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Use of *Tenebrio molitor* larvae meal and the prebiotic chitosan in pig nutrition

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Abstract

The growing global demand for sustainable and efficient food production has intensified interest in alternative feed resources that can support livestock performance while reducing environmental impact. As the world population is projected to surpass 10 billion by 2050, the pressure on agriculture and animal production systems will continue to increase, particularly for high-quality protein sources such as pork. Traditional feedstuffs, including soybean meal and fishmeal, are facing limitations in availability, cost stability, and environmental sustainability. Issues such as deforestation linked to soybean cultivation, marine overexploitation affecting fishmeal production, volatility in international trade, and regulatory restrictions on animal by-products further complicate feed formulation within the European Union. In this context, finding new protein-rich feed ingredients has become essential for ensuring the future strength and sustainability of livestock production systems.

Insects have emerged as a promising solution to these challenges due to their high nutritional value, excellent feed conversion efficiency, low environmental footprint, and compatibility with circular bioeconomy principles. *Tenebrio molitor* (yellow mealworm) larvae, in particular, are characterized by high levels of digestible protein, lipids, essential amino acids, vitamins, minerals, and bioactive compounds such as chitin and antimicrobial peptides. EU regulatory developments, most notably the 2021 authorization of processed insect protein for poultry and swine, have accelerated interest in their potential use in monogastric nutrition. Importantly, the nutritional and functional value of insect meals can be modulated through the larvae's rearing substrate, offering a unique opportunity to enrich the profile of insect-derived feed ingredients by incorporating plant-based bioactive compounds. This represents an innovative area of research with limited available data, particularly regarding the use of enriched-substrate insects in pig nutrition.

Chitosan, a deacetylated derivative of chitin, has gained increasing attention as a functional feed additive with prebiotic, antimicrobial, and immunomodulatory properties. Its potential to improve gut microbiota composition, support digestive health, and enhance growth performance makes it highly valuable during the critical early-weaning period in pigs. Despite its promising effects, its combined use with insect meal has never before been evaluated in early growing pigs. This dissertation was designed to fill these gaps by investigating both the nutritional and functional potential of *T. molitor* larvae meal, produced on conventional or phytochemical-enriched

substrates, and by evaluating the combined impact of conventional insect meal and chitosan supplementation.

Two experimental feeding trials were conducted: one in early-growing pigs and one in finishing pigs. Together, these trials aimed to assess the effects of insect meal and chitosan on zootechnical parameters, gut microbiota, health biomarkers, and meat quality traits. On the first trial, sixty early-growing pigs were allocated into five dietary treatments: a control diet, two diets containing 10% *T. molitor* meal produced on conventional or phytochemical-enriched substrates, a diet supplemented with chitosan, and a combined conventional insect meal–chitosan diet. The whole experimental trial lasted 42 days. Results revealed that the combined use of conventional *T. molitor* meal and chitosan significantly improved growth performance, demonstrating a synergistic effect. Gut microbiota composition was altered by insect-based diets and chitosan supplementation, with significant reductions in pathogenic bacterial groups and increase of beneficial microbial populations. Blood biomarkers remained largely unaffected, except for an increase in cholesterol levels in pigs receiving enriched-substrate larvae meal, a result that requires further investigation. Meat quality traits were also positively influenced, with improved oxidative stability, elevated total phenolic content, favorable modifications in fatty acid composition, and beneficial shifts in meat microbial populations. These findings collectively demonstrate that the use of insect meal, especially when combined with chitosan, can function as an effective strategy to support gut health, growth performance, and meat quality during the early-growing phase.

The second trial was conducted in finishing pigs, providing the first published evidence on the use of *T. molitor* meal during the final phase of swine production. Eight-teen pigs were allocated to three treatments: a control diet and diets including insect meal derived from conventional or enriched substrates. Throughout the experimental period, measurements were evaluated for growth performance, blood biomarkers, fecal microbial profiles, and meat quality characteristics. While growth performance was not significantly affected by insect meal supplementation at this stage, profound effects were observed on gut microbiota composition, including reductions in Enterobacteriaceae populations and increases in beneficial anaerobic bacteria. Notably, several meat quality characteristics improved, including higher antioxidant capacity, and increased collagen content in belly cuts.

A central innovation of this dissertation lies in the use of *T. molitor* larvae reared on phytochemical-enriched substrates. The enrichment of the rearing substrate with medicinal aromatic plant residues, rich in antioxidant and antimicrobial compounds, successfully transferred bioactive properties into the resulting insect meal. This led to measurable improvements in meat oxidative stability in both early-growing and finishing pigs, while enhancements in the fatty acid profile were observed in the early-growing pigs. These findings show a promising opportunity to create more targeted insect-based feed ingredients by enriching the larvae's substrate. This approach also supports the circular bioeconomy by turning plant by-products into valuable and more functional feed.

Another major contribution is the first-time evaluation of the combined use of *T. molitor* meal and chitosan in early-weaned pigs. The current trial demonstrates that the combination of bioactive feed ingredients administered during the post-weaning phase had synergistic improvements performance, gut microbiota, and meat oxidative stability. Such approaches directly align with the consumers' need to reduce antibiotic use.

Overall, this dissertation provides a comprehensive evaluation of insect meal and chitosan as innovative feed ingredients for swine nutrition. By improving zootechnical parameters, gut microbial profile, blood biomarkers, and meat quality characteristics, it offers a robust scientific proof supporting the usage of insect-derived proteins and functional bioactive compounds in pig diets. The results highlight that *T. molitor* larvae meal, particularly when produced on enriched substrates, can serve as a sustainable and nutritionally effective substitute to conventional protein sources. Furthermore, chitosan supplementation in early-growing pigs presents clear advantages that require further study in combination with different insect species.

In conclusion, this dissertation represents a significant contribution to establishing environmentally responsible and superior feed strategies for contemporary swine production. By successfully integrating insect meal and natural bioactive compounds, the findings open new avenues for simultaneously optimizing animal health and enhancing the quality of the produced meat. To fully understand this knowledge, future research should determine the ideal dosage, study the long-term effects on the pigs' health, and explain the path of nutrients and compounds moving from the feed, through the insects, and into the pigs.

Περίληψη

Η αυξανόμενη παγκόσμια ζήτηση για βιώσιμη και αποδοτική παραγωγή τροφίμων έχει ενισχύσει σημαντικά το ενδιαφέρον για καινοτόμες πηγές ζωοτροφών υψηλής διατροφικής αξίας. Τέτοιου είδους ζωοτροφές κρίνονται απαραίτητες προκειμένου να διατηρηθεί η παραγωγικότητα των ζώων, ενώ παράλληλα επιδιώκεται και η μείωση του περιβαλλοντικού αποτυπώματος της κτηνοτροφίας. Με τον παγκόσμιο πληθυσμό να αναμένεται να υπερβεί τα 10 δισεκατομμύρια έως το 2050, η πίεση στα γεωργικά και ζωικά παραγωγικά συστήματα εντείνεται, προκειμένου να εξασφαλισθούν πρωτεϊνικές πηγές υψηλής θρεπτικής αξίας, όπως το χοιρινό κρέας. Οι παραδοσιακές πηγές ζωοτροφών, όπως το σογιάλευρο και το ιχθυάλευρο, αντιμετωπίζουν σοβαρά προβλήματα που αφορούν τη διαθεσιμότητα, την αστάθεια των τιμών και το περιβαλλοντικό αποτύπωμα. Πιο συγκεκριμένα, η αποψίλωση των δασών για την εξυπηρέτηση των αναγκών καλλιέργειας σόγιας, η θαλάσσια υπεραλίευση που υπονομεύει την παραγωγή ιχθυάλευρου, οι γεωπολιτικές αστάθειες στο διεθνές εμπόριο, καθώς και οι κανονιστικοί περιορισμοί στη χρήση ζωικών υποπροϊόντων, δυσχεραίνουν περαιτέρω τη διαμόρφωση ασφαλών και σταθερών διατροφικών στρατηγικών, ιδίως εντός της Ευρωπαϊκής Ένωσης. Ως εκ τούτου, η διερεύνηση καινοτόμων πρωτεϊνούχων ζωοτροφών καθίσταται πλέον επιτακτική ανάγκη για τη διασφάλιση και την ενίσχυση των σύγχρονων συστημάτων ζωικής παραγωγής.

Την τελευταία δεκαετία, τα έντομα έχουν αναδειχθεί ως μια πολλά υποσχόμενη εναλλακτική λύση για την αντιμετώπιση των προκλήσεων στις ζωοτροφές, χάρη στην υψηλή διατροφική τους αξία, τον εξαιρετικό δείκτη μετατρεψιμότητας της τροφής και τη συμβατότητά τους με τις αρχές της κυκλικής οικονομίας. Οι προνύμφες *Tenebrio molitor* (σκουλήκι των αλεύρων) χαρακτηρίζονται από υψηλά επίπεδα εύπεπτης πρωτεΐνης, λιπιδίων, απαραίτητων αμινοξέων, βιταμινών, μετάλλων, καθώς και βιοδραστικών συστατικών όπως η χιτίνη και τα αντιμικροβιακά πεπτίδια. Οι πρόσφατες νομοθετικές ρυθμίσεις της Ε.Ε., με κορυφαία αυτή του 2021, η οποία επιτρέπει τη χρήση πρωτεϊνών από συγκεκριμένα είδη εντόμων, σε σιτηρέσια πτηνών και χοιρινών, έχουν επιταχύνει το ενδιαφέρον για την εφαρμογή τους στη διατροφή μονογαστρικών ζώων. Ιδιαίτερη έμφαση πρέπει να δοθεί στο ότι η θρεπτική και λειτουργική αξία των εντόμων μπορεί να τροποποιηθεί από το υπόστρωμα εκτροφής που χρησιμοποιείται. Αυτό αποτελεί ένα καινοτόμο ερευνητικό πεδίο, ωστόσο τα διαθέσιμα επιστημονικά δεδομένα παραμένουν περιορισμένα, ειδικά όσον αφορά την επίδραση των εμπλουτισμένων υποστρωμάτων στα σιτηρέσια των χοίρων.

Η χιτοσάνη, ένας αποακετυλιωμένος πολυσακχαρίτης που παράγεται από τη χιτίνη, έχει προσελκύσει έντονο επιστημονικό ενδιαφέρον ως λειτουργικό πρόσθετο ζωοτροφών. Διαθέτει πρεβιοτικές, αντιμικροβιακές και ανοσορρυθμιστικές ιδιότητες και μπορεί να βελτιώσει την εντερική μικροχλωρίδα, την υγεία του εντέρου και τους ρυθμούς ανάπτυξης των παραγωγικών ζώων. Σύμφωνα με την υφιστάμενη δημοσιευμένη βιβλιογραφία, η συνδυαστική χρήση αλεύρων εντόμων του είδους *T. molitor* και χιτοσάνης δεν έχει εξεταστεί ποτέ ξανά σε απογαλακτισμένα χοιρίδια.

Στα πλαίσια αυτής της διδακτορικής διατριβής, πραγματοποιήθηκαν δύο διατροφικοί πειραματισμοί: ένας σε απογαλακτισμένα χοιρίδια και ένας σε χοίρους τελικής πάχυνσης. Οι πειραματισμοί αξιολόγησαν την επίδραση του αλεύρου εντόμων *T. molitor*, το οποίο εκτράφηκε σε συμβατικό υπόστρωμα ή υπόστρωμα εμπλουτισμένο με αρωματικά και φαρμακευτικά φυτά της ελληνικής χλωρίδας, με ή χωρίς χιτοσάνη, στις ζωοτεχνικές επιδόσεις, στη μικροχλωρίδα του εντέρου, στους αιματολογικούς και βιοχημικούς δείκτες και στα ποιοτικά χαρακτηριστικά του παραγόμενου κρέατος.

Στον πρώτο διατροφικό πειραματισμό, 60 απογαλακτισμένα χοιρίδια τυχαία κατανεμήθηκαν σε πέντε ομάδες: μία ομάδα μαρτύρων, δύο ομάδες που έλαβαν άλευρο εντόμων *T. molitor* (συμβατικό ή εμπλουτισμένο), μία ομάδα με χιτοσάνη και μία ομάδα που διατράφηκε με τον συνδυασμό συμβατικού αλεύρου εντόμων και χιτοσάνης. Η συνολική διάρκεια του διατροφικού πειραματισμού ήταν 42 ημέρες. Τα αποτελέσματα έδειξαν ότι ο συνδυασμός *T. molitor* και χιτοσάνης βελτίωσε σημαντικά την ανάπτυξη των χοιριδίων, υποδεικνύοντας συνεργιστική δράση. Η μικροχλωρίδα του εντέρου τροποποιήθηκε σημαντικά, με μείωση παθογόνων βακτηρίων και αύξηση ωφέλιμων πληθυσμών, ενώ οι αιματολογικοί δείκτες παρέμειναν σε φυσιολογικά επίπεδα, με εξαίρεση την αύξηση της χοληστερόλης στην ομάδα που έλαβε το εμπλουτισμένο εντομάλευρο. Η ποιότητα του κρέατος βελτιώθηκε ως προς την αντιοξειδωτική ικανότητα, την περιεκτικότητα σε φαινολικές ενώσεις, και τους μικροβιακούς πληθυσμούς στα παραγόμενα τεμάχια κρέατος.

Ο δεύτερος πειραματισμός κάλυψε ένα σημαντικό κενό στη διεθνή βιβλιογραφία, εστιάζοντας στην πρωτοποριακή εφαρμογή του εντόμου *T. molitor* σε χοίρους τελικής πάχυνσης, μία προσέγγιση που δεν αναφέρεται σε προηγούμενες δημοσιευμένες μελέτες. Συνολικά 18 χοίροι κατανεμήθηκαν τυχαία σε τρεις ομάδες: μία ομάδα μαρτύρων, μία ομάδα όπου χορηγήθηκε

σιτηρέσιο με συμβατικό *T. molitor* και μία ομάδα όπου χορηγήθηκε σιτηρέσιο με το εμπλουτισμένο *T. Molitor*. Παρόλο που οι ζωοτεχνικές επιδόσεις δεν διαφοροποιήθηκαν σημαντικά, παρατηρήθηκαν ουσιαστικές μεταβολές στη μικροχλωρίδα του εντέρου, συμπεριλαμβανομένης της μείωσης των πληθυσμών Enterobacteriaceae και της αύξησης των ωφέλιμων αναερόβιων βακτηρίων. Επιπλέον, βελτιώθηκαν ποιοτικά χαρακτηριστικά του παραγόμενου κρέατος, όπως αυξημένη αντιοξειδωτική ικανότητα και ενισχυμένη περιεκτικότητα σε κολλαγόνο στα τεμάχια της κοιλιάς.

Η βασική καινοτομία της παρούσας διδακτορικής διατριβής έγκειται στην αξιοποίηση εντόμων που αναπτύσσονται σε εμπλουτισμένα υποστρώματα με αρωματικά και φαρμακευτικά φυτά της ελληνικής χλωρίδας, με στόχο τη μεταβίβαση λειτουργικών βιοδραστικών ιδιοτήτων αυτών στο παραγόμενο άλευρο εντόμων. Αυτό είχε ως αποτέλεσμα την ενίσχυση της οξειδωτικής σταθερότητας του παραγόμενου κρέατος και τη βελτίωση της λιπιδικής σύστασης αυτού. Εξίσου σημαντική καινοτομία αποτελεί η πρώτη λεπτομερής αξιολόγηση της συνδυαστικής χορήγησης αλεύρου εντόμων *T. molitor* και χιτοσάνης σε απογαλακτισμένα χοιρίδια. Αυτός ο συνδυασμός απέδειξε πολυδιάστατα οφέλη, βελτιώνοντας ταυτόχρονα την ανάπτυξη των απογαλακτισμένων χοιριδίων, την υγεία του εντέρου και τα ποιοτικά χαρακτηριστικά του κρέατος

Συνολικά, η παρούσα διδακτορική διατριβή παρέχει μια ολοκληρωμένη αξιολόγηση των λειτουργικών και διατροφικών δυνατοτήτων του αλεύρου εντόμων *T. molitor* και της χιτοσάνης στη σύγχρονη χοιροτροφία. Η έρευνα συμβάλλει ουσιαστικά στην ανάπτυξη βιώσιμων και καινοτόμων διατροφικών στρατηγικών. Τα ευρήματα καταδεικνύουν ότι άλευρο εντόμων αποτελεί μια βιώσιμη και θρεπτική πηγή πρωτεϊνούχων ζωοτροφών, ιδιαίτερα όταν προέρχεται από εμπλουτισμένα υποστρώματα, καθώς μεταφέρει βιοδραστικές ιδιότητες στο τελικό προϊόν, ενώ η χιτοσάνη αναδεικνύεται ως ένα αποτελεσματικό λειτουργικό πρόσθετο, παρουσιάζοντας ιδιαίτερη αξία στην ευαίσθητη φάση του απογαλακτισμού των χοιριδίων, όπου επιφέρει συνεργιστικά οφέλη στους ρυθμούς ανάπτυξης και στους βιοδείκτες υγείας.

Chapter 1: Introduction

1.1. Introduction

The swine industry is rapidly growing and has become one of the most dynamic areas in global animal agriculture. As of April 2024, there is an estimated 760.14 million pigs worldwide, with numbers slightly decreasing from around 778 million heads the previous year [1]. China leads by a wide margin with around 427.43 million pigs, accounting for approximately 45% of the global pig population, followed by the European Union with 132.14 million pigs (about 15%) and the United States with 76.35 million pigs (about 8%) [2, 3]. Other countries making notable contributions to global pig farming include Brazil, Russia, Canada, Mexico, and South Korea, which together with the top three countries hold the majority of the global pig population (Figure 1.1)[2, 3]. These numbers highlight the critical importance of pig farming in the global meat market and its significant economic impact in many regions.

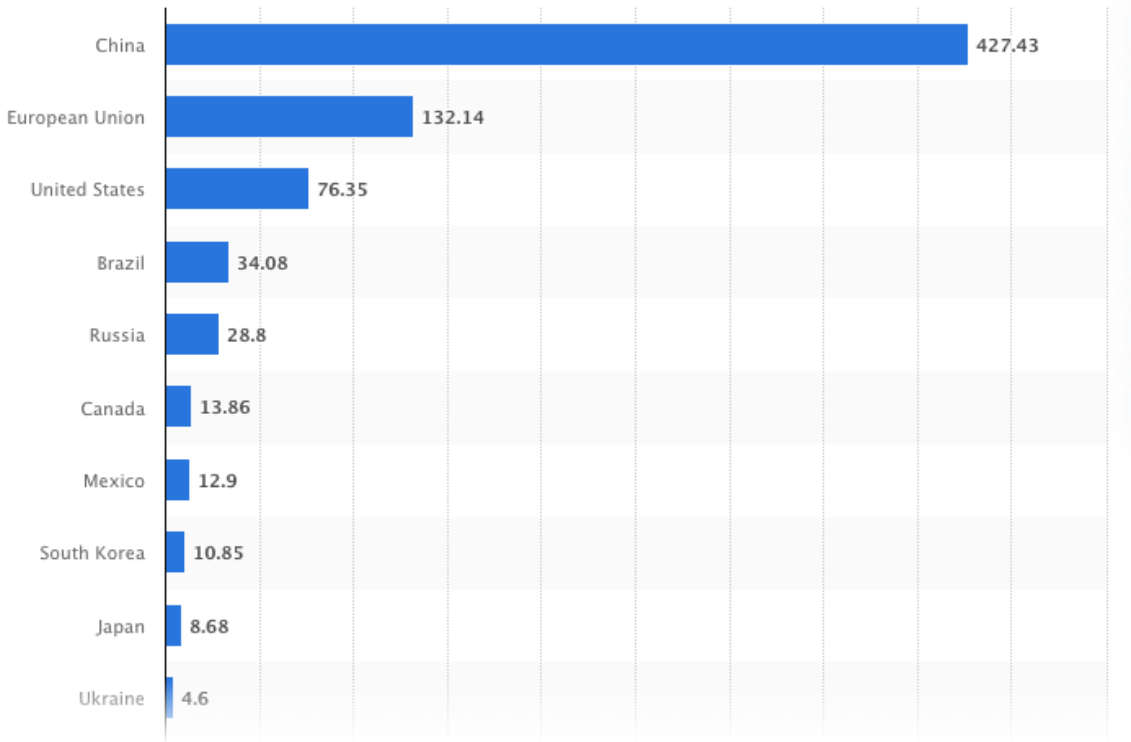

Figure 1.1. Number of pigs worldwide in 2025, by leading country (in million head)
Reference: Statista (2025)[2]

By 2050, the global human population is expected to reach approximately 9.7 billion, leading to a projected 56% surge in worldwide food demand [4]. This rapid population growth poses serious challenges for global food security, especially when it comes to ensuring a stable supply of animal-based proteins like meat [5]. Among livestock species, pigs are one of the most commonly raised animals and play a vital role in meeting protein needs across various regions and cultures [6]. The Food and Agriculture Organization of the United Nations [7] reports that pork is the most widely consumed meat in the world, accounting for about 34% of total meat consumption, followed closely by poultry at 33% and beef at 20%.

In 2019, the global pork meat market was valued at around $236.11 billion, and it's projected to grow to $257.87 billion by 2027, with a compound annual growth rate of 3.9% between 2021 and 2027 [8]. This growth is being driven by changing dietary preferences worldwide and better access to pork products for consumers. The wider availability of packaged pork has also helped boost sales across various markets. However, the increasing popularity of plant-based diets and the introduction of stricter animal welfare laws could present obstacles to further market expansion. On the other side, rising consumer interest in organic pork and clean-label options is expected to open up new opportunities for the pork industry [6].

The importance of the pig industry in today's society is better understood by examining the entire pig value chain [9]. This chain covers every stage, from raising and feeding pigs to processing pork and distributing it to consumers. At each point in the process, skilled professionals play a vital role, ensuring if the industry operates efficiently and remains profitable [10]. Beyond its contribution to food production, each part of the chain supports economic growth and generates valuable employment opportunities [11].

In 2021, pig meat ranked as the 119th most traded product globally, with a total trade value of $36.9 billion, representing a slight decline of 1.27% from $37.4 billion in 2020. This trade accounted for about 0.18% of all global commerce. The top exporters of pig meat were Spain ($6.47 billion), the United States ($5.84 billion), Germany ($3.96 billion), Canada ($3.27 billion), and Denmark ($3.19 billion). Meanwhile, the largest importers were China ($8.17 billion), Japan ($4.48 billion), Italy ($2.07 billion), South Korea ($1.77 billion), and Mexico ($1.62 billion) [12].

According to the United States Department of Agriculture (USDA, 2024) changes in regional demand and supply have strongly influenced global pork trade, creating larger trade gaps for

countries that rely heavily on imports [13]. An analysis of 2022 net export data, calculated as exports minus imports, presents significant trade deficits in several nations. For instance, Greece reported a net deficit of $532.9 million, a 17.2% increase compared to 2020, ranking 11th among countries with the largest pork trade deficits, just behind major importers like Japan, mainland China, and Italy [14].

1.1.1. The pig industry in the new era

As a major pillar of global livestock production, the swine sector is critical for securing an adequate supply of high-quality animal protein. However, contemporary pig production systems are increasingly constrained by a complex interplay of structural, biological, and socio-economic factors. Heterogeneous and progressively stringent regulatory frameworks, rapid but uneven adoption of new technologies, variability in housing, nutrition, and biosecurity practices, and shifting consumer expectations regarding animal welfare, environmental sustainability, and product safety collectively intensify the pressure on the industry [15-17].

1.1.2. Disease Management

One of the most critical challenges facing the swine industry is the control and prevention of infectious diseases. Diseases such as African Swine Fever (ASF) [18], Porcine Epidemic Diarrhea virus (PEDv) [19], and Porcine Reproductive and Respiratory Syndrome (PRRS) [20] have led to significant losses in pig populations around the world. These outbreaks not only compromise animal health but also create serious economic disruptions by reducing pork supply, inflating prices, and impacting trade flows. For example, ASF outbreaks in Asia and Eastern Europe have resulted in the culling of millions of pigs, severely affecting domestic production and global supply chains [21]. When outbreaks like these occur, they often lead to serious trade consequences, countries may impose import bans, livestock movement can be delayed, and producers are forced to invest heavily in biosecurity measures to control the spread [22].

1.1.3. Antibiotic Resistance

The swine industry has been recognized as a significant contributor to the global issue of antibiotic resistance. For years, antibiotics were commonly used in pig farming to promote growth and prevent disease [23, 24], but this practice has contributed to the emergence of antibiotic-resistant bacteria [25]. In response, many governments have introduced stricter regulations and revised policies to address the issue [26]. As a result, producers are increasingly turning to alternative

methods to maintain animal health and promote growth, such as improved biosecurity, vaccination programs, and feed additives [27-29].

1.1.4. Animal Welfare

In the contemporary era of widespread information access, consumers are more informed than ever about where their food comes from and the ethical practices behind its production. This heightened awareness has led to increasing concern about the humane treatment of farm animals [30]. As a result, certain industry practices, such as the use of gestation crates and procedures like tail docking and castration without anesthesia, have faced growing attention. These concerns pressed the swine industry to adopt more ethical, welfare-focused production standards [31].

