks if their concentrations exceed regulatory limits (Alexander et al., 2010).

Freeze-drying uses sublimation under reduced pressure, effectively preserving texture, nutritional value, aroma, and colour, making it suitable for high-value insect products (Hernández-Álvarez et al., 2021). Although freeze-drying helps retain heat-sensitive nutrients like vitamin B12 (Hernández-Álvarez et al., 2021; Lenaerts et al., 2018), it is energy-intensive and costly (Parniakov et al., 2022). Some studies, however, report increased lipid oxidation and reduced protein levels in freeze-dried yellow mealworms, indicating that it does not inherently prevent oxidative deterioration (Bogusz et al., 2024; Keil et al., 2022; Kröncke et al., 2019; Lenaerts et al., 2018).

Oven drying is a conventional preservation method which removes water at elevated temperatures and offers good scalability for industrial applications (Hernández-Álvarez et al., 2021). However, excessive heat exposure can detrimentally affect the nutritional quality

of the product, leading to substantial reductions in vitamins B2, B3, and B12 (Bawa et al., 2020), PUFA levels (Dobermann et al., 2019), as well as decreased protein solubility (Kröncke et al., 2018). Additionally, browning, caused by both enzymatic and non-enzymatic reactions, occurs at higher temperatures, though blanching before drying can mitigate this effect (Purschke et al., 2018).

Microwave drying, which uses electromagnetic waves to evaporate moisture rapidly, also reduces drying time significantly (Hernández-Álvarez et al., 2021). However, similar to oven drying, it can decrease protein solubility (Kröncke et al., 2018) and result in substantial vitamin losses, particularly vitamins B2, B3, and B12 (Bawa et al., 2020; Lenaerts et al., 2018). Although microwave-dried insects exhibit lighter browning compared to oven-dried products, the effect on sensory attributes remains a consideration (Bawa et al., 2020).

Fluidised bed drying uses an upward flow of hot air to suspend insects, enhancing heat and mass transfer for efficient drying (Hernández-Álvarez et al., 2021). The method allows continuous, large-scale production with relatively short drying times. However, this method, like other thermal treatments, may reduce protein solubility and cause browning (Kröncke et al., 2018; Purschke et al., 2018).

Vacuum drying, performed at lower temperatures than other thermal methods, helps preserve heat-sensitive compounds but is time-consuming and not ideal for large-scale production (Hernández-Álvarez et al., 2021). Similar to freeze-drying, it increases susceptibility to lipid oxidation (Kröncke et al., 2018).

1.1.5.2 Insect meal production

Insects can be processed into homogeneous forms, such as paste or powder, through wet or dry milling (Ojha et al., 2021). Wet milling, applied to fresh or blanched insects, is common at the industrial level due to easier handling of fluid material (Dossey et al., 2016). However, wet-milled material is microbiologically unstable and must be stored below 5 °C for rapid use (De Smet et al., 2019). In contrast, dry milling produces stable powder with an extended shelf life, allowing room temperature storage and easier commercialisation (Ojha et al., 2021). While wet milling offers processing benefits, dry milling provides superior stability and market flexibility.

A subsequent drying step is necessary for shelf-stable insect-based products. Spray drying is often preferred for producing homogeneous powder quickly while preserving nutritional properties (Son et al., 2019). In contrast, drum drying is more suitable for high-fat substrates, though it may suffer from uneven heat transfer (Dossey et al., 2016; J. Tang et al., 2003). Other drying methods include oven, freeze, solar, smoke, microwave-assisted, fluidised-bed, and vacuum drying, each having its pros and cons, as explained above. The choice of technique balances microbial safety, nutritional preservation, scalability, cost, and product stability.

Insect meal can be whole-fat or defatted, with fat removal achieved through physical (Arsiwaalla & Aarts, 2015), mechanical (Tschirner & Simon, 2015), or chemical methods (Ravi et al., 2019; Son et al., 2019). Defatting may occur before or after milling. Milling prior to defatting can release enzymes that promote lipid and protein hydrolysis (Ojha et al., 2021). Physical defatting (e.g., decanting and centrifuging) results in a fat-containing stream, an aqueous stream containing protein-derived matter and another stream containing solids, such as chitin (Ojha et al., 2021). It is environmentally friendly, producing non-toxic, safe products for food, feed, and pharmaceuticals (Arsiwaalla & Aarts, 2015). Mechanical defatting involves oil extraction using pressure, followed by milling (Tschirner & Simon, 2015). In contrast, solvent defatting is applied to dried and milled material to enhance solvent penetration (Ojha et al., 2021; Ravi et al., 2019). Although

more efficient, solvent extraction raises environmental and sensory concerns, as it can reduce flavour, colour, and consumer acceptability, particularly when solvents like n-hexane are used (Son et al., 2019). Overall, defatting increases protein content and amino acid concentrations, while preventing lipid breakdown and extending shelf life (Kim et al., 2022; Marasca et al., 2025).

Recent advancements highlight eco-friendly technologies like high hydrostatic pressure (HHP), pulsed electric fields (PEF), ultrasound (US), and cold atmospheric pressure plasma (CAPP) for sustainable processing with minimal environmental impact (Z. Liang et al., 2024; Ojha et al., 2021). These methods aim to enhance both product safety and quality, while improving processing efficiency. However, their application in edible insect processing remains limited and requires further research.

1.1.5.2.1 Fermentation

Fermentation is a process assisted by either uncontrolled (natural or spontaneous) or controlled (by starter culture) microorganisms that convert large molecules into smaller ones through diverse oxidation or reduction mechanisms (Castro-López et al., 2020). During this process, the stimulation and/or improvement of the production of valuable compounds, such as essential amino acids, easily digestible proteins, essential fatty acids, alcohols, and vitamins, is conducted (Mishra et al., 2017; Srivastava, 2018). Since edible insects differ in the composition of traditionally fermented foods (fruits and vegetables), it is essential to use an appropriate selection of bacterial or fungal starter cultures that utilise both inorganic and/or organic sources of nitrogen in order to achieve a suitable substrate metabolism and acidification (Mouritsen et al., 2017; V. Singh et al., 2017). Additionally, since insects' bodies contain rigid exoskeletons, this can compromise the microorganism's accessibility to the substrate and affect the fermentation process. To address this issue, it has been suggested that edible insects be pre-treated through milling, blanching, or boiling prior to initiating fermentation (Kewuyemi et al., 2020).

Although research on the fermentation of edible insects remains limited, preliminary findings indicate potential benefits. For example, including (i) enhancing sensory qualities such as flavour, aroma, and texture; (ii) increasing nutritional value by improving nutrient bioavailability; (iii) eliminating anti-nutritional factors; (iv) increasing levels of bioactive compounds, such as amino acids, polyphenols, organic acids, (v) promoting intestinal health by incorporating beneficial microorganism; and (vi) extending shelf life and reducing microbiological risks by inhibiting pathogenic microorganisms (Castro-López et al., 2020; Di Cagno et al., 2013; Gaggia et al., 2011).

1.2 Tomatoes and Treated Wastewater

Tomatoes are among the most popular horticultural crops worldwide. Tomato fruits can be consumed either fresh or processed in many forms, such as tomato juice, soup, paste, puree, ketchup, sauce, and salsa. Tomato fruits are also nutritious and a rich source of lycopene, ß-carotene, vitamin C, provitamin A, folate, minerals (e.g., potassium), and secondary metabolites including flavonoids, phytosterols, and polyphenols. Due to its global importance, widespread consumption, and nutritional quality, the tomato (*Solanum lycopersicum* L.) was chosen as a model plant.



Figure 1.3: Tomato as a model plant.

1.2.1 Treated wastewater reuse

Water scarcity is a critical concern in an increasing number of regions, including the south and south-western USA, north-eastern Brazil, central Argentina, Northern and Eastern Africa, the Middle East, the Caucasus, northern India, northern China, and southern Europe (García-Ruiz et al., 2011; Huggins et al., 2022). Even in countries like Slovenia with significant rainfall, some regions are still impacted by seasonal water shortages. Zalokar et al. (2021), using monthly discharge data from 46 gauging stations spanning from 1961 to 2016, found that the frequency and intensity of hydrological droughts have increased in recent decades. Both Kobold (2022) and Zalokar et al. (2021) identified spring and summer as particularly critical periods for water deficits. Furthermore, they reported that droughts in Slovenia are regional in nature, affecting specific areas rather than the entire country uniformly.

Since 2000, the Slovenian Environment Agency (ARSO) has recorded 7 droughts that affected Slovenia at the scale of a natural disaster (Figure 1.4). Droughts are also occurring in regions and during seasons that have not previously experienced such problems. An additional risk to agriculture is the rapid onset of summer droughts ("flash droughts") during heat waves. Projections indicate that the trend of increasing frequency and intensity of agricultural drought will continue (Sušnik et al., 2025).

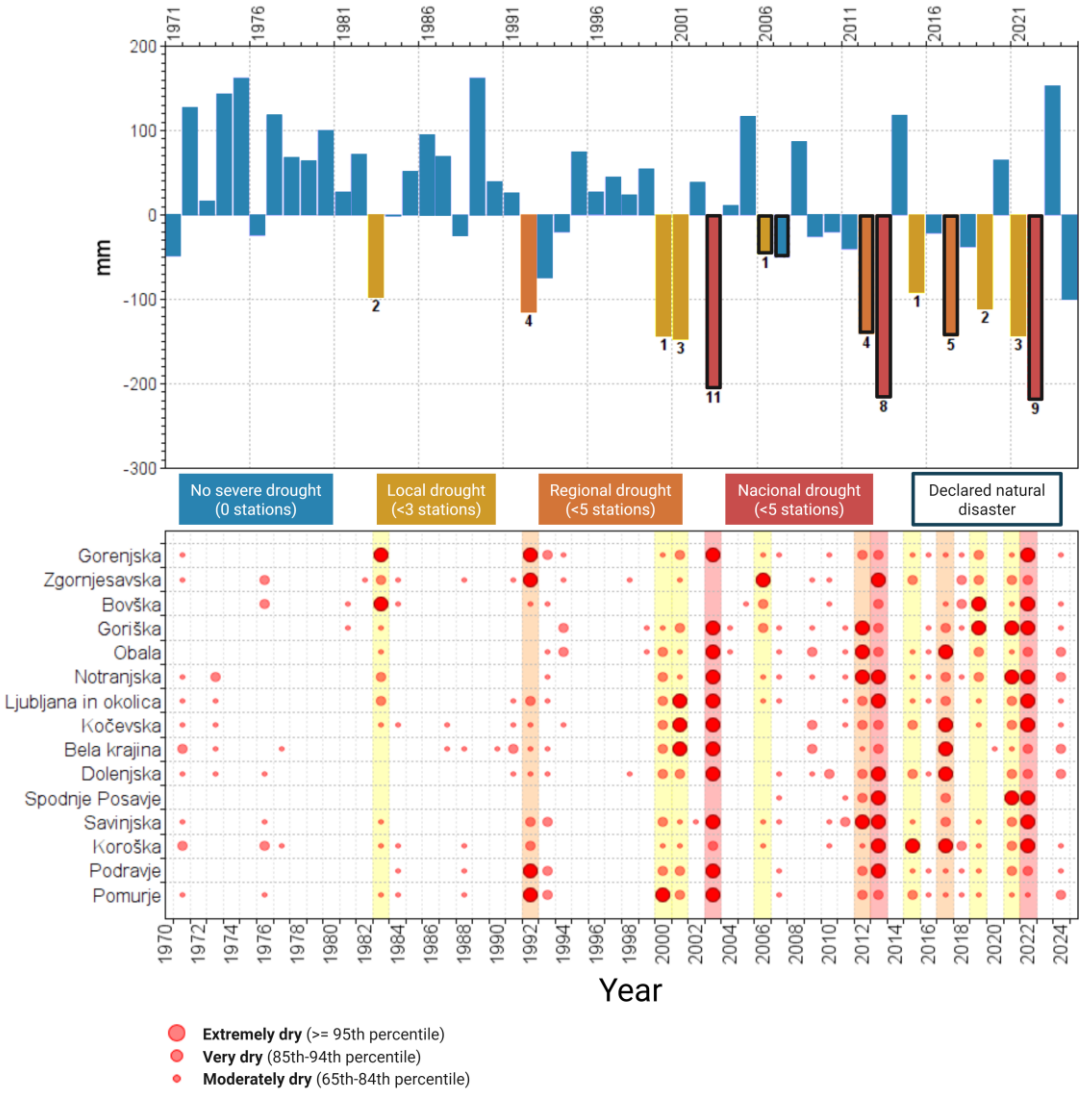


Figure 1.4: Average summer meteorological water balance for Slovenia, along with the corresponding number of regions experiencing extreme agricultural drought and their spatial extent (above), and the intensity of agricultural drought by region, expressed in percentile classes (below) for the period 1971 to 2024. Adopted by the Slovenian Environment Agency 2025 (Sušnik et al., 2025).

Since agriculture is the major consumer of water globally (accounting for 70% of abstracted water worldwide), it is severely affected by the water crisis (Christou et al., 2024). Therefore, it is crucial to maintain food security by using non-conventional water resources of adequate quality in the agriculture sector. Treated wastewater is a sustainable alternative for mitigating irrigation water scarcity, especially in areas where conventional water resources are limited or absent.

Currently, nearly 380 billion m^3 of urban wastewater are generated each year globally, with projections of a 51% increase by 2050, due to population growth and increased urbanisation (Qadir et al., 2020). Presently, less than 20% of the global wastewater generated is treated, ranging from 8 to 70% in low- and high-income countries, respectively (UNESCO, 2017). The volume of treated wastewater reused in agriculture is even lower (2-15%), as most is used for other purposes, such as recharging aquifers or discharged to

downstream environments, such as rivers and lakes (Christou et al., 2024). The challenges of using treated wastewater in agriculture lie in limited social awareness and acceptance, the presence of various microbiological and chemical contaminants (including chemicals of emerging concern and microplastics) in treated effluents, and the willingness of governmental and intergovernmental organisations to invest and subsidise this practice (Cipolletta et al., 2021; UNESCO, 2017).

Despite the challenges, several countries have already been successfully reusing treated wastewater in agriculture. Israel can be considered a pioneer in treated wastewater reuse for agriculture, with more than 87% of the produced effluents directly reused for irrigation, providing more than half of the agricultural sector's total irrigation needs (Marin et al., 2017). The success in increasing the use of treated wastewater by the Israeli agricultural sector is attributed to a centralised water system, water quotas for farmers, allocation of funds for necessary infrastructure constructions, funding of research to assess the impacts of intensive use of treated wastewater on crops and soil and implementation of strict regulation regarding health and agronomic quality of treated wastewater (Christou et al., 2024). Another example is the USA, particularly California, Florida, Texas, and Arizona, where TWW reuse in agriculture is supported by comprehensive regulations and guidelines. It is, however, used to irrigate citrus orchards and high-value vegetables, including artichokes, broccoli, cauliflower, celery, and lettuce (Ritter, 2021). Also, China and Australia have successfully adopted treated wastewater reuse (X. Liu et al., 2020; Radcliffe, 2022; Radcliffe & Page, 2020).

In Europe, TWW is mainly used in agriculture in Mediterranean countries, including Cyprus, Spain, Italy, Greece, France, and Portugal. Cyprus reuses nearly 80% of all tertiary-treated wastewater for both direct and indirect agricultural irrigation (Christou et al., 2024). Spain reuses 11% of treated wastewater, with 73% of this volume for agriculture. The TWW reuse for agriculture is very regional, and is the highest in the Region of Murcia and Region of Almeria (91%), the Valencian Community (43%), the Canary (24%) and Balearic (45%) archipelagos (Villacorta-Ranera et al., 2025). Italy has the potential to use 23% of TWW for agriculture, but currently reuses only 4% (Collivignarelli et al., 2026; *Utilitalia*, 2022). Although TWW irrigation is practised in other countries, it is not as widespread (Christou et al., 2024; *Minimum Requirements for Water Reuse*, 2024). To date, there is no TWW reuse in Slovenia.

1.2.1.1 European legislation

In Europe, TWW reuse is regulated under Regulation (EU) 2020/741, which became applicable on June 26, 2023. The regulation establishes the minimum requirements for water reuse, including: i) harmonised minimum water quality requirements for the safe reuse of treated urban wastewater in agricultural irrigation, ii) harmonised minimum monitoring requirements, iii) risk management provisions to assess and address potential additional health risks and possible environmental risks, iv) permitting requirements, and v) provisions on transparency, whereby key information on every water reuse project is made available to the public (*Water Reuse*, 2024).

More specifically, treated wastewater can be classified into four classes, A to D, which must meet the criteria for *E. coli*, biological oxygen demand, total suspended solids, turbidity, *Legionella,* and intestinal nematodes. The exact requirements and explanations of the classes are given in Table 1.6 and Table 1.7. The preparation of a risk management plan is also a mandatory component of any treated urban wastewater reuse system intended for agricultural irrigation. It aims to ensure that treated wastewater is used and managed safely to protect human and animal health and the environment. A water reuse risk management plan should be established based on the five to six elements:

- Description of the entire water reuse system from the entry into the urban wastewater treatment plant to the point of use.
- Identification of all parties involved in the water reuse systems, including their roles and responsibilities.
- Identification of potential hazards, including the presence of micropollutants and pathogens, and the potential for hazardous events.
- Identification of environments and populations at risk, and the exposure routes to the identified potential hazards.
- Risk assessment must evaluate potential hazards to the environment, human, and animal health, considering the nature, duration, and severity of risks, as well as affected populations and environments. It should follow the precautionary principle, comply with relevant EU and national legislation, and integrate guidelines for food, feed, and worker safety. The assessment may be based on existing scientific studies and data.
- Additional requirements for water quality and monitoring when necessary and appropriate to ensure adequate protection of the environment and human and animal health, in particular when there is clear scientific evidence that the risk originates in the water. Additional requirements may in particular concern heavy metals, pesticides, disinfection by-products, pharmaceuticals, other substances of emerging concern (including micro pollutants and micro plastics), and antimicrobial resistance (Regulation (EU) 2020/741, 2020).

Table 1.6: Classes of reclaimed water quality and permitted agricultural use and irrigation method.

Minimum reclaimed water quality class	Crop category (*)	Irrigation method
A	All food crops consumed raw, where the edible part is in direct contact with reclaimed water, and root crops consumed raw	All irrigation methods
B	Food crops consumed raw, where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops, and non-food crops, including crops used to feed milk- or meat-producing animals	All irrigation methods
C	Food crops consumed raw, where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops, and non-food crops, including crops used to feed milk- or meat-producing animals	Drip irrigation (**) or other irrigation method that avoids direct contact with the edible part of the crop
D	Industrial, energy, and seeded crops	All irrigation methods (***)
(*) If the same type of irrigated crop falls under multiple categories of Table 1, the requirements of the most stringent category shall apply.		

(**) Drip irrigation (also called trickle irrigation) is a micro-irrigation system capable of delivering water drops or tiny streams to the plants and involves dripping water onto the soil or directly under its surface at very low rates (2–20 L/hour) from a system of small-diameter plastic pipes fitted with outlets called emitters or drippers.
(***) In the case of irrigation methods which imitate rain, special attention should be paid to the protection of the health of workers or bystanders. For this purpose, appropriate preventive measures shall be applied.

Table 1.7: Reclaimed water quality requirements for agricultural irrigation.

Reclaimed water quality class	Indicative technology target	Quality requirements				
		E. coli (number/100 ml)	BOD_5 (mg/l)	TSS (mg/l)	Turbidity (NTU)	Other
A	Secondary treatment, filtration, and disinfection	≤ 10	≤ 10	≤ 10	≤ 5	Legionella spp.: < 1 000 CFU/L where there is a risk of aerosolisation Intestinal nematodes (helminth eggs): ≤ 1 egg/L for irrigation of pastures or forage
B	Secondary treatment, filtration, and disinfection	≤ 100	In accordance with Directive 91/271/EEC (Annexe I, Table 1)	In accordance with Directive 91/271/EEC (Annexe I, Table 1)	-	
C	Secondary treatment, filtration, and disinfection	≤ 1 000			-	
D	Secondary treatment, filtration, and disinfection	≤ 10 000			-	

1.2.2 Influence of treated wastewater on the agro-environment and plants

The practice of using TWW offers many advantages and disadvantages that can impact the overall sustainability and productivity of agricultural systems. Treated wastewater contains essential macronutrients such as nitrogen, potassium, and phosphorus, which can act as fertilisers and, importantly, reduce reliance on commercial fertilisers (Rusănescu et al., 2022). However, this practice must be monitored to prevent environmental pollution (Christou et al., 2024).

1.2.2.1 Salinity

A possible drawback of using TWW for soil irrigation is that it may lead to increased soil salinity. TWW often contains elevated levels of salts, which can accumulate in the soil and inhibit crop growth (Bakari et al., 2022). High salt accumulation and/or the addition of particulate organic matter from TWW in soil can result in deterioration of soil structure, leading to unfavourable physical and hydraulic soil properties and consequently reduced water and oxygen availability to plants (Leuther et al., 2018; L. Wang et al., 2024). Also, TWW irrigation can contaminate groundwater situated below irrigation sites (Benaafi et al., 2024). To reduce the potential risk, salt input into the sewage system has to be reduced.

1.2.2.2 Potentially toxic elements

Particular attention should be given to boron, a component in industrial detergents, which is transferred to sewage and TWW (Fatnassi et al., 2025). When present in irrigation water, B can induce phytotoxic effects in crops even at low concentrations (Zhao et al., 2023). Since conventional wastewater treatment does not effectively remove B, source control is essential (Dorta-Santos et al., 2016). Treated wastewater also contains potentially toxic elements (Pb, Cd, Ni, Cr, As), which are usually within safe limits for agricultural use. However, long-term application of treated wastewater can lead to excessive build-up of potentially toxic elements in soil and crops, posing environmental and health risks (Othman et al., 2021). Additionally, TWW can carry pathogens that harm farmers, infect crops, and pose a risk to human and animal health through the food chain (Becerra-Castro et al., 2015). Therefore, adequate treatment and rigorous monitoring of TWW quality are crucial to ensure safe and sustainable reuse (Christou et al., 2024).

1.2.2.3 Contaminants of emerging concern

Another problematic aspect is the presence of CECs. Contaminants of emerging concern are compounds that can be newly identified or have been present in the environment for a long time. They can be either anthropogenic or naturally occurring chemicals or biological agents. What defines them is their recognition as potentially hazardous to humans and ecosystems, based on newly available data or improved detection methods (F. Wang et al., 2024). They include pharmaceuticals and personal care products, per- and polyfluoroalkyl substances, emerging pathogens, cyanotoxins and other natural toxins, pesticides, industrial chemicals, micro/nano plastics, nanomaterials, antibiotic resistance genes, and other exogenous substances whose impacts on humans and the environment are still not well understood (Cousins et al., 2022; Puri et al., 2023; F. Wang et al., 2024). CECs enter wastewater treatment through industrial and municipal wastewater, where they are mineralised or partially degraded and/or transformed into various transformation products and end up as complex mixtures in wastewater effluent and sludge (Petrie et al., 2015).

Irrigation with wastewater can lead to CEC residue accumulation in agricultural soils following transportation and potential transformation (by biotic and abiotic factors) (Ben Mordechay et al., 2022; Christou et al., 2017). If CECs are not degraded or retained in soil, they can contaminate groundwater (Kampouris et al., 2022). Also, they can be absorbed by crop plants and accumulated within their tissues (Christou, Papadavid, et al., 2019). Upon entering the food web, they can bioaccumulate in other organisms and humans, potentially causing toxic effects (Paltiel et al., 2016; Schapira et al., 2020).

The mechanisms underlying CEC uptake by plants, as well as their accumulation in different plant tissues, including edible ones, have been studied under controlled conditions. The studies showed that CECs in plants can induce transcriptomic and metabolomic rearrangements that impact normal plant physiology and morphology, indicating stress response (Christou et al., 2016; Mansilla et al., 2024). Moreover, CECs can induce oxidative stress and disrupt phytohormone regulation, further impairing plant growth and development (Mansilla et al., 2021). In some cases, CECs can also be metabolised and detoxified in plant cells, which involves three phases:

- Phase I, enzymes modify the compound via oxidation, reduction, or hydrolysis.
- Phase II, the metabolite is conjugated with polar molecules (e.g., sugars, amino acids, glutathione) to enhance solubility.
- Phase III, conjugates are sequestered or excreted from the cell into vacuoles, resulting in their excretion from the cell and, ultimately, from the plant (Mansilla et al., 2021).

The CEC uptake in plants is affected by plant species, soil physicochemical properties, and environmental conditions that influence evapotranspiration (Christou, Papadavid, et al., 2019).

1.2.2.4 Micro and nanoplastics

Although the removal of micro and nanoplastics from wastewater is high (up to 95%), since they are retained in sludge, TWW is still an important source of micro and nanoplastics (J. Li et al., 2018). Micro and nanoplastics can be colonised by wastewater microorganisms, resulting in the formation of dynamic biofilms, known as plastispheres, which interact with co-existing contaminants in the wastewater treatment plant (WWTP), including CECs (P. Liu et al., 2023; Xia et al., 2022). Wastewater plastispheres can enhance the persistence of antibiotic resistance elements (antibiotic-resistant bacteria, antibiotic-resistant genes, and mobile genetic elements) and bacterial pathogens by favouring their microenvironment and horizontal gene transfer and limiting their inactivation by disinfection processes, thus accelerating their toxicological impacts (Junaid et al., 2022; P. Liu et al., 2023; Manoli et al., 2022). In addition, micro(nano)plastics can accumulate in soil fauna, wildlife, and plants, causing adverse effects (Cui et al., 2023; F. Wang et al., 2022). The uptake and accumulation of micro(nano)plastics in cultivated plants, along with other contaminants in TWW and irrigated soil, can induce phytotoxic effects, negatively impacting plant growth and development (Naziri et al., 2023). Moreover, the accumulation of micro(nano)plastics in the edible parts of crop plants can further contribute to their biomagnification in the food chain, with potential human health risks (Y. Liu et al., 2023).

1.2.2.5 Influence of CEC on crop quality

Although significant research has explored the uptake and accumulation of CECs from TWW and spiked irrigation solutions in crops, only a few studies have examined their impact on crop quality and biochemical parameters. To the author's knowledge, only three studies have specifically investigated the effects of CECs, which can be found in TWW, from spiked irrigation solutions, but no studies have yet focused on the influence of CECs from TWW itself. Christou, Kyriacou, et al. (2019) evaluated the effects of three pharmaceutically active compounds, both individually and in combination, in irrigation water, on various quality attributes of tomato fruits. These included colouration, acidity, soluble solids content, lycopene, ascorbic acid, phenols, antioxidant activity, and sugar content. The study revealed significant changes in soluble solids and carbohydrate content caused by individual pharmaceuticals. However, no additive or synergistic effects were observed when combined. Hurtado et al. (2017) examined the effects of 11 CECs on lettuce and identified alterations in relative concentrations of amino acids, carboxylic acids, and sugars. Chen et al. (2018) evaluated the response of ten different rice varieties to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), a predominant congener of polybrominated diphenyl ethers (PBDEs), in a hydroponic system. The metabolic response was variety-specific: the tolerant variety exhibited stronger metabolic activity and a more effective antioxidant defence system, with significant upregulation of amino acids, organic acids, saccharides, and secondary metabolites (polyols, glycosides) to counteract oxidative stress, while the susceptible variety showed a decrease in most metabolites, indicating its vulnerability.

Based on current research, it is important to note that the effect of CECs on crop quality is highly variable, depending on factors such as plant species, CEC type and concentration, and the specific metabolite affected. Due to a lack of data, it is challenging, if not impossible, to draw general conclusions that would apply universally across different metabolite groups (e.g., amino acids, sugars, organic acids), plant species, or types of contaminants. Each combination of plant and CEC may yield distinct effects on crop

quality, underscoring the need for context-specific analyses rather than broad generalisations.

1.2.3 Tomato fruit quality

Tomato quality is a multidimensional concept that includes physical, chemical, nutritional, and sensory attributes. The following chapters will focus on the quality characteristics of red tomato varieties.

1.2.3.1 Physical quality

Physical attributes refer to the external characteristics of tomatoes, which are key to consumer perception and market grading. High-quality tomatoes should be uniform, with no deformities and defects, such as cracks, bruises, or blossom-end rot (Molelekoa et al., 2025). Their bright red colour signifies full ripeness and desirable flavour, as well as the presence of lycopene, the pigment responsible for the red shade and known for its antioxidant properties (Barrett & Anthon, 2008). Colour is also a major quality component in tomato and is an important focus of breeders. The main factors affecting pigment concentration and colour development are cultivar, growing region, and season. At the same time, during post-harvest storage, temperature is the most critical environmental factor influencing biological processes, including red colour development and fruit softening (Tadesse et al., 2015).

Another important physical quality parameter of tomato is texture, which is perceived through touch, whether the fruit is picked up by hand or placed in the mouth and chewed. The texture of tomato fruit is determined by turgor pressure, the composition of individual plant cell walls, and the middle lamella that holds cells together (Barrett et al., 2010). Tomatoes are composed of approximately 93-95% water and 5-7% total solids, of which 80-90% is soluble and 10-20% insoluble.

Insoluble material derived from cell walls is the greatest contributor to texture (Barrett et al., 2010). Cell walls are principally composed of three classes of polysaccharides: cellulose, hemicellulose, and pectin. In tomato, the ripening-associated changes occur in the pectic polysaccharides, which play an important role in cell-to-cell adhesion. During ripening, pectic polymers degrade due to the action of pectin-metabolising enzymes (D. Wang et al., 2018). Factors that affect the textural properties of tomatoes are either production-related or tissue-specific. Production-related factors include:

(1) cultivar or variety,
(2) maturity at harvest and degree of ripeness,
(3) cultural practices, including the use and type of fertiliser, application of certain hormones, amount of water, and degree of sun exposure, and
(4) environmental stresses on the tomato plant prior to harvesting, such as drought, salinisation, water stress, chilling, and freezing.

Tissue-specific or structure-related factors that affect the textural properties of tomatoes include the following:

(1) chemical composition of the cell wall,
(2) activity of softening-related enzymes,
(3) turgor pressure,
(4) cell shape and size distribution,
(5) the amount and distribution of intercellular spaces, and the proportions and arrangements of specialised tissues such as vascular, epidermal, and locular tissues (Amr & Raie, 2022).

1.2.3.2 Chemical quality

The key chemical quality parameters of tomatoes are titratable acidity and total soluble solids. Titratable acidity affects the flavour, preservation, and processing characteristics of both fresh and processed tomato products (Amr & Raie, 2022). A higher titratable acidity improves the safety and shelf stability of tomato products by reducing microbial growth, thereby allowing shorter heat treatments during processing (Vieira et al., 2020). The sugar-to-acid ratio is a major determinant of taste quality, influencing the perceived sweetness, sourness, bitterness, and tartness that collectively define consumer acceptability of tomato products (Anđelini et al., 2023). During ripening, titratable acidity reaches its maximum at the beginning of ripening, then decreases as the fruit approaches the ripe stage and continues to decrease with over-maturity (Anthon et al., 2011). Citric acid is the primary contributor to titratable acidity in tomatoes, followed by smaller amounts of malic and glutamic acids (Villena et al., 2023).

Total soluble solids (TSS) are a key indicator of tomato quality. For processed tomatoes, TSS is a key indicator of quality and competitiveness. Processing tomatoes should have at least 5% TSS, while each 1% increase is equivalent to a 25% reduction in raw material consumption (Gur & Zamir, 2015). Consequently, the processed tomato market has clear quantitative minimum requirements for TSS, aiming to achieve extremely high TSS levels to optimise production costs.

In contrast to processed tomatoes, the fresh tomato market emphasises the balance between TSS and acidity, aiming to provide consumers with the best flavour experience (Xu et al., 2025). TSS are commonly measured using a refractometer, which expresses the percentage of soluble compounds in the fruit juice (Amr & Raie, 2022). Fresh tomatoes consist mainly of sugars and organic acids, along with smaller amounts of amino acids, ascorbate, minerals, pectins, and phenolic compounds. Among these constituents, sugars, acids, phenols, and minerals play a central role in determining the characteristic taste and flavour of tomatoes, with sugars contributing the most quantitatively (Figàs et al., 2015). The TSS content is influenced by both the tomato cultivar and the stage of ripeness at harvest (Beckles, 2012). In general, TSS, along with glucose and fructose, the primary contributors to soluble solids, increase during ripening as starch is converted into sugars. However, slight declines may occur in overripe fruits (Young et al., 1993).

1.2.3.3 Nutritional and sensorial quality

Nutritional quality of tomatoes relates to sugars, organic acids, carotenoids, polyphenols, amino acids, fatty acids, while sensory quality relates mainly to volatile organic compounds.

1.2.3.3.1 Sugars

Sugars are among the most important nutritional components of tomatoes, accounting for 3-6% of tomato weight, depending on the variety and cultivar (Abdul-Hammed et al., 2012; Ágoston et al., 2017; Amr & Raie, 2022). As mentioned above, they play a significant role in taste and texture. The main sugars present in tomatoes are glucose, fructose, and sucrose.

During ripening, glucose and fructose increase significantly, and sucrose also forms, as starch stored in the fruit is broken down into sugars by enzymes (Kanayama, 2017). Sugar content is affected by many environmental factors, including temperature, light exposure, soil quality, irrigation practices, fertiliser use, and stress factors. Water, low temperature, salinity, and oxidative stress often increase sugar accumulation, while nutrient deficiencies, low light, and high-temperature stress typically reduce accumulation (Casals et al., 2021; Dasgan et al., 2021; Gruda, 2005; Marsic et al., 2018).

1.2.3.3.2 Organic acids

Organic acids are another critical factor in the sweet-and-sour taste of tomatoes. The most abundant organic acids present in ripe fruits are citric acid and malic acid, while shikimic and fumaric acids are present in traces (Marsic et al., 2018). Their content ranges from 0.3% to 0.6%, while their ratio varies significantly across tomato cultivars (Felföldi et al., 2021). The content of organic acids is important for processing and storage, as they determine the fruit's pH and, in sufficient amounts (pH<4.5), prevent the development of spoilage microorganisms (Bergougnoux, 2014).

As with sugars, the amount of organic acid increases during ripening. At all stages, citric acid is the dominant organic acid, but unripe green tomatoes may contain significant amounts of malic acid, whereas its content in ripe fruits is relatively low. After ripening, citric acid content decreases, while malic acid content remains relatively stable (Agius et al., 2018). Other factors influencing the organic acid composition of tomatoes include variety, climate, location, agricultural practices, and stress factors (Hernández et al., 2014). Stress factors such as salinity and drought increase organic acid levels (Habibi et al., 2021; Marsic et al., 2018). Similar to soluble sugars, these compounds accumulate as part of osmotic adjustment mechanisms that help maintain cell turgor, water retention, and metabolic activity under water-deficit conditions (Farooq et al., 2009).

1.2.3.3.3 Vitamins

Tomatoes are an excellent source of vitamins, including vitamin B-complex, A, E, and K, with vitamin C being the most abundant (Ali et al., 2020). Many of them serve as enzyme cofactors (vitamins B-complex, A, K, and C), while vitamins E and C also function as biological antioxidants; vitamin A functions as a hormone and also serves as a photoreceptive cofactor in vision. In contrast, vitamins E, K, C, and B-complex also serve as cofactors in oxidation-reduction reactions (Combs & McClung, 2017). Vitamin content varies significantly depending on variety and cultivar, ripeness, storage conditions, and processing. During ripening, vitamin C increases to the half-mature stage and then slightly decreases at the full-ripe stage, while in the postharvest period, it gradually decreases (Antala et al., 2025; X. Li et al., 2025). Studies have shown that various abiotic stress conditions, such as drought and salinity, influence vitamin C accumulation in tomatoes (Mellidou et al., 2021).

1.2.3.3.4 Carotenoids

Tomatoes contain several types of carotenoids, with lycopene being the predominant one, accounting for more than 80% of the total carotenoids in fully ripe fruits. Other carotenoids in tomatoes include ß-carotene, which accounts for approximately 5−7%, and trace amounts of α-carotene and lutein (Marsic et al., 2018). These carotenoids are plant pigments that protect tomatoes from photo-oxidative damage and serve as natural antioxidants for humans, helping to combat cellular oxidative stress (Ali et al., 2020).

The concentrations of carotenoids in tomatoes can vary depending on factors such as variety, cultivar, ripening stage, storage conditions, and processing methods. Carotenoids increase during ripening and, after the full-ripe stage, remain stable for some time before slowly degrading (González-Casado et al., 2018; Lin, 2005). Moreover, high-temperature stress leads to the degradation and inhibition of lycopene biosynthesis, while low temperatures can slow both fruit ripening and lycopene production (Dasgan et al., 2021). In contrast, increased salinity has been shown to enhance lycopene content (Marsic et al., 2018). During processing, lycopene remains relatively stable, except at very high temperatures and long heating times (Shi et al., 2008).

1.2.3.3.5 Polyphenols

More than 170 phenolic compounds are present in tomato in low concentrations and differ by variety and cultivar (Paolo et al., 2018; Slimestad & Verheul, 2009). They can be divided into groups based on their core structure: hydroxycinnamic acids, flavanones, flavonols, anthocyanins, and flavonol glycosides (Martí et al., 2016). The most abundant polyphenol is naringenin chalcone, followed by naringenin, quercetin, rutin, chlorogenic acid, kaempferol, and myricetin (Martí et al., 2016). Accumulation of polyphenols in tomato fruit is tissue-specific, with flavonoids almost entirely restricted to the peel (98%). At the same time, a range of hydroxycinnamic acids (coumaric, ferulic, and caffeic acids) and their conjugates with sugars and quinic acid have been reported in both skin and pulp (Moco et al., 2007). Concentrations of different polyphenols vary with maturity stage, agricultural practices, season, light, temperature, stress conditions, and storage conditions (Marsic et al., 2018; Slimestad & Verheul, 2009). Polyphenols are potent antioxidants and have been reported to interfere with the initiation, promotion, and progression of cancer (Ramos, 2008).

1.2.3.3.6 Amino acids

Tomatoes are a rich source of amino acids, with essential amino acids accounting for about 40% of the total protein content (Ali et al., 2020). Amino acids are fundamental for various bodily functions in humans, including maintaining cellular structure, nutrient transport and storage, wound healing, and tissue repair (Elango & Laviano, 2017). In tomatoes, amino acids are found both as part of proteins and as free amino acids. Among the total amino acids, glutamic acid is the most abundant, while cystine is the least, and leucine is the most prevalent essential amino acid, with methionine being the least (Ali et al., 2020).

Free amino acids, such as glutamate, γ-aminobutyric acid, glutamine, and aspartic acid, make up 80% of the total free amino acids in tomatoes (Kader et al., 1978). During ripening, the concentration of specific free amino acids changes significantly. Glutamate, aspartate, phenylalanine, and alanine increase as the fruit ripens and turns red, while γ-aminobutyrate, glutamine, serine, and valine decrease (Osorio et al., 2011). Glutamate is particularly important for the characteristic "umami" flavour in ripe tomatoes, and its levels are higher in cultivated varieties compared to wild species (Sorrequieta et al., 2010). Environmental factors, agricultural practices, and stress conditions notably influence amino acid levels in tomato fruits (Fibiani et al., 2022; Fusco et al., 2025). For instance, salinity stress significantly increases free proline levels, whereas drought stress increases concentrations of phenylalanine, asparagine, serine, and valine (Agius et al., 2022; Shu et al., 2024).

1.2.3.3.7 Fatty acids

Although fatty acids are present in tomatoes in low quantities, they play important roles, including energy storage, cell membrane structure, signalling molecules, and aroma precursors (Kachroo & Kachroo, 2009; S. Wang et al., 2023). Through these roles, they influence flavour, texture, nutritional value, and storage stability, making them important determinants of overall tomato quality. The highest fatty acid in tomato fruit is linoleic acid, followed by oleic and palmitic (Saini et al., 2017). Tomatoes contain the essential fatty acids linoleic and α-linolenic acids, which are vital for human health, as they regulate inflammation, support cardiovascular and brain function, and serve as precursors to biologically active long-chain fatty acids (Sala-Vila et al., 2022). During ripening, there is an increase in linoleic and α-linolenic acids and overall polyunsaturated fatty acids (Saini et al., 2017). Overall, fatty acid composition in tomato fruits varies depending on the variety and ripening stage, agricultural practices, environmental conditions, and stress

conditions (Asakura et al., 2021; Gao et al., 2025; L. Li et al., 2024; Staveckienė et al., 2023).

1.2.3.3.8 Volatile organic compounds

Tomato volatiles are crucial components of fruit flavour, providing the characteristic aroma that complements sweetness and acidity. More than 400 volatile compounds have been identified in tomatoes, but only a limited number, including aldehydes, alcohols, esters, ketones, lactones, and terpenoids, make significant contributions to flavour perception (Kaur et al., 2023). These volatiles originate from three primary biosynthetic pathways derived from lipids, amino acids, and terpenoid precursors (S. Wang et al., 2023). Tomato aroma is typically described as a complex blend of green, grassy, fruity, floral, and earthy notes, arising from the combination of lipid-derived aldehydes (fresh-green), amino acid-derived compounds (sweet-fruity and floral), and carotenoid-derived volatiles (fruity-flowery) that together create the characteristic fresh tomato scent (Kaur et al., 2023; S. Wang et al., 2023). The volatile organic compound profile continuously changes throughout fruit development, from early growth, fruit ripening, and the postharvest period. It is influenced by genetic factors, agricultural practices (grafting, irrigation and water quality, growing media, fertilisation, green house/open field), environmental conditions (temperature, light, soil), stress conditions (herbivory or pathogen attack, salt stress, drought, temperature extremes) and postharvest treatment (storage, postharvest chemical application, processing) (Distefano et al., 2022; Erika et al., 2022; Ji et al., 2025; S. Wang et al., 2023).

1.2.3.3.9 Minerals

Beyond their organic constituents, tomatoes are a significant dietary source of essential minerals, as 200 g of fresh tomato provides 10% of the adequate intake for adults and about 5-7% of the reference daily intake for P and Mg (Erba et al., 2013). While some nutrients, such as N, P, K, Ca, Mg, and S, are needed in large amounts by tomato for normal growth and reproduction, others, such as Fe, Cu, Zn, Mn, B, Mo, and Cl, are needed in small amounts (Sainju et al., 2003).

Nutrients influence various physiological and biochemical processes, for instance, N is vital for protein and amino acid synthesis, P supports root development and early fruit setting, while K promotes the growth and development of tomato plants and enhances fruit quality and flavour (Allen, 1973; Song et al., 2025; Yusuf et al., 2023). Calcium maintains cell wall integrity and prevents disorders like blossom-end rot, whereas Mg contributes to chlorophyll formation (Ho, 2005; Ishfaq et al., 2022). As a result, tomatoes are regularly fertilised with N, P, and K and occasionally with Ca and Mg (Sainju et al., 2003). Other nutrients are not generally applied unless a plant deficiency occurs. For greenhouse-grown tomatoes, the growth media other than soil are fortified with all nutrients (Xiong et al., 2017). The concentration and balance of elements depend on factors such as soil nutrient availability, pH, fertilisation practices, genotype, environmental and stress conditions (Hernández Suárez et al., 2007; Jin et al., 2025; Rodríguez-Ortiz et al., 2022; Sellitto et al., 2019). Both nutrient deficiencies and excesses can negatively affect tomato yield and quality, as well as lead to environmental issues like nitrate leaching (Mao et al., 2024). Therefore, periodic soil and plant analyses are essential to ensure optimal fertilisation rates that sustain productivity while protecting environmental quality. (Sainju et al., 2003).

1.2.3.3.10 Potential effects of treated wastewater and CECs on tomato

The influence of TWW and CECs on tomato fruit quality remains insufficiently explored. Furthermore, to date, there have been no studies investigating the mechanisms by which

TWW and CECs affect tomato metabolism and quality. While the effects of increased salinity and elevated potentially toxic elements associated with TWW irrigation have been discussed in this chapter, another important aspect is the potential impact of CECs on tomato fruit metabolism, particularly through the induction of oxidative stress. Based on current knowledge of plant responses to reactive oxygen species (Wei et al., 2025), CEC-induced oxidative stress may accelerate early ripening processes, affecting fruit colour by altering carotenoid synthesis and accumulation. It may also influence fruit texture by promoting cell wall degradation, leading to softening. Furthermore, oxidative stress could alter key quality-related metabolites, including organic acids, the conversion of starch to soluble sugars, vitamins, volatile compounds, and pigments, thereby modifying flavour and nutritional value. Excessive oxidative stress could accelerate tomato ripening beyond optimal levels or cause oxidative damage during ripening, potentially affecting fruit drop, postharvest behaviour, pigment synthesis, softening dynamics, and overall shelf life.

Based on knowledge from aquatic plants (J. Liang et al., 2024) and soybean seedlings (S. Wang et al., 2015), bisphenols and related CECs could affect tomato via additional molecular mechanisms. For example, exposure to bisphenol analogues could alter gene transcription, protein translation, and endogenous metabolic pathways. Possible structural damage, often visible at early stages of exposure, is associated with inhibited photosynthesis and disrupted plant growth. Moreover, as endocrine-disrupting compounds, bisphenols could disrupt tomato hormonal balance, since phytohormones play central roles in stress signalling, resistance responses, and metabolic regulation. All of those considerations are based on limited studies investigating the effects of bisphenols in other plant species and should therefore be interpreted with caution. They primarily serve as preliminary hypotheses and indicate potential directions for future research rather than definitive conclusions for tomatoes.

1.3 Explainable Machine Learning in Food Sciences

Nutritional quality assessments and metabolomics analyses generate large, complex datasets, making it challenging to process and interpret them to extract meaningful patterns and to find clear answers to research hypotheses. Food data statistics usually include basic descriptive statistics and hypothesis testing (t-tests, ANOVA, nonparametric tests), confidence interval estimation, regression analysis, and multivariate analysis (PCA, factor analysis, cluster analysis) (Eftimov et al., 2017; R. Singh et al., 2023). However, these methods have their shortcomings.

Descriptive statistics offer only a summary snapshot of the data and do not identify causal relationships or explain the mechanisms underlying observed patterns. Hypothesis testing is constrained by underlying assumptions, such as normality and homoscedasticity, that are often violated in real-world data, and its outcomes are sensitive to sample size and the selected significance threshold, indicating statistical association, which can lead to spurious associations rather than causation. Regression analysis further assumes linear relationships between variables and independence and normality of residuals, which limits its ability to represent complex or non-linear interactions unless extended or modified. Multivariate techniques, while powerful, often require advanced statistical expertise for correct interpretation, assume linearity, and are highly sensitive to data quality, outliers, and methodological choices, all of which can substantially influence results (R. Singh et al., 2023). These limitations have motivated the emergence of explainable machine learning as a powerful alternative, capable of capturing complex nonlinear relationships while providing transparent, interpretable insights into the underlying drivers of food-related data.

Explainable machine learning, also known as explainable artificial intelligence (XAI), comprises a set of computational methods that make the decision-making processes of complex models transparent and interpretable to humans. Unlike traditional "black box" machine learning algorithms, explainable approaches allow researchers to understand how input variables contribute to model outputs, identify key features driving predictions, and reveal hidden relationships within high-dimensional datasets (Linardatos et al., 2020).

Explainable machine learning has evolved alongside broader machine learning development, emerging in response to growing concerns about the opacity of increasingly complex models. Early machine learning and statistical approaches, such as linear regression and decision trees, were inherently interpretable but limited in their ability to model complex patterns (Hastie et al., 2009; Quinlan, 1986). With the rise of more powerful "black-box" models in the 2000s and 2010s, including neural networks, support vector machines, and deep learning, predictive performance improved substantially, but interpretability declined, raising concerns in high-stakes domains such as healthcare, finance, and law (Bishop, 2006; Burrell, 2016; LeCun et al., 2015). These concerns led to the emergence of XAI in the mid-2010s, focusing on developing methods that can explain model predictions without sacrificing accuracy, exemplified by techniques such as LIME (Local Interpretable Model-agnostic Explanations) proposed by Ribeiro et al. (2016) and SHAP (SHapley Additive exPlanations) by Lundberg & Lee (2017). More recently, the field has expanded in response to ethical, legal, and societal demands for transparency, fairness, and accountability (Barocas et al., 2023; Mehrabi et al., 2022), reinforced by regulatory frameworks such as the GDPR (General Data Protection Regulation) (Goodman & Flaxman, 2017). That led to advances in counterfactual explanations, fairness-aware models, and human-centred interpretability approaches (Barredo Arrieta et al., 2020; Miller, 2019; Wachter et al., 2017).

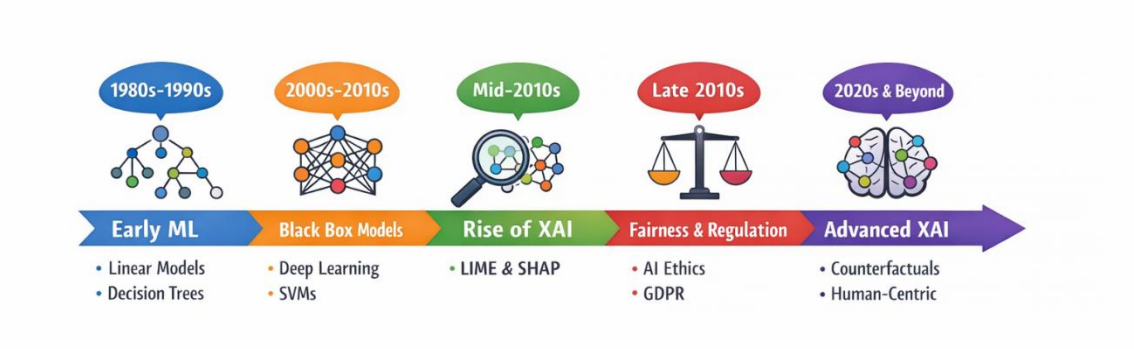


Figure 1.5: The evolution of explainable machine learning.

1.3.1 Applications of explainable machine learning in food chemistry and agriculture

Explainable machine learning has been successfully applied across a wide range of food-related challenges, offering insights into various domains such as food origin tracing, crop yield prediction, sensorial quality, and food safety. In the context of tracing the origin of food products, Huang (2023), Kang (2023, 2024) and Sun (2022) utilised explainable models like eXtreme Gradient Boosting (XGBoost), Light Gradient-Boosting Machine (LightGBM) and SHAP to identify the origin of lotus parts, mussels, Chinese oysters, and sea cucumbers based on metabolomic profiles, stable isotope ratios and elemental compositions, respectively. These models provided accurate, interpretable recommendations on food authenticity, with SHAP facilitating understanding of the model's outputs by revealing the most influential features.

In agricultural applications, Murakami et al. (2021), Newman (2021), and Sihi, (2022) employed a range of machine learning models, including neural networks (NN), random forest (RF), support vector machine regression (SVR), partial least squares regression (PLS), cubist regression (CB), XGBoost, and LIME, to predict crop yields based on environmental variables. These models demonstrated high prediction accuracy, with R^2 values exceeding 0.8, indicating their robustness. Murakami et al. (2021) identified that minimum air temperature and precipitation during the grain-filling period were crucial factors in yield prediction. Newman (2021) highlighted that key influences on crop performance include precipitation, air temperature, soil conditions, and management inputs, such as chemical and fertiliser applications. Meanwhile, Sihi (2022) emphasised the importance of growing degree days as a key climatic factor and found that water-holding capacity was among the most significant soil properties affecting crop yield variability. These findings underscore the intricate relationships between environmental variables, agricultural practices, and crop yields, providing valuable insights for improving agricultural practices in response to evolving environmental conditions.

In food chemistry, Maroni (2022) used RF and LightGBM models, coupled with SHAP, to classify sweeteners and bitterants based on their chemical descriptors, offering a rational approach to the design and screening of food additives. Similarly, Minamikawa (2022) employed RF, multiple linear regression (MLR), and Bayesian networks to explore the relationship between fruit morphology and sensory attributes, identifying key morphological features that directly correlate with fruit quality traits, such as peeling ease and fruit hardness.

Kha et al. (2023) developed a prediction model using XGBoost and SHAP to investigate drug-food constituent interactions, identifying critical chemical reactions between food compounds and drugs that could affect drug efficacy. This model was based on a vast dataset, including over 13,000 drugs and 70,000 food compounds, and yielded valuable insights into adverse interactions at the atomic level.

In another example, Puram (2024) used machine learning models, including regression trees and random forests, to analyse user reviews from food-sharing apps and identified key challenges such as app design, service responsiveness, and ethical behaviour. Their findings were interpreted using accumulated local effects and partial dependence plots, revealing critical areas for improvement to enhance the user experience, including service responsiveness, app design, food variety, and unethical behaviour.

The ability of explainable machine learning to simplify and interpret complex datasets makes it a promising tool for future studies exploring the impact of treated wastewater and CECs on tomato fruit quality under different treatment conditions. This approach is particularly valuable within the framework of this doctoral research, which aims to integrate metabolomic and elemental data to provide a deeper, more interpretable understanding of how various treatments influence tomato quality.

1.4 Consumer Acceptance

1.4.1 Consumer acceptance of edible insects

Entomophagy, the practice of eating insects, has been a part of the human diet for thousands of years, with evidence of their consumption found in prehistoric archaeological sites (Evans et al., 2015; Sutton, 1995). It has been a common practice across Africa, Asia, Latin America, and Oceania (Olivadese & Dindo, 2023). It is estimated that around 2 billion people consume insects (Van Huis, 2013), although this number has not been updated recently and could be overestimated, since there are no statistics available up to

date (Van Huis et al., 2022). Also, when estimating the global population that consumes insects, considerations should be made regarding who is classified as an insect consumer, what proportion of each national population consumes insects, how insect consumption changes over time, and which edible insects are considered part of the diet (Van Huis et al., 2022).

Despite the long history of entomophagy in several countries (Olivadese & Dindo, 2023), insects are met with significant scepticism, safety concerns, and unfamiliarity in Western societies (Kröger et al., 2022). Research indicates that the acceptability of insect-based foods is influenced by many factors, including personal sociodemographic characteristics, psychological factors, familiarity with insects, and the marketing mix (product, price, promotion, and place) (Alhujaili et al., 2023).

1.4.1.1 Personal factors

Sociodemographic factors influencing the acceptance of edible insects include age, gender, education, household income, and household size (Alhujaili et al., 2023). Overall, younger and more educated individuals, particularly those aged 20–40, show greater openness to entomophagy, and urban residents tend to be more accepting than rural populations (Kulma et al., 2020; Sogari et al., 2019). Regarding gender, men generally display higher acceptance of insect-based foods, regardless of the visibility of insects in products (Verbeke, 2015). However, other studies report no significant gender differences (Sogari et al., 2019). The influence of income and household size is less consistent: higher-income households in China and Poland were more willing to consume insects (A.-J. Liu et al., 2019; Orkusz et al., 2020), while larger households showed greater acceptance in China but lower acceptance in Kenya (A.-J. Liu et al., 2019; Pambo et al., 2018).

Psychological factors are divided into three groups: i) emotions and attitudes, ii) social influence, and iii) wider availability (Alhujaili et al., 2023). Emotions and attitudes play a crucial role, with disgust, food neophobia, and rejection of food produced using new technologies working against insect acceptability as food. On the other hand, positive attitudes, such as curiosity about insects and interest in insects as food, positively influence people's willingness to try insect-based foods. Also, people who are concerned about the health and environmental impacts of insects are more willing to consume insects than those who are not (Alhujaili et al., 2023; Mina et al., 2023; Rehman & Ogrinc, 2024).

Social influence plays a key role in consumers' acceptance of edible insects, as people tend to conform to others' behaviours and opinions. Studies show that positive social norms, such as peers or experts endorsing insect-based foods, can significantly increase willingness to try them. In contrast, negative social influence from family or friends reduces acceptance (Berger et al., 2019). For example, when students believed their peers had tried roasted mealworms, participation rose from 53% to 81%, highlighting the strong effect of social conformity on entomophagy (Jensen & Lieberoth, 2019).

Limited availability of insect-based products in food retailers remains a major barrier to consumption, as consumers are more willing to buy or eat them when they are readily accessible (Verneau et al., 2020). Difficulties in finding such products often discourage regular consumption, even among interested consumers. Moreover, even with market availability, the acceptance develops gradually and is influenced by perceived behavioural control, which was the case in Belgium, where only 11% of consumers had tried insect-based foods two years after their introduction (Van Thielen et al., 2019).

Familiarity plays a key role in increasing consumer acceptance of edible insects. This acceptance is often rooted in cultural traditions stemming from indigenous practices or past experiences with food insecurity (Raheem et al., 2019). For those without such traditions, learning about the environmental and nutritional benefits of entomophagy and

familiarity with foreign cuisines may encourage a more positive attitude towards insect-based foods (Cicatiello et al., 2016). Similarly, learning how to prepare them at home increases consumers' openness and willingness to incorporate insects into their diet (Balzan et al., 2016).