1.1.5. Environmental Impact

The environmental footprint of the swine industry is receiving great attention as global concerns about climate change and ecological sustainability continue to rise. Key environmental challenges include greenhouse gas emissions [32], deforestation linked to the cultivation of feed crops [33], and water pollution from fertilizer runoff [34]. Addressing these issues is critical for the long-term sustainability of pig farming and maintaining public trust. As such, adopting proactive, science-based strategies is essential to minimizing the industry's environmental footprint and align swine production with broader environmental and societal goals [35, 36].

1.1.6. Feed Costs

Pig farming is closely linked to the grain market, making the industry especially vulnerable to fluctuations in grain prices [37]. Feed represents the largest cost in pig production, so price volatility can severely affect profitability [38]. As producers try to manage and forecast feed expenses in an unpredictable global market, this creates significant economic challenges. In response, many producers turn to strategies such as advance sales agreements, exploring alternative feed sources, and adopting methods to improve feed efficiency and reduce overall costs [39, 40].

1.1.7. Trade Restrictions

The worldwide supply of pork products is impacted by trade obstacles, which continue to present major difficulties for the swine industry [41]. These barriers arise from several factors, including export bans during disease outbreaks [42], variations in national food safety and animal welfare regulations [43], and the use of taxes and other restrictive trade restrictions [44].

1.1.8. Biosecurity

Biosecurity plays a critical role in disease prevention within the swine industry and is essential for maintaining both herd health and economic stability. To prevent the introduction and transmission of infectious agents between and within pig farms, strict biosecurity measures must be established [45]. Even a single mistake can lead to widespread outbreaks, threatening animal welfare and triggering major financial losses throughout the entire production chain [46]. As a result, the long-term sustainability and resilience of the industry rely heavily on the consistent application and evolution of strict biosecurity protocols [47].

1.1.9. Pigs and Related Global Food Systems: Benefits and Health Considerations

Pigs play a significant role in food security due to their efficient feed conversion ratio, turning feed into meat more effectively than many other livestock animals [48]. This efficiency helps in producing a larger amount of food from a limited resource. Their relatively short gestation period (about 114 days) and ability to produce large litters mean a faster supply of meat to the market [49]. Their adaptability to diverse rearing systems, from intensive commercial operations to smallholder farms, further underscores their value, particularly in developing regions [50]. Particularly in these countries, pigs are essential to family food security and profits, and they also provide manure, which improves crop yields and soil fertility [51]. Nutritionally, pork is a rich source of high-quality protein, B vitamins, and essential minerals such as iron and zinc [52]. However, in order to guarantee that the advantages can be achieved without sacrificing long-term viability, sustainable and humane practices must be adopted due to the environmental, ethical, and public health concerns of pork production.

On the other hand, the excessive and improper consumption of pork, especially in the form of processed meats like bacon and sausages, has raised notable public health concerns. The International Agency for Research on Cancer (IARC) has classified processed meat as carcinogenic to humans, linking it specifically to a higher chance of developing colorectal cancer [53]. Moreover, the high levels of sodium and preservatives commonly found in processed pork products have been linked to elevated risks of hypertension and cardiovascular diseases [54]. The consumption of undercooked pork also poses a parasitic risk, notably from *Trichinella spiralis*, which can lead to trichinosis, a serious foodborne illness [55]. Additionally, some pork cuts contain

a lot of saturated fat, which has been linked to higher cholesterol and a greater risk of heart disease [56].

1.2. Nutritional Needs of Growing and Finishing Pigs

1.2.1. Stages of Pig Growth

The growth and development of pigs can be categorized into distinct stages, each characterized by specific growth rates and metabolic transitions. During the weaning stage, which usually occurs around 3 weeks of age and last for several weeks, piglets exhibit daily weight gains of 200-500 grams [57]. During this period, piglets undergo a critical dietary transition from milk to solid feed, demanding significant adaptation of the digestive system, including enhanced enzyme production to efficiently break down complex carbohydrates and proteins [58]. In the growing stage (10-20 weeks), pigs experience accelerated muscle hypertrophy, with daily gains increasing to 500–800 grams. During this phase, metabolic energy is predominantly allocated toward lean tissue growth, and the gastrointestinal tract continues to develop to maximize nutrient absorption [57]. The finishing stage (from 20 weeks of age to market weight) is characterized by daily weight gains of 800–1100 grams or more, as pigs approach their target market weights, typically ranging between 90 and 120 kilograms [59]. As animals approach their genetic growth potential, lean tissue deposition slows down, and a greater proportion of nutrients is directed toward fat deposition [60].

1.2.2. Specific Nutritional Components Needed in Each Stage

Pigs, like other livestock species, pass through distinct growth stages, each characterized by specific physiological and metabolic changes that require targeted nutritional strategies. During the weaning stage, the transition from sow milk to solid feed represents a critical period requiring careful dietary formulation. Protein requirements are particularly elevated at this stage, ranging from approximately 20 to 24%, reflecting the vital role of amino acids in supporting rapid muscle growth and overall development [59]. Lipids also constitute an important dietary component, recommended to be included in the diet at 5 to 7%, to offset the lower energy content of solid feed compared to sow milk and satisfy the piglets' metabolic energy needs [61]. Concurrently, mineral nutrition plays a fundamental role in skeletal development, with calcium and phosphorus being essential macrominerals for bone mineralization. Trace minerals such as zinc and copper are supplemented due to their critical functions in maintaining intestinal integrity, modulating immune responses, and enhancing disease resistance [62]. Vitamins A, D, and E are equally indispensable,

supporting immune function, vision, and musculoskeletal development during this vulnerable phase. Additionally, specific amino acids, including lysine, methionine, and threonine, are emphasized for their indispensable roles in protein synthesis and metabolic regulation [63].

During the growing stage, pigs experience significant physiological and morphological changes that demand precise nutritional adjustments to support their development. Dietary protein levels should be moderated to approximately 16–18%, reflecting the ongoing requirements for muscle hypertrophy and skeletal growth (NRC, 2012). Concurrently, lipid inclusion is typically reduced to 3–5%, providing sufficient energy to sustain the animals' increased physical activity while maintaining dietary balance (NRC, 2012). A critical nutritional consideration at this stage is the maintenance of an appropriate calcium-to-phosphorus ratio, which is essential for optimizing bone mineralization and preventing metabolic skeletal disorders [64]. Vitamins and amino acids remain essential, but their amounts and ratios must be adjusted to align to the pigs' changing nutritional requirements [65].

By the finishing stage, the primary goal becomes preparing the pigs for market or breeding purposes. As such, protein intake should be reduced to around 13–15% to ensure a balanced muscle-to-fat ratio [59]. Fat content in the diet is moderated to 2.5–4% to support energy needs while limiting excessive fat deposition [59]. While energy requirements remain high, caution is exercised to avoid excess fat accumulation, which could negatively impact carcass and meat quality [66]. Continuous monitoring of mineral, vitamin, and amino acid intake is essential to ensure pigs reach their growth targets, maintain health, and produce high-quality meat [65, 67].

1.3. Traditional Diets and Their Limitations

1.3.1. Common Ingredients in Swine Diets

Corn (maize) is one of the most widely used cereal grains in swine nutrition, valued for its high energy content, excellent digestibility, and consistent nutrient profile. The primary energy source in corn is starch, which supports the metabolic demands of growing and finishing pigs [68]. On average, corn contains approximately 8–9% crude protein, 3.5–4.5% fat, and 70–72% carbohydrates. It also contains moderate amounts of essential B vitamins, including niacin, pantothenic acid, and folate, as well as minerals such as phosphorus and magnesium [59]. While it is relatively low in lysine and other essential amino acids, corn pairs effectively with protein-rich ingredients like soybean meal to create a balanced diet [67]. Its palatability, global availability,

and compatibility with feed processing technologies, make it a key ingredient in modern swine feeding programs [69]. As a result, corn remains the main feedstuff for cost-effective and nutritionally balanced pig diets around the world.

Barley serves as another valuable grain in swine feeding formulations. While it provides substantial energy mainly through carbohydrates, barley generally has slightly lower energy content than corn due to its elevated fiber content [70]. With a protein content of about 10–12%, barley offers a suitable amino acid composition for pigs [59, 71]. However, its utilization requires careful management due to high beta-glucan levels, a type of soluble fiber known to increase digestive viscosity and potentially impair nutrient absorption [72]. Effective incorporation of barley into pig diets often requires processing methods, such as grinding, and strategic formulation depending on barley variety and pig age [71].

Soybean meal is recognized prominently as the main protein source in pig feed formulations, characterized by a protein content ranging between 44–48% [59]. This plant-based ingredient contain essential amino acids such as lysine, tryptophan, and threonine, indispensable for muscle growth and development [73]. Additionally, soybean meal contributes significantly to mineral content, notably phosphorus and potassium, and provides substantial levels of B vitamins [59]. Together with corn, soybean meal serves as a key dietary component, addressing the energy and protein requirements of swine.

Fishmeal, derived from processed fish or by-products, has traditionally been utilized as a superior protein supplement in pig feeds [74]. It features an exceptional amino acid profile, including critical amino acids like lysine, methionine, and threonine [59], and also provides valuable omega-3 fatty acids, vitamins, and minerals [75]. However, the challenges associated with sustainability, fluctuating quality, and rising costs have led to more limited inclusion rates in current pig feeds [76]. Therefore, careful selection and strict quality controls are essential to ensure nutritional benefits while keeping animals healthy.

1.3.2. Limitations of Traditional Diets

Price fluctuations of primary feed ingredients, particularly corn and soybean meal, presents significant economic challenges for swine producers. Factors influencing these variations include climatic conditions, global demand shifts, trade policies, and biofuel production requirements [77].

Due to the significant dependence on these ingredients, fluctuations in their prices can substantially impact farm profitability and long-term viability.

1.3.3. Environmental Impact of Large-Scale Crop Farming

Extensive cultivation practices, notably soybean farming in South America, are major factors of deforestation, leading to severe biodiversity loss [78]. Furthermore, substantial water resources are required for corn and soybean cultivation, intensifying pressure on freshwater resources [79]. Other environmental issues include water pollution from pesticides and fertilizers runoff, threatening aquatic ecosystems [80], and soil degradation caused by nutrient loss from repeated farming [81].

1.3.4. Potential for Disease Transmission

Corn is highly vulnerable to fungal contamination that can lead to mycotoxin production, negatively affecting swine health by reducing growth rates and increasing disease susceptibility [82, 83]. Similarly, raw soybeans contain anti-nutritional substances such as trypsin inhibitors, lectins, and phytates, which reduce nutrient absorption. Although heat treatment and other processing methods can significantly reduce these compounds, they cannot be completely removed [84].

1.4. The Rise of Alternative Protein Sources: An Overview

1.4.1. Microalgae

Alternative protein sources are becoming increasingly important in swine nutrition as producers seek more sustainable, cost-effective, and reliable options to replace traditional proteins like soybean meal and fishmeal [85]. Among the most promising alternatives are microalgae such as *Spirulina* and *Chlorella*, which contain remarkably high protein levels, typically ranging from 50% to 70%, along with essential fatty acids, vitamins, and trace minerals [86, 87]. Beyond their nutritional value, algae production offers notable sustainability benefits. These microorganisms can be cultivated on non-arable land, require significantly less freshwater than conventional crops, and produce a considerably smaller environmental footprint [88]. In addition, certain algae species are natural sources of omega-3 fatty acids, which contribute to improved animal health and potentially enhance meat quality [89].

Despite their potential, several challenges limit the widespread adoption of algae as a primary protein source in pig diets. The high costs associated with algae production, especially in closed systems like photobioreactors, creates barriers to commercial scalability [90]. Moreover, algae's unique sensory characteristics can impact feed palatability, which may reduce feed intake and performance in swine if not properly managed [91, 92]. Further research is needed to optimize algae processing, improve flavor profiles, and investigate long-term effects on pig health and productivity.

1.4.2. Rapeseed Meal

Rapeseed meal, a major by-product of vegetable oil and biofuel production [93], has gained increasing attention as a locally available protein source for pig diets. With a crude protein content ranging from 33.7% to 35.6% (as fed) [94], Rapeseed meal offers a valuable opportunity to diversify feed formulations and reduce dependence on imported protein ingredients. However, its use in swine nutrition has traditionally been limited by high levels of fiber and several anti-nutritional factors, including glucosinolates, sinapine, tannins, and erucic acid [95, 96]. Glucosinolates are of particular concern, as their degradation products can decrease feed intake, alter thyroid hormone synthesis [96], and consequently affect metabolism and performance. Indeed, even when total glucosinolate levels fall below the recommended limit of 2.1 mmol/kg, pigs fed rapeseed meal have shown increased thyroid gland weight [93]. Phenolic compounds such as tannins can further reduce protein digestibility and disrupt protein metabolism [97]. Similar physiological effects, including elevated thyroid weight but normal circulating thyroid hormone levels, have been reported in pigs receiving 6–10% rapeseed meal during the growing–finishing period [98]. Nevertheless, considerable progress in plant breeding has led to the development of improved rapeseed varieties, characterized by very low erucic acid and glucosinolate concentrations in the defatted meal [94].

1.4.3. Grain Legume Seeds

Grain legumes, including fava beans, peas, lupins, and chickpeas, represent an important category of alternative protein sources for pig nutrition. These seeds provide moderate protein levels (20–30% CP), substantial amounts of soluble and insoluble fiber, slowly digestible starch, essential micro- and macronutrients, vitamins, and various bioactive phytochemicals such as flavonoids and other antioxidants [99]. Among them, yellow lupin contains the highest crude protein

concentration (324–381 g/kg DM), although most legumes are characteristically low in sulfur-containing amino acids (methionine and cystine) and, in some cases, tryptophan when compared with soybean meal [99]. Their broader use in pig feeding has traditionally been constrained by the presence of anti-nutritional factors, including tannins, protease inhibitors, alkaloids, lectins, pyrimidine glycosides (vicine and convicine), and saponins [99, 100]. These compounds can impair palatability (tannins, alkaloids), reduce nutrient digestibility (tannins, lectins, protease inhibitors), or exert toxic effects (alkaloids), while high levels of galactosides may lead to flatulence, excessive fermentation, and diarrhea in pigs [99]. Many legumes, particularly lupins, also contain considerable amounts of non-starch polysaccharides, which can slow digesta passage and depress feed intake and growth performance, whereas the high neutral detergent fiber content of their hulls further reduces nutrient digestibility [97, 100]. Additionally, the complex structural conformation of legume proteins may contribute to their reduced susceptibility to proteolysis, thereby limiting their digestibility [99, 100]. Nevertheless, recent advances in plant breeding have produced cultivars with improved nutritional profiles [99], and several processing techniques—including decortication, soaking, extrusion, cooking, germination, and enzyme supplementation—have been shown to effectively enhance their nutritional value [99, 101].

1.4.4. Microbial Cell Protein

Microbial cell protein, derived from bacteria, fungi, yeasts, or algae, has gained increasing attention as a sustainable alternative protein source due to its rapid production rates and the ability to utilize low-cost substrates such as food-industry by-products, agricultural residues, and forestry waste [102]. A well-studied methane-oxidizing microbial consortium composed of *Methylococcus capsulatus*, *Alcaligenes acidovorans*, *Bacillus brevis*, and *Bacillus firmus* can efficiently convert methane into high-quality biomass with minimal dependence on soil and water resources [103]. Microbial cell protein typically contains around 70% crude protein [104] and provides an amino acid profile comparable to fishmeal and soybean meal [103], although with slightly lower lysine and higher tryptophan concentrations [105]. Several studies have demonstrated its nutritional value for growing pigs [106-108], and recent evidence indicates that microbial cell protein may offer superior standardized ileal digestible essential amino acid content relative to digestible lysine compared with fishmeal [105]. Nevertheless, research on its use in weaned piglets remains limited [109, 110].

Despite concerns regarding its high nucleic acid content, typically a constraint for human consumption due to the risk of hyperuricemia, gout, and kidney stones [111, 112], these issues are largely irrelevant in pigs. Unlike humans, pigs efficiently convert uric acid to allantoin, which is readily excreted [113]. Moreover, nucleotides play an essential role in periods of rapid cellular turnover, including intestinal development and immune function in young piglets. Consequently, dietary nucleotides from microbial cell protein may confer beneficial effects during the vulnerable post-weaning period by supporting gut maturation, immune competence, and metabolic resilience [114, 115].

1.4.5. Cultured Meat

Cultured meat, an innovative protein obtained by cultivating animal cells in controlled environments, has the potential to produce foods like seafood and organs [116]. While it promises to match conventional meat in nutrition and sensory properties in order to address ethical and environmental issues [117, 118], its future viability relies on overcoming significant technological challenges regarding cell lines, culture media, and scalable bioreactors [119]. Although industrial-scale production remains difficult, regulatory progress is moving quickly, with recent approvals for human and animal consumption approved in Singapore, the United States, Israel, Hong Kong, and the United Kingdom [120-123]. It is important to highlight that, based on current literature, data regarding the use of cultivated meat exists only for pet feed applications, and not for its use in monogastric animals.

1.4.6. Insects

Insects are becoming a promising alternative to conventional livestock feed ingredients like fishmeal and soybean meal. Their use in animal diets supports more sustainable production by turning low-value organic waste, such as fruits, vegetables, and even manure, into high-quality protein [124]. In addition to their nutritional and health benefits for animals, insect farming has a much smaller environmental footprint. When raised on organic waste, insects generate fewer greenhouse gas emissions and require significantly less land and water compared to traditional feed crops. One of their greatest advantages lies in their impressive feed conversion efficiency, which is linked to their poikilothermic physiology. However, it’s worth mentioning that insect farming can sometimes demand more energy than growing conventional feed crops [124].

Among the most commonly farmed insect species for animal feed are black soldier fly larvae (*Hermetia illucens*) and mealworms (*Tenebrio molitor*). In aquaculture, insect-based meals have shown strong potential, with inclusion rates of up to 30% performing comparably to traditional fishmeal without negative effects [125]. In poultry diets, replacing conventional protein sources with up to 30% insect meal has also proven effective; however, higher levels may lead to nutritional imbalances or metabolic concerns [126, 127]. For pigs, inclusion rates between 10% and 20% of insect-based ingredients, such as yellow mealworm or black soldier fly, are generally recommended [128]. In the pet food industry, which accounts for about 3% of global feed use, insects are gaining popularity as a protein source, largely due to their hypoallergenic qualities [124].

The nutritional quality of insect-based feed can vary widely, influenced by factors such as the insect species, its stage of development, the type of rearing substrate, and the processing methods used [129]. Because of this variability, it is essential to ensure consistent nutrient profiles for reliable feed performance. Although insects show a great potential as a sustainable protein source, current production levels are still too low to meet the global demand for animal feed. Cost is another challenge at present, as insect meal tends to be more expensive than conventional feed ingredients. To address this, researchers suggest using underutilized organic waste as rearing substrates, which could help reduce production costs and improve sustainability [130]. In addition, insect farming faces unique challenges that differ from traditional livestock systems, including the need for better automation, more efficient bioconversion processes, effective disease management strategies, and clearer research and regulatory frameworks [131].

Regulations around insect-derived feed products are evolving in response to growing interest in alternative protein sources. Within the European Union, the use of insects in aquaculture feeds was approved in 2017, and this authorization was extended to include poultry and swine feeds in 2021 [132]. Following this extension, the use of insect-based animal proteins from eight specific insect species was formally approved by EU Member States in April 2021. The authorized species were *Musca domestica* (L.), *T. molitor* (L.), *H. illucens* (L.), *Alphitobius diaperinus* (Panzer), *Acheta domesticus* (L.), *Bombyx mori* (L.), *Gryllodes sigillatus* (Walker), and *Gryllus assimilis* (F.) [132].More recently, in 2024, the European Commission clarified that live insects may also be used as feed for non-ruminant livestock, such as pigs and poultry, provided they are reared on

substrates that meet EU feed legislation requirements [133]. Industry groups and ongoing research into pathogen transmission continue to shape regulatory developments. Despite progress, achieving uniformity in the quality and supply of insect-based feeds remains a persistent challenge. To solve this issue, the industry needs to safely use organic waste and have clear, science-based regulations [124].

However, despite their inclusion in the list of authorized species, scientific literature regarding the specific application of *A. domesticus* (L.), *G. sigillatus* (Walker), and *G. assimilis* (F.) in swine nutrition is currently unavailable. In contrast, there is a significant number of experimental studies documenting the use of *Ptecticus tenebrifer* and *Zophobas morio* in pig diets, offering valuable insights into their potential as alternative feed ingredients.

1.4.7. *Hermetia illucens* Larvae

The Black Soldier Fly (*Hermetia illucens*) has emerged as one of the most promising alternative protein sources for sustainable pig nutrition due to its favorable amino acid profile and high nutritional value [134]. *H. illucens* larvae and prepupae products, whether full-fat, partially defatted, or fully defatted, contain substantial crude protein (35.9%–48.1% in full-fat meal; approximately 51%–59% in defatted meals) and ether extract levels ranging from 9% to 48% [74, 135-139]. Their standardized ileal digestibility values for key amino acids such as lysine, methionine, and threonine are comparable to soybean meal, with only slight reductions reported for arginine and methionine depending on fat removal [139]. Across multiple swine studies, inclusion of *H. illucens* larvae meal, typically at 8%–10%, has not negatively influenced growth performance, gut morphology, or blood parameters in weaned pigs, and in some cases has enhanced feed efficiency, diet palatability, and nutrient digestibility [137, 138, 140]. In growing and finishing pigs, partial or complete replacement of fishmeal or soybean meal with *H. illucens* larvae meal has maintained or improved final body weight, average daily gain, carcass traits, and even sensory attributes such as flavor and juiciness of pork loin [74, 141-143].

Beyond their nutritional contributions, *H. illucens* larvae exert functional benefits on gut health and immunity, further supporting their inclusion in swine diets. Diets containing *H. illucens* larvae meal (4%–10%) have been shown to beneficially modulate the cecal and colonic microbiota, increasing populations of *Lactobacillus* and short-chain fatty acid–producing bacteria, thereby enhancing gut maturity and intestinal barrier integrity [144-146]. Such responses include higher

concentrations of butyrate, upregulation of anti-inflammatory cytokines, and increased expression of tight-junction proteins such as zonula occludens-1 and occludin [144]. Importantly, *H. illucens* supplementation does not trigger systemic inflammatory responses and does not impair villus structure or mucosal health in piglets [137, 146]. These combined nutritional and functional effects position *H. illucens* as a sustainable, health-promoting ingredient for modern pig production systems. Nevertheless, future research is needed to define optimal inclusion levels, evaluate long-term effects on pork quality, and further explore consumer acceptance and industry-scale feasibility.

1.4.8. *Musca domestica* Larvae

The housefly (*Musca domestica*), commonly known as the maggot, has emerged as another highly promising insect species for use in livestock nutrition due to its biological efficiency, rapid life cycle, and capacity to grow on a wide range of organic waste materials, making it a particularly sustainable source of high-quality protein [147]. *M. domestica* larvae meal is characterized by a robust nutritional profile, containing on average 50% crude protein, with reported values ranging from 39.2% to 64.0%, as well as substantial ether extract levels averaging 23.5% (20.8%–25.3%) [147]. Such nutrient richness suggests strong potential for replacing more conventional animal-derived proteins. Indeed, experimental studies in pigs support this: Dankwa et al. (2000) demonstrated that the substitution of fishmeal (10.8%) with 10% *M. domestica* larvae meal in diets for weaned pigs over a 10-week period resulted in no adverse effects on growth performance or carcass characteristics, indicating that *M. domestica* larvae can effectively maintain productivity when used as a major protein replacement [148]. Beyond basic nutrient content, *M. domestica* larvae also exhibit favorable amino acid digestibility characteristics. Tan et al. (2020) reported that *M. domestica* meal contains higher levels of amino acids, crude protein, and ether extract than *H. illucens* meal, while standardized ileal digestibility values for nearly all amino acids, except methionine and cysteine, were superior in *M. domestica* meal compared with *H. illucens* meal [149]. These findings collectively highlight *M. domestica* larvae as a nutritionally valuable and environmentally sustainable alternative protein source, with promising applicability in swine feeding programs [134].

1.4.9. *Alphitobius diaperinus* Panzer

Alphitobius diaperinus is often regarded as a poultry pest [150], it develops faster than *T. molitor* [151] and offers a nutritional profile superior to soybean meal. Specifically, its larvae are rich in crude protein (57.7g/100g DM) and lipids (26.3g/100g DM), with essential amino acid levels, such as lysine (37.5g/kg DM) and methionine (14.6 g/kg DM) [152]. In a study on 48 piglets where *A. diaperinus* meal replaced soybean meal up to 9%, no adverse effects on growth or meat quality were found. The only observed change was a linear increase in polyunsaturated fatty acids in backfat, supporting *A. diaperinus* meal as a safe alternative protein source [152].