1.4.1.2 Elements of the marketing mix

The marketing of edible insects can be enhanced through the 4Ps of the marketing mix: product, price, promotion, and place (McCarthy, 1960). Understanding how each factor shapes consumer acceptance is essential for developing strategies that encourage wider adoption of insect-based foods.

Consumer acceptance of edible insect products is influenced by the insect species used, sensory attributes, product form, and perceived benefits (Alhujaili et al., 2023). Insect species used in the insect-based products can affect taste. Preferred species vary across countries, with crickets, grasshoppers, and mealworms often favoured (Payne, 2015; Simion et al., 2019; Tuccillo et al., 2020). Acceptance tends to increase when insects are processed and invisible in the final product (Bartkowicz & Babicz-Zielińska, 2020; Cicatiello et al., 2020; Collins et al., 2019; Wilkinson et al., 2018).

Taste and texture are decisive factors: good taste and familiar textures promote repeated consumption, while unpleasant flavours deter it (Tan et al., 2016). The choice of food carrier matters, since consumers tend to prefer insects in familiar forms such as pasta, meatballs, or snacks rather than in unconventional foods (Ardoin & Prinyawiwatkul, 2021; Lombardi et al., 2019; Poortvliet et al., 2019). Moreover, they are more willing to try insect-based foods if they are flavoured (Wilkinson et al., 2018). Studies on preferences for sweet or savoury edible insect products yield mixed results, with savoury products performing better in some studies (Lombardi et al., 2019) and sweet products in others (Laureati et al., 2016).

Opinions are also divided on whether insects should be served as appetisers, main courses, or desserts (Caparros Megido et al., 2014). Perceived nutritional value, healthiness, and sustainability can further improve acceptance, especially among environmentally aware consumers (Dagevos, 2021; Nyberg et al., 2020; Petersen et al., 2020; Tuccillo et al., 2020), though concerns about food safety or contamination may reduce willingness to try insect-based products (Castro & Chambers, 2019; A.-J. Liu et al., 2019; Wilkinson et al., 2018).

Price affects consumers' perceptions of both quality and value. Berger et al. (2018) found that consumers are willing to pay up to 80% more for insect-based products when they are associated with premium quality. In contrast, House (2016) and Rehman & Ogrinc (2024) reported that consumers are more likely to purchase insect-based products if their prices are similar to or lower than those of equivalent conventional foods. Conversely, lowering prices may reduce perceived quality, showing that price and quality expectations are closely linked (Wilkinson et al., 2018).

Effective promotion strategies should align messaging with consumer values (Alhujaili et al., 2023). Marketing that emphasises the health and sustainability benefits of edible insects can increase willingness to try them (Van Thielen et al., 2019). Emotional and value-driven appeals (e.g., "good for you and the planet") are often more persuasive than purely informational claims (Onwezen et al., 2019). Clear communication about product safety and transparency in ingredient information also enhances trust and encourages regular consumption (Alemu et al., 2017; Van Thielen et al., 2019).

Availability remains a key factor in consumer acceptance. Consumers prefer to purchase insect-based foods from mainstream supermarkets, followed by health stores and catering outlets, indicating that wider distribution could normalise their consumption (Alemu et

al., 2017; Clarkson et al., 2018a; Van Thielen et al., 2019). Increasing product visibility and accessibility is therefore critical for expanding the market (Van Thielen et al., 2019).

1.4.2 Consumer acceptance of treated wastewater reuse and crops irrigated with treated wastewater

Public acceptance is crucial for the successful implementation of treated wastewater for crop irrigation and later crop consumption. Therefore, it is necessary to evaluate a producer's acceptance of using TWW for irrigation, consumers' acceptance of buying and consuming the crops, and the factors that influence their acceptance. Public resistance has been a significant factor in the failure of many wastewater reuse projects (Verhoest et al., 2022). Therefore, extensive perception assessment should be performed before policy and decision-makers move forward with the use of TWW (Akpan et al., 2020).

1.4.2.1 Factors negatively influencing public acceptance

Social acceptance of TWW reuse is constrained by a range of interrelated psychological, health-related, environmental, and institutional factors. Disgust-based reactions, commonly referred to as the "yuck factor," represent one of the strongest barriers to acceptance and persist regardless of the actual quality of treated wastewater (Garcia-Cuerva et al., 2016; Obijianya et al., 2025; Verhoest et al., 2022). Perceived health risks, particularly concerns about pathogens, chemical contaminants, and long-term health effects, significantly reduce consumers' willingness to consume crops irrigated with TWW (Deh-Haghi et al., 2021; Verhoest et al., 2022) and also discourage farmers from using TWW due to fears of adverse impacts on their own health, their families, and the surrounding environment (Deh-Haghi et al., 2020).

Preference for reuse options involving minimal human contact further reflects these concerns, as people tend to favour applications such as toilet flushing, electricity generation, construction, or car washing, while potable and food-related uses are least preferred (Akpan et al., 2020b). Environmental risks also influence acceptance, with farmers in Jordan expressing concern that the high salinity of TWW could degrade soil structure and long-term soil productivity (Carr et al., 2010). Institutional trust is another critical determinant, as lower trust in responsible authorities is associated with greater resistance to TWW reuse (Al-Rikabi et al., 2025). In contrast, endorsement by medical professionals, academics, experts, and government bodies increases confidence in reuse schemes, in contrast to endorsements from farmers, industrial actors, local municipalities, or media sources, which are viewed with greater scepticism (Akpan et al., 2020b). Finally, inadequate access to information and limited awareness of TWW treatment and reuse processes remain major barriers to acceptance (Deh-Haghi et al., 2021), compounded in some regions by cultural and religious biases against wastewater reuse (Garcia-Cuerva et al., 2016).

1.4.2.2 Factors positively influencing public acceptance

Economic and environmental benefits are key drivers of public acceptance of TWW reuse for irrigation. Reduced irrigation costs and lower reliance on chemical fertilisers have been shown to positively influence consumer attitudes, with cost savings representing a particularly strong incentive for adoption (Al-Rikabi et al., 2025). In Iraq, cost reduction was identified as the primary motivation for TWW reuse across multiple applications, especially among lower-income groups (Al-Rikabi et al., 2025), while in Iran, consumers demonstrated a greater willingness to purchase crops irrigated with TWW when prices were lower than those of crops irrigated with freshwater (Deh-Haghi et al., 2021).

Environmental benefits further reinforce acceptance, as water conservation behaviour, reduced pressure on freshwater resources, mitigation of environmental damage, and a sense of collective effectiveness in environmental protection are all associated with more positive attitudes toward TWW reuse (Al-Rikabi et al., 2025; Verhoest et al., 2022). Awareness amplifies these effects by lowering perceived health and contamination risks and weakening emotional resistance, such as the "yuck factor," through improved understanding of treatment processes and safety standards (Deh-Haghi et al., 2021; Verhoest et al., 2022). At the same time, greater awareness enhances recognition of the environmental and agronomic benefits of TWW, including freshwater conservation and nutrient recovery, thereby strengthening trust in reuse systems and increasing overall acceptance and adoption (Al-Rikabi et al., 2025; Savari et al., 2025).

1.4.2.3 Influence of demographic factors

Consumer acceptance of TWW reuse is also shaped by demographic factors, although their effects vary across contexts. Al-Rikabi et al. (2025) found that women, older individuals, and low-income groups in Iraq exhibited greater reservations toward TWW reuse, particularly due to perceived health risks such as disease transmission and concerns about treatment quality; this was attributed to higher health risk awareness among women and the elderly, and to lower trust in water authorities among economically disadvantaged groups. Similarly, Wester et al. (2015) reported that women in the United States with lower levels of education were more likely to feel uncomfortable with TWW reuse. In contrast, Deh-Haghi et al. (2021) showed that men in Iran were less willing to accept and pay for products irrigated with TWW, while Darabee et al. (2025) observed that higher education levels positively influenced perceptions of TWW reuse. Garcia-Cuerva et al. (2016) further demonstrated that education significantly affects support for water reuse, alongside other socio-demographic factors such as economic status, ethnicity, income, and urban versus rural residence, while age and sex had no statistically significant effect.

1.4.2.4 Future directions

Al-Rikabi et al. (2025) argue that public policies and awareness programs in Iran should prioritise groups that show greater resistance to TWW reuse, particularly women, older individuals, and low-income populations, by strengthening institutional transparency and providing credible health information, thereby reducing the trust gap and enhancing community acceptance. They further emphasise that policy design should align with the social and economic characteristics of local populations to enhance the effectiveness of sustainable water management strategies. Similarly, Deh-Haghi et al. (2020, 2021) highlight the need to raise stakeholders' awareness and actively engage them to increase public acceptance of TWW technologies, while also recommending assessments of local water needs and clear communication about how specific farms and crops can benefit from TWW reuse. In addition, Garcia-Cuerva et al. (2016) suggest a gradual implementation approach, whereby TWW is first introduced in applications with higher social acceptability, such as street cleaning, car washing, irrigation of parks and sports fields, or toilet flushing, allowing acceptance of more sensitive uses, including food crop irrigation and residential lawn watering, to increase as public knowledge and familiarity with the system grow.

Up to this point, the factors that may positively or negatively influence producers' willingness to use TWW for irrigation, as well as consumers' willingness to purchase food produced in this way, and the future directions have been discussed. However, two additional aspects warrant particular attention. First, in regions affected by severe water scarcity, the use of TWW or desalinated water is often not a matter of preference but of necessity, as these may represent the only feasible options for sustaining agricultural

production. In such contexts, the relevant question is therefore not whether these alternative water sources should be used, but how their use can be managed safely and effectively. Second, consumers may already be purchasing foods produced with TWW irrigation without being aware of it. This is especially relevant for fruits and vegetables imported from water-scarce regions, including Israel, Cyprus and southern Spain, where the likelihood of irrigation with reclaimed water is relatively high. Because such products are generally not labelled according to irrigation source, consumers are often unaware of the production water used, which highlights a potential discrepancy between stated attitudes toward TWW reuse and actual purchasing behaviour.

Chapter 2

Aims and Hypotheses

This doctoral thesis was motivated by Europe's reliance on imported protein, growing water scarcity, and the transition towards circular food production systems. It therefore investigates two sustainable food production strategies: the use of farmed edible insects as an alternative protein source, and the cultivation of tomatoes irrigated with treated wastewater (Figure 2.1).

Despite the EU's approval of insects as safe novel foods and as a rich protein source, the quality of farmed edible insects requires further research, specifically on how different insect species, sexes, and developmental stages can be selected to yield the most optimal insects for insect-based feed and food. Therefore, the first objective will be to characterise farmed edible insect species across different sexes and developmental stages and to determine differences between them.

Another aspect, which is gaining increasing attention in the research community and industry, is the use of different processing techniques to improve not only safety but also nutritional quality, sensorial aspects, and shelf-life of insect-based products. Fermentation is one such method with significant potential to improve the nutritional and functional properties of edible insects. However, variations in insect species, fermentation conditions, and analytical approaches limit direct cross-study comparisons. Therefore, a substantial knowledge gap remains regarding how different fermentation cultures and conditions influence the nutritional, functional, and safety characteristics of various insect species. Accordingly, the second objective will be to study how the fermentation of common farmed edible insects with different fermentation cultures influences their quality and oxidation stability.

As discussed in the introduction, the use of treated wastewater can benefit crops by supplying essential macronutrients. However, it can also increase soil salinity and introduce potentially toxic elements, CECs, and micro- and nanoplastics. Although the effects of CECs and potentially toxic elements on the safety of such irrigated crops have already been studied to some extent, the effects of CECs on crop quality remain poorly understood. There is a significant knowledge gap regarding how a wide range of different CECs influence crop quality, not only individually but also in mixtures, which can result in additive and synergistic effects. Different growing media can also result in different effects. The third objective will be to determine how CECs from TWW influence tomato quality grown in soil and in soil-less (hydroponic) systems.

Since metabolomic studies generate large amounts of data, basic statistics alone are insufficient to decode complex interactions. One solution is to use explainable machine learning, which has begun to be used in food science, but has yet to be applied to determining the influence of treated wastewater and CECs on tomato fruit quality. The fourth objective will be to identify those key metabolites or elements that contribute to

the differentiation of tomatoes grown under different conditions in soil and soil-less media using explainable machine learning.

To achieve these objectives, this thesis tests the following hypotheses:

H1: Species, sex, and stage of development have a statistically significant influence on the metabolic profile of edible insects.

H2: Fermentation of edible insects has a measurable effect on their nutritional value and oxidation stability compared to non-fermented ones.

H3: Exposure of tomato plants to CECs, treated wastewater, or sludge has a measurable effect on tomato quality and statistically significantly alters the tomato metabolome.

H4: An explainable machine learning approach can more effectively identify those key metabolites that contribute to the differentiation of tomatoes grown under different conditions in soil and soil-less media than standard statistical methods.

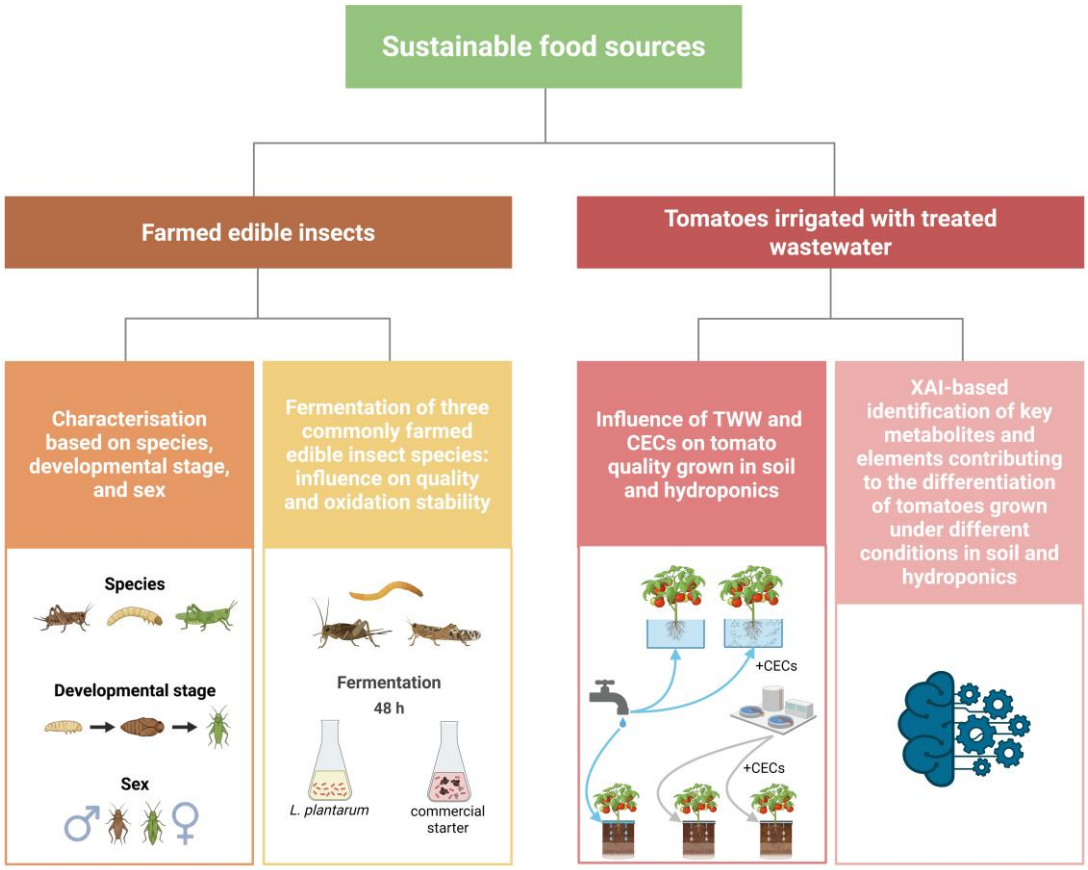


Figure 2.1: Overview of the thesis.

Chapter 3

Publications

3.1 Scientific Review Paper: "Quality, Safety and Authenticity of Insect Protein-Based Food and Feed: Insights from the INPROFF Project"

This chapter presents the paper entitled Quality, safety and authenticity of insect protein-based food and feed: insights from the INPROFF Project" by David Heath, Anja Vehar, Lenka Kouřimská, Martin Kulma, Petra Škvorová, Hana Šubrtová Salmonová, Barbora Lampová, Nayyer Rehman, Polona Jamnik, Barbara Jeršek, Matjaž Červek, Nataša Poklar Ulrih, Ester Heath, and Nives Ogrinc. The paper was published in the journal Exploration of Foods and Foodomics in 2024. This paper reviews the nutritional quality and safety of edible farmed insects from the point of view of the Czech-Slovenian bilateral project: Quality, Safety and Authenticity of Insect Protein-based Food and Feed (INPROFF). It describes four topics: i) nutritional enhancement of insects, ii) safety and quality, iii) authenticity, traceability, and consumer acceptance, and iv) metrological challenges of analysing insects.

The first chapter examines strategies for enhancing the nutritional profile of insect biomass by optimising feed substrates, including the use of alternative feedstocks and fermentation-based approaches. Insects are naturally rich in proteins, lipids, minerals, and bioactive compounds; however, their composition varies considerably with species, developmental stage, sex, and diet. The chapter also highlights the potential economic advantages of rearing insects on food and agricultural by-products, while noting that such substrates must be formulated into nutritionally balanced diets to ensure stable biomass quality. Furthermore, it emphasises that high-quality feed can enrich insects with valuable compounds such as carotenoids and omega-3 fatty acids. Fermentation is presented as an additional strategy to enhance the nutritional, functional, sensory, and safety attributes of insect-derived products.

The second chapter summarises the chemical, biological, and environmental risks to which insects are exposed. Insects can accumulate a variety of contaminants, including toxic elements, pesticide residues, mycotoxins, micro- and nanoplastics, and organic pollutants such as PCBs (polychlorinated biphenyls), PAHs (polycyclic aromatic hydrocarbons), and PFAS (per- and polyfluoroalkyl substances). The biotransfer, bioaccumulation, and biotransformation of these contaminants from feed to insects, and how this uptake varies with species, developmental stage, and rearing conditions, remain insufficiently explored. Similar gaps exist regarding the influence of processing methods on contaminant persistence. Although targeted analytical methods dominate current monitoring, the chapter emphasises the need for advanced tools, such as suspect and non-

target screening with high-resolution mass spectrometry, to characterise the full chemical exposome. Biological risks, including spore-forming bacteria, parasites, food-borne viruses, and insect-specific viruses, also require attention. Research on microbial safety and shelf-life extension of insect-based foods and feeds is still in its early development.

The third chapter highlights growing concerns about fraud in the insect supply chain and the limited availability of analytical tools tailored to insect products. Stable isotope-ratio analysis ($\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$) shows strong potential for verifying diet, geographical origin, trophic level, and product formulation. Early studies demonstrate the feasibility of discriminating origins and detecting adulteration, but broader datasets and improved reference materials are needed.

The last chapter emphasises the many meteorological challenges associated with analysing edible insects, particularly the scarcity of appropriate reference materials for insects, limited interlaboratory comparisons, calibration difficulties in LC-MS (liquid chromatography-mass spectrometry) analysis, matrix effects, and the need for harmonisation in non-target screening workflows. Accurate measurement frameworks will be essential to support regulatory acceptance, risk assessment, and certification schemes. This paper makes an important contribution to science by critically examining knowledge gaps in the fields of nutritional quality, chemical and microbiological safety of edible insects, authenticity testing, and metrology in edible insect research.

As joint first author, I contributed to the conceptualisation, co-authored the section on the authenticity and traceability of edible insects, was responsible for visual materials, and shared responsibility for final editing.

Exploration of Foods and Foodomics

Open Exploration

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Quality, safety and authenticity of insect protein-based food and feed: insights from the INPROFF Project

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Abstract

This paper reviews the nutritional quality and safety of edible farmed insects from the point of view of the Czech-Slovenian bilateral project: Quality, Safety and Authenticity of Insect Protein-based Food and Feed (INPROFF). Insects as a sustainable source of dietary protein for animal feed and even humans, when integrated into the European agrifood system, could offer a solution to Europe's feed protein deficit and help alleviate environmental pressure from increasing protein demand, such as declining availability of land, water, marine and energy resources, the overuse of pesticides and reduced biodiversity. However, despite a growing interest in the European Union (EU) in farming edible insects, many economic, scientific, technological, and social barriers remain. In response, Slovenia, represented by the Jožef Stefan Institute, the Biotechnical Faculty of the University of Ljubljana, and Jata Emona d.o.o. (the leading supplier of feedstuffs for the Slovenian market), a country with no history of insect rearing but an interest in alternative protein, joined with the Czech University of Life Sciences Prague, representing the Czech Republic—a country with a history of insect rearing and research into edible insects—to establish INPROFF a three-year bilateral project that aims to close the knowledge gap regarding the quality and safety of insect-based products and boost the farmed insect food and feed value chain. Specifically, it comprises three thematic pillars: (P1) nutritional enhancement, (P2) safety and quality and (P3) authenticity, traceability, and consumer acceptance. The paper also discusses the gaps in the metrological challenges of analysing

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insects, which will be critical for ensuring safety, quality, and sustainability. The paper finds that although much work has been done, many exciting avenues remain for new research.

Keywords

Farming insects, alternative protein, quality, fermentation, safety, authenticity, traceability, metrology

Introduction

As demand for sustainable protein intensifies, many food scientists propose using farmed insect protein for animal feed and even for human consumption. Moreover, in 2013, the Food and Agriculture Organisation (FAO), a part of the United Nations, published a report recommending consuming insects as a sustainable solution to the shortage of world food supplies [1] and are now generally accepted as a sustainable way to help alleviate Europe's feed protein deficit having a significant role in the EU's protein strategy [2–5].

Unlike wild-caught insects in many parts of the world, farmed insects in Europe are considered (legally) no different from other farmed animals and must comply with National and EU legislation, and although a full review of the EU legislation is beyond the scope of this paper, the growth of the farmed insect industry will be significantly impacted by the legislation. Historically, given that insects were not consumed in the countries that make up the EU, they are covered by Regulation (EU) 2015/2283 on Novel Foods [6], which governs the authorisation and marketing of novel foods, including insects, within the EU following an assessment by the European Food Safety Authority (EFSA) and, as of the time of writing, it includes yellow mealworm (*Tenebrio molitor*), house cricket (*Acheta domesticus*), migratory locust (*Locusta migratoria*) and the buffalo worm (*Alphitobius diaperinus*). Producers must also comply with, among others, regulations regarding general principles and standards governing food and feed safety (Regulation [European Commission (EC)] 178/2002), hygiene rules [Regulation (EC) 852/2004], animal health [Regulation (EU) 2016/429], proper labelling with regards allergen information, nutrition labelling, and country of origin labelling [Regulation (EU) 1169/2011]; importation [Regulation (EU) 2019/626], and use of animal by-products [Regulation (EU) 142/2011] [7–12].

Strict regulations also exist regarding animal feed, including placing feed on the market concerning safety, labelling, and traceability requirements [Regulation (EC) No 767/2009] and a catalogue of authorised feed materials, which includes insects and their products, that can be used in animal feed formulations [Regulation (EU) No 68/2013] [13, 14]. Moreover, in the EU, insect protein is classified as processed animal protein (PAP), and its use, production, and labelling are strictly controlled under specific rules [Regulation (EU) 2017/893] [15]. Insects approved for animal feed in aquaculture include black soldier fly (*Hermetia illucens*), yellow mealworm, common housefly (*Musca domestica*), lesser mealworm (*Alphitobius diaperinus*), house cricket, field cricket (*Gryllus assimilis*) and, as of 2024, the silkworm (*Bombyx mori*) pupae are authorised for use in aquaculture feed. The recent changes to the regulations allowing certain insect PAPs in pig and poultry feed represent a boon for the farmed insect industry. However, ruminants still cannot feed insects due to transmissible spongiform encephalopathies (TSEs). Overall, many producers and promoters of edible insects feel that legislation regarding what substrate (e.g., food waste) insects can be fed is restrictive and is holding back the field [2]. A detailed review of all the regulations relating to EU law is published by the International Platform of Insects for Food and Feed (IPIFF) [16].

Even though insects are now on the market and have passed scrutiny by EFSA, there remain many gaps in the knowledge regarding farmed insects and many challenges in improving their economic viability (feed modification) and acceptability to the consumer. In response, in 2021, the Jožef Stefan Institute (JSI), the Biotechnical Faculty of the University of Ljubljana (UL-BF), the Czech University of Life Sciences Prague (CZU) and Emona Nutrition Research & Development Department of Jata Emona d.o.o. (ENR-JE), Slovenia's leading feed producer established a joint three-year bilateral research project INPROFF: Quality, Safety and Authenticity of Insect Protein-based Food and Feed. The project, jointly funded by the Slovenian Research

Agency (ARIS) and The Czech Science Foundation (GAČR), is designed to generate new knowledge and stimulate scientific cooperation. It employs various strategies to close the knowledge gap regarding the quality and safety of insect-based products. The project focuses on the yellow mealworm, house cricket and migratory locust. These were chosen because they are already on the market and are relevant as both food and feed.

At the project's core are three thematic pillars: (P1) nutritional enhancement, (P2) safety and quality and (P3) authenticity, traceability and consumer acceptance, all of which are underpinned by one horizontal activity: (H1) insect production and characterisation (Figure 1). Pillar I seeks to optimise the nutritional quality of insect biomass by investigating the rearing of insects on both conventional and alternative feeds and through fermentation of their biomass. Pillar II is concerned with the safety of farmed insects by developing advanced analytical protocols to assess their chemical and biological safety. Finally, Pillar III is about developing forensic tools to verify the authenticity of insect-based products, promoting transparency and trust in the market, as well as seeking to understand consumer attitudes and acceptance towards eating insects. Moreover, the project is intended to catalyse future collaborations for continual advancements in insect farming research and innovation.

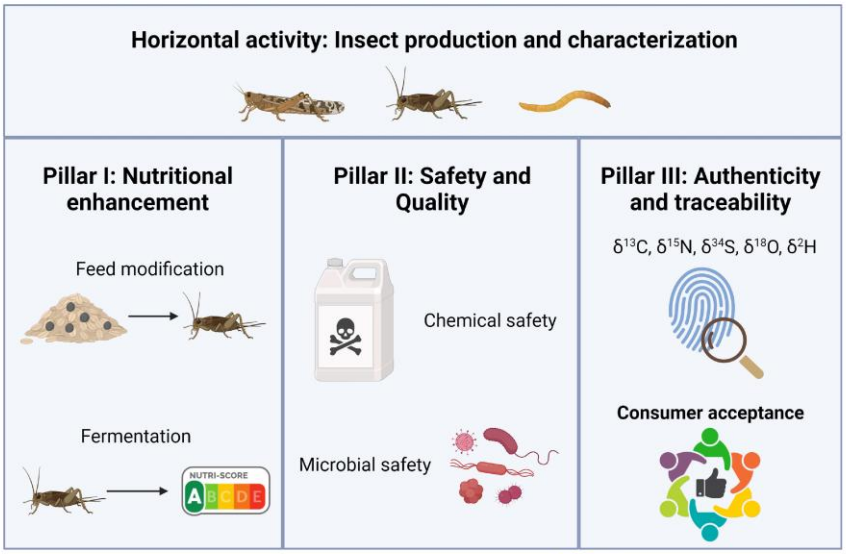


Figure 1. Thematic pillars of the project. Created with BioRender.com

In this paper, we review the current state-of-the-art and challenges regarding farmed edible insects pertaining to the three thematic pillars of the INPROFF project. Among four primary academic peer-reviewed databases: Google Scholar, Scopus, Web of Science, and Science Direct, Scopus was selected as it is one of the leading international and prominent databases, familiarity, and delivered a significant number of hits for the search term "edible insects" (Table 1). The search term "edible insects" although it includes wild-caught species not covered by this review, was a better catch-all term than "farmed insects" and "entomophagy". The search was restricted to the following fields: Title, Abstract, and Author Keywords. Acceptance criteria included all articles published in English between 2004 and 2024. The analysis identified over 2,000 papers going back 20 years (Table 1). A comprehensive list of search terms is provided in the supplementary information (Table S1), while Table S2 presents the search results (hits) for the term "edible insects" along with the studied species. The findings indicate that the most studied species is "*Tenebrio molitor*".

Table 1. Search results (hits) for the search term "edible insects" in different databases and results of connected terms performed in Scopus (Search fields: article title, abstract, keywords)

Search term	Hits (2004–2024)
"Edible insects"	
Google Scholar	1,710
Scopus	2,011
Web of Science	1,401
Science Direct	673
"Insects"	236,679
"Edible insects"	2,011
"Entomophagy"	866
"Farmed insects"	43
"Edible insects" AND …	
"Nutrition" (Pillar I)	315
"Safety" (Pillar II)	362
"Authenticity" (Pillar II)	12
"Metrology"	0

When the number of publications is plotted against years, the results reveal how scientific interest in edible insects has increased significantly since 2014 and the publication of the FAO's paper on *Edible Insects Future Prospects for Food and Feed Security* by van Huis et al. [1] (Figure 2). The retrieved articles were further probed using targeted keywords tailored to each pillar theme to identify significant knowledge gaps, and then they were systematically scrutinised for inclusion in the review. Regarding the different thematic pillars, safety, followed closely by nutrition, produced similar hits. The search term "authenticity" produced a significantly smaller number of hits, while the term "metrology" produced no hits (Table 1). The full results are presented in the supplementary (Table S1).

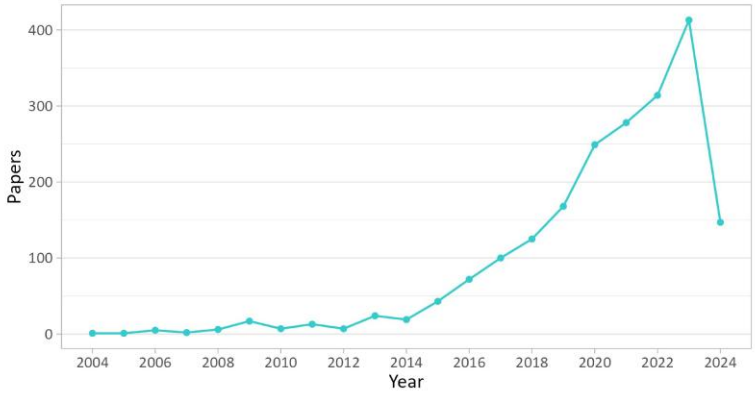


Figure 2. Plot of number of papers vs. publication year (Search fields: article title, abstract, keywords: "edible insects", data: Scopus)

Pillar I: enhancing the nutritional quality of insect biomass

As mentioned, as part of a more sustainable European protein strategy, farmed insect protein content is a viable option for becoming more self-sufficient in protein production for feed and human consumption. Pillar I aims to achieve two objectives: enhancing the nutritional and functional value of insect biomass and adapting insect feed composition to include agri-byproducts, thereby enhancing their nutritional quality. For instance, the INPROFF project explores how fermentation can boost nutritional values, sensorial

properties, and safety/shelf life. It also looks at ways to modify insect feed by adding agricultural by-products to make insects more economically viable and competitive with conventional protein sources, such as soybeans.

The literature review reveals that farmed insects are a rich source of easily digestible quality protein [≈ 40–75 g/kg of dry matter (DM)] equivalent to meat [17, 18]. They can also provide most of the essential amino acids, including lysine, leucine, phenylalanine and tryptophan, fat and lipids (approx. 20–55 g/100 g) [19, 20] along with several essential fatty acids with total polyunsaturated fatty acids (PUFA) accounting for 3–50% [20]. They also contain fibre (8.5–27 g/100 g) and carbohydrates, mostly chitin (2.7 mg and 49.8 mg per kg of fresh weight), as well as minerals (Ca, K, P, Na, Mg, Cu, Fe, Zn, and Mn) and vitamins A, D, E, K, and C and B groups vitamins [20]. Insects also represent an excellent sustainable source of chitin (5–15% w/w) [21]. The latter values were obtained using a reliable electrophoresis assay developed within INPORFF, which overcomes the inherent problem of being unable to distinguish between chitin and cellulose.

Bioactive compounds like antioxidant enzymes and peptides are also present in insects [22], some of which have therapeutic potential [23]. For instance, cantharidin from blister beetles can induce apoptosis in cancer cells by affecting nucleic acid and protein metabolism in hepatocellular carcinoma cells. Cecropin peptides found in some insects have shown cytotoxic effects against leukaemia and lymphoma cells by forming pores in their plasma membranes [24]. Traditional Chinese medicine frequently utilises insect extracts to treat various ailments, including cancer [23]. Not all bioactive compounds are necessarily beneficial. Insects belong to foods with high levels of total purine (3.23–13.22 g/kg DM) and uricogenic purines (adenine and hypoxanthine: (1.55–8.86 g/kg edible DM), which contain amounts analogous to meat (3.61–5.53 g/kg meat DM) and are a problem for those who suffer from gout and kidney stones [25]. From this perspective, the study's authors conclude that the study insects make unsuitable alternatives for low-purine diets.

Although polyphenols are commonly associated with plants, insects also contain polyphenolic compounds, which act as antioxidants with anti-inflammatory, anti-cancer, antityrosinase, antigenotoxic, and pancreatic lipase inhibitory activity [26]. Insects also possess polyphenols serving as pigments and chemical defences, and they can synthesise and store non-dietary phenolic compounds through sclerotisation [27]. They also have the capability to selectively absorb and accumulate flavonoids in their bodies from the larval stage via their host plant, so their diet is important in determining the type and amount of polyphenols present [26]. Research into the content and specific bioactive properties of insect-derived polyphenols remains ongoing.

Nutritionally, the case for insects is strong; however, more rigorous analyses are needed, including assessments of insect nutrients not thoroughly investigated (particularly vitamins), the presence of non-nutrients and anti-nutrients and how they influence the digestibility and bioavailability of nutrients in various species and processed products. A lack of such studies also means there is currently a limited understanding of how insect-based foods affect the human and animal gut microbiome [28]. Imbalances in the human gut microbiome are linked to metabolic and non-communicable diseases, gastrointestinal conditions, allergies, asthma, and even neuropsychiatric disorders [29]. An even more significant gap exists (search hits = 8) in our understanding of the insect gut microbiome.

Insects are also promoted as a more sustainable option than conventional non-plant protein sources [30–32]. For example, the rearing of insects through vertical farming, which, unlike locusts, is particularly suited to the mealworm, requires far less land (3.6 m^2/kg of fresh insects to 94.7 m^2/kg of dry insects), significantly less water (0.42–0.82 m^3 of water depleted per 1 kg of fresh insects); have highly efficient feed conversion rates (50–70%); produce less waste and amenable to further processing and rearing in modern production systems [33–35]. The result is lower greenhouse gas emissions (2.3–3.1 kg CO_2 eq per kg of fresh insects) than other non-plant protein sources [36, 37]. Insects, those in larvae form, can also serve as aqua feed alternatives, replacing traditional non-sustainable options like fish meal [3].

The nutritional content of insects varies based on numerous factors, which means that it can be improved using specific measures in farming technology to improve the efficiency of the animal's diet [38]. For insects to compete economically with other sources of animal feed, it will be necessary to rear them on by-products and waste from the food industry and agriculture, i.e., transforming low-value organic by-products into high-value food or feed [32, 39, 40], thereby reducing costs and increasing the economic viability of insect-based feed [41]. However, this will require the development of nutritionally balanced insect diets. Similarly, insects can also be a source (bio-factories) of other valuable secondary commodities for pharmaceuticals, biofuels and lubricants [42, 43], and their waste (frass) can be used as fertiliser [44, 45].

Farming edible insects allows an opportunity to manipulate the nutrient content of insects via their feed [46, 47]. For example, the transfer of beneficial substances such as carotenoids or omega-3 fatty acids (n-3) from feed to insects has been observed [38, 48]. These fatty acids, omega 3 (n-3) and omega 6 (n-6), are required in mammalian diets because mammals cannot synthesise these de novo. Although essential for health, Western diets contain too little n-3 and too much n-6 PUFA. Increasing the beneficial fatty acids amounts of n-3 PUFA, which would result in more favourable n-6/n-3 ratios, would benefit animal feed and humans since it can reduce coronary heart disease, cancer, autoimmune and inflammatory diseases [49]. For example, Škvorová et al. [47] have shown that rapeseed cake can substitute soybean meal in feeding the field cricket, resulting in higher crude protein levels and lower amounts of palmitic acid.

Fermentation is an effective method for enhancing the nutritional properties of certain foods and is being investigated to extend their shelf life [50, 51], microbiological safety and sensory characteristics [50, 52, 53]. It is also being used as a way of increasing the efficiency of their original bioactive compounds (functional value) via their release or transformation by lactic acid bacteria (LAB) as well as enriching the substrate in their metabolites, e.g., polysaccharides, bioactive peptides, vitamin B12, short-chain fatty acids [54, 55]. These transformations are associated with improving the bioaccessibility and bioavailability of food components and their health-related properties [55].

Moreover, fermentation can also increase the digestibility and stability of waste before being utilised as insect feed by converting food and by-product wastes into ingredients for artificial insect diets [51, 56]. *Companilactobacillus* appears most promising, resulting in a significant increase in free glutamic and aspartic acid. These compounds are responsible for the umami flavour of fermented foods and might stimulate consumer acceptance of insects and their consumption [57]. Hadj Saadoun et al. [58] applied lactic acid fermentation of waste to the puparia and dead adults of black soldier flies. The authors showed that the fermented puparium has a high antimicrobial activity against three pathogenic strains: *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli*. The authors also reported a shift in lipid composition from that typical of insects to one containing fatty acids, while the LAB strains could modify the biomass's protein composition depending on the specific LAB strain and the substrate.

Pillar II: safety and quality

Insect products regulated as novel foods or as feed are considered safe, having undergone an extensive risk assessment process, and, in the EU, where insects are allowed on the market, have to adhere to strict EU regulations [59, 60]. For example, [Regulation (EU) 2023/915] establishes maximum permitted levels in food for the specific contaminants [e.g., toxic elements (cadmium, lead, mercury, inorganic tin, arsenic), plant toxins (erucic acid)], mycotoxins (e.g., aflatoxins, ochratoxin A, fumonisins, and citrinin), processing contaminants [e.g., 3-MCPD, dioxins, dioxin-like polychlorinated biphenyls (PCBs) and nondioxin-like PCBs, polycyclic aromatic hydrocarbons (PAH), and polyfluorinated substances (PFAS)], and others (e.g., melamine and nitrate) [61]. However, despite the work on looking at the safety of insects [32, 46, 62–70], like with any new food or feed, there will be known and unknown so-called emerging risks [70]. A key part of the INPROFF project involves characterising less well-studied contaminants' chemical and biological safety using state-of-the-art instrumentation. This task involves performing target analysis and developing nontarget screening workflows, which have yet to be fully utilised in the literature. The project also

investigates the effect of specific bioactive substances and their cytotoxicity using in vitro gastrointestinal cell cultures.

Farmed insects, fed on cereal-based feeds, could be exposed to toxic elements, pesticide residues, mycotoxins and other biologically produced toxins and biohazards (bacteria, viruses, fungi, parasites), food chemicals and additives of natural or non-natural origin, many of which have the potential to bioaccumulate and physical foreign matter for example from the degradation of production equipment [66, 68, 71, 72]. Modern intensive farming production methods, even for minilivestock (insects), offer many routes for potential sources of contamination across the entire food supply chain, particularly given the many unknowns associated with scaling up insect production systems [70, 73, 74]. For insects destined for feed (e.g., black soldier fly), the risk of exposure will be amplified if, in the future, insects are reared on agri-byproducts, mixed wastes from processing plants, supermarkets and even restaurants [75].

Chemical contamination, especially the biotransfer, bioaccumulation, and biotransformation of contaminants from feed to food [76] and how their uptake is affected by species, life stages, and rearing environment [77] is also underexplored. The same is true concerning the effect that processing methods have on the presence of chemical hazards, even though there is some evidence that processing methods like boiling, frying and roasting increase the safety of edible insects [71]. However, conventional cooking conditions will most likely not be an effective way to eliminate contaminants such as per- and polyfluorinated substances (PFAS), so-called "forever chemicals" [78, 79] with a high potential to bioaccumulate [80]. Other potential risks addressed in food/feed but not yet studied concerning insect rearing include, for example, the uptake of micro and nano plastics from feed [81, 82], which could by themselves be problematic or act as carriers for other contaminants [83, 84]. Reducing such risks will require greater control over their feed and environment and the development of high throughput put and methods capable of detecting a broader range of compounds.

Among the now large number of publications, the majority are target analyses of a few compounds or multi-residue analyses [68, 85]. Targeted methods focus on detecting and quantifying pre-defined compounds or analytes in a sample, which can miss other potentially hazardous groups of contaminants present in the samples. In response, many scientists have turned to suspect and nontarget screening methods to define the chemical characterisation of particular samples [74, 80, 86].

The sheer number of potential organic chemical contaminants and their mixtures, which are constantly changing, also raises problems in assessing risk, and focusing on only a few specific target compounds could mean other hazards go undetected. For this reason, attention is turning towards exploring alternative analytical workflows, such as suspect and non-targeted ones, as part of an exposome concept [87]. Suspect screening involves searching for compounds on a pre-defined list of "suspect" compounds or compound classes, often based on their chemical properties or structural features [88, 89]. Such approaches offer a more comprehensive assessment by detecting unexpected contaminants or adulterants, enhancing the detection of emerging risks that may not be accounted for in targeted analyses, i.e., non-targeted analysis aims to screen and identify compounds present in a sample without prior knowledge. This ability to perform such analysis is partly due to the introduction of Time-of-Flight (ToF) and Orbitrap-mass spectrometry (MS) high-resolution benchtop instruments, which have the advantage of using full-scan acquisition mode with high sensitivity, high resolving power and accurate mass measurement, allowing the rapid characterisation of potentially thousands of compounds [88, 90, 91].

Ideally, all three approaches—targeted, suspect, and nontarget—are sequentially performed on the same set of samples. However, few insect-related studies have been published using the described approaches [74, 80, 86]. For instance, Poma et al. [74] performed targeted screening of organic contaminants [flame retardants, PCBs, dichlorodiphenyltrichloroethane (DDT), dioxin compounds and selected pesticides] and additional suspect pesticide screening of edible insects and insect-based food intended for human consumption. The organic chemical mass fractions of targeted compounds were typically lower than in common animal products. In contrast, the untargeted screening analysis revealed the presence of vinyl toluene, tributylphosphate (present in 75% of the samples), and pirimiphos-methyl

(identified in 50% of the samples)—frequently identified contaminants that would have been missed with a targeted approach. Li and Bischel [80] also performed target and suspect screening to evaluate the bioaccumulation of trace organic contaminants in black soldier fly insect larvae fed on almond hulls, a by-product from the almond industry. Their study showed that the four targeted per- and polyfluoroalkyl substances (PFAS), perfluorobutanoic acid (PFBA), perfluorooctanoic acid (PFOA), perfluorobutanesulfonic acid (PFBS) and perfluorooctanesulfonic acid (PFOS), did not bioaccumulate in the larvae. However, employing a nontarget approach uncovered the presence of other PFAS compounds, specifically hydrogen-substituted polyfluoroalkyl carboxylic acids (H-PFCA), which were found to bioaccumulate in the larvae and were correlated with their presence in the feed (almond husks).

Similarly, Macan Schönleben et al. [86] performed target and suspect pesticide screening of 52 edible insects from Asia, Africa, and Europe. Fifty-six pesticides were determined using target (30) and suspect (26) screening approaches. Notably, in the case of targeted screening, the neonicotinoid imidacloprid had the highest detection frequency (DF, 70%), followed by fipronil (48%). The findings indicated that the levels of dichlorvos and fipronil were in the same order of magnitude as specific maximum residue limit (MRL) values for meat. Most detected compounds were below the defined MRLs. However, it underscores the necessity for further extensive investigations to provide a comprehensive chemical characterisation of potential risks relating to edible insects as well as the need to establish (MRL) values for edible insects. These limited studies that do not focus entirely on target analysis show the importance of suspect screening for providing valuable insights into food/feed chemical characterisation and facilitating a more proactive and holistic approach to ensuring the safety of farmed insects. However, the full potential of applying a nontarget approach is still to be explored.

Biological risks must also be considered when contemplating the use of insects. The microbiota of insects, found in the gut and other anatomical parts, is complex. While the microbiota of some commercially essential insects has been analysed [50, 92–95], more work in this area remains to be done. This task is essential, as insects tend to be consumed entirely, with a few exceptions. Although most pathogenic microbes that infect insects are harmless to humans due to phylogenetic differences, insects can be vectors or carriers of harmful microbes, primarily when insects are reared under poorly controlled hygienic conditions [96]. Among the most harmful bacteria that inhabit all three insect species analysed within the INPROFF project (yellow mealworm, house cricket, and migratory locust), *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus* could be mentioned. Several bacterial species have been associated with edible insects, both farm-reared and wild-caught, and some of these are not only pathogenic and opportunistic bacteria, but they can also reduce the shelf-life of edible insects [97].

Endospore-forming bacteria in edible insects are also a significant food safety concern as the heat-resistant spores may withstand the standard processing methods adopted for edible insects, like drying, boiling, and deep-frying [98]. In this context, the *Bacillus cereus* group seems to be one of the most suitable parameters for describing food safety and microbial quality [99]. So far, risks associated with foodborne viruses, like hepatitis A, hepatitis E and norovirus, from consuming edible insects are low. However, care must be taken not to introduce the viruses in insect production units through their feed and unsafe handling practices. Insect species deemed fit for mass production may be vectors for parasites, although this risk may not be exceptionally high and is poorly documented [100, 101]. Contamination of edible insects post-processing is also an area of consideration [102].

The study of the pathogenicity of insect-specific viruses in humans is also an area for further study. Insect viruses are assumed to be specific to insect species and cannot replicate in vertebrate cells. Despite this limited specificity, there is a negligible risk of new strains of mammalian-specific viruses emerging due to recombination and rearrangement of genetic material, potentially leading to host switching. Although pathogenic viruses of vertebrates cannot replicate in insects, edible insects can still passively transmit these pathogens [63, 103]. Research into extending the shelf-life of processed or farmed insect-derived foods and feeds and microbial studies on farmed edible insects is still in its early stages [104]. Similarly, there is a gap in our understanding of other microbial risks, e.g., protists [105]. Most microbes in or on insects are not

considered harmful to human consumers. However, the microbial safety of insect-based foods intended for human consumption is still under debate, as insects could be vectors of potentially pathogenic bacteria.

Pillar III: authenticity, traceability, and consumer acceptance

The European Commission estimates that the total cost of food fraud globally is about 40 billion Euros annually [106]. Without the necessary tools, a nascent insect farming industry is ripe for economic fraud, such as product adulteration and mislabelling. The mapping of the insect supply chain in the study performed by Traynor et al. [70] illustrates the complexity that exists. This situation is worsened by the lack of standard procedures for determining the authenticity of insect products [107]. The literature review reveals a significant literature gap when searching "edible insects" and "authenticity" (hits = 12), with few studies investigating the potential fraud in edible insect food supply chains. INPROFF was partly set up to fill this gap by taking advantage of the IJS food authenticity and traceability expertise and applying it to farmed insect products. This task is made possible due to significant progress in developing accurate tools for determining the species-level authenticity of food and detecting adulterants [108–111].

One such tool involves the use of the stable isotope approach. Stable isotopes of light elements (expressed in δ^2H, δ^{13}C, δ^{15}N, δ^{18}O, and δ^{34}S) have been widely used to investigate food authenticity and origin [112], although for farmed edible insects for food and feed its use is limited to a few examples and is more widespread in insect ecology [113]. Nevertheless, the information obtained in ecological studies contributes to the knowledge needed to determine the authenticity of insects for food and feed. For example, the δ^{13}C values are strongly influenced by diet, providing insights about whether the insects fed on C3 or C4 plants [114]. The δ^{34}S values are also related to the food insects consume. The values are influenced by geology, microbial processes in the soil (including aerobic and anaerobic growing conditions), the distance from the sea ("sea spray effect") and fertilisation techniques [115, 116]. The δ^{15}N values of the animals are strongly related to their trophic level [117]. Herbivorous species are expected to have a lower δ^{15}N than carnivorous ones, as ^{15}N accumulates along the trophic chain [112]. The δ^2H and δ^{18}O of water are strictly related parameters [118] that depend on latitude, altitude, and even distance from the sea [119]. For plants, the correlation between δ^2H and δ^{18}O is still valid but not that strict, as the only source of hydrogen is the water absorbed through the stomata [120]. As the sources of oxygen and hydrogen of the insects are both the water they drink and the plants they eat, their δ^2H and δ^{18}O are influenced by such factors.

Pianezze et al. [121] analysed 40 samples of edible insects and four insect-based food items, providing δ^{13}C, δ^{15}N, δ^{34}S, δ^{18}O, and δ^2H data. Due to ingredient and flavour additions, they found δ^2H and δ^{18}O helpful in differentiating natural from seasoned insects. Other parameters, like δ^{13}C, δ^{15}N and δ^{34}S, showed promise in characterising insect diet, geographical origin, and trophic level. Macan Schönleben et al. [86] studied 52 edible insect species from Asia, Africa, and Europe, focusing on δ^{13}C, δ^{15}N, δ^{34}S, and δ^2H for food authenticity and traceability. Linear discriminative analysis differentiated geographical areas, with African samples showing distinct profiles, and accurately classified pesticide presence based on δ^{34}S values at a 76% rate.

INPROFF is developing a crucial component in food authenticity and traceability: establishing a reference dataset based on genuine samples, as highlighted by Donarski et al. [122]. This dataset will serve as a benchmark against which insect-based products in the market can be compared. Moreover, many aspects must be considered when developing such a database, including insect species, diet, environment, developmental stage, and processing techniques.

A study by Fuso et al. [123] further evaluates the analytical tools currently available to authenticate insects and their rearing substrate. The study emphasises the need for specialised analytical methods to detect rearing substrate residues in insect products or to reveal metabolomic changes caused by different feeds. A novel method for authenticating edible insect powders was recently introduced by Tata et al. [124] based on obtaining direct analysis in real-time high-resolution MS (DART-HRMS) signatures, marking it the first application of ambient MS to screen novel foods. The study successfully differentiated between four

test species (house cricket, yellow mealworm, migratory locust and silkworm) with a cross-validation accuracy of 100% for all training samples. A distinct metabolic fingerprint was observed for house cricket in particular. Additionally, Poma et al. [125] explored the potential of entometabolomics in food systems, marking a crucial step towards understanding edible insects' metabolic profiles that can be used to identify the authenticity of insect food.

Consumer acceptance

For humans, the eating of insects, termed "entomophagy", is not new and many species are already consumed by much of the world's population. The literature commonly quotes two billion plus people who regularly consume insects and suggests that insects could become a regular part of the Western diet [64]. However, entomophagy is not a cultural norm in Europe, and convincing those not accustomed to eating insects remains a significant hurdle to their acceptance. However, research is dedicated to making insects more palatable [126]. Today, insect-based products such as pasta, snacks, and protein bars are available on the European market, projected to be two billion euros/year by 2030 [127].

Food scientists have identified various factors influencing consumer acceptance of insect consumption, among which food neophobia and disgust remain the primary barriers to eating insects [128–130]. However, familiarity, tradition, and sensory attributes also affect the willingness to consume insects across different geographical locations and cultures [131]. While Western countries emphasise nutritional value and sustainability, sensory qualities and cultural practices play a more substantial role in regions where entomophagy has a longstanding tradition [132, 133].

The availability of information and prior experience with edible insects can positively shape consumer attitudes [134–136]. In Western societies, incorporating insects into familiar food products and communicating their environmental and nutritional benefits can enhance their acceptance [126, 131]. Additionally, focusing on product development that aligns with the sensory expectations of consumers [137, 138] and sparks curiosity about edible insects [139, 140] can lower the initial resistance towards them. Within the INPROFF project, a study performed by Kulma et al. [141] found that in the case of the Czech Republic, a significant percentage of those taking part described their entomology experience as positive (43.8%), 11.8% stated they regularly consumed insects, a small fraction (6.7%) reported negative experiences, and 37.7% remained neutral. Also, the data showed consumer preference leaned towards insect powder and processed whole insects such as crickets, katydids, and locusts, with cockroaches being the least favoured.

Metrological approach and challenges

All three pillars of the INPROFF project rely on analytical methods, and ensuring robustness between and comparability of the data obtained requires using appropriate metrological tools. Metrology is critical in ensuring that food products meet safety standards and regulatory requirements across the food supply chain, which can only be achieved through accurate, consistent, and traceable measurements. Typically, these include quality reference standards, validated standardised methods, standardised sampling practices, proven calibration methods and, where possible, matrix-specific certified reference materials (CRMs) in addition to reference materials (RMs), and interlaboratory comparisons are used to achieve comparability of analytical results [142]. Despite its importance in food research, this literature review found no specific studies (zero hits) applying metrology to farmed insects or significant discussion regarding its importance to the field.

For food products, including edible insect products, metrology is the foundation for supporting accurate labelling of food products, including nutritional information, ingredient lists, allergens, and expiration dates [143]. As the determinants of general nutritive quality (Pillar I), proximates such as lipids, carbohydrates, proteins, and minerals must be considered. Here, the standards are general reference methods collected under the International Organization for Standardization (ISO) system [144]. Moreover, within lipids and proteins, the content of essential components, such as essential amino acids and essential fatty acids, are determined by amino acid analysis and multidimensional gas chromatography [145]. In the

case of trace nutrients such as elements and vitamins, the methodology is much less standardised and under ongoing development [143].

Analytical methods for many known organic contaminants (Pillar II) in food have undergone extensive validation and standardisation [146]. Among the "new age" pollutants, pesticides are probably the most studied and multi-trace residue analysis methods are preferred over single-residue methods [147]. However, as already discussed earlier under Pillar II, the range of these pollutants is not complete and is expanding as the knowledge of the risks of consuming novel foods such as insects increases. Also, contaminants can transform in, for example, animal-derived food and predicting the structure and toxicity of their transformation products is a developing trend [148].

In trace analysis, quantitation is also more susceptible to analytical errors and statistical imprecision and demand methods with increased sensitivity and specificity, which, especially for the latter, is effectively achieved through chromatography (most commonly liquid) hyphenated to MS. However, although liquid chromatography-MS (LC-MS) is a highly specific and sensitive analytical technique, matrix effects are often caused by the alteration of ionisation efficiency of target analytes in the presence of co-eluting compounds in the same matrix and often impede signal intensity and even mass accuracy. Accordingly, the evaluation of matrix effects is explicitly suggested by regulative bodies like the United States Food and Drug Administration (US FDA) guidelines on bioanalytical analysis [149].

One of the most common approaches to account for uncertainty issues in target analysis is using an internal standard (IS). These are typically suitable surrogate compounds with properties similar to the target analyte, such as chemical structure, solubility, and volatility. In MS, they are ideally isotopically labelled chemicals (^{13}C, ^{15}N, or ^{2}H) since they are absent in samples but have the same characteristics. In MS-based target analysis of organic micropollutants, the addition of IS is used to compensate for losses of physicochemically similar compounds when internal calibration approaches are used. When targeting more extensive groups of contaminants, the set of IS should ideally cover the whole range of the chemical space to be explored (retention time, mass range, ionisation mode, and physicochemical properties). However, besides being impossible to account for all substances potentially present in the sample, only a limited number of compounds are commercially available in isotope-labelled form, and high price also limits their application [150].

The trend towards non-targeted high-resolution MS (HRMS) also raises metrological issues. Regarding nontarget analysis and suspect screening, methods focus on compound identification rather than quantitation. One of the most important scopes of NTA is that it can directly support chemical risk characterisation but must provide quantitative estimates. Even though limited quantitative NTA methods exist, they do not fully consider estimation uncertainty, and most nontarget approaches still need harmonisation and validation [151, 152]. Equally, in the case of new threats such as from nanoparticles and micro-/nanoplastics, as also discussed in Pillar II, their detection and quantification are limited by the complexity of the food matrix absence of suitable standards, making their identification and quantification challenging and represent major bottlenecks [153].

From a microbial risk perspective, appropriate ISO norms and standardised procedures exist to determine biological safety for classical food hygiene parameters [154]. For example, total aerobic and anaerobic bacterial counts, counts of *Staphylococcus aureus*, *E. coli* and *Enterobacteriaceae*, *Clostridium perfringens*, spore-forming bacteria, enterococci, and the presence of *Salmonella* were determined. *Bacillus cereus* (a microbial quality indicator) was detected according to ČSN EN ISO 7932 [155], identified by 16S rDNA sequencing, and its pathogenic potential was studied by detecting virulence-associated genes.

Another challenge is maintaining consistency and reproducibility in constructing microbial communities, especially in highly diverse microbial communities as in the case of fermentation [156, 157] or studying the effects of consuming insects on human gut microbiome [28] human and feed modification on the insect microbiome [41, 158] or for biotechnological applications [159]. Another trend that calls for the establishment of metrological tools is highlighted by Mattarozzi et al. [160], namely, the rapid development of point-of-need devices and low-cost portable testing, frequently intended for use by non-

experts. While this trend benefits the food industry enormously (e.g., faster results, on-site testing, improved food safety, cost reductions, enhanced traceability, and empowering non-experts), it leaves many metrological questions open regarding accuracy and reliability, standardisation, calibration, validation, and regulatory acceptance [160].

Metrology is particularly important for Pillar III since accurate measurements are essential to support the certification systems that ensure authenticity, prevent misleading claims, and prevent adulteration. Semiautomated and fast stable isotope-ratio analysis (SIRA) in continuous-flow mode with an elemental analyser (EA) interfaced with isotope-ratio MS (IRMS) has become a routine and invaluable tool for food authenticity and traceability (Pillar III) [161–163]. Several standardised analytical techniques have been officially recognised as well-established, validated and acknowledged by the Association of Official Agricultural Chemists (AOAC), the European Committee for Standardization (CEN), or EU methods [162]. Importantly, accurate measurements of relative stable isotope ratios of hydrogen (expressed as δ^{2}H), carbon (δ^{13}C), nitrogen (δ^{15}N), oxygen (δ^{18}O), and sulfur (δ^{34}S) require normalisation using at least two isotopic RMs with contrasting isotopic compositions to (i) anchor δ-values to conventional isotope—delta scales and (ii) compensate for differences in responses of analytical instrumentation, which commonly compress isotope—delta scales [164–166]. The new guidelines for reporting HCNOS isotopic delta values with JSI participation were recently published [167]. While authenticity identification usually works successfully with one or two stable isotope parameters, geographical origin determination often requires multiple stable isotope parameters and even the inclusion of elemental composition or heavier isotopes such as strontium. Other metrological tools include participation in interlaboratory [168] and the Consultative Committee for Metrology in Chemistry and Biology (CCQM) organised assessment [169] and development of new RMs of plant and animal origin [170].

From a metrological perspective and across all three thematic pillars, there is a notable deficiency in suitable RMs and interlaboratory trials, including collaborative trials, which aim to verify the performance of an analytical method, and proficiency testing, which focuses on assessing participants' performance [146]. RMs (and CRMs) represent one of the main metrological tools supporting reliable measurements regarding calibration, method validation and quality control, guaranteeing metrological traceability, method validation and quality control [146]. Ideally, RMs must be chemically as similar as possible to the sample to account for matrix effects, i.e., based on the studied insects.

While there has been an increase in the production of new RMs in recent years, there remains a shortage of RMs tailored explicitly for the agrifood sector despite the persistent demand to create new RMs with varied matrix/analyte combinations to meet evolving analytical needs. This necessity arises from several factors, including advancements in analytical techniques, the emergence of new parameters of interest (e.g., nanoparticles and micro/nanoplastics), the evolution of methods for food profiling for quality, authenticity, and traceability purposes, and the imperative to study emerging contaminants as discussed in this review highlights the emerging needs for new RMs. Addressing these methodological gaps presents a significant opportunity for future research [143, 146].

Conclusions

In this review, we addressed the current progress and challenges relating to farmed insects as food and feed, identified through the lens of the INPROFF project's core themes: nutrition enhancement, quality and safety, and authenticity. Three things quickly become apparent. The first is that interest in farming insects in a European context continues to grow; second, that much research has been and is being done, resulting in a significant body of research; and third, but just as important, there remains much to be learnt, and farming insects creates unique issues. The argument for accepting insects as alternative proteins for feed is economical, while as food, the issues remain regarding their negative perception. Both could be resolved, for example, in the former case by substitution and fortification (valorisation) of feed and the latter by incorporating insects as ingredients and developing new foods with increased functionality as added value or by improving their sensorial qualities thereby sidestepping any potential neophobia and possibly by also taking a more heuristic approach to increase acceptance.

The safety of farmed insects still poses nutritional, chemical, and biological challenges and gaps in our understanding to be filled, something that will become more significant if insects are reared on by-products of agriculture and waste streams from food processing. More work is needed to understand the transfer of contaminants from raw materials during production and processing, including exogenous and endogenous nonbiological and biological risks. The large number of potential contaminants also drives the development of nontarget analytical workflows based on high-resolution mass spectrometry methods. Also important is addressing the gaps in metrological challenges of analysing insects (and novel foods in general), which will be critical for ensuring safety, quality, and sustainability as they continue to gain prominence in the food industry. Finally, authenticity and adulteration are only now being addressed. Fortunately, many authenticating tools developed for other food groups based on chemical, isotopic and genetic methods can be adapted to farmed insects.

Abbreviations

AOAC: Association of Official Agricultural Chemists

ARIS: Slovenian Research Agency

CCQM: Consultative Committee for Metrology in Chemistry and Biology

CEN: European Committee for Standardization

CRMs: certified reference materials

CZU: Czech University of Life Sciences Prague

DART-HRMS: direct analysis in real-time high-resolution mass spectrometry

DDT: dichlorodiphenyltrichloroethane

DM: dry matter

EA: elemental analyser

EC: European Commission

EFSA: European Food Safety Authority

EU: European Union

FAO: Food and Agriculture Organisation

GAČR: The Czech Science Foundation

H-PFCA: hydrogen-substituted polyfluoroalkyl carboxylic acids

HRMS: high-resolution mass spectrometry

INPROFF: Quality, Safety and Authenticity of Insect Protein-Based Food and Feed

IPIFF: International Platform of Insects for Food and Feed

IRMS: isotope-ratio mass spectrometry

IS: internal standard

ISO: International Organization for Standardization

JSI: Jožef Stefan Institute

LAB: lactic acid bacteria

LC-MS: liquid chromatography-mass spectrometry

MRL: maximum residue limit

MS: mass spectrometry

PAH: polycyclic aromatic hydrocarbons

PAP: processed animal protein

PCBs: polychlorinated biphenyls

PFAS: polyfluorinated substances

PFBA: perfluorobutanoic acid

PFBS: perfluorobutanesulfonic acid

PFOA: perfluorooctanoic acid

PFOS: perfluorooctanesulfonic acid

PUFA: polyunsaturated fatty acids

RM: reference materials

SIRA: stable isotope-ratio analysis

ToF: Time-of-Flight

TSEs: transmissible spongiform encephalopathies

US FDA: United States Food and Drug Administration

Supplementary materials

The supplementary material for this article is available at: https://www.explorationpub.com/uploads/Article/file/101041_sup_1.pdf.

Declarations

Author contributions

DH: Conceptualisation, Investigation, Writing—original draft, Writing—review & editing. NO: Conceptualisation, Investigation, Writing—original draft, Writing—review & editing. All other authors, LK, MK, PŠ, AV, HŠS, BL, NR, PJ, BJ, MČ, NPU, and EH were responsible for writing and editing. All authors have reviewed and approved the submitted manuscript.

Conflicts of interest

Nives Ogrinc who is a guest editor for the special issue "Metrological Aspects in the Analysis of Nutrients, Functional Compounds, Additives and Contaminants in Food and Feed" of *Exploration of Foods and Foodomics* had no involvement in the decision-making or the review process of this manuscript. The other authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

Not applicable.

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3.2 Scientific Paper: "Nutritional Composition of Farmed Insects: Impact of Species, Developmental Stage, and Sex"

This chapter presents the paper entitled "Nutritional composition of farmed insects: impact of species, developmental stage, and sex" by Anja Vehar, Doris Potočnik, Lidija Strojnik, Tea Zuliani, David Heath, Marjeta Mencin, Urška Vrhovšek, Petra Škvorová, Lenka Kouřimská, Martin Kulma, Ester Heath, and Nives Ogrinc. The paper was published in the Journal of Insets as Food and Feed (A') in 2025. This paper describes the nutritional composition of seven insect species: *Schistocerca gregaria* (desert locust), *Locusta migratoria* (migratory locust), *Tenebrio molitor* (yellow mealworm), *Acheta domesticus* (house cricket), *Gryllus assimilis* (Jamaican field cricket), *Alphitobius diaperinus* (lesser mealworm), and *Blaberus discoidalis* (discoid cockroach), differing in sex and developmental stage. The aim was to determine nutritional differences among species, developmental stages, and sexes.

Insects were obtained from the insect-rearing facility at the Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences in Prague. The selected species, the desert locust and the discoid cockroach, were separated by sex (male and female). The migratory locust and the Jamaican field cricket were separated according to age (adult and subadult), and the yellow mealworm and lesser mealworm were separated according to form (larvae and pupae). All of them were analysed for fatty acids (GC-FID), amino acids (GC-MS), total nitrogen (OH/CNS pyrolizer/EA-TCD), elements (ICP-MS) and polyphenols (LC-MS/MS).

This study confirms the nutritional potential of all seven farmed insects as valuable sources of essential fatty acids, amino acids, and minerals. Notably, fatty acid content varied across species and developmental stages, with adult insects generally exhibiting a better fatty acid profile for human consumption than subadults. This variation in fatty acid composition suggests metabolic differences between species and physiological changes that occur during growth. Furthermore, sex-based differences were evident in both the desert locust and the discoid cockroach, with males exhibiting a slightly higher fatty-acid quality, including higher essential fatty acid and polyunsaturated fatty acid content, indicating distinct reproductive energy demands of each sex.

Amino acid profiles also differed significantly across species and developmental stages, although no sex-based differences were found. Similarly, differences in elemental composition were observed between species, developmental stages, and sexes, reflecting the diverse metabolic processes, physiological needs during growth, and specific biological roles of the insects.

Regarding polyphenol content, the study found that the insect species tested can be considered a low-to-medium source of polyphenols (on a dry-weight basis) compared to conventional polyphenol-rich foods. Polyphenol levels varied among species, with locusts and cockroaches showing higher concentrations than other species. Developmental stage also influenced polyphenol concentrations, with pupae having higher levels than larvae in both yellow mealworms and lesser mealworms. At the same time, adult Jamaican field crickets exhibited higher total polyphenol concentrations than subadults. In discoid cockroaches, males had higher polyphenol levels than females. Although polyphenol concentrations were generally low, these findings suggest potential bioactive benefits and open avenues for further research into insect-derived antioxidants. These results contribute to an understanding of how rearing conditions and the biological characteristics of farmed

insects influence their nutritional quality, with implications for optimising insect-farming practices to enhance their value as a sustainable food source.

As the first author of the manuscript, I conducted the analyses of amino acids, fatty acids and polyphenols, analysed complete data, prepared all visual materials, and was responsible for both the drafting and finalisation of the manuscript. The findings were presented as a poster presentation at the INSECTA 2023 International Conference, September 13-14, 2023, in Magdeburg, Germany and as an oral presentation at the 7th IMEKO FOODS conference, October 25-27, 2023, in Paris, France.

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RESEARCH ARTICLE

Nutritional composition of farmed insects: impact of species, developmental stage, and sex

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Abstract

In this study, seven insect species, *Schistocerca gregaria* (desert locust), *Locusta migratoria* (migratory locust), *Tenebrio molitor* (yellow mealworm), *Acheta domesticus* (house cricket), *Gryllus assimilis* (Jamaican field cricket), *Alphitobius diaperinus* (lesser mealworm) and *Blaberus discoidalis* (discoid cockroach), differing in sex and developmental stage, were analysed for fatty acid, amino acid, elemental and polyphenolic composition. Results confirm that the studied insects are a good source of fatty (polyunsaturated/saturated fatty acids ratio: <1.3±0.0) and amino acids (total essential amino acids <25.6±1.0 g/100 g dry weight) and elements. Although as many as 59 individual polyphenols were detected at low levels, each species contained 8–34 polyphenols. The total polyphenols content ranged from 7.6–511 mg/kg dry weight, with female desert locust (511 mg/kg dry weight, 19), adult migratory locust (288 mg/kg dry weight, 34) and discoid cockroach, both male (449 mg/kg dry weight, 21) and female (357 mg/kg dry weight, 20) having the largest amount and number of polyphenols. Results showed that species, developmental stage and sex significantly influence the biochemical parameters of the insects. The research findings will contribute to optimising insect rearing, improving the quality of insect biomass as a nutritional source and furthering their integration as food and feed.

Keywords

amino acids – farmed insects – fatty acids – minerals – quality – polyphenols

1 Introduction

Edible insects are a highly diverse and nutritionally rich source of macronutrients and micronutrients, receiving increasing attention as a sustainable alternative source of protein for food and feed (Ordoñez-Araque and Egas-Montenegro, 2021). Research on their nutrient content has shown that their nutritional profiles vary among

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species, primarily influenced by environmental factors such as geographic (for wild-caught) and climatic conditions, as well as diet, and while taxonomic proximity is relevant, it appears to play only a comparatively minor role (Meyer-Rochow *et al.*, 2021; Oonincx and Finke, 2021). Additionally, their nutritional composition varies across developmental stages, with significant shifts in carbohydrates, proteins, fats, fibre, ash, and minerals observed among various insect orders, including Coleoptera, Hymenoptera, Orthoptera, and Blattodea (Meyer-Rochow *et al.*, 2021). These changes are largely influenced by whether the insect follows a holometabolous or hemimetabolous developmental pathway.

In holometabolous insects, fat content generally increases during the larval stage, while protein and moisture levels decline as energy reserves are stored for metamorphosis. During pupation, these fat stores are utilized, resulting in lower fat and higher protein content in the adult stage compared to larvae, a pattern observed in European honeybee drones (*Apis mellifera*; Ghosh *et al.*, 2020), Italian honeybees (*Apis mellifera ligustica*; Ghosh *et al.*, 2016), yellow mealworms (*Tenebrio molitor*; Ramos-Elorduy *et al.*, 2002), American palm weevils (*Rhynchophorus phoenicis*; Opara *et al.*, 2012), and palm beetles (*Oryctes rhinoceros*; Omotoso, 2018). In contrast, hemimetabolous insects exhibit a gradual increase in fat content throughout nymphal growth, accompanied by a decrease in protein and moisture. However, as these insects transition into adulthood, fat stores decline, particularly in females, due to reproductive demands and flight activity, while protein content rises. This pattern has been documented in variegated grasshoppers (*Zonocerus variegatus*; Ademolu *et al.*, 2010), Mediterranean field crickets (*Gryllus bimaculatus*; Anand and Lorenz, 2008; Lorenz and Anand, 2004), and several cockroach species, including the Argentine wood roaches (*Blaptica dubia*), discoid cockroaches (*Blaberus discoidalis*), Turkestan cockroaches (*Blatta lateralis*; Kulma *et al.*, 2016), and Death's head cockroaches (*Blaberus craniifer*; Kulma *et al.*, 2020). Additionally, environmental factors, such as drought and overwintering periods, may further influence nutrient composition, affecting the availability and utilization of fat, protein, and moisture reserves (Ghosh *et al.*, 2017).

The nutritional value of insects may also vary between sexes. For instance, female house crickets are reported to contain more lipids and have a higher energy value, while male crickets contain slightly higher protein content (Kulma *et al.*, 2019). Conversely, female silkworm larvae have higher levels of both lipids and proteins than males (Kotake-Nara *et al.*, 2002; Mahesh *et al.*, 2015; Trivedy *et al.*, 2008), and female variegated grasshoppers contain more protein, carbohydrate, fat, and ash than males (Ademolu *et al.*, 2017). These findings indicate that sex-specific differences may be common among edible insects and warrant further exploration.

The importance of bioactive compounds in human diets is also an increasingly important topic in nutrition. Compounds such as phenolics and flavonoids are particularly notable for their beneficial properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial, and antibacterial effects, as well as their roles in enzyme activity and metabolic regulation (David-Birman *et al.*, 2018; Escobar-Ortiz *et al.*, 2022; Hayakawa *et al.*, 2022; Issaoui *et al.*, 2020; Niedzwiecki *et al.*, 2016; Sadat *et al.*, 2022; Sajadimajd *et al.*, 2020). Polyphenols are also important in animal feed for several reasons, including antioxidant properties, product quality improvement, digestive and other health benefits and reduced dependency on synthetic additives (Bešlo *et al.*, 2023; Gessner *et al.*, 2017; Vasta *et al.*, 2019). However, despite the potential health benefits of these compounds, research into the phenolic content of insects, especially its relationship with diet and developmental stage, remains limited (Aiello *et al.*, 2023; Nino *et al.*, 2021b). The mechanisms by which insects acquire or synthesise phenolic compounds, such as through the sclerotisation process (Sugumaran, 2010), and the impact of insect class and diet on polyphenol levels remain areas for further investigation (Aiello *et al.*, 2023). Furthermore, identifying insect species richest in phenolic compounds and clarifying how these levels vary with sex and development could lead to a better understanding of their benefit for human and animal health.

This study aims to examine how species, developmental stage, sex, and diet influence the nutritional and bioactive composition of farmed insects. It examines variations in their fatty acid and amino-acid profiles, elemental composition, and, importantly, their polyphenolic content across seven farmed insect species: desert locust, migratory locust, yellow mealworm, house cricket, Jamaican field cricket, lesser mealworm and the discoid cockroach.

2 Materials and methods

Insect rearing

For this study, seven species: desert locust (*Schistocerca gregaria*, DL), migratory locust (*Locusta migratoria*, ML), yellow mealworm (*Tenebrio molitor*, YM), house cricket (*Acheta domesticus*, HC), Jamaican field cricket (*Gryllus assimilis*, JFC), lesser mealworm (*Alphitobius diaperinus*, LM) and the discoid cockroach (*Blaberus discoidalis*, DC) were selected based on their status as novel foods and feed by the EU (YM, ML, HC, LM, JFC) or because they are promising candidates for future approval (DL, DC) (Commission regulations (EU) 2021/882, 2021/1975, 2022/169, 2022/188, 2023/5, 2023/58, 2021/1372). All species are suitable for rearing in insectaries and were obtained from the insect-rearing facility at the Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences in Prague.

Specimens of the desert locust and discoid cockroach were separated according to sex (male and female), species of the migratory locust and the Jamaican field cricket were separated according to age (adult and subadult) and yellow mealworm and lesser mealworm according to form (larvae and pupae). They were reared using the rack system and maintained at 27±1 °C with a relative humidity of 40–50% and a lighting regime consisting of a 12:12 h (light:dark) photoperiod. While no extra heating devices were used for cockroaches, crickets, locusts, and mealworms, heating foils and 40 W bulbs were used to create warm zones for locusts. The cockroaches, crickets and locusts were reared in plastic boxes (56×39×28 cm, IKEA, Prague, Czech Republic) equipped with egg trays (Schubert Partner, Prague, Czech Republic) and secured using a lid comprising approximately 80% aluminium anti-insect mesh.

The insects were fed *ad libitum*. Petri dishes with chicken feed (77.9% wheat, 17.6% soybean meal, 1.8% rapeseed oil, and 2.7% minerals and vitamin premix; particle size <1 mm, CF) for cockroaches and crickets or wheat bran (WB) for locusts were provided in case of shortage or every third day when the unconsumed substrate was removed (if present). The locusts were additionally provided grass hay (H) and fresh Poaceae grass (PG). Furthermore, Petri dishes containing water gel (Oslavan, Náměšť nad Oslavou, Czech Republic) were provided daily for cockroaches, crickets and locusts. Crickets, cockroaches and locusts were kept in the same container from the day after hatching until the harvest at the adult or penultimate instar stage. The mealworm colonies were reared using breeding trays (60×40×12.5 cm, Beekenkamp, Maasdijk, The Netherlands) and fed by a mixture of wheat bran and chicken feed in a 4:1 ratio. The water gel was provided daily directly onto the feed substrate as a source of moisture. Insects exclusively reared on the analysed batch of substrates are denoted by an asterisk (*). The diets used in this study are commonly used for rearing insects. Mealworms were harvested when the first pupae appeared. Before freeze-killing, the experimental insects were fasted for 24 hours. Samples of insects and fresh grass were lyophilised (Trigon Plus lyophiliser, Čestlice, Czech Republic), homogenised (A10; IKA Werke, Staufen, Germany) and stored in the freezer until analysis. The dry matter content was 30% for all insects and was calculated from the mass of the sample before and after lyophilization.

Chemicals and reagents

For fatty-acid analysis, dichloromethane (CH_2Cl_2), methanol (MeOH), and hexane (C_6H_{14}) for organic residue analysis were sourced from J.T. Baker (Deventer, The Netherlands). Sodium hydroxide (NaOH, puriss. p.a., ACS reagent, reag. Ph. Eur., ≥98%, pellets) and a 14% boron trifluoride in methanol solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ) was prepared using the Milli-Q System (Merck Millipore, Watertown, MA, USA). Additionally, a Supelco 37 Component FAME Mix in dichloromethane, containing methyl esters of 37 fatty acids, was obtained from Supelco (Bellefonte, PA, USA).

Standards for amino acids were acquired as follows: L-valine (≥98%), L-leucine (≥98%), L-isoleucine (≥98%), L-threonine (≥98%), L-serine (≥99%), L-proline (≥99%), L-methionine (≥98%), L-glutamic acid (≥99%), L-phenylalanine (≥99%), L-lysine (≥98.0%), L-histidine (≥99%) and L-tyrosine (≥98%) were all sourced from Sigma-Aldrich. Additionally, standards for alanine (≥98%), glycine (≥98%), and aspartic acid (≥98%) were purchased from Fluorochem (UK). The internal standard for amino acids, Stable Isotope Labelled Amino Acid Mix Solution 1, containing labelled variants of 17 amino acids, was obtained from Supelco (Switzerland). Hydrochloric acid (37%) was sourced from VWR Chemicals (France), while thioglycolic acid (≥99%) and phenol (≥99%) were purchased from Sigma-Aldrich. All other reagents used in the analyses of amino acids were provided in the EZ Amino Acid Analysis Kit (Phenomenex, USA).

For the polyphenol analysis, methanol (≥99.9%, MeOH), acetonitrile (≥99.9%, MeCN) and chloroform (99.0–99.4%, $CHCl_3$) were obtained from Honeywell. Formic acid (≥99.0%, HCOOH) was obtained from

Sigma-Aldrich (Finland). Standards for polyphenols analysis ellagic acid, esculetin, gallic acid, ferulic acid, quercetin-3-glucoside, quercetin-3-rhamnoside, scopoletin, umbelliferone were purchased from Fluka, syringic acid from Alfa Aesar, daphnetin from Biomedicals, 2,5-dihydroxybenzoic acid, cinnamic acid, naringenin, rosmarinic acid, sinapyl alcohol, syringaldehyde from Aldrich, 4-hydroxybenzoic acid, vanillin from Carlo Erba, 4-hydroxy-3-methoxybenzoic acid, apigenin, esculin, kaempferol, luteolin, naringenin-7-glucoside, phlorizin dihydrate, protocatechuic acid, robinin from Roth, catechin, daidzein, isorhamnetin, isorhamnetin-3-glucoside, isorhamnetin-3-o-rutinoside, kaempferol-3-glucoside, kaempferol-3-o-rutinoside, myricetin, myricitrin, o-coumaric acid, quercetin-3-galactoside, quercetin-3-glucopyranoside, quercetin-3,4-diglucoside, quercetin-4-glucoside, quercetin-3-glucuronide, rutin, syringetin-3-O-galactoside, syringetin-3-O-glucoside, trilobatin from Extrasynthese, quercetin-3-acetylglucoside from ICC Indofine, 4-aminobenzoic acid sodium salt, anthranilic acid, apigenin-7-glucoside, apiin, arbutin, caffeic acid, catechol, chlorogenic acid, coniferyl alcohol, coniferyl aldehyde, cryptochlorogenic acid, hesperidin, luteolin-7-O-glucoside, neochlorogenic acid, orientin, p-coumaric acid, pterostilbene, resveratrol, sinapic acid, taxifolin from Sigma, 2,4-dihydroxybenzoic acid, phloretin, procyanidin A2 from Sigma-Aldrich, 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, fertaric acid, quercetin, quercetin-3-O-arabinoglucoside, naringenin, phloretin, procyanidin B3, sieboldin from TransMIT, acetovanillone from SAFC and trans-cinnamic-d5 acid from CDN isotopes, while transcoutaric acid was isolated in the lab. For the elemental composition analysis, nitric acid (65% Suprapur® for trace analysis, HNO_3) and hydrogen peroxide (30% Suprapur, for trace analysis, H_2O_2) were obtained from Supelco.

Fatty-acid analysis

Freeze-dried insect and feed samples (200 mg) were weighed directly into screw-cap vials, and 500 µl of CH_2Cl_2 and 3 ml of 0.5 M NaOH in MeOH were added for total lipid extraction. Samples were then purged with N_2, heated for 10 min at 90 °C and rapidly cooled in an ice bath. Once cool, 3 ml of BF_3 in MeOH was added to generate the fatty acid methyl esters (FAMEs). The extract was then purged with N_2 and heated for 10 min at 90 °C. After cooling, the FAMEs were extracted using 3 ml of Milli-Q water and 1.5 ml of hexane, which was transferred directly to a GC vial and stored at −20 °C. Samples were prepared in duplicates.

The characterisation of FAMEs was performed using an Agilent 6890N (Network GC System, Agilent Technology, Santa Clara, CA, USA) gas chromatograph fitted with a flame ionisation detector (GC-FID). Separation was achieved on a CP-Sil 88, 100 m×0.25 mm×0.20 µm, on a capillary column (Agilent J&W, EU). The temperature program was as follows: 100 °C (held 5 min) to 180 °C at 8 °C/min (held 9 min), ramped to 230 °C at 1 °C/min (held 1 min). The carrier gas was helium in constant flow mode (1.3 ml/min). The injection volume was 1 µl, with a split ratio of 10:1. The injector was set to 260 °C and the detector to 250 °C. The individual fatty acids were identified and quantified by comparing their retention times with a standard Supelco 37 component FAME Mix (Supelco) and expressed in weight percent of total fatty acids. Procedural blanks were analysed with each set of samples. The Supelco 37 component FAME mix standard was analysed after every ten samples to verify the stability of the analytical system. Results were expressed as the percentage of each fatty acid with respect to the total fatty-acid content. This percentage was calculated using the peak area of the samples corrected with the respective correction factors (Christie, 1982).

Total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), total n–3 and n–6, as well as the PUFA/SFA and n–6/n–3 ratios, were calculated. For all samples, the index of atherogenic (IA), index of thrombogenic (IT), and hypocholesterolemic/hypercholesterolemic ratio (h/H) were also determined for the total lipid content according to the following equations (Chen and Liu, 2020):

$$\mathrm{IA} = \big(\mathrm{C12{:}0} + (4 \times \mathrm{C14{:}0}) + \mathrm{C16{:}0}\big) / (\Sigma\mathrm{MUFA} + \Sigma\mathrm{n{-}6} + \Sigma\mathrm{n{-}3}) \quad (1)$$

$$\mathrm{IT} = (\mathrm{C14{:}0} + \mathrm{C16{:}0} + \mathrm{C18{:}0}) / \big((0.5 \times \Sigma\mathrm{MUFA}) + (0.5 \times \Sigma\mathrm{n{-}6}) + (3 \times \Sigma\mathrm{n{-}3}) + (\mathrm{n{-}3/n{-}6})\big) \quad (2)$$

$$\mathrm{h/H} = (\mathrm{C18{:}1} + \Sigma\mathrm{PUFA}) / (\mathrm{C12{:}0} + \mathrm{C14{:}0} + \mathrm{C16{:}0}) \quad (3)$$

Amino-acid analysis

A known weight of lyophilised insect sample (50 mg) was weighed into hydrolysis micro-reaction vessels, and 2.5 ml of 4% thioglycolic acid in 6 M HCl and 25 µl of liquid phenol (heated at 80 °C) was added, and the sample vortexed. Samples were then gently purged with N_2 for 5 min, and the vials were sealed and heated at 110 °C

for 24 h. During hydrolysis, glutamine was converted to glutamic acid, and asparagine was converted to aspartic acid, so the reported values for glutamic and aspartic acid are the sum of glutamine and glutamic acid and asparagine and aspartic acid, respectively. After hydrolysation, samples were filtered through CHROMAFIL Xtra PTFE, 0.45 µm syringe filters (Macherey-Nagel, Germany). The amino-acid analysis of protein hydrolysates was done using the EZ:faast™ kit. First, 50 µl of filtered hydrolysate was transferred into the sample preparation vial, and 100 µl of 1 M Na_2CO_3 solution was added and vortexed. For the insects, 30 µl of a pH-adjusted mixture (pH 2) was transferred into a new vial, while 70 µl was used for the feed. For freeze-dried Poaceae grass and hay, the entre pH-adjusted hydrolysate was used.

Fifty microliters of internal standard (200 nmol/ml) was added to each vial and the contents vortexed. Afterwards, the sample was slowly passed through the attached sorbent tip using a 1.5 ml syringe. Milli-Q water (200 µl) was added to the sample preparation vial, mixed thoroughly, and then slowly passed through the sorbent tip once more. The accumulated liquid was discarded, and the sorbent tip detached.

The compounds of interest were then eluted using 200 µl of a mixture of 0.33 M NaOH/N-propanol/3-picoline (3:1.6:0.4, v/v/v). Elution was achieved by attaching the sorbent tip to a 0.6 ml syringe and pulling the plunger back halfway. The sorbent was wet with the eluting medium and ejected from the tip into the sample preparation vial. The step was repeated until all sorbent particles were expelled into the sample preparation vial. After that, 50 µl propyl chloroformate/chloroform/iso-octane (1:3:1, v/v/v) mixture was added. The content in the vial was emulsified by vortexing for 5 s. The reaction was allowed to proceed for 1 min, then repeated. Afterwards, 100 µl of a mixture of iso-octane/chloroform (9:1, v/v) was added into the preparation vial, vortexed and allowed to proceed for 1 min. The organic layer (100 µl) was transferred into a GC autosampler vial, evaporated under a gentle stream of N_2 (max. 10 min) and then redissolved in 100 µl in iso-octane/chloroform (4:1, v/v). The reconstituted sample was then transferred into an insert and analysed by GC-MS. Samples were prepared in duplicates.

Analyses were performed using a 7890B series gas chromatograph (GC) coupled to a 5977A single quadrupole mass selective detector (Agilent). Chromatographic separation was achieved using a ZB-AAA GC column (10 m×0.25 mm; Phenomenex) with helium as the carrier gas (1.1 ml/min). Samples (1.5 µl) were injected in split mode 1:15 at 250 °C. The GC oven temperature program was as follows: an initial temperature of 110 °C was ramped at 30 °C/min to 320 °C. The total runtime was 7 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. The source temperature was 240 °C, quad 180 °C, auxiliary 310 °C, the scan range was 45-440 *m/z*. The target compounds were identified and quantified using selected ion monitoring (SIM mode). The monitored SIM ions and retention times (RTs) for the derivatised AAs and internal standards are presented in Table S1. The data were processed using MassHunter software (Agilent Technologies).

The ratio of essential to total amino acids was calculated as E/T (%), and the essential amino-acid index (EAAI) was calculated according to (FAO, 2013) based on the following essential amino acids: valine, leucine, isoleucine, threonine, phenylalanine+tyrosine, methionine, histidine and lysine, using Equation 4. Due to method limitations, the calculations did not include cystine and tryptophan.

$$\text{EAAI} = \left(\frac{\text{g of lysine in 100g of analysed protein}}{\text{g of lysine in 100g of reference protein}} \times (\text{etc. for other 7 EAA})\right)^{1/8} \times 100 \quad (4)$$

The index is based on the determined content of all previously mentioned amino acids compared to those of a reference protein composition for older children (≥3 years) and adults (FAO, 2013). The essential amino-acid score (AAS) was calculated as follows:

$$\text{AAS} = \frac{\text{mg amino acid per g of protein}}{\text{mg amino acid per g of reference protein}} \times 100 \quad (5)$$

The amino acid with the lowest AAS is considered the limiting amino acid.

Total nitrogen analysis

Total nitrogen was determined on the Vario PYRO Cube (OH/CNS Pyrolyser/Elemental Analyzer) (IsoPrime, Cheadle, Hulme, UK) with the thermal conductivity detector (TCD). Approximately 5.5 mg of the sample was weighed directly into a tin capsule, sealed using tweezers and placed into the autosampler of the elemental analyser. Total nitrogen (expressed in %) was computed from the absolute nitrogen content and the

sample weight according to the formula (6):

$$c = \frac{a \times 100}{w} \quad (6)$$

where c is total nitrogen concentration (%), a is absolute nitrogen content (mg), and w is the weight of the sample (mg). The absolute nitrogen content was determined by external calibration using the following RM reference materials: USGS91 with 1.5±0.04% of N, USGS61 with 28.8% of N, USGS88 with 16.66±0.45% of N, USGS89 with 15.78±0.19% of N and UGS43 with, 15.3% of N.

Elemental analysis

A sample of known weight (0.10–0.15 g) was weighed into a pre-cleaned Teflon digestion vial along with 1 ml of HNO_3 and 250 µL H_2O_2. The sample was then subjected to microwave digestion (Ultrawave, Single Reaction Chamber Microwave Digestion System, Milestone, Italy). The program was as follows: heating to 240 °C in 20 min and holding for 15 min at 100 bars at a max power of 1500 W. The digested solution was quantitatively transferred into a polyethylene-graduated tube and diluted to 10 ml using Milli-Q water. Before analysis, samples were diluted with Milli-Q in a ratio 1:5 and 1:10. The determination of the selected elements (Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Sb, Pb) was performed using an inductively coupled plasma mass spectrometer (ICP-MS, 7700x Agilent Technologies, Tokyo, Japan). Quantification was achieved using external calibration, and samples were prepared in duplicates. Blanks and selected reference materials (Oyster tissue NIST 1566b, Typical diet 1548b) were also prepared in the same way as the samples to ensure the quality of the results. The LOD was calculated as three times the standard deviation of the blank samples.

A risk assessment for toxic elements was performed based on an adult (70 kg) ingesting 100 g of fresh insects. The intake of each element was first calculated and then compared with reference values (Table S5) obtained from OpenFoodTox: EFSA's chemical hazards database (Carnesecchi *et al.*, 2023). Based on the measured concentrations of essential elements and their reference daily intakes (RDI, Table S6), daily intake values (%) were calculated for consuming 100 g of fresh insects per day.

Polyphenol analysis

Extraction of polyphenols was adapted from Vrhovsek *et al.* (2012). Briefly, 200 mg of lyophilised insect sample was weighed into a 2 ml microcentrifuge tube, then 1200 µl of the mixture MeOH/H_2O (2:1, v/v), 800 µl of chloroform and 200 µl of H_2O were added. Samples were spiked with 20 µl of the internal standard trans-cinnamic-d_5 acid (50 mg/l), shaken on an orbital shaker (IKA Trayster digital) at 80 rpm for 15 min and then centrifuged at 15000 × g for 5 min at 4 °C. The upper aqueous fraction was transferred into a 2.0 ml microcentrifuge tube. Again, 1200 µl of the mixture MeOH/H_2O (2:1, v/v) was added into the first microcentrifuge tube, shaken and centrifuged under the same conditions. The upper aqueous fraction was collected and dried using the EZ-2 personal evaporator (Genevac, UK) at 38 °C. The sample was then resuspended in 100 µl of MeOH/H_2O (2:1, v/v), vortexed, sonicated in an ultrasonic bath for 5 min at 59 KHz, centrifuged at 15000 × g for 5 min at 4 °C, filtered through 0.2 µm RC membrane filter (filter vials) and transferred into 2 ml LC-MS vial with glass insert and stored at –20 °C before analysis.

Feed samples were treated the same as insect samples, except only 100 mg of lyophilised sample was weighed into a 2 ml microcentrifuge tube, then 600 µl of the mixture MeOH/H_2O (2:1, v/v), 400 µl of chloroform and 100 µl of H_2O were added. After transferring the upper aqueous fraction, only 600 µl of the mixture MeOH/H_2O (2:1, v/v) was added. The dried sample was resuspended in 250 µl of MeOH/H_2O (2:1, v/v). Hay and dried grass samples were treated the same as feed samples, except the dried samples were resuspended in 400 µl of MeOH/H_2O (2:1, v/v).

All samples were analysed using a Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Xevo triple quadrupole mass spectrometer, TQMS. Separation was achieved at 40 °C using a Waters Acquity HSS T3 column 100 mm×2.1 mm, 1.8 µm. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow rate was 0.4 ml/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume was 2 µl. After each injection, the needle was rinsed with 600 µl of weak wash solution (water/methanol, 90:10) and 200 µl of strong wash solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.

The analyser was operated in multiple reaction monitoring (MRM) mode under electrospray ionisation (ESI). The capillary voltage was 3.5 kV in positive mode and

–2.5 kV in negative mode; the source was kept at 150 °C; desolvation temperature was 500 °C; cone gas flow was 50 l/h; and desolvation gas flow was 800 l/h. Chromatograms were analysed using Waters MassLynx 4.1 and TargetLynx software. The results were normalised based on the response of the IS in the sample to that of the IS (50 mg/l) injected separately.

Statistical analyses

Data processing and visualisation were performed using R-based software (version 4.2.3). All data sets were checked for normal distribution using the Shapiro-Wilk test and for homoscedasticity of the variances using Levene's test or Brown-Forsythe test. Based on the number of sample groups, significant differences were assessed using a *t*-test or Mann–Whitney *U*-test and a One-way ANOVA or Kruskal-Wallis test. Significant differences in fatty acid, amino acid, and elemental content were assessed for sex, developmental stage, and species. Sex-based differences were assessed only in discoid cockroaches and desert locusts, while developmental stage differences were evaluated between larvae and pupae. Comparisons between adults and subadults were limited due to an insufficient number of samples. For this reason, samples of yellow mealworms, yellow mealworms*, and lesser mealworms were grouped into one group, and the pupal yellow mealworm and lesser mealworm samples were grouped into a second group to obtain sufficient measurements (n>3) for statistical analysis. Significant differences based on species were determined between all species and species harvested as adults (desert locust, migratory locust, Jamaican field cricket house cricket and discoid cockroach) and species harvested as larvae (yellow and lesser mealworms). Groups containing less than three samples were not statistically analysed (Table S8).

3 Results and discussion

Fatty-acid composition

The fatty-acid composition of feed (Table 1, Table S7) contained a total of 11 FA above 1%, including five SFA (C14:0 or myristic acid, C16:0 or palmitic acid, C18:0 or stearic acid, C20:0 or arachidic acid, C22:0 behenic acid), three MUFAs (C16:1 or palmitoleic acid, C18:1 *cis* 9 or oleic acid, C20:1 *cis* 11 or *cis*-11-eicosenoic acid), and three PUFAs (C18:2 *cis*, *cis* 9,12 or linoleic acid (LA), C18:3 all *cis* 9,12,15 alpha or α-linolenic acid (ALA), C20:5 all *cis* 5,8,11,14,17 or eicosapentaenoic acid (EPA)). Their relative concentrations (>1%) depend on the type of feed and are as follows for chicken feed: oleic>LA>palmitic>stearic>ALA>behenic>*cis*-11-eicosenoic acids, for wheat bran: LA>palmitic>oleic>ALA>stearic acid, for wheat bran and chicken feed mix (4:1): linoleic>oleic>palmitic>ALA>stearic acid, for hay: palmitic>ALA>LA>oleic>stearic>arachidic>EPA>myristic acid, and for dried grass: ALA>palmitic>LA>oleic>stearic>palmitoleic>arachidic acid. The different feed types were also rich in the essential fatty acids LA (15.1±4.3% to 56.2±15.9%) and ALA (1.9±0.5% to 53.9±15.2%), whose relative concentrations were dependent on feed type. They also contained lower amounts of EPA (ND to 1.8±0.5%) and docosahexaenoic acid (DHA) (ND to 0.7±0.2%). Although EPA and DHA can be synthesised in humans from ALA, due to low conversion efficiency, foods and feed rich in EPA and DHA are recommended (Burdge and Wootton, 2002; Burdge *et al.*, 2002; Das, 2013).

A total of seven FAs were detected in insects (Table 1, Table S7) in amounts >1%, including three SFAs (myristic, palmitic, stearic, behenic acid), two MUFAs (palmitoleic and oleic acid) and two PUFAs (LA and ALA). Their relative levels (>1%) were as follows: oleic>palmitic>LA>stearic>ALA>myristic>palmitoleic acid. All insects contained both essential fatty acids LA (7.9±0.1% to 32.8±0.0%) and ALA (0.2±0.0 to 13.9±0.7%), while EPA and DHA were present in lower amounts, i.e. ND to 0.5±0.0% and ND to 0.8±0.0%, respectively. Overall, the concentrations of essential fatty acids agree with published data for desert locust (LA: 24.67±0.25%, ALA: 10.14±0.02%) by Kinyuru (2021), migratory locust (LA: 16.96±0.08%, ALA: 10.47±0.11%) by Perez-Santaescolastica *et al.* (2023), yellow mealworm (LA: 16.63±1.41% to 33.8%, ALA: 0.23±0.09% to 1.43%) by Dreassi *et al.* (2017), Finke (2015, 2002) and Perez-Santaescolastica *et al.* (2023), house cricket (LA: 26.26% to 36.67±0.14%, ALA: 0.9% to 5.13%) by Finke (2015, 2002), Kulma *et al.* (2019) and Perez-Santaescolastica *et al.* (2023), Jamaican field cricket (LA: 31.79±2.49% to 37.72±2.57%, ALA: 0.68±0.11% to 8.68±2.24%) by Starčević *et al.* (2017), lesser mealworm (LA: 22.57±0.10%, ALA: ND) by Perez-Santaescolastica *et al.* (2023) and discoid cockroach (LA: 15.3% to 17.9%, ALA: 1.7% to 1.9%) by Kulma *et al.* (2016).

Fatty-acid profiles were evaluated using PUFA/SFA and n–6/n–3 ratios, along with indices of atherogenicity (IA), thrombogenicity (IT), and the hypocholesterolemic/hypercholesterolemic ratio (h/H) (Table 1). For a healthy human diet, consumption of foods with a PUFA/SFA ratio close to one is recommended, as this is associated with a reduction in cardiovascular risk, while

TABLE 1 Contents of the main fatty acids (FA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n–6 fatty acids, n–3 fatty acids, n–6/n–3 ratio, index of atherogenicity (IA), index of thrombogenicity (IT), and hypocholesterolemic/hypercholesterolemic ratio (h/H) in insects and feed

		Myristic acid (%)	Palmitic acid (%)	Palmitoleic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (LA,%)	α-linolenic acid (ALA,%)	SFA (%)	MUFA (%)	PUFA (%)	PUFA/SFA	n–6 (%)	n–3 (%)	n–6/n–3	IA	IT	h/H
Insects	DL (male)	1.6±0.1	20.7±0.5	0.8±0.0	10.7±0.3	36.9±0.2	14.7±0.2	11.6±0.0	34.0±0.4	38.3±0.1	27.7±0.3	0.8±0.0	14.9±0.2	12.8±0.0	1.2±0.0	0.4±0.0	0.5±0.0	2.9±0.1
	DL (female)	2.0±0.2	27.1±0.7	0.6±0.0	13.2±0.4	40.3±0.2	7.9±0.1	5.0±0.0	44.6±0.7	42.0±0.6	13.4±0.2	0.3±0.0	8.2±0.2	5.3±0.0	1.6±0.0	0.6±0.0	1.0±0.0	1.8±0.0
	ML (subadult)	1.5±0.0	20.3±0.0	0.7±0.0	12.3±0.0	44.8±0.2	12.0±0.0	5.0±0.3	35.7±0.0	46.1±0.1	18.2±0.1	0.5±0.0	12.4±0.1	5.7±0.2	2.2±0.1	0.4±0.0	0.7±0.0	2.9±0.0
	ML (adult)	1.3±0.0	17.8±0.0	1.0±0.0	11.5±0.0	44.6±0.2	13.8±0.0	6.7±0.4	31.8±0.1	46.1±0.3	22.1±0.2	0.7±0.0	14.5±0.0	7.6±0.3	1.9±0.1	0.3±0.0	0.6±0.0	3.4±0.0
	YM (larvae)	3.2±0.0	21.0±0.3	1.6±0.0	4.5±0.1	48.7±0.6	18.2±0.2	0.5±0.0	30.2±0.3	50.9±0.5	19.0±0.2	0.6±0.0	18.3±0.2	0.6±0.0	29.2±0.3	0.5±0.0	0.8±0.0	2.8±0.0
	YM (pupae)	3.3±0.0	18.0±0.2	1.5±0.0	3.9±0.1	43.0±0.5	28.8±0.4	0.2±0.0	26.0±0.2	44.8±0.5	29.2±0.3	1.1±0.0	29.0±0.3	0.2±0.0	138.3±5.5	0.4±0.0	0.7±0.0	3.3±0.0
	HC	0.7±0.0	24.4±0.3	0.9±0.0	11.3±0.1	24.1±0.0	32.3±0.0	0.7±0.0	38.5±0.7	25.9±0.1	35.6±0.6	0.9±0.0	34.3±0.7	1.3±0.1	25.9±1.7	0.4±0.0	1.1±0.0	2.4±0.0
	JFC (adult)	0.7±0.0	23.2±0.3	2.1±0.0	6.9±0.1	28.9±0.0	32.8±0.0	1.4±0.0	32.2±0.3	31.5±0.1	36.3±0.2	1.1±0.0	34.2±0.1	2.1±0.1	16.2±0.6	0.4±0.0	0.8±0.0	2.7±0.0
	LM (larvae)	0.8±0.0	28.8±0.1	0.6±0.1	8.2±0.4	39.6±0.1	18.3±0.2	1.0±0.1	39.7±0.1	40.4±0.2	19.9±0.0	0.5±0.0	18.8±0.2	1.0±0.2	18.7±4.2	0.5±0.0	1.2±0.0	2.0±0.0
	LM (pupae)	1.3±0.0	27.6±0.1	0.7±0.1	7.6±0.3	42.7±0.1	17.1±0.2	0.4±0.0	38.1±0.1	43.9±0.1	18.0±0.2	0.5±0.0	17.6±0.2	0.4±0.0	43.3±0.4	0.5±0.0	1.1±0.0	2.1±0.0
	DC (male)	0.6±0.0	14.6±0.2	1.3±0.0	11.9±0.1	49.1±0.1	17.4±0.0	0.7±0.0	28.4±0.5	51.7±0.1	19.9±0.5	0.7±0.0	18.7±0.1	1.2±0.6	18.7±10.1	0.2±0.0	0.7±0.0	4.5±0.1
	DC (female)	0.6±0.0	20.6±0.3	2.5±0.0	11.6±0.2	51.1±0.6	10.4±0.1	0.7±0.0	33.8±0.1	54.2±0.4	12.0±0.5	0.4±0.0	11.1±0.2	1.0±0.3	12.2±3.8	0.4±0.0	0.9±0.0	3.0±0.0
	HC*	0.6±0.0	26.2±0.3	0.9±0.0	8.6±0.1	28.4±0.0	32.2±0.0	1.4±0.0	35.9±0.1	29.8±0.1	34.1±0.0	0.9±0.0	32.5±0.0	1.5±0.0	21.3±0.4	0.4±0.0	1.0±0.0	2.3±0.0
	YM*	3.4±0.2	17.7±0.7	1.4±0.0	3.8±0.1	42.5±0.7	28.6±0.1	1.0±0.0	26.0±0.9	44.2±0.8	29.8±0.0	1.1±0.0	28.7±0.0	1.1±0.1	26.4±1.4	0.4±0.0	0.6±0.0	3.4±0.2
	ML*	1.4±0.0	18.6±0.0	0.8±0.0	10.5±0.0	23.6±0.1	27.7±0.1	13.9±0.7	32.7±0.2	24.9±0.3	42.5±0.6	1.3±0.0	28.0±0.1	14.5±0.7	1.9±0.1	0.4±0.0	0.4±0.0	3.3±0.0
Feed	CF (HC*)	0.1±0.0	11.5±3.3	0.2±0.1	2.5±0.7	55.8±15.8	19.8±5.6	3.3±0.9	17.7±5.0	57.5±12.0	24.8±7.0	1.4±0.0	20.4±5.8	4.3±1.2	4.7±0.0	0.1±0.0	0.1±0.0	7.3±2.5
	WB, CF (4I, YM*)	0.1±0.0	18.3±5.2	0.2±0.0	1.1±0.3	19.6±5.5	54.3±15.4	4.8±1.3	20.2±5.7	20.6±5.8	59.2±11.5	3.1±1.5	54.3±12.9	4.9±1.4	12.0±6.0	0.2±0.1	0.1±0.0	4.5±1.6
	CF (ML*)	0.1±0.0	13.7±3.9	0.2±0.1	3.0±0.9	61.5±17.4	14.0±4.0	1.9±0.5	20.4±5.8	63.3±10.4	16.3±4.6	0.8±0.0	14.1±4.0	2.2±0.6	6.4±0.0	0.2±0.1	0.1±0.1	5.9±2.1
	WB (ML*)	0.1±0.0	20.3±5.7	0.2±0.0	1.1±0.3	16.1±4.6	56.2±15.9	4.4±1.2	20.2±5.7	20.6±5.8	59.2±11.5	3.1±1.5	54.3±12.9	4.9±1.4	12.0±6.0	0.2±0.1	0.1±0.0	4.5±1.6
	H (ML*)	1.4±0.4	35.0±9.9	0.8±0.2	3.4±1.0	5.0±1.4	17.0±4.8	30.9±8.7	44.1±12.5	5.8±1.5	50.0±13.9	1.2±0.7	17.3±4.7	32.8±9.2	0.5±0.0	0.8±0.4	2.0±1.8	1.6±0.8
	PG (ML*)	0.7±0.2	18.5±5.2	1.3±0.4	2.6±0.7	3.4±1.0	15.1±4.3	53.9±15.2	25.4±7.2	4.8±1.4	69.8±8.5	2.9±1.2	15.3±4.3	54.5±12.9	0.3±0.2	0.3±0.1	0.2±0.2	3.9±1.5

DL, desert locust; ML, migratory locust; YM, yellow mealworm; HC, house cricket; JFC, Jamaican field cricket; LM, lesser mealworm; DC, discoid cockroach; CF, chicken feed; WB, wheat bran; H, hay; PG, Poaceae grass.

* Insects reared exclusively on the analysed batch of substrates.

a ratio <0.33 can have an atherogenic effect (Czernichow *et al.*, 2010; Hamułka *et al.*, 2018). A high dietary n–6/n–3 value is related to an increased risk of developing coronary heart disease and cancer, among others; therefore, ratios below four are advisable (Milićević *et al.*, 2014). Lower values of IA (<0.5) and IT (<0.5) are related to a reduced risk of developing cardiovascular disease, and higher levels of h/H (>1.0) are considered desirable (Chen and Liu, 2020).

The differences in fatty-acid profiles among the species studied are pronounced (Table S8). Based on the PUFA/SFA ratio, yellow mealworm (pupae and larvae), both house crickets, and the Jamaican field cricket (adult) can be considered a good source of these fatty acids, whereas data suggest that desert locust (female) might have an atherogenic effect. However, this would require further study. Also, the PUFA/SFA values determined for house cricket (0.9±0.0) are slightly higher than those reported by Kulma *et al.* (2019), which ranged from 0.6–0.7. Only the desert locust (male and female) and migratory locust (subadult and adult) had an n–6/n–3 ratio value below four. The n–6/n–3 values of male (1.2±0.0) and female desert locust (1.6±0.0), adult (1.9±0.1) and subadult migratory locust (2.2±0.1), adult Jamaican field cricket (16.2±0.6), larval lesser mealworm (18.7±4.2) and larval yellow mealworm (26.4±1.4 to 29.2±0.3) are similar to those reported by Kinyuru (2021), Perez-Santaescolastica *et al.* (2023), Taghi Gharibzahedi and Altintas (2023), Starčević *et al.* (2017), and Lawal *et al.* (2022).

The IA, IT, and h/H ratios varied significantly among species (Table S8). The IA index ranged from 0.2±0.0 to 0.6±0.0, aligning with the reported ranges for meat (0.165–1.32) and fish (0.21–1.41) by Chen and Liu (2020). Similarly, the IT index ranged from 0.4±0.0 to 1.2±0.0, consistent with the reported ranges for meat (0.288–1.694) and fish (0.14–0.87) by the same study. Likewise, the h/H ranged from 1.8±0.0 to 4.5±0.1 and was in the same range as most fish and meat, i.e. 1.54–4.83 and 1.27–2.786, respectively (Chen and Liu, 2020). The values IA, IT, and h/H for both samples of house cricket, yellow mealworm (larvae), migratory locust (adult), migratory locust*, and lesser mealworm (larvae) were also similar to those reported in the literature (Kulma *et al.*, 2019; Lawal *et al.*, 2022; Taghi Gharibzahedi and Altintas, 2023; Perez-Santaescolastica *et al.*, 2023). In terms of their IA, IT and h/H, the migratory locust (adult) and the discoid cockroach (male) would have the most beneficial impact on human health among the insects studied.

Depending on the stage of development, the larval insects had significantly higher levels of margaric (0.2±0.0% to 0.6±0.8%) and ALA (0.5±0.0% to 1.0±0.1%) compared to the pupal insects (margaric: ND to 0.1±0.0%, ALA: 0.2±0.0 to 0.4±0.0%), while significantly lower concentrations of elaidic acid (ND) compared to the pupal insects (ND to 0.1±0.0%). Regarding omega fatty acids, the larval insects had significantly higher n–3 fatty acids (0.6±0.0% to 1.1±0.1%) and significantly lower n–6/n–3 ratio (18.7±4.2 to 29.2±0.3) compared to the pupal insects (0.2±0.0 to 0.4±0.0 and 43.3±0.4 to 138.3±5.5, respectively). Also, Dreassi *et al.* (2017) reported lower n–6/n–3 ratios in larval yellow mealworms and stated that for that reason, larvae represent the preferred developmental stage for the human diet. Although there was an insufficient number of samples to analyse the differences between adult and subadult insects statistically, it can be seen from the data that migratory locust subadults have more SFA (35.7±0.0%) than adult insects (31.8±0.1% to 32.7±0.2%). In comparison, both adult migratory locusts and adult Jamaican field crickets have more PUFA (22.1±0.2% to 42.5±0.6%) and consequently a higher PUFA/SFA (0.7±0.0 to 1.3±0.0) ratio than subadult migratory locusts (18.2±0.1 and 0.5±0.0, respectively).

These differences can be attributed to changes in metabolic needs during insect development, which affect fatty-acid synthesis and storage. For example, SFA may dominate in early developmental stages as they provide readily available energy for rapid growth (Huis, 2013; Rumpold and Schlüter, 2013). Later, as insects approach adulthood, the proportion of unsaturated fatty acids often increases, as these contribute to maintaining cellular integrity, preparing for reproduction and maintaining survival-related functions. In the case of larvae, the different fatty composition reflects not only the metabolomic requirements but also the need to accumulate reserves for maintenance during metamorphosis (Ewald *et al.*, 2020). Specifically, total fat is increased during the larval stage and then consumed when the insect is in the pupal stage (Ramos-Elorduy *et al.*, 2002; Ghosh *et al.*, 2016, 2020; Dreassi *et al.*, 2017).

Regarding sex differences, the male desert locusts and discoid cockroaches had significantly higher levels of LA (14.7±0.2% to 17.4±0.0%) and DHA (0.1±0.0% to 0.8±0.0%) than females (7.9±0.1% to 10.4±0.1% and ND, respectively). They also had significantly higher levels of n–6 (14.9±0.2% to 18.7±0.1%), PUFA (19.9±0.5% to 27.7±0.3%) and consequently higher PUFA/SFA (0.7±

0.0 to 0.8±0.0) ratio compared to females (n–6: 8.2± 0.2% to 11.1±0.2%, PUFA: 12.0±0.5% to 13.4±0.2%, PUFA/SFA: 0.3±0.0 to 0.4±0.0), which is in contrast to silkworm pupae, as higher content of PUFA was observed in females (Kotake-Nara *et al.*, 2002). Female insects also had a less desirable significantly higher IT (0.9±0.0 to 1.0±0.0) compared to males (0.5±0.0 to 0.7±0.0). According to the results, male insects have a slightly better nutritional value based on fatty-acid profile compared to females. Kulma *et al.* (2019) investigated the effect of sex on the nutritional value of house crickets, and although they found differences between males and females in total fat content, the proportions of fatty acids in both sexes were similar. Other studies have shown that sex-based differences in insect fatty-acid profiles often arise from the distinct reproductive energy demands of each sex. For example, migratory insects like the female fall armyworm (*Spodoptera frugiperda*) adapt their lipid contents first for migration but then redirect them toward egg production after migration, influencing the fatty-acid profile (Xu *et al.*, 2024), while female German cockroaches (*Blattella germanica*) display specific unsaturated fatty acids that act as sex pheromones, attracting males (Pei *et al.*, 2021).