1.4.10. Silkworm pupae

Silkworm pupae, known by various names such as Silkworm pupae meal, defatted or non-defatted meal, and specific regional varieties like Eri or Muga [153], represent a significant by-product of the silk industry. While 90% of global production derives from the domesticated mulberry silkmoth (*Bombyx mori*) [153], silk is also produced from Saturniidae species such as *Samia cynthia ricini* and *Antheraea* spp. [154, 155]. During processing, pupae are killed via boiling, drying, or NaOH treatment to preserve the cocoon [156, 157], generating approximately 8 kg of wet pupae for every 1 kg of raw silk [156]. Nutritionally, the meal is protein-dense (50% to >80% DM) with notably high lysine (6-7% of protein) and methionine (2-3% of protein) levels, though it contains fewer minerals (3-10% DM) than other animal by-products [158]. In pig nutrition, research indicates Silkworm pupae meal can effectively replace conventional proteins; Brazilian study showed that replacing 100% of soybean meal with undefatted Silkworm pupae meal maintained growth performance, though substitution rates above 50% reduced intake, likely due to palatability or energy density, while improving feed conversion [159]. Furthermore, specific trials with Muga silkworm pupa powder in Large White Yorkshire grower pigs demonstrated that replacing soybean meal at 2% and 4% levels significantly improved average daily gain and feed conversion efficiency [160]. This substitution not only reduced feed costs per kg of gain but also positively influenced nutrient digestibility and hematological parameters, including red blood cells and hemoglobin counts.

1.4.11. *Ptectious tenebrifer* Larvae

The larvae of *Ptecticus tenebrifer*, commonly referred to as mealworms in some regions, have also gained attention as a potentially valuable alternative protein source in swine nutrition. Their

nutrient composition is characterized by 48.2% crude protein, 29.5% ether extract, 6.06% crude fiber, 6.82% crude ash, 5.4% total lysine, and 1.5% total methionine on an as-fed basis, indicating a well-balanced profile of essential amino acids and energy-rich lipids [161]. This composition places *P. tenebrifer* larvae among the insect species with considerable potential to replace traditional protein ingredients such as fishmeal in pig diets. Supporting this, Ao and Kim [161] reported that the inclusion of 2% dried *P. tenebrifer* larvae, used to replace an equivalent proportion of fishmeal in weaned pig diets, did not negatively affect growth performance over a five-week feeding period. Furthermore, the apparent total tract digestibility of dry matter, nitrogen, and gross energy remained unchanged, demonstrating that the nutritional efficiency of the diet was maintained.

1.4.12. *Zophobas morio* Larvae

The superworm (*Zophobas morio*), also known as the giant mealworm, is a member of the darkling beetle family and is traditionally reared as feed for birds and reptiles, but it has recently attracted interest as a potential protein source for swine diets. Nutritionally, *Z. morio* larvae contain 7.4% total nitrogen, 39.3% ether extract, 5.3% crude ash, 2.7% total lysine, 8% total methionine, 11.2% neutral detergent fiber, and 6.4% acid detergent fiber on a dry matter basis, illustrating a lipid-rich profile with notable levels of essential amino acids [162]. Their protein digestibility also appears highly favorable: the apparent ileal digestibility values of amino acids and crude protein from diets containing 5% *Z. morio* larvae were comparable to those obtained with plasma protein, *T. molitor* larvae, and black soldier fly larvae when tested in early-weaned pigs at day 28 [136]. Similarly, in weaned pigs at day 56, no differences were observed in the ileal digestibility of dry matter, amino acids, or crude protein between diets containing 5% *Z. morio* larvae and those containing *5% plasma protein*, indicating that *Z. morio* can match the digestibility of high-quality conventional protein sources. Beyond digestibility, functional benefits have also been reported. Liu et al. (2020) demonstrated that supplementation of 5% *Z. morio* larvae modulated the activation of mRNA expression related to sodium-coupled neutral amino acid transporter 2 through the mTOR signaling pathway, suggesting enhanced intracellular amino acid transport and potential impacts on metabolic regulation [163].

1.4.13. *Tenebrio molitor* Larvae

T. molitor larvae, among other insect species, are well-recognized for their favorable nutritional properties, including high levels of protein and fat [164-166], good digestibility [167, 168], and acceptability [169, 170]. They also offer functional health benefits due to components such as chitin and antimicrobial peptides, which contribute to immune modulation and pathogen resistance [171]. *T. molitor* larvae are relatively easy to breed and possess a consistent protein composition, making them suitable for stable production [151]. As a result, they are widely reared for use in pet foods, zoo animal diets, and as feed ingredients for aquaculture species, pigs, and poultry [169, 172].

T. molitor, a globally distributed pest that affects flour, grains, and stored food products, belongs to the darkling beetle family [172]. Its life cycle consists of four distinct stages: egg, larva, pupa, and adult. Under optimal conditions at 25°C, females can lay approximately 500 eggs, which typically hatch into larvae within 3 to 9 days [173-175](Figure 1.2). The larval phase, characterized by a light yellow-brown coloration, can last from 1 to 8 months depending on environmental factors [173, 174]. At a temperature of 18°C, the pupal stage lasts approximately 5 to 28 days, while adults generally live for 2 to 3 months [173, 175]. Larvae typically reach lengths of 2.0–3.5 cm or more [176], whereas adult beetles can grow up to about 1 cm in length [173].

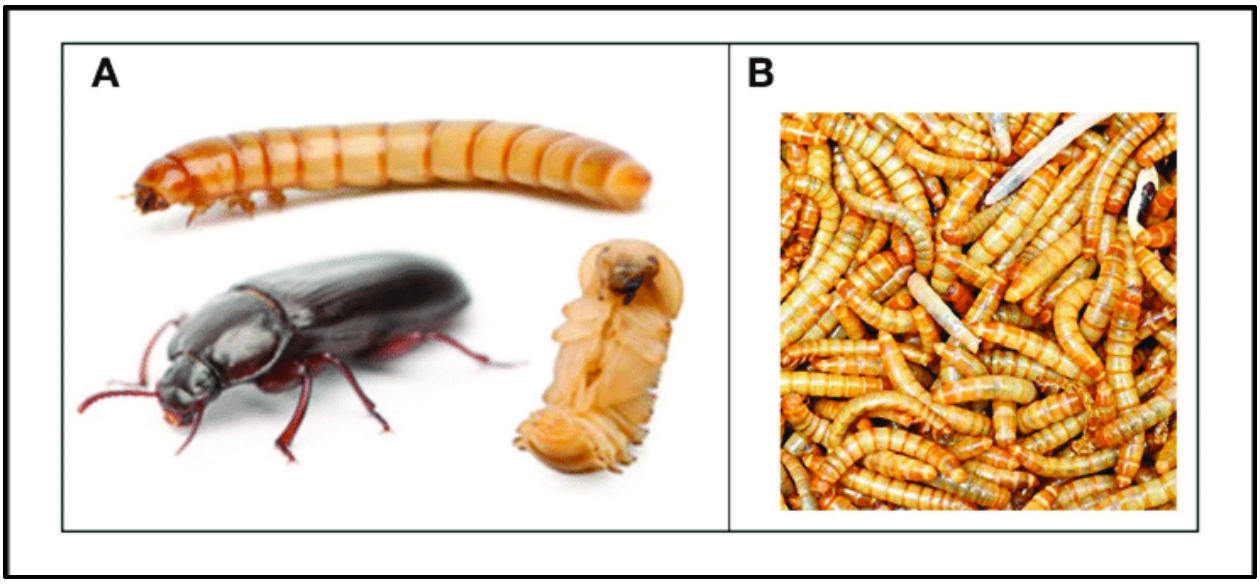

Figure 1.2. The mealworm *T. molitor*. Life cycle showing larva, pupa and adult.
Reference: De Loof et al. (2019)[177]

T. molitor larvae have an omnivorous diet that includes both plant and animal-based materials, such as meat and feathers. A protein content of approximately 20%, derived from ingredients like soybean flour, skimmed milk powder, and yeast, is generally recommended for optimal growth [172, 176, 178]. In industrial rearing, their diet typically consists of cereal by-products such as wheat, oats, or maize bran, supplemented with fruits and vegetables to ensure hydration. Although the basic nutritional composition of *T. molitor* larvae, specifically protein, fat, and moisture, remains consistent when fed cereal-based diets [172], some dietary modifications can influence the fatty acid profile. For instance, diets rich in unsaturated fatty acids can increase levels of caprylic acid [179], while feeding on plant waste can lead to higher protein and lower fat content [174].

Studies have demonstrated that the larvae's overall composition is significantly affected by diet type [180], emphasizing the importance of dietary optimization to achieve desirable nutritional results. Notably, *T. molitor* larvae exhibit a feed conversion ratio (FCR) of approximately 3 on high-protein diets, making them comparable to poultry (FCR: 2.0) and pigs (FCR: 3.6), and substantially more efficient than beef cattle (FCR: 7.8) [151]. Furthermore, because nearly 100% of the insect body is edible, the adjusted FCR of *T. molitor* surpasses the effective edible yield of both poultry (1.7) and pork (2.3) [181].

The composition of the larval diet also plays a crucial role in determining reproductive efficiency and growth performance. Rumbos et al. (2020) observed enhanced reproductive outcomes and larval development in diets rich in carbohydrates, such as wheat bran and white flour [180]. Therefore, formulating the feed composition for *T. molitor* is essential for achieving both nutritional quality and efficient large-scale production [182].

1.4.14. Processing of *Tenebrio molitor* Larvae

According to the IPIFF (2019), insect processing for animal feed typically follows a two-step approach: first, slaughtering, commonly done by heating or freezing, and then post-slaughter processing, which involves drying and grinding the insects into meal [183]. These steps are critical for both ensuring food safety and preserving the nutritional value of the final product. During the slaughter phase, methods like blanching, freezing, chilling, or drying are often used, each helping to improve shelf life and make storage and transport of *T. molitor* larvae more efficient. An increasing number of studies are focused on identifying processing methods that effectively ensure safety while preserving key nutrients.

After slaughter, the drying stage is particularly critical due to the larvae's high initial moisture content—approximately 68%—which can promote enzymatic degradation, microbial spoilage, or oxidative changes [184]. To prevent these issues, it's generally recommended to reduce the moisture content to about 4–5% [185]. Several drying methods are commonly used for this purpose, including oven drying, vacuum drying, freeze-drying, and microwave drying [184, 186, 187]. According to Kröncke et al. (2019) the nutritional value of *T. molitor* larvae remains consistent across these different drying techniques [184]. Moreover, each method effectively reduced water activity to levels that inhibit microbial growth [188].

Prior to grinding, *T. molitor* larvae may undergo additional treatments such as defatting or hydrolysis (Figure 1.3). These treatments allow the larvae to be used in different forms as animal feed ingredients, including whole ground (full-fat) meal [169, 172], defatted meal, or hydrolyzed meal [168]. Defatting is particularly important for enhancing storage stability and processing efficiency, as full-fat larvae contain high levels of fat (25–35%) and fatty acids (10–30%), which are prone to oxidation during drying and storage [189]. A variety of defatting methods are employed, including mechanical pressing, organic solvent extraction, and supercritical CO_2

extraction, each offering different levels of fat removal efficiency and varying impacts on the preservation of nutrients. [190-192].

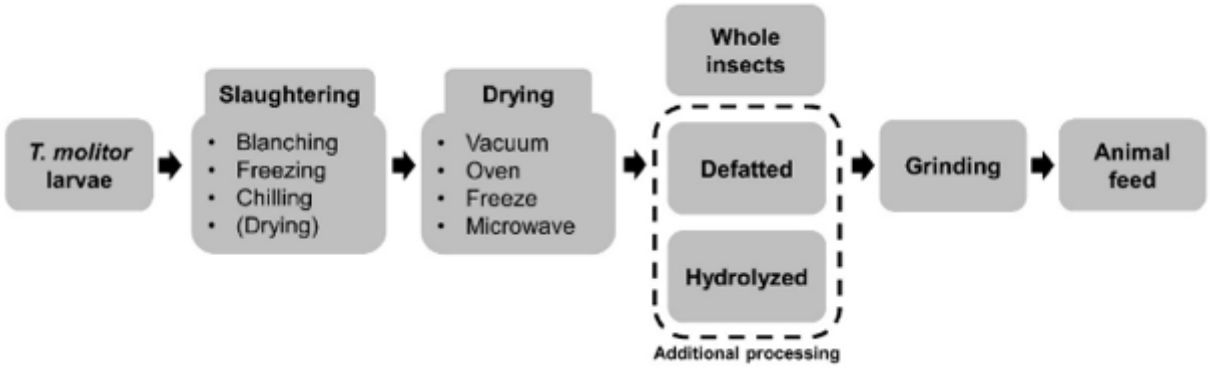

Figure 1.3. Scheme of *T. molitor* larvae processing for animal feed.

Reference: Hong et al. (2020) [182]

1.4.15. Nutritional Profile of the *T. molitor*

T. molitor larvae are becoming an increasingly popular ingredient in animal feed, largely due to their higher production efficiency compared to adult insects. Before being incorporated into feed formulations, the larvae are typically dried and ground, a process that not only extends shelf life but also improves their ease of handling and mixing. Additionally, oil extraction from the larvae produces a nutrient-rich by-product known as larval meal, which can serve as a valuable supplementary feed ingredient [182].

Meal produced from *T. molitor* larvae is notably rich in crude protein, typically ranging from 44% to 69% on dry matter basis, with an average content of about 52.4% [165]. This value exceeds that of soybean meal, which averages 49.4%, but remains below fishmeal, which contains about 67.5% crude protein [59]. However, it is important to note that conventional protein estimates for insects often use a nitrogen-to-protein conversion factor (kp) of 6.25, which may overestimate true protein content due to the presence of non-protein nitrogen components, such as chitin [193]. Chitin, a structural polysaccharide composed of glucosamine and N-acetylglucosamine, is indigestible and inflates nitrogen values. Recent studies propose using alternative kp values for more accurate estimation, including 4.74 [194], 4.75 [195], and 5.41 [196]. Using the kp value of 5.41 for these larvae, their crude protein content averages 47.2%, varying from 43.9% to 51.0% [182]. This

corrected value of protein level closely matches with soybean meal and remains below that of fishmeal [59].

In terms of lipid composition, the average crude fat content of *T. molitor* larvae varying from 23% to 47% on dry matter basis, depending on the processing method [165]. These levels are substantially higher than those found in soybean meal (3%) and fishmeal (11-17%) [178]. Conversely, the larvae have an average crude ash content of 4.2% on dry matter basis, which is lower than soybean meal (7.2%) and fishmeal (17.2%), with reported values ranging from 2.65% to 6.99% [59, 197].

T. molitor larvae contain measurable levels of dietary fiber, typically evaluated through parameters such as crude fiber, acid detergent fiber (ADF), and neutral detergent fiber (NDF) [198, 199]. The primary source of fiber in these larvae is the chitin-rich cuticle. On average, the crude fiber content of *T. molitor* larvae is 7.43% on dry matter basis, with values ranging between 4.19% and 22.35% [197]. Reported ADF and NDF contents are approximately 7.20% (as fed basis) and 17.4% (on dry matter basis), respectively [200, 201].

The fibrous structure of *T. molitor* larvae consists predominantly of chitin, interlinked with proteins, lipids, and other biological components [202]. Chitin is a linear polysaccharide composed of β-(1-4)-linked N-acetyl-D-glucosamine units, structurally analogous to cellulose, which is composed of β-(1-4)-linked D-glucopyranose units. Because chitin is similar to cellulose, ADF measurements, when adjusted for amino acids, can be used to estimate how much chitin insects contain [203].

T. molitor larvae are notable for their rich and well-balanced protein and amino acid profile, positioning them as an environmentally sustainable alternative to conventional protein sources such as soybean meal and fishmeal [182]. Among the essential amino acids, leucine, valine, and lysine are the most abundant, while histidine, methionine, and tryptophan are present in lower concentrations. Reported ranges for specific amino acids include lysine (1.58%–5.76%), methionine (0.52%–2.20%), threonine (1.57%–4.29%), and tryptophan (0.02%–1.86%), on dry matter basis [182]. Compared to soybean meal, *T. molitor* larvae contain higher concentrations of lysine, methionine, threonine, tryptophan, valine, and isoleucine [182]. Although the lysine, methionine, and threonine levels in *T. molitor* are slightly lower than those found in fishmeal, the

larvae provide superior levels of tryptophan, valine, and isoleucine [182]. This amino acid composition enhances their suitability as a high-quality feed ingredient for monogastric animals.

T. molitor larvae contain a wide variety of fatty acids, with unsaturated fatty acids (UFA) making up the majority of their lipid profile. Saturated fatty acids (SFA) include myristic acid (C14:0), which ranges from 2.12% to 5.21%, palmitic acid (C16:0) from 9.33% to 17.21%, and stearic acid (C18:0) from 0.26% to 3.06%. Key UFAs present include palmitoleic acid (C16:1) ranging from 9.33% to 17.24%, oleic acid (C18:1n9) from 40.78% to 49.71%, linoleic acid (C18:2n6) from 24.19% to 35.58%, linolenic acid (C18:3n3) from 0.35% to 2.27%, γ-linoleic acid (C18:3n6) from 0.03% to 1.85%, and eicosenoic acid (C20:1n9) from 0.06% to 0.39% (values expressed on a dry matter basis) [182].

The total SFA content in *T. molitor* larvae ranges between 22.3 and 23.3g, while UFAs constitute approximately 77.7 to 78.5g [204, 205]. This unsaturated fat composition is comparable to that of poultry meal and fishmeal [167]. Notably, *T. molitor* larvae are rich in polyunsaturated fatty acids (PUFAs), including omega-3 and omega-6 fatty acids. Specifically, omega-3 content ranges from 46.1 to 47.3g, while omega-6 content ranges from 31.1 to 31.6g (values expressed per 100g) [204, 205].

T. molitor larvae also offer a valuable mineral profile. The calcium content ranges from 0.04% to 0.50%, while phosphorus is present at higher levels, between 0.70% and 1.04%. Sodium and potassium are also notable, with contents ranging from 0.21% to 0.36% and 0.85% to 1.12%, respectively. As for trace minerals, iron concentrations in the larvae span from 63.0 to 100.0 mg/kg, zinc ranges between 102.0 and 117.4 mg/kg, and copper content varies from 12.3 to 20.0 mg/kg (values expressed on as fed basis)[189, 196, 204, 205]. This mineral composition enhances the larvae's nutritional value, supporting their suitability as a balanced feed ingredient.

Chitin, a major structural component of the insect exoskeleton, is recognized as an indigestible form of fiber with beneficial immunomodulatory properties [206]. It is recognized as one of the most valuable components of insects, supporting animal health by enhancing immune function, providing antimicrobial effects, and positively influencing gut microbiota [207]. Its presence has been associated with improved immune function and overall animal performance [208]. The quantity and structure of chitin can vary depending on the insect species and developmental stage. Among commonly used insect species, *T. molitor* larvae tend to have relatively lower chitin

content. For instance, Adámková et al. (2017) reported chitin levels of 13g/100g in *T. molitor* larvae and 12g/100g in pupae (as fed basis) [209]. Other studies have noted a wider range in larvae, with values averaging 6.41% and spanning from 4.92% to 13.0% (as fed basis) [182].

Furthermore, chitosan, a deacetylated derivative of chitin with well-established biomedical applications, has also been identified in *T. molitor*. Research by Jin et al. (2016) reported chitosan levels of approximately 11.56 mg/g in *T. molitor* larvae (as fed basis), suggesting their potential role as functional components in animal feed [169, 210].

The nutritional composition of *T. molitor* larvae is significantly influenced by the composition of the substrate on which they are reared. Diets rich in protein have been shown to enhance the protein content of larvae [151, 211]. Similarly, modifying the substrate's fat content, for example increasing fat from 0.46% to 9.34%, can lead to an elevation in unsaturated fatty acid levels within the larvae [212]. Mineral uptake is also substrate-dependent, with larvae accumulating minerals directly from their diet, thus emphasizing the importance of carefully designed feeding substrates to optimize mineral content [213].

Conversely, *T. molitor* meal typically contains minimal amounts of certain essential nutrients and bioactive compounds such as polyphenols [214]. However, recent findings have demonstrating that it is feasible to enrich insect larvae with bioactive compounds through dietary formulation. For instance, supplementing the substrate with specific herbs or plant-derived materials can significantly enhance the antioxidant and functional compound profile of the larvae [214]. Overall, substrate composition plays a crucial role in modulating the nutritional and functional properties of *T. molitor*, offering a viable strategy for optimizing larvae composition to meet specific dietary or functional requirements.

1.4.16. Previous Studies: *Tenebrio molitor* in Pig Diets

Research investigating the incorporation of *T. molitor* larvae or their derived meal in pig diets remains relatively limited when compared to studies focused on broiler chickens. This gap may be attributed to the higher feed intake requirements of pigs, which demand greater quantities of insect-derived protein. Notably, Jin et al. (2016) demonstrated that increasing dietary inclusion of *T. molitor* larvae from 0% to 6%, while maintaining consistent metabolizable energy, crude protein, lysine, and methionine levels, led to significant improvements in growth performance parameters among weaning pigs during the initial five weeks post-weaning [169]. The authors attributed these

enhancements to improved feed palatability, likely due to the distinctive flavor of the larvae, which stimulated increased feed intake. Furthermore, higher inclusion levels were associated with improved nutrient digestibility and favorable shifts in blood biomarkers, indicating enhanced protein utilization efficiency.

Meyer et al. (2020) investigated the inclusion of up to 10% *T. molitor* larvae meal in the diets of weaning pigs and reported no significant impact on key growth performance indicators [128]. However, they observed alterations in specific plasma amino acid concentrations, suggesting metabolic responses to dietary supplementation. Importantly, plasma biochemical markers remained largely unaffected by increasing the larvae meal content from 0% to 10%.

In a related study, Ringseis et al. (2021) examined the influence of *T. molitor* meal in the diets of growing pigs and found no major changes in oxidative stress parameters, except for an increase in catalase activity in skeletal muscle tissue. This effect may suggest that insect meal supplementation boosted antioxidant activity in specific tissues [215].

Cho et al. (2023) explored the potential of replacing spray-dried plasma protein (SDPP) with full-fat *T. molitor* (FFTM) or hydrolyzed *T. molitor* (HTM) larvae meal in weaned pig diets [216]. Their findings revealed that pigs fed FFTM or HTM exhibited similar or even higher levels of serum immunoglobulins (IgA and IgG) compared to those receiving SDPP, supporting the use of *T. molitor* meal as a functional and immunologically effective protein alternative in early pig nutrition.

Recent studies have explored the possibility of substituting fishmeal with *T. molitor* larvae or their meal in the diets of weaning pigs. Ao et al. (2020) demonstrated that replacing fishmeal entirely with 2% *T. molitor* larvae did not adversely affect growth performance or related physiological parameters [217]. Similarly, Ko et al. (2020) found no significant differences in growth or nutrient digestibility when fishmeal was replaced with defatted *T. molitor* larvae meal in weaning pig diets [218]. Interestingly, the substitution was associated with a notable increase in serum immunoglobulin G (IgG) levels after 14 days, suggesting a potential enhancement in immune response.

Yoo et al. (2019) reported that growing pigs fed a diet containing 9.95% *T. molitor* larvae exhibited significantly higher apparent ileal digestibility (AID) of several amino acids, including lysine,

histidine, arginine, and cystine, compared to those fed an equivalent level of fishmeal [167]. Moreover, in the same study the standardized ileal digestibility (SID) of arginine and cystine was superior in the *T. molitor*-supplemented group. In a related study, Cho et al. (2020) evaluated pigs fed 10% defatted *T. molitor* larvae meal and hydrolysate, finding comparable AID values for lysine, methionine, and threonine between the groups [168]. Collectively, these findings suggest that a 10% inclusion of *T. molitor* larvae can offer digestibility advantages over conventional protein sources such as fishmeal and poultry meal. Based on current evidence, *T. molitor* larvae may be effectively incorporated at up to 6% in weaning pig diets and up to 10% in growing pig diets [182].

To our knowledge, no published studies have evaluated the effects of incorporating *T. molitor* larvae into the diets of fattening or finishing pigs. This lack of research may be attributed to both the high cost and limited availability of *T. molitor* larvae, as well as the significantly larger feed intake requirements in this phase of swine production. As the insect farming sector continues to develop and scale, there is a critical need for well-designed studies exploring the efficacy, optimal inclusion rates, and economic feasibility of *T. molitor*-based feed ingredients for growing-finishing pigs.

1.5. Feed Additives in swine nutrition

Modern pig production systems are increasingly influenced by the need to reduce antibiotic usage as growth promoters, a trend highlighted by Jacela et al. (2010)[219]. This transition has encouraged the adoption of advanced breeding and management practices that focus on unlocking the genetic potential of hybrid breeds through enhanced welfare standards and precision nutrition. To support these objectives, contemporary swine nutrition relies heavily on the inclusion of functional feed additives designed to optimize feed efficiency and promote overall animal health [220].