Although the statistical evaluation was not possible since there were insufficient samples, the variations in fatty-acid concentrations between the different types of feed did not directly match the variations in the fatty-acids found in insects fed on specific feeds. The data suggests that the insect species has a more significant influence on fatty-acid composition than the type of feed itself, agreeing with Boukid *et al.* (2021). However, it must be stressed that this study was not primarily designed to demonstrate the effect of different diets on the nutritional value of insects, and more research is needed to draw firmer conclusions. Nevertheless, there are a number of studies involving Jamaican field cricket, Mediterranean flour moth (*Ephestia kuehniella*), yellow mealworm, black soldier fly, house cricket, lesser mealworm and African edible bush cricket (*Ruspolia differens*), all of which demonstrate that diet influences the fatty-acid profile of insects (Rutaro *et al.*, 2018; Truzzi *et al.*, 2020; Boukid *et al.*, 2021; Oonincx and Finke, 2021; Rossi *et al.*, 2022; Škvorová *et al.*, 2024).

Total nitrogen content and amino-acid composition

The total nitrogen content (Table 2) ranged from 8.34± 0.04 g/100 g DW (pupal yellow mealworm) to 13.0±0.1 g/100 g DW (male discoid cockroach). Taking into account that arginine, cystine and tryptophan were not determined in this study, the total amino-acid content ranged from 37.3±1.1 g/100 g DW (subadult Jamaican field cricket) to 48.4±2.1 g/100 g DW (adult migratory locust). The total essential amino-acid content (Table 3) ranged from 19.5±0.4 g/100 g DW (subadult Jamaican field cricket) to 25.6±1 g/100 g DW (pupal lesser mealworm), while the essential to total amino acids (E/T) ranged from 49.6±0.3% (female desert locust) to 56.6±1.6% (pupal lesser mealworm). The essential amino-acid index (EAAI) ranged from 163±1% (female desert locust) to 187±5% (pupal lesser mealworm). Analysed insects were the highest in valine (3.29±0.03 to 4.83±0.16 g/100 g DW), leucine (3.35±0.02 to 4.82±0.17 g/100 g DW), alanine (2.81±0.15 to 5.53±0.34 g/100 g DW), aspartic (3.29±0.08 to 5.87±0.36 g/100 g DW) and glutamic acid (3.97±0.09 to 5.26±0.02 g/100 g DW), but lower in methionine (0.498±0.011 to 0.972± 0.061 g/100 g DW) and histidine (0.986±0.012 to 1.95± 0.03 g/100 g DW), agreeing with published data (Finke, 2002, 2015; Yi *et al.*, 2013; Kulma *et al.*, 2019; Perez-Santaescolastica *et al.*, 2023; Škvorová *et al.*, 2024). According to the amino-acid score (Table S4), methionine was the limiting amino acid in all species, which is in line with previous studies (Araújo *et al.*, 2024; Škvorová *et al.*, 2024), while some studies recognised cystine together with methionine (Finke, 2002; Sánchez-Muros *et al.*, 2014; Oibiokpa *et al.*, 2018; Khatun *et al.*, 2021), isoleucine (Oibiokpa *et al.*, 2018), threonine (Oibiokpa *et al.*, 2018), phenylalanine (Kulma *et al.*, 2020), tryptophan and leucine (Köhler *et al.*, 2019) as limiting amino acids. The analysed insects had 4–10% higher E/T values (Table 3) compared to E/T values for house cricket, migratory locust, lesser and yellow mealworm, as reported by Perez-Santaescolastica *et al.* (2023).

Species differed significantly in their alanine, glycine, leucine, isoleucine, serine, phenylalanine, histidine content, E/T, EAAI, total N and total AA content. The reasons behind these differences could be attributed to various factors such as species-specific metabolism, dietary differences, environmental factors (temperature, humidity, habitat) and adaptations to specific ecological roles or functions (flight, reproduction, or survival mechanisms) (Hughes, 2010; Meyer-Rochow *et al.*, 2021; Oonincx and Finke, 2021).

Depending on the stage of development, the only significant difference found was in the levels of alanine and glycine, which were significantly higher in the larval insects (alanine: 3.33±0.00 to 3.53±0.01 g/100 g DW, glycine: 2.43±0.03 to 2.68±0.03 g/100 g DW) than in the pupae (alanine: 2.81±0.15 to 3.20±0.12 g/100 g DW, glycine: 2.43±0.08 to 2.48±0.07 g/100 g DW), while tyrosine was significantly higher in the pupal

TABLE 2 Total nitrogen, total amino acids and nitrogen-to-protein conversion factor

	Total N (g/100 g DW)	Total AA (g/100 g DW)*	Kp**
DL (female)	10.3±0.9	46.9±2.6	4.97
ML (adult)	11.2±0.0	48.4±2.1	4.70
YM (larvae)	8.60±0.44	41.3±0.6	5.22
YM (pupae)	8.34±0.04	40.6±2.5	5.29
HC	12.1±0.2	46.8±3.1	4.22
JFC (subadult)	8.81±0.31	37.3±1.1	4.60
JFC (adult)	10.2±0.2	44.3±0.5	4.71
LM (larvae)	9.24±0.12	43.5±0.2	5.12
LM (pupae)	10.3±0.0	45.2±0.5	4.77
DC (male)	13.0±0.1	42.7±0.2	3.57
DC (female)	11.7±0.4	40.7±0.9	3.78
HC*	9.29±0.16	41.3±2.0	4.83
YM*	9.17±0.13	44.0±0.1	5.21
ML*	10.8±0.2	44.7±0.6	4.49

* Arginine, cystine+cysteine, tryptophan were not determined.
** The calculation of the factor takes accounts for the assumption that 8% of the protein nitrogen originates from non-determined amino acids.

insects (3.93±0.20 to 4.47±0.12 g/100 g DW) than larvae (3.09±0.03 to 3.98±0.11 g/100 g DW). Although statistical comparison was limited by sample size, the data indicate that the adult migratory locust and Jamaican field cricket have higher essential (22.5±0.3 to 24.2±0.9 g/100 g DW) and non-essential amino-acid concentrations (21.8±0.2 g/100 g DW to 24.2±1.1 g/100 g DW) than subadults (EAA: 19.5±0.4 g/100 g DW, NEAA: 17.8±0.8 g/100 g DW), making them a superior amino-acid source for human consumption. Similar findings were reported for yellow mealworm and house cricket (Finke, 2002, 2007; Yu *et al.*, 2021). While a proper statistical comparison of amino-acid profiles between sexes was not possible, no apparent differences were observed, consistent with (Kulma *et al.*, 2019), who found that only protein content varied by sex in house crickets.

Numerous studies have demonstrated that the Jones' factor (6.25) significantly overestimates the insect protein content (Table S2). In this study, the nitrogen-to-protein conversion factor (Kp) was determined by calculating the total nitrogen and total amino-acid content while considering that, based on a literature review (Table S3), 8% of the protein nitrogen originates from amino acids not analysed in this study. The obtained Kp values, ranging from 3.57 in male discoid cockroaches to 5.29 in pupal yellow mealworms, fall on the lower end of the range of values reported in the literature (Table S2). Notably, the Kp values for discoid cockroaches were particularly low (3.57–3.78), likely due to their elevated non-protein nitrogen content, which may be associated with the accumulation of uric acid in the fat body. In cockroaches, surplus nitrogen from a protein-rich diet is stored as uric acid within the fat body (Mullins and Cochran, 1975; Cerreta *et al.*, 2022). During periods of protein deficiency, this uric acid functions as a nitrogen reservoir, enabling amino-acid biosynthesis through the activity of their endosymbiotic microorganisms (Patino-Navarrete *et al.*, 2014).

Elemental composition

Elemental concentrations in insects and their feed in fresh weight (FW) are presented in Tables 4 and 5, including macro elements: Na, Mg, K, Ca; micro elements: Zn, Al, Cu, Fe, Mn, Rb, Sr, Mo, Ni and trace elements: V, Cr, Co, As, Se, Ag, Cd, Sb and Pb. Daily intake values for 100 g fresh insects are presented in Table 6. In general, insect consumption would provide the best supply of the following essential elements: Zn (25.6±3.3 to 112.5±14.5%), Cu (26.2±1.2 to 233.2±10.8%), Fe (9.2±0.4 to 14.4±0.6%) and Mn (6.0±0.7 to 86.4±9.6%). The quality of the substrate when rearing insects for livestock and human consumption is a key factor in ensuring the feed safety (Purschke *et al.*, 2017). According to maximum levels established by Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed for Cu (15–35 mg/kg FW), Fe (30 mg/kg FW), Cd (2 mg/kg FW), Pb (10 mg/kg FW), As (2 mg/kg FW) and Hg (0.1 mg/kg FW) in the feedstuff, all the insects tested would be suitable for feed

TABLE 3 Concentrations of amino acids (g/100 g DW), essential and nonessential amino-acid content (g/100 g DW) and essential total amino-acids ratio (%) in insects and feed

		Alanine	Aspartic acid	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Proline	Serine	Valine	Threonine	Tyrosine	Total EAA	Total NEAA	E/T (%)
Insects	DL (female)	5.39±0.30	4.33±0.14	4.93±0.12	3.11±0.05	1.22±0.06	2.06±0.11	4.37±0.17	3.32±0.10	0.575±0.015	1.76±0.03	3.47±0.17	2.38±0.05	4.38±0.25	2.46±0.15	3.10±0.14	23.2±1.4	23.6±1.2	49.6±0.3
	ML (adult)	5.53±0.34	4.29±0.01	5.08±0.37	3.15±0.05	1.30±0.02	2.27±0.04	4.82±0.17	3.52±0.08	0.609±0.04	1.82±0.09	3.88±0.12	2.25±0.07	4.83±0.16	1.99±0.10	3.04±0.17	24.2±0.9	24.2±1.1	50.0±0.3
	YM (larvae)	3.53±0.01	3.85±0.01	4.33±0.04	2.62±0.05	1.44±0.01	1.98±0.00	3.81±0.05	3.11±0.05	0.498±0.01	1.66±0.06	3.17±0.09	2.07±0.06	3.89±0.04	2.25±0.02	3.09±0.03	21.7±0.4	19.6±0.2	52.6±0.2
	YM (pupae)	2.81±0.15	4.04±0.13	4.24±0.20	2.48±0.07	1.41±0.06	1.99±0.10	3.78±0.15	2.81±0.15	0.518±0.032	1.88±0.11	2.49±0.07	2.14±0.08	3.81±0.20	2.26±0.04	3.93±0.20	22.4±1.5	18.2±1	55.2±0.3
	HC	3.60±0.14	5.87±0.36	3.97±0.09	2.82±0.08	1.19±0.04	2.32±0.11	4.05±0.16	3.65±0.23	0.972±0.061	2.26±0.14	3.24±0.14	3.08±0.16	3.88±0.17	3.13±0.17	2.74±0.11	24.2±1.7	22.6±1.4	51.7±0.2
	JFC (subadult)	3.53±0.10	3.29±0.08	4.15±0.27	2.47±0.00	0.986±0.012	1.60±0.02	3.35±0.02	3.11±0.16	0.516±0.004	1.46±0.02	2.57±0.04	1.82±0.04	3.29±0.03	2.13±0.00	3.05±0.06	19.5±0.4	17.8±0.8	52.2±0.6
	JFC (adult)	4.07±0.11	4.66±0.03	4.51±0.07	2.73±0.05	1.20±0.00	1.96±0.03	4.12±0.03	3.73±0.04	0.744±0.004	1.80±0.01	3.04±0.07	2.78±0.02	3.75±0.09	2.53±0.03	2.66±0.07	22.5±0.3	21.8±0.2	50.8±0.1
	LM (larvae)	3.33±0.00	4.38±0.06	3.97±0.19	2.43±0.03	1.68±0.50	2.18±0.03	3.67±0.01	3.98±0.07	0.650±0.009	2.36±0.04	2.81±0.01	2.24±0.00	3.92±0.09	1.91±0.06	3.98±0.11	24.3±0.3	19.2±0.1	55.9±0.4
	LM (pupae)	3.20±0.12	4.21±0.17	4.77±0.77	2.43±0.08	1.95±0.30	2.04±0.05	3.61±0.14	3.76±0.22	0.666±0.037	2.64±0.07	2.74±0.08	2.29±0.03	3.99±0.11	2.45±0.05	4.47±0.12	25.6±1.0	19.6±0.5	56.6±1.6
	DC (male)	4.07±0.16	3.66±0.07	4.73±0.39	3.15±0.02	1.56±0.02	1.75±0.01	3.51±0.04	3.00±0.04	0.616±0.013	1.65±0.01	2.99±0.04	2.15±0.02	4.15±0.08	2.36±0.13	3.31±0.08	21.9±0.1	20.8±0.4	51.4±0.6
	DC (female)	3.34±0.05	4.08±0.04	4.27±0.50	2.79±0.00	1.46±0.01	1.73±0.02	3.44±0.03	3.10±0.03	0.631±0.005	1.81±0.00	2.66±0.03	2.24±0.01	3.89±0.02	2.16±0.27	3.09±0.00	21.3±0.3	19.4±0.7	52.4±0.5
	HC*	3.80±0.10	4.43±0.23	4.35±0.11	2.55±0.04	1.15±0.04	1.92±0.10	3.80±0.19	3.44±0.17	0.657±0.036	1.73±0.05	2.71±0.06	2.39±0.02	3.81±0.18	1.82±0.04	2.73±0.10	21.1±1.3	20.2±0.8	51.0±0.6
	YM*	3.52±0.04	4.41±0.01	4.24±0.11	2.68±0.03	1.55±0.01	2.12±0.01	4.12±0.01	3.68±0.09	0.623±0.004	1.95±0.02	3.10±0.03	2.11±0.12	4.08±0.06	2.44±0.07	3.38±0.02	24.0±0.1	20.1±0.2	54.4±0.3
	ML*	4.27±0.03	4.19±0.02	5.26±0.02	2.90±0.00	1.22±0.01	1.97±0.03	4.10±0.04	3.55±0.19	0.687±0.012	1.79±0.08	3.15±0.03	2.45±0.07	3.97±0.01	2.39±0.04	2.82±0.04	22.5±0.5	22.2±0.2	50.3±0.3
Feed	CF (HC*)	0.690±0.026	1.74±0.13	3.19±0.16	0.891±0.008	0.470±0.018	0.728±0.033	1.52±0.02	1.38±0.22	0.302±0.022	0.989±0.003	1.18±0.03	0.889±0.099	1.11±0.04	0.661±0.034	0.648±0.028	7.81±0.6	8.58±0.65	47.7±0.0
	WB, CF (4:1, YM*)	0.591±0.013	0.878±0.011	2.49±0.06	0.837±0.014	0.406±0.004	0.440±0.025	1.01±0.01	0.749±0.012	0.138±0.003	0.630±0.000	0.884±0.015	0.638±0.028	0.851±0.035	0.475±0.017	0.451±0.003	5.15±0.09	6.32±0.17	44.9±1.1
	CF (ML*)	0.527±0.010	1.18±0.03	2.86±0.05	0.745±0.018	0.394±0.008	0.594±0.010	1.21±0.03	0.885±0.030	0.145±0.003	0.781±0.015	1.00±0.03	0.719±0.011	0.927±0.012	0.530±0.007	0.500±0.015	5.96±0.19	7.03±0.21	45.9±0.1
	WB (ML*)	0.610±0.007	0.898±0.006	2.33±0.03	0.863±0.008	0.409±0.004	0.419±0.006	1.00±0.01	0.729±0.007	0.144±0.000	0.628±0.041	0.873±0.014	0.628±0.005	0.852±0.002	0.479±0.006	0.452±0.003	5.11±0.11	6.2±0.01	45.2±0.6
	H (ML*)	0.451±0.006	0.674±0.021	0.336±0.029	0.453±0.005	0.138±0.001	0.314±0.000	0.697±0.007	0.444±0.004	0.081±0.002	0.433±0.002	0.378±0.009	0.255±0.001	0.576±0.004	0.277±0.002	0.290±0.001	3.25±0.03	2.55±0.04	56.1±0.2
	PG (ML*)	0.867±0.010	1.41±0.02	0.820±0.094	0.631±0.009	0.245±0.002	0.493±0.008	1.05±0.01	0.802±0.018	0.178±0.003	0.624±0.015	0.574±0.002	0.497±0.007	0.920±0.000	0.452±0.003	0.454±0.005	5.21±0.05	4.79±0.12	52.1±0.9

DL, desert locust; ML, migratory locust; YM, yellow mealworm; HC, house cricket; JFC, Jamaican field cricket; LM, lesser mealworm; DC, discoid cockroach; CF, chicken feed; WB, wheat bran; H, hay; PG, Poaceae grass.

* Insects reared exclusively on the analysed batch of substrates.

(The European Parliament and the Council, 2002). The risk assessment showed that the consumption of 100 g of fresh insects per day does not pose a risk to an adult human.

The elemental composition of insects is depends on diet, growing environment and processing mechanisms (Haque *et al.*, 2021). The elemental concentrations in the insects in this study (Tables 4 and 5) agree with data for yellow mealworm, house cricket and the migratory locust obtained by Finke (2015, 2002) and Machado (2024). The results indicate different elemental compositions based on species, developmental stage, and sex. Among insect species, house crickets had the highest levels of Na (2028±60 mg/kg FW), Ca (676±29 mg/kg FW), Fe (23.1±0.9 mg/kg FW) and Se (111±9 µg/kg FW), adult migratory locusts the highest concentrations of Zn (124±16 mg/kg FW) and Cu (30.3±1.4 mg/kg FW), adult Jamaican field crickets Mn (25.9±2.9 mg/kg FW), male discoid cockroaches K (9622±516 mg/kg FW) and yellow mealworms* Mg (1193±47 mg/kg FW). Species differed significantly in their Na, Mg, K, Ca, Al, Cu, Fe, Mn, Rb, Mo, Co, As, Se, Cd, Sb, and Pb content. Meyer-Rochow *et al.* (2021) also reported species-specific patterns but found that differences in the elemental composition of insects are primarily attributable to geographic and ecological factors since elements are obtained from dietary sources.

Depending on the stage of development, larvae of the lesser mealworm and both yellow mealworms had significantly higher concentrations of Zn (49.1±6.3 to 96.8±12.5 mg/kg FW), Rb (0.285±0.022 to 0.818±0.062), Sr (0.358±0.024 to 1.43±0.09 mg/kg FW), Ni (0.115±0.007 to 0.232±0.015 mg/kg FW), Co (14.8±0.9 to 19.0±1.1 µg/kg FW) and Cd (18.2±5.0 to 35.1±9.7 µg/kg FW) compared to pupal insects (Zn: 28.2±3.6 to 30.9±4 mg/kg FW, Rb: 0.160±0.012 to 0.292±0.022, Sr: 0.25±0.017 to 0.269±0.018 mg/kg FW, Ni: 0.032±0.002 to 0.046± 0.003 mg/kg FW, Co: 7.89±0.46 to 11.8±0.7 µg/kg FW and Cd: 7.11±1.96 to 10.3±2.8 µg/kg FW). Although a statistical comparison of adult (both migratory locusts, Jamaican field cricket) and subadult insects (Jamaican field cricket) was not possible due to insufficient number of samples, the data show that adult insects had higher concentrations of Mg (298±12 to 348±14 mg/kg FW), Zn (59.7±7.7 to 124±16 mg/kg FW), Cu (6.19±0.29 to 30.3±1.4 mg/kg FW), and Mo (0.261±0.013 to 0.296± 0.015 mg/kg FW) compared to subadults (Mg: 213±8, Zn: 48.2±6.2, Cu: 5.05±0.23, Mo: 0.152±0.007 mg/kg FW), while they have lower concentrations of Sr (0.359±0.024 to 0.62±0.041 mg/kg FW) compared to subadults (0.807±0.053 mg/kg FW), suggesting that in general adult insects contain more elements than subadults. Until now, there has been little research on the elemental composition of insects at different stages of development. Ghosh *et al.* (2020) reported an increase in mineral content with the developmental stage of male bees, while Carolyne *et al.* (2017) suggested that house crickets should be harvested at 9–11 weeks as the larvae are in their best nutritional condition at that age based on elemental composition.

In terms of sex, female desert locusts and discoid cockroaches had significantly higher concentrations of Mn (3.99±0.44 to 4.84±0.54 mg/kg FW), Co (22.0±1.3 to 25.1±1.5 µg/kg FW) and Se (73.4±6.1 to 96.5±8.0 µg/kg FW) compared to male insects (Mn: 2.41±0.27 to 3.05±0.34 mg/kg FW, Co: 13.7±0.8 to 16.8±1.0 µg/kg FW and Se: 39.7±3.3 to 68.9±5.7 µg/kg FW). To date, no differences in elemental composition based on the sex of the insects have been reported, making it difficult to compare these results.

Polyphenols

Sixty-one polyphenols were detected in the tested insect's feed (Supplementary Material 2), ranging from <LOQ to 1050 mg/kg DW, with each feed type containing 16 (Wheat bran ML*) to 54 different polyphenols (Hay ML*). Except for hay (2254 mg/kg DW) and Poaceae grass (461 mg/kg DW), the total concentrations of polyphenols in feed were relatively low, 66.2–146 mg/kg DW (Figure 1). Neochlorogenic acid (132 mg/kg DW), chlorogenic acid (1050 mg/kg DW), orientin (139 mg/kg DW) and hesperidin (249 mg/kg DW) were detected in the highest concentrations in hay and Poaceae grass-fed to ML*.

Fifty-nine polyphenols were detected in insects (Supplementary Material 2), with individual species containing between eight and 34 polyphenols. The following insects were found to have the highest total polyphenols: desert locust (adult female) at 511 mg/kg DW, migratory locust (adult) at 288 mg/kg DW, discoid cockroach (male) at 449 mg/kg DW, and discoid cockroach (female) at 357 mg/kg DW. Total concentrations in other insects ranged from 7.6 mg/kg DW (Jamaican field cricket, subadult) to 91.1 mg/kg DW (lesser mealworm, pupae). Among the polyphenolic compounds identified, 3,5-dihydroxybenzoic acid (303 mg/kg DW), protocatechuic acid (114 mg/kg DW), and quercetin (172 mg/kg DW) were found in the highest concentrations, particularly in desert locusts (females) and discoid cockroaches (both males and females).

Thus far, the polyphenols profiles were determined only for house crickets (292.37±1.37 to 2100 mg gal-

TABLE 4 Concentrations of essential elements in insects and feed (FW)

	Sample	Ca (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	K (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	Se (µg/kg)	Zn (mg/kg)
Insects	DL (male)	209±9	17.7±0.8	14.9±0.6	2744±147	269±11	3.05±0.34	790±23	39.7±3.3	56.3±7.3
	DL (female)	237±10	28.7±1.3	15.9±0.6	2937±157	338±13	4.84±0.54	1011±30	96.5±8.0	45.2±5.8
	ML (adult)	237±10	30.3±1.4	17.5±0.7	2930±157	311±12	2.94±0.33	950±28	61.6±5.1	124±16
	YM (larvae)	164±7	7.06±0.33	21.5±0.9	3221±173	817±32	2.83±0.31	580±17	33.0±2.7	96.8±12.5
	YM (pupae)	112±5	4.68±0.22	16.6±0.7	2390±128	532±21	2.21±0.24	440±13	33.6±2.8	28.2±3.6
	HC	676±29	8.82±0.41	22.2±0.9	4965±266	461±18	19.3±2.1	2028±60	111±9	62.4±8.1
	JFC (subadult)	435±19	5.05±0.23	16.4±0.7	3274±175	213±8	14.6±1.6	1158±34	65.0±5.4	48.2±6.2
	JFC (adult)	450±20	6.19±0.29	14.7±0.6	3068±164	298±12	25.9±2.9	1138±33	77.5±6.5	59.7±7.7
	LM (larvae)	158±7	5.04±0.23	17.7±0.7	2899±155	413±16	3.83±0.42	573±17	26.0±2.2	54.3±7.0
	LM (pupae)	188±8	5.80±0.27	22.5±0.9	3018±162	446±17	4.58±0.51	628±18	45.0±3.7	30.9±4.0
	DC (male)	333±14	5.00±0.23	21.1±0.9	9622±516	342±13	2.41±0.27	1388±41	68.9±5.7	77.5±10.0
	DC (female)	470±20	3.41±0.16	22.0±0.9	3765±202	347±14	3.99±0.44	1401±41	73.4±6.1	63.0±8.1
	HC*	506±22	7.16±0.33	23.1±0.9	3282±176	301±12	11.5±1.3	1236±36	91.2±7.6	66.1±8.5
	YM*	162±7	7.17±0.33	19.7±0.8	3331±179	1193±47	3.98±0.44	961±28	52.7±4.4	49.1±6.3
	ML*	259±11	14.3±0.7	22.1±0.9	3540±190	348±14	1.81±0.20	444±13	56.3±4.7	64.4±8.3
Feed	CF (HC*)	3076±134	61.7±2.9	64.7±2.6	2690±144	537±21	49.5±5.5	551±16	96.9±8.1	31.1±4.0
	WB, CF (4:1, YM*)	414±18	3.83±0.18	45.5±1.9	3806±204	1244±49	40.5±4.5	56.2±1.6	18.7±1.6	29.9±3.9
	CF (ML*)	3068±133	4.68±0.22	121±5	2960±159	552±22	63.1±7.0	337±10	101±8	64.2±8.3
	WB (ML*)	235±10	3.78±0.18	39.3±1.6	3727±200	1148±45	41.5±4.6	3.71±0.11	3.31±0.28	32.6±4.2
	H (ML*)	721±31	1.27±0.06	16.7±0.7	5393±289	297±12	30.7±3.4	4.28±0.13	7.18±0.6	5.24±0.68
	PG (ML*)	888±39	2.62±0.12	350±14	5113±274	440±17	110±12	46.85±1.38	96.4±8	9.78±1.26

DL, desert locust; ML, migratory locust; YM, yellow mealworm; HC, house cricket; JFC, Jamaican field cricket; LM, lesser mealworm; DC, discoid cockroach; CF, chicken feed; WB, wheat bran; H, hay; PG, Poaceae grass.

* Insects reared exclusively on the analysed batch of substrates.

TABLE 5 Concentrations of non-essential elements in insects and feed (FW)

	Sample	Ag (μg/kg)	Al (mg/kg)	As (μg/kg)	Cd (μg/kg)	Co (μg/kg)	Cr (μg/kg)	Ni (mg/kg)	Mo (mg/kg)	Pb (μg/kg)	Rb (mg/kg)	Sb (μg/kg)	Sr (mg/kg)	V (μg/kg)
Insects	DL (male)	<1.4	22.2±1.5	22.6±0.6	19.1±5.3	16.8±1.0	65.2±8.8	0.134±0.009	0.366±0.018	8.38±1.25	1.57±0.12	4.15±0.5	0.477±0.032	8.05±0.12
	DL (female)	961±60	17.1±1.1	25.8±0.6	27.9±7.7	25.1±1.5	35.3±4.7	0.385±0.024	0.350±0.017	3.61±0.54	1.20±0.09	5.65±0.68	0.710±0.047	5.81±0.09
	ML (adult)	5.91±0.37	18.3±1.2	18.2±0.5	10.5±2.9	15.3±0.9	98.2±13.2	0.088±0.006	0.296±0.015	14.8±2.2	3.13±0.24	2.27±0.27	0.359±0.024	12.0±0.2
	YM (larvae)	<1.4	3.96±0.27	21.5±0.5	32.3±8.9	19.0±1.1	49.5±6.6	0.207±0.013	0.299±0.015	<0.1	0.818±0.062	0.48±0.06	1.43±0.09	39.2±0.6
	YM (pupae)	<1.4	2.63±0.18	13.1±0.3	10.3±2.8	7.89±0.46	27.9±3.7	0.032±0.002	0.22±0.011	<0.1	0.292±0.022	<0.24	0.250±0.017	2.21±0.03
	HC	<1.4	15.7±1.0	7.30±0.18	3.90±1.07	18.8±1.1	67.2±9.0	0.094±0.006	0.442±0.022	8.60±1.29	2.32±0.18	<0.24	0.475±0.031	18.6±0.3
	JFC (subadult)	<1.4	21.7±1.5	5.00±0.12	5.92±1.63	25.5±1.5	107±14	0.063±0.004	0.152±0.007	23.0±3.4	1.44±0.11	1.07±0.13	0.807±0.053	12.5±0.2
	JFC (adult)	<1.4	19.5±1.3	9.08±0.23	7.22±1.99	19.5±1.1	27.1±3.6	0.073±0.005	0.261±0.013	13.5±2.0	1.82±0.14	<0.24	0.581±0.038	9.01±0.13
	LM (larvae)	<1.4	4.94±0.33	4.81±0.12	18.2±5.0	14.8±0.9	240±32	0.115±0.007	0.204±0.010	3.53±0.53	0.285±0.022	<0.24	0.358±0.024	8.20±0.12
	LM (pupae)	<1.4	15.0±1.0	5.99±0.15	7.11±1.96	11.8±0.7	50.9±6.8	0.046±0.003	0.186±0.009	6.94±1.04	0.160±0.012	<0.24	0.269±0.018	10.7±0.2
	DC (male)	<1.4	24.5±1.6	5.02±0.12	1.24±0.34	13.7±0.8	34.0±4.6	0.060±0.004	0.234±0.012	5.91±0.88	2.35±0.18	5.40±0.65	0.720±0.048	7.44±0.11
	DC (female)	<1.4	26.3±1.8	8.52±0.21	2.22±0.61	22.0±1.3	31.3±4.2	0.081±0.005	0.347±0.017	30.5±4.6	2.54±0.19	1.85±0.22	0.968±0.064	18.1±0.3
	HC*	<1.4	36.7±2.5	5.70±0.14	5.13±1.41	11.5±0.7	26.8±3.6	0.052±0.003	0.449±0.022	38.6±5.8	0.911±0.069	<0.24	0.733±0.049	27.4±0.4
	YM*	<1.4	0.53±0.04	15.16±0.38	35.1±9.7	16.8±1.0	8.76±1.18	0.232±0.015	0.320±0.016	<0.1	0.489±0.037	<0.24	0.716±0.047	8.18±0.12
	ML*	<1.4	23.5±1.6	27.2±0.7	6.06±1.67	13.5±0.8	35.7±4.8	0.280±0.018	0.261±0.013	20.6±3.1	0.993±0.076	197±24	0.620±0.041	19.6±0.3
Feed	CF (HC*)	<1.4	31.3±2.1	27.7±0.7	9.44±2.60	126±7	102±14	0.406±0.026	1.11±0.05	44.4±6.6	1.10±0.08	4.62±0.56	3.83±0.25	221±3
	WB, CF (4:1, YM*)	<1.4	4.16±0.28	8.61±0.21	30.7±8.5	15.7±0.9	11.9±1.6	0.405±0.026	0.529±0.026	3.54±0.53	2.04±0.16	<0.24	1.92±0.13	8.9±0.13
	CF (ML*)	<1.4	81.2±5.4	36.1±0.9	9.05±2.49	160±9	693±93	0.882±0.056	1.38±0.07	119±18	1.41±0.11	10.4±1.3	3.69±0.24	195±3
	WB (ML*)	<1.4	1.72±0.12	7.61±0.19	33.2±9.1	14.5±0.8	8.80±1.20	0.406±0.026	0.241±0.012	2.18±0.33	2.28±0.17	<0.24	1.70±0.11	3.00±0.04
	H (ML*)	<1.4	19.0±1.3	5.61±0.14	19.7±5.4	12.2±0.7	24.1±3.2	0.210±0.013	0.310±0.015	9.97±1.49	4.19±0.32	2.73±0.33	2.57±0.17	31.5±0.5
	PG (ML*)	4.91±0.31	535±36	206±5	36.6±10.1	158±9	738±99	0.800±0.051	0.268±0.013	465±70	1.19±0.09	28.0±3.4	6.76±0.45	879±13

DL, desert locust; ML, migratory locust; YM, yellow mealworm; HC, house cricket; JFC, Jamaican field cricket; LM, lesser mealworm; DC, discoid cockroach; CF, chicken feed; WB, wheat bran; H, hay; PG, Poaceae grass.

* Insects reared exclusively on the analysed batch of substrates.

TABLE 6 Daily intake values (%) for 100 g of fresh insects

	RDI (mg/day)								
	Ca (1000)	Cu (1.3)	Fe (16)	K (3500)	Mg (300)	Mn (3)	Na (3000)	Se (0.07)	Zn (11)
DL (male)	2.1±0.1	136.2±6.3	9.3±0.4	7.8±0.4	9.0±0.4	10.2±1.1	4.0±0.1	5.7±0.5	51.1±6.6
DL (female)	2.4±0.1	221.0±10.2	10±0.4	8.4±0.4	11.3±0.4	16.1±1.8	5.1±0.1	6.1±0.5	41.1±5.3
ML (adult)	2.4±0.1	233.2±10.8	10.9±0.4	8.4±0.4	10.4±0.4	9.8±1.1	4.7±0.1	8.8±0.7	112.5±14.5
YM (larvae)	1.6±0.1	54.3±2.5	13.4±0.5	9.2±0.5	27.2±1.1	9.4±1.0	2.9±0.1	4.7±0.4	88.0±11.4
YM (pupae)	1.1±0.0	36.0±1.7	10.4±0.4	6.8±0.4	17.7±0.7	7.4±0.8	2.2±0.1	4.8±0.4	25.6±3.3
HC	6.8±0.3	67.9±3.1	13.9±0.6	14.2±0.8	15.4±0.6	64.4±7.1	10.1±0.3	15.8±1.3	56.8±7.3
JFC (subadult)	4.4±0.2	38.8±1.8	10.2±0.4	9.4±0.5	7.1±0.3	48.7±5.4	5.8±0.2	9.3±0.8	43.8±5.6
JFC (adult)	4.5±0.2	47.6±2.2	9.2±0.4	8.8±0.5	9.9±0.4	86.4±9.6	5.7±0.2	11.1±0.9	54.2±7.0
LM (larvae)	1.6±0.1	38.7±1.8	11.1±0.5	8.3±0.4	13.8±0.5	12.8±1.4	2.9±0.1	3.7±0.3	49.4±6.4
LM (pupae)	1.9±0.1	44.6±2.1	14.1±0.6	8.6±0.5	14.9±0.6	15.3±1.7	3.1±0.1	6.4±0.5	28.1±3.6
DC (male)	3.3±0.1	38.4±1.8	13.2±0.5	27.5±1.5	11.4±0.4	8.0±0.9	6.9±0.2	9.8±0.8	70.5±9.1
DC (female)	4.7±0.2	26.2±1.2	13.8±0.6	10.8±0.6	11.6±0.5	13.3±1.5	7.0±0.2	10.5±0.9	57.3±7.4
HC*	5.1±0.2	55.1±2.6	14.4±0.6	9.4±0.5	10.0±0.4	38.4±4.3	6.2±0.2	13.0±1.1	60.1±7.8
YM*	1.6±0.1	55.2±2.6	12.3±0.5	9.5±0.5	39.8±1.6	13.3±1.5	4.8±0.1	7.5±0.6	44.6±5.8
ML*	2.6±0.1	110.0±5.1	13.8±0.6	10.1±0.5	11.6±0.5	6.0±0.7	2.2±0.1	8.0±0.7	58.5±7.5

DL, desert locust; ML, migratory locust; YM, yellow mealworm; HC, house cricket; JFC, Jamaican field cricket; LM, lesser mealworm; DC, discoid cockroach; RDI, reference daily intake.

* Insects reared exclusively on the analysed batch of substrates.

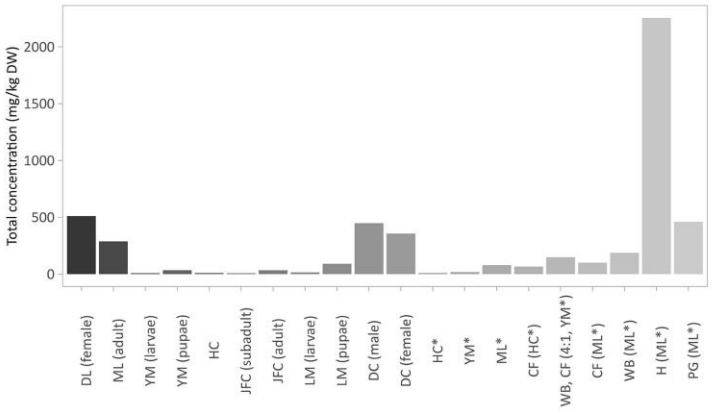


FIGURE 1 Histogram showing total concentrations of polyphenols in insects and feed.

lic acid/100 g), lesser mealworms (283.26±0.63 mg gallic acid/100 g) and yellow mealworms (273.15±2.03 mg gallic acid/100 g) by Gumul *et al.* (2023) and Nino *et al.* (2021a), while this study identified polyphenols in four other species. The polyphenol profiles of house cricket, lesser, and yellow mealworms determined in this study differed from those of previous studies, while the total concentrations are hard to compare since the Folin–Ciocalteu reagent method was not used in this study. Although there is no universal classification, based on research (Rothwell *et al.*, 2013), foods can be broadly divided into low (<10 mg/kg), medium (100-1000 mg/kg) and high (>1000 mg/kg) concentrations of polyphenols. In this regard, the studied insects (dry weight basis) would be classified as low- and medium-concentration foods.

The results show how different polyphenols are found in different insect species, and those with the

highest total concentrations of polyphenols also contain the largest number of different polyphenols. It is reasonable to assume that differences between species can have an impact on polyphenol concentrations in insects, as each species has a different genetic basis, develops specific metabolic pathways, has to adapt to its environment in its own way, has specific dietary references, defence mechanisms and differences in physiological functions (e.g. mating and reproduction).

With regard to developmental stage, pupae had higher total polyphenol concentrations than larvae in both the yellow mealworms (26 mg/kg) and the lesser mealworms (76 mg/kg), while the adult Jamaican field crickets had higher total polyphenol concentrations than the subadults (26 mg/kg). The number of polyphenols was comparable in lesser mealworm larvae and pupae (n = 10 and n = 12) and yellow mealworm larvae and pupae (n = 9 and n = 10), while ten polyphenols were present in either larval or pupal lesser mealworm and seven in yellow mealworm larvae/pupae. Adult Jamaican crickets (n = 11) contained three polyphenols more than subadult crickets (n = 8), while nine polyphenols were present in only one of them. While direct comparative studies are lacking, the number, variety and concentrations of polyphenols can vary according to developmental stage, as insects at different life stages (larva, pupa, adult) often require different nutrients and defences against oxidative stress due to changes in physiology, metabolism and nutritional needs, which can affect polyphenol synthesis.

Sex differences in polyphenol concentrations were evident in the discoid cockroach, with male insects having higher total polyphenol concentrations by 92 mg/kg. The number of individual polyphenols in males (n = 21) and females (n = 20) were similar, while 11 of them were present either in males or females. Even though there is little evidence of this in the literature, sex differences could affect polyphenol concentrations, as males and females often have different biological roles, needs, and energy expenditures, which can result in different polyphenol use or synthesis (Nino *et al.*, 2021b).

The total polyphenol concentrations of the insects, whose diets were also analysed, followed the order lesser mealworm*>yellow mealworm*>house cricket*, the same order as found for their diets. The same trend was observed for the number of individual polyphenols detected: lesser mealworm* (n = 29)>yellow mealworm* (n = 15)>house cricket* (n = 8). The much lower concentration and variety of polyphenols in insects compared to their feed suggest that insects metabolise polyphenols from their feed into other compounds. A possible explanation for low concentrations in insects could be the use of a processed diet instead of a fresh-plant diet, which could affect the insect's absorption of phenolic compounds. For example, Burghardt *et al.* (2001) observed that the absorption of phenolics was higher when the common blue butterfly (*P. icarus*) was fed natural host plants compared to an experimental diet. Also, given that the majority of the reared insects, with the exception of locusts, in this study were fed processed chicken feed and wheat bran instead of a fresh plant diet, absorption could have been affected. This finding is supported by the fact that both desert and migratory locusts were fed with hay and fresh grass containing the highest total polyphenol concentrations. Notably, the discoid cockroaches also had a high total polyphenol content even though they were fed processed feed. A possible reason is the *de novo* synthesis of polyphenols through the endogenous process of sclerotisation and melanisation (Nino *et al.*, 2021b; Torres-Castillo and Olazarán-Santibáñez, 2023). Although this study shows that insects are low in polyphenols, the possibility of increasing bioactive compounds through feed manipulation remains.

4 Conclusions

This study confirms the nutritional potential of seven farmed insects (desert locust, migratory locust, yellow mealworm, house cricket, Jamaican field cricket, lesser mealworm and discoid cockroach) as sources of essential fatty acids, amino acids, and minerals. With regards to fatty-acid content, differences were observed among species and between developmental stages, showing that adult insects have a better fatty-acid profile for the human diet than subadults. The fatty-acid composition of insects suggests metabolic differences between species and physiological changes during growth. Sex-based differences were evident for both desert locust and discoid cockroach, with males exhibiting a slightly higher fatty-acid quality in terms of essential fatty acids and polyunsaturated fatty-acids content, indicating distinct reproductive energy demands of each sex. Also, amino-acid profiles differed significantly across species and developmental stages but not between sexes. Differences between species, developmental stages and sexes were also found for elements, reflecting metabolic differences between species, physiological needs during growth and different biological roles, highlighting the complexity of nutrient dynamics in insects.

Regarding polyphenols, the data indicate that insect species tested could be considered a low to medium source (dry weight basis) compared to conventional polyphenol-rich foods. Levels of polyphenols also varied between species, with locusts and cockroaches being higher in total polyphenols. With regard to developmental stage, pupae had higher total polyphenol concentrations than larvae in both the yellow mealworm and the lesser mealworm, while the adult Jamaican field crickets had higher total polyphenol concentrations than the subadults. Only for the discoid cockroach did the males have a higher total polyphenol content than females. While polyphenol concentrations were generally low, the findings suggest potential bioactive benefits and open avenues for further exploration of insect-derived antioxidants. Finally, although the study is limited in sample size, these insights add to the body of data for optimising the rearing of farmed insects to enhance their nutritional quality.

Supplementary materials

Data is available on https://doi.org/10.1163/23524588-bja10234 under Supplementary Materials.

Author contributions

Anja Vehar: conceptualization, methodology, investigation, writing (original draft); Doris Potočnik: methodology, investigation; Lidija Strojnik: methodology, investigation; Tea Zuliani: investigation; David Heath: writing (review and editing), funding acquisition; Marjeta Mencin: investigation; Urška Vrhovšek: methodology; Petra Škvorová: investigation; Lenka Kouřimská: funding acquisition; Martin Kulma: investigation; Ester Heath: supervision; Nives Ogrinc: supervision, writing (review and editing), funding acquisition.

Conflict of interest

The authors declare no conflict of interest.

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3.3 Scientific Paper: "Evaluation of Nutritional Quality and Oxidation Stability of Fermented Edible Insects"

This chapter presents the paper entitled "Evaluation of Nutritional Quality and Oxidation Stability of Fermented Edible Insects" by Anja Vehar, Doris Potočnik, Marjeta Mencin, Mojca Korošec, Blaž Ferjančič, Marta Jagodic Hudobivnik, Polona Jamnik, Ajda Ota, Lenka Kouřimská, Martin Kulma, David Heath, and Nives Ogrinc. The paper was published in Foods (A'') in 2025. This paper describes the fermentation of three edible insect species, yellow mealworm, house cricket, and migratory locust, using *Lactobacillus plantarum* and commercial starter culture for 48 h, and its influence on their nutritional quality.

Nutritional quality was assessed in terms of proximate composition following official AOAC (Association of Official Agricultural Chemists) methods, fatty acids using GC-FID, amino acids using GC-MS, elements using ICP-MS, and oxidation stability using an oxidation stability tester. The results showed that fermentation effects depended on both the insect species and the fermentation conditions.

Fermentation with *L. plantarum* reduced total dietary fibre in both yellow mealworms and house crickets. At the same time, an increase in non-protein nitrogen was observed for both species. Other parameters, such as total nitrogen, protein nitrogen, and total fat content, remained largely unaffected by the fermentation process. Despite these changes, no significant improvements in the insects' fatty acid profiles were observed, likely due to the relatively short fermentation period used in this study.

Regarding amino acids, fermentation had a mixed effect. While specific amino acids, such as ornithine, showed beneficial increases, the fermentation process reduced the levels of essential amino acids, including lysine, methionine, and threonine. These amino acids are often limiting in plant-based diets, making their reduction less desirable.

The effects of fermentation on mineral composition were also variable. In some cases, fermentation increased the levels of essential elements, including Fe, K, Mg, Mn, Na, P, and Zn. However, reductions in other elements, including Zn, Se, and Cu, were observed. These changes in mineral composition appeared to reflect relative concentration effects, underscoring the complexity of the fermentation process.

Oxidation stability, an important functional property of food products, also varied among insect species. Fermentation decreased oxidation stability in both yellow mealworm and migratory locust, while a significant improvement was observed in house cricket. This variation highlights the complex interplay between insect species, microbial cultures, and processing conditions in determining both the nutritional and functional outcomes of fermented insect products.

This study fills a significant gap in the current literature, as there is limited data on the effects of fermentation on yellow mealworm and house cricket under these conditions. Also, no prior research has explored the fermentation of migratory locusts, making this work a novel contribution to the field.

As first author, I conducted the fatty acid and amino acid profiling, was responsible for all data analysis, prepared all visual materials, and was responsible for both the drafting and finalisation of the manuscript. The findings were presented as an oral presentation at INSECTA 2024, May 14-16, 2024, in Potsdam, Germany, and as a poster presentation at Insectsplus 2025, May 12-14, 2025, in Cloppenburg, Germany.

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Evaluation of Nutritional Quality and Oxidation Stability of Fermented Edible Insects

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Abstract

Fermentation, a traditional method for enhancing nutritional value and functionality, has significant potential for improving the quality, safety and acceptability of farmed insect products. In this study, yellow mealworm, house cricket and migratory locust were fermented using *Lactobacillus plantarum* and a commercial starter culture for 48 h. Samples were analyzed for proximate composition, amino and fatty acid profiles, elemental composition and oxidation stability. Fermentation reduced total dietary fiber in yellow mealworm (33%) and house cricket (12%), and increased non-protein nitrogen (38% and 16%), while total and protein nitrogen remained unaffected. Fatty acid profiles also remained unchanged, whereas the amino acid composition varied depending on the species and fermentation culture. Essential mineral concentrations varied depending on species and fermentation culture Fe (19–23%), K (25%), Mg (12–23%), Mn (36–378%), Na (20–49%) and P (22%) increased, levels of Se (15%), and Cu (16%) decreased, while Zn levels showed inconsistent trends among treatments. Oxidation stability of yellow mealworm (41–42%) and migratory locust (21–29%) decreased, but improved for house cricket (153–167%). Overall, fermentation enhanced the nutritional value of edible insects, although the extent of improvement varied by species and fermentation culture.

Keywords: edible insects; fermentation; nutritional value; amino acids; fatty acids; elemental composition; oxidation stability

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1. Introduction

Edible insects represent a diverse and nutrient-rich source of macro- and micronutrients, increasingly recognized as a sustainable alternative to traditional animal-based protein [1]. Compared to conventional livestock, farmed insects have a significantly lower

environmental footprint. For example, they require 75–95% less water and 80–90% less land. They also emit up to 80% fewer greenhouse gases while providing high-quality protein with a complete amino acid profile, essential fatty acids, and key micronutrients such as copper, iron, magnesium, zinc, and B vitamins [2]. In addition, insect farming is adaptable to vertical systems, generates minimal noise, and produces almost no waste, as insect frass can be used as an organic fertilizer [3,4]. Moreover, utilizing agricultural by-products and food waste as insect feed can further reduce production costs, addressing a major barrier to market entry [5–9], while contributing to food waste reduction in regions where such practices are allowed under local regulations [10–14].

The European Commission also recognizes the potential of insects as sustainable sources of food and feed by approving several species as novel foods under various regulations. Approved species include yellow mealworm (*Tenebrio molitor*; Commission Regulation 2021/882, 2022/169, 2025/89) [15–17], migratory locust (*Locusta migratoria*; Commission Regulation 2021/1975) [18], house cricket (*Acheta domesticus*; Commission Regulations 2022/188, 2023/5) [19,20], and lesser mealworm (*Alphitobius diaperinus*; Commission Regulation 2023/58) [21]. Furthermore, Commission Regulation 2021/1372 authorizes eight insect species for feed in poultry, pig, and fish farming [22]. These include tropical house cricket (*Gryllodes sigillatus*), black soldier fly (*Hermetia illucens*), Jamaican field cricket (*Gryllus assimilis*), house fly (*Musca domestica*), and silkworm moth (*Bombyx mori*), in addition to the aforementioned species.

Although insects have a long history of widespread consumption in several countries [23], they are met with significant skepticism, safety concerns, and unfamiliarity in Western societies [24,25]. One of the main factors influencing consumer perception is related to product characteristics, such as taste, price, texture, appearance, smell, the degree of insect visibility, and the proportion of insect-based ingredients [24]. However, these characteristics can be changed and improved using differing processing techniques, such as blanching, boiling, curing, drying, fermentation, freeze-drying, deep frying, marination, roasting, smoking and stewing, which can also reduce microbial loads and extend insect product shelf life [8,26–29].

Among these methods, fermentation is a widely recognized technique for improving the nutritional, functional, and sensory qualities of various food products [30]. The process involves spontaneous or controlled (starter culture-assisted) microbial activity that breaks down large molecules into smaller ones through various oxidation–reduction reactions. It can serve multiple purposes, including (i) enhancing sensory qualities such as flavor, aroma, and texture; (ii) increasing nutritional value by improving nutrient bioavailability; (iii) eliminating anti-nutritional factors; (iv) increasing levels of bioactive compounds, such as amino acids, polyphenols, organic acids, (v) promoting intestinal health by incorporating beneficial microorganism; and (vi) extending shelf life and reducing microbiological risks by inhibiting pathogenic microorganisms [30–33].

Recent studies have begun to explore the potential of fermentation for improving the nutritional and functional properties of edible insects, particularly species such as yellow mealworm [34–37], black soldier fly [38–41], house cricket [42,43], desert locust [36] and Mexican grasshopper [37]. Various microbial cultures, primarily lactic acid bacteria, *Bacillus* spp., and *Aspergillus* spp., have been used, with reported improvements in protein digestibility, amino acid profiles, fatty acid composition, antioxidant activity, shelf life and microbiological safety.

Changes in proximate composition and nutrient metabolism appear consistently across species, although the underlying mechanisms differ. For example, lactic acid fermentation of black soldier fly larvae and related products has been shown to modify proximate composition, amino acid and fatty acid profiles, reduce chitin content, and show antimicrobial

activity against pathogens [38]. Also, similar studies using *Bacillus subtilis*, *Aspergillus niger*, *Lactobacillus crispatus* and *Pichia kudriavzevii*, report shifts in carbon, amino acid, and fatty acid metabolism as well as changes in bioactive components and microbial community structure [39–41]. In house cricket, *Lactobacillus plantarum* fermentation has been reported to modify proximate composition, organic acids, alcohols, fatty and amino acid profiles, volatile compounds, mineral content, and the composition of the microbiome [43,44]. Other approaches, including the use of methods that mimic Thai fermented shrimp paste, have highlighted effects on peptide content, mineral composition, amino acids, and in vitro protein digestibility [42]. In yellow mealworm, fermentation with lactic acid bacteria, commercial meat starter cultures, and *Aspergillus oryzae* has been reported to modify proximate composition, amino acid and fatty acid profiles, antioxidant activity, and microbiome composition [44], along with microbial counts, sugar consumption, organic acid production, and sensory properties [26,34–37].

While these studies demonstrate the potential of fermentation to improve the nutritional and functional quality of edible insects, variations in insect species, fermentation conditions, and analytical approaches limit direct cross-study comparisons. Moreover, there remains a substantial knowledge gap regarding how different fermentation cultures and conditions influence the nutritional, functional, and safety characteristics of various insect species.

The present study investigates the effects of fermentation using *Lactobacillus plantarum* and a commercial starter culture on three farmed edible insect species: yellow mealworm, migratory locust, and house cricket, in terms of proximate composition, amino acid and fatty acid profiles, elemental composition, and oxidation stability. This work addresses an existing knowledge gap, as data on yellow mealworm and house cricket under such fermentation conditions remain limited, and, to the authors' knowledge, no studies on fermented migratory locust have yet been reported.

2. Materials and Methods

2.1. Chemicals and Reagents

Dichloromethane (CH_2Cl_2), methanol (MeOH), and hexane (C_6H_{14}) for organic residue analysis were sourced from J. T. Baker B.V. (Deventer, The Netherlands). Sodium hydroxide (NaOH) (puriss. p.a., ACS reagent, ≥98%) and 14% boron trifluoride in methanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (≥99.5%) was obtained from Merck, Germany. Ultrapure water (18.2 MΩ) was prepared using the Milli-Q System (Merck Millipore, Watertown, MA, USA). A Supelco 37 Component FAME Mix in dichloromethane, containing methyl esters of 37 fatty acids, was obtained from Supelco (Bellefonte, PA, USA). Amino acid standards (≥98%) used for analysis included L-valine, L-leucine, L-isoleucine, L-threonine, L-serine, L-proline, L-methionine, L-glutamic acid, L-phenylalanine, L-lysine, L-histidine, and L-tyrosine, sourced from Sigma Aldrich. Standards for alanine, glycine, and aspartic acid were purchased from Fluorochem (Derbyshire, UK). The internal standard, Stable Isotope Labelled Amino Acid Mix Solution 1, containing labelled variants of 17 amino acids, was obtained from Supelco (Buchs, Switzerland). Hydrochloric acid (37%) was sourced from VWR Chemicals (Fontenay-sous-Bois, France), while thioglycolic acid (≥99%, France) and phenol (≥99%, USA) were purchased from Sigma Aldrich. All other reagents for amino acid analysis were provided in the EZ Amino Acid Analysis Kit from Phenomenex (Torrance, CA, USA). For elemental composition analysis, nitric acid (65% Suprapur® for trace analysis, HNO_3) and hydrogen peroxide (30% Suprapur, for trace analysis, H_2O_2) were obtained from Supelco. For insect fermentation, De Man, Rogosa and Sharpe (MRS) broth was obtained from Merck (Darmstadt, Germany), and sodium chloride (NaCl) was obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Insect Rearing

Three edible insect species, the yellow mealworm, the house cricket, and the migratory locust, were selected based on their approval as novel foods by the EU (Commission Regulations 2021/882, 2021/1975, 2022/169, 2022/188, 2025/89). All species were sourced from the insect-rearing facility at the Faculty of Agrobiology, Food, and Natural Resources, Czech University of Life Sciences, Prague and were maintained under controlled conditions.

House crickets and mealworms were reared in a rack system under controlled conditions at 27 ± 1 °C, 40–50% relative humidity, and a 12:12 h light–dark photoperiod. Migratory locusts were maintained at the same temperature and photoperiod, but at a lower relative humidity of 30%. The humidity was controlled using a DeLonghi dehumidifier (Treviso, Italy) and measured daily with a Comet C3120 thermohygrometer (Comet system, Rožnov pod Radhoštěm, Czech Republic). No additional heating was used for house crickets and yellow mealworms, while migratory locusts were provided with heating foils and 40 W bulbs to create warm zones. House crickets and migratory locusts were reared in plastic boxes (56 × 39 × 28 cm, IKEA, Prague, Czech Republic), fitted with egg trays (Schubert Partner, Prague, Czech Republic) and secured with 80% aluminum anti-insect mesh lids. Yellow mealworms were reared in 60 × 40 × 12.5 cm breeding trays (Beekenkamp, Maasdijk, The Netherlands).

Insects were fed *ad libitum*. House crickets received chicken feed (77.9% wheat, 17.6% soybean meal, 1.8% rapeseed oil, 2.7% mineral and vitamin premix; particle size < 1 mm), while migratory locusts were provided wheat bran, grass hay, and fresh Poaceae grass. Mealworms were fed on a 4:1 mixture of wheat bran and chicken feed. Water gel (Oslavan, Náměšť nad Oslavou, Czech Republic) was supplied daily for house crickets and migratory locusts. House crickets and locusts were maintained in the same containers from hatching until harvest at the adult stage, while mealworms were harvested upon first pupal emergence.