These additives include feed enzymes, prebiotics, probiotics, organic acids, and herbs all of which have demonstrated efficacy in enhancing nutrient utilization, correcting dietary deficiencies, and modulating gut microflora [221-225]. The role of gut microflora is particularly important in monogastric species such as pigs, which lack a proventriculus. This anatomical limitation reduces the pigs' ability to digest fiber and makes them more sensitive to anti-nutritional compounds in feed, highlighting the need for targeted nutritional strategies [226-228].

1.5.1. Feed Enzymes

In the context of pig nutrition, the inclusion of enzymatic preparations is especially critical for piglets due to the underdevelopment of their digestive enzyme systems and unstable intestinal microflora [40, 220]. The immature gastrointestinal tract of piglets often results in lower digestion efficiency and feed conversion ratio, creating an environment conducive to the proliferation of pathogenic microorganisms, which can lead to post-weaning diarrhea. Consequently, nutritional strategies for piglets emphasize the need of highly digestible, low-fiber feed formulations.

Nevertheless, findings from a series of studies, have demonstrated that targeted supplementation with feed enzymes can mitigate these limitations. Studies by Vahjen et al. (2007), Hanczakowska et al. (2012), and Chen et al. (2016) have shown that the inclusion of specific enzymes not only reduces feed costs but also enables the use of less digestible raw materials [223, 226, 229]. Among the most commonly used feed components for pigs are cereal grains such as barley, which contain high levels of non-starch polysaccharides (NSPs) [230]. These NSPs can reduce nutrient absorption; however, enzymatic additives such as xylanase and beta-glucanase have been proven to improve digestibility. For instance, Sterk et al. (2007) reported that the inclusion of xylanase in weaned piglet diets significantly enhanced the digestibility of crude protein, fat, fiber, and NSPs, thereby improving overall feed efficiency [231].

1.5.2. Prebiotics

Prebiotics constitute an important functional component of swine nutrition, acting as selectively fermentable substrates that enhance the growth and metabolic activity of beneficial gut microbiota while indirectly suppressing pathogenic taxa [232]. In pigs, dietary prebiotics have been shown to modulate both the composition and quantitative profiles of intestinal microbial communities, typically favoring lactic acid–producing and other commensal bacteria at the expense of opportunistic and enteropathogenic species. As a result, their inclusion in the diet is frequently associated with reduced colonization and shedding of key foodborne and enteric pathogens, including *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni* and *Clostridium perfringens* [220].

Among the most studied prebiotics are mannooligosaccharides (MOS), which have demonstrated beneficial effects in weaning piglets. Research by Agazzi et al. (2020) and Zhou et al. (2020) highlighted the capacity of MOS to stabilize gut health during the post-weaning transition [233,

234]. Supplementation of piglet diets with 0.1% to 0.2% of MOS has been associated with reduced incidences of diarrhea and improved fecal consistency, as observed by Grela et al. (2006) and Castillo et al. (2008)[235, 236].

Furthermore, prebiotics rich in fructooligosaccharides have been shown to support gastrointestinal development in piglets. These compounds not only mitigate post-weaning diarrhea but also enhance intestinal morphology by promoting villus elongation, ultimately improving feed conversion efficiency [237, 238]. The collective evidence underscores the significance of prebiotics as functional feed additives in enhancing gut health and performance in swine, particularly during the vulnerable weaning period.

1.5.3. Probiotics

Probiotics are functional feed additives composed of live microbial strains that are naturally present in the gastrointestinal tract and, when administered in adequate amounts, confer health benefits to the host [239]. In swine nutrition, probiotics typically include lactic acid bacteria such as *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Pediococcus acidilacti*, *Enterococcus faecium*, and *Bacillus subtilis* [220]. These beneficial microbial strains support gastrointestinal health, particularly under conditions of microbial imbalance, such as during diarrheal episodes or after prolonged antibiotic treatment.

Studies by Guerra et al. (2007) and Bernárdez et al. (2008) demonstrated that supplementation with probiotics including *P. acidilacti*, *L. lactis*, *L. casei*, and *E. faecium* significantly reduced the severity of diarrhea in weaning piglets and led to improved average daily weight gain [240, 241]. Notably, the improvements in growth performance observed with probiotic supplementation were found to be comparable to those achieved with traditional antibiotic treatments. Furthermore, evidence from Zhang et al. (2020) and Lin et al. (2020) demonstrates that probiotic supplementation in lactating sows improves the health outcomes of their piglets, highlighting the significant and extended benefits of maternal dietary interventions [242, 243]. Collectively, these findings support the utility of probiotics as a viable alternative to antibiotics for promoting gut health, improving nutrient utilization, and supporting performance in swine production systems.

1.5.4. Organic acids / Preservatives

Organic acids, often employed as feed preservatives, play a pivotal role in maintaining the quality and safety of swine diets. These compounds are effective in inhibiting the growth of pathogenic bacteria and molds during storage, while simultaneously contributing to improved gastrointestinal function in pigs [244, 245]. Commonly used acids include short-chain organic acids such as propionic, formic, lactic, sorbic, fumaric, citric, and orthophosphoric acids, in addition to medium-chain fatty acids like caprylic and capric acids [220].

The primary mode of action of these acids involves reducing the pH of both the feed and the gastrointestinal tract, creating an unfavorable environment for pathogenic microorganisms. Moreover, certain organic acids possess the ability to penetrate bacterial cell membranes, leading to intracellular acidification and subsequent bacterial inactivation [244-246].

Beyond their preservative function, organic acids have been shown to positively impact animal performance. Research by Balasubramanian et al. (2016) and Devi et al. (2016) demonstrated that supplementation of organic acids in the diets of lactating sows improved feed digestibility and feed conversion efficiency, while also enhancing the health and growth of their offspring [228, 247]. These findings underscore the multifaceted benefits of incorporating organic acids into swine nutrition strategies.

1.5.5. Phytobiotics/Plant extracts/Plant bioactive compounds

The ban on antibiotic growth promoters and the strict regulations on the use of antibiotics in livestock production within the European Union have increased the need for natural alternatives to maintain animal health and performance [248, 249]. Consequently, there is growing interest in natural bioactive compounds derived from plants, commonly referred to as phytobiotics, as safe and sustainable feed additives [250]. The main activities and various applications of aromatic plants, extracts, and essential oils are summarized in Figure 1.4. [251]. Their use is also increasing because of the expansion of organic farming, which requires non-antibiotic options [250]. Herbs, plant extracts, plant bioactive compounds, and phytobiotic blends have long been recognized for their medicinal and nutritional value due to the presence of glycosides, alkaloids, saponins, flavonoids, tannins, pectins, and organic acids [252, 253]. These biologically active components have antioxidant and antimicrobial activities and enhance immune responses in animals [254, 255].

Dietary supplementation with essential oils, plant extracts, or mixtures containing these bioactive compounds has been shown to positively influence gut physiology and digestion [256]. Concentrated products made from single plant species or mixed herbal blends are now commonly available for different animal types and growth stages [257-259]. Even at low inclusion levels, these additives can improve health status and productivity by modulating digestive enzyme activity and promoting beneficial gut microbial populations. Furthermore, plant-based supplements can help animals handle stress, lower harmful gas emissions (like ammonia and CO_2), and improve the overall environment in animal housing [260-263]. Research also indicates that appropriately formulated herbal mixtures can enhance meat quality through improved oxidative stability and sensory parameters [249, 264, 265].

Young pigs, especially during weaning, are very sensitive to digestive problems and infections; therefore, adding phytobiotics to their diet at this stage is very helpful [226, 266, 267]. Several studies have shown that herbs stimulate feed intake and growth in piglets while maintaining gut microbial balance by suppressing pathogenic bacteria such as *E. coli* and reducing diarrhea incidence [268-273]. In fattening pigs, phytobiotic supplementation, especially with garlic, has been reported to enhance feed efficiency, average daily gain, and final body weight, while also improving hematological parameters [274-280]. Collectively, these findings highlight the potential of plant-derived additives as effective, natural alternatives to antibiotics in promoting animal health, productivity, and product quality.

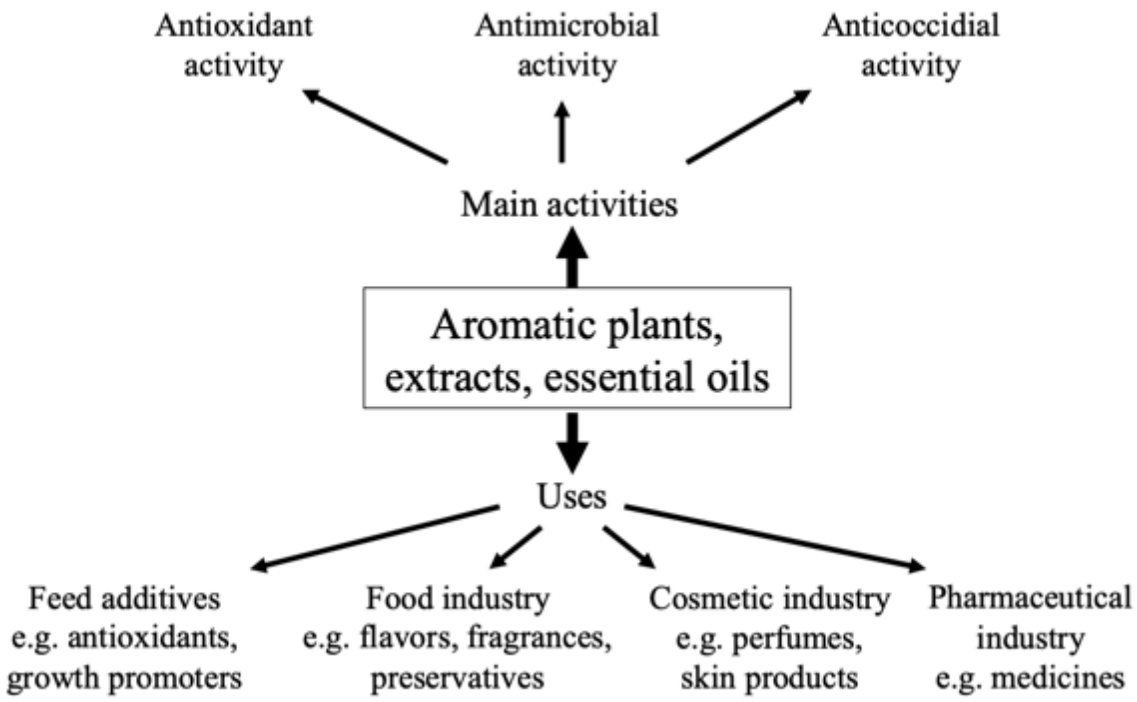

Figure 1.4. Activities and uses of aromatic plants
Reference: Christaki et al. (2012)[251]

1.5.6. Chitosan as feed additive

In contemporary intensive swine production, a wide variety of feed additives are commonly used to maintain animal health, support metabolic function, and enhance performance outcomes. Among these additives are organic acids, feed enzymes, probiotics, prebiotics, and botanical extracts. A relatively recent addition to this list is chitosan, a prebiotic compound derived from chitin, which has gained attention for its potential functional properties in animal nutrition [281].

Chitosan is a non-toxic polyglucosamine composed of β-(1,4)-linked 2-acetamido-D-glucose and 2-amino-D-glucose units (Figure 1.5). Although rarely found in its native form in nature — being present in select fungi species — chitosan is predominantly produced via the deacetylation of chitin, a naturally occurring biopolymer found in the exoskeletons of arthropods such as shrimps, crabs, and insects [282, 283]. This transformation, which involves treating chitin with concentrated sodium hydroxide at high temperatures, converts it into chitosan, making it soluble in acidic

environments and therefore more suitable for use in animal nutrition than its original form [284, 285].

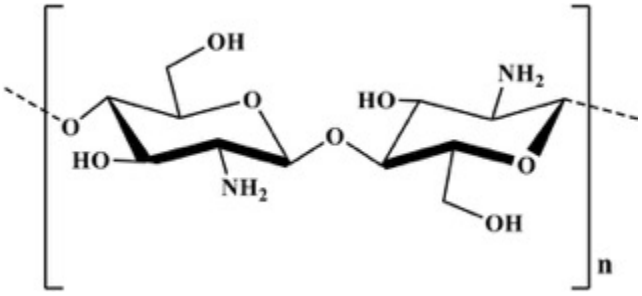

Figure 1.5. Chemical structure of chitosan
Reference: Uyanga et al. (2023)[286]

Moreover, chitosan can be enzymatically or chemically depolymerized into chito-oligosaccharides (COS), which are characterized by improved bioavailability and functional efficacy. Methods for producing COS include acid hydrolysis, mechanical processes, and enzymatic degradation [287]. These derivatives have been explored for their prebiotic potential and their capacity to modulate gut microbiota, making chitosan and its derivatives promising alternative for integration into swine feed as functional nutritional components.

Chitosan and its oligosaccharide derivatives contain reactive functional groups, such as amino and hydroxyl groups, which play a key role in their wide range of biological effects (Figure 1.6). In contrast to its precursor chitin, chitosan exhibits a range of health-promoting properties. These include antimicrobial effects [288], anti-inflammatory activity [289], antioxidative potential [290], antitumor effects [291], immunostimulatory functions [292], and hypocholesterolemic effects [293]. These features suggest that chitosan could be a valuable health-enhancing feed additive for livestock, potentially serving as a substitute for feed antibiotics [281].

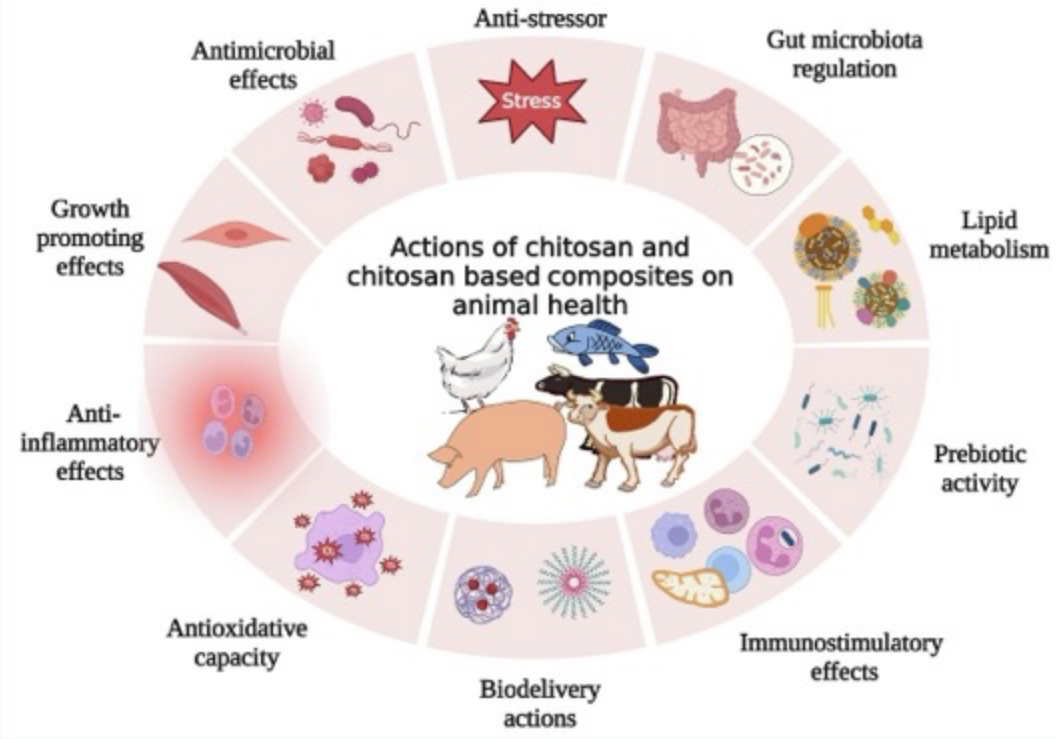

Figure 1.6. Schematic illustration of the actions of chitosan and chitosan-based composites to modulate the biological and physiological responses of animals

Reference: Uyanga et al. (2023)[286]

1.5.7. Chitosan in Pig Diets

The weaning phase represents a critical period in swine production, during which piglets are highly susceptible to environmental, nutritional, and immunological stressors. These stressors can disrupt metabolic homeostasis, often leading to gastrointestinal disturbances, reduced growth performance, and elevated morbidity and mortality rates. Chito-oligosaccharides (COS), as derivatives of chitosan, have shown potential in mitigating these challenges. For instance, Liu et al. (2008) reported that dietary supplementation with 0.01% or 0.02% COS significantly enhanced feed intake, body weight gain, and feed conversion efficiency in weaned piglets [294]. Additionally, COS supplementation improved nutrient digestibility and was associated with favorable changes in intestinal morphology, including increased villus height and a higher villus-to-crypt ratio in both the ileum and jejunum. Supporting these findings, Tang et al. (2005) suggested that COS may promote growth by influencing endocrine activity, as early-weaned piglets fed a diet with 0.025% COS showed increased serum levels of growth hormone and insulin-like growth factor 1 [295].

In a study conducted by Xu et al. (2013), the effects of dietary COS at varying inclusion levels, specifically 0.01%, 0.05%, 0.10%, and 0.20%, were evaluated in weaned pigs to assess its impact on growth performance, small intestinal morphology, and serum concentrations of growth hormone (GH)[296]. The study aimed to examine the potential regulatory mechanisms of COS on piglet growth through endocrine modulation and improvements in gut morphology. The results revealed a significant quadratic response in body weight gain across the different COS levels, with the most pronounced improvements observed at a dietary inclusion of 0.05%. Similarly, serum GH levels and morphological parameters, such as villus height and villus-to-crypt ratios in the duodenum, jejunum, and ileum, also followed a quadratic trend, peaking at the 0.05% supplementation level.

In another experiment by the same team, Xu et al. (2013) reported that dietary inclusion of COS at concentrations ranging from 0.01% to 0.20% significantly increased the relative weight and length of the duodenum and enhanced jejunal length in weaned piglets, suggesting beneficial effects on intestinal development [297]. Based on these findings, a subsequent study by Xu et al. (2014) confirmed the positive impact of COS supplementation within the same concentration range on growth performance in weaned pigs [298]. These improvements were attributed to enhanced nutrient digestibility, particularly of dry matter, crude protein, calcium, and phosphorus. Furthermore, COS supplementation was associated with elevated jejunal amylase activity, indicating improved digestive enzyme function. Supporting this, Chen et al. (2009) observed that COS levels of 0.25% and 0.50% resulted in improvements in body weight gain and the apparent digestibility of dry matter and nitrogen [299].

Yin et al. (2008) investigated the immunomodulatory effects of dietary COS administered at a concentration of 0.025% in early-weaned piglets [300]. Their findings indicated that weaning stress significantly suppressed immune function, as evidenced by reduced serum concentrations of antibodies and cytokines. However, COS supplementation neutralized these effects, leading to upregulated gene expression of interleukin-1β (IL-1β) in the jejunal mucosa and mesenteric lymph nodes. Furthermore, pigs receiving COS exhibited elevated serum levels of key immune markers, including IL-1β, IL-2, IL-6, IgA, IgG, and IgM. These results suggest that dietary COS can enhance cell-mediated immunity in early-weaned piglets, likely through modulation of cytokine expression and stimulation of antibody production.

Yang et al. (2012) investigated the effects of dietary supplementation with COS at concentrations of 0.02%, 0.04%, and 0.06% on the caecal microflora of weaned piglets [301]. The results indicated that pigs receiving 0.04% COS had increased populations of *Bifidobacteria* and *Lactobacilli*, along with a reduced presence of *Staphylococcus aureus* by day seven post-weaning. Furthermore, a 0.06% COS supplementation resulted in a continued elevation of *Bifidobacteria* by day fourteen. The authors proposed two potential mechanisms underlying the antimicrobial activity of COS: first, the positively charged NH_3^+ groups on the COS glucosamine monomers may damage bacterial membranes, leading to leakage of intracellular components; second, COS may indirectly modulate the gut microbiota by selectively promoting beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, which outcompete and suppress pathogenic species like *S. aureus*.

In a similar study, Chen et al. (2009) evaluated the immunomodulatory effects of dietary COS at a 0.50% inclusion level in weanling pig diets, comparing responses between non-challenged pigs and those subjected to an *E. coli* lipopolysaccharide (LPS) challenge [299]. The study found that COS supplementation led to a reduction in rectal temperature and circulating cortisol levels, along with an increase in insulin-like growth factor-1 (IGF-1) concentrations following the LPS challenge. However, COS had no significant impact on lymphocyte counts. Based on their findings, they concluded that dietary COS exerted only a minimal modulating effect on the inflammatory stress markers in weanling pigs.

Liu et al. (2008) investigated the potential of COS at a dietary inclusion level of 0.016% to serve as an alternative to the antibiotic cyadox in mitigating the effects of *E. coli* infection in weaned pigs [293]. While COS supplementation yielded several health benefits—including elevated plasma IGF-I concentrations, reduced numbers of IgA-positive cells in the intestinal mucosa, lower diarrhea incidence, and partial alleviation of infection-associated symptoms—it did not match the antibiotic's efficacy in preserving growth performance under pathogenic challenge. In contrast, a subsequent study by Xiao et al. (2013) demonstrated that dietary COS at 0.03%, comparable to chlortetracycline, effectively reduced intestinal inflammation and enhanced growth performance in piglets challenged with enterotoxigenic *E. coli*, a common cause of post-weaning diarrhea [302]. COS supplementation was associated with increased intraepithelial lymphocyte counts, improved villus morphology, enhanced feed conversion ratio, and modulation of key protein expression pathways, supporting its potential as a functional alternative to antibiotic feed

additives. Additionally, in a 70-day trial on growing pigs with an average starting weight of 31 kg, Han et al. (2007) reported that dietary COS (0.10% or 0.30%) significantly improved immune responses, as evidenced by elevated antibody titers following vaccination [303].

Liu et al. (2008) examined the effects of dietary COS on the fecal microbiota of weaned pigs and found that supplementation with 0.01% or 0.02% COS significantly reduced diarrhea incidence and *E. coli* counts, while concurrently increasing *Lactobacillus* populations in fecal samples [294]. Similarly, Han et al. (2007) reported that higher COS inclusion levels (0.30% and 0.40%) inhibited the growth of pathogenic bacteria in growing pigs averaging 25 kg in body weight [304]. Additional studies by Yan et al. (2011) and Wang et al. (2009) were in align with these findings, highlighting COS's potential to selectively reduce fecal *E. coli* concentrations without adversely affecting beneficial bacterial populations such as *Lactobacillus* [305, 306].

Research examining the effects of chitosan supplementation on hematological parameters in swine remains limited. In a study conducted by Zhou et al. (2012) involving weanling pigs, dietary inclusion of COS at levels of 0.10% and 0.20% resulted in a reduction in blood lymphocyte concentrations, while erythrocyte and leukocyte counts remained unaffected [307]. In contrast, a study by Yan et al. (2011) reported that dietary supplementation with 0.30% COS in weaned pigs led to an increase in lymphocyte levels without significantly altering other hematological indices [305].

In a study conducted on growing pigs, Wang et al. (2009) investigated the effects of dietary supplementation with 0.50% COS on selected blood biochemical parameters [306]. The results indicated that COS inclusion led to a significant increase in serum high-density lipoprotein (HDL) cholesterol levels, suggesting a potential cardiovascular benefit. However, other key serum lipid indicators, including total cholesterol and triglyceride concentrations, remained unaffected by the supplementation.

Multiple studies have demonstrated that COS can positively influence digestive efficiency and nutrient uptake in pigs. Specifically, COS supplementation has been shown to enhance ileal digestibility, increase adsorption capacity, and stimulate enterocyte proliferation, collectively improving the digestion and absorption of nutrients [308]. However, other findings also suggest that the efficacy of COS may depend on dosage and purity. For instance, low doses of high-purity COS have been associated not only with growth-promoting effects but also with a reduction in

villus height in sections of the small intestine, such as the jejunum and duodenum [309, 310]. In a study evaluating the physiological effects of low-dose COS supplementation in weaned piglets, Yang et al. (2016) reported significant improvements in serum immunoglobulin G and calcium concentrations, as well as increased levels of select amino acids in the intestinal mucosa [310].

Earlier investigations by the same research group suggested that dietary supplementation with COS may induce alterations in gastric pH, modulate specific immune parameters, and cause morphological changes in the gut of weaned piglets [309]. While most research has concentrated on the effects of COS in weaned piglets, relatively few studies have investigated its impact on mature pigs or sows. One such study by Egan et al. (2015) examined sows with an average body weight of approximately 70 kg and found that dietary COS supplementation resulted in reductions in several parameters, including final body weight and the efficiency of certain digestive processes [311]. Additionally, research by Xie et al. (2015, 2016) explored the maternal effects of COS when administered during gestation and lactation [312, 313]. Their findings indicated that COS supplementation in sows led to beneficial outcomes, including improved average daily weight gain, enhanced amino acid concentrations in milk, and favorable alterations in both plasma and hepatic biomarkers in suckling piglets.