All insects were starved for 24 h prior to euthanasia by freeze-killing (−20 °C for 24 h). Samples were lyophilized (Trigon Plus, Čestlice, Czech Republic), homogenized (A10; IKA Werke GmbH & Co. KG, Staufen, Germany), and stored at −20 °C until further analysis. The dry matter content (%) of fresh insects for further calculations was determined by calculating the mass difference before and after lyophilization and was 30%.

2.3. Insect Fermentation

The insect samples (yellow mealworm, house cricket and migratory locust) were subjected to lactic acid fermentation using *L. plantarum* and a commercial starter culture for meat processing. *Lactobacillus plantarum* IM 527 was obtained from the Institute of Dairy Science and Probiotics, Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Slovenia. An aliquot of *L. plantarum* stock culture (100 µL) was inoculated into 20 mL of sterilized liquid MRS medium and incubated on a rotary shaker (150 rpm, 30 °C, 12 h) until the late exponential phase was reached. The obtained broth (2 mL) was then centrifuged (10,000× *g*, 5 min) and washed twice with a saline solution to obtain the inoculum suspension.

A stock suspension of a commercial starter culture SM-194 Bactoferm® (Chr Hansen A/S, Hørsholm, Denmark) was prepared according to the manufacturer's instructions by dissolving 0.125 g of the culture in 5 mL of sterile saline solution. The starter culture comprised *Staphylococcus xylosus*, *Debaryomyces hansenii*, *Lactobacillus sakei*, *Staphylococcus carnosus*, and *Pediococcus pentosaceus*.

Both fermentation types were performed using 8 g of lyophilized and milled insect material, placed in 50 mL polyethylene centrifuge tubes and then capped. For house cricket and migratory locust, a substrate-to-sterile double distilled H_2O ratio of 1:2 (8 g:16 mL)

was used, whereas a ratio of 1:1.5 (8 g:12 mL) was applied for yellow mealworm. Samples were then inoculated with the *L. plantarum* or commercial starter culture suspension (240 µL for house cricket and migratory locust; 200 µL for yellow mealworm) and incubated under static conditions at 30 °C for 48 h.

Fermentation was performed in triplicate. To monitor the fermentation process, samples were collected at 0, 24, and 48 h, and the pH and colony-forming units (CFU) were measured. A reduction in pH (3–30%), accompanied by an increase in CFU count (29–47%) over the fermentation period, indicated active microbial growth and metabolic activity, confirming successful fermentation. Following fermentation, all samples were lyophilized (72 h, −53 °C, 0.280 mbar) and stored at −20 °C until further analysis.

2.4. Determination of Proximate Composition

Proximate composition of yellow mealworm and house cricket, both unfermented and fermented with *L. plantarum*, was determined using official AOAC methods for macronutrient analysis, though due to insufficient sample amounts, the analysis was limited to these two species. This decision was based on several practical considerations: (1) proximate composition requires relatively large sample quantities compared to other analyses in this study; (2) initial difficulties in rearing migratory locust resulted in limited biomass; and (3) the insectarium's capacity to produce large-scale insect biomass was constrained. Consequently, yellow mealworm and house cricket were selected as model species due to their current market relevance, relative ease of rearing, and established production protocols. *Lactobacillus plantarum* was chosen for fermentation as it is one of the most extensively studied lactic acid bacteria, particularly in the context of food fermentation.

The moisture content was measured following AOAC 950.46 [45], and dry matter was calculated as 1—the moisture content. Ash, total fat, and protein contents were determined using AOAC 920.153, 991.36, and 928.08 [45], respectively. Protein content was calculated from nitrogen (Kjeldahl method) using a factor of 6.25, after subtracting non-protein nitrogen (NPN).

The NPN extracts were prepared using a modified DeVries et al. (2017) method [46,47]. A known amount (2.00 g) of sample was mixed with 10 mL distilled water and 10 mL 24% trichloroacetic acid, homogenized (Ultra-Turrax, 13,500 rpm, 30 s), sonicated (10 min), centrifuged (4000 rpm, 10 min), and filtered with filter paper discs (grade 389, 125 mm diameter, 8–12 µm pore size, 100% cotton linters, Sartorius, Germany). NPN was determined from the clear filtrate (5 g) by the Kjeldahl method.

Dietary fiber content was determined using the enzymatic–gravimetric AOAC 991.43 method [45], employing heat-stable α-amylase, protease, and amyloglucosidase (Merck KGaA, Germany). Insoluble dietary fiber was recovered by filtration, while soluble dietary fiber was precipitated from filtrates with 96% ethanol. Ash and protein contents of residues were corrected, and total dietary fiber was the sum of insoluble and soluble fractions. Available carbohydrate content (g/100 g) was calculated using Equation (1) [48]:

$$\text{Available carbohydrate} = 100\ \text{g}/100\ \text{g} - (\text{water} + \text{ash} + \text{protein} + \text{fat} + \text{fiber}) \quad (1)$$

where protein is calculated as total nitrogen × 6.25, thereby accounting for both protein and non-protein nitrogen fractions.

The energy value (EV, kJ/100 g) was calculated as the sum of the energy values for each macronutrient using the following equation [48]:

$$\text{EV} = \text{EV}_{\text{protein}} + \text{EV}_{\text{fat}} + \text{EV}_{\text{available carbohydrates}} + \text{EV}_{\text{dietary fibre}} \quad (2)$$

The energy value of each nutrient was calculated by multiplying its content (g/100 g) by the corresponding energy conversion factor: 17 kJ/g for protein and available carbohydrates, 37 kJ/g for fat, and 8 kJ/g for dietary fiber [48].

2.5. Fatty Acid Analysis

Freeze-dried insect samples (200 mg) were weighed into screw-cap vials. To extract total lipids, 500 µL of dichloromethane and 3 mL of 0.5 M sodium hydroxide in methanol were added. The samples were purged with nitrogen gas, heated for 10 min at 90 °C, and then rapidly cooled in an ice bath. After cooling, 3 mL of boron trifluoride in methanol was introduced to convert the lipids into fatty acid methyl esters (FAMEs). The extract was then purged with nitrogen and heated for 10 min at 90 °C. Once cooled, the FAMEs were extracted using 3 mL Milli-Q water and 1.5 mL hexane. The organic phase was transferred to a GC vial and stored at −20 °C. All samples were prepared in duplicate.

All samples were analyzed using an Agilent 6890N gas chromatograph (Network GC System, Agilent Technology, Santa Clara, CA, USA) with a flame ionization detection (FID). Separation was achieved on a CP-Sil 88 capillary column (100 m × 0.25 mm × 0.20 µm, Agilent J&W). The temperature program was as follows: 100 °C (5 min), ramped to 180 °C at 8 °C/min (9 min), and then increased to 230 °C at 1 °C/min (1 min). Helium was used as the carrier gas in constant flow mode (1.3 mL/min), with an injection volume of 1 µL and a split ratio of 10:1. The inlet and detector were set to 260 °C and 250 °C, respectively.

Fatty acids were identified and quantified by comparing their retention times with the Supelco 37-component FAME mix standard. The results were expressed as relative weight percentages of total fatty acids. Procedural blanks were analyzed alongside each batch of samples, and the FAME mix standard was run after every ten samples to verify system stability.

Fatty acids were categorized as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), with a specific focus on n − 3 and n − 6 fatty acids. The PUFA/SFA and n − 6/n − 3 ratios were also calculated. Additionally, the atherogenic index (IA), thrombogenic index (IT), and hypocholesterolemic/hypercholesterolemic ratio (h/H) for the total lipid content were calculated according to the following equations [49]:

$$\mathrm{IA} = \frac{\mathrm{C12:0} + (4 \times \mathrm{C14:0}) + \mathrm{C16:0}}{\Sigma\mathrm{MUFA} + \Sigma\mathrm{n-6} + \Sigma\mathrm{n-3}} \quad (3)$$

$$\mathrm{IT} = \frac{\mathrm{C14:0} + \mathrm{C16:0} + \mathrm{C18:0}}{(0.5 \times \Sigma\mathrm{MUFA}) + (0.5 \times \Sigma\mathrm{n-6}) + (3 \times \Sigma\mathrm{n-3}) + (\mathrm{n-3/n-6})} \quad (4)$$

$$\mathrm{h/H} = \frac{\mathrm{C18:1} + \Sigma\mathrm{PUFA}}{\mathrm{C12:0} + \mathrm{C14:0} + \mathrm{C16:0}} \quad (5)$$

2.6. Amino Acid Analysis

A known weight of lyophilized insect sample (50 mg) was placed into hydrolysis micro-reaction vessels, and 2.5 mL of 4% thioglycolic acid in 6M HCl and 25 µL of liquid phenol (heated to 80 °C) were added. The mixture was then vortexed. The samples were then purged with nitrogen for 5 min, sealed, and heated at 110 °C for 24 h. During hydrolysis, glutamine was converted to glutamic acid, and asparagine to aspartic acid; therefore, reported values for glutamic and aspartic acids represent the combined total of these amino acids. After hydrolysis, the samples were filtered through CHROMAFIL Xtra PTFE 0.45 µm syringe filters (Macherey-Nagel, Düren, Germany).

Amino acid analysis was performed using the EZ:faastTM kit. First, 50 µL of the filtered hydrolysate was transferred into a sample preparation vial, followed by the addition

of 100 µL of 1 M Na_2CO_3 solution and vortexing. A 30 µL aliquot of the pH-adjusted mixture (pH 2) was then transferred into a new vial, and 50 µL of internal standard (200 nmol/mL) was added, followed by vortexing. The sample was then passed through a sorbent tip using a 1.5 mL syringe. After adding 200 µL of Milli-Q water into the vial, the sample was passed through the sorbent tip again, and the liquid was discarded. The sorbent tip was then detached.

The compounds of interest were eluted using 200 µL of a NaOH/N-propanol/3-picoline mixture (3/1.6/0.4, $v/v/v$). This elution was performed by attaching the sorbent tip to a 0.6 mL syringe, pulling back the plunger halfway, and wetting the sorbent with the eluting medium before expelling the liquid into the sample vial. A 50 µL aliquot of propyl chloroformate/chloroform/iso-octane (1:3:1, $v/v/v$) was added, and the contents were emulsified by vortexing for 5 s. The reaction was allowed to proceed for 1 min and repeated once more. After this, 100 µL of a 9:1 (v/v) iso-octane/chloroform mixture was added and vortexed for 1 min. One hundred µL of the organic layer was transferred into a GC autosampler vial, evaporated under nitrogen for 10 min, and reconstituted in 100 µL iso-octane/chloroform (4:1, v/v). The reconstituted sample was transferred into a GC-MS vial with a glass insert and analyzed. All samples were prepared in duplicate.

Samples were analyzed on a 7890B series GC coupled to a 5977A single quadrupole mass selective detector (Agilent, USA). Separation was achieved using a ZB-AAA GC column (10 m × 0.25 mm, Phenomenex, USA), with helium as the carrier gas (1.1 mL/min). A volume of 1.5 µL of the sample was injected in split mode (1:15). The GC oven temperature program was 110 °C (1 min), then ramped at 30 °C/min to 320 °C (total runtime: 7 min). The inlet and transfer line were set to 250 °C and 260 °C, respectively. The mass spectrometer was operated in electron impact (EI) mode at 70 eV, with a source temperature of 240 °C, a quadrupole temperature of 180 °C, and an auxiliary temperature of 310 °C. The scan range was 45–440 m/z, and the compounds were identified and quantified using selected ion monitoring (SIM) mode (Table S1). The data were processed using MassHunter software (version 10.0., Agilent Technologies, USA).

The ratio of essential to total amino acids (E/T, %) and the essential amino acid index (EAAI) were determined according to FAO (2013) [50] based on valine, leucine, isoleucine, threonine, phenylalanine + tyrosine, methionine, histidine, and lysine. Cystine and tryptophan were excluded from the calculations due to method limitations, since acid hydrolysis degrades tryptophan and cystine. The formula for EAAI is as follows:

$$\mathrm{EAAI} = \sqrt[8]{\frac{\mathrm{goflysinein100\ gofanalysedprotein}}{\mathrm{goflysinein100\ gofreferenceprotein}}} \times (\mathrm{etc.forother\ 7EAA}) \times 100 \quad (6)$$

The index is based on the sum of all amino acids compared to those of the reference protein for older children (≥3 years) and adults [50]. The amino acid with the lowest amino acid score (AAS) is considered the limiting amino acid. The AAS was calculated according to the following equation.

$$\mathrm{AAS} = \frac{\mathrm{mgaminoacidper\ g\ ofprotein}}{\mathrm{mgaminoacidper\ g\ ofreferenceprotein}} \times 100 \quad (7)$$

2.7. Elemental Analysis

Sample digestion for elemental analysis was performed following Vehar et al. (2025) [51]. Briefly, a known quantity of lyophilized insect sample (0.10–0.15 g) was placed into a pre-cleaned Teflon digestion vial. Subsequently, 1 mL of HNO_3 and 250 µL of H_2O_2 were added to each vial. All samples were microwave-digested using an Ultrawave Single Reaction Chamber Microwave Digestion System (Milestone, Italy). The digestion

program involved heating the samples to 240 °C over 20 min, followed by a 15-min hold at 100 bars with a maximum power of 1500 W. After digestion, the digestate was quantitatively transferred to a polyethylene-graduated tube and diluted to a final volume of 10 mL with Milli-Q water. For measuring macro elements, samples were further diluted with 5% HNO_3.

The determination of selected elements (Ag, Al, As, B, Ba, Ca, Cd, Co, Cs, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sb, Se, Sn, Sr, U, V, and Zn) was conducted using an Agilent 8800 triple quadrupole inductively coupled plasma mass spectrometer, QQQ-ICP-MS (Agilent, Japan, Tokyo). Different reaction gases, H_2, He and O_2 were used. Internal standards (Y, Rh, Sc, and Gd) were added online in all modes, and external calibration was used to quantify the results. All samples were prepared in duplicate. Additionally, blanks and certified reference materials (SRM NIST 1566b Oyster tissue, Typical Diet NIST 1548b) were prepared in each run in the same manner to ensure the quality and accuracy of the results. The limit of detection (LOD) was calculated as three times the standard deviation of the blank samples.

A risk assessment for toxic elements was performed based on an adult (70 kg) ingesting 100 g of fresh insects. The intake of each element was first calculated (Table S6) and then compared with reference values (Table S4) obtained from OpenFoodTox: EFSA's chemical hazards database [52]. Based on the concentrations of essential elements and their respective reference daily intakes (RDI; Table S5), the percentage contribution to daily intake for adults was reported, based on a consumption scenario of 100 g of fresh insects per day.

2.8. Oxidation Stability Analysis

To assess the oxidation stability, a known amount (3.5 g) of lyophilized sample was placed in the test chamber of a RapidOxy 100 Oxidation Stability Tester (Anton Paar, Austria). The induction time was measured at 120 °C and 700 kPa, with continuous monitoring of oxygen consumption. The induction time was recorded when the pressure drop reached 10%, indicating oxidation stability. The measurement error is 2–3%.

2.9. Statistical Analysis

Data processing and visualization were performed using R-based software (version 4.2.3). All data sets were checked for normal distribution using the Shapiro-Wilk test and for homoscedasticity using Levene's test. For proximate composition, where only yellow mealworm and house cricket before and after fermentation with *L. plantarum* were analyzed, significant nutritional parameters were assessed using pairwise comparisons. Depending on the assumptions for parametric testing (normality and homoscedasticity of variance), either a *t*-test or the Mann-Whitney *U* test was applied. If the assumptions were not met, the Mann-Whitney *U* test was used; otherwise, the *t*-test was applied. For all other analyses, where three insect species were evaluated before and after fermentation with *L. plantarum* and a commercial starter culture, significant nutritional and compositional parameters were assessed using one-way ANOVA or the Kruskal-Wallis test, depending on whether the assumptions for parametric testing were satisfied. When assumptions were violated, the Kruskal-Wallis test was applied; otherwise, one-way ANOVA was used. The statistical significance level was 0.05 with $p \leq 0.05$, indicating rejection of the null hypothesis. Fermented and unfermented samples from the same species were grouped to ensure reliable statistical comparisons between species. However, due to the insufficient number of samples, a statistical comparison between fermented and unfermented samples was not possible.

3. Results and Discussion

3.1. Proximate Composition

Due to limited sample availability, the proximate composition was assessed only for yellow mealworm and house cricket before and after 48 h fermentation with *L. plantarum*. Proximate composition in terms of ash, total fat and proteins (Table 1) was comparable to those obtained by Cho et al. (2018), Khatun et al. (2021), Kulma et al. (2019), Messina et al. (2019) and Yi et al. (2013), while Kittibunchakul, Whanmek, and Santivarangkna (2023) and Vasilica et al. (2022) report higher values for total protein and lower values for total fat [35,42,43,53–56]. Statistical analyses (Table S2) showed that yellow mealworm had statistically significantly higher ($p < 0.05$) ash concentrations (4.35 to 4.50 g/100 g) and lower total nitrogen content (8.95 to 9.15 g/100 g) compared to house cricket (ash: 4.23 to 4.30 g/100 g, total nitrogen: 9.24 to 9.30 g/100 g).

Table 1. Proximate composition of unfermented and fermented (48 h) yellow mealworm and house cricket (lyophilized).

	YM	YMP	HC	HCP
Dry matter [g/100 g]	97.8 ± 0.1	96.3 ± 0.1	97.9 ± 0.1	97.3 ± 0.0
Water [g/100 g]	2.20 ± 0.06	3.70 ± 0.08	2.06 ± 0.08	2.68 ± 0.02
Ash [g/100 g]	4.35 ± 0.02	4.50 ± 0.01	4.30 ± 0.04	4.23 ± 0.02
Total fat [g/100 g]	28.2 ± 1.9	24.4 ± 2.3	22.9 ± 4.6	23.6 ± 2.7
Insoluble dietary fiber [g/100 g]	7.05 ± 0.35	4.38 ± 0.10	6.19 ± 0.53	5.22 ± 0.30
Soluble dietary fiber [g/100 g]	0.025 ± 0.029	0.366 ± 0.259	0.178 ± 0.198	0.366 ± 0.290
Total dietary fiber [g/100 g]	7.08 ± 0.33	4.75 ± 0.35	6.37 ± 0.35	5.59 ± 0.05
Total nitrogen [g/100 g]	9.15 ± 0.03	8.95 ± 0.01	9.24 ± 0.01	9.30 ± 0.03
Non-protein nitrogen [g/100 g]	0.061 ± 0.002	0.196 ± 0.005	0.069 ± 0.001	0.179 ± 0.002
Protein nitrogen [g/100 g]	9.09 ± 0.02	8.75 ± 0.02	9.17 ± 0.01	9.12 ± 0.03
Proteins (6.25) [g/100 g]	56.8 ± 0.1	54.7 ± 0.1	57.3 ± 0.1	57.0 ± 0.2
Available carbohydrates [g/100 g]	0.98 ± 1.90	6.71 ± 2.37	6.64 ± 4.62	5.85 ± 2.66
Energy value [kJ/100 g]	2082 ± 3	1985 ± 3	1985 ± 7	1984 ± 4

YM—yellow mealworm, YMP—yellow mealworm fermented using *L. plantarum*, HC—house cricket, HCP—house cricket fermented using *L. plantarum*.

The results show a decrease in total dietary fiber content following fermentation in both insect species (33% for yellow mealworm and 12% for house cricket), primarily due to a reduction in insoluble dietary fiber (38% and 16%, respectively). Although the values for soluble dietary fiber are very low and therefore imprecise, the measurement showed a slight increase in the yellow mealworm (from 0.025 g/100 g to 0.366 g/100 g). The increase in soluble fiber after fermentation with *L. plantarum* has been previously reported in other commodities, as the enzymatic breakdown of insoluble fiber during fermentation leads to its conversion into soluble forms [57–59].

Regarding nitrogen composition, total nitrogen and protein nitrogen remained unchanged in yellow mealworm and house cricket, while non-protein nitrogen increased (221% and 159%, respectively). Despite the rise in non-protein nitrogen, the total nitrogen and protein nitrogen contents stayed constant, suggesting that microbial activity was breaking down proteins, but not enough to reduce the protein content in the insects significantly. Instead, the nitrogen is likely redistributed into various forms, such as amino acids and peptides, maintaining the overall nitrogen balance. This redistribution has already been shown, for example, in *Broussonetia papyrifera* fermented using *L. plantarum* during ensiling [59].

Following fermentation, only yellow mealworm exhibited an increase in available carbohydrates (from 0.98 g/100 g to 6.71 g/100 g), while no significant differences were observed in protein content, energy value, or total fat for either species. The significant increase in available carbohydrates may be due to the fermentation process breaking down

complex polysaccharides and fibers into simpler sugars, which are then released as available carbohydrates [60]. The lack of significant changes in protein content, energy value, or total fat suggests the limited impact of *L. plantarum* on protein and fat molecules, as these macronutrients are more resistant to microbial degradation compared to carbohydrates and fibers. Additionally, the fermentation conditions might not have been optimized for significant lipid breakdown or protein denaturation, which can require more specialized conditions or longer fermentation periods.

Comparison with existing literature on edible insects is complicated by the variability in experimental design, insect species, fermentation culture, and analytical methodology in the limited number of available studies. For instance, Cho et al. (2018) fermented raw and defatted yellow mealworm using a soy sauce fermentation process involving *Aspergillus oryzae* and *Bacillus licheniformis*, reporting increased total and protein nitrogen levels after six days, which remained stable over the 13- and 20-day fermentation period [35]. Liu et al. (2022) fermented black soldier fly larvae for four days with *Bacillus subtilis* and *Aspergillus niger*, observing a reduction in actual protein (40%) and fat (30%) content [39]. In the study by Kittibunchakul et al. (2023), house cricket was fermented using a traditional Thai shrimp paste method, employing Kapi as a starter culture, which predominantly contains lactic acid bacteria [42]. After 4 weeks of fermentation, they noted significant reductions in energy (24%), protein (23%), fat (21%), and carbohydrates (43%). Meng et al. (2023), who fermented black soldier fly larvae for 24 h using *Lactobacillus crispatus* and *Pichia kudriavzevii* cultures, also observed a decrease in crude fat content, while crude protein remained unchanged [40]. Also, Pérez-Rodríguez, Ibarra-Herrera, and Pérez-Carrillo (2023) fermented yellow mealworm and Mexican grasshopper with *Aspergillus oryzae*, finding that after four days, protein content decreased (49%, 16%, respectively) in both species, while fat content increased (14%, 119%, respectively), and crude fiber remained unaffected [37].

3.2. Fatty Acid Composition

A total of seven FAs were detected in amounts >1% in the studied insects (Table 2), including three SFAs (myristic, palmitic, stearic), two MUFAs (palmitoleic and oleic acid) and two PUFAs (linoleic—LA and α-linoleic acid—ALA). Their relative levels (>1%) for yellow mealworm were as follows: oleic > LA > palmitic > myristic > stearic > palmitoleic > ALA. The relative levels for house cricket were: oleic > LA > palmitic > stearic > ALA > palmitoleic > myristic acid, while for migratory locust: LA > oleic > palmitic > stearic > ALA > myristic > palmitoleic. All insects contained both essential fatty acids, LA (26.4% to 31.4%) and ALA (0.959% to 9.10%). Overall, the relative concentrations of essential fatty acids are consistent with previously published data, where reported concentrations range from 13.8% to 27.7% for LA and 6.7% to 13.9% for ALA in migratory locust; 16.6% to 33.8% for LA and 0.23% to 1.43% for ALA in yellow mealworm; and 26.26% to 36.67% for LA and 0.7% to 5.13% for ALA in house cricket [51,54,61–64]. Statistical analyses showed that insect species significantly differ ($p < 0.05$) in most fatty acids, except for arachidic, 11-eicosenoic acid and lignoceric acid (Table S2).

All investigated insect species demonstrated favorable PUFA/SFA ratios, ranging from 1.13 to 1.16 for yellow mealworm, 0.935 to 0.993 for house cricket, and 1.14 to 1.23 for migratory locust. These values are consistent with previous reports for edible insects [43,51], where PUFA/SFA close to 1 is considered beneficial for cardiovascular health due to their role in improving plasma lipid profiles and reducing low-density lipoprotein (LDL) cholesterol levels. At the same time, a ratio < 0.33 can have an atherogenic effect [65–67]. Among the species examined, migratory locust exhibited the most optimal n-6/n-3 fatty acid ratio (3.14–3.29), which falls within the range (<4) for lowering chronic disease risk [68]. In contrast, yellow mealworm and house cricket displayed considerably higher ratios (26.6–

27.9 and 17.1–17.5, respectively), reflecting a predominance of n-6 fatty acids that has also been reported for these species in earlier studies [51,64,69].

Table 2. Contents of the main fatty acids (>1%) saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-6 fatty acids, n-3 fatty acids, n-6/n-3 ratio, index of atherogenicity (IA), index of thrombogenicity (IT), hypocholesterolemic/hypercholesterolemic ratio (h/H) in unfermented and fermented insects.

	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Myristic acid [%]	3.79 ± 0.02	3.76 ± 0.05	3.76 ± 0.01	0.504 ± 0.005	0.533 ± 0.006	0.525 ± 0.003	1.29 ± 0.01	1.41 ± 0.00	1.41 ± 0.01
Palmitic acid [%]	16.0 ± 0.0	16.2 ± 0.2	16.2 ± 0.1	23.8 ± 0.2	25.0 ± 0.1	24.7 ± 0.2	19.8 ± 0.1	20.9 ± 0.0	20.9 ± 0.1
Palmitoleic acid [%]	1.68 ± 0.00	1.67 ± 0.00	1.67 ± 0.00	0.763 ± 0.013	0.795 ± 0.003	0.794 ± 0.028	0.805 ± 0.013	0.801 ± 0.002	0.813 ± 0.019
Stearic acid [%]	3.20 ± 0.00	3.35 ± 0.03	3.34 ± 0.01	8.61 ± 0.01	8.81 ± 0.02	8.81 ± 0.09	9.59 ± 0.03	9.83 ± 0.04	9.87 ± 0.04
Oleic acid [%]	46.5 ± 0.0	46.0 ± 0.3	46.0 ± 0.1	31.9 ± 0.5	31.4 ± 0.0	31.6 ± 0.1	28.2 ± 0.3	28.0 ± 0.0	27.9 ± 0.0
Linoleic acid [%]	26.4 ± 0.0	26.5 ± 0.0	26.5 ± 0.0	31.4 ± 0.1	30.8 ± 0.0	30.9 ± 0.2	29.8 ± 0.2	28.4 ± 0.0	28.5 ± 0.0
α-linolenic acid [%]	1.00 ± 0.01	0.959 ± 0.002	0.968 ± 0.000	1.85 ± 0.01	1.77 ± 0.01	1.77 ± 0.01	9.06 ± 0.06	9.06 ± 0.00	9.10 ± 0.00
SFA [%]	23.9 ± 0.0	24.4 ± 0.3	24.4 ± 0.1	33.6 ± 0.4	34.9 ± 0.0	34.6 ± 0.3	31.6 ± 0.1	33.1 ± 0.1	33.1 ± 0.1
MUFA [%]	48.5 ± 0.0	47.9 ± 0.3	48.0 ± 0.1	32.9 ± 0.5	32.4 ± 0.0	32.6 ± 0.1	29.5 ± 0.2	29.4 ± 0.0	29.2 ± 0.1
PUFA [%]	27.6 ± 0.0	27.7 ± 0.0	27.7 ± 0.0	33.4 ± 0.1	32.6 ± 0.0	32.8 ± 0.2	38.9 ± 0.2	37.5 ± 0.0	37.7 ± 0.1
PUFA/SFA	1.16 ± 0.00	1.13 ± 0.01	1.14 ± 0.00	0.993 ± 0.009	0.935 ± 0.002	0.947 ± 0.015	1.23 ± 0.00	1.14 ± 0.00	1.14 ± 0.01
n-6 [%]	26.6 ± 0.0	26.7 ± 0.0	26.7 ± 0.0	31.6 ± 0.1	30.9 ± 0.0	31.0 ± 0.2	29.9 ± 0.2	28.5 ± 0.0	28.6 ± 0.1
n-3 [%]	1.00 ± 0.01	0.959 ± 0.002	0.968 ± 0.000	1.85 ± 0.01	1.77 ± 0.01	1.77 ± 0.01	9.06 ± 0.06	9.06 ± 0.00	9.10 ± 0.00
n-6/n-3	26.6 ± 0.2	27.9 ± 0.1	27.6 ± 0.0	17.1 ± 0.0	17.4 ± 0.1	17.5 ± 0.1	3.29 ± 0.05	3.14 ± 0.00	3.14 ± 0.01
IA	0.414 ± 0.001	0.417 ± 0.007	0.417 ± 0.002	0.390 ± 0.005	0.417 ± 0.002	0.411 ± 0.006	0.367 ± 0.003	0.399 ± 0.000	0.399 ± 0.003
IT	0.342 ± 0.000	0.342 ± 0.004	0.343 ± 0.001	0.601 ± 0.006	0.631 ± 0.003	0.624 ± 0.009	0.509 ± 0.003	0.542 ± 0.001	0.542 ± 0.003
h/H	3.68 ± 0.01	3.63 ± 0.05	3.64 ± 0.02	2.68 ± 0.04	2.51 ± 0.01	2.55 ± 0.04	3.16 ± 0.02	2.92 ± 0.00	2.92 ± 0.02

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture. SFA, MUFA, PUFA, n-6, n-3, IA, IT, h/H are calculated based on all detected fatty acids (Table S3).

All insects presented a favorable low index of atherogenicity (IA < 0.5), with values of 0.414–0.417 for yellow mealworm, 0.390–0.417 for house cricket, and 0.367–0.399 for migratory locust. However, only the yellow mealworm demonstrated a thrombogenicity index (IT) below 0.5 (0.342–0.343), compared to house cricket (0.601–0.631) and migratory locust (0.509–0.542). Lower values of IA (<0.5) and IT (<0.5) are associated with a reduced risk of developing cardiovascular disease [49,70]. Finally, all species exhibited a desirable hypocholesterolemic/hypercholesterolemic ratio (h/H > 1), with yellow mealworm showing the highest values (3.63–3.68), followed by migratory locust (2.92–3.16) and house cricket (2.51–2.68). A higher h/H ratio is associated with a more favorable lipid profile, as hypocholesterolemic fatty acids (PUFA and MUFA) promote the reduction of serum cholesterol, whereas hypercholesterolemic fatty acids (mainly C12:0, C14:0, C16:0) elevate it [49,71].

Interestingly, despite successful fermentation, the fatty acid profile of the insect species remained unaffected compared to unfermented insects, regardless of the insect species or fermentation culture used. This finding contrasts with those of other previous studies, although those studies involved different insect species and fermentation cultures. For instance, Liu et al. (2022) fermented black soldier fly larvae for four days using *Bacillus subtilis* and *Aspergillus niger*, resulting in a 25-fold increase in free short-chain fatty acids post-fermentation [39]. Similarly, Hadj Saadoun et al. (2020) fermented prepupae, puparia, and dead adults of black soldier flies with lactic acid bacteria (*Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*) for 72 h, noting a reduction in typical fatty acids like lauric acid (C12:0) and an increase in minor fatty acids such as short-chain fatty acids, odd-chain fatty acids, and branched-chain fatty acids, which are characteristic of bacterial cell walls [38]. Vasilica et al. (2022) fermented insect flour for 48 h using *L. plantarum* and spontaneous

fermentation, observing that controlled fermentation enriched SFA and MUFA, while PUFA decreased during fermentation [43].

Several possible explanations exist for the lack of change in the fatty acid profiles observed in this study. First, the relatively short fermentation duration (48 h) may not have been sufficient for microbial activity to alter lipid components significantly. Second, the microbial strains employed—*L. plantarum* and a commercial starter culture composed of *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Lactobacillus sakei*, *Pediococcus pentosaceus*, and *Debaryomyces hansenii*—are primarily adapted to metabolize carbohydrates and amino acids, rather than lipids [72–74]. Consequently, their enzymatic potential for lipid transformation is limited. Third, the processing of insect substrates prior to fermentation could have influenced microbial access and activity. For example, Aleknavičius et al. (2022) reported higher microbial abundance and metabolic activity in non-processed fermented insects compared to processed forms, which may enhance interactions with lipid components [75].

3.3. Amino Acid Composition

Taking into account that arginine, cystine and tryptophan were not determined in this study, the total amino acid content (Table 3) ranged from 38.3 g/100 g DW (MLM) to 50.6 g/100 g DW (MLP), while total essential amino acid content ranged from 19.6 g/100 g DW (MLM) to 24.4 g/100 g DW (YM). Essential to total amino acids (E/T) ranged from 44.1% (MLP) to 54.6% (YMP), while essential amino acid index (EAAI, Table 4) ranged from 150 (MLP) to 184 (YMP). Analyzed insects were the highest in valine (3.81−4.58 g/100 g DW), alanine (3.38−4.71 g/100 g DW), glutamic acid (2.74−8.09 g/100 g DW), leucine (3.61−4.23 g/100 g DW), aspartic acid (2.95−4.66 g/100 g DW), but lower in methionine (0.478−0.774 g/100 g DW), ornithine (0.250−3.20 g/100 g DW) and histidine (0.973−1.64 g/100 g DW). Results align with previously reported values for yellow mealworm, house cricket and migratory locust [51,54,63,64,76].

Table 3. Concentrations of amino acids (g/100 g DW), essential and nonessential amino acid content (g/100 g DW) and essential total amino acids ratio (%) in unfermented and fermented insects.

	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Alanine	3.38 ± 0.12	3.90 ± 0.06	3.84 ± 0.02	4.04 ± 0.07	3.98 ± 0.16	4.07 ± 0.15	4.71 ± 0.23	4.64 ± 0.17	4.54 ± 0.56
Aspartic acid	4.66 ± 0.30	3.52 ± 0.10	3.84 ± 0.09	4.34 ± 0.15	3.89 ± 0.08	4.01 ± 0.15	4.01 ± 0.21	3.60 ± 0.14	2.95 ± 0.08
Glutamic acid	4.67 ± 0.48	2.84 ± 0.10	2.74 ± 0.09	4.09 ± 0.54	3.76 ± 0.33	3.88 ± 0.23	3.63 ± 0.39	8.09 ± 0.31	3.30 ± 0.18
Glycine	2.65 ± 0.09	2.82 ± 0.03	2.77 ± 0.04	2.78 ± 0.14	2.66 ± 0.07	2.69 ± 0.01	2.83 ± 0.08	3.08 ± 0.15	2.69 ± 0.09
Histidine	1.57 ± 0.06	1.64 ± 0.02	1.60 ± 0.02	1.14 ± 0.01	0.973 ± 0.018	1.14 ± 0.04	1.20 ± 0.05	1.46 ± 0.05	1.05 ± 0.06
Isoleucine	2.10 ± 0.05	2.25 ± 0.05	2.19 ± 0.02	1.94 ± 0.01	1.92 ± 0.04	1.96 ± 0.04	2.03 ± 0.08	2.25 ± 0.05	1.91 ± 0.07
Leucine	4.12 ± 0.16	4.23 ± 0.09	4.16 ± 0.06	4.03 ± 0.05	3.88 ± 0.08	3.95 ± 0.10	4.05 ± 0.17	4.14 ± 0.08	3.61 ± 0.16
Lysine	3.58 ± 0.21	2.56 ± 0.09	2.51 ± 0.06	3.87 ± 0.06	3.05 ± 0.04	3.49 ± 0.04	3.83 ± 0.17	3.25 ± 0.38	3.01 ± 0.10
Methionine	0.670 ± 0.036	0.677 ± 0.039	0.652 ± 0.003	0.774 ± 0.027	0.742 ± 0.016	0.748 ± 0.026	0.672 ± 0.045	0.660 ± 0.024	0.478 ± 0.020
Ornithine	<0.250	0.537 ± 0.037	0.542 ± 0.112	<0.250	0.699 ± 0.227	0.774 ± 0.234	<0.250	3.20 ± 1.10	1.15 ± 0.24
Phenylalanine	1.97 ± 0.10	1.99 ± 0.02	2.03 ± 0.06	1.93 ± 0.07	1.78 ± 0.03	1.81 ± 0.02	2.04 ± 0.08	1.92 ± 0.20	1.66 ± 0.05
Proline	3.00 ± 0.12	3.20 ± 0.06	3.11 ± 0.04	2.92 ± 0.06	2.76 ± 0.09	2.86 ± 0.10	3.14 ± 0.09	4.03 ± 0.07	2.80 ± 0.16
Serine	2.73 ± 0.08	2.05 ± 0.06	2.09 ± 0.07	2.14 ± 0.33	1.88 ± 0.07	1.93 ± 0.09	2.32 ± 0.10	1.66 ± 0.32	1.31 ± 0.41
Threonine	2.43 ± 0.11	1.88 ± 0.02	1.92 ± 0.03	2.09 ± 0.17	1.90 ± 0.04	1.88 ± 0.08	2.35 ± 0.14	2.02 ± 0.39	1.40 ± 0.45
Tyrosine	3.84 ± 0.13	3.07 ± 0.07	3.12 ± 0.05	3.16 ± 0.06	2.40 ± 0.08	2.56 ± 0.11	3.53 ± 0.02	2.01 ± 0.28	2.42 ± 0.48
Valine	4.11 ± 0.18	4.40 ± 0.07	4.36 ± 0.01	3.90 ± 0.04	3.81 ± 0.09	3.91 ± 0.16	4.38 ± 0.15	4.58 ± 0.06	4.05 ± 0.22
Total EAA	24.4 ± 1.0	22.7 ± 0.3	22.5 ± 0.1	22.8 ± 0.3	20.5 ± 0.4	21.4 ± 0.5	24.1 ± 0.8	22.3 ± 0.9	19.6 ± 0.7
Total AA	45.5 ± 1.5	41.6 ± 0.3	41.5 ± 0.3	43.1 ± 0.9	40.1 ± 0.3	41.7 ± 1.0	44.7 ± 1.2	50.6 ± 1.5	38.3 ± 1.7
E/T [%]	53.6 ± 0.7	54.6 ± 0.4	54.4 ± 0.3	52.9 ± 0.7	51.0 ± 0.7	51.5 ± 0.3	53.8 ± 0.5	44.1 ± 1.6	51.1 ± 0.5

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture.

Table 4. Amino acid scores and essential amino acid index [%].

	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Histidine	2.15 ± 0.07	2.47 ± 0.03	2.41 ± 0.03	1.65 ± 0.02	1.52 ± 0.02	1.70 ± 0.02	1.67 ± 0.07	1.80 ± 0.09	1.71 ± 0.03
Isoleucine	1.54 ± 0.02	1.80 ± 0.02	1.76 ± 0.02	1.50 ± 0.04	1.60 ± 0.02	1.57 ± 0.01	1.51 ± 0.02	1.49 ± 0.05	1.66 ± 0.02
Leucine	1.54 ± 0.01	1.73 ± 0.03	1.70 ± 0.01	1.58 ± 0.02	1.64 ± 0.03	1.61 ± 0.01	1.53 ± 0.02	1.39 ± 0.04	1.59 ± 0.01
Lysine	1.75 ± 0.07	1.37 ± 0.04	1.34 ± 0.04	1.99 ± 0.03	1.69 ± 0.01	1.86 ± 0.05	1.90 ± 0.04	1.43 ± 0.15	1.75 ± 0.02
Methionine	0.669 ± 0.014	0.74 ± 0.04	0.714 ± 0.006	0.816 ± 0.013	0.842 ± 0.018	0.816 ± 0.010	0.682 ± 0.027	0.593 ± 0.015	0.568 ± 0.010
Phenylalanine + Tyrosine	3.36 ± 0.02	3.20 ± 0.06	3.27 ± 0.01	3.11 ± 0.03	2.75 ± 0.04	2.76 ± 0.04	3.28 ± 0.05	2.05 ± 0.13	2.80 ± 0.21
Threonine	2.33 ± 0.08	1.97 ± 0.02	2.01 ± 0.02	2.11 ± 0.16	2.06 ± 0.04	1.96 ± 0.04	2.28 ± 0.07	1.73 ± 0.31	1.60 ± 0.57
Valine	2.32 ± 0.05	2.71 ± 0.04	2.70 ± 0.02	2.32 ± 0.02	2.44 ± 0.04	2.40 ± 0.05	2.51 ± 0.09	2.32 ± 0.05	2.71 ± 0.04
EAAI [%]	179 ± 2	184 ± 2	182 ± 1	177 ± 2	172 ± 2	174 ± 1	177 ± 2	150 ± 6	164 ± 6

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture.

According to amino acid scores calculated based on reference protein composition for older children (≥3 years) and adults [50], methionine is limiting amino acid for all unfermented and fermented insects, which is in line with previous studies [77,78], while some studies recognized cystine together with methionine [53,62] and tryptophan [76] as limiting amino acids in insect species analyzed in this study. Also, nitrogen to protein conversion factor (Kp) was determined for yellow mealworm (5.41), yellow mealworm fermented with *L. plantarum* (5.06), house cricket (5.05) and house cricket fermented with *L. plantarum* (4.67), which was comparable to Kp factors for yellow mealworm (4.64–7.35) and house cricket (4.17–5.71) reported in previous studies [51,56,62–64,79–86]. When calculating Kp, it was taken into account that unmeasured amino acids have an 8% contribution to total amino acids [51], and ornithine was excluded from the total amino acids.

Species differed significantly ($p < 0.05$, Table S2) in their alanine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, tyrosine and valine, while total essential and total amino acids were similar. The reasons for these differences could be attributed to various factors such as species-specific metabolism, dietary differences, environmental factors (temperature, humidity, habitat) and adaptations to specific ecological roles or functions (flight, reproduction, or survival mechanisms) as suggested in previous studies [87–89].

The observed changes in amino acid profiles following fermentation varied significantly across insect species and microbial cultures. In the case of the yellow mealworm, fermentation with both *L. plantarum* and the commercial starter culture resulted in comparable trends, i.e., an increase in alanine (14–15%) and ornithine (115–117%), but reductions in several key amino acids, including aspartic acid (18–25%), glutamic acid (39–41%), lysine (28–30%), serine (24–25%), threonine (21–23%) and tyrosine (19–20%). These results contrast with those of An et al. (2019), who detected an increase in aspartic and glutamic acid when yellow mealworm was fermented with *L. plantarum* [34].

In the case of the house cricket, both fermentation with *L. plantarum* and the commercial starter culture produced similar changes in the amino acid profile. An increase was observed only in ornithine content (115–117%), while several essential and non-essential amino acids decreased. Specifically, reductions were noted for aspartic acid (8–10%), histidine (0–15%), lysine (10–21%), serine (10–12%), threonine (9–10%), and tyrosine (19–24%). Additionally, the total essential amino acid content decreased by 6–10%. These results suggest a potentially adverse impact on protein quality and overall nutritional value, particularly due to the loss of essential amino acids. These results contradict the findings of Kittibunchakul et al. (2023) and Vasilica et al. (2022), who reported exclusively an increase in amino acids after fermentation with lactic acid bacteria and *L. plantarum* [42,43]. Kittibunchakul et al. (2023) mimicked the process of making shrimp paste Kapi, which also involves salting the substrate and a longer fermentation time, which could be the

reason for the differences compared to this study, while Vasilica et al. (2022) used the same fermentation time (48 h) and the same *L. plantarum* culture as in this study.

In the case of the migratory locust, the effects of fermentation with *L. plantarum* and the commercial starter culture differed considerably. Fermentation with *L. plantarum* resulted in increased levels of glutamic acid (123%), histidine (22%), isoleucine (11%), ornithine (1181%), and proline (28%), as well as an overall rise in total amino acid content (13%). However, this treatment also led to reductions in aspartic acid (10%), lysine (15%), serine (28%), threonine (14%), tyrosine (43%), and the essential-to-total amino acid (E/T) ratio (18%). In contrast, fermentation with the commercial starter culture resulted in a substantial increase only in ornithine (359%), while most other amino acids declined in concentration. Notable reductions were observed for aspartic acid (27%), histidine (13%), leucine (11%), lysine (21%), methionine (29%), phenylalanine (19%), proline (11%), serine (44%), threonine (40%), tyrosine (31%), as well as total essential amino acids (19%) and total amino acid content (14%). Overall, the results from both fermentation treatments suggest a potentially adverse impact on protein quality and nutritional value, largely due to the depletion of multiple essential amino acids.

As observed in all three insect species, ornithine concentrations significantly increased following fermentation with both microbial cultures. A potential explanation for this could be the involvement of the arginine deiminase (ADI) pathway, which is strongly associated with lactic acid bacteria [90]. In this pathway, arginine is initially converted to citrulline through the action of the enzyme arginine deiminase. Subsequently, citrulline is converted to ornithine by the enzyme ornithine carbamoyl transferase. This pathway plays a crucial role in bacterial survival, particularly under acidic or nitrogen-limited conditions, and also contributes to adenosine triphosphate (ATP) production through the conversion of arginine, with ammonia and carbon dioxide as by-products [91].

Overall, the nutritional consequences of these shifts are mixed. Although increases in amino acids like ornithine are beneficial, the reduction of essential amino acids, such as lysine, methionine, and threonine, is more concerning. Lysine, methionine + cystine, threonine and tryptophan dominate as limiting amino acids in plant-based diets [92], so their reduction could impair protein quality if insect meals are used in food or feed applications. The results also underscore the importance of strain selection and species compatibility in fermentation design to avoid the degradation of nutritionally important amino acids.

3.4. Elemental Composition

Elemental concentrations of fermented and unfermented insects are presented in Tables 5–7 and daily intake values for 100 g of fresh insects for adults in Table 8. The elemental concentrations of unfermented insects are in agreement with data obtained by Finke (2002, 2015), Machado et al. (2024), and Vehar et al. (2025), indicating that species differ significantly ($p < 0.05$, Table S2) in most elements, except for Na, Ni, and Zn [51,62,63,93]. Species-specific patterns in elemental composition have been reported before and are attributed to growing environments (geographic and ecological factors) and processing mechanisms [51,88,94]. However, elements are mainly obtained from dietary sources.

Due to the small number of samples, differences in elemental concentrations were not statistically evaluated; instead, a 10% threshold value was used to confirm the presence of differences, which depended on the insect species and the fermentation culture used. In the case of the yellow mealworm, fermentations with *L. plantarum* and commercial starter culture resulted in increased elemental concentrations. Fermentation with *L. plantarum* increased Al (61%), V (29%), Ni (27%), Ag (34%), Cd (16%), Sn (26%), Sb (14%), Cs (29%), Hg (15%) and U (19%), while commercial starter culture increased Mn (96%), Ag (85%), Cd

(13%), Cs (28%), Hg (14%) and U (11%). In case of house cricket, *L. plantarum* increased B (37%) and Ag (14%) and decreased Al (31%), V (16%), Co (14%), Zn (14%), Cd (12%), Hg (20%) and Pb (21%), while commercial starter culture increased B (18%), Mg (12%), Mn (42%), Ni (11%), Sb (19%) and decreased Al (29%), Zn (12%), Cd (15%) and Pb (16%). In the case of the migratory locust, both fermentation cultures mainly increased the concentrations of elements. *L. plantarum* increased Na (20%), Mn (36%), Fe (23%) and decreased Se (15%), while commercial starter culture increased Na (49%), Mg (23%), P (22%), K (25%), Mn (378%), Fe (19%), Co (20%), Zn (12%), Rb (12%) and Sn (25%) and decreased Cu (16%) and Ag (19%). In general, fermentation resulted in increased concentrations of essential elements Fe (19–23%), K (25%), Mg (12–23%), Mn (36–378%), Na (20–49%), P (22%), decreased Se (24%) and Cu (16%), while Zn increased in migratory locust fermented with commercial starter culture (12%) and decreased in house cricket fermented with both cultures (12–14%).

Table 5. Makro elements (g/kg FW) in unfermented and fermented insects.

Elements	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Ca	0.155 ± 0.016	0.148 ± 0.005	0.154 ± 0.004	0.379 ± 0.030	0.368 ± 0.012	0.414 ± 0.002	0.271 ± 0.025	0.277 ± 0.006	0.305 ± 0.033
K	2.71 ± 0.15	2.66 ± 0.00	2.62 ± 0.14	2.90 ± 0.02	2.79 ± 0.03	3.00 ± 0.15	3.13 ± 0.00	3.11 ± 0.23	3.90 ± 0.02
Mg	1.04 ± 0.05	0.960 ± 0.043	1.00 ± 0.05	0.216 ± 0.007	0.213 ± 0.006	0.242 ± 0.015	0.268 ± 0.016	0.272 ± 0.023	0.329 ± 0.015
Na	0.388 ± 0.023	0.383 ± 0.007	0.396 ± 0.025	1.07 ± 0.01	1.04 ± 0.01	1.13 ± 0.02	0.232 ± 0.004	0.279 ± 0.009	0.346 ± 0.030
P	2.79 ± 0.10	2.67 ± 0.06	2.69 ± 0.15	2.14 ± 0.02	2.01 ± 0.00	2.19 ± 0.04	2.07 ± 0.05	2.12 ± 0.21	2.51 ± 0.02
S	1.04 ± 0.07	1.03 ± 0.02	1.02 ± 0.03	1.38 ± 0.01	1.34 ± 0.04	1.39 ± 0.00	1.09 ± 0.04	1.08 ± 0.10	1.14

YM: yellow mealworm, YMP: yellow mealworm fermented using L. plantarum, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using L. plantarum, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using L. plantarum, MLM: migratory locust fermented using commercial starter culture.

Table 6. Mikro elements (mg/kg FW) in unfermented and fermented insects.

Elements	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Al	0.607 ± 0.133	0.803 ± 0.070	0.646 ± 0.052	24.9 ± 4.1	17.2 ± 3.5	17.8 ± 2.9	23.3 ± 8.9	37.6 ± 26.7	27.1 ± 6.6
B	1.11 ± 0.17	1.05 ± 0.06	1.03 ± 0.01	0.257 ± 0.036	0.351 ± 0.024	0.303 ± 0.024	0.337 ± 0.029	0.356 ± 0.001	0.329
Cu	5.07 ± 0.33	4.95 ± 0.03	4.95 ± 0.26	5.96 ± 0.12	5.67 ± 0.11	6.27 ± 0.38	10.0 ± 0.4	10.5 ± 0.4	8.44 ± 0.02
Fe	13.8 ± 1.3	12.9 ± 0.5	12.9 ± 0.5	17.4 ± 0.3	15.7 ± 0.8	18.4 ± 0.3	15.7 ± 0.1	19.3 ± 1.7	18.7 ± 0.0
Mn	2.93 ± 0.25	2.88 ± 0.06	5.75 ± 0.22	11.4 ± 0.2	10.8 ± 0.1	16.2 ± 0.7	0.775 ± 0.048	1.05 ± 0.20	3.70 ± 1.23
Mo	0.332 ± 0.003	0.353 ± 0.010	0.336 ± 0.006	0.249 ± 0.001	0.255 ± 0.000	0.251 ± 0.004	0.311 ± 0.063	0.267 ± 0.001	0.277 ± 0.025
Ni	0.147 ± 0.021	0.187 ± 0.030	0.16 ± 0.00	0.0645 ± 0.0063	0.0743 ± 0.0187	0.0716 ± 0.0012	0.256 ± 0.033	0.235 ± 0.004	0.254 ± 0.014
Rb	0.239 ± 0.014	0.226 ± 0.004	0.237 ± 0.018	0.967 ± 0.019	0.883 ± 0.021	0.975 ± 0.012	0.798 ± 0.001	0.796 ± 0.038	0.986 ± 0.013
Sr	0.542 ± 0.075	0.525 ± 0.010	0.551 ± 0.052	0.590 ± 0.046	0.556 ± 0.058	0.614 ± 0.027	0.697 ± 0.057	0.662 ± 0.023	0.759 ± 0.036
Zn	40.0 ± 3.4	39.3 ± 0.2	40.4 ± 3.3	70.2 ± 5.4	60.2 ± 6.8	61.7 ± 1.2	35.5 ± 0.5	35.4 ± 2.7	39.8 ± 0.3

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture.

Table 7. Trace elements (µg/kg FW) in unfermented and fermented insects.

Elements	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Ag	0.507 ± 0.029	0.681 ± 0.044	0.937 ± 0.116	0.866 ± 0.028	0.989 ± 0.012	0.850 ± 0.202	2.62 ± 0.54	2.71 ± 0.37	2.11 ± 0.04
As	15.4 ± 2.3	16.1 ± 0.6	16.1 ± 0.4	4.81 ± 2.00	5.09 ± 1.32	4.01 ± 0.75	6.97 ± 0.40	7.57 ± 1.22	19.4 ± 18.5
Cd	25.6 ± 0.5	29.8 ± 0.8	29.0 ± 0.4	11.2 ± 1.3	9.82 ± 0.40	9.52 ± 0.37	12.7 ± 8.0	15.6 ± 3.1	11.2 ± 4.4
Co	17.5 ± 1.6	18.0 ± 0.8	17.3 ± 0.1	23.8 ± 0.7	20.4 ± 0.7	21.6 ± 0.8	8.60 ± 1.00	10.6 ± 3.4	10.3 ± 0.4
Cs	0.382 ± 0.016	0.494 ± 0.052	0.490 ± 0.076	4.46 ± 0.04	4.29 ± 0.39	4.53 ± 0.10	3.68 ± 2.87	1.86	4.35 ± 3.25
Hg	0.282 ± 0.033	0.325 ± 0.002	0.321 ± 0.004	0.641 ± 0.042	0.516 ± 0.025	0.617 ± 0.068	1.06 ± 0.23	1.24 ± 0.18	0.970 ± 0.230
Pb	2.16 ± 0.16	2.34 ± 0.10	2.06 ± 0.03	33.2 ± 0.2	26.3 ± 3.2	28.0 ± 1.1	31.4 ± 5.4	43.2 ± 18.0	34.0 ± 2.67
Sb	0.532 ± 0.018	0.607 ± 0.034	0.493 ± 0.034	1.37 ± 0.20	1.41 ± 0.12	1.63 ± 0.16	2.55 ± 0.41	3.37 ± 0.81	2.78 ± 0.58
Se	42.4 ± 0.6	45.4 ± 0.1	43.6 ± 1.7	98.0 ± 2.3	99.7 ± 4.6	95.1 ± 6.8	83.7 ± 10.1	71.0 ± 5.70	88.9 ± 1.1
Sn	6.13 ± 1.29	7.72 ± 0.09	7.70 ± 1.42	15.7 ± 2.1	14.9 ± 2.9	13.6 ± 1.2	10.1 ± 0.4	12.9 ± 4.0	12.6 ± 2.9
U	23.6 ± 0.4	28.2 ± 1.4	26.2 ± 0.5	31.6 ± 8.4	28.4 ± 2.7	27.8 ± 0.6	1.29 ± 0.21	1.55 ± 0.45	1.21 ± 0.23
V	52.9 ± 7.5	61.6 ± 0.0	58.9 ± 1.1	63.8 ± 9.1	53.9 ± 1.6	56.7 ± 0.7	18.2 ± 4.8	23.5 ± 6.0	17.7 ± 0.7

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture.

Table 8. Daily intake values (%) for 100 g of fresh insects, calculated for adults.

	RDI (mg/day)	YM	YMP	YMM	HC	HCP	HCM	ML	MLP	MLM
Ca	1000	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.0	3.8 ± 0.3	3.7 ± 0.1	4.1 ± 0.0	2.7 ± 0.3	2.8 ± 0.1	3.1 ± 0.3
Cu	1.3	39.0 ± 2.6	38.1 ± 0.2	38.1 ± 2.0	45.9 ± 0.9	43.6 ± 0.8	48.3 ± 2.9	76.9 ± 3.0	81.1 ± 2.9	64.9 ± 0.1
Fe	16	8.6 ± 0.8	8.1 ± 0.3	8.1 ± 0.5	10.8 ± 0.2	9.8 ± 0.5	11.5 ± 0.2	9.8 ± 0.0	12.1 ± 1.1	11.7 ± 0.0
K	3500	7.7 ± 0.4	7.6 ± 0.0	7.5 ± 0.4	8.3 ± 0.0	8.0 ± 0.1	8.6 ± 0.4	8.9 ± 0.0	8.9 ± 0.7	11.2 ± 0.1
Mg	300	34.7 ± 1.7	32.0 ± 1.4	33.5 ± 1.6	7.2 ± 0.2	7.1 ± 0.2	8.1 ± 0.5	8.9 ± 0.6	9.1 ± 0.8	11.0 ± 0.5
Mn	3	9.8 ± 0.8	9.6 ± 0.2	19.2 ± 0.7	38.0 ± 0.6	36.1 ± 0.3	53.9 ± 2.3	2.6 ± 0.2	3.5 ± 0.7	12.3 ± 4.1
Na	2000	1.9 ± 0.1	1.9 ± 0.0	2.0 ± 0.1	5.4 ± 0.0	5.2 ± 0.0	5.7 ± 0.1	1.2 ± 0.0	1.4 ± 0.0	1.7 ± 0.1
P	500	55.8 ± 2.0	53.4 ± 1.2	53.8 ± 3.0	42.8 ± 0.4	40.2 ± 0.1	43.8 ± 0.8	41.4 ± 1.1	42.3 ± 4.1	50.3 ± 0.5
Se	0.07	6.1 ± 0.1	6.5 ± 0.0	6.2 ± 0.2	14.0 ± 0.3	14.2 ± 0.7	13.6 ± 1.0	12.0 ± 1.4	10.1 ± 0.8	12.7 ± 0.2
Zn	11	36.3 ± 3.1	35.7 ± 0.2	36.7 ± 3	63.8 ± 4.9	54.7 ± 6.2	56.1 ± 1.1	32.3 ± 0.4	32.1 ± 2.5	36.2 ± 0.3

YM: yellow mealworm, YMP: yellow mealworm fermented using *L. plantarum*, YMM: yellow mealworm fermented using commercial starter culture, HC: house cricket, HCP: house cricket fermented using *L. plantarum*, HCM: house cricket fermented using commercial starter culture, ML: migratory locust, MLP: migratory locust fermented using *L. plantarum*, MLM: migratory locust fermented using commercial starter culture.