1.6. Enrichment of *Tenebrio molitor* with bioactive compounds

Rearing *T. molitor* on substrates enriched with aromatic and medicinal plants native to the Greek flora offers an innovative and sustainable strategy with multiple benefits for animal nutrition and health. Herbs such as oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) are rich in bioactive compounds—particularly phenolics, flavonoids, and essential oils—that have been extensively studied for their potent antioxidant, antimicrobial, and anti-inflammatory properties [251]. By incorporating these plants into the larvae's rearing substrate, the nutritional and functional profile of *T. molitor* can be enhanced, potentially yielding insect meal with increased nutrient density and added bioactive value (Figure 1.7).

This biofunctional enhancement holds particular promise for swine nutrition, especially during the critical post-weaning phase, when piglets experience heightened physiological stress and are vulnerable to gastrointestinal disorders such as post-weaning diarrhea [58]. The use of phytochemical-enriched *T. molitor* meal could offer a natural dietary solution to support gut health, strengthen immune function, and mitigate inflammation, reducing reliance on antibiotics or

synthetic additives. Moreover, bioactive compounds consumed through the larvae may be absorbed and metabolized by pigs, offering systemic health benefits and possibly reducing the incidence of metabolic disorders.

Beyond animal health, there are important implications for meat quality and consumer health. Diets enriched with phytochemical-fed larvae may influence the fatty acid composition, oxidative stability, and sensory attributes of pork, potentially enhancing its flavor, shelf life, and nutritional value. Such innovations align with the growing consumer demand for functional animal products that prioritize both animal welfare and human health. Additionally, this approach complements circular bioeconomy models by valorizing local botanical resources and integrating them into a sustainable insect farming system. Nevertheless, this concept presents a promising way to combine the use of traditional Mediterranean plant and modern pig feeding practices.

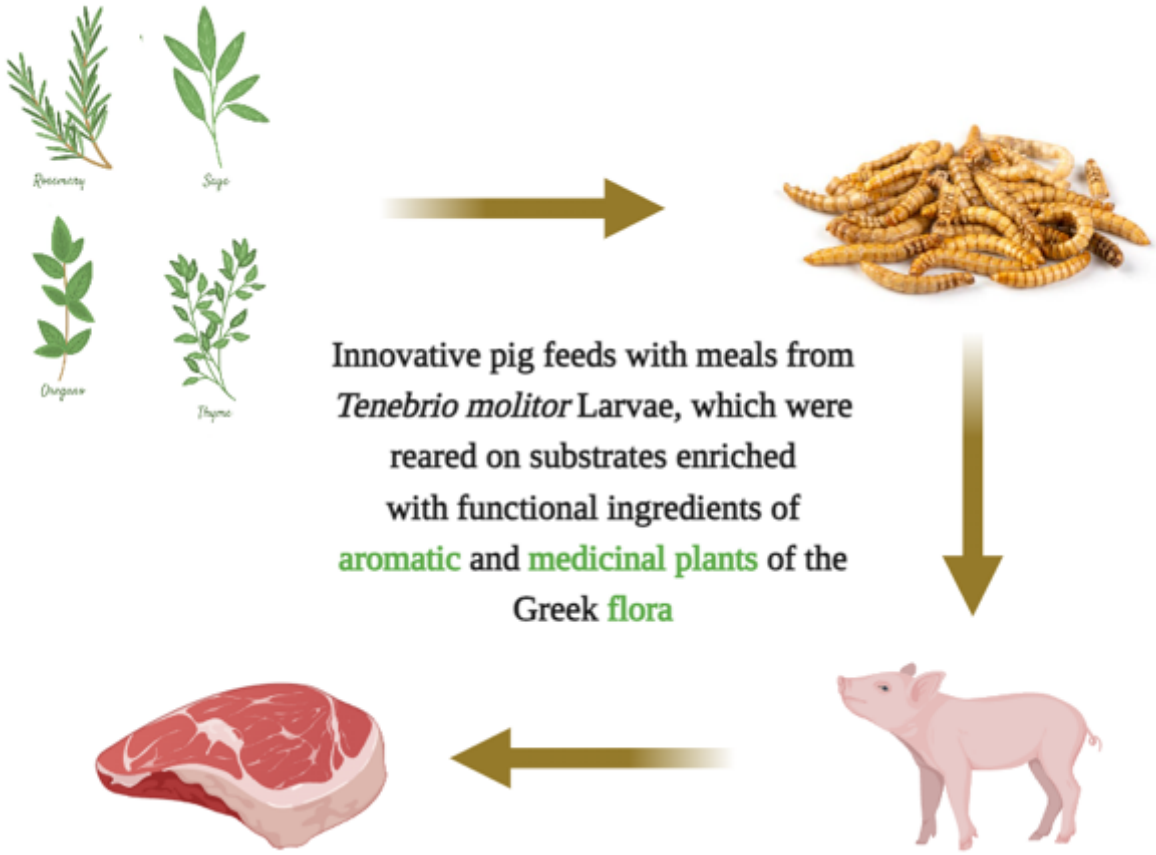

Figure 1.7. Use of innovative pig feeds on growing and finishing pig diets

1.7. Synergistic Effects: *Tenebrio molitor* and Chitosan

The integration of *T. molitor* in combination with chitosan into livestock feed formulations represents a promising innovation in animal nutrition. This supplementation approach offers a comprehensive strategy to enhance nutrient utilization and support animal health. The synergistic potential of *T. molitor*, rich in high-quality protein, essential fatty acids, and bioactive compounds, and chitosan which is recognized for its antimicrobial, immunomodulatory, and digestive-enhancing properties, positions this combination as a valuable alternative to conventional feed additives.

Chitosan is widely recognized for its capacity to promote a balanced gut microbiota, a key factor in enhancing nutrient absorption and maintaining intestinal health [314]. Notably, *T. molitor*

naturally contains chitosan within its exoskeleton, adding further value for its inclusion in animal feed formulations. This endogenous source of chitosan may further amplify the gut health benefits typically associated with external supplementation. Additionally, *T. molitor* offers a favorable nutritional profile, rich in high-quality protein and essential amino acids, which are vital for optimal growth and physiological function in livestock [151]. When combined with the gut-modulating properties of chitosan, these nutrients are likely to be more efficiently utilized, thereby supporting improved growth performance and overall animal well-being.

In conclusion, incorporating *T. molitor* in combination with chitosan into livestock diets offers a promising, holistic strategy for improving animal health, performance, and welfare. This approach aligns with current trends in natural feed innovation and sustainable farming practices. Given its potential to enhance immune function, optimize nutrient utilization, and reduce reliance on pharmaceutical interventions, this synergistic combination merits further investigation and could play a key role in the future of livestock nutrition.

1.8. Defining the potential of using *T. molitor* insect meal in pig diets

This PhD dissertation investigated the potential of incorporating *T. molitor* insect meal—reared either on conventional or phytochemically enriched substrates—and its combination with chitosan into swine diets. The primary objective was to evaluate the effects of these dietary interventions on important aspects of pig production, including zootechnical performance, health biomarkers, gut microbiota composition, and meat quality characteristics. To address these aims, two distinct feeding trials were conducted:

1. **First trial:** Investigated the nutritional effects of *T. molitor* larvae meal, reared either on conventional or phytochemically enriched substrates, in diets for early-growing pigs. This trial also evaluated the combined supplementation of conventional *T. molitor* meal with the prebiotic chitosan.
2. **Second trial:** Extended the investigation to lately finishing pigs, evaluating the nutritional contribution of *T. molitor* meal reared on both conventional and enriched substrates.

The novelty and significance of this dissertation lie in addressing critical gaps in current knowledge. According to our knowledge, while different insect meals has been explored as a protein source in weaned pigs, no previous research has evaluated the combined effects of *T.*

molitor larvae meal and chitosan in early-weaned piglets, and *T. molitor* meal has not been tested in finishing pig diets. This dissertation systematically investigates these interventions and provides robust evidence that nutrient-rich alternatives can effectively replace conventional protein sources, supporting healthy growth, efficient production, and environmentally sustainable pig farming. The scientific contributions of this work have been presented through two peer-reviewed publications and multiple conference presentations, addressing the main research questions.

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Chapter 2: Investigating the use of *Tenebrio molitor* larvae reared with different substrates, and dietary chitosan supplementation as feed ingredients in growing pig diets

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1. *Utilization of Tenebrio molitor larvae reared with different substrates as feed ingredients in growing pigs.* Zacharis, C., Bonos, E., Giannenas, I., Skoufos, I., Tzora, A., Voidarou, C. C., Tsinas, A., Fotou, K., Papadopoulos, G., Mitsagga, C., Athanassiou, C., Antonopoulou. E. & Grigoriadou, K. (2023). Veterinary Sciences 10: 393.
2. *Combined dietary supplementation of Tenebrio molitor larvae and chitosan in growing pigs: a pilot study.* Zacharis, C., Bonos, E., Voidarou, C., Magklaras, G., Fotou, K., Giannenas, I., Giavasis, I., Mitsagga, C., Athanassiou, C., Antonopoulou, C., Grigoriadou, K., Tzora A. & Skoufos, I. (2024). Veterinary Sciences, 11(2), 73.

2.1. Introduction

The rapid growth of the global population and rising living standards have greatly raised the demand for sustainable food production, particularly for high-quality protein sources [1-5]. In the European Union, local feed production remains insufficient, leading to dependence on imported protein ingredients such as soybean meal and fishmeal [6-9]. However, limited arable land and marine overexploitation have restricted their availability, while price fluctuations and strict regulations on animal by-products further complicate feed formulation [10, 11]. As a result, the search for sustainable and nutritionally rich protein alternatives has become a key priority for animal production systems.

Insects, particularly *Tenebrio molitor*, have emerged as a promising solution due to their high protein and lipid content, efficient feed conversion, and ability to be reared on organic by-products with a low environmental footprint [12-17]. The recent EU authorization of processed insect proteins for use in poultry and pig feeds [12] has further increased interest in their use. Studies show that *T. molitor* meal can enhance pig growth, feed efficiency, and gut health, though its nutritional composition depends on the rearing substrate [14-21]. Enriching insect diets with residues of medicinal and aromatic plants may further improve their bioactive properties [21]. Moreover, chitosan, a derivative of chitin naturally present in *T. molitor* larvae, has demonstrated prebiotic and immunomodulatory benefits in pigs [22-25]. This chapter therefore evaluates the effects of replacing fishmeal with *T. molitor* meal, reared on either conventional or phytochemical-enriched substrates, combined with chitosan supplementation, on growth performance, gut health, blood parameters and meat quality in early-growing pigs.

2.2. Materials and Methods

2.2.1. Experimental Design, Animals, and Diets

The experimental protocol for this trial was reviewed and approved by the Ethics and Research Ethics Committee of the University of Ioannina of Greece (protocol number 56652, 26 November 2021).

The experimental trial was performed on a commercial swine farm in the area of Epirus (Greece). Initially, 132 crossbred growing pigs (¼ Large White, ¼ Landrace, and ½ Duroc; 34 days of life) were examined to be clinically healthy by a veterinarian (Figure

2.1). From this initial pool, a total of sixty (60) growing pigs (30 males and 30 females) with an average initial body weight of 8.39 ± 0.82 kg were randomly chosen and allocated into five distinct groups (groups A, B, C, D, and E; six males and six females per group) and housed in separated indoor pens with slatted plastic floors, heating, and mechanical ventilation. Each pig was uniquely identified with ear tags (Figures 2.2 & 2.3). Throughout the experiment, environmental factors including ambient temperature and humidity were constantly monitored (24–26 ◦C and 60–70%, respectively). Moreover, throughout the trial, the pigs had free access to fresh water and the feeds.

Figure 2.1. First day of the experimental trial: Allocation of the growing pigs into five distinct groups

Figures 2.2 & 2.3. Growing pigs during the experimental trial

Two insect meals of *T. molitor* were used, which were reared on two different substrates. The first meal ("Conventional") was created from insects reared in a conventional substrate, while the second meal ("Enriched") was created from insects reared in a substrate partially enriched (20%) with plant material from residues of distillation of medicinal aromatic plants: Greek oregano (*Origanum vulgare* subsp. *hirtum*), thymus (*Thymus vulgaris*), sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*) and their essential oils, linseed (*Linum usitatissimum*), sea fennel (*Crithmum maritimum*), and olive residues after the process. Insects were reared for a period of four months in total, starting from newly hatched larvae until the stage of late-instar larvae, i.e., prior to pupation, as suggested by Rumbos et al. (2021), which was the instar that was used in the feeding trials [26]. The insects were kept frozen (−20 ◦C) until being used for the preparation of the pig diets (Figure 2.4). For chitosan supplementation, dry high-purity chitosan with a molecular weight of 250,000 daltons (GP8523-1000, Glentham Life Sciences Ltd., Corsham, UK) was procured.

Figure 2.4. *T. molitor* larvae before grinding

Group A (Control) received a standard maize–barley–soybean meal–based diet formulated according to the recommendations of the National Research Council [27] and the Premier Nutrition atlas [28]. In Group B, fishmeal was fully replaced by the "Conventional" *Tenebrio molitor* larvae meal, incorporated at 10% of the total diet. In Group C, fishmeal was similarly substituted by the "Enriched" *T. molitor* larvae meal, maintaining the same inclusion level (10%). Group D was fed the control diet supplemented with high-purity chitosan powder at a concentration of 0.5 g/kg. Finally, Group E received a combination of the "Conventional" *T. molitor* larvae meal (10%) and chitosan (0.5 g/kg) (Figure 2.5).

Figure 2.5. Raw materials used for experimental feed: maize and *T. molitor* larvae

All five diets were formulated to be isocaloric and isonitrogenous. On the day of feed preparation, frozen *T. molitor* larvae were thawed, weighed, ground using a hammer mill, and mixed with the remaining ingredients in a horizontal mixer, following the standard procedure used on the commercial farm. According to Jin et al. (2016), dried *T. molitor* larvae contain on average 11.56 g/kg of chitosan; this value was used to estimate the chitosan content in the diets of Groups B, C, and E [15]. The total phenolic content of the diets was analyzed with the Folin–Ciocalteu method as described by Vasilopoulos et al. (2022)[29]. Table 2.1 presents the chemical composition of *T. molitor* whole larvae meal. Table 2.2 presents the ingredients and chemical composition of the five diets.

Table 2.1. Chemical composition of *Tenebrio molitor* whole larvae meal.

Chemical Composition, g/kg as Fed	***T. molitor* Whole Larvae Meal**
Dry matter	271.6
Digestible energy (DE, MJ/kg)	7.6
Crude protein	169.8
Crude fiber	22.0
Ether extract	123.0
Ash	13.0
Acid detergent fiber (ADF)	23.0
Neutral detergent fiber (NDF)	52.0
Chitin	11.56
Lysine	10.0
Meth + Cyst	5.0
Methionine	0.3
Cystine	0.2
Threonine	10.0
Tryptophan	1.9
Calcium	1.0
Total phosphorus	3.0

Table 2.2. Ingredients and chemical composition of the diets.

Ingredients, g/kg as Fed	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**
Maize	336.0	205.4	205.4	335.5	204.9
Barley	347.0	347.0	347.0	347.0	347.0
Wheat middlings	30.0	30.0	30.0	30.0	30.0
Soybean meal (47% crude protein)	168.0	188.8	188.8	168.0	188.8
Soybean oil	19.0	54.8	54.8	19.0	54.8
Vitamin and mineral premix [1]	60.0	60.0	60.0	60.0	60.0
Fishmeal (72% crude protein)	30.0	0.0	0.0	30.0	0.0
"Conventional" *T. molitor* meal	0.0	100.0	0.0	0.0	100.0
"Enriched" *T. molitor* meal	0.0	0.0	100.0	0.0	0.0
Chitosan	0.0	0.0	0.0	0.5	0.5
Benzoic acid	3.0	3.0	3.0	3.0	3.0
Zn oxide	3.0	3.0	3.0	3.0	3.0
Salt	2.0	2.0	2.0	2.0	2.0
Monocalcium phosphate (22% P)	2.0	6.0	6.0	2.0	6.0
Calculated analysis, g/kg as fed					
Dry matter	884.2	841.6	841.6	884.2	841.6
Digestible energy (DE, MJ/kg)	13.6	13.6	13.6	13.6	13.6
Crude protein	186.6	186.5	186.5	186.6	186.5
Crude fiber	34.5	34.9	34.9	34.5	34.9
Ether extract	39.4	79.0	79.0	39.4	79.0
Ash	52.8	54.1	54.1	52.8	54.1
Acid detergent fiber (ADF)	39.5	39.8	39.8	39.5	39.8
Neutral detergent fiber (NDF)	114.0	109.0	109.0	114.0	109.0
Chitosan	0.000	1.156	1.156	0.500	1.656
Total Lysine	12.3	12.2	12.2	12.3	12.2
Total Methionine and Cystine	7.7	7.4	7.4	7.7	7.4
Total Methionine	4.9	4.6	4.6	4.9	4.6
Total Cystine	2.8	2.8	2.8	2.8	2.8
Total Threonine	6.2	6.5	6.5	6.2	6.5
Total Tryptophan	2.0	2.1	2.1	2.0	2.1
Calcium	5.6	5.5	5.5	5.6	5.5
Total phosphorus	5.0	5.3	5.3	5.0	5.3
Sodium	3.0	2.9	2.9	3.0	2.9
Chloride	5.2	4.9	4.9	5.2	4.9
Potassium	6.7	6.4	6.4	6.7	6.4

[1] Supplied per kg diet: 15,000 IU retinol, 50 mcg 25-hydroxyvitamin D3, 9.96 mg tocopherol, 10.02 mg menadione, 3 mg thiamine, 10.02 mg riboflavin, 6 mg pantothenic acid, 6 mg pyridoxine, 40.02 mcg cobalamin, 100 mg ascorbic acid, 35 mg nicotinic acid, 300 mcg biotin, 1.5 mg folic acid, 375 mg choline chloride, 200 mg iron (II) sulfate monohydrate, 90 mg copper sulfate pentahydrate, 60 mg manganese sulfate monohydrate, 100 mg zinc sulfate monohydrate, 2 mg calcium iodate, 300 mg sodium selenide, 150 mg L-selenomethionine–selenium, 1500 FYT 6-phytase, 80 U β-1,4-endoglucanase, 70 U β-1,3 (4)-endoglucanase, 270 U β-1,4-endoxylanase, 5000 mg benzoic acid, 40.8 mg butylated hydroxytoluene, 3.5 mg propyl gallate.

The feeding trial lasted 42 days. Throughout this period, the growing pigs were weighed individually on the 1st, 21st, and 42nd days using a Mini-L 3510 animal scale (Zigisis, Chalkidiki, Greece). Daily records were maintained for feed intake and any cases of disease or mortality. The performance parameters that were evaluated during the trial included average weight, average weight gain, average feed intake, and average feed conversion ratio (FCR, calculated as kg of feed intake per kg of live weight gain) for three specific periods: 1–21 days, 21–42 days, and the overall period of 1–42 days (Figure 2.6). On the 42nd day, six animals were randomly selected from each pen, were processed in a nearby commercial abattoir, and their carcasses were stored.

Figure 2.6. Final body weight measurement of growing pigs on the last day of the trial

2.2.2. Determination of Fecal Microbial Populations

Fresh fecal (stool) samples were gathered on the last day (42nd) of the trial from each pig to analyze [30] and determine their bacterial profile. Initially, 1 g of a fresh fecal (stool) sample was homogenized with 9 mL of sterile peptone water solution at 0.1%. The Miles and Misra Plate Method (surface drop) was applied for the bacterial enumeration. The samples were serially diluted via 12-fold dilutions (from 10−1 to 10−12) using standard 96-well plates. Then, 10 μL of each dilution was inoculated on media and incubated properly. Specifically, total aerobic and anaerobic bacterial counts

were determined using plate count agar medium (Oxoid, Basingstoke, UK), while plates were incubated at 30 ◦C aerobically for 48 h and at 37 ◦C anaerobically for 48–72 h, respectively. MacConkey and Kanamycin aesculin azide (KAA) agar (Merck, Darmstadt, Germany) were, respectively, used for the isolation of Enterobacteriaceae and Enterococcaceae. All plates were incubated aerobically at 37 ◦C for 24–48 h. De Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and M17 agar (Lab M Limited, Lancashire, UK) were used for the isolation and enumeration of Lactobacillaceae, while media were incubated at 37 ◦C for 48 h in anaerobic conditions. For bacterial counts, typical colonies from an appropriate dilution were counted, and counts were expressed as colony-forming units (CFU) × log per 1 g wet weight sample. Typical colonies grown on different media were then described and subcultured (Figure 2.7).

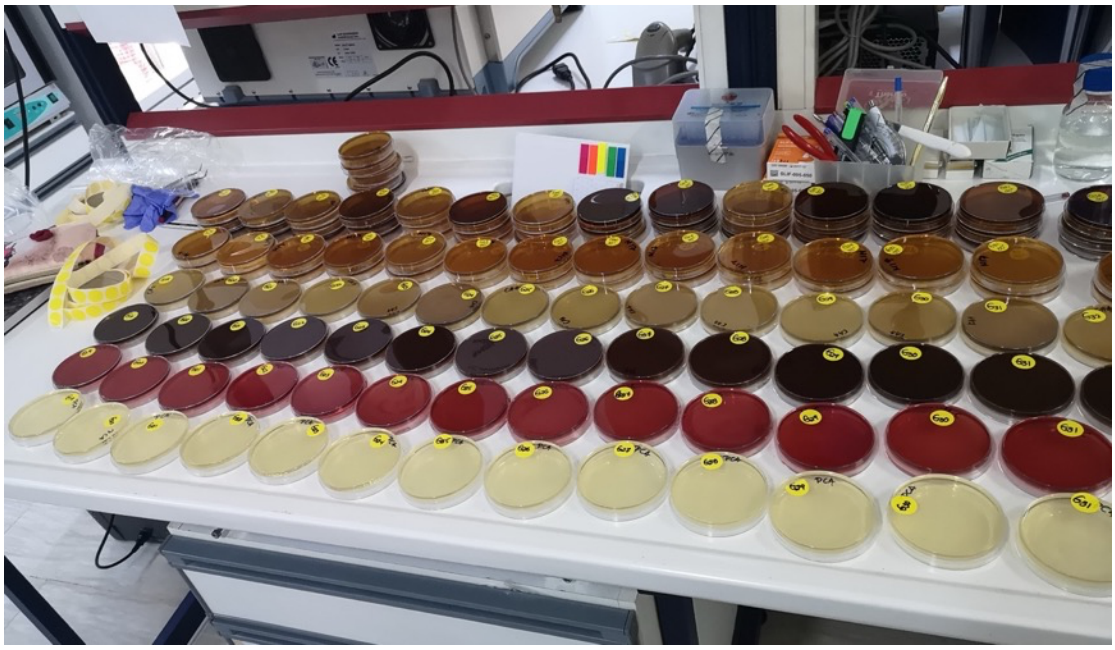

Figure 2.7. Array of different agar media used for the isolation and subculturing of bacterial populations

All bacterial populations were identified at family level by the automated Vitek 2 compact system (bioMérieux, Marcy l'Etoile, France), which provides reliable and accurate results for a wide range of Gram-positive and Gram-negative bacteria [31](Figure 2.8). For the identification of Enterobacteriaceae, Enterococcaceae, and Lactobacillaceae, the Vitek 2 Gram-Negative identification card (ID-GN) (bioMérieux, Marcy l'Etoile, France), the Vitek 2 Gram-Positive identification card (ID-GP)

(bioMérieux, Marcy l'Etoile, France), the CBC and ANC identification cards (bioMérieux, Marcy l'Etoile, France), and the Vitek 2 ANC ID card (bioMérieux, Marcy l'Etoile, France) were used, respectively.

Figure 2.8. Identification of the bacterial populations using the "Vitek 2 compact system"

2.2.3. Hematological and Biochemical Analysis of Blood Samples

On the 42nd day of the experiment, the feeders of the pigs were emptied four hours before blood sampling. For the determination of hematological and biochemical parameters, blood samples were taken from six growing pigs per treatment prior to slaughter. For blood collection, 4 mL of blood was collected from the jugular vein of the pigs and placed in vacutainer tubes with ethylenediaminetetraacetic acid (EDTA). Hematological parameters (WBC, White Blood Cells; Lym, Lymphocytes; Mon, Monocytes; Gra, Granulocytes; RBC, Red Blood Cells; Hct, Hematocrit; Hb, Hemoglobin; and THR, Thrombomodulin) were determined using an automated analyzer MS4 (Melet Schloesing Lab, Osny, France) and biochemical parameters (ALB, Albumine; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CHOL, Cholesterol; CK, Creatine kinase; GLU, Glucose; TBIL, Total Bilirubin; and TRIG, Triglycerides) in serum using the IDEXX VETTEST 8008 (IDEXX LAB, Westbrook, ME, USA)(Figures 2.9 & 2.10).