Vasilica et al. (2022) examined essential elements, Cr and Ni, in fermented house cricket using atomic absorption spectrophotometry after ash recovery in 20% HCl [43]. They found that *L. plantarum* increased all elemental concentrations after 24 h, while spontaneous sourdough fermentation increased all except P and Zn, which is only partially consistent with our findings. The authors attributed the increase to a reduction in pH, which may activate phytase and reduce mineral-binding compounds, such as phytates and tannins [95]. In contrast, the present study measured total elemental content after acid digestion with ICP-MS. Therefore, the observed increases likely reflect relative concentration effects, such as a reduction in dry matter during fermentation [96], which concentrates remaining minerals, as well as microbial accumulation or redistribution within the biomass. These effects appear to be species- and culture-dependent.

The results show that consuming 100 g of fermented and unfermented insects in fresh weight (FW) would contribute a substantial portion of the recommended daily intake for adults of Cu (38.1–81.1%), P (40.2–55.8%), and Zn (32.1–63.8%). Specifically, 100 g of fermented and unfermented yellow mealworm would provide 32.0–34.7% of the recommended daily intake of Mg, while house cricket would supply 36.1–53.9% of the requirement for Mn. Daily intake values for all essential elements, except Na and Zn, differ statistically significantly between species ($p < 0.05$, Table S2). A risk assessment indicates that consuming 100 g of fresh fermented or unfermented insects poses no health risk to adults. Furthermore, based on the maximum levels established by Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed for Cu (15–35 mg/kg FW), Fe (30 mg/kg FW), Cd (2 mg/kg FW), Pb (10 mg/kg FW), As (2 mg/kg FW) and Hg (0.1 mg/kg FW) in the feedstuff, all tested insects comply with feed safety standards [97].

3.5. Oxidation Stability

Numerous studies have explored the impact of fermentation on oxidation stability in various food products, highlighting its potential to enhance the shelf life and stability of lipids and bioactive compounds [98–101]. However, limited research has been conducted specifically on the oxidation stability of edible insects, particularly under the fermentation conditions used in this study. Given the constraints on sample material, oxidation stability measurements were conducted with a single measurement per condition (Figure 1). Among the three insect species analyzed, yellow mealworm exhibited the highest oxidation stability (induction time = 209 min), followed by migratory locust (78 min), then house cricket (66 min). The effect of fermentation on oxidation stability depended on the species. In yellow mealworm, fermentation notably reduced the induction time by 42% with *L. plantarum* and by 41% with the commercial starter culture. A similar, though less pronounced

decrease, was observed for migratory locust, with induction times reduced by 21% and 29%, respectively. In contrast, house cricket exhibited improved oxidation stability after fermentation, with an increase in induction time of 153% with *L. plantarum* and 167% with the commercial starter culture.

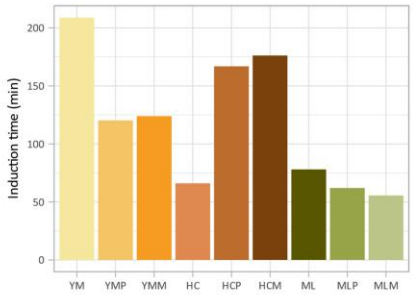


Figure 1. Induction times for insects before and after fermentation (YM: yellow mealworm, YMP: YM + *L. plantarum*, YMM: YM + commercial starter culture, HC: house cricket, HCP: HC + *L. plantarum*, HCM: HC + commercial starter culture, ML: migratory locust, MLP: ML + *L. plantarum*, MLM: ML + commercial starter culture).

These findings suggest that the impact of fermentation on lipid oxidation stability is primarily dependent on the insect species, while the type of microbial culture used plays a comparatively minor role. Similar observations were reported by Borremans, Smets, and Van Campenhout (2020), who noted the onset of secondary lipid oxidation in yellow mealworm during lactic acid fermentation, with further progression over time [102]. This finding aligns with the results of the current study, which found that fermentation reduced the oxidation stability of yellow mealworm. Bernardo and Conte-Junior (2024) also emphasized that the oxidation stability of edible insects is strongly influenced by species-specific lipid composition and the processing methods used [103]. Insects vary in PUFA content, which contributes to differences in susceptibility to oxidation. In this study, yellow mealworm contained the lowest amount of PUFA (Table 2), potentially explaining its initially higher oxidation stability compared to the other two species, except for fermented house cricket, which showed an unexpected increase in induction time.

Processing steps, such as freezing, freeze-drying, cooking and grinding, can also affect oxidation stability. These treatments may alter the nutritional and physicochemical properties of food products through physical, thermal or chemical mechanisms, consequently decreasing their oxidation stability [104]. In the present study, all insect samples were killed by freezing, freeze-dried, ground, and stored frozen prior to fermentation. These methods are known to increase sample porosity and the specific surface area, making the lipids more vulnerable to oxidation [53,103,105].

Overall, the observed changes in oxidation stability were influenced not only by fermentation but also by prior processing methods. Treatments such as freezing, freeze-drying, and grinding likely contributed to increased lipid exposure and susceptibility to oxidation. Thus, oxidation stability in fermented insect products reflects the combined effects of species-specific lipid composition, fermentation conditions, and processing techniques, all of which should be considered in product development.

4. Conclusions

This study demonstrated that the effects of fermenting three edible insect species (yellow mealworm, house cricket, and migratory locust), with *L. plantarum* and a commercial

starter culture, are highly dependent on both the insect species and the specific fermentation conditions. Fermentation resulted in a decrease in total dietary fiber and an increase in non-protein nitrogen in both yellow mealworm and house cricket, while other parameters, such as total nitrogen, protein nitrogen, and total fat, remained largely unaffected. No notable improvements were observed in fatty acid profiles, likely due to the relatively short fermentation time. Although increases in specific amino acids, such as ornithine, were beneficial, the reduction of essential amino acids, including lysine, methionine, and threonine, which are often limiting in plant-based diets, was less desirable. Fermentation had mixed effects on mineral composition, enhancing the levels of essential elements like Fe, K, Mg, Mn, Na, P, and Zn in some cases, while reducing others, including Zn, Se, and Cu, with changes reflecting relative concentration effects. Oxidation stability also varied, decreasing in yellow mealworm and migratory locust but improving significantly in house cricket.

These findings highlight the complex interplay between insect species, microbial cultures, and processing conditions in determining nutritional and functional outcomes. Given the limited sample size in this study, future research should include larger sample sets and longer fermentation periods to understand better the mechanisms involved and optimize fermentation strategies for edible insect products. Additionally, it would be valuable to assess bioaccessibility and elemental speciation in future studies to explore how these factors influence the nutritional bioavailability of key micronutrients in fermented insect products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods14172929/s1, Table S1: Monitored ions for amino acid analysis; Table S2: Significant differences in all measured parameters between all species and yellow mealworm vs. house cricket for nutritional composition (* = $p < 0.05$, x = not statistically analyzed); Table S3: Fatty acid profile (%); Table S4: Reference values for risk assessment; Table S5: Population reference intake and adequate intake values; Table S6: Intake values calculated for the consumption of 100 g of fresh insects for risk assessment.

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Abbreviations

The following abbreviations are used in this manuscript:

AAS	amino acid score
ADI	arginine deiminase
ALA	α-linolenic acid
AOAC	Association of Official Agricultural Chemists
ATP	adenosine triphosphate
CFU	colony-forming units
DW	dry weight
E/T	essential amino acid to total amino acid ratio
EAA	essential amino acid
EAAI	essential amino acid index
EFSA	European Food Safety Authority
EI	electron impact
EU	European Union
EV	energy value
FAME	fatty acid methyl ester
FAO	Food and Agriculture Organization
FID	flame ionization detection
FW	fresh weight
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
h/H	hypocholesterolemic/hypercholesterolemic ratio
HC	house cricket
HCM	house cricket fermented using commercial starter culture
HCP	house cricket fermented using *L. plantarum*
IA	atherogenic index
IT	thrombogenic index
LA	linoleic acid
LOD	limit of detection
ML	migratory locust
MLM	migratory locust fermented using a commercial starter culture
MLP	migratory locust fermented using *L. plantarum*
MRS	De Man, Rogosa and Sharpe broth
MUFA	monounsaturated fatty acid
n-3	omega-3 fatty acids
n-6	omega-6 fatty acids
NPN	non-protein nitrogen
NIST	National Institute of Standards and Technology
PUFA	polyunsaturated fatty acid
QQQ-ICP-MS	triple quadrupole inductively coupled plasma mass spectrometer
RDI	reference daily intake
SFA	saturated fatty acid
SIM	selected ion monitoring
spp.	species
SRM	standard reference material
YM	yellow mealworm
YMM	yellow mealworm fermented using commercial starter culture
YMP	yellow mealworm fermented using *L. plantarum*

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3.4 Scientific Paper: “Contaminant Uptake in Wastewater Irrigated Tomatoes”

This chapter presents the paper entitled “Contaminant uptake in wastewater irrigated tomatoes” by Ana Kovačič, Eirini Andreasidou, Anže Brus, Anja Vehar, Doris Potočnik, Marta Jagodic Hudobivnik, David Heath, Marina Pintar, Nina Kacjan Maršić, Nives Ogrinc, Urška Blaznik, and Ester Heath. The paper was published in the Journal of Hazardous Materials (A'') in 2023. This study investigated the uptake of CECs and potentially toxic elements (PTEs) in tomato fruits.

Tomatoes were grown in two ways: hydroponically in nutrient solution or in spiked nutrient solution, and in lysimeters irrigated with potable water, treated wastewater, or spiked treated wastewater. Nutrient solution and treated wastewater were spiked with 14 CECs at 0.1 mg/L, derived from industrial chemicals, pharmaceuticals, and common stimulants. This concentration was selected to reflect environmentally relevant levels. The spiking was performed to ensure the presence of CECs in both matrices, enabling a controlled assessment of their uptake under different conditions, particularly in cases where the original solutions might not contain detectable levels of these compounds. CECs and PTEs were analysed in ripe lyophilised tomato fruits using LC-MS/MS and ICP-MS.

Results showed the uptake of bisphenol S (0.034–0.134 μg/kg FW), 2,4 bisphenol F (<LOQ–0.081 μg/kg FW), and naproxen (<LOQ–0.137 μg/kg FW), with bisphenol S detected at the highest concentrations. All three compounds were present at significantly higher levels in hydroponically grown tomatoes.

Elemental analysis revealed significant differences between hydroponically grown tomatoes and those cultivated in lysimeters for all elements except Se, Rb, and Hg. Notably, higher concentrations of Mn, Cu, and Zn in hydroponic samples with spiked nutrient solutions indicate that the addition of CECs influenced elemental composition in hydroponics, whereas no such differences were observed in lysimeter-grown tomatoes irrigated with TWW and spiked TWW. Additionally, tomatoes grown in lysimeters irrigated with TWW showed significantly higher concentrations of B and Na compared to those irrigated with potable water, suggesting increased uptake from TWW. At the detected levels, both CECs and PTEs were associated with low chronic dietary exposure.

The contribution of this study to science lies in demonstrating how different irrigation regimes influence the uptake of CECs and how CECs, in turn, influence the uptake of PTEs in crops. The findings provide valuable evidence that, if properly monitored, reusing TWW for irrigation can pose a minimal risk, although more studies need to be performed for a definite answer on the safety of TWW irrigation providing more information about CECs, including the formation of metabolites and transformation products, possible synergistic effects and different agricultural practices. This research contributes to the broader field of sustainable agriculture by offering insights that can inform policymaking and guide the development of future legislative frameworks for water resource management in agriculture.

In this study, I analysed the data concerning the elemental composition of tomatoes, performed the principal component analysis, conducted the dietary exposure and health risk assessments, created the figures, and wrote the corresponding sections of the manuscript.

The findings from this study were presented as an oral presentation at a) 4th Jožef Stefan International Postgraduate School Students' Conference, June 1-3 2022, in Kamnik, Slovenia, b) 2nd ISO-FOOD Symposium from Food Source to Health, April 24-26, 2023, in Portorož, Slovenia, c) 18th International Conference on Chemistry and the Environment, June 11-15 June, 2023, in Venice, Italy, d) EuroAnalysis 2023: analytical probing of

complex systems, August 27-31, 2023, in Geneva, Switzerland, e) BFestival 14, February 2024, in Ljubljana, Slovenia and as a poster presentation at ICWRR2024 International Conference on Wider Uptake of Water Resource Recovery from Wastewater Treatment and IWA-RMTC 2024 7th IWA – Regional Membrane Technology Conference, June 18-21, 2024, in Palermo, Italy.

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Research Article

Contaminant uptake in wastewater irrigated tomatoes

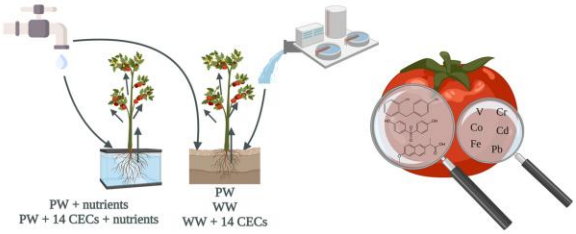
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HIGHLIGHTS

- Uptake study of 14 CECs and 27 elements in tomatoes grown in soil/soil-less media.
- Bisphenol S, 2,4 bisphenol F, and naproxen were detected in fruits.
- CECs presence and growing condition affect the elemental composition of tomatoes.
- Contaminants at determined levels showed low dietary chronic exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

As population growth and climate change add to the problem of water scarcity in many regions, the argument for using treated wastewater for irrigation is becoming increasingly compelling, which makes understanding the risks associated with the uptake of harmful chemicals by crops crucial. In this study, the uptake of 14 chemicals of emerging concern (CECs) and 27 potentially toxic elements (PTEs) was studied in tomatoes grown in soil-less (hydroponically) and soil (lysimeters) media irrigated with potable and treated wastewater using LC-MS/MS and ICP-MS. Bisphenol S, 2,4 bisphenol F, and naproxen were detected in fruits irrigated with spiked potable water and wastewater under both conditions, with BPS having the highest concentration (0.034–0.134 μg kg^{-1} f. w.). The levels of all three compounds were statistically more significant in tomatoes grown hydroponically (<LOQ - 0.137 μg kg^{-1} f. w.) than in soil (<LOQ - 0.083 μg kg^{-1} f. w.). Their elemental composition shows differences between tomatoes grown hydroponically or in soil and tomatoes irrigated with wastewater and potable water. Contaminants at determined levels showed low dietary chronic exposure. When the health-based guidance values for the studied CECs are determined, results from this study will be helpful for risk assessors.

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1. Introduction

Worldwide, many regions are experiencing an increase in water scarcity [27]. Even in countries with abundant water resources, uneven distribution can lead to water shortages, particularly during drier months [17]. For example, in the Mediterranean region, it is estimated that *"20% of the population lives under constant water stress and in summer, over 50% of the population is affected by water stress"* [11]. Agriculture remains the leading consumer of available freshwater, representing more than 70% of total usage. Thus, alternative water resources must be sought to complement conventional freshwater sources [33]. For instance, treated wastewater as a non-conventional water resource is already utilised globally for irrigated edible crops, e.g., cereals or leafy, root and fruit vegetables [29]. This practice yields certain benefits apart from saving freshwater by providing valuable nutrients, organic matter, and essential elements for crops [7].

Currently, the EU also favours utilising wastewater as an additional method to preserve available water resources and promote a circular economy. It also acknowledges that treated wastewater represents a reliable water supply with the potential benefits of utilising treated wastewater endorsed through a broad framework, including regulation (EU) 2020/741, which defines the minimum requirements for water reuse in agriculture [36]. This framework outlines the current European-wide minimum standards, including *E. Coli* levels, biochemical oxygen demand, total suspended solids and turbidity, with different concentrations depending on reclaimed water quality class. Many current conventional Wastewater Treatment Plants (WWTPs) are also redefining themselves as Water Resource Recovery Facilities (WRRFs) with the implementation of technical innovations, not only focusing on treated water but also on the production of nutrients, energy and other valuable bio-based materials from wastewater [23].

Despite this, doubts remain among many experts since it is known that conventional water treatment technologies are not able to completely remove Chemicals of Emerging Concern (CECs) or Potentially Toxic Elements (PTEs) [2,28]. The utilisation of reclaimed wastewater for irrigation may contribute to the introduction of CEC and PTEs in agroecosystems and their crop uptake [15,37]. Thus, an excess supply of macronutrients, CECs and trace elements in reclaimed water could affect plant growth and crop quality [24]. The bioaccumulation of CECs and PTEs in edible plants parts can also pose a health risk due to their subsequent introduction into the food chain [1,9,4,31]. For these reasons, a proper risk assessment is needed when considering using treated wastewater for irrigation.

Various vegetables have been studied under controlled conditions, including greenhouse experiments, field trials, and hydroponic systems [24,32,38]. However, there is still a significant knowledge gap regarding the uptake of PTEs and CECs in vegetables when using treated municipal wastewater. For example, most existing studies focus on either elements or small groups of organic contaminants, the most common being pharmaceuticals (e.g., carbamazepine, diclofenac, and ibuprofen), and only look at a single growing system [25,35,10]. Moreover, current research typically fails to consider the potential interactions between PTEs and CECs, and how they affect each other's accumulation and uptake in vegetables.

This paper examines the uptake of fourteen organic contaminants, including pharmaceuticals, hormones, and psychoactive substances, with some of them, e.g., BPA alternatives, being studied for the first time. It also assesses the occurrence of twenty-seven elements in tomatoes irrigated with treated wastewater. The tomato plant (*Solanum lycopersicum L. Rally*) was chosen as its roots, stems, leaves, and edible fruits make it an ideal research plant, and they are one of the most widely cultivated and consumed vegetables globally. We also compared two growing systems – soil (lysimeters) and soil-less (hydroponics) media – to explore the impact of different growing regimes and contaminant and element levels. A reliable and robust ultrasound-assisted extraction method was developed and optimised to facilitate this. Finally, dietary exposure and health risk assessments were estimated for the studied PTE and CECs. The findings thus provide a systematic insight into the uptake of PTE and CECs, offering valuable information for setting regulations regarding wastewater reuse in agriculture.

2. Materials and methods

2.1. Reagents and chemicals

General information about the investigated CECs is presented in the Supplementary information (Table SI-1). Bisphenol A (BPA; > 97%) was purchased from Merck (Darmstadt, Germany), isomers of bisphenol F (22BPF; 98%, 24BPF; 98% and 44BPF; > 98%), bisphenol S (BPS; > 98%), caffeine (CAF; 99%), ibuprofen (IB; > 98%), naproxen (NP; 98%), ketoprofen (KP; > 98%), diclofenac (DF; 97%), were purchased from Sigma-Aldrich (Steinheim, Germany), whereas estrone (E1; 99%), 17β-estradiol (β-E2; 98%), 17α-ethynyl estradiol (EE2; 98%) were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Isotopically labelled surrogate standards: BPA (BPA-d_{16}, 98%), CAF ($^{13}C_3$-caffeine, 99%), DF ($^{13}C_6$-DF, 99.9%) were purchased from Sigma-Aldrich (Steinheim, Germany), IB (IB-d_3; 99.4%) from C/D/N Isotopes Inc. (Quebec, Canada), 17β-estradiol ($^{13}C_2$-βE2) from Camprto Scientific (Berlin, Germany) and BPS ($^{13}C_{12}$-BPS) and 44BPF ($^{13}C_{12}$-44BPF) from CanSyn Chem. Corp. (Toronto, Canada). All analytical grade solvents were obtained from J. T. Baker (Deventer, Netherlands). Formic acid (FA; 99%) and ammonium fluoride (NH_4F, 99.8%) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Ultrapure water (18.0 MΩ cm at 25 °C) was prepared using the Milli-Q water purification system (Merck Millipore, Darmstadt, Germany).

Stock solutions (≈ 1.0 mg mL^{-1}) for CECs were prepared by dissolving each compound in methanol, and working standards (10, 100 and 1000 ng mL^{-1}) were prepared by appropriate serial dilutions of the stock solutions. Similarly, an internal standard solution (1.0 μg mL^{-1}) was prepared from a solution (1.0 mg mL^{-1}) of isotopically labelled compounds (BPA-d_{16}, $^{13}C_3$-caffeine, $^{13}C_6$-DF, IB-d_3, $^{13}C_2$-βE2, $^{13}C_{12}$-BPS and $^{13}C_{12}$-44BPF). All standards were prepared in methanol and stored in amber glass vials at 4 °C. Samples were filtered through 0.2 μm regenerated cellulose membrane syringe filters (Phenomenex, Torrance, USA).

For the elemental analysis, the additional reagents were obtained: nitric acid (HNO_3, 67 – 69% Superpure – for trace analysis) purchased from CARLO ERBA (Val de Reuil, France), a NIST 3113 Mercury (Hg) standard solution, Periodic table mix 1 for ICP (TraceCERT®, 33 elements) and a mixture of single elements: K, S, P, Mg, Mo, Sn and Sb (TraceCERT®) which were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Mixtures containing the single elements: Na (Certipur®) and U were purchased from MERCK (Darmstadt, Germany) and Honeywell, Fluka (Seelze, Germany), respectively. Reference materials: SRM 1573a (Tomato Leaves), SRM 1547 (Peach Leaves) and SRM 1570a (Spinach Leaves) were acquired from NIST (National Institute of Standards and Technology) (Gaithersburg, MD, USA).

The stock solution for the calibration curve of elemental analysis was prepared by dissolving a known weight of each compound in 5% HNO_3 to give the appropriate concentrations, i.e., for Hg from 0.1 ng g^{-1} up to 10 ng g^{-1} (0.1, 0.5, 1, 5 and 10 ng g^{-1}); for "mix 1 for ICP" from 0.1 ng g^{-1} up to 250 ng g^{-1} (0.1, 0.5, 1, 5, 10, 50, 100 and 250 ng g^{-1}). To achieve a higher calibration, single element standards Na, Ca, K, S, P and Mg (10,000, 5000, 2000 and 1000 ng g^{-1}) and Mo, Sn, Sb and U (10,000 and 1000 ng g^{-1}) were mixed with the stock solution. Measured concentrations were 0.1, 0.5, 1, 10 and 100 ng g^{-1}.

2.2. Experimental design and treatments

The study consists of two experiments using hydroponic tubs (Exp. 1) and lysimeters (Exp. 2) performed over one growing season in 2020.

Tomato *Solanum lycopersicum L. Rally* was grown in a greenhouse under controlled agronomic conditions until seedlings (with 5–6 leaves; eight weeks after sowing) were ready to be transferred to the experiments (Fig. 1a and b). Wastewater for plant irrigation was obtained from a local wastewater treatment plant in Ajdovščina, Slovenia, which treats municipal and industrial wastewater for a population of approximately 42,000 (8400 m^3/day).

Exp.1: Hydroponics

Seedlings were transplanted to 60-L covered hydroponic tubs in late June 2020. Tubs (120 cm × 60 cm) were covered with a sheet of Styrodur® (20 mm) in which six plants were fixed in prepared holes (φ50 mm) using inert polyester foam. The mean day and night temperatures were recorded hourly using a USB data logger (DL-120TH; Voltcraft, Hirschau, Germany). The daily mean temperature was between 16 °C and 29 °C and remained above 14 °C during the night. The relative humidity was between 56% and 98%.

In total, the experiment (Fig. 1a) included five tubs, each containing six plants: i) two tubs which served as controls, where plants were exposed to standard Hoagland Arnon nutrient solution prepared using potable water, containing the following ionic composition: 14.0 mM NO_3^-, 1.0 mM NH_4^+, 1.0 mM $H_2PO_4^-$, 6.0 mM K^+, 4.0 mM Ca^{2+}, 2.0 mM SO_4^{2-}, 2.0 mM Mg^{2+}, 10 μM Mn^{2+}, 5.0 μM Zn^{2+}, 30 μM B^{3+},15 μM Fe^{3+}, 0.75 μM Cu^{2+}, and 0.5 μM Mo^{6+}. The pH of the nutrient solution was adjusted to pH 6 by adding 74% H_2SO_4; electrical conductivity was 2 dS m^{-1}. The nutrient solution was pumped from the storage tub at a flow rate of 4 L h^{-1} using a drip irrigation system with one emitter per plant. The nutrient solution was circulated continuously and supplemented after a 50% drop with freshly prepared nutrient solution. Controls (two tubs, each containing six plants) were grown under CEC-free conditions (HPW), while three tubs (six plants each) contained potable water with added fertiliser and spiked with CEC at 0.1 mg L^{-1} (HSPW).

Exp.2: Lysimeter experiments

Seedlings were transferred to a lysimeter (WRRF in Ajdovščina; 45.8764 N, 13.9064 E, 106 m a.s.l.) on 19/5/2020. Ajdovščina has a

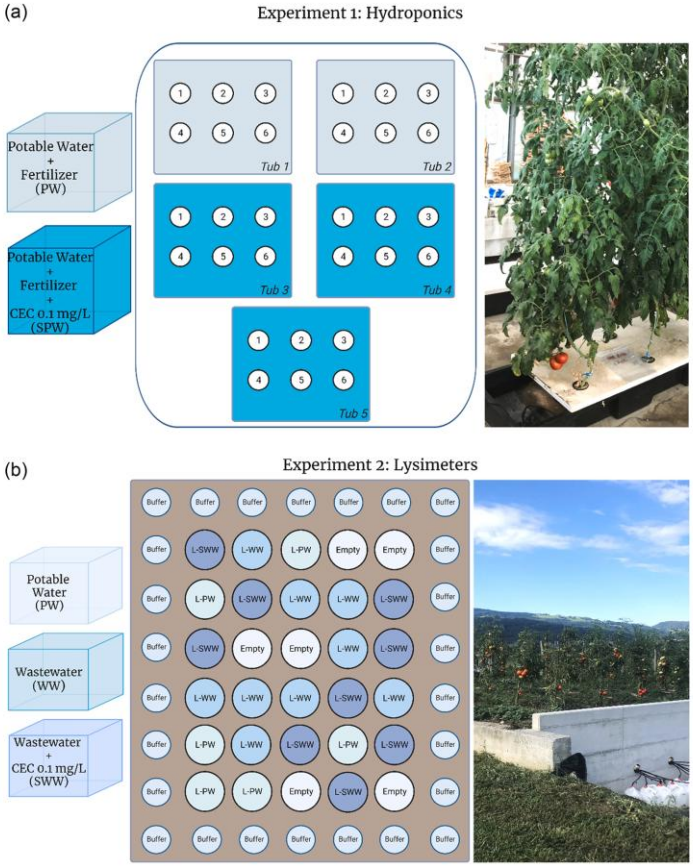


Fig. 1. a. Hydroponics experimental design (Exp.1). **b.** Lysimeter experimental design (Exp.2).

Mediterranean climate. The average air temperature from May to September 2020, measured at the nearest meteorological station Bilje (45.8964 N, 13.6245 E, 55 m a.s.l.), 21.7 km of areal distance from Ajdovščina, was 17.1, 20.3, 23.0, 23.2 and 19 °C. The sum of monthly precipitation measured at the nearest precipitation station Zalošče (45.8875 N, 13.7476 E, 75 m a.s.l.), 12.7 km of areal distance from Ajdovščina, was 108.4, 193.5, 118.0, 145.1, and 339.7 mm.

The tomatoes were grown under three sets (Fig. 1b) of conditions: i) six lysimeters served as a control, where plants were irrigated with potable water (LPW); nine lysimeters irrigated with wastewater from the WWTP (LWW), and nine lysimeters were irrigated with wastewater spiked with CEC at 0.1 mg L^{-1} (LSWW). Tomatoes in lysimeters were surrounded by a buffer zone, providing equal exposure to sun and wind. The media was freshly prepared on day zero and again at 2.5 months. Each lysimeter (40 cm in diameter, 100 cm and positioned 100 cm apart) contained one plant and local soil, i.e., Eutric brown soil on flysch (Table SI-3). The lysimeters were covered with a 2 mm thick plastic cover to prevent evaporation from the soil and rainwater from entering the lysimeters. Irrigation was achieved using an automated drip system (2 L h^{-1}). Plants were watered at 0.5 L per plant per day between 19/6–23/6, 1 L per day between 25/6–16/7 and 2 L every second day between 16/7–10/9. The total amount of irrigation water was 36 L.

All plants were fertilised at transplanting with 5 g N (KAN, 39%), 6.6 g P (Superphosphate 0:26:0; 26% P), and 17 g K (K_2SO_4; 50% K), corresponding to 70, 60, and 300 kg of nutrients per hectare, respectively. Additionally, the plants were fertilised with 5 g N (KAN, 39% N) three times at one-month intervals.

2.3. Sample collection and preparation

Tomato fruits were hand-harvested at the fully ripe stage. For the hydroponic experiments, the harvesting season was divided into three periods: (1st) 24/8–10/9, (2nd) 14/9–22/9 and (3rd) 28/9–9/10. Similarly, for lysimeter experiments, the first harvest was performed on 4/8, the second on 28/8 and the third on 11/9/2020.

During each harvesting, the ripened fruits were collected, washed with water, blotted dry and stored whole in plastic bags at − 20 °C. Frozen samples (in 60/600 g PE containers) were cut into pieces, freeze-dried and ground to a powder. Composite samples were prepared as follows: 1) all harvested fruits per plant (three or two plants selected) and per treatment (COM), 2) all harvested fruits per harvest (1st, 2nd and 3rd harvest, S1-S3) per treatment, 3) all harvested fruits per treatment from three or two plants per treatment (plants from tubs or lysimeters; P1-P3).

For determining CECs, each composite sample was prepared and analysed in triplicate. Briefly, 0.25 g of freeze-dried composite sample was weighed in a 10 mL centrifuge tube and spiked with the isotope-labelled internal standard mixture (V_{add} = 25 µL; c = 1 µg mL^{-1}). Ultrapure water (1 mL) was added, and the sample was vortexed (30 s) and shaken overnight (IKA KS 4000, 200 mot rpm). Afterwards, a 1:1 mixture of ethyl acetate:acetonitrile (4 mL) was added, and the mixture was sonicated (20 min) and centrifuged at 9000 rpm for 20 min. The supernatant was transferred to a 10 mL glass vial, and the residue was again suspended in 4 mL of ethyl acetate:acetonitrile (1:1) and further sonicated and centrifuged. The solvent from the combined extracts was removed using N_2 (40 °C). Once dry, the extracts were reconstituted in methanol:ultrapure water (0.5 mL, 1:1), centrifuged (9000 rpm, 20 min), filtered (0.2 µm regenerated cellulose membrane filters) and stored at −20 °C.

Samples of growth media spiked with CECs (100 mL) were analysed based on Kovačič et al. [18]. Briefly, 1 mL aliquot of water sample was diluted with 2 mL of ultrapure water, spiked with the internal standard mixture (30 µL, 1 µg mL^{-1}), and acidified (pH = 3) with 0.1 mL HCl_{aq} (c = 0.12 M). The samples were then loaded onto Oasis Prime HLB SPE cartridges (60 mg sorbent, 3 mL) at 2 mL min^{-1} using a Vacuum Manifold, washed with 1 mL of 10% $MeOH_{aq}$ and dried under vacuum (30 min). The elution step was performed using 5% formic acid in ethyl acetate (3 × 0.6 mL). Dried extracts (N_2, 40 °C) were reconstituted in a 1:1 solution of methanol:ultrapure water (1.0 mL), filtered through (0.2 µm) and stored at − 20 °C.

The samples for elemental analysis were prepared by weighing a known amount (0.1 g) of the freeze-dried tomato composite into pre-cleaned Teflon digestion vials to which 1 mL of 67 – 69% HNO_3 was added. The samples were digested using the ULTRAWAVE, Single Reactor Chamber Microwave Digestion System (MILESTONE, Italy). The program was as follows: heating to 240 °C for 20 min and held for 15 min at max 100 bar and max power: 1500 W. The digests were then diluted to 10 mL using Milli-Q water. Before analysis, samples were diluted with 5% HNO_3 in a ratio of 1:5 and 1:10.

2.4. Determination of CECs in tomato fruits

All samples were analysed using an UHPLC-Qtrap (Shimadzu Nexera X2 UHPLC, Kyoto, Japan) coupled to a linear ion trap quadrupole 4500 QqLIT-MS/MS (Sciex, Framingham, MA, USA). The separation was achieved at 40 °C using a Poroshell 120 EC-C18 column (100 × 3 mm; 2.7 µm particle size, Agilent Technologies, USA). The mobile phase was ultrapure water with 1 mM NH_4F and methanol with 1 mM NH_4F at a flow rate of 0.3 mL min^{-1}. The analyser was operated under electrospray ionisation (ESI) in negative mode with the following settings: curtain gas: 35 psi; collision gas: medium; source temperature: 550 °C; ion spray voltage: − 4.5 kV; ion source gas 1: 35 psi; ion source gas 2: 20 psi. The injection volume was 5 µL. The mobile phase gradient was as follows: 0 min, 70% B; 0–2 min, 80% B; 2–4 min, 85% B; 4–7 min, 100% B; 7.50 min; 100% B; 7.50–11.50 min; 70% B. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Transitions were optimised for the compound-dependent parameters (scheduled MRM), including declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP). Retention times and MS/MS optimised conditions are summarised in Table SI-2. Chromatograms were analysed using Analyst v1.6.3.

The method was validated for linearity, range, trueness, precision, the lowest limit of detection (LOD) and quantification (LOQ), analyte recovery, matrix effect and robustness following Eurachem guidelines [22]. The matrix-matched method was validated for four tomato-based matrices: three different tomato varieties collected from the supermarket (cherry, plump and beef tomato) and their pooled sample. All validation parameters were measured at low and high calibration levels, 2.5 and 15 ng g^{-1} for 13 CECs and 15 and 150 ng g^{-1} for BPA, respectively. Quality control samples (low calibrant) were included after every 20th injection. Procedural blanks (n = 4) samples were prepared and systematically included in each batch of analyses, while solvent blanks were analysed to evaluate potential carry-over. All samples were blank-corrected.

2.5. Elemental analysis of tomato fruits

The elemental analysis was performed as described by Potočnik et al. [30]. Briefly, B, Na, Mg, Al, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Cs, Ba, Hg, and Pb were analysed after digestion using an Agilent 8800 triple quadrupole inductively coupled plasma mass spectrometer, QQQ-ICP-MS (Agilent, USA). Internal standards (Y, Rh, Sc, and Gd) were added online in all modes, and external calibration was used to quantify the results. Blank samples and selected reference materials: SRM 1573a (Tomato Leaves), SRM 1547 (Peach Leaves) and SRM 1570a (Spinach Leaves) were added within each batch of samples. The LOD was calculated as three times the standard deviation of the blank samples and was for macro elements Mg 20, S 150, P 20, K 175 and Ca 1000 ng g^{-1} f.w., for microelements, B 18.5, Na 45, Al 12.5, Mn 0.5, Fe 8.5, Ni 0.75, Cu 0.75, Zn 9.0, Rb 0.25, Sr 0.5, Mo 0.25, Ba 0.25 ng g^{-1} f.w. and for trace elements V 0.02, Cr 0.35, Co 0.05, As 0.04, Se 0.25, Ag 0.02, Cd 0.01, Cs 0.05, Hg 0.015, Pb 2.0 ng g^{-1} f.w.

2.6. Dietary exposure and health risk assessment

Dietary exposure of the detected CECs and PTEs was estimated for children (toddlers) and adults by combining data on CEC and PTE concentration in composite samples of fresh tomatoes (Table SI-5) with the corresponding consumption levels from the EFSA Comprehensive European Food Consumption Database [34]. These are the most complete and detailed data currently available at the EU level. Only surveys appropriate for calculating chronic exposure were selected to assess repeated exposure (21 surveys for toddlers and 39 for adults). Dietary exposure in fresh tomatoes was assessed deterministically; average exposure was based on detected concentration and average consumption data, while high exposure was based on detected concentration and high consumption (97.5-percentile) data.

A temporary health-based guidance value has been derived only for BPS (DNEL 200 μg (kg day)$^{-1}$). Its ongoing assessment may change as BPS is suspected to affect human reproductive and hormonal systems [12]. The derived no-effect level (DNEL) is the level of exposure above which a human should not be exposed to a substance and was compared with the dietary exposure estimates for BPS.

The potential non-carcinogenic risk to human health resulting from consuming PTEs in tomatoes was assessed using the hazard quotient (HQ) approach calculated according to Eq. (1).

$$HQ = \frac{DE}{RfD} \quad (1)$$

DE is the dietary exposure (μg (kg day)$^{-1}$), and RfD is the reference dose (μg (kg day)$^{-1}$), Table SI-6). An HQ < 1 indicates the absence of significant non-carcinogenic health risk, whereas an HQ > 1 denotes a possibility of adverse health effects. The DE was calculated as follows:

$$DE = C_m \times C_d \quad (2)$$

where C_m (μg g^{-1} f.w.) is the concentration of PTE in fresh samples and C_d is the consumption of fresh tomatoes, where data are different on an individual level (toddlers and adults). In case the latest reference dose was available as a tolerable daily intake level (μg day^{-1}), an average weight of a toddler of 12 kg and an adult of 70 kg was assumed [34].

Since the concentrations of PTEs were measured in freeze-dried samples as ng g^{-1} d.w., all units were converted to ng g^{-1} f.w. (Eq. 3).

$$c_{f.w.} = \frac{c_{d.w.} \times (100 - W)}{100} \quad (3)$$

Here $c_{f.w.}$ is the concentration of PTEs based on fresh weight, $c_{d.w.}$ concentration based on dry weight and W [%] content of water in tomato, which is 95%.

2.7. Data processing and analysis

Data processing and visualisation were performed using R-based software (version 3.6.1), SigmaPlot 14 (Systat Software GmbH) and SIMCA (Sartorius). All data sets were checked for normal distribution using the Shapiro-Wilk test and for homoscedasticity of the variances using Levene’s test or Brown-Forsythe test. The influence of compound type, growing conditions and harvesting time on the uptake of CECs was assessed using the Wilcoxon Ran-Sum or Kruskal-Wallis test at the significance level of $p \leq 0.05$, Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). A significant difference in elemental concentrations of control samples (HPW, LPW) and hydroponics samples (HPW, HSPW) was assessed using the t-test or Mann-Whitney U test, while in the case of lysimeter samples, it was assessed using One-way ANOVA or Kruskal-Wallis test.

3. Results and discussion

3.1. Method optimisation and validation

3.1.1. Optimisation of sample preparation for CECs extraction from tomatoes

The mixture of standards and internal standards solution was added in methanol to 0.25 g of lyophilised tomato and covered with 1 mL of different solvents (methanol, ultrapure water, acetonitrile). The sample was then thoroughly mixed and shaken in the dark at room temperature until all of the solvent had evaporated (12 hrs), ensuring the homogeneity of the added compounds in the analysed tomato sample. For ultrasound-assisted extraction of CECs from tomato fruit samples, different solvent combinations of ethyl acetate, methanol, acetonitrile, and the addition of formic acid, including different ratios and volumes (3–10 mL), were tested. For this purpose, a 50 ng g^{-1} concentration of each target CEC was added to 0.25 g of fruit sample. Ethyl acetate/acetonitrile (1/1) gave the highest extraction efficiency (42–127%) for all CECs, and thus, it was selected for further testing. Different solvents (ultrapure water, methanol, acetonitrile, 0.25–1 mL) for spiking tomato samples with labelled and unlabelled CECs were also tested. The supernatant was centrifuged and processed as described in Section 2.3.

Ultrasound-assisted extraction (internal standards covered with 1 mL in ultrapure water, two times 4 mL of ethyl acetate:acetonitrile = 1:1) of dry tomato fruit samples was also compared with QuEChERS extraction of the fresh tomato fruit. The obtained analyte recovery and method precision using QuEChERS were lower, and the matrix effect higher (RE=30–110%, method repeatability RSD=3–33%, ME= ± 10–3000%) when compared to the optimised ultrasound-assisted extraction method (RE=65–92%, method repeatability RSD = <15%, ME= ± 1–86%).

3.1.2. Method validation

Method validation was performed on four tomato-based matrices 1) beef tomato, 2) plump tomato, 3) cherry tomato and 4) pooled tomato matrix. Accordingly, all samples were blank-corrected. Sensitivity was evaluated in terms of LOD and LOQ, calculated as three times and ten times the standard deviation of the baseline (standard addition) divided by the slope of the calibration curves, respectively. Using the LOQ as the lowest calibration standard, LOQs were 0.5 or 1.0 ng g^{-1} (0.025 or 0.05 μg kg^{-1} f.w.) for 13 CECs and 40 ng g^{-1} (2.0 μg kg^{-1} f.w.) for BPA. The linearity of the method was obtained using spiked matrix-matched samples at different calibration points across the working range.

The calibration range for the analysis consisted of seven/eight calibration points (0.5/1–25/50 ng g^{-1} for 13 CECs and 40–500 ng g^{-1} for BPA) using the LOQ as the lowest calibration level, whereas the concentration of each of the internal standards remained fixed (25 ng g^{-1}). Each calibrant was prepared in triplicate. The coefficient of determination (R^2) was > 0.99 for all tested CEC, except for EE2 ($R^2 = 0.98$) when using a linear calibration curve. The analyte recovery is reported as the ratio between peak areas of analyte/internal standard spiked before and after extraction, whereas internal standard spiked after sample extraction is used to cover instrument signal variations. The mean recoveries were 64.7–91.5% and 55.6–94.0% at the low and high levels. The matrix effect (± 1–86%) was based on a comparison between the analyte peak area normalised with the internal standard when the analyte mixture was added to tomato extract compared to when added to the final reconstituted solvent (methanol:ultrapure water=1:1). Precision was expressed in terms of method repeatability, calculated as the relative standard deviation (RSD) of three parallel samples ($n = 3$), and instrumental repeatability, determined as the RSD of three consecutive injections of the same sample ($n = 3$). The RSDs of the calibrants were < 15%. Instrument precision was < 10%, except for naproxen at the higher level (19.7%). Trueness was expressed as [(experimental value - spiked value)/spiked value] ($n = 3$) and was less than 15% at both levels. The method’s robustness was tested by

comparing the slope (k) of four blank corrected calibration curves. The calculated RSD of k_1, k_2, k_3 and k_4 was less than 20%, except for ketoprofen (25%), which also had the highest variations in ME between different tomato-type matrices. The developed method is robust for any tomato variety and can be used to quantify the tested CECs. The uptake of investigated CEC in this study was quantified using a matrix-matched calibration line from pooled tomato samples. All validation results are summarised in Table 1. No potential carry-over was observed within analysed batches, and the RSD of quality control samples was below 10%.

3.2. CECs in the irrigation water

The amounts of individual CECs in the spiked irrigation media after 1.5 months varied between 50 and 100 μg L^{-1}, except for E1 (<50 μg L^{-1}). After 2.5 months, BPA, caffeine, naproxen, ketoprofen, and ibuprofen remained above 50% of the initial theoretical concentration (c_i = 60–80 μg L^{-1}), which may be a result of degradation processes. Water samples were also taken from hydroponic tubs before and after refilling. The concentration of CEC before refilling was (except for naproxen) < 10 μg L^{-1}, in line with the concentration determined in irrigation media after refilling. Consequently, 1 m^3 irrigation media was freshly prepared after 2.5 months and analysed after four months of field experiments. Most CECs were > 50 μg L^{-1} in the water from the reservoir (m^3) and hydroponic tubs. The concentrations of CECs in all controls (without CECs) were <LODs.

3.3. Uptake of CECs in tomato fruit

All concentrations (Table SI-4) are given on a fresh weight basis (μg kg^{-1} f. w.), considering the composition of a ripe tomato: 95% water and 5% dry matter [16]. The RSDs of composite samples were < 20% (n = 3), confirming the homogeneity of the samples. Interestingly, out of the 14 compounds selected in this study, BPS, 24BPF, and naproxen were detected in tomato fruits grown in spiked media under both experiments (Fig. 2). No significant difference in the amount of CECs in tomatoes between different harvests (p-value = 0.754) was observed. A Wilcoxon Ran-Sum test showed a significant difference (p-value = 3.36E-13) in the uptake of CECs between tomatoes grown hydroponically or in soil, with more significant uptake when grown hydroponically.

The PCA, performed on the whole data set, grouped samples into three groups 1) hydroponically grown tomatoes without CEC, 2) hydroponically grown tomatoes with CEC, and 3) soil-grown tomatoes (Fig. 3). The data set obtained from different growing conditions with added CEC was formed into two groups, hydroponically and tomatoes grown in lysimeters, using the OPLS-DA method (Fig. SI-2). Slight separation in OPLS-DA of non-spiked (LWW and LPW) from spiked (LSWW) conditions is also observed (Fig. SI-2), while no separation between lysimeters treated with non-spiked wastewater (LWW) and potable water (LPW) was observed (Fig. SI-3) suggesting that reusing treated wastewater can be considered a reliable water supply in agriculture. The concentrations of BPS were higher in tomatoes grown hydroponically (0.071–0.134 μg kg^{-1} f. w.). However, in soil-grown tomatoes, BPS was also above LOQ (0.034–0.070 μg kg-1 f. w.), while 24BPF (<LOQ-0.081 μg kg^{-1} f. w.) and naproxen (0.050–0.137 μg kg^{-1} f. w.) were present above LOQ only in the case of hydroponically grown tomatoes. The results of the Kruskal Wallis test show a difference (p-value = 6.09E-14) in the uptake between compounds. The average concentrations of CECs present in hydroponically and soil-grown tomatoes were 0.08 ± 0.03 μg kg^{-1} f. w. and 0.06 ± 0.01 μg kg^{-1} f. w., respectively.

The uptake of BPS and 24BPF has not been previously examined in the literature. However, in line with Bueno et al. [6], naproxen was ≤LOQ in tomato fruits irrigated with reclaimed water under field conditions with added CECs (1 μg L^{-1}). Contrary to our findings, the authors observed the uptake of caffeine and diclofenac. Most studies that

Table 1
Method performance of a robust analytical method for determining 14 CECs in tomato fruit sample, including method repeatability, instrumental repeatability, trueness, linearity, extraction efficiency, matrix effect, LOD, LOQ and robustness.

Compound	Method repeatability		Instrumental repeatability		Trueness		Linearity			Extraction efficiency		Matrix effect		LOD	LOQ	LOD	LOQ	Robustness
	(RSD)		(RSD)		(%)					(%)		(%)		[ng g^{-1} d.w.][a]	[ng g^{-1} d.w.[a]]	[μg kg^{-1} f.w.][b]	[μg kg^{-1} f.w.][b] *	RSD
	LC	HC	LC	HC	LC	HC	R^2	[ng g^{-1} d.w.][a]	[μg kg^{-1} f.w.][b] *	LC	HC	LC	HC					k_1-k_4
BPS	14.6	6.8	2.0	0.5	13.6	1.0	0.99	0.5–25	0.025–1.25	66.6	66.1	3.0	3.0	0.04	0.5	0.0020	0.025	4.4
BPA	10.3	3.5	3.5	3.5	9.1	6.7	0.99	40–500	2–25	94.0	91.5	22.4	37.1	4.1	40.0	0.21	2.0	16.5
22BPF	12.9	15.1	5.9	6.1	6.5	4.4	0.99	0.5–25	0.025–1.25	89.3	81.4	86.0	87.4	0.003	0.5	0.0002	0.025	20.5
24BPF	10.5	12.9	5.8	5.5	11.7	4.9	0.99	0.5–25	0.025–1.25	83.6	78.3	29.6	34.7	0.004	0.5	0.0002	0.025	21.1
44BPF	13.6	8.2	5.5	6.9	8.4	8.3	0.99	0.5–25	0.025–1.25	84.0	76.1	9.4	2.0	0.02	0.5	0.0010	0.025	5.2
Caffeine	9.3	12.1	4.9	9.8	12.7	6.0	0.99	0.5–50	0.025–2.25	79.9	85.2	83.6	4.7	0.02	0.5	0.0010	0.025	3.5
Ketoprofen	11.8	14.0	0.7	3.0	13.9	2.4	0.99	1.0–50	0.05–2.25	72.9	88.6	80.6	27.9	0.27	2.5	0.014	0.05	24.9
Ibuprofen	13.8	7.4	2.2	6.8	3.7	2.0	0.99	1.0–25	0.05–1.25	76.5	70.3	84.6	6.5	0.24	1.0	0.012	0.050	6.1
Naproxen	7.5	10.2	19.3	2.0	9.6	0.9	0.99	0.5–25	0.025–1.25	80.6	82.2	75.4	46.2	0.02	0.5	0.0010	0.025	7.3
E1	6.5	5.8	5.8	3.8	3.7	0.8	0.99	0.5–25	0.025–1.25	81.7	88.0	1.4	7.3	0.008	0.5	0.0004	0.025	7.2
β-E2	12.8	6.9	2.7	4.5	41.5	3.2	0.99	0.5–25	0.025–1.25	80.7	83.7	8.2	5.0	0.08	0.5	0.0040	0.025	3.8
EE2	11.7	6.9	6.8	5.6	4.2	11.9	0.98	1.0–50	0.05–2.25	79.3	87.7	27.8	10.9	0.39	1.0	0.020	0.050	4.1
E3	8.2	8.5	9.3	5.5	14.0	8.7	0.99	0.5–25	0.05–1.25	55.6	64.7	7.2	3.5	0.10	0.5	0.0050	0.025	2.9
Diclofenac	6.5	7.8	1.6	1.6	9.1	1.0	0.99	0.5–25	0.05–1.25	84.0	81.0	83.5	6.1	0.05	0.5	0.0025	0.025	3.0

[a] Concentration in ng g^{-1} dry weight [ng g^{-1} d.w.], [b] *Concentration in μg kg^{-1} fresh weight [μg kg^{-1} f.w.]

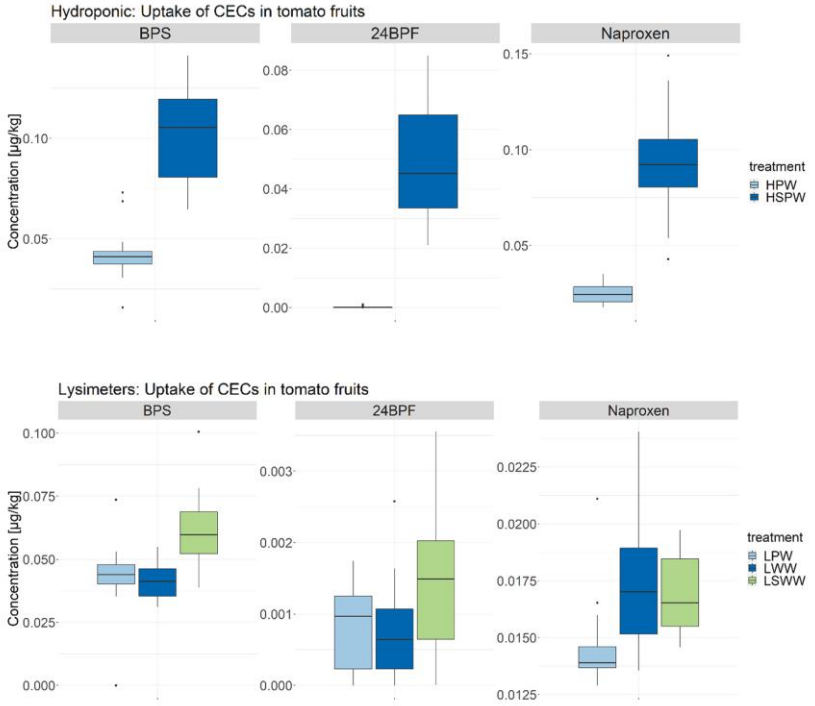


Fig. 2. Levels of CECs (µg kg^{-1} f. w.) in composite samples of hydroponically (HPW, HSPW) or soil-grown tomatoes (LPW, LWW, LSWW) under different conditions (treated with potable or wastewater in the absence or presence of CECs).



Fig. 3. Principal Component Analysis (PCA) of the whole data set (detected concentration of CECs from the composite tomato samples). Score plot PC1 vs PC2.

investigated caffeine uptake do report its presence in tomato fruits [25, 3]; however, this was not the case in our study. A possible explanation could be different studied concentrations and compositions of compounds added in growing media and field conditions (e.g., soil type). The uptake of diclofenac was also not observed in the study by Christou et al. [10], where they performed hydroponic cultivation using water with added pharmaceuticals. In the case of soil irrigation and prolonged WW irrigation, diclofenac was determined in tomato fruits 11.63 μg kg^{-1} f.w. Christou et al. [8]. Unlike Lu et al. [19], who studied hydroponically grown tomatoes with the addition of CECs (c_i = 50 μg L^{-1}) in wastewater and Margenat et al. [24], who studied tomatoes grown in peri-urban agriculture irrigated with no addition of CECs in reclaimed water, we did not observe the uptake of BPA nor 44BPF, respectively.

Given that the irrigated (lysimeters) and nutrient (hydroponics) medium were spiked with compounds at the same concentration level, it is interesting that only BPS, 24BPF and naproxen were determined in tomato fruits in both experiments. The absence of most of the added compounds in irrigation media in tomato fruits can be explained by the greater water flow (translocation) to leaves, leading to a higher accumulation of CECs in leaves than in the fruit, as observed by Martinez-Piernas et al. [25]. Secondly, CECs taken up by the plant can be metabolised to phase I metabolites (e.g., hydroxylation), as well as forming phase II metabolites, e.g., conjugation of the parent compound or phase II metabolites with glucose, glucuronic acid, malonic acid [26]. The metabolisation of the parent compounds supports our observation, e.g., the absence of parent compounds in tomato fruits.

3.4. Occurrence of elements in tomato fruit

The results of mean elemental concentrations in tomatoes from different growing conditions are presented in Fig. 4, including macro elements: K, Ca, Mg, P and S; microelements: Na, Rb, Fe, Zn, B, Cu, Mn, Al, Mo, Ba, Ni and Sr and trace elements: Cr, Pb, Se, Cd, Cs, Co, V, As, Ag and Hg. Within recent literature, a wide range of elemental concentrations in tomatoes has been reported that depend on variety, substrate, climate and irrigation-fertilisation strategies. Our results of HPW indicate a similar concentration range to the study performed by Gundersen et al. [14] in Sager et al. [21] in Austria, except for Mo, Ni, Cd, Cr, and Pb and Al, Fe and Rb, respectively (Table SI-5). Giorgia et al. [13] had lower macro and micro elements concentrations in Sicily but higher or comparable concentrations of trace elements. Concentrations of microelements in Morocco were much lower than in our study, while Cd was comparable and Pb higher [20]. Tomatoes from Salvador had higher concentrations of potentially toxic elements: Al, Co, Cr, Hg and V [5]. The concentrations in tomatoes irrigated with treated wastewater are comparable to or lower than those in the literature, where the concentrations for microelements ranged from 0.095 mg kg^{-1} f.w. (Mo) to 30 mg kg^{-1} f.w. (Fe) (Fig. 4), and for the trace elements from 0.454 μg kg^{-1} f.w. (Hg) to 0.361 mg kg^{-1} f.w. (Pb) (Table SI-6).