Figure 2.9. Preparation of growing pig blood samples for further analysis

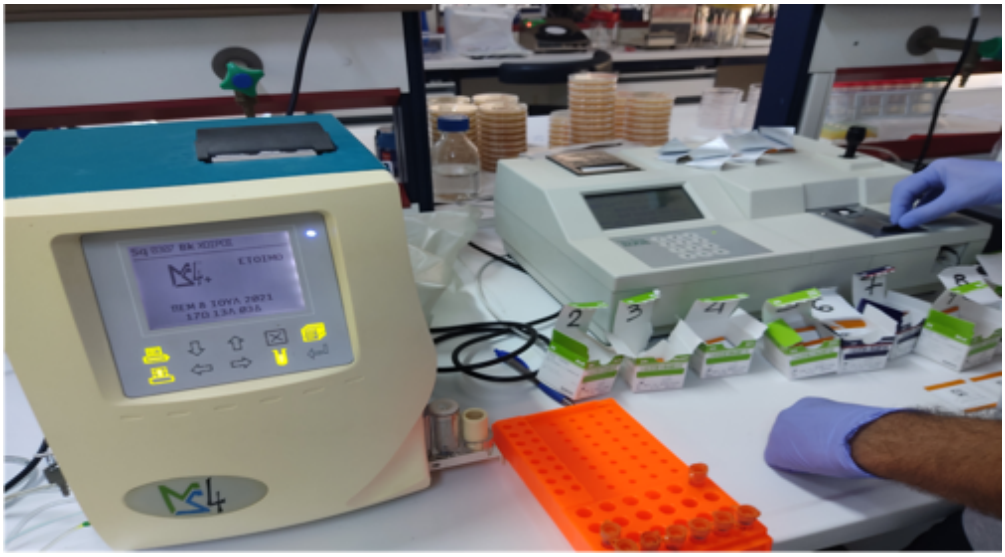

Figure 2.10. Analysis of hematological and biochemical parameters in growing pig blood

2.2.4. Collection of Meat Samples

The pigs were transported to the nearby commercial abattoir and then were processed following the regulations set forth by the national guidelines [32](Figure 2.11 & 2.12). During the processing, the carcasses were weighed and then meat samples were

collected from four specific muscle areas: ham (*biceps femoris* and *semimembranosus* muscles), shoulder (*trapezius* and *triceps branchi* muscles), belly (*external abdominal* and *oblique* muscles), and boneless steak (*longissimus thoracis* muscle) (Figure 2.13 & 2.14).

Figure 2.11. Transportation of growing pigs to the nearby commercial abattoir

Figure 2.12. Growing pig carcasses after the processing

Figures 2.13 & 2.14. Meat samples packaged and prepared for further analysis or storage

2.2.5. Chemical Analysis of Meat Samples

All collected meat cuts were stored at −20 ◦C to preserve their freshness and prevent any spoilage. On the day of the analysis, subsamples of 200g were cut, unfrozen, and minced using a commercial meat mincer (Bosch, Gerlingen, Germany). Moisture, crude protein, fat, collagen, and ash content were analyzed with a "FoodScanTM Lab analyzer" (FOSS, Hillerod, Denmark), following the AOAC 2007.04 guidelines [33, 34](Figure 2.15).

Figure 2.15. Chemical analysis of meat samples using the "FoodScanTM Lab analyzer"

2.2.6. Microbial Analysis of Meat Samples

Microbial populations were identified and enumerated in meat samples from shoulder, belly, and boneless steak samples. From each sample, 10 g of meat were collected and homogenized in a Bagmixer 400 (Interscience, Saint-Nom-la-Bretèche, France) with 90 mL of sterile Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK). Each sample was 10-fold diluted using glass tubes with 9 mL of sterile MRD. From the appropriate dilution, either 1 mL or 0.1 mL were inoculated in Petri dishes for the enumeration of the bacterial counts. The tested microorganisms were: *Escherichia coli*, which was cultivated on Tryptone Bile X-Glucuronide (TBX) agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37 ◦C for 24 h; Sulfite-Reducing Clostridia, which were cultivated on Perfringens Agar Base (Oxoid, Basingstoke, UK) and incubated at 37 ◦C for 48 h under anaerobic conditions using anaerobic jars with the addition of Anaerocult A (Oxoid, Basingstoke, UK); *Staphylococcus aureus* and *Staphylococcus* sp. That were spread on Baird Parker agar (Oxoid, Basingstoke, UK), which was supplemented with egg yolk tellurite (50 mL/1 L substrate) and incubated under aerobic conditions at 37 ◦C for 48 h; Total Mesophilic Counts that were measured in Plate Count Agar (PCA) (Oxoid, Basingstoke, UK) at 30 ◦C for 48 h under aerobic conditions; and *C. jejuni* that was spread on Campy Blood Free Selective Medium (CCDA) (Acumedia–Lab M, Lansing, MI, USA) with Campylobacter selective supplement under microaerophilic conditions in an incubator with 10% CO_2 at 37 ◦C for 72 h. All samples were examined for the presence of *Salmonella* spp. and *Listeria monocytogenes* per 25 g of meat using, respectively, the ISO 6579:2002 and ISO 4833:2001 methods [35, 36]. The Petri dishes were incubated in Binder BD 115 thermostable incubators [37].

2.2.7. Total Phenolic Analysis of Meat Samples

For the measurement of the total polyphenols of the meat samples (shoulder, belly, and boneless steak), a modified Folin–Ciocalteu method was used [38]. According to this method, 0.2 g/L of gallic acid (Merck, Darmstadt, Germany) was diluted in 100 mL of distilled water. The stock solution was used to prepare the standard solutions of 0.005, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 g/L of gallic acid. From each standard solution, 0.2 mL was transferred into a 50 ml falcon tube and mixed with 10.8 mL of distilled water, 8 mL of Na_2CO_3 (75 g Na_2CO_3 in 1 L distilled water) (Penta Chemicals, Prague, Czech Republic), and 1 mL of the Folin–Ciocalteu reagent (PanReac AppliChem, Darmstadt,

Germany). A control sample was prepared in which 0.2 mL of distilled water was added instead of a standard solution to calibrate the UV-Vis spectrophotometer (DR 5000, Hach Lange, Ames, IA, USA). All tubes were homogenized in a vortex, and they were placed in a dark cabinet for 1 h at room temperature. After the incubation, the control was used to calibrate the UV-Vis spectrophotometer (DR 5000, Hach Lange) at 750 nm, and then all the standard solutions were measured (Figure 2.16). A standard curve of concentration of gallic acid and absorbance was constructed using Microsoft Excel software, and the R^2 was 0.9989. The above procedure was followed to measure the total polyphenols in the meat.

Then, 5 g of shoulder, belly, or boneless steak meat were homogenized in a blender with 10 mL of distilled water and filtered with filter paper. A quantity of 0.2 mL of the filtrate was transferred into 50 mL falcon tubes and mixed with 10.8 mL of distilled water, 8 mL of Na_2CO_3 (75 g/L solution), and 1 mL of the Folin–Ciocalteu reagent. A blank sample was prepared in which 0.2 mL was added instead of the sample in order to calibrate the UV-Vis spectrophotometer. All tubes were mixed in a vortex and placed in a dark cabinet at room temperature for 1 h. After the incubation, the blank sample was used to calibrate the spectrophotometer at 750 nm, and then all the samples were measured.

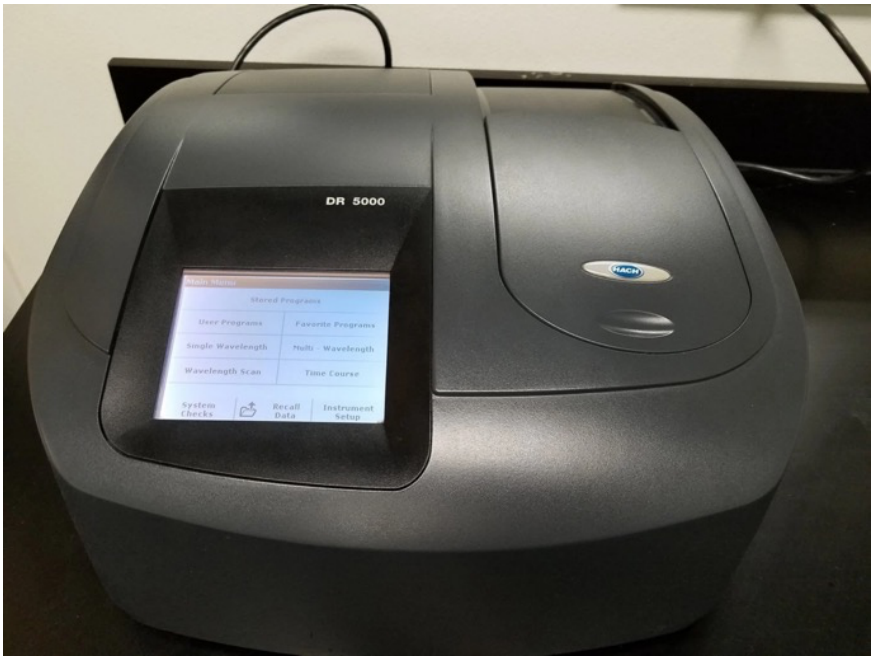

Figure 2.16. Total phenolic analysis of meat samples using the device "Hach Lange DR 5000 UV-Vis spectrophotometer"

2.2.8. Oxidative Stability Analysis of Meat Samples

For the measurement of lipid oxidation in the meat, a modified method by Dias et al. (2020) was used [39]. Shoulder, belly, and boneless steak meat cuts were used to measure lipid oxidation using the 2-thiobarbituric acid method (TBARS). From each sample, 5 g of meat was homogenized with 25 mL of trichloroacetic acid in a blender, transferred into a glass bottle, and left for 20 min. Then, the samples were filtered with filter paper, and 5 mL of the filtrate was transferred into glass tubes with 5 mL of 2-thiobarbituric acid. A blank sample was prepared, replacing the sample with 5 mL of trichloroacetic acid. All tubes were mixed in a vortex and placed in a water bath at 60 ◦C for 15 min. The samples were measured in a UV-Vis spectrophotometer after calibration with the blank sample at 532 nm.

2.2.9. Color and pH Analysis of Meat Samples

The chromatic profiles of the meat cuts (shoulder, belly, and boneless steak) were assessed using the "Hunter scale" (L*, a*, and b* values)(Figure 2.17) using a "CAM-System 500" analyzer (Lovibond, Amesbury, UK) and following the method described by Bonos et al. (2022)[37].

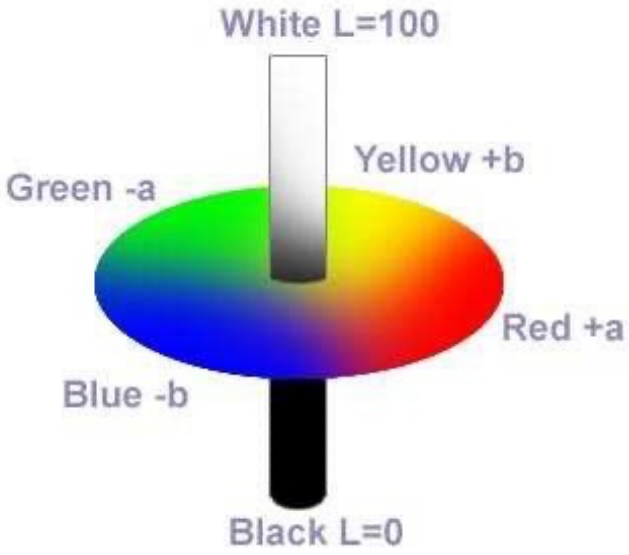

Figure 2.17. Schematic representation of the Hunter L*, a*, and b* color scale.

The pH levels of these meat cuts were assessed using a "Hanna Instruments pH meter" (Woonsocket, RI, USA), as described by Van de Perre et al. (2010)[40].

2.2.10. Meat Fatty Acid Analysis

To analyze the fatty acid profile of the shoulder meat samples, the processing method described by O'Fallon et al. (2007) was followed [41]. The separation and quantification of the methyl esters were carried out by following the procedure of Skoufos et al. (2016)[42]. The analysis was conducted using a "TraceGC device Model K07332" (Thermofinigan, Thermoquest, Milan, Italy), which was equipped with a flame ionization detector (FID) (Figure 2.18).

Figure 2.18. Fatty acid analysis of meat samples using the device "TraceGC device Model K07332"

2.2.11. Statistical Analysis

The basic study design was a RCB (random complete block design), and each ear tagged pig was considered an experimental unit. Log-transformation (log10) of microbiology data was performed prior to analysis. Data homogeneity was tested using Levene's test. Experimental data were analyzed by one-way analysis of variance (one-way ANOVA) or the Krushar–Wallis test, depending on the data format, using the SPSS v20 statistical package (IBM, Armonk, NY, USA) [43]. The Tukey's test was used for post-hoc comparisons between the three treatment groups. The significance level for all tests was set at 5% ($p \leq 0.05$). Values of p between 0.05 and 0.10 ($0.05 < p \leq 0.10$) were considered to have tendencies to differ.

2.3. Results

2.3.1. Total Phenolic Count

The total phenolic content of the feed in the control group (Group A) was 30.71 mg of gallic acid equivalents (GAE)/L of extract. The feed of Group B, which contained the

"Conventional" *T. molitor* meal, showed a total phenolic content of 47.67 mg GAE/L of extract, while the feed of Group C, containing the "Enriched" *T. molitor* meal, showed a value of 28.05 mg GAE/L of extract. The feed of Group D, supplemented with chitosan, had a total phenolic content of 29.23 mg GAE/L of extract, whereas Group E, which combined the "Conventional" *T. molitor* meal with chitosan, presented a total phenolic content of 43.39 mg GAE/L of extract.

2.3.2. Performance Parameters

The results of the examined diets on the performance parameters of the growing pigs are presented in Table 2.3. Based on the results presented in the table, no significant differences ($p > 0.10$) in initial body weight were observed among the experimental groups on day 0, indicating that all pigs started the trial at a similar weight. On day 21, pigs in Group B showed a significantly higher ($p \leq 0.05$) body weight compared to the control Group A, while Group E also exhibited a significantly higher ($p \leq 0.05$) body weight compared to Group A and D. On day 42, Group E had a higher body weight ($p \leq 0.05$) compared to Group D. During the first period (days 1–21), pigs in Group B showed a significantly higher ($p \leq 0.001$) weight gain compared to Groups A and D, while Group E achieved the greatest ($p \leq 0.001$) gain compared to Groups A, C, and D. During the second period (days 21–42), no significant differences ($p > 0.10$) in weight gain were observed among treatments. Over the entire experimental period (days 1–42), Group E had a significantly higher ($p \leq 0.05$) total weight gain compared to Group D. The feed intake and feed conversion ratio were within the expected ranges for the commercial pig farm that housed the experimental trial. Concerning carcass parameters, no significant differences ($p > 0.10$) were observed among treatments.

Table 2.3. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on performance parameters of growing pigs.

Body weight on day (kg)	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**SEM**	**P**
0	8.41	8.51	8.42	8.31	8.31	0.105	0.974
21	14.77^{a}	16.86^{bc}	16.04^{abc}	15.15^{ab}	17.46^{c}	0.250	0.001
42	24.86^{ab}	24.98^{ab}	25.29^{ab}	23.12^{a}	26.96^{b}	0.381	0.030
Weight gain for the period (kg)							
1 to 21 days	6.36^{a}	8.35^{bc}	7.63^{ab}	6.84^{a}	9.15^{c}	0.201	<0.001
21 to 42 days	10.09	8.13	9.25	7.97	9.61	0.319	0.138
1 to 42 days	16.45^{ab}	16.48^{ab}	16.88^{ab}	14.81^{a}	18.63^{b}	0.366	0.021
Feed intake per pig for the period (kg)							
1 to 21 days	14.56	14.02	14.05	13.53	13.87	-	-
21 to 42 days	21.19	20.46	20.49	19.65	20.25	-	-
1 to 42 days	35.75	34.48	34.54	33.18	34.12	-	-
Feed conversion ratio (FCR) for the period (kg feed / kg weight gain)							
1 to 21 days	2.29	1.68	1.84	1.98	1.52	-	-
21 to 42 days	2.10	2.52	2.22	2.47	2.30	-	-
1 to 42 days	2.17	2.09	2.05	2.24	1.82	-	-
Carcass parameters							
Carcass weight (kg)	14.94	15.66	16.80	16.72	18.26	0.448	0.168
Carcass percentage (%)	0.63	0.63	0.63	0.74	0.67	0.018	0.249

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. ^{a,b,c} Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$).

2.3.3. Fecal Microbial Populations

The effects of dietary supplementation on fecal microbial species are shown in Table 2.4. On day 42, total aerobic bacteria counts were significantly lower ($p < 0.001$) in Groups C, D, and particularly in Group E compared to the Groups A and B. Total anaerobes tended to be higher ($0.05 < p \leq 0.10$) in Group E compared to Group C. Enterobacteriaceae counts also tended to be lower ($0.05 < p \leq 0.10$) in Group E compared to Group B. No significant differences ($p > 0.10$) were found among groups for Enterococcaceae. However, Lactobacillaceae populations were significantly higher ($p < 0.05$) in Group E compared to Group B presented the lowest.

Table 2.4. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on fecal microflora populations of growing pigs.

Day 42 (Log10 CFU/g)	Group A	Group B	Group C	Group D	Group E	SEM	P
Total aerobes	8.34^{c}	8.63^{c}	7.49^{b}	7.43^{b}	6.64^{a}	0.117	<0.001
Total anaerobes	8.56^{xy}	8.74^{xy}	8.39^{x}	8.93^{xy}	9.23^{y}	0.101	0.085
Enterobacteriaceae	6.46^{xy}	6.90^{y}	6.38^{xy}	6.08^{xy}	5.89^{x}	0.121	0.092
Enterococcaceae	4.04	4.06	4.09	3.87	3.88	0.089	0.911
Lactobacillaceae	8.12^{ab}	6.96^{a}	7.09^{ab}	7.44^{ab}	8.60^{b}	0.193	0.028

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. $^{a, b, c}$ Means (n = 12 per treatment) with no common superscript differ significantly ($p \leq 0.05$). $^{x, y}$ Means (n = 12 per treatment) with no common superscript tend to differ ($0.05 < p \leq 0.10$).

2.3.4. Blood Parameters

Table 2.5 presents the impact of dietary supplementation on hematological and biochemical parameters. No significant differences ($p > 0.10$) were observed in hematological and biochemical parameters among the five groups, expect of cholesterol which was statistically significant higher ($p < 0.001$) in Group C compared to the other groups.

Table 2.5. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on blood parameters of growing pigs.

Hematological parameters	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**SEM**	**P**
WBC (m/mm3)	23.47	22.03	21.70	23.44	21.00	0.945	0.913
Lym (%)	34.33	35.48	37.37	38.82	39.08	0.826	0.292
Mon (%)	9.35	7.58	7.65	7.63	8.80	0.278	0.127
Gra (%)	56.32	56.93	54.98	55.08	53.45	0.967	0.839
RBC ($x10^6$/µL)	6.32	6.62	6.13	5.87	6.96	0.143	0.127
Hct (%of red blood cells)	35.02	36.32	34.28	34.98	37.98	0.815	0.659
Hb (g/dl)	11.87	12.27	11.82	11.48	14.10	0.331	0.082
THR ($x10^3$ /µL)	329.50	325.50	296.00	325.33	378.83	16.260	0.637
Biochemical parameters							
ALB (g/dL)	2.63	2.57	2.47	2.42	2.52	0.522	0.745
ALT (U/L)	117.33	115.33	122.83	123.83	126.50	3.409	0.848
AST (U/L)	69.50	74.83	70.33	47.38	68.83	3.331	0.066
CHOL (mg/dL)	75.00[a]	70.00[a]	92.00[b]	74.50[a]	76.66[a]	1.962	0.001
CK (U/L)	1189.50	1014.00	1050.67	1017.83	1221.00	113.935	0.968
GLU (mg/dL)	92.17	98.17	10.17	100.83	92.00	9.838	0.922
TBIL (mg/dL)	0.09	0.12	0.09	0.07	0.13	0.011	0.506
TRIG (mg/dL)	49.00	48.17	55.50	63.33	54.83	2.027	0.113

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. WBC, White Blood Cells; Lym, Lymphocytes; Mon, Monocytes; Gra, Granulocytes; RBC, Red Blood Cells; Hct, Hematocrit; Hb, Hemoglobin; THR, Thrombocytes; ALB, Albumine; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CHOL, Cholesterol; CK, Creatine kinase; GLU, Glucose; TBIL, Total bilirubin. [a, b] Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$).

2.3.5. Microbiological, Chemical, and Oxidative Stability Analysis of the Meat

The chemical composition of ham, boneless steak, shoulder, and belly meat cuts from pigs fed the experimental diets is presented in Table 2.6. In ham meat, moisture content in Group D tended to be higher ($0.05 < p \leq 0.10$) than in Group B. In belly meat, ash content was significantly influenced by the dietary treatments ($p < 0.05$), with Group A showing a higher ash percentage compared to Group E. All other chemical composition parameters were not significantly affected ($p > 0.10$) by dietary treatments.

Table 2.6. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on meat chemical composition of growing pigs.

Chemical composition (%) Ham meat	Group A	Group B	Group C	Group D	Group E	SEM	P
Fat	2.64	3.20	3.17	2.94	3.06	0.134	0.720
Protein	19.56	20.06	19.66	19.82	20.07	0.101	0.400
Moisture	76.89^{xy}	76.09^{x}	76.83^{xy}	76.93^{y}	76.32^{xy}	0.123	0.080
Collagen	1.02	0.89	0.89	1.11	1.03	0.033	0.130
Ash	0.98	0.97	0.95	1.03	1.04	0.023	0.750
Boneless steak meat							
Fat	3.18	2.57	2.77	2.75	2.98	0.106	0.440
Protein	19.80	20.61	20.26	20.34	20.47	0.119	0.242
Moisture	75.97	76.05	76.26	76.22	75.79	0.108	0.667
Collagen	1.17	1.08	1.10	1.20	1.27	0.338	0.421
Ash	1.05	0.98	0.90	0.96	0.93	0.021	0.157
Shoulder meat							
Fat	5.22	5.50	5.53	5.41	5.91	0.161	0.770
Protein	18.43	18.21	17.95	18.42	18.32	0.108	0.597
Moisture	75.56	75.55	75.50	75.42	75.17	0.143	0.926
Collagen	1.31	1.33	1.16	1.21	1.10	0.039	0.288
Ash	0.97	0.90	0.93	0.93	0.94	0.018	0.851
Belly meat							
Fat	9.87	8.61	9.54	8.61	8.99	0.228	0.770
Protein	16.93	17.55	17.10	17.33	17.49	0.147	0.597
Moisture	72.27	72.89	72.44	73.09	72.84	0.176	0.926
Collagen	1.66	1.67	1.52	1.62	1.50	0.054	0.288
Ash	1.00^{b}	0.90^{ab}	0.87^{ab}	0.93^{ab}	0.81^{a}	0.021	0.039

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. $^{a, b}$ Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$). $^{x, y}$ Means (n = 6 per treatment) with no common superscript tend to differ ($0.05 < p \leq 0.10$).

The microbial profile of shoulder, belly, and boneless steak meat from pigs fed the experimental diets is presented in Table 2.7. In shoulder meat, *Escherichia coli* counts were significantly lower ($p < 0.001$) in Groups B and E compared to the other three treatments. Similarly, *Clostridium spp.* populations were significantly reduced ($p < 0.05$) in Group B compared to the control Group A and Group C. *C. jejuni* and *Staphylococcus spp.* counts showed a tendency to decrease ($0.05 < p \leq 0.10$) in Groups B and E respectively, compared to the control. In belly meat, *Clostridium spp.* Counts were significantly lower ($p < 0.05$) in Group B compared to Groups A and C. In boneless steak meat, total microbial counts tended to decrease ($0.05 < p \leq 0.10$) in Group E compared to the Group C.

Table 2.7. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on meat microbial populations of growing pigs.

Shoulder Meat Microbiota (Log_{10} CFU/g)	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**SEM**	**P**
Total microbes	5.93	5.11	5.23	5.15	5.51	0.135	0.384
Escherichia coli	4.27^{b}	2.44^{a}	4.11^{b}	3.95^{b}	2.60^{a}	1.952	<0.001
Clostridium spp.	3.24^{b}	2.01^{a}	3.25^{b}	2.69^{ab}	2.38^{ab}	0.150	0.022
Campylobacter jejuni	3.44^{y}	2.33^{x}	3.09^{xy}	3.13^{xy}	2.47^{xy}	0.149	0.070
Staphylococcus spp.	4.80^{y}	4.61^{xy}	4.63^{xy}	4.27^{xy}	4.21^{x}	0.081	0.083
Staphylococcus aureus	2.60	2.45	2.38	1.96	2.63	0.097	0.220
Belly meat microbiota (Log10 CFU/g)							
Total microbes	6.04	6.20	6.33	5.92	6.03	0.123	0.869
Escherichia coli	4.31	3.37	3.88	3.62	3.17	0.181	0.316
Clostridium spp.	3.24^{b}	2.01^{a}	3.25^{b}	2.32^{ab}	2.38^{ab}	0.165	0.034
Campylobacter jejuni	4.17	4.09	4.33	4.15	3.61	0.142	0.590
Staphylococcus spp.	4.80	4.61	4.63	4.27	4.21	0.081	0.630
Staphylococcus aureus	2.40	2.46	2.18	2.22	1.93	0.120	0.691
Boneless steak meat microbiota (Log10 CFU/g)							
Total microbes	4.35^{xy}	4.35^{xy}	4.76^{y}	4.12^{xy}	3.90^{x}	0.101	0.068
Escherichia coli	2.74	2.08	2.10	1.89	1.58	0.880	0.366
Clostridium spp.	1.45	1.47	1.61	1.53	1.63	0.324	0.891
Campylobacter jejuni	3.32	2.98	2.86	2.85	2.59	0.135	0.568
Staphylococcus spp.	3.09	2.33	2.79	3.07	2.47	0.162	0.481
Staphylococcus aureus	2.91	2.11	2.32	2.07	1.80	0.188	0.435

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. $^{a, b}$ Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$). $^{x, y}$ Means (n = 6 per treatment) with no common superscript tend to differ ($0.05 < p \leq 0.10$).