Statistical analyses (Table SI-7) showed that all the elements between HPW and LPW tomatoes are significantly different, except Se, Rb and Hg. Concentrations of Na, Mg, P, S, K and Mo were significantly higher in HPW tomatoes, indicating their uptake from the applied fertiliser. In comparison, concentrations of Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Ag, Cd, Cs, Ba, and Pb were significantly higher in LPW,

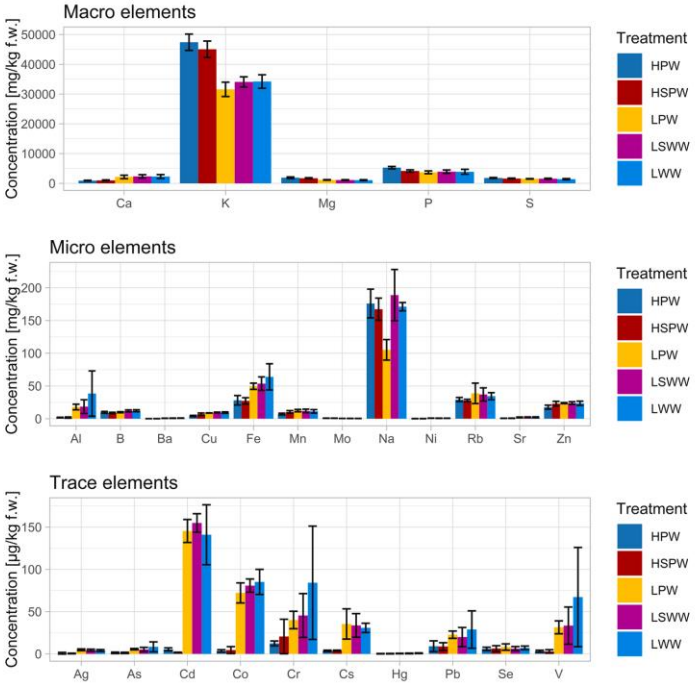


Fig. 4. Mean concentrations of macro, micro and trace elements in different treatments.

indicating their higher concentrations in soil and their uptake into the tomato fruits. The most remarkable level of 27 times higher in LPW than HPW was observed for Cd. A similar observation was also noted in the study performed by Gundersen et al. [14]. Possible explanations are 1) a more accessible form of Cd can be present in the soil, 2) more intensive microbiological activity in the soil can promote uptake of Cd in the plant, and 4) the root system in the soil is bulkier and allows higher utilisation of Cd from the substrate. The separation of the HPW and LPW tomatoes was also confirmed with OPLS-DA analysis (Fig. SI-4). Hydroponics tomato comparison showed significantly higher Mg, P, S and Cd concentrations in HPW tomatoes.

In comparison, concentrations of Mn, Cu and Zn were significantly higher in HSPW, which could be the consequence of the altered uptake of elements by added CECs, i.e., reducing the uptake of the first group of elements and increasing the uptake of the second group. The separation of the HPW and HSPW tomato samples by OPLS-DA can be seen in Fig. SI-5. Comparing tomato samples grown in lysimeters (Fig. SI-6), only B and Na were significantly higher in LWW tomato samples, and no elements were significantly higher in LPW samples, indicating higher uptake of B and Na from the treated wastewater. Surprisingly, there were no significantly different concentrations of elements between LWW and LSWW tomato samples (Fig. SI-7). Therefore, there is no evidence that CECs addition affects the uptake of elements from treated wastewater. The PCA (Fig. 5) performed on the whole data set of measured elements in tomato fruit grouped the samples grown in HPW, HSPW and LPW, which was also confirmed with OPLS-DA (SI-2.2) but did not separate tomatoes grown in LWW and LSWW. There were two outliers in the LWW group, named LWW_S1_COMP and LWW_30_COMP, which are the composite sample from the first sampling and the composite sample by plant 30. Both have significantly higher Al, V, and Cr and significantly lower concentrations of P and Zn than other LWW samples.

3.5. Dietary exposure and health risk assessment

Average chronic exposure to the detected CECs (24BPF, BPS, naproxen) from consuming fresh hydroponically produced tomatoes for children (toddlers: 1–3 years) was 0.01–0.13 ng (kg bw day)$^{-1}$, 0.02–0.27 ng (kg bw day)$^{-1}$, 0.02–0.26 ng (kg bw day)$^{-1}$ with high exposure of 0.07–0.48 ng (kg bw day)$^{-1}$, 0.15–0.97 ng (kg bw day)$^{-1}$, 0.14–0.93 ng (kg bw day)$^{-1}$. The highest exposure for children was 0.97 ng (kg bw day)$^{-1}$ for BPS. Results for chronic exposure of adults were lower and average consumption of fresh tomatoes resulted in exposures of 0.01–0.08 ng (kg bw day)$^{-1}$, 0.01–0.15 ng (kg bw day)$^{-1}$, 0.01–0.15 ng (kg bw day)$^{-1}$. Exposure levels for high consumers were in the range of 0.05–0.26 ng (kg bw day)$^{-1}$, 0.09–0.51 ng (kg bw day)$^{-1}$, 0.09–0.50 ng (kg bw day)$^{-1}$.

The selected population groups are less exposed via tomatoes grown in soil than soil-less media, which is expected since the CEC concentrations are also lower. Only the concentration of BPS was measured above the LOQ. The average chronic exposure to BPS for children was 0.02–0.16 ng (kg bw day)$^{-1}$, and for adults, 0.01–0.09 ng (kg bw day)$^{-1}$. The highest estimated exposure of BPS was 0.59 ng (kg bw day)$^{-1}$ for toddlers as high consumers of fresh tomatoes. By comparing the highest exposure estimates of BPS with the currently derived no-effect level of 200 μg (kg bw day)$^{-1}$, there is no health concern for any age group. However, such an assessment has a provisional status. The obtained exposure results will be helpful in risk characterisation when the health-based guidance values for the studied CECs are determined.

Regulation (EC) 1881/2006 of the European Commission has set maximum levels for certain toxic elements in vegetables. For example, the maximum level of 0.05 mg kg^{-1} of Cd and 0.10 mg kg^{-1} for Pb were established. Our results indicate the highest estimated daily intake (EDI) of 16.1 ng (kg_{bw} day)$^{-1}$ for Cd in LSWW and 3.01 ng (kg_{bw} day)$^{-1}$ for Pd in LWW (Table SI-9) that are negligible concerning daily intake budgets. Further, average and high chronic exposures to detected PTEs from fresh tomatoes grown in all five different treatments and hazard quotients for toddlers and adults are given in Table SI-9 and Table SI-10. The data show that all the calculated hazard quotients for each PTE in each treatment were < 1, and the hazard index was < 1 for each treatment, indicating the absence of significant non-carcinogenic health risk in the case of consuming tomatoes from all five different treatments.

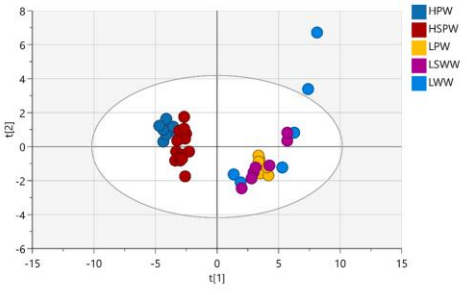


Fig. 5. Principal Component Analysis (PCA) results for the whole data set of elements in tomato fruit (determined concentrations from the composite tomato samples). Scores Scatter Plot t[1] vs t[2].

4. Conclusion

The present study provides new evidence on the levels of CECs in tomato fruits produced under soil and soil-less growing conditions using treated wastewater, as well as the influence of those chemicals on PETs occurrence. Bisphenol S, 24BPF and naproxen out of the 14 spiked CECs were detected with higher uptake in the case of hydroponically grown tomatoes. Importantly, we showed that the elemental composition in tomatoes was affected by the addition of CECs. Based on CEC uptake and elemental composition, no health risk from consuming tomatoes grown hydroponically or in soil was detected. However, our data also revealed how different irrigation regimes affect the uptake of chemicals. The results of this study add to the argument that, if properly monitored, reusing treated wastewater for irrigation can be a safe approach in agriculture and can support the decisions of policymakers and stakeholders in creating future legislative frameworks related to sustainable water resource management in agriculture. Nevertheless, more studies need to be performed for a definite answer on the safety of wastewater reuse for irrigation, providing more information about CECs, including the formation of metabolites and transformation products, possible synergistic effects and different agricultural practices.

Credit authorship contribution statement

Ana Kovačič: Conceptualization, Investigation, Formal analysis, Data curation, Methodology, Visualization, Writing – original draft. **Eirini Andreasidou:** Writing – original draft. **Anže Brus:** Formal analysis, Data curation. **Anja Vehar:** Formal analysis, Data curation, Writing – original draft. **Doris Potočnik:** Formal analysis, Data curation, Writing – original draft. **Marta Jagodic Hudobivnik:** Formal analysis. **David Heath:** Methodology, Conceptualization, Writing – review & editing. **Marina Pintar:** Supervision, Conceptualization, Methodology, Writing – review & editing. **Nina Kacjan Maršič:** Supervision, Conceptualization, Methodology, Writing – review & editing. **Nives Ogrinc:** Supervision, Writing – review & editing. **Urška Blaznik:** Formal analysis, Data curation, Writing – original draft. **Ester Heath:** Supervision, Conceptualization, Writing – review & editing, Project administration, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Novelty statement

The use of treated wastewater (TWW) for agricultural production is compelling, particularly as the supply of clean freshwater decreases, leading to water scarcity in many regions. Thus, understanding the risks associated with potentially introducing harmful chemicals into crops from TWW irrigation is crucial to protect health and the environment. The present study provides novel evidence on the uptake of 14 chemicals of emerging concern and 27 potentially toxic elements in tomato fruits produced in soil and soil-less conditions irrigated with potable and treated wastewater. The results add to the argument that the reuse of treated wastewater for irrigation can be a safe approach in agriculture and provide essential information for supporting the decisions of policymakers and stakeholders regarding treated water reuse.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.130964.

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3.5 Scientific Paper: "Influence of Contaminants of Emerging Concern on the Primary and Secondary Metabolites in Hydroponically Grown Tomatoes"

This chapter presents the paper entitled "Influence of contaminants of emerging concern on the primary and secondary metabolites in hydroponically grown tomatoes" by Anja Vehar, Nina Kacjan Maršić, Špela Železnikar, Doris Potočnik, Lidija Strojnik, Maja Mikulič Petkovšek, Helena Šircelj, Damijana Kastelec, Ana Kovačič, Marina Pintar, David Heath, Ester Heath, and Nives Ogrinc. The paper was published in Food Chemistry (A'') in 2025. The study aimed to evaluate how CECs influence the metabolism of hydroponically grown tomato fruits.

Tomatoes were grown in a nutrient solution and a spiked nutrient solution. Nutrient solution was spiked with 14 CECs at 0.1 mg/L, derived from industrial chemicals, pharmaceuticals, and common stimulants. Ripe lyophilised tomato fruits were analysed for sugars (HPLC-RI), organic acids (HPLC-UV), carotenoids (HPLC-DAD), polyphenols (HPLC-DAD-MS/MS), amino acids (GC-MS), fatty acids (GC-FID), and volatile organic compounds (HS SPME GC-MS).

Results showed that CEC exposure led to a statistically significant increase in tomato yield but did not significantly affect the levels of sugars, organic acids, carotenoids, polyphenols, or fatty acids. However, exposed tomatoes exhibited lower concentrations of specific amino acids, including valine, leucine, aspartic acid, and methionine, suggesting potential stress responses to contaminant exposure. Moreover, exposure to CECs significantly altered the volatile organic compound (VOC) profile. Several of the altered VOCs are associated with plant stress, suggesting that CEC exposure may induce physiological responses. However, changes were also observed in VOCs that are not typically linked to stress, indicating a more complex interaction between CEC exposure and tomato metabolism.

This study is particularly relevant to the use of TWW in hydroponic systems. It is among the first to contribute to understanding how CECs influence the quality of tomato fruits.

As joint first author, my contributions included assisting with sample collection and preparation, conducting the amino acid, fatty acid, and volatile organic compound analyses, processing the complete dataset, generating the visual materials, and drafting and finalising the manuscript.

The findings were presented as an oral presentation at a) 4th Jožef Stefan International Postgraduate School Students' Conference, June 1-3 2022, in Kamnik, Slovenia, b) 2nd ISO-FOOD Symposium from Food Source to Health, April 24-26, 2023, in Portorož, Slovenia, c) 18th International Conference on Chemistry and the Environment, June 11-15 June, 2023, in Venice, Italy, d) EuroAnalysis 2023: analytical probing of complex systems, August 27-31, 2023, in Geneva, Switzerland, e) BFestival 14, February 2024, in Ljubljana, Slovenia and as a poster presentation at ICWRR2024 International Conference on Wider Uptake of Water Resource Recovery from Wastewater Treatment and IWA-RMTC 2024 7th IWA – Regional Membrane Technology Conference, June 18-21, 2024, in Palermo, Italy.

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Influence of contaminants of emerging concern on the primary and secondary metabolites of hydroponically grown tomatoes

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ABSTRACT

The practical application of treated wastewater for irrigation depends not only on its safety but also on its potential impact on crop quality. This study investigated the effects of 14 contaminants of emerging concern (CECs) on the metabolic profile of tomatoes (*Solanum lycopersicum* L.) grown hydroponically. Results showed that compared to the controls, exposure to CECs significantly increased yield (48–154 %) but did not affect the content of sugars, organic acids, carotenoids and polyphenols. However, exposed tomatoes had significantly lower concentrations of specific amino acids, including valine, leucine, aspartic acid and methionine (7–14 %), while fatty acids remained unchanged. Additionally, CECs significantly altered the volatile organic compound profile of tomato fruit. Overall, these findings suggest that exposure to CECs can affect tomato quality. This study is particularly relevant in the context of using CEC-contaminated treated wastewater in hydroponic systems.

1. Introduction

Agriculture accounts for the largest share of water use globally, representing approximately 87 % of total freshwater consumption. Moreover, around 60 % of global freshwater withdrawals are allocated specifically for irrigation purposes (Wu et al., 2022). Consequently, the use of treated wastewater (TWW) is increasingly recognized as a sustainable strategy to supplement water supplies and protect natural resources. For example, TWW reuse has been identified as a high-priority solution in the European Water Innovation Partnership's Strategic Implementation Plan in order to ensure a safe and predictable source of water whilst lowering the pressure on water bodies and enhancing the EU's ability to adapt to climate change (Alcade-Sanz & Gawlik, 2017). In the EU, only 2.4 % of all treated municipal wastewater is treated and reused so far. While a few Member States reclaim nearly all of their treated wastewater (up to 89 %), the majority either reuse only a small fraction (as little as 5 %) or do not engage in water reuse at all (Directorate-General for Environment, 2023). This pattern reflects the global situation, where of the approximately 400 km^3 of wastewater generated annually, less than 20 % is treated, and only 2–15 % is reused for irrigation, depending on the region (Christou et al., 2024).

An additional benefit of using treated wastewater for irrigation is its potential to enhance plant growth and yield due to its high macro- and micronutrient content (Ofori et al., 2021). However, there are concerns. For instance, reusing TWW can contribute to soil salinization, potentially resulting in reduced water uptake by plants (Singh, 2021). Treated wastewater can also be contaminated with toxic elements, pathogen microorganisms, and contaminants of emerging concern (CECs), i.e., residues of industrial chemicals, pharmaceuticals, personal care products, biocides, pesticides, veterinary medicinal products, food and feed additives, drugs, and stimulants that are not removed during conventional wastewater treatment (Deviller et al., 2020; Ofrydopoulou et al., 2022; Renau-Pruñonosa et al., 2020). The presence of such compounds has led to concerns about CECs entering the human food chain through TWW irrigation.

In Europe, comprehensive guidelines and regulations have been introduced to mitigate the health and environmental risks associated with TWW reuse. Regulation (EU) 2020/741 sets minimum

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requirements for the safe reuse of TWW in agricultural irrigation, focusing on *E. coli* levels, biochemical oxygen demand, total suspended solids and turbidity (EU, 2020). While the regulation acknowledges the potential risks posed by CECs to public health and the environment, it does not specify explicit quality standards for them. Instead, it requires water reuse risk management plans to address the presence of CECs as part of a broader risk assessment approach, ensuring that reclaimed water is safe for its intended use (EU, 2020).

Several studies have explored the uptake of CECs in crops irrigated with TWW, with most research conducted in soil-based systems outdoors or in greenhouses (Christou, Papadavid, et al., 2019). These studies consistently demonstrate that crops grown in CEC-contaminated substrates and irrigated with TWW containing CECs can accumulate CECs in their tissue. The data indicate that the capacity of crops to absorb and retain CECs generally follows a decreasing trend: leafy vegetables > root vegetables > cereals and fodder crops > fruit vegetables, while the uptake and accumulation of CECs in fruit trees remain underexplored. Furthermore, published studies reveal that many factors influence the ability of crops to uptake and accumulate CECs, including plant physiological traits, species and genotype, the physicochemical properties of CECs, as well as climatic conditions and soil characteristics (Christou, Papadavid, et al., 2019).

Soil can act as a sink for persistent CECs, which may be released into the soil solution during irrigation or rainfall, subsequently taken up by irrigated or rainfed plants, or leached into groundwater (Ben Mordechay et al., 2023; Christou et al., 2017; Martínez-Piernas et al., 2019). In contrast, hydroponic systems—soilless plant cultivation techniques using water-based nutrient solutions—eliminate soil contamination and minimize environmental pollution through water recirculation. They also offer key benefits such as improved water and nutrient efficiency, precise nutrient delivery, reduced soil-borne issues, and space-saving potential (Pomoni et al., 2023; Rajendran et al., 2024). However, despite these advantages, hydroponic systems do not prevent the uptake and accumulation of CECs from treated wastewater in plants (McGinnis et al., 2019).

Although significant research has explored the uptake and accumulation of CECs, only two studies have examined their impact on crop quality and investigated metabolomic changes. Hurtado et al. (2017) analysed the effect of 11 CECs on metabolomic changes in lettuce, identifying alterations in amino acids, carboxylic acids, and sugars. Similarly, Christou, Kyriacou, et al. (2019) evaluated the effects of three pharmaceutically active compounds, individually and as a mixture, in irrigation water on various quality attributes. These attributes included colouration, acidity, soluble solids content, lycopene, ascorbic acid, polyphenols, antioxidant activity, and sugar content. The study revealed significant changes in soluble solids and carbohydrate content caused by individual pharmaceuticals, but no additive or synergistic effects were observed when they were combined. Despite these findings, significant gaps remain in understanding how CECs influence the metabolic profile of other crops, other key metabolites such as amino acids, fatty acids, and volatile organic compounds, as well as their effects on crop aroma and flavour.

This study evaluated the impact of 14 CECs (representing industrial chemicals, common stimulants, anti-inflammatory drugs and estrogens) on the metabolic profile of hydroponically grown tomatoes (*Solanum lycopersicum* L.). By comparing a standard nutrient solution with one spiked with CECs, the aim was to isolate the effects of these micropollutants and assess their impact on the metabolic profile of tomato fruits. This research is situated within the broader context of increasing global water scarcity and the need to reduce agricultural water consumption, which accounts for the majority of global freshwater use. The reuse of TWW in agriculture offers a potential solution to these challenges; however, the presence of CECs in TWW raises concerns, not only about crop safety but also food quality. By investigating the quality effects of CECs on tomato fruits, this study contributes to a better understanding of the risks and opportunities associated with using TWW in hydroponic crop production systems.

2. Materials and methods

2.1. Plant material and growth conditions (experimental design)

The experiment was conducted in a greenhouse at the Biotechnical Faculty in Ljubljana, Slovenia (46° 2′ 58.23″ N, 14° 28′ 30.75″ E, 298 m above sea level) from June to October 2020. A commercial tomato hybrid variety, 'Rally' (Enza Zaden, Netherlands), from the beef-type group *Solanum lycopersicum* L., was used. The experiment consisted of five tubs: (a) two control tubs with plants ($n = 12$) grown in potable water (HPW) with an added Arnon-Hoagland nutrient solution and (b) three tubs with plants ($n = 18$) exposed to potable water with a nutrient solution spiked with a CEC mix at 0.1 mg L^{-1} (HSPW). Model CECs included industrial chemicals (BPA, BPS, 22BPF, 24BPF, 44BPF), common stimulants (caffeine), anti-inflammatory drugs (ketoprofen, ibuprofen, naproxen, diclofenac) and estrogens (E1, β-E2, EE2, E3), with log K_{ow} ranging from −0.07 to 4.91. The model CECs were selected based on their frequent detection in Slovenian wastewater and receiving waters (Česen et al., 2019; Česen & Heath, 2017; Česen, Heath, et al., 2018; Česen, Lenarčič, et al., 2018; Škufca, Kovačič, Griessler Bulc, & Heath, 2021; Škufca, Kovačič, Prosenc, et al., 2021; Vehar et al., 2022).

Tomato seedlings were initially cultivated in plug trays containing a peat-based substrate. Once the plants had developed 5–6 true leaves, the substrate was rinsed of the root system, and the seedlings were transferred to the hydroponic system. At the time of transfer, tomato plants were healthy, uniform, and free from visible signs of disease or pest infestation. Full details about the nutrient solution and greenhouse conditions can be found in the Supplementary Material, Chapter S1. A spiking solution was added twice during the growing season (early June and early August), while the nutrient solution was replenished every 7–10 days.

2.2. Sample collection and preparation

Fruits were harvested every 7 to 10 days when fully ripe, resulting in eight pickings (Fig. S1). In order to assess the influence of CEC exposure duration, the harvesting period was divided into four intervals: 24 Aug–7 Sep (1st period), 14 Sep (2nd period), 18–22 Sep (3rd period), and 28 Sep–9 Oct (4th period). The four intervals were defined based on the number of ripe fruits harvested, which varied due to temperature-dependent ripening. Elevated summer temperatures delayed ripening, whereas the more moderate conditions beginning in September accelerated fruit maturation, leading to an uneven distribution of harvests. Each fruit was weighed at harvest, and the total yield per plant (g $plant^{-1}$) was recorded. After weighing, fruits were rinsed with water, blotted dry and stored in plastic bags at −20 °C.

Harvested tomatoes were frozen, diced with a ceramic knife, freeze-dried and ground. Composite samples for sugars, organic acids, ascorbic acid, carotenoids and polyphenol analysis were prepared from all fruits harvested per treatment across six plants per replication and harvest period. For amino acid, fatty acid and elemental analysis, composite samples were prepared as follows: (1) all harvested fruits per tub; (2) all harvested fruits per harvest period per treatment; (3) all harvested fruits per treatment. Volatile organic compound analyses were conducted on defrosted individual tomato pieces. For dry matter determination, 2 g of the frozen sample was freeze-dried for 24 h in a Gamma 2.20 lyophiliser (Christ, Germany). The water content (%) was calculated from the difference between the masses before and after freeze-drying.

2.3. Analytical methods

Detailed information on reagents, extraction procedures, and analytical protocols for the determination of sugars, organic acids, carotenoids, polyphenols, amino acids, fatty acids, and volatile organic

compounds (VOCs) is provided in the Supplementary material (Sections S2 and S3). Briefly, sugars and organic acids were determined according to the method of Mikulic-Petkovsek et al. (2012). Freeze-dried samples were extracted with Milli-Q water, and the supernatants were analysed using high-performance liquid chromatography (HPLC, Thermo Scientific Vanquish, USA). Sugars were quantified using HPLC with refractive index detection (HPLC-RI). Separation was achieved using a Rezex ECM monosaccharide column (300 × 7.8 mm, Ca^{2+}, Phenomenex, USA) and water as the mobile phase. Organic acids were quantified by HPLC with UV detection (HPLC-UV) using a Rezex ROA-organic acid H^+ column (8 %, 300 × 7.8 mm, Phenomenex, USA) and 4 mM H_2SO_4 as the mobile phase. Compounds were identified based on retention times and quantified using external standards.

Carotenoids were quantified following a modified method of Šircelj et al. (2005). Compounds were extracted from freeze-dried samples using acetone. Analysis was performed by HPLC-DAD (Dionex UltiMate 3000, Thermo Scientific, USA) using a Gemini C18 column (150 × 4.6 mm, 3 µm, Phenomenex, USA), with two mobile phases: A (acetonitrile/methanol/water) and B (acetone/ethyl acetate), under gradient elution. Compounds were identified by their retention times and quantified using external standards.

Individual soluble polyphenols were extracted based on the method of Mikulic-Petkovsek et al. (2013). Freeze-dried samples were extracted using 80 % methanol containing 3 % (*v*/v) formic acid. Polyphenols were analysed by HPLC-DAD-MS/MS (Dionex UltiMate 3000, Thermo Scientific, USA and LTQ XL Thermo Scientific, USA) using a Gemini C18 column (150 × 4.6 mm, 3 µm, Phenomenex, USA), with solvent A (3 % acetonitrile, 0.1 % formic acid in water) and solvent B (3 % water, 0.1 % formic acid in acetonitrile) as the mobile phases. Mass spectra and retention time matching were used for compound identification, and quantification was performed using standard curves.

For amino acid determination, samples were hydrolysed for 24 h at 110 °C in 4 % thioglycolic acid in 6 M HCl with added phenol. Solid-phase extraction and derivatisation were performed using the EZ:faast™ amino acid analysis kit (Phenomenex, USA), followed by GC-MS (7890 B GC & 5977 A Series MSD, Agilent Technologies, USA) analysis using a ZB-AAA GC column (10 m × 0.25 mm, Phenomenex, USA). Quantification was performed against known amino acid standards.

Fatty acids were analysed following total lipid extraction using dichloromethane and 0.5 M sodium hydroxide in methanol. Fatty acid methyl esters (FAMEs) were prepared by transesterification with boron trifluoride (BF_3) in methanol, then extracted with hexane and Milli-Q water. FAMEs were separated and analysed by gas chromatography with flame ionization detection (GC-FID, Agilent 6890 N, USA) using a CP-Sil 88 capillary column (100 m × 0.25 mm × 0.20 µm, Agilent J&W, USA). Identification and quantification were performed by comparing retention times to those of a Supelco 37 Component FAME Mix standard. Results were expressed as weight per cent of total fatty acids.

Volatile organic compounds were extracted using headspace solid-phase microextraction (HS SPME) from defrosted, homogenised samples, employing a DVB/CAR/PDMS fibre (50/30 µm thickness, Sigma-Aldrich, USA). VOCs were analysed by GC-MS (7890 B GC & 5977 A Series MSD; Agilent Technologies, USA) using a VFWAXms capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J & W, USA). Identification was based on comparing authentic standards, calculated retention indices (RIs), and mass spectral data from the NIST 14 library. Semi-quantitative data (relative percentages of total volatiles) were calculated from peak areas of the total ion current (TIC) profiles.

2.4. Statistical analysis

The effects of two factors were examined: (a) exposure to a nutrient solution without CECs (HPW) vs. exposure to a nutrient solution with added CECs (HSPW), and (b) sampling period. Three tubs were used for HSPW and two for HPW, with six plants per tub as experimental units. Data were processed using R-based software (versions 4.2.3 and 3.2.6), SigmaPlot 14 (Systat Software GmbH), XLSTAT (version 2016.1.1, Lumivero), and SIMCA 17 (Sartorius). Normality was checked with the Shapiro-Wilk test, and homoscedasticity was checked with Levene's test. Significant yield differences for each sampling period and significant metabolite contents were assessed through pairwise comparisons using *t*-tests or Mann-Whitney *U* tests, depending on the conditions for the safe use of the parametric tests (normality, homoscedasticity of variance). If one condition has not been satisfied, we use the Mann-Whitney *U* test; otherwise, a *t*-test was used. In addition, repeated measures ANOVA was used for the time-dependent measurements. The statistical significance level was 0.05 ($p \leq 0.05$ meant the null hypothesis was rejected). OPLS-DA was used for volatile organic compounds, and Mann-Whitney *U* tests with step-wise methods were used to reduce variables.

3. Results and discussion

3.1. Effects of plant exposure to CECs on fruit yield

Fruit yield data from individual harvest periods are expressed as the mean mass (g) of marketable fruit per plant (Fig. 1). Statistical analyses showed a significant enhancement in growth depending on the sampling period. In the first and fourth sampling periods, HSPW tomatoes had a statistically significantly higher yield compared to the control (HPW). In plants, CECs are taken up by roots and redistributed via the vascular system through transpiration-induced mass flow or a symplastic pathway using transporters and channels (Miller et al., 2016). These compounds maintain bioactivity within the plant, leading to varying physiological responses depending on the plant species, environmental conditions, CEC properties, and exposure concentrations (Hurtado et al., 2017). Therefore, CECs may have toxic effects at high concentrations, negatively impacting growth and yield. However, at low concentrations, CECs can induce hormesis, a phenomenon where mild stress enhances plant growth, including increased biomass and elongation of roots, stems, and leaves by stimulating metabolic pathways and improving nutrient uptake (Migliore, Godeas, et al., 2010; Migliore, Rotini, et al., 2010). In this study, the increase in yield can be attributed to this hormetic effect, as the CECs were present within a specific concentration range that promoted plant growth.

3.2. Effects of plant exposure to CECs on sugars, organic acids and carotenoids

No significant effects of CECs on glucose and fructose content were observed (Fig. 2). The levels of both reduced sugars remained stable throughout the growth period, ranging from 14.0 ± 1.2 mg kg^{-1} FW to 16.3 ± 0.3 mg kg^{-1} FW for glucose and from 16.8 ± 1.7 mg kg^{-1} FW to 18.7 ± 2.3 mg kg^{-1} FW for fructose. In contrast, Christou, Kyriacou, et al. (2019) observed significant changes in carbohydrate content (glucose and fructose) in tomato fruits when exposed individually to diclofenac, sulfamethoxazole, and trimethoprim at environmentally relevant concentrations (10 µg L^{-1} each). The exposure of the plants to a mixture of the three compounds showed no significant effects on carbohydrates, except for glucose content, which indicated no clear additive or synergistic effects. When Christou et al. (2016) exposed alfalfa (*Medicago sativa* L.) plants to four common, individually applied pharmaceutically active compounds (10 µgL^{-1}) (diclofenac, sulfamethoxazole, trimethoprim, 17a-ethinylestradiol) and their mixture, they noted that CEC-mediated effects are species-dependent and influenced by the physico-chemical properties of the CECs, with potential variation across different plant tissues.

Among the organic acids analysed (Fig. 2) in the tomato fruits, citric acid was the most abundant, followed by malic and ascorbic acid, while fumaric acid was detected only in trace amounts (0.001–0.002 g kg^{-1} FW), bellow limit of quantification (LOQ; 0.0111 g kg^{-1} FW). The higher levels of citric acid compared to malic acid indicate that the tomatoes were fully ripened at the time of sampling, as citrate synthesis increases

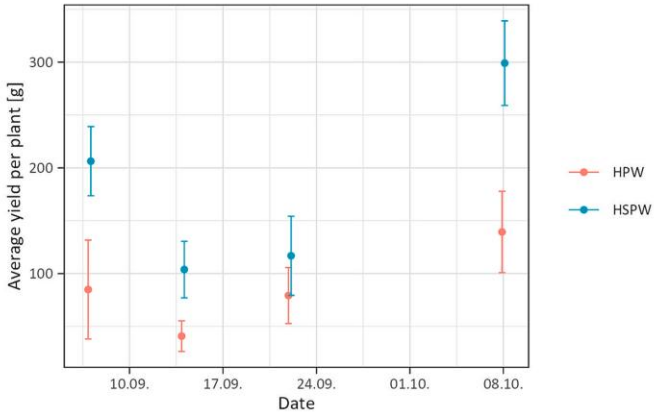


Fig. 1. Marketable yields in each harvest period for both treatments.

during ripening and peaks at full maturity. Malic acid levels are known to increase until the orange fruit stage (approximately one week before full ripeness) but then sharply decrease as it is consumed during respiration during the final stages of ripening (Batista-Silva et al., 2018). Similarly, the observed low fumaric acid levels further support full ripeness of the tomatoes, as fumaric acid concentrations are highest during early fruit development and significantly decline as ripening progresses (Carrari & Fernie, 2006).

Overall, no significant effect of CEC exposure on organic acid content was observed. However, the harvest period significantly influenced malic acid levels, as tomatoes from the third harvest period had statistically significantly higher malic acid content compared to those from the second harvest period. This difference likely reflects a lower ripening stage of the fruits from the third harvest period, which coincided with a drop in average daily air temperature from 25 °C to 19 °C and reduced sunlight exposure from 8–9 h to 5–6 h per day. These environmental changes are known to influence the ripening process strongly and likely slowed fruit maturation (Batista-Silva et al., 2018; Carrari & Fernie, 2006; Gautier et al., 2008).

Also, ascorbic acid levels decreased throughout the experiment, with the highest levels observed in fruits from the first harvest period. These values were significantly higher than those from the second harvest period. The ascorbic acid content in tomatoes is known to fluctuate throughout the year, primarily influenced by environmental factors, agricultural practices, and fruit ripening (Dumas et al., 2003; Gautier et al., 2008). Temperature and light exposure are the most critical environmental factors regulating ascorbic acid synthesis (Massot et al., 2010). Increased light exposure enhances biosynthesis in leaves and fruits through upregulation of gene expression and enzyme activity in the ascorbic acid biosynthetic pathway. In this study, higher average daily temperatures and longer sunshine duration before the first harvest (Fig. 2) likely contributed to the higher ascorbic acid content in the fruits than those from subsequent harvests.

Four lipophilic pigments were analysed in tomato fruits: lycopene (1.29 ± 0.22–4.14 ± 0.54 mg kg^{-1} FW) and β-carotene (1.80 ± 0.21–2.74 ± 0.14 mg kg^{-1} FW) were present in higher concentrations, while α-carotene and lutein were detected only in trace amounts (0.100–0.300 mg kg^{-1} FW and 0.010–0.130 mg kg^{-1} FW, respectively), bellow LOQ (0.480 mg kg^{-1} FW). Lycopene content was unaffected by both CEC exposure and harvest period, while β-carotene content was significantly influenced by the harvest period ($p = 0.0234$), with lower levels observed in fruits harvested later in the experimental period compared to earlier harvests. Gene expression studies have shown that pharmaceutically active compounds can affect transcript abundance related to sucrose and carotenoid biosynthesis and catabolism (Christou, Kyriacou, et al., 2019). Despite the significant effects of pharmaceutically active compounds on the gene expression of enzymes involved in lycopene biosynthesis, they did not significantly alter lycopene levels in fruits. This observation may be attributed to differences in the gene expression and enzymatic activity of phytoene synthase and phytoene desaturase (Schmidt & Redshaw, 2015) or the critical role of post-transcriptional regulation in modulating carotenoid synthesis (Enfissi et al., 2017).

Lycopene is a key antioxidant in fresh tomato fruits responsible for their red colour (Dumas et al., 2003). During ripening, carotenoid concentrations increase substantially by a factor of 10–14, primarily due to lycopene accumulation (Fraser et al., 1994). Lycopene content in fresh tomatoes typically ranges from 40 to 140 mg kg^{-1} FW under typical growing conditions (Dasgan et al., 2021; Dumas et al., 2003; Gautier et al., 2008). However, in unfavourable conditions, exceptionally high temperatures can reduce lycopene content to as low as 1 mg kg^{-1} FW (Dumas et al., 2003). In the present study, lycopene levels were lower, while β-carotene levels were higher than those reported in other studies (Bramley, 2002;Dasgan et al., 2021; Dumas et al., 2003). This discrepancy can likely be attributed to the high temperatures recorded during the growing period (average daily temperatures of 23–28 °C, with maximums of 35–38 °C). This is because lycopene synthesis is inhibited at temperatures above 32 °C, and levels decrease drastically, whereas β-carotene content is less affected (Dasgan et al., 2021; Dumas et al., 2003). Furthermore, temperatures above 35 °C are known to stimulate the enzymatic conversion of lycopene to β-carotene, explaining the observed β-carotene in our study (Baqar & Lee, 1978; Dasgan et al., 2021; Dumas et al., 2003).

3.3. *Effects of plant exposure to CECs on polyphenols*

Polyphenols, products of secondary metabolism, play a vital role in defining the organoleptic, nutritional, and pharmacological properties of tomato fruits (Gómez-Romero et al., 2010; Zhu et al., 2022). Statistical analysis revealed no significant effect of plant exposure to CECs or harvest time on polyphenols levels (Fig. 3). These findings align with those of Christou, Kyriacou, et al. (2019), who similarly observed no significant impact of exposure to three pharmaceutically active compounds on the lycopene, phenolic, or ascorbic acid content of tomato

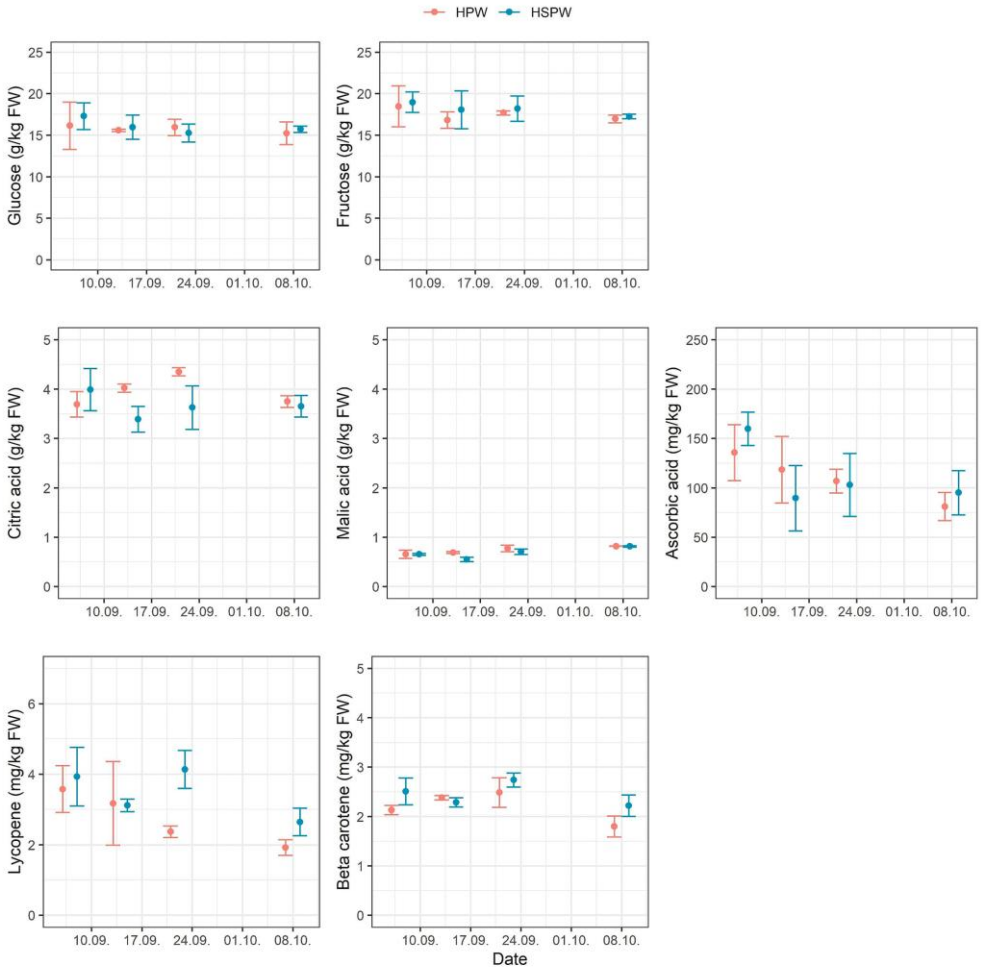


Fig. 2. Charts showing the glucose, fructose, citric acid, malic acid, ascorbic acid and lycopene and beta carotene content in tomato fruits in each harvest period for each treatment.

fruits.

Polyphenols from three groups were analysed in tomato fruits in this study: hydroxycinnamic acids (51.0 ± 5.8–64.4 ± 6.0 mg kg^{-1} FW), flavanones (19.0 ± 5.0–26.9 ± 7.2 mg kg^{-1} FW), and flavonols (5.41 ± 0.10–7.70 ± 1.01 mg kg^{-1} FW). Among the hydroxycinnamic acids, caffeic acid derivatives and caffeic acid hexosides were the predominant compounds, suggesting that the tomatoes were at a deep red stage of ripeness at harvest. The low chlorogenic acid content is consistent with its concentration decreasing to 60 % of that in green fruit during ripening, as it is converted into caffeic acid derivatives or caffeic acid glucosides (Gautier et al., 2008).

Naringenin hexosides and quercetin 3-O-rutinoside, along with kaempferol hexosides and rutinosides, were identified as the main constituents of the flavanones and flavonols groups, respectively. The concentration of polyphenols in our study was lower than that found in the cherry tomato cultivar grown in a greenhouse in southern France, as reported by Gautier et al. (2008). The results of the present study show a similar concentration range of flavonoids and slightly lower levels of hydroxycinnamic acid compared to tomatoes (*Solanum esculentum*) from Almeria, as analysed by Gómez-Romero et al. (2010). Additionally, in a study where tomatoes were irrigated with 1 mM Zn, 1 mg L^{-1} caffeine, and a Zn + caffeine combination, much higher concentrations of 4-coumaric acid, caffeic acid, and ferulic acid were reported compared to the present study's findings (Vichi et al., 2024).

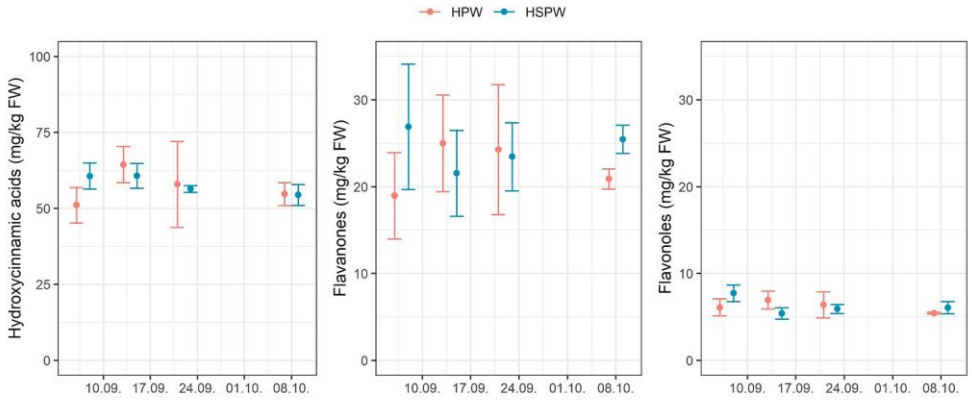


Fig. 3. Chart showing the content of hydroxycinnamic acids, flavanones and flavonols in tomato fruits in each harvest period for each treatment.

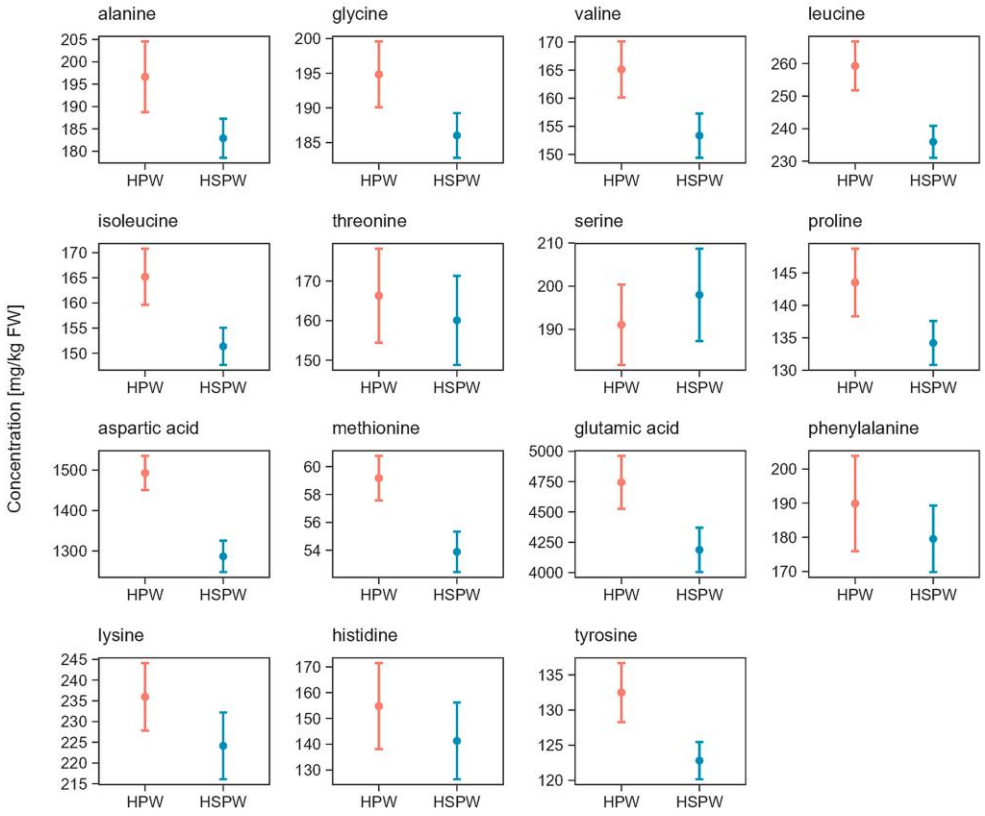


Fig. 4. Effect of added CECs on amino acid concentrations in tomato fruits.

3.4. Effects of plant exposure to CECs on amino acids profile

The highest concentrations of amino acids detected (Fig. 4) were glutamic acid (up to 5853 mg kg^{-1} FW) and aspartic acid (up to 1680 mg kg^{-1} FW). Due to the method used in this study, asparagine was converted to aspartic acid and glutamine to glutamic acid, meaning the reported values represent the sum of both. Other amino acids were detected at concentrations below 293 mg kg^{-1} FW, while hydroxyproline and hydroxylysine were below LOQ in all samples. The total amino acid content was higher in HPW tomatoes (8491 mg kg^{-1} FW) than in HSPW tomatoes (7597 mg kg^{-1} FW). Compared to the results of Ali et al. (2020), the concentrations in this study were similar, except for aspartic acid (approximately 15 % higher) and glutamic acid (approximately 40 % higher), which may be due to the conversion process. Statistical analysis (Table S3) revealed significantly higher concentrations of valine (197 ± 8 mg kg^{-1} FW), leucine (259 ± 7 mg kg^{-1} FW), aspartic acid (1493 ± 42 mg kg^{-1} FW), and methionine (59.1 ± 1.6 mg kg^{-1} FW) in HPW tomatoes compared to HSPW tomatoes (valine: 183 ± 4 mg kg^{-1} FW, leucine: 236 ± 8 mg kg^{-1} FW, aspartic acid: 1286 ± 39 mg kg^{-1} FW, methionine: 53.9 ± 1.5 mg kg^{-1} FW).

Amino acids play various roles in tomatoes and change fruit development and ripening. For instance, aspartic acid increases while valine decreases as fruit development progresses, and asparagine exhibits typical parabolic curves, peaking around the breaker stage. These amino acids are also linked to the organoleptic qualities of the fruit; for example, changes in glutamic acid directly influence taste perception, while other amino acids contribute to flavour through their involvement in aroma synthesis (Oms-Oliu et al., 2011).

In addition, amino acids can serve as indicators of plant stress, including drought, salinity, cold and heat stress, oxidative stress, toxic elements stress, and biotic stress (D'Mello, 2019; Heinemann & Hildebrandt, 2021; Ingrisano et al., 2023; Rodrigues-Corrêa & Fett-Neto, 2019; Sita & Kumar, 2020). The study by Heinemann and Hildebrandt (2021) suggests that plants catabolize proteins and use amino acids as alternative substrates in respiratory pathways activated when coping with energy shortages, such as drought or low light conditions. This ability could explain why the total amino acid content in HPW tomatoes was higher than in HSPW tomatoes, as previously mentioned. Amino acid degradation generates tricarboxylic acid cycle intermediates that serve as substrates for mitochondrial respiration.

The oxidation of amino acids with complex structures, such as branched-chain and aromatic amino acids, provides energy for ATP synthesis comparable to those derived from glucose (Hildebrandt et al., 2015). While further evidence on the degradation pathways is still needed, the breakdown of leucine, isoleucine, and valine produces acetyl coenzyme A and CO_2 (Heinemann & Hildebrandt, 2021). Methionine, a key player in stress resistance, is particularly susceptible to oxidation by reactive oxygen species, with its sulphur being oxidized to sulfoxide. Through the repair of methionine sulfoxide by methionine sulfoxide reductases, methionine can act as a scavenger, protecting cells from oxidative stress (Campbell et al., 2016). The significantly lower concentrations of methionine in HSPW tomatoes may indicate increased oxidative stress or a reduced capacity for oxidative stress resistance in these plants.

Asparagine, aspartate, glutamate, and glutamine are common nitrogen carriers, playing a primary role in the recycling, storage, and transport of nitrogen in germinating seeds, vegetative organs, and senescing tissues (Gaufichon et al., 2016). Studies have shown that their concentrations vary depending on the type of stress, tissue, and plant (Han et al., 2021). While the significantly lower concentrations of valine, leucine, aspartic acid, and methionine observed in this study may suggest a negative influence of added CECs on HSPW tomatoes, further research is needed to confirm this.

3.5. Effects of plant exposure to CECs on fatty acids profile

The highest relative concentrations were found for linoleic acid (LA), followed by palmitic acid, α-linolenic acid (ALA), oleic acid, stearic acid, eicosapentaenoic acid (EPA), and lignoceric acid, with all other fatty acids present at concentrations below 1 % (Fig. S2). Tomatoes contained two n-6 fatty acids, LA and dihomo-γ-linolenic acid, and four n-3 fatty acids, including ALA, eicosatrienoic acid, docosadienoic acid, and EPA (Table 1). They also contained the essential fatty acids LA and ALA, as well as EPA, but lacked docosahexaenoic acid DHA. While EPA and DHA can be synthesized from ALA, the conversion efficiency is low, making it advisable to consume foods rich in EPA and DHA (Burdge et al., 2002; Burdge & Wootton, 2002; Das, 2013). An optimal n-6/n-3 ratio in the diet is important for managing inflammation and other biological processes, with a recommended range of 1:1 to 5:1 (Gonzalez-Becerra et al., 2023). The tomatoes studied, with an n-6/n-3 ratio of approximately 2.65, could significantly contribute to a healthy diet. Compared to the results of Ali et al. (2020), the content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were comparable.

Literature suggests that stress caused by drought, salinity, heat, toxic elements, nutrient deficiencies, and oxidative stress typically reduces the ratio of PUFA to SFA (Maurya, 2020). In the case of biotic stress, studies have shown decreased levels of both SFA and PUFA (Ortega et al., 2019). In this study, statistical analyses revealed that HPW and HSPW tomatoes did not significantly differ in the content of individual fatty acids, SFA, MUFA, PUFA, or n-6 and n-3 fatty acids. Therefore, adding CECs to HSPW tomatoes did not influence fatty acid content.

3.6. Effects of plant exposure to CECs on volatile organic compounds profile

In this study, 67 samples (21 HPW, 46 HSPW) were analysed, and 115 compounds were identified at three different levels by comparing mass spectra (MS, eight compounds), retention indices (RI, 78 compounds), or by using standards (27 compounds, STD, Supplementary material 2). The VOC profile included acyclic, cyclic, and heterocyclic hydrocarbons, alcohols, phenols, ethers, aldehydes, ketones, carboxylic acids, esters, and lactones, as well as compounds containing nitrogen and sulphur. The detected VOCs were predominantly derived from biosynthetic pathways, with lipids, terpenoids, and amino acids serving as precursors. The VOCs with the highest relative concentrations were 6-methyl-5-hepten-2-one (4.59–17.8 %), eugenol (2.10–20.2 %), (*E*)-6,10-dimethyl-5,9-undecadien-2-one (0.00–10.2 %), 2-methoxyphenol (2.06–12.4 %), (E)-citral (1.33–6.36 %), 2-isobutylthiazole (0.708–6.55 %), 6-methyl-3,5-heptadiene-2-one (1.64–4.83 %), (Z)-3,7-dimethyl-2,6-octadienal (0.689–3.68 %), phenylethyl alcohol (1.19–5.46 %), (E)-2-octenal (0.445–3.88 %), and methyl salicylate (0.151–22.9 %).

Most of the identified VOCs have been confirmed in the literature as present in tomatoes, except for 22 compounds which may have resulted

Table 1
Contents of linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), saturated (SFA), monounsaturated (MUFA), polyunsaturated fatty acids (PUFA), n-6 and n-3 fatty acids and their ratio.

	HPW	HSPW
LA (%)	39.9 ± 2.0	39.8 ± 2.1
ALA (%)	11.3 ± 1.5	13.8 ± 1.6
EPA (%)	2.10 ± 1.00	1.80 ± 0.80
SFA (%)	39.6 ± 4.1	38.4 ± 4.7
MUFA (%)	6.40 ± 1.70	6.10 ± 2.00
PUFA (%)	55.8 ± 4.7	55.5 ± 4.8
n-6 (%)	40.6 ± 2.5	39.9 ± 2.3
n-3 (%)	15.2 ± 2.2	15.6 ± 2.5
n-6/n-3	2.70 ± 1.10	2.60 ± 1.00

from the addition of CECs, contamination from the greenhouse or sample preparation laboratories, or naturally occurring in tomatoes at low concentrations and not yet reported in the literature. Six of these compounds showed significant differences between HPW and HSPW tomatoes. Isoamyl isovalerate was found only in HPW, while methyl octanoate and diethyl phthalate were present at higher concentrations in HSPW. Additionally, benzonitrile, heptanoic acid, and dimethyl nonanedioate were detected only in HSPW tomatoes, likely due to the addition of CECs. In total, 28 VOCs differed significantly between HPW and HSPW tomatoes (Supplementary Material 2), with 14 exhibiting higher concentrations in HPW and 15 in HSPW, indicating that CECs influence VOC production. Most of the significantly different compounds primarily originate from fatty acid or lipid metabolism (16), followed by amino acid metabolism (5), predominantly from phenylalanine and carotenoid metabolism (2). The specific metabolic pathway could not be determined for four significantly different compounds. Six of these compounds have been identified as stress indicators. Diethyl phthalate is most likely a contaminant (CID 6781., 2025).

An OPLS-DA model (Fig. 5) was created using 29 VOCs (variables) and 59 samples to differentiate between HPW and HSPW tomatoes. The model showed an R2 of 0.535, a Q2 of 0.376, and an accuracy of 85.07 %. Eight samples were excluded from the OPLS-DA analysis. Of these, three (1 HPW and 2 HSPW) contained significantly higher concentrations of methyl salicylate, two (1 HPW and 1 HSPW) had significantly higher concentrations of eugenol, one HSPW sample had a significantly higher concentration of eugenol and lacked (E)-6,10-dimethyl-5,9-undecadien-2-one, and one HSPW sample was missing (E)-6,10-dimethyl-5,9-undecadien-2-one but contained a significantly higher concentration of (2-nitroethyl)-benzene. Methyl salicylate plays a major role in the indirect defence response of tomatoes (Ament et al., 2010) and is considered undesirable in ripe fruits when accumulated to high levels (Sapkota et al., 2023). Eugenol, detected in salt-stressed plant emissions (Midzi et al., 2022), also signals cold and drought tolerance regulation (Zhao et al., 2022). The findings suggest that the excluded tomatoes experienced more stress than the others.

Among the 29 selected VOCs, 12 showed significant differences between HPW and HSPW tomatoes, with 7 of these related to plant stress based on the literature (Supplementary material 2). While no studies have focused on VOCs in crops affected by CECs, VOCs have been found to indicate various biotic and abiotic stresses, such as drought, salinity, heat, cold, CO_2 increase, ozone, excessive light, and mechanical damage (Midzi et al., 2022). This study demonstrates that CECs exert stress on tomatoes, significantly alter the VOC profile, and cause differences in compounds that are not directly associated with stress in the literature.

4. Conclusions

This study confirms that exposure to CECs influences the metabolic profile of hydroponically grown tomatoes, although environmental factors and stress have a more pronounced impact. Additionally, CEC exposure led to a statistically significant increase in tomato yield but did not significantly affect the levels of sugars, organic acids, carotenoids, or polyphenols. However, exposed tomatoes exhibited lower concentrations of specific amino acids, including valine, leucine, aspartic acid, and methionine, suggesting potential stress responses to contaminant exposure. While no significant differences were observed in the fatty acid profile between exposed and non-exposed tomatoes, exposure to CECs significantly altered the VOC profile. Several of altered VOCs are associated with plant stress, suggesting that CEC exposure may induce physiological responses. However, changes were also observed in VOCs that are not typically linked to stress, indicating a more complex interaction between CEC exposure and tomato metabolism.

Overall, this study is particularly relevant in the context of using treated wastewater in hydroponic systems. However, while it demonstrates measurable metabolic effects in hydroponically grown tomatoes exposed to a specific mixture of model CECs, further research is needed to explore potential synergistic and antagonistic effects of other CECs, their metabolites and transformation products. In addition, using non-target metabolomics and transcriptomic studies would help identify the metabolic pathways most affected by CEC exposure and provide a more mechanistic understanding of the observed metabolic shifts. Given that hydroponic cultivation may influence tomato exposure to CECs differently than soil-based systems, repeating the experiment in conventional soil cultivation would provide valuable comparative insights. Furthermore, since VOC alterations may affect sensory properties, conducting sensory studies would offer a deeper understanding of the implications for tomato quality and consumer perception.