The effects of the experimental diets on meat oxidative stability, pH, and color characteristics of growing pigs are presented in Table 2.8. Regarding total phenolic content, shoulder meat from Groups B and E exhibited significantly higher ($p < 0.05$) phenolic values compared to the control Group A. Similarly, in belly meat, total phenols tended to increase ($0.05 < p \leq 0.10$) in Group E compared to the control Group. Concerning lipid oxidation (TBARS values), shoulder meat from Groups B, D, and E showed significantly lower ($p = 0.007$) malondialdehyde levels than the control Group. In boneless steak meat, TBARS values tended to decrease ($0.05 < p \leq 0.10$) in Group D compared to Group B. In terms of pH, belly meat exhibited significantly lower ($p < 0.05$) pH values in Groups D and E compared to the control Group A and Group B and on Group C compared to Group A. Boneless steak meat from Groups C and E tended to have higher ($0.05 < p \leq 0.10$) pH values than the Group B. Regarding L* value of meat color, which represents lightness, boneless steak meat from Group B showed a tendency ($0.05 < p \leq 0.10$) for higher L* values compared to Group C.

Table 2.8. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on meat oxidative stability, pH, and color characteristics of growing pigs.

Total Phenols (g/L)	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**SEM**	**P**
Shoulder meat	1.96 ^{a}	5.31^{b}	3.85 ^{ab}	4.54 ^{ab}	5.27^{b}	0.389	0.023
Belly meat	1.83 ^{x}	2.04 ^{xy}	2.34^{xy}	2.36 ^{xy}	2.38^{y}	0.077	0.076
Boneless steak meat	3.54	5.25	4.82	4.34	4.18	0.231	0.169
TBARS (mg MDA/kg)							
Shoulder meat	0.06^{b}	0.03^{a}	0.05^{ab}	0.03^{a}	0.03^{a}	0.004	0.007
Belly meat	0.05	0.05	0.05	0.05	0.05	0.002	0.939
Boneless steak meat	0.114^{xy}	0.127^{y}	0.126^{xy}	0.108^{x}	0.126^{xy}	0.003	0.092
pH							
Shoulder meat	5.84	5.76	5.76	5.73	5.76	0.016	0.016
Belly meat	5.96^{c}	5.94^{bc}	5.86^{ab}	5.85^{a}	5.85^{a}	0.014	0.014
Boneless steak meat	5.95^{xy}	5.87^{x}	6.08^{y}	6.02^{xy}	6.08^{y}	0.028	0.059
Color L*							
Shoulder meat	63.22	61.50	60.22	58.68	57.86	0.824	0.248
Belly meat	64.40	58.64	61.10	62.94	62.52	0.874	0.302
Boneless steak meat	72.14^{xy}	72.32^{y}	67.54^{x}	71.76^{xy}	67.72^{xy}	0.788	0.089
Color A*							
Shoulder meat	15.14	14.48	16.56	17.02	13.86	0.709	0.612
Belly meat	13.92	15.94	15.78	13.60	14.80	0.588	0.664
Boneless steak meat	8.08	7.14	7.60	5.26	6.68	0.446	0.366
Color B*							
Shoulder meat	12.32	13.24	12.18	12.94	12.00	0.295	0.668
Belly meat	10.12	11.54	11.22	12.16	11.08	0.381	0.338
Boneless steak meat	14.98	16.08	18.28	17.16	19.68	0.639	0.150

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. $^{a, b, c}$ Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$). $^{x, y}$ Means (n = 6 per treatment) with no common superscript tend to differ ($0.05 < p \leq 0.10$). Lightness (L*), redness (a*), and yellowness (b*) values.

The effects of dietary supplementation on the shoulder meat fatty acid profile are presented in Table 2.9. C14:0 (Myristic) fatty acid was significantly lower ($p \leq 0.01$) in groups B, D, and E compared to the control group A. C15:0 (Pentadecanoic) fatty acid tended to be lower ($0.05 < p \leq 0.10$) in group B compared to group A. C15:1 (cis-10-Pentadecenoic) fatty acid was lower ($p \leq 0.05$) in group D compared to group A. C16:1 (Palmitoleic) fatty acid was significantly higher ($p \leq 0.01$) in groups D and E compared to groups A, B and C. C17:1 (cis-10-Heptadecenoic) fatty acid was significantly higher ($p \leq 0.01$) in group B compared to groups C, D, and E. C18:0 (Stearic) fatty acid tended to be lower ($0.05 < p \leq 0.10$) in group E compared to the control group A. C18:1n9t (Elaidic) fatty acid was slightly higher ($p \leq 0.05$) in group C compared to group A. C18:1n9c (Oleic) fatty acid was significantly higher ($p \leq 0.01$) in groups D and E compared to groups B, and C. C18:2n6c (Linoleic) fatty acid was significantly higher ($p \leq 0.05$) in groups B, C, and E compared to the other two groups. C20:0 (Arachidic) fatty acid tended to be higher ($0.05 < p \leq 0.10$) in groups C, D, and E compared to group A. C18:3n3 (α-Linolenic) fatty acid tended to be higher ($0.05 < p \leq 0.10$) in group D compared to the control group. C21:0 (Henicosanoic) fatty acid was higher ($p \leq 0.05$) in groups B and E compared to the control. C22:0 (Behenic) fatty acid was significantly lower ($p \leq 0.05$) in groups D and E compared to groups A and B. Total saturated fatty acids (ΣSFA) were lower ($p \leq 0.01$) in group E compared to control group A, while total monounsaturated (ΣMUFA) were significantly higher ($p \leq 0.01$) in groups D and E. Both total polyunsaturated fatty acids (ΣPUFA) and n6 (omega-6) fatty acids were significantly higher ($p \leq 0.01$) in groups B, C and, E compared to the other two groups. Finally, n3 (omega-3) fatty acids tended to be higher ($0.05 < p \leq 0.10$) in Group D compared to the control group.

Table 2.9. Effect of dietary addition of *Tenebrio molitor* larvae meal and chitosan on shoulder meat fatty acid composition of growing pigs.

Shoulder Meat Fatty Acids	Group A	Group B	Group C	Group D	Group E	SEM	P
C14:0 (Myristic)	0.30^{b}	0.06^{a}	0.17^{ab}	0.09^{a}	0.07^{a}	0.026	0.001
C15:0 (Pentadecanoic)	0.29^{y}	0.05^{x}	0.22^{xy}	0.15^{xy}	0.13^{xy}	0.027	0.054
C15:1 (cis-10-Pentadecenoic)	2.01^{b}	1.64^{ab}	1.40^{ab}	0.62^{a}	0.96^{ab}	0.163	0.026
C16:0 (Palmitic)	28.40	26.89	25.90	27.90	25.57	0.640	0.141
C16:1 (Palmitoleic)	0.09^{a}	0.84^{ab}	1.48^{bc}	4.02^{d}	2.32^{c}	0.372	0.010
C17:0 (Heptadecanoic)	0.50	0.30	0.21	0.20	0.27	0.050	0.104
C17:1 (cis-10-Heptadecenoic)	0.53^{ab}	0.82^{b}	0.48^{a}	0.26^{a}	0.39^{a}	0.057	0.003
C18:0 (Stearic)	12.43^{y}	10.49^{xy}	11.37^{xy}	10.93^{xy}	9.29^{x}	0.369	0.065
C18:1n9t (Elaidic)	0.047^{a}	0.060^{ab}	0.090^{b}	0.077^{ab}	0.086^{ab}	0.006	0.037
C18:1n9c (Oleic)	23.38^{ab}	21.78^{a}	20.16^{a}	25.42^{b}	26.11^{b}	0.650	0.001
C18:2n6t (Linolelaidic)	0.06	0.07	0.07	0.05	0.06	0.005	0.549
C18:2n6c (Linoleic)	24.70^{a}	29.28^{b}	32.01^{b}	24.71^{a}	29.08^{b}	0.830	0.019
C18:3n6 (γ-Linolenic)	0.07	0.05	0.08	0.09	0.18	0.016	0.112
C20:0 (Arachidic)	0.66^{x}	1.05^{xy}	1.16^{y}	1.29^{y}	1.33^{y}	0.074	0.089
C18:3n3 (a-Linolenic)	0.23^{x}	0.42^{xy}	0.47^{xy}	0.52^{y}	0.43^{xy}	0.035	0.097
C20:1n9c (cis-11-Eicosenoic)	0.05	0.03	0.02	0.01	0.02	0.005	0.263
C21:0 (Henicosanoic)	0.40^{ab}	0.56^{b}	0.35^{a}	0.44^{ab}	0.59^{b}	0.030	0.010
C20:2 (cis-11,14-Eicossadienoic)	0.38	0.33	0.28	0.32	0.25	0.024	0.426
C22:0 (Behenic)	5.48^{b}	5.28^{b}	4.08^{ab}	2.91^{a}	2.84^{a}	0.345	0.024
Σ SFA (Total Saturated)	48.47^{c}	44.69^{bc}	43.46^{ab}	43.90^{ab}	40.10^{a}	0.797	0.001
Σ MUFA (Total Monounsaturated)	26.10^{ab}	25.17^{a}	23.63^{a}	30.42^{c}	29.89^{bc}	0.786	0.001
Σ PUFA (Total Polyunsaturated)	25.44^{a}	30.15^{b}	32.91^{b}	25.69^{a}	30.01^{b}	0.839	0.001
n6 (omega 6) Fatty Acids	24.83^{a}	29.39^{b}	32.16^{b}	24.85^{a}	29.33^{b}	0.833	0.023
n3 (omega 3) Fatty Acids	0.23^{x}	0.42^{xy}	0.47^{xy}	0.52^{y}	0.43^{xy}	0.035	0.097

Group A, commercial diet; Group B, diet containing 10% "Conventional" *T. molitor* meal; Group C, diet containing 10% "Enriched" *T. molitor* meal; Group D, diet supplemented with 0.5 g/kg chitosan; Group E, diet containing 10% "Conventional" *T. molitor* meal and 0.5 g/kg chitosan; SEM, standard error of the mean. $^{a, b, c, d}$ Means (n = 6 per treatment) with no common superscript differ significantly ($p \leq 0.05$). $^{x, y}$ Means (n = 6 per treatment) with no common superscript tend to differ ($0.05 < p \leq 0.10$).

2.4. Discussion

Over the past several decades, there has been a significant enhancement in the growth rate of growing pigs. This has led to achieving greater body weight in a shorter period of time and an improved feed conversion ratio. However, the practice of early weaning—around 3–4 weeks—presents both nutritional and environmental challenges and can often negatively affect the gut functionality, immunity, feed efficiency, and overall growth performance of the piglets [44]. In response, the industry has tried to improve the formulations of the diets for piglets, trying to achieve high palatability and digestibility by utilizing highly digestible animal protein sources, such as insect meals, and promote good growth and health. These diets are expected to fulfill the dietary needs and promote the growth of the gastrointestinal and immune systems in young pigs [45]. To our knowledge, the present study is the first to investigate the effects of both "Conventional" and "Enriched" *T. molitor* larvae meals, as well as the combined use of the "Conventional" meal with chitosan, in the diets of growing pigs. The results of the trial suggest that employing a combination of *T. molitor* larvae meal (100 g/kg) and chitosan (0.5 g/kg) can completely replace 30 g/kg fishmeal in the diets of early weaned growing pigs, simultaneously improving their performance parameters. Moreover, the inclusion of either the "Conventional" or the "Enriched" *T. molitor* larvae meal did not adversely affect the pigs' growth performance. Some previously published studies have already demonstrated improvements in zootechnical parameters when *T. molitor* was incorporated into growing pig diets [14, 15]. However, it is important to highlight that not all studies have reported consistent results. For instance, Meyer et al. (2020) indicated a negative impact on pig growth performance when *T. molitor* was included in the final diet at a 10% rate [17]. From this perspective, chitosan appears pivotal to the pigs' enhanced performance. This improvement in zootechnical parameters is likely due to the additional chitosan supplementation combined with the chitin contained in the *T. molitor* larvae; notably, chitosan, due to its molecular structure, resists mammalian enzyme digestion and reaches the large intestine mostly unaltered. In the present experiment, while Group A had no chitosan content, the other groups had chitosan added either as part of the dried larvae meal or in pure form. More specifically, this combined addition increased the amount of chitosan in Group E by 43% compared to Groups B and C. Chitosan is considered to possess prebiotic properties and is known to enhance gut health and consequently animal performance.

Diets enriched with chitosan and tested in the growing pigs resulted in an enhanced growth rate, while this effect was attributed to the elevated levels of digestive enzymes (such as amylase) and the improved health status of the epithelium of the small intestine [46, 47]. In addition, these results suggest a potential boost in nutrient digestibility. Another important consideration is that insects not only recycle waste into protein but also have a lower carbon footprint and an efficient feed conversion ratio [48]. However, in the EU, the utilization of catering or animal waste as insect feed is prohibited [49]. Based on these results, using *T. molitor* larvae offers a promising alternative protein source for pig farming with improved sustainability processes.

During the weaning transition, piglets often face both various external environmental pressures and internal physiological challenges [50]. Weaned piglets, in particular, are vulnerable to pathogens because their intestinal immune system is not fully developed, which is often identified as an imbalance in the growth of intestinal beneficial versus potentially pathogenic microorganisms [51]. Currently, considerable research efforts are focused on seeking effective feed additives that can regulate the gut microbiota and alleviate intestinal inflammation. The gut microbiota is a complex and critical system that plays a vital role in numerous physiological functions of the host, including intestinal structure, barrier function, immune response, and overall health [52, 53]. A primary factor influencing intestinal microbiota is the nutrient composition of the diet, particularly the incorporation of antimicrobial compounds, whether they are of natural or synthetic origin [54]. In this study, we investigated the bacterial composition of the pig feces using a culture-dependent techniques analysis. The combined supplementation of *T. molitor* larvae meal and chitosan in the diets resulted in a reduction of Enterobacteriaceae and total aerobic bacterial counts, while concurrently increasing total anaerobic bacteria and Lactobacillaceae populations. These findings are consistent with previous studies in which chitosan alone was used as a feed additive, suggesting a beneficial modulation of the gut microbiota [24, 55]. Based on these results, supplementation of chitosan appears to have a positive impact on gut microbial populations. This is supported by the decrease in total aerobic counts, which typically include various potentially pathogenic microorganisms [56]; from the increase in total anaerobic bacteria, which are capable of producing short-chain fatty acids [57]; and from the reduction in Enterobacteriaceae, which include some of the most pathogenic bacteria in pig production [58], leading to substantial economic losses. These

modifications may be associated with chitosan, which can act as a distinct nutrient for certain families of intestinal microbes. Consequently, chitosan supplementation can cause changes in the types of microbial fermentation metabolites produced within the intestinal lumen [59]. Moreover, insect meals are rich in antimicrobial compounds that can inhibit the growth of pathogenic bacteria, such as methicillin-resistant Staphylococcus aureus [60]. Additionally, Choi et al. (2013) report that the use of insect-derived antimicrobial peptides, such as AMP-P5, in monogastric animal diets yields results on the intestinal microbiota comparable to those of antibiotics [61]. Furthermore, in the present experiment, the combined use of *T. molitor* and chitosan increased Lactobacillaceae counts and decreased total aerobic bacteria. Lactobacillaceae is a family of bacteria that can have several beneficial effects on gut health, immune support, and nutrient absorption [62]. The stress of weaning pigs often leads to changes in their gut bacteria: a decrease in beneficial bacteria like the Lactobacillus group and a reduction in microbial diversity create favorable conditions for the growth of harmful bacteria like *Clostridium* spp., *Prevotella* spp., and *E. coli* [63]. Notably, in our experiment, the combined supplementation with *T. molitor* larvae meal and chitosan had a beneficial effect on the examined fecal microbiota. This suggests that this combination can potentially alleviate post-weaning stress in growing pigs.

Hematological (WBC, Lym, Mon, Gra, RBC, Hct, Hb, and THR) and most biochemical parameters (ALB, ALT, AST, CK, GLU, TBIL, and TRIG) were not affected by the addition of the two *T. molitor* meals (except of total cholesterol) in the present trial and were within the physiological reference intervals reported for swine [64]. This could be a clear biomarker of the adequate quality of the tested diets, which contributed to the maintenance of the animal's health status. These results are in accordance with Ao et al. (2019), who tested dietary *T. molitor* larvae in the diets of growing pigs [65]. An increase in the count of blood platelets has been reported by Chia et al. (2019) when they supplemented the feeds of growing pigs with 50% *T. molitor*, which may be attributed to the high digestibility of insect-based protein and high levels of minerals such as iron [66]. Moreover, some recent studies have examined other insect meals (*H. illucens*) in broiler and pig diets and did not identify any detrimental effects on blood chemical parameters [67-69].

The presence of microbes in meat is strictly connected to its overall quality and safety for consumption. Although muscle tissues are usually sterile in live animals, under commercial processing conditions, their meat is contaminated during slaughter, cutting, and storage. In the present study, all measured levels of microbial contamination fell within acceptable safety parameters. Additionally, we found no evidence of harmful bacteria such as *Salmonella* spp. or *Listeria monocytogenes* in any of the 25 g meat samples analyzed. The only statistically significant result was the reduction in *E. coli*, *C. jejuni, Clostridium spp., and Staphylococcus spp.* especially in the shoulder meat samples when both *T. molitor* larvae meals and chitosan was used. The reported antimicrobial activity of insect meals is linked to their rich content of antimicrobial peptides [70]. Additionally, according to Chen et al. (2022), there is a remarkable association between the intestinal microbial populations and the quality of swine meat, suggesting that the diet of an animal has the potential to influence not only the gut microbial communities but also the bacterial metabolites and, consequently, the overall quality of the meat during storage [71]. Also, it has been documented that elevated populations of beneficial gut bacteria are positively associated with superior meat quality [72].

Meat quality is vital to the economic viability of pig farming, as it directly influences the meat's capacity for extended storage and further processing [73]. The impact of alternative production systems on the chemical composition of pig meat is not consistently supported by the existing literature. Generally, physical activity affects certain meat quality traits, such as muscle metabolism and post-slaughter pH levels, more than it does the meat's chemical composition [74]. When there are changes in the meat's chemical composition, they are often due to management factors like feed composition, feed intake, and the metabolic energy used for maintenance [75]. Recent studies have specifically explored how alternative production systems influence the chemical properties of meat [76]. In our study, the dietary use of *T. molitor* and chitosan did not affect the main parameters of the chemical composition of the meat cuts, such as fat, protein, and collagen. It can be noted that the use of chitosan increased the moisture content of the ham. In addition, the ash content of belly was decreased by the combined use of *T. molitor* and chitosan. The increase in moisture content in meat may be attributable to its inverse relationship with fat content, as originally outlined by Callow (1948)[77]. These two factors are closely connected to the meat's juiciness. In

pigs, a higher percentage of lean meat is associated with a higher level of ash content and a lower level of intramuscular fat [78]. The present results diverge from previous research [79] that suggested the inclusion of *T. molitor* in pig feed leads to higher percentages of protein and fat in pork meat. In conclusion, the present research found that using *T. molitor* and chitosan in pig feed does not have a negative impact on meat quality. It should be noted that these ingredients offer promising opportunities for further study, particularly in exploring how they affect the deposit of minerals in muscle tissue.

It is well established that the animal diet plays a significant role in shaping the physicochemical properties of the produced meat [80, 81]. Conducting tests to measure the antioxidant capacity is particularly valuable for assessing the antioxidant status of meat from animals that have been given different types of feed [82]. In the present experiment, the total phenolic value was increased by the supplementation of *T. molitor* and chitosan in shoulder and belly meat cuts. Respectively, MDA content was reduced in shoulder meat cuts. It appears that there is a relationship between the content of dietary phenols in the meat and its resistance to oxidative damage. These observations are in agreement with previous studies, which reported that the use of *T. molitor* in monogastric animal diets can improve the oxidative stability of the meat [29]. Xu et al. (2018) reported that incorporating chitosan into the diets of weaned piglets led to an increase in total antioxidant capacity along with a reduction in the levels of MDA and cortisol in serum [46], which is in agreement with our results where chitosan supplementation reduced MDA content on boneless steak meat cut. However, supplementing *T. molitor* into the feeds of growing pigs did not influence the thiobarbituric acid reactive substances in ham cuts [20]. These results indicate the potential of both *T. molitor* and chitosan to protect growing pigs from oxidative stress by enhancing the functions of their antioxidant defense systems.

The quality of swine meat is intricately connected to the pH levels in the edible tissue [83]. Additionally, consumer decisions to purchase meat are frequently influenced by the visual appeal of its color [84]. The combined use of *T. molitor* and chitosan led to contrasting effects on pH values in different meat cuts: it lowered the pH in belly meat while raising it in boneless steak cuts. However, it is important to note that the pH levels for all examined meat cuts fell within the preferred acceptable ranges [85]. In addition, meat color is an important acceptability parameter for consumers since they often reject

products that vary from what they expect to be “normal” [37]. One of the factors that can affect pork meat color is the pigment content of the diet [76]. The present study shows only a tendency for alterations in L* color values of boneless steak meat cuts, particularly with “Conventional” *T. molitor* supplementation. All the other meat color parameters (L*, A*, and B* values) did not differ between the treatments; therefore, the added insect meals did not affect the overall pigment content of the diets. The specific underlying mechanisms for the above effects are unknown, and the published research about the effect of insect meals on pig meat quality is still very limited. According to Yu et al. (2019), there is evidence that dietary chitin and its derivatives, chitosan and chito-oligosacharides, can improve some pork meat parameters such as drip loss and color [7].

The fatty acid profile of meat from monogastric animals, such as pigs, is directly shaped by the specific types of fats included in their diet [86]. In the current study, the fatty acid composition of the meat was altered by the dietary supplementation of both *T. molitor* and chitosan. Supplementation only with *T. molitor* resulted in reduced levels of total saturated and monounsaturated fatty acids and elevated levels of total polyunsaturated and omega-6 fatty acids in shoulder meat cuts. Combined use of *T. molitor* and chitosan reduced the levels of total saturated fatty acids and increased the levels of total monounsaturated, total polyunsaturated and omega-6 fatty acids. According to Siemianowska et al. (2013), the fatty acid profile of *T. molitor* larvae is notably rich in monounsaturated fatty acids, specifically oleic, elaidic, linoleic, and eicosapentaenoic acids [87]. Moreover, the fatty acid composition of *T. molitor* larvae and meals can be improved through modification of the rearing substrate [88]. Our results are in agreement with recent studies, which also reported a decrease in saturated fatty acids (SFA) and an increase in polyunsaturated and omega-3 fatty acids in the produced meat of growing pigs when *H. illucens*, *T. molitor*, and *A. diaperinus* larvae meals were added in the diets [7, 89, 90]. In contrast, there is limited research on the effect of chitosan supplementation on pork meat fatty acids. Chitosan supplementation decreased total saturated and polyunsaturated and increased total monounsaturated and omega-3 fatty acids in the shoulder meat cut. A reasonable explanation could be that chitosan not only boosts the synthesis of short-chain fatty acids [91] but also improves lipid metabolism [92].

2.5. Highlights from the statistical comparison between enriched and conventional *T. molitor* larvae meals

Statistical analysis was conducted on three of the five initially defined experimental groups: the control group, the group receiving conventional *T. molitor* larvae, and the group receiving enriched *T. molitor* larvae. The analysis was performed using one-way ANOVA to evaluate the effects of conventional and enriched *T. molitor* on the diets of early-growing pigs. The key findings are summarized below:

Main Findings:

- **Growth performance:** No significant differences were observed among the groups ($p > 0.05$).
- **Fecal microbiota:** Significant shifts in microbial composition and balance were detected ($p < 0.001$).
- **Blood parameters:** Total cholesterol was significantly reduced in the insect-fed groups ($p < 0.001$), while other blood indices remained unchanged.
- **Meat quality:**
    - Total phenolic content was increased ($p < 0.05$).
    - Fatty acid profile was improved ($p < 0.001$), suggesting potential health benefits.
    - No significant effects were observed on meat color or proximate composition.
    - Specific microbial populations in the meat were significantly altered ($p < 0.001$).