Abbreviations

ALA	α-linolenic acid
CAR	carboxen
CEC	contaminant of emerging concern
DHA	docosahexaenoic acid

(*continued on next page*)

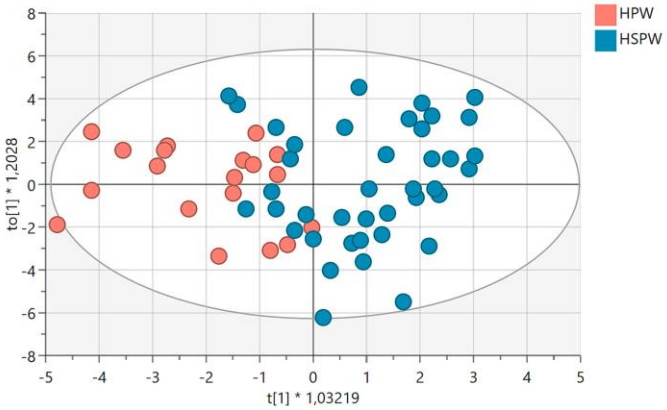


Fig. 5. OPLS-DA model based on selected VOCs.

(*continued*)

DVB	divinylbenzene
EC	electrical conductivity
EFSA	European Food Safety Authority
EI	electron impact
EPA	eicosapentaenoic acid
ESI	electrospray ionization
FAME	fatty acids methyl ester
FW	fresh weight
GC-FID	gas chromatography with flame ionization detector
GC-MS	gas chromatography coupled to mass spectrometry
HPLC	high-performance liquid chromatography
HPW	hydroponics potable water
HSPW	hydroponics spiked potable water
LA	linoleic acid
LOD	limit of detection
LOQ	limit of quantification
ME	matrix effect
MS	mass spectrum
MUFA	monounsaturated fatty acid
n-3	omega 3 fatty acid
n-6	omega 6 fatty acid
OPLS-DA	orthogonal projections to latent structures discriminant analysis
PDMS	polydimethylsiloxane
PUFA	polyunsaturated fatty acid
RI	retention index
RSD	relative standard deviation
RT	retention time
SE	standard error
SFA	saturated fatty acid
SIM	selected ion monitoring
SPME	solid-phase microextraction
TIC	total ion current
TWW	treated wastewater
UV	ultraviolet
WHO	World Health Organization

CRediT authorship contribution statement

A. Vehar: Writing – original draft, Visualization, Investigation, Formal analysis. **N. Kacjan Maršić:** Writing – original draft, Visualization, Investigation. **Š. Železnikar:** Writing – review & editing, Investigation. **D. Potočnik:** Methodology, Formal analysis. **L. Strojnik:** Methodology. **M. Mikulič Petkovšek:** Formal analysis. **H. Šircelj:** Formal analysis. **D. Kastelec:** Visualization, Formal analysis. **A. Kovačič:** Writing – review & editing. **M. Pintar:** Writing – review & editing. **D. Heath:** Writing – original draft. **E. Heath:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **N. Ogrinc:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this work, the authors used ChatGPT for translation and grammar purposes. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145226.

Data availability

Data will be made available on request.

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3.6 Scientific Paper: "Explainable Machine Learning for Assessing Tomato Quality Irrigated with Treated Wastewater"

This chapter presents the paper entitled "Explainable machine learning for assessing tomato quality irrigated with treated wastewater" by Anja Vehar, Jan Drole, Nina Kacjan Maršić, Barbara Koroušić Seljak, Ester Heath, and Nives Ogrinc. The paper was submitted to the journal Food Chemistry (A'') in 2026 and is currently under review. The study aimed to identify the key metabolites and elements that differentiate between various irrigation regimes, including TWW and CECs, using explainable machine learning.

Tomatoes were cultivated in two ways: hydroponically in nutrient solution or spiked nutrient solution, and in lysimeters irrigated with potable water, TWW, or spiked TWW. Nutrient solution and treated wastewater were spiked with 14 CECs at 0.1 mg/L, derived from industrial chemicals, pharmaceuticals, and common stimulants. Ripe lyophilised tomato fruits were analysed for sugars (HPLC-RI), organic acids (HPLC-UV), carotenoids (HPLC-DAD), polyphenols (HPLC-DAD-MS/MS), amino acids (GC-MS), fatty acids (GC-FID), and elements (ICP-MS). Classification machine learning models (random forests and decision trees) were used to distinguish between treatments. The SHAP method was used to identify key metabolites and elements driving treatment differentiation at the global and local levels.

Despite the limited sample size, the classification models performed well, achieving 75-88% accuracy in distinguishing treatments. At the global level, the elements Cs, Cd, P, Co, and margaric acid were the most important for prediction. At the local level, key features included elements (Cd, Co, Cs, Cu, P, Na), ascorbic acid, and specific fatty acids (palmitic, margaric, oleic, linoleic, behenic acid), which varied depending on the treatment. The method also proved valuable for identifying which specific features led to a sample's false classification, providing clear reasoning for the error.

To the author's knowledge, this is the first study to apply XAI techniques in this context, specifically to identify the biochemical parameters most crucial for distinguishing among the five treatments. Furthermore, the approach can highlight a smaller set of features sufficient for classifying samples into distinct groups, potentially reducing the number of analyses, material costs, and time, while ensuring robust model performance.

As joint first author, I was responsible for the amino acid and fatty acid analyses, data collection, graphical abstract design, and manuscript drafting and finalising.

The findings were presented as an oral presentation at the 11th International Symposium on Recent Advances in Food Analysis, November 5-8, 2024, in Prague, Czech Republic, and as a poster presentation at the 4th Food Chemistry Conference, October 14-16, 2025, in Glasgow, United Kingdom.

Explainable machine learning for assessing tomato quality irrigated with treated wastewater

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Abstract

This study investigated how different treatments affect element uptake and metabolism in tomatoes grown in soil (lysimeters) and soilless (hydroponics) systems. Soil-grown tomatoes were irrigated with potable water, treated wastewater, and treated wastewater spiked with 14 contaminants of emerging concern (CECs). These model CECs, spiked at 0.1 mg/L, included bisphenols, non-steroidal anti-inflammatory drugs, estrogens, and caffeine. Hydroponically grown tomatoes were grown in nutrient solution, with or without CECs. Tomato quality was assessed by analysing sugars, organic acids, polyphenols, carotenoids, amino acids, fatty acids, and elemental composition. Classification machine learning models were applied, achieving 75-88% accuracy in distinguishing treatments. The SHAP method identified key metabolites (ascorbic, palmitic, margaric, oleic, linoleic, behenic acid) and elements (Cd, Co, Cs, Cu, P, Na) driving treatment differentiation. This study demonstrates how explainable machine learning can decode complex metabolic interactions, providing insights into how treated wastewater and CECs influence tomato quality.

Keywords (1-7)

Tomato, contaminant of emerging concern, treated wastewater, primary and secondary metabolite, element, explainable machine learning, SHAP

1. Introduction

Agriculture accounts for 87% of global water consumption and 60% of freshwater withdrawals, primarily for irrigation (Wu et al., 2022). Treated wastewater (TWW) reuse is increasingly promoted as a sustainable solution to supplement water supplies and protect natural resources. Globally, less than 20% of wastewater is treated, and only 2–15% of this is reused for irrigation in agriculture, depending on the region (Christou et al., 2024). In Europe, the European Water Innovation Partnership's Strategic Implementation Plan recognises TWW reuse as a key strategy to reduce water body pressure and improve climate adaptation (Alcade-Sanz & Gawlik, 2017). Despite its potential, only 2.4% of treated municipal wastewater in the EU is currently reused, with significant variation among member states (Directorate-General for Environment, 2023).

Although TWW has been shown to improve crop growth and yield due to its nutrient content (Ofori et al., 2021), concerns persist regarding soil salinisation and the presence of toxic elements, pathogenic microorganisms and contaminants of emerging concern (CECs). These include industrial chemicals, pharmaceuticals, personal care products, biocides, pesticides, veterinary medicinal products, food and feed additives, drugs, and stimulants that are not entirely removed during conventional wastewater treatment (Deviller et al., 2020; Ofrydopoulou et al., 2022). Such compounds can be taken up by plants and enter the food chain. In Europe, EU Regulation 2020/741 establishes minimum safety standards for TWW reuse; however, it does not set specific limits for CECs; instead, it requires a risk-based management plan (Regulation (EU) 2020/741, 2020).

Evidence shows that crops irrigated with TWW containing CECs can accumulate these compounds in edible tissues. Leafy vegetables show the highest uptake, followed by root vegetables, cereals and fodder crops, and fruit vegetables (Christou, Papadavid, et al., 2019). Bueno et al. (2022) reported that the levels (μg/kg) of pharmaceuticals in tomato plants followed the order: root > tomato > soil > leaf, while pesticide levels exhibited a different pattern, with the order being root > soil > leaf > tomato. Overall, the extent of uptake and accumulation of CECs in plants depends on a combination of factors such as plant’s genotype and physiological state, soil fauna, soil pore water chemistry, physico-chemical properties of CECs and environmental perturbations (Christou, Papadavid, et al., 2019).

Different agriculture systems, such as soil-based and hydroponic cultivation, offer distinct advantages and disadvantages for crop quality when exposed to TWW. Soil-based cultivation faces long-term risks from the accumulation of persistent contaminants like heavy metals and CECs in soil, uneven nutrient and contaminant distribution, and often requires higher pesticide use, which can further affect food safety (Farvardin et al., 2024; Gomiero, 2018). On the other hand, it benefits from the natural filtration properties of soil, which can degrade or immobilise some contaminants, and supports greater resilience to environmental stress due to more extensive root systems (Karlova et al., 2021; Singer & Brown, 2018).
Hydroponic systems allow for precise control of nutrients and water quality and reduce the risk of soil-borne contaminants (Pomoni et al., 2023). Additionally, the controlled environment minimises pesticide use and reduces vulnerability to pests and diseases. However, hydroponic systems are susceptible to waterborne contaminants, including CECs, which can be directly absorbed by plants, altering their biochemical profiles and potentially reducing crop quality

(Beltrán et al., 2021). Both systems require careful management to mitigate the risks of CEC exposure while maintaining food quality and safety.

Although significant research has explored the uptake and accumulation of CECs in crops, only a few studies have examined their impact on crop quality and biochemical parameters. For example, Vehar et al. (2025) investigated the effects of 14 CECs on the metabolic profile of hydroponically grown tomato fruits. They found that exposure to CECs significantly increased tomato yield and altered amino acid and volatile organic compound profiles, without affecting sugars, organic acids, carotenoids, polyphenols, or fatty acids. Christou, Kyriacou, et al. (2019) evaluated the effects of three pharmaceutically active compounds, both individually and in combination, in irrigation water, on various quality attributes of tomato fruits. These included colouration, acidity, soluble solids content, lycopene, ascorbic acid, phenols, antioxidant activity, and sugar content. The study revealed significant changes in soluble solids and carbohydrate content caused by individual pharmaceuticals. However, no additive or synergistic effects were observed when combined. Hurtado et al. (2017) examined the effects of 11 CECs on lettuce and identified alterations in relative concentrations of amino acids, carboxylic acids, and sugars. Chen et al. (2018) evaluated the response of ten different rice varieties to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), a predominant congener of polybrominated diphenyl ethers (PBDEs), in a hydroponic system. The metabolic response was variety-specific: the tolerant variety exhibited stronger metabolic activity and a more effective antioxidant defence system, with significant upregulation of amino acids, organic acids, saccharides, and secondary metabolites to counteract oxidative stress, while the susceptible variety showed a decrease in most metabolites, indicating its vulnerability. Despite these insights, understanding how CEC uptake influences quality and sensory attributes remains challenging due to the complexity of the data. Advanced computational approaches, particularly explainable machine learning, offer a means to unravel these intricate relationships, while conventional statistics like hypothesis testing and basic data analysis may struggle to capture the complexity of modern datasets.

Explainable Machine Learning (XML) refers to techniques and methods in machine learning that make the decisions, predictions and processes of a machine learning understandable to humans, enabling transparency, interpretability and explainability of the model's outcomes (Bernardo et al., 2023). It is being applied more and more in food science such as tracing food origins using stable isotopes, elemental composition, and metabolomic profiles (Huang, 2023; Kang, 2023, 2024; Sun, 2022), predicting crop yields under varying environmental conditions (Murakami et al., 2021; Newman, 2021; Sihi, 2022), classifying sweeteners and bitterants (Maroni, 2022), identifying relationships between fruit morphology and sensory-evaluated quality attributes (Minamikawa, 2022), predicting drug-food constituent interactions (Kha et al., 2023), and investigating challenges influencing food-sharing app usage (Puram, 2024).

In this paper, we compare tomatoes grown in two different tomato cultivation systems: soil-based (lysimeters) and soilless (hydroponics), where tomatoes were grown in nutrient solution, spiked nutrient solution and irrigated with potable water, treated wastewater and spiked treated wastewater. To analyse the data, we applied XML methods, enabling a deeper understanding of the complex effects of various factors on tomato quality. To the authors' knowledge, this is the first study to use XML techniques in this context, specifically to identify which biochemical parameters are most important for distinguishing among the five applied treatments.

2. Materials and methods

2.1. Experimental design

The experimental design, as described by Kovačič et al. (2023), consisted of two experiments conducted in soil and hydroponics over one growing season in 2020. The soil experiment was conducted in lysimeters at the test field located at Ajdovščina wastewater treatment plant (106 m a.s.l.; 45°52'34.14" N, 13°54'26.23" E) from May to September 2020. The climate was Mediterranean, with monthly air temperatures from 17.1–23.2 °C and total precipitation of 108.4–339.7 mm. Tomatoes (*Solanum lycopersicum* L. Rally) were grown under three irrigation regimes: potable water (LPW), treated wastewater (LWW), and treated wastewater spiked with 0.1 mg/L CEC (LSWW). The wastewater used for irrigation was sourced from the local treatment plant in Ajdovščina, Slovenia, which has a treatment capacity of 8,400 m³/day (equivalent to 42,000 population equivalents) for municipal and industrial wastewater. Lysimeters (40 cm × 100 cm; diameter × height) were filled with local soil, i.e., eutric brown soil developed on flysch parent material typical of the Vipava Valley region (southwestern Slovenia) and irrigated via an automated drip system. Plants received 36 L of water in total, and were fertilised periodically throughout the experiment. The CECs studied in both soil and hydroponic experiment were from the groups of industrial chemicals (BPS, BPA, 22BPF, 24BPF, 44BPF), anti-inflammatory drugs (ketoprofen, ibuprofen, naproxen, diclofenac), estrogens (E1, β-E2, EE2, E3) and a common stimulant (caffeine).

The hydroponic experiment was conducted in a greenhouse at the Biotechnical Faculty in Ljubljana (298 m above sea level; 46° 2' 58.23" N, 14° 28' 30.75" E) from June to October 2020. Seedlings were transplanted into 60 L covered tubs (120 × 60 cm) with six plants per tub. Two tubs with standard nutrient solution (HPW) served as controls, while three tubs received standard solution spiked with 0.1 mg/L CEC (HSPW). Conditions included mean daily temperatures of 16–29 °C and 56–98% humidity. Nutrient solution was continuously circulated and supplemented as needed.

2.2. Sample collection and preparation

The tomato fruits were hand-harvested at full ripeness. In the hydroponic experiments, the harvesting season was divided into three periods: (1) August 24 to September 10, (2) September 14 to September 22 and (3) September 28 to October 9. Similarly, in the lysimeter experiments, the first harvesting was carried out on August 4, the second on August 28 and the third on September 11, 2020. During each harvest, ripe fruits were harvested, rinsed with water, blotted dry and stored whole in plastic bags at -20 °C. The frozen samples were cut into pieces, freeze-dried and ground into powder. The composite samples were prepared as follows: (1) all harvested fruits per plant and per treatment, (2) all harvested fruits per period (first, second and third periods) per treatment, (3) all harvested fruits per treatment (plants from tubs or lysimeters).

2.3. Analytical methods

Detailed information on reagents, extraction procedures, and analytical protocols for the determination of sugars, organic acids, carotenoids, polyphenols, amino acids, fatty acids, and elements, is adapted from Kovačič et al. (2023) and Vehar et al. (2025). Briefly, sugars and organic acids were determined according to the method of Mikulic-Petkovsek et al. (2012). Freeze-dried samples were extracted with Milli-Q water, and the supernatants were analysed using high-performance liquid chromatography (HPLC, Thermo Scientific Vanquish, USA).

Sugars were quantified using HPLC with refractive index detection (HPLC-RI). Separation was achieved using a Rezex ECM monosaccharide column (300 × 7.8 mm, Ca^{2+}, Phenomenex, USA) and water as the mobile phase. Organic acids were quantified by HPLC with UV detection (HPLC-UV) using a Rezex ROA-organic acid H^+ column (8%, 300 × 7.8 mm, Phenomenex, USA) and 4 mM H_2SO_4 as the mobile phase. Compounds were identified by retention time and quantified using external standards.

Carotenoids were quantified following a modified method of Šircelj et al. (2005). Compounds were extracted from freeze-dried samples using acetone. Analysis was performed by HPLC-DAD (Dionex UltiMate 3000, Thermo Scientific, USA) using a Gemini C18 column (150 × 4.6 mm, 3 µm, Phenomenex, USA), with two mobile phases: A (acetonitrile/methanol/water) and B (acetone/ethyl acetate), under gradient elution. Compounds were identified by their retention times and quantified using external standards.

Individual soluble polyphenols were extracted based on the method of Mikulic-Petkovsek et al. (2013). Freeze-dried samples were extracted using 80% methanol containing 3% (v/v) formic acid. Polyphenols were analysed by HPLC-DAD-MS/MS (Dionex UltiMate 3000, Thermo Scientific, USA and LTQ XL Thermo Scientific, USA) using a Gemini C18 column (150 × 4.6 mm, 3 µm, Phenomenex, USA), with solvent A (3% acetonitrile, 0.1% formic acid in water) and solvent B (3% water, 0.1% formic acid in acetonitrile) as the mobile phases. Mass spectra and retention time matching were used for compound identification, and quantification was performed using standard curves.

For amino acid determination, samples were hydrolysed for 24 h at 110 °C in 4% thioglycolic acid in 6 M HCl with added phenol. Solid-phase extraction and derivatisation were performed using the EZ:faast™ amino acid analysis kit (Phenomenex, USA), followed by GC-MS (7890 B GC & 5977 A Series MSD; Agilent Technologies, USA) analysis using a ZB-AAA GC column (10 m × 0.25 mm; Phenomenex, USA). Quantification was performed against known amino acid standards.

Fatty acids were analysed following total lipid extraction using dichloromethane and 0.5 M sodium hydroxide in methanol. Fatty acid methyl esters (FAMEs) were prepared by transesterification with boron trifluoride in methanol, then extracted with hexane and Milli-Q water. Compounds were analysed by gas chromatography with flame ionisation detection (GC-FID, Agilent 6890N, USA) using a CP-Sil 88 capillary column (100 m × 0.25 mm × 0.20 µm, Agilent J&W, USA). Identification and quantification were achieved by comparing retention times with those of the Supelco 37 Component FAME Mix standard. Results were expressed as weight per cent of total fatty acids.

Elemental analysis was conducted following the method described by Potočnik et al. (2021). Elements were analysed after digestion using an Agilent 8800 QQQ-ICP-MS (Agilent, USA). Internal standards were introduced online in all modes, and external calibration was employed for quantification. Each batch included blank samples and certified reference materials (tomato leaves SRM 1573a, peach leaves SRM 1547, spinach leaves SRM 1570a).

2.4. Explainable machine learning pipeline

An XML approach was developed to identify the biochemical parameters most influential in distinguishing between five different tomato cultivation systems. A multi-class classification scenario (Sahare & Gupta, 2012) was implemented, in which tomato samples were classified

into five categories: 'HPW', 'LWW', 'LPW', 'HSPW', and 'LSWW'. The collected dataset was then split using stratified k-fold cross-validation to ensure proportional class representation in both the training and test subsets.

In this study, 70 candidate features were extracted for each observation, but only 41 samples were available and distributed across five classes (HSPW = 13, HPW = 9, LSWW = 7, LWW = 6, LPW = 6). As the number of features (70) significantly exceeded the number of samples (41), there was a risk of unstable estimates and redundancy due to correlated predictors. To manage potential multicollinearity and avoid information leakage, feature redundancy was handled during cross-validation by selecting a subset of approximately uncorrelated features. Specifically, within each cross-validation split, the absolute Pearson correlation matrix of features was computed on the training portion and transformed into a dissimilarity matrix. Agglomerative hierarchical clustering with Ward linkage was then performed on the dissimilarity matrix (Ackermann et al., 2014), and the resulting dendrogram was cut into 30 clusters. From each cluster, a single representative feature was selected: the one with the highest cumulative absolute correlation to other features in the cluster, ensuring that only one feature per cluster was used in downstream modelling.

Two classification models, Random Forest and Decision Tree, were evaluated using traditional performance metrics for multi-class classification. SHapley Additive exPlanations (SHAP) were employed to interpret the best performing models' predictions by quantifying each feature's marginal contribution and interactions at both the overall and the individual sample level (Van den Broeck et al., 2022). By examining the results across multiple training and testing splits, consistently recurring patterns unlikely to arise by chance were identified. Also, the robustness of the model explanations was analysed to assess whether the choice of training data influences the results.

3. Results and discussion

3.1. Feature selection

Before training the explainable classifier, feature selection was performed by combining correlation analysis with clustering, selecting a single representative feature from each cluster (see section 2.4). Figure 1 displays the Pearson correlation matrix for the features in the first training fold. The matrix revealed several groups with strong positive correlations (red), such as a cluster of amino acids (glycine, valine, leucine, isoleucine, proline, tyrosine) in the upper left corner, which exhibited strong intra-group correlations. Similarly, elements showed positive correlations in the lower-right section of the matrix. Sugars, particularly glucose and fructose, also demonstrated a strong correlation with each other. In contrast, within their respective groups, organic acids, carotenoids, and polyphenols showed no significant correlations.

Several compounds also exhibited correlations across different groups. For instance, flavonols correlated with specific amino acids, such as alanine, aspartic acid, and glutamic acid, as well as with elements such as Mg, K, Ca, Co, Ni, Sr, Mo, Ag, Cd, Cs, and Ba. Additionally, the amino acids alanine, aspartic acid, and glutamic acid, which correlated with flavonols, also shared correlations with elements including Mg, K, Co, Ni, Mo, Cd, and Cs. The remaining correlations were mostly negative or near zero (blue), suggesting that these feature groups were either largely independent or inversely related.

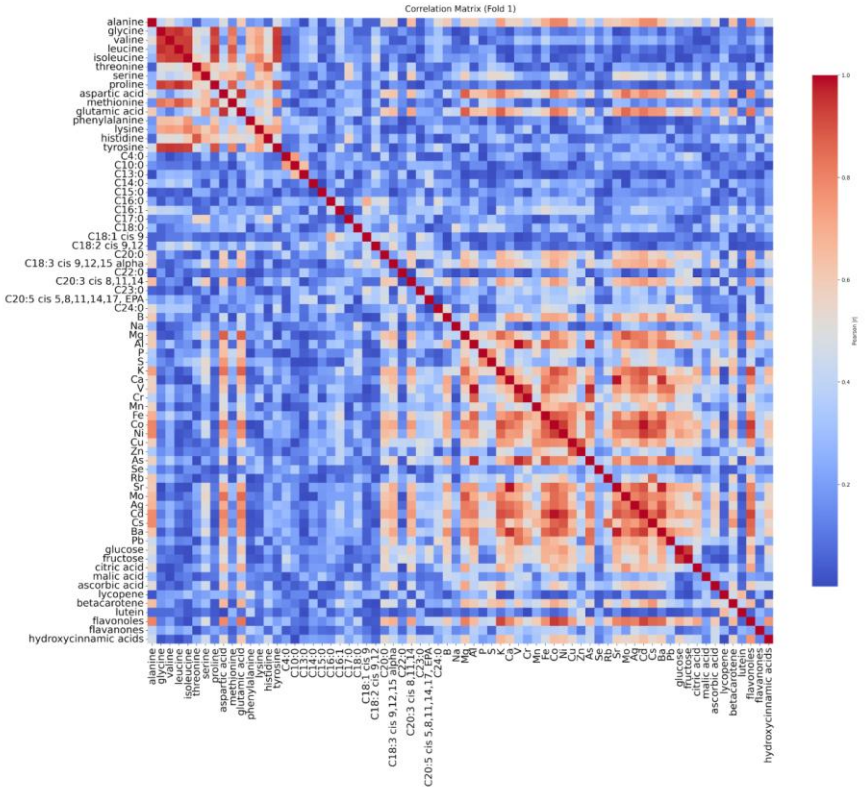


Figure 1. Pearson correlation matrix of the features in the first training fold.

A correlation matrix was then created and used to perform clustering, with 30 clusters to ensure that the number of features remained lower than the number of samples. From each cluster, a single representative feature was selected as the one with the highest cumulative absolute correlation with the other features in that cluster (Figure S1).

As previously explained, the evaluation was conducted across five train–test folds to ensure the robustness of the modelling approach. To assess the stability of the correlation patterns and the features selected for explainable modelling, the correlation matrices for all five folds are presented (Figure S2), and the corresponding clustering results (Figure S3), highlighting the features selected from each cluster. Both figures reveal consistent patterns across the training folds, indicating that the choice of training samples has little influence on feature selection.

A union of all 30 features selected from each fold resulted in 45 distinct features: 18 in all five folds, seven in four folds, three in three folds, six in two folds, and 11 in only one fold (Figure S4). Each fold contained some unique features. Most features consistently selected across all folds were fatty acids, with four elements, as well as representatives of polyphenols, sugars,

carotenoids, and amino acids. These features show very low correlation with other compounds or elements, as indicated by the correlation matrices (Figure S2), suggesting that they provide independent biochemical information.

To further assess the overlap of features selected across folds, the Jaccard similarity was calculated (Figure S5). This metric compares the size of the intersection to the size of the union of two sets and, in this context, quantifies the similarity of feature portfolios across folds, with higher values indicating greater overlap. The results showed that the highest similarity occurred between the first and fourth folds (71%). In comparison, all other fold comparisons exceeded 50%, indicating a robust and consistent set of features with predictive power. These results demonstrated that the number of required chemical analyses could be significantly reduced, as a smaller set of features, identified through their correlations, provided a robust and sufficient set for further analysis.

3.2. Multi-class classification

The goal was to classify the tomato samples into one of five predefined classes: HPW, LWW, LPW, HSPW, and LSWW. Decision Trees and Random Forests were selected due to their strong performance on tabular data (Uddin & Lu, 2024). Their performance was compared against a baseline defined by the frequency of the majority class in the training set, with models considered superior when their performance exceeded the baseline. Table 3.1 presents the performance metrics achieved by the two models and the baseline, including accuracy and weighted recall, precision, and F1-score. The metrics were aggregated across the five folds and are reported as mean values with their corresponding standard deviations. Accuracy measures the proportion of samples that are correctly classified. However, it can be misleading if some classes have many more samples than others. Precision indicates the proportion of samples assigned to a given class that truly belong to that class, while recall measures the proportion of samples from that class that are correctly identified. The F1-score combines precision and recall into a single measure, balancing false positives and false negatives. For multi-class problems, these metrics can be averaged in two ways: macro, which gives equal weight to all classes, or weighted, which gives more weight to larger classes with more samples. Weighted averaging was used here to account for class imbalance and provide a more representative assessment of model performance.

Table 3.1. Performance metrics of the two models and the baseline, including accuracy, weighted recall, precision, and F1-score

Model	Accuracy	Precision	Recall	F1
Random Forest	0.778 ± 0.148	0.754 ± 0.185	0.778 ± 0.148	0.744 ± 0.173
Decision Tree	**0.875 ± 0.112**	**0.883 ± 0.113**	**0.875 ± 0.112**	**0.867 ± 0.122**
Baseline	0.319 ± 0.069	0.107 ± 0.042	0.319 ± 0.069	0.159 ± 0.056

As shown in Table 3.1, both models (DT and RF) performed substantially better than the baseline, indicating that training a machine learning model provides a clear advantage over predicting the majority class. Across both models, the DT consistently outperforms the RF model in all reported metrics and demonstrates greater robustness, as evidenced by the lower standard deviations. The distributions of accuracy and weighted F1-score for each model

across the five folds (Figure S6 and S7) also show that the DT model was more robust, with changes in the feature portfolio across folds affecting the metrics by only ±12%.

3.3. Global and local explanations

Next, the best-performing model (in this case DT) was selected, and an explainability analysis was conducted by calculating the Shapley values. SHapley Additive exPlanations (SHAP) provided a unified approach to interpreting machine learning models by assigning each feature a contribution value toward a prediction. At the global level, SHAP values were aggregated across all samples to reveal the overall importance of each feature and its average impact on the model's output, enabling the identification of features that consistently drive predictions. At the local level, SHAP values explained individual predictions by showing how each feature contributed positively or negatively to the model's decision for a specific sample. This dual perspective allowed both a broad understanding of model behaviour and a detailed explanation of individual outcomes.

3.3.1. Global explanations

A summary of the global feature importances across all samples, aggregated across all folds (Figure 2), shows that the most important features, including Cs, Cd, and Co, play a key role in distinguishing all five classes. Additionally, Na is particularly influential in distinguishing LPW, LWW, and LSWW, while P is critical for differentiating HPW and HSPW. Furthermore, C17:0 is specifically relevant for separating LSWW and LWW.

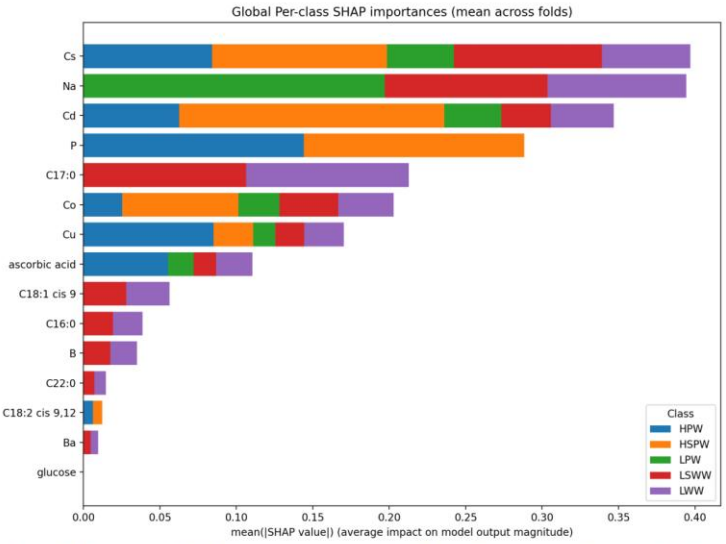


Figure 2. Summary of global feature importances for all samples, aggregated across all folds.

Global feature importances were analysed for each fold (Figures S8-S12), revealing consistent patterns across folds. The most comparable outcomes were observed in folds two, three and four. In fold one, Co was the most important feature; in folds two, three, and four, Cs was the most important, while in fold five, Cd was the most important. Referring back to the correlation

matrix for fold one, Co, Cs and Cd were positively correlated and therefore conveyed similar information.

The main sources of Co, Cs, and Cd in this study were soil (in lysimeters) and treated wastewater, as reflected in the concentration sequence of the individual elements (Supplementary material – data). The concentrations for Cs were LPW > LSWW > LWW > HSPW > HPW, for Cd LSWW > LPW > LWW > HPW > HSPW, and for Co LWW > LSWW > LPW > HSPW > HPW. Although Na did not contribute to the differentiation of hydroponic treatments, it played a significant role in distinguishing among lysimeter treatments. Irrigation with treated wastewater increased soil Na concentrations (Simhayov et al., 2023), which subsequently leaded to elevated Na levels in the fruit. High concentrations of Na in soil could also harm plants by creating osmotic stress. Furthermore, the presence of CECs appeared to enhance Na uptake, as reflected in the observed concentration gradient: LSWW > LWW > LPW.

Unlike Na, P distinguished hydroponic treatments. The concentration gradient HPW > HSPW indicated reduced P uptake in the medium with added CEC. This finding suggests that CECs in hydroponics might influenced the P uptake by possibly affecting plant stress responses (McGinnis et al., 2019), chemical balances and microbiome in the root zone (Disciglio et al., 2015; Muscarella et al., 2024; Zhang et al., 2025), which influence the uptake of P. The margaric acid (C17:0) distinguished samples irrigated with treated wastewater and spiked treated wastewater, with a concentration gradient of LSWW > LWW. Unlike the elements, fatty acids are not directly uptaken from the soil but are synthesised in plants, in this case tomato fruits. Margaric acid is generally a very low-abundance fatty acid in most plants. Although direct evidence for margaric acid is lacking, differences in other fatty acids suggest that microbial interactions and environmental stress can influence plant metabolism (He & Ding, 2020; Walley et al., 2013), potentially contributing to shifts in margaric acid levels. These observations support the idea that the addition of CECs induces stress in tomato plants, potentially disrupting normal metabolic pathways, including lipid biosynthesis and fatty acid composition.

3.3.2. Local explanations

After providing the global explanations, the patterns associated with accurate predictions for each class were analysed separately across the five folds. SHAP force plots visualise how individual features push a model's prediction higher or lower relative to the baseline, showing the direction and magnitude of each feature's contribution for a single sample. Figure 3 shows the local feature importance for a true prediction on the HPW sample, randomly selected from the test set for each of the five testing folds. The patterns showed that a small subset of features, specifically certain elements and biochemical compounds, consistently drove accurate predictions for this class. Among these, P, Co, Cu, Cs, Cd, ascorbic acid and linoleic acid (C18:2 cis 9,12) emerged as most influential. The consistent appearance of these variables across all five folds suggests that they represented a distinct chemical signature of the HPW class. Since P, Cu and Co are regulated in plants (Dong et al., 2025; Hu et al., 2021; Khan et al., 2023; Wairich et al., 2022), they may reflect physiological or metabolic processes unique to HPW class, while elements like Cs and Cd could be environmental markers, since they have no known function for plant nutrition (Burger & Lichtscheidl, 2018) or are even toxic for plants (Hassan et al., 2024). The detection of ascorbic acid and linoleic acid, both essential

nutrients for humans and key metabolic indicators in plants (He & Ding, 2020; Mellidou et al., 2021), further strengthened the hypothesis that HPW samples possess a distinct biochemical fingerprint, setting them apart from other classes.



Figure 3. Local feature importance for a true prediction of the HPW samples across five folds.

Figure 4 shows the local feature importance for a true prediction of the HSPW sample that is randomly selected from the test set of each of the five testing folds. The small subset of features that consistently drove true predictions for HSPW samples consisted of P, Co, Cu, Cs, and linoleic acid (C18:2 cis 9,12), all of which were important also for HPW samples. Given that the HPW and HSPW tomato samples differed only by the presence of added CECs in the latter, it is reasonable that their key features remained broadly similar. Both classes shared the same biochemical foundation, and the observed differences likely arose from variations in the concentrations rather than the type of elements and metabolites. The introduction of CECs may elicit subtle metabolic adjustments, including alterations in nutrient assimilation, redox homeostasis, and lipid metabolism. These responses could influence the levels of P, Cu, and Co through their roles in enzymatic regulation (Hu et al., 2021; Khan et al., 2023; Wairich et al., 2022), while inducing oxidative stress that affects compounds like linoleic acid (He & Ding, 2020).



Figure 4. Local feature importance for a true prediction of the HSPW samples across five folds.

The small subset of features important for making a true prediction of the LPW samples (Figure 5) includes Co, Cs, Cd, Na and ascorbic acid, most of which were also identified as important features in HPW and HSPW samples, except for Na. Na appears as a significant feature in samples grown in soil (LPW, LWW, LSWW) but is less important in hydroponic treatments. One reason could be irrigation with treated wastewater (LWW), which increases Na soil concentrations (Ungureanu et al., 2020), and the addition of CECs during LSWW treatment, which may influence Na uptake. The same is also true in the concentration gradient: LSWW > LWW > LPW. Regarding the other elements, samples grown in soil have significantly higher concentrations, due to higher levels in soil compared to hydroponic media. Additionally, treated wastewater may contribute to variations in soil elemental concentrations, thereby affecting the uptake of these elements into tomato fruits.



Figure 5. Local feature importance for a true prediction of the LPW sample across five folds.

The small subset of features important for making a true prediction of the LWW samples (Figure 6) included Co, Cs, Cd, Na, Ba, ascorbic acid, palmitic acid (C16:0), margaric acid (C17:0) and behenic acid (C22:0). Ba appeared among the elements for the first time. Its concentration gradient was LWW > LSWW > LPW, underscoring its importance for LWW tomatoes. Elevated Ba concentrations could indicate anthropogenic contamination (Nogueira et al., 2010). In LWW samples, palmitic, margaric, and behenic acids also appeared as significant features, suggesting that wastewater may affected fatty acid metabolism in tomatoes.

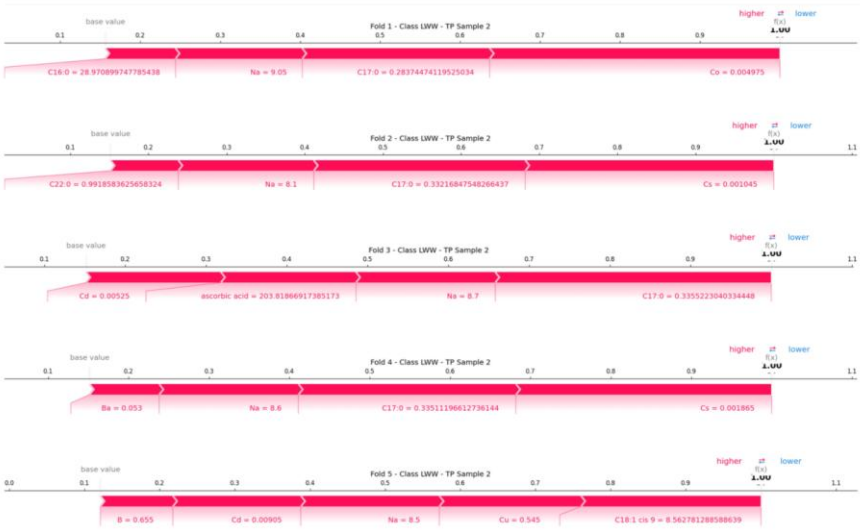


Figure 6. Local feature importance for a true prediction of the LWW sample across five folds.

Figure 7 shows the local feature importance for a true prediction on randomly selected LSWW samples from the test set of each of the four testing folds. In the fold three, we did not obtain a true prediction for this class, which is the reason why only four plots are presented. The small subset of features included Co, Cs, Cd, Cu, Na, and palmitic acid (C16:0), margaric acid (C17:0) and oleic acid (C18:1 cis 9). All elements were important for the samples from other groups, indicating that CECs, in this case, influenced and slightly altered the uptake of these elements. Palmitic and margaric acids appeared earlier, while oleic acid appeared for the first time, indicating that added CECs also might affected fatty acid metabolism. Overall, all local explanations reveal consistent patterns across the folds for each true prediction within each class. This analysis also enables insights for each analysed sample rather than relying solely on high-level statistical outcomes across all samples.

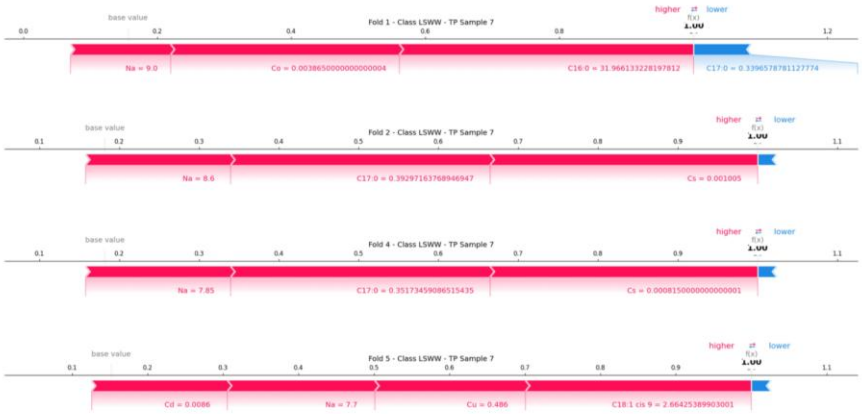


Figure 7. Local feature importance of selected examples for a true prediction of the LSWW sample across four folds.

Additionally, the local feature importances can help identify the variables that contribute to false predictions, offering insights into what may have gone wrong in the experiment, analysis, or modelling process. To illustrate this, an instance labelled as LSWW from the fold three, which is missing in Figure 7, is presented (Figure 8) and the variable that contributed to its misclassification as LWW, in this case, was margaric acid (C17:0).

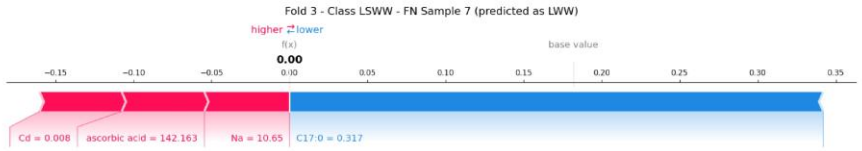


Figure 8. Local feature importances of a selected LSWW sample from fold three, which was falsely predicted as LWW sample.

4. Conclusions

This study demonstrated that a feature selection approach, multi-class classification, and both global and local explanations are highly effective for metabolomic studies with multiple sample classes and a large number of features, even when working with a relatively small sample size. Despite the limited sample number, the models achieved strong performance, successfully identifying the most significant features contributing to class separation. Local importances provided insights into which elements or compounds played a key role in classifying each sample. At the global level, the elements Cs, Cd, P, Co, and margaric acid were the most important for prediction. At the local level, key features included elements (Cd, Co, Cs, Cu, P, Na), ascorbic acid, and specific fatty acids (palmitic, margaric, oleic, linoleic, behenic acid), which varied depending on the treatment. The method also proved valuable in identifying how specific features falsely classified a sample, providing clear reasoning for its false classification. Based on these results, it can be concluded that for classifying samples into distinct groups, analysis of a smaller set of elements and metabolites would be sufficient,

significantly reducing the number of analyses, material costs, and time, while maintaining robust model performance.

Abbreviations

BDE-47	2,2',4,4'-tetrabromodiphenyl ether
CEC	contaminant of emerging concern
DT	Decision Tree
FAME	fatty acids methyl ester
GC-MS	gas chromatography with mass spectrometry
HPLC-DAD	high-performance liquid chromatography with a diode array detector
HPLC-DAD-MS/MS	high-performance liquid chromatography with a diode array detector and tandem mass spectrometry
HPLC-RI	high-performance liquid chromatography with refractive index detection
HPLC-UV	high-performance liquid chromatography with ultraviolet detection
HPW	hydroponics potable water
HSPW	hydroponics spiked potable water
LPW	lysimeters potable water
LSWW	lysimeters spiked treated wastewater
LWW	lysimeters treated wastewater
ML	machine learning
PBDEs	polybrominated diphenyl ethers
QQQ-ICP-MS	inductively coupled plasma tandem mass spectrometry
RF	Random Forest
SHAP	SHapley Additive exPlanations
SRM	standard reference material
TWW	treated wastewater
XML	explainable machine learning

CRediT authorship contribution statement

A. Vehar: Writing – original draft, Investigation, Visualisation, **J. Drole**: Writing – original draft, Formal analysis, **T. Eftimov**: Writing - Review & Editing, Methodology, Supervision, **N. Kacjan Maršić**: Investigation, **B. Koroušič Seljak**: Supervision, **E. Heath**: Writing - Review & Editing, Supervision, Funding acquisition, **N. Ogrinc**: Writing - Review & Editing, Supervision, Funding acquisition

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this work, the authors used ChatGPT for translation and grammar purposes. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Given her role as Regular Editor and Guest Editor, prof. dr. Nives Ogrinc had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

Appendix A. Supplementary data

Supplementary material 1.docx

Data availability

Data on Zenodo:
Code: https://github.com/jan3657/tomato_sample_explainable_classifier

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Chapter 4

Conclusions

This PhD thesis advances knowledge on the quality of two sustainable food sources: farmed edible insects and tomatoes irrigated with TWW. In the case of edible insects, it systematically evaluates the influence of species, developmental stage, and sex on their nutritional composition. Furthermore, it investigates the application of fermentation as a processing strategy, demonstrating its potential to modify quality attributes and oxidation stability, while highlighting its species- and culture-dependent effects.

For tomatoes, the thesis examines the impact of TWW and CEC uptake on fruit quality, with particular emphasis on differences between hydroponic and soil-based cultivation systems. Given the complexity and high dimensionality of the metabolomic and elemental datasets, an explainable machine learning approach was applied to identify the key metabolites and elements driving the differentiation between treatments.

This research has resulted in six papers: five published and one currently under review in an A'' SCI international journal. Among the published papers, four appeared in SCI international journals (A'' and A') and one in a non-SCI journal. Authorship contributions include two first-author publications, three joint-first authorships, and one co-authorship. Furthermore, the findings have been disseminated through 12 presentations at international conferences.

The main conclusions of this thesis can be summarised into four research topics as follows:

1. **Nutritional composition characterisation of edible insects:** Farmed edible insects were demonstrated to be a valuable source of essential fatty acids, amino acids, and minerals. Species, developmental stage, and sex significantly influenced fatty acid and polyphenol composition, while amino acid profiles did not differ significantly between sexes, confirming the first hypothesis: “H1: Species, sex and stage of development have a statistically significant influence on metabolic profile of edible insects.” These differences reflect variations in metabolic processes, physiological requirements during growth, biological functions, and reproductive energy demands. Overall, these findings enhance the understanding of how rearing conditions and intrinsic biological characteristics influence the nutritional quality of farmed insects, providing a basis for optimising insect production systems and improving their value as a sustainable food source.

- **Influence of fermentation on the quality of edible insects:** Fermentation was shown to be species- and fermentation culture-specific, with effects less consistently positive than commonly reported in the literature. In general, fermentation with *L. plantarum* did not affect the proximate composition of yellow mealworm and house cricket. However, it led to a reduction in total dietary fibre and an increase in non-protein nitrogen. Also, no significant improvements in the

insects' fatty acid profiles were observed. Fermentation increased ornithine, but reduced essential amino acids, such as lysine, methionine, and threonine, which are often limiting in plant-based diets, making their reduction less desirable. The changes in mineral composition appeared to reflect relative concentration effects, underscoring the complexity of the fermentation process. Oxidation stability was decreased in both the yellow mealworm and migratory locust, while a significant improvement was observed in the house cricket. All the variations highlight the complex interplay between insect species, microbial cultures, and processing conditions in determining the nutritional and functional outcomes of fermented insect products, thereby confirming the second hypothesis: "H2: Fermentation of edible insects has a measurable effect on their nutritional value and oxidation stability compared to non-fermented ones."

2. **Influence of TWW and CECs on tomato quality grown in hydroponics and soil:** This thesis demonstrated that CEC uptake effects are highly dependent on concentration, growing medium, and matrix (nutrient solution or TWW). Higher concentrations of bisphenol S, 2,4-bisphenol F, and naproxen in tomatoes grown in spiked nutrient solution were associated with increased concentrations of Mn, Cu, and Zn compared to those grown in a standard nutrient solution, indicating that the presence of CECs influenced elemental composition in hydroponics.

   In contrast, the uptake of these compounds in lysimeter-grown tomatoes was lower than in hydroponics, which is reflected in the absence of significant differences in elemental composition between tomatoes irrigated with TWW and those irrigated with spiked TWW. However, irrigation with TWW resulted in significantly higher concentrations of B and Na than with potable water, suggesting greater uptake from TWW.

   The evaluation of quality parameters in hydroponic systems showed that CECs significantly increased yield without affecting sugars, organic acids, carotenoids, polyphenols, or fatty acids. However, tomatoes grown in spiked nutrient solutions exhibited significantly lower concentrations of valine, leucine, aspartic acid, and methionine, along with a significantly altered VOC profile. These changes may be attributed to stress induced by CEC exposure or to the endocrine-disrupting properties of the detected compounds. Overall, exposure to CECs and TWW has a measurable effect on tomato quality and significantly alters the tomato metabolome. This confirms the third hypothesis: "H3: Exposure of tomato plants to CECs, treated wastewater, or sludge has a measurable effect on tomato quality and statistically significantly alters the tomato metabolome."

3. **Explainable machine learning approach for classification of tomatoes grown under different conditions:** The explainable machine learning approach proved to be an effective tool to identify key metabolites and elements, which contribute to the classification of tomatoes from five different treatments in hydroponics (nutrient solution and spiked nutrient solution) and soil (potable water, TWW and spiked TWW). This confirms the fourth hypothesis: "H4: An explainable machine learning approach can more effectively identify those key metabolites that contribute to the differentiation of tomatoes grown under different conditions in soil and soil-less media than standard statistical methods." Despite the limited number of samples, the classification models achieved high performance (75-88% accuracy), while the SHAP method identified key metabolites and elements at the global and local level. At the global level, the elements Cs, Cd, P, Co, and margaric acid were the most important for prediction, while at the local level, key features included elements (Cd, Co, Cs, Cu, P, Na), ascorbic acid, and specific fatty acids (palmitic, margaric, oleic, linoleic, behenic acid). The method also proved

valuable for identifying which specific features led to a sample's false classification, providing clear reasoning for its classification. Furthermore, the approach can highlight a smaller set of features sufficient for classifying samples into distinct groups, potentially reducing the number of analyses, material costs, and time while ensuring robust model performance.

Although edible insects and tomatoes irrigated with treated wastewater represent two distinct scientific topics and production systems, they are connected by their common contribution to the development of sustainable food systems, circular resource use, and future food security. Both systems address pressing global challenges, including increasing food demand, resource scarcity, climate change, and the need to reduce the environmental footprint of agriculture. Edible insects represent an alternative animal-derived food source with the potential to contribute to protein security while reducing the environmental burden associated with conventional protein production. In parallel, the use of treated wastewater in tomato cultivation represents a resource-efficient agricultural strategy that supports wastewater reuse in the context of increasing water scarcity. Together, these two research areas address complementary aspects of future food production: the diversification of sustainable food sources and the optimisation of agricultural resource use.

Importantly, this thesis demonstrates that the sustainability of alternative food systems cannot be evaluated only in terms of environmental benefits. Their successful implementation also depends on a detailed understanding of food quality, nutritional value, processing effects, and food safety. For edible insects, the findings provide practical insights for insect producers by demonstrating how species, developmental stage, and sex influence nutritional composition, thereby supporting informed decisions in production systems. They also offer valuable guidance for food processors by highlighting how fermentation affects the quality and stability of insect-based products.

In the case of tomatoes, the results contribute to a better understanding for both policymakers and farmers of how treated wastewater and associated contaminants influence tomato quality, supporting more informed and sustainable water reuse strategies. Furthermore, the application of explainable machine learning proved to be a powerful and effective approach for analysing complex metabolomic datasets, with strong potential for broader use in future food and agricultural research.

Chapter 5

Future work

Although this thesis answers many research questions, it also raises new scientific questions and highlights several areas that require further investigation. Regarding insect characterisation, additional species beyond the seven analysed in this study show strong potential for farming and warrant further evaluation, including the house fly, black soldier fly, and silkworm. To enable robust statistical analysis, future studies should include a larger sample size. Furthermore, as this thesis examined the nutritional composition across developmental stages for only a limited number of species, primarily due to challenges in sample collection, future research should aim to characterise the nutritional composition of all species across developmental stages and sexes. A more comprehensive nutritional assessment should also be conducted, including proximate composition, vitamins, antioxidant capacity, peptides, and chitin. In addition, integrating VOC analysis with sensory evaluation would provide valuable insights into the relationship between chemical composition and consumer perception.

Regarding the processing of insects using fermentation, future work should focus on optimising fermentation conditions, particularly fermentation time. A broader range of microbial cultures, such as *Bacillus subtilis*, *Aspergillus niger*, *Lactobacillus crispatus*, *Pichia kudriavzevii*, and *Aspergillus oryzae*, should be investigated, along with different insect species. Oxidation stability should be assessed using a larger number of samples to improve statistical robustness. Additional analyses, including vitamins and antioxidant capacity, would further enhance understanding of fermentation effects. Moreover, analysing VOCs before and after fermentation, together with sensory evaluation of fermented insect meal and insect-based products enriched with fermented ingredients, would provide important insights into product quality and consumer acceptance.

Regarding the uptake of CECs by tomatoes, several directions for future research have been identified. In this PhD, experiments were conducted using a mixture of 14 CECs at an environmental concentration of 0.1 mg/L (worst-case scenario) in hydroponic nutrient solutions, while in lysimeters, TWW and TWW spiked with CEC (0.1 mg/L) were applied. In both cases, only 14 CECs were targeted in all matrices, including aqueous media, soil, and tomatoes. Given that, besides these targeted CECs, a wide variety of nontargeted compounds, including nontargeted CECs and their metabolites/transformation products, are commonly present in wastewater, future studies should investigate a broader range of compounds across different concentration levels, as their effects on the tomato metabolome are likely both compound- and concentration-dependent. In addition, these compounds should be included in risk assessment as well as potential synergistic or antagonistic interactions.

Accordingly, further research should also focus on the effects of all CEC residues on tomato quality and metabolism. Again, the application of untargeted high-resolution mass

spectrometry combined with modelling approaches could provide a more comprehensive understanding of tomato metabolomic responses to CEC exposure. Moreover, experimental designs should be expanded to include both hydroponic and soil-based systems under comparable conditions (e.g., irrigation with TWW and spiked TWW in hydroponics, and spiked potable water in lysimeters) to assess the influence of the growing system on CEC uptake and plant metabolomic responses.

An additional research avenue concerns the use of wastewater sludge as a soil amendment. Given that wastewater treatment plants generate substantial amounts of sludge rich in organic matter, its agricultural application could provide insights into the behaviour of sludge-associated CECs and their transformation products. Due to differences in physicochemical interactions between CECs and sludge compared to water, their bioavailability, mobility, and plant uptake may vary significantly, warranting further investigation, particularly in relation to tomato quality.

Finally, explainable machine learning is a promising tool for analysing complex datasets generated in such studies. Future work should ensure a sufficient number of samples (preferably more than six per group) to enable robust model development. Additional value could be achieved by validating models using samples collected across multiple growing seasons, allowing the assessment of model robustness and the influence of seasonal variability. Furthermore, integrating machine learning with multi-omics approaches would provide deeper insights into the complex interplay between environmental factors, biochemical processes, and product quality. Such interdisciplinary approaches will be crucial for supporting the transition toward sustainable and circular food systems.

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Publications Related to the Thesis

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Biography

Anja Vehar initiated her academic training at the University of Ljubljana's Faculty of Chemistry and Chemical Technology in 2014. She obtained her B.Sc. degree in Chemical Engineering in 2018, defending her thesis titled "Influence of Cavitational Pretreatment on the Relief of Ultrafiltration Modules." She continued her specialisation at the same institution, earning a M.Sc. in Chemical Engineering in 2021 with a research focus on "Monitoring the Removal Efficiency of Bisphenols from Wastewater in a Municipal Wastewater Treatment Plant."

Currently, Ms Vehar is a doctoral candidate in the Ecotechnologies program at the Jožef Stefan International Postgraduate School in Ljubljana. Her dissertation research, conducted at the Department of Environmental Sciences, is supervised by Prof. Dr Nives Ogrinc and co-supervised by Prof. Dr. Ester Heath. Her doctoral research focuses on circular food systems, evaluating the nutritional quality of sustainable food sources, specifically farmed insects as an alternative protein and tomatoes irrigated with reclaimed wastewater as an example of resource recovery as primary case studies.

Throughout her doctoral studies, she has gained extensive technical proficiency in GC-MS for amino acid analysis, GC-FID for fatty acid analysis, and HS-SPME-GC-MS for the analysis of volatile organic compounds. Her expertise also includes advanced data processing and statistical evaluation, supplemented by specialised training in stable isotopes analysis, non-target analysis, and RStudio programming. An active contributor to the scientific community, Ms Vehar has presented her findings at 27 international conferences. In 2022, she was recognised as the Best Science Communicator at the 14th Jožef Stefan International Postgraduate School Conference for her work on the quality attributes of tomatoes irrigated with treated wastewater. Her academic career is further distinguished by four international exchanges, including tenures at the Slovak University of Technology (2017/2018), the University of Zagreb (2019), the Fondazione Edmund Mach in Italy (2023), and the Czech University of Life Sciences Prague (2024).