2.6. Highlights from the statistical comparison between conventional *T. molitor* larvae meal and chitosan

Statistical analysis was conducted on four of the five initially defined experimental groups: the control group, the group receiving conventional *T. molitor* larvae meal, the group receiving chitosan, and the group receiving a combination of conventional *T. molitor* larvae meal and chitosan. The analysis was performed using two-way ANOVA to evaluate the effects of the larvae meal, chitosan, and their interaction, and was the second publication of the experiment. The key findings are summarized below:

Main Findings:

- **Growth performance:** *T. molitor* supplementation significantly enhanced overall growth ($p < 0.05$), while chitosan alone had no effect ($p > 0.05$). The combined treatment improved specific zootechnical parameters ($p < 0.05$).
- **Blood parameters:** *T. molitor* increased red blood cell counts ($p < 0.05$), whereas chitosan increased lymphocyte counts ($p < 0.05$).
- **Fecal microbiota:** Chitosan and the combined treatment significantly modulated gut microbial populations ($p < 0.05$).
- **Meat quality:**
    - *T. molitor* enhanced phenolic content, oxidative stability, and fatty acid profile ($p < 0.05$).
    - Chitosan improved phenolic content, oxidative stability, fatty acid composition, and meat color ($p < 0.05$).
    - The combination of *T. molitor* and chitosan affected meat color and fatty acid profile ($p < 0.05$).

2.7. Conclusions

This study evaluated the effects of two distinct *Tenebrio molitor* larvae meals—one produced on a conventional substrate and another on a plant-enriched substrate—as well as the combined use of *T. molitor* larvae meal and chitosan in the diets of growing pigs. Our results are particularly encouraging, demonstrating not only enhanced growth parameters but also beneficial effects on gut microbial populations, hematological parameters, and some identified effects in meat quality parameters (such as lipid oxidation and fatty acid composition). While further investigation is needed to fully validate the efficacy of the combined use of insect meals and chitosan in swine nutrition, our study provides strong evidence for the benefits of this innovative dietary approach.

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Chapter 3: Investigating the use of *Tenebrio molitor* larvae reared with different substrates as feed ingredients in finishing pig diets

3.1. Introduction

The agriculture sector relies on efficient and safe production systems to supply high-quality food. Growing awareness among consumers and producers regarding animal welfare, soil restoration, environmental impact, and water use has heightened scrutiny of livestock practices [1]. With the global population projected to exceed 10 billion by 2050 [2], the demand for food, particularly pork meat, which is expected to increase by 105% between 2010 and 2050 [3], create significant challenges for the agricultural and feed industries. Key protein sources for pig diets, such as fishmeal, processed animal proteins, soybeans, and milk by-products, are affected by fluctuating prices, reliance on imports, and regulatory restrictions on the use of animal tissue due to bovine spongiform encephalopathy concerns [1, 4-7]. These factors underscore the urgent need to identify alternative high-quality protein sources for sustainable pig nutrition.

Edible insects, including *Tenebrio molitor* larvae, offer a promising solution due to their high-quality protein, fats, minerals, vitamins, chitin, and antimicrobial peptides [8-12]. Insects can be efficiently reared on diverse substrates, including agro-industrial by-products, promoting circular economy principles while requiring less land and water than conventional livestock [1] [13] [1, 14]. EU regulations now permit the use of insect-derived proteins from eight species in poultry and pig feeds [15]. *T. molitor* larvae are especially attractive due to their digestibility, short production cycle, balanced amino acid profile, and high nutrient content comparable to soybean meal [16-19]. The nutritional composition of larvae can be modulated by their rearing substrate [20, 21], and this study explores the novel approach of enriching substrates with medicinal aromatic plants to enhance the bioactive and nutritional value of *T. molitor* meals. These meals were subsequently tested in finishing pig diets to evaluate effects on performance, gut microbiota, health, and meat quality, representing a novel approach that according to our knowledge has not been previously explored in the literature.

3.2. Materials and Methods

3.2.1. Trial Design

The experimental trial was conducted in a commercial swine farm, in the Epirus region of Greece. Eighteen (9 males and 9 females) clinically healthy crossbreed finishing pigs (¼ Large White, ¼ Landrace, and ½ Duroc) at 135 days of age were chosen. Ear tags were used to identify each pig individually and each pig was used as an experimental unit throughout the trial [22]. These animals, with initial average body weight of 89.67 ± 2.52 kg, were randomly allocated (3 males and 3 females) to three treatment groups (A, B and, C). Each group was placed in separate pens (Figures 3.1, 3.2 & 3.3).

Figure 3.1. Finishing pigs during the experimental trial

Two types of insect meals derived from *T. molitor* larvae were utilized, each reared with different feed materials. Both the "Conventional" and "Enriched" *T. molitor* larvae meals

used in this study were produced from the same insects reared and processed as described in Section 2.2.1.

The first group (Control - A) received a conventional maize-barley-based diet formulated based on the guidelines of the NRC (2012) and the Premier Nutrition database (2014)[23, 24]. In Group B's diet, the "Conventional" insect meal was included at a level of 6.0%, while in Group C's diet, the "Enriched" insect meal was incorporated at the same inclusion rate of 6.0%. In both Group B and Group C, the insect meals replaced 50% of the soybean meal. All three feeds were carefully designed to be isocaloric and with the same amounts of essential amino acids. Table 1 shows the dietary components and the nutrient profile of these three feeds. The pigs of all three groups had ad libitum access to food and water during the trial.

Figure 3.2. Finishing pigs during the experimental trial

Figure 3.3. Management of finishing pigs during the experimental trial

Table 3.1. Dietary components and nutrient profile of the three feeds used in the experimental trial.

	Groups		
Ingredients, g/kg as fed	**A**	**B**	**C**
Maize	650.0	648.5	648.5
Barley	50.0	50.0	50.0
Wheat middlings	150.0	150.0	150.0
Soybean meal (47% crude protein)	120.0	60.0	60.0
"Conventional" *T. molitor* meal	0.0	60.0	0.0
"Enriched" *T. molitor* meal	0.0	0.0	60.0
Vitamin and mineral premix [1]	10.0	10.0	10.0
Amino acid premix [2, 3]	2.5	4.0	4.0
Calcium carbonate	11.5	11.5	11.5
Salt	6.0	6.0	6.0
Calculated analysis, g/kg as fed			
Dry matter	875.4	855.3	855.3
Digestible energy (DE, MJ/kg)	13.6	13.7	13.7
Crude protein	143.5	137.2	137.2
Crude fiber	34.9	35.9	35.9
Ether extract	35.4	35.5	35.5
Ash	44.6	44.1	44.1
Acid detergent fiber (ADF)	42.5	39.2	39.2
Neutral detergent fiber (NDF)	134.1	128.6	128.6
Total Lysine	8.7	8.9	8.9
Total Methionine and Cystine	5.0	5.5	5.5
Total Methionine	2.7	3.0	3.0
Total Cystine	2.3	2.5	2.5
Total Threonine	5.1	5.5	5.5
Total Tryptophan	1.5	1.5	1.5
Calcium	6.1	5.9	5.9
Total phosphorus	3.8	3.4	3.4
Sodium	2.6	2.5	2.5
Chloride	4.2	4.2	4.2

[1] Provided per kg complete diet: 6,500 International Unit (IU) retinyl acetate; 1,200 IU cholecalciferol; 12.5 mcg 25-hydroxycholecalciferol; 60 mg alpha-tocopherol acetate; 2 mg menadione nicotinamide bisulphite; 2 mg thiamine mononitrate; 7 mg riboflavin; 25 mg pantothenic acid; 3 mg pyridoxine hydrochloride; 25 mcg cyanocobalamin; 25 mg nicotinic acid; 1 mg folic acid; 0.15 mg biotin; 300 mg choline chloride; 108 mg Fe from ferrous sulphate monohydrate; 25 mg Cu from copper sulphate; 48 mg Mn from manganese oxide; 84 mg Zn from zinc oxide; 1.2 mg I from calcium iodate; 0.24 mg Se from sodium selenite; 700 mg methionine; 100 mg L-tryptophan; 2730 L-Lysine mg HCl; 1,182.02 mg L-threonine; 1,500 phytase units (FYT; 6-fytase); 200 fungal xylanase units (FXU; endo-1,4-β-xylanase). [2] Provided per kg complete diet of group A: 871.88 mg L-lysine HCl; 824.74 mg L-threonine; 98.87 mg L-tryptophan; 44 mg DL-methionine. [3] Provided per kg complete diet of

groups B and C: 1395.01 mg L-lysine HCl; 1319.58 mg L-threonine; 158.20 mg L-tryptophan; 70.4 mg DL-methionine.

The whole experimental trial lasted 35 days. The pigs of groups B and C were fed the experimental diets from day 1 to day 28, whereas from day 29 to day 35 all three groups were fed the commercial diet of control group. Throughout the experimental period, each pig was individually weighed on days 1, 14, 28 and 35 using a Mini-L 3510 scale designed for adult pigs (Zigisis, Chalkidiki, Greece). Data on feed consumption were recorded every day. Additionally, bodyweight, average feed consumption per treatment, and feed to gain ratio were evaluated for the intervals of 1–14 days, 14–28 days, and the overall period of 1–28 days (Figure 3.4). On the final day of the dietary trial, all pigs were humanely sacrificed at a nearby commercial abattoir, where meat samples were taken and frozen prior to analysis.

Figure 3.4. Body weight measurement of the finishing pigs at the end of the experimental trial

3.2.2 Investigation of Fecal Microbiota

Fresh fecal samples were collected from each pig on days 1, 28, and 35 of the trial (the latter being seven days after the termination of the administration of the experimental diets) to evaluate individual bacterial profiles (Figure 3.5). The detailed procedure followed for sample collection and microbiological analysis is described in Section 2.2.2.

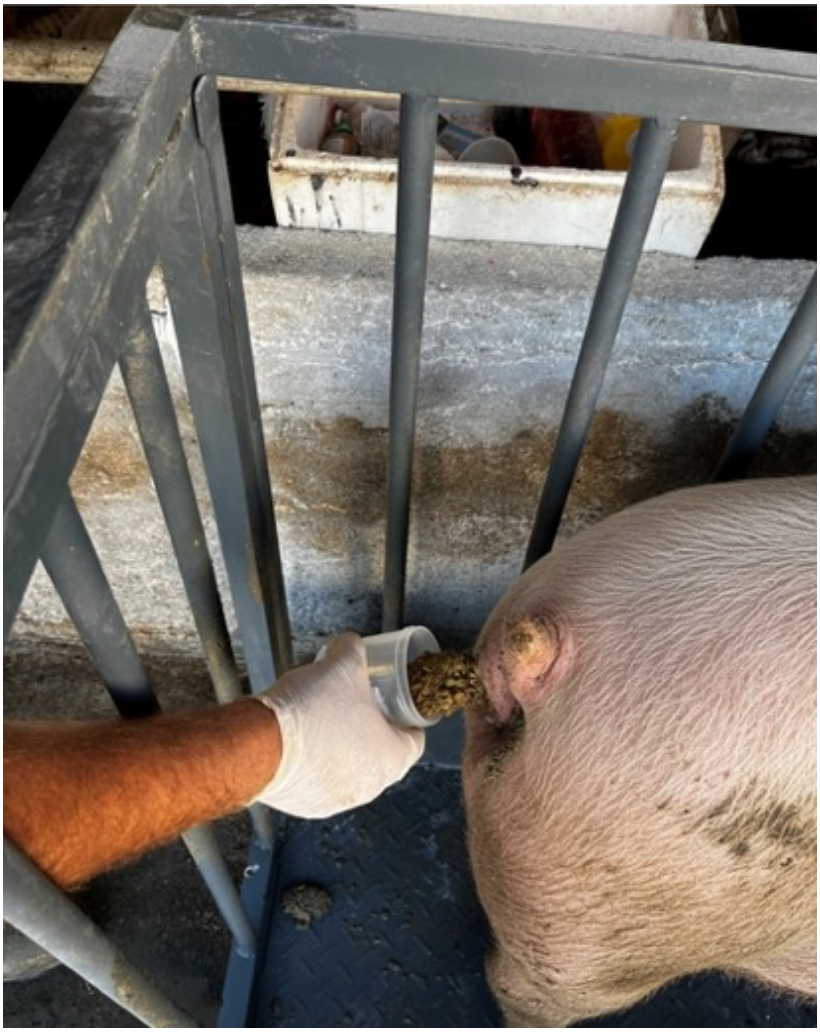

Figure 3.5. Fresh fecal sampling from finishing pigs during the experimental trial

Bacterial isolates were identified using the Bruker MALDI Biotyper system (Bruker Daltonik, Leipzig, Germany). Isolates and control strains from agar plates were analyzed via MALDI-TOF MS using a Microflex LT instrument (Bruker Daltonik), following established protocols [25, 26]. Briefly, bacterial cultures were overlaid with 1 µL of matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Prague, Czech Republic), dispersed in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich), prior to being air-dried. The MALDI Biotyper 3.0 software tool (Bruker Daltonik), which contains a library of 6,903 reference spectra, was used to analyze mass spectra. ID scores of 1.700–1.999 indicated probable genus identification, scores of 2.000–2.299 indicated secure genus identification with probable species identification, and scores of 2.300–3.000 indicated highly probable species identification [27]. These scores were based on the manufacturer's criteria for identification (Figure 3.6).

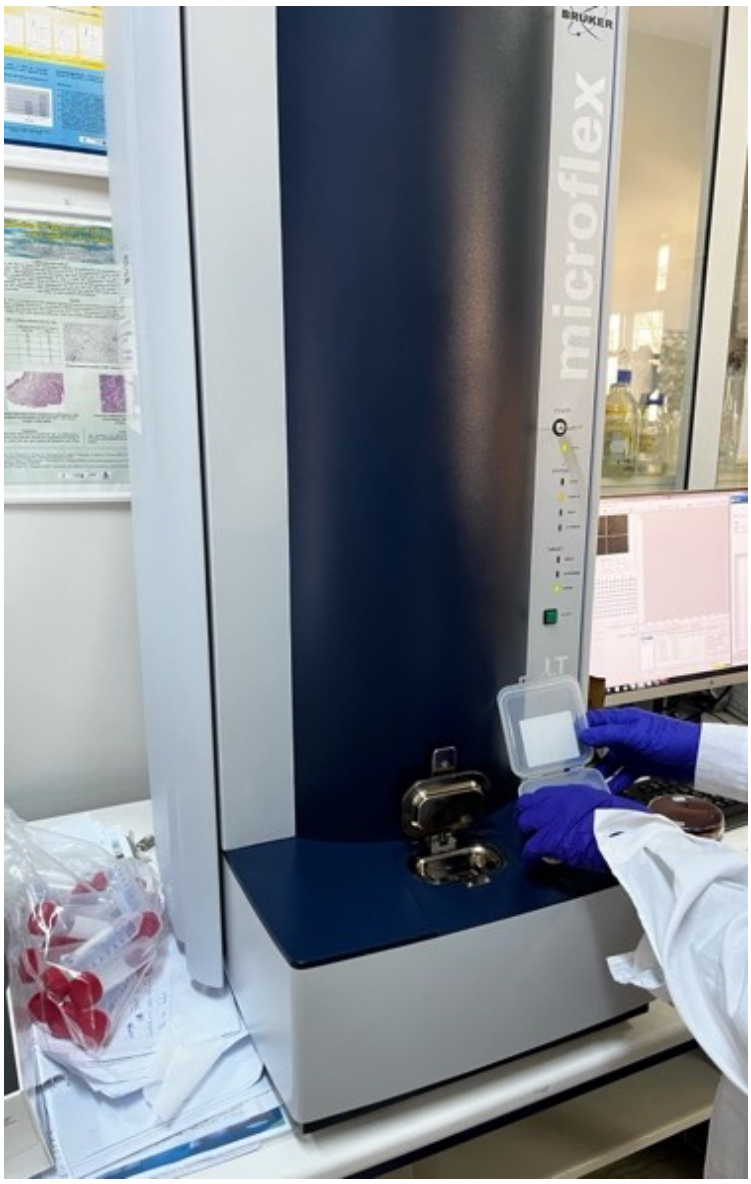

Figure 3.6. Identification of the bacteria isolates using the "Bruker MALDI Biotyper system"

3.2.3. Investigation of Blood Samples

On the 28th day of the dietary trial, the feed was removed from the feeders 4 hours before collecting blood samples from every finishing pig in order to examine biochemical and hematological markers. From each animal, 4 mL of blood was drawn from the jugular vein and distributed to different vacutainer tubes (without anticoagulant; with ethylenediaminetetraacetic acid - EDTA; with heparin). The detailed procedure followed for the analysis of biochemical and hematological markers is described in Section 2.2.3.

3.2.4. Sampling of Meat Cuts

At the final day of the experiment, all animals were slaughtered at a nearby abattoir according to national regulations [28], with considerations to minimize pre-slaughter stressors (transfer, handling and electrical stunning) [29](Figure 3.7). Sampling of meat cuts from the shoulder (*trapezius* and *triceps brachii* muscles) and belly (*external abdominal* and *oblique* muscles) was performed, and all samples were refrigerated prior to further analysis.

Figure 3.7. Finishing pig carcasses after the processing

3.2.5. Microbial Examination of Meat

Meat samples from the belly and shoulder were examined to identify and quantify microbial populations, following the procedure described in Section 2.2.6.

3.2.6. Chemical Analysis of Meat

Meat samples obtained during slaughter were stored at −20 °C until processing for chemical analysis, following the procedure described in Section 2.2.5.

3.2.7. Investigation of Total Polyphenols, Lipid Oxidation, and Total Antioxidant Activity in Meat Samples

The determination of total phenolic compounds in meat samples (belly and shoulder) was performed following the procedure described in Section 2.2.7.

Malondialdehyde (MDA) levels were measured during refrigerated storage in shoulder and belly meat using a method outlined in Section 2.2.8.

Meat extracts were prepared using the above-mentioned procedure for measuring total phenol content. Determination of Total Antioxidant Capacity TAC was conducted by the phosphomolybdate method using as described by Prieto et al. (1999)[30]. Results were expressed as a percentage of TAC.

3.2.8. Statistical Analysis

The statistical analysis for this experimental trial was conducted following the same procedure described in Section 2.2.11.

3.3. Results

3.3.1. Effect on Performance Parameters

The effects of the tested diets on the performance parameters of finishing pigs are shown in Table 2. No significant differences ($p > 0.10$) were observed in body weight or weight gain between the groups. Feed intake for the overall period (Days 1 to 35) were within typical ranges for this swine farm (A=3.65, B=3.66, and C=3.63 kg/day). Similarly, feed conversion ratio (FCR) for the overall period were also within expected ranges (A=3.34, B=3.47, and C=3.45 kg feed/ kg gain).

Table 3.2. Effect of dietary *Tenebrio molitor* larvae meal supplementation in the performance parameters of finishing pigs.

Body Weight on Day (kg)	Group A	Group B	Group C	SEM	P
1	88.30	90.10	90.60	0.650	0.340
14	105.12	104.42	104.48	0.839	0.940
28	119.78	119.40	119.98	1.241	0.852
35	128.05	127.80	128.24	1.603	0.994
Weight gain for the period (kg)					
1 to 14 days	16.82	14.32	13.88	0.657	0.142
14 to 28 days	14.66	14.98	15.50	0.531	0.810
28 to 35 days	7.48	8.40	8.26	0.700	0.864
1 to 35 days	36.90	37.70	37.64	1.354	0.969

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal; SEM, standard error of the mean.

3.3.2. Effect on Fecal Microflora

Insect meal supplementation influenced the fecal microflora (Table 3)(Figure 3.8). On day 1, no significant differences ($p > 0.10$) were observed in bacterial populations among the groups. On day 28, Enterobacteriaceae populations in group B were significantly higher ($p \leq 0.05$) compared to the other two groups. In contrast, no significant differences ($p > 0.10$) were noted for the other bacterial populations analyzed (Enterococcaceae, Lactobacillaceae, Bifidobacteriaceae, Total Anaerobes, and Total Aerobes). On day 35, group A showed significantly higher Enterobacteriaceae populations ($p \leq 0.001$) compared to the other two groups. Additionally, Total Anaerobes populations in group B were significantly higher ($p \leq 0.001$) than those in the other groups. The remaining bacterial populations (Enterococcaceae, Lactobacillaceae, Bifidobacteriaceae, and Total Aerobes) showed no significant differences ($p > 0.10$) between the groups at this time point.

Table 3.3. Effect of dietary *Tenebrio molitor* larvae meal supplementation on the fecal microbial populations of finishing pigs at three different time points.

Day 1 (Log_{10} CFU/g)	**Group A**	**Group B**	**Group C**	**SEM**	**P**
Enterobacteriaceae	6.48	5.43	5.61	0.268	0.243
Enterococcaceae	4.41	4.93	4.48	0.160	0.350
Lactobacillaceae	7.40	7.84	7.77	0.136	0.399
Bifidobacteriaceae	5.95	5.45	5.75	0.132	0.318
Total Anaerobes	8.14	8.09	7.91	0.166	0.604
Total Aerobes	6.68	6.18	6.08	0.134	0.154
Day 28 (Log_{10} CFU/g)					
Enterobacteriaceae	5.55[a]	6.17[b]	5.27[a]	0.142	0.019
Enterococcaceae	4.89	4.38	4.46	0.161	0.406
Lactobacillaceae	9.90	9.88	9.48	0.183	0.596
Bifidobacteriaceae	5.04	4.72	4.77	0.144	0.653
Total Anaerobes	11.18	10.96	11.78	0.053	0.149
Total Aerobes	6.01	6.33	5.61	0.160	0.128
Day 35 (Log_{10} CFU/g)					
Enterobacteriaceae	6.45[b]	5.13[a]	4.91[a]	0.194	<0.001
Enterococcaceae	4.24	4.20	4.00	0.205	0.893
Lactobacillaceae	8.92	9.10	9.97	0.299	0.222
Bifidobacteriaceae	5.21	5.26	5.46	0.105	0.619
Total Anaerobes	10.22[a]	11.30[b]	10.29[a]	0.136	<0.001
Total Aerobes	6.41	5.92	5.62	0.214	0.454

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. [a, b] Means with different superscripts are significantly different ($p \leq 0.05$).

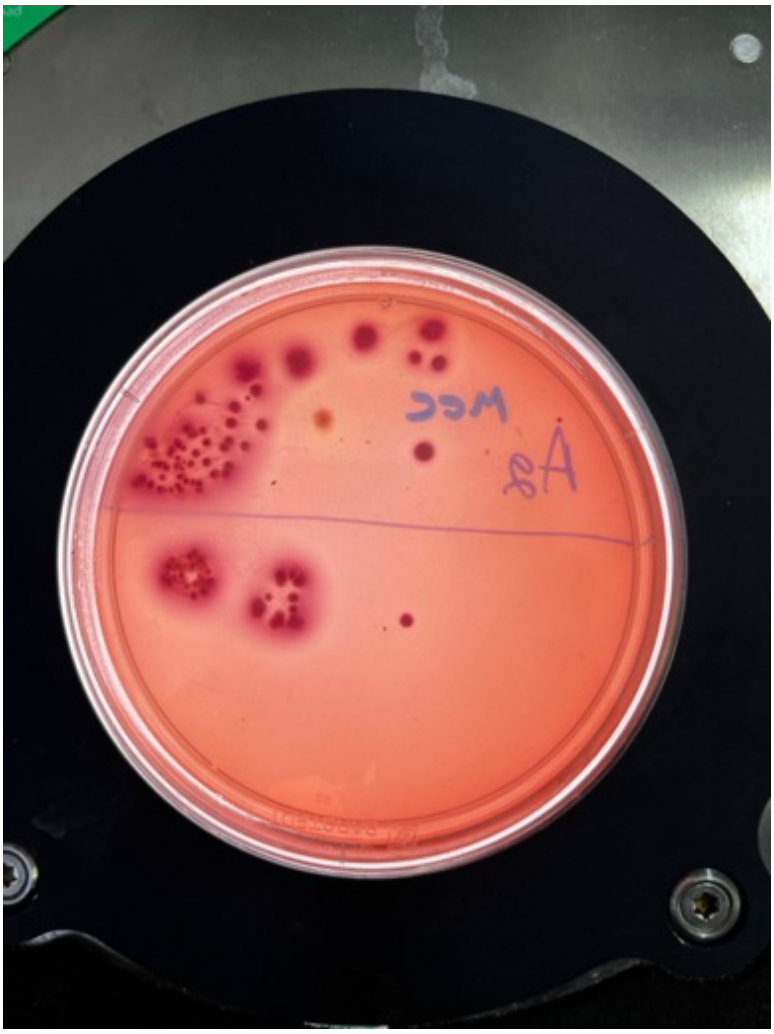

Figure 3.8. Cultured bacterial colonies on MacConkey agar plate

In addition to the analysis at the family level of pig feces, a comprehensive identification of the numerous bacterial strains was performed using the Bruker MALDI Biotyper system. This advanced approach allowed for accurate characterization of the microbial composition. Table 4 presents the results of the analysis.

On Day 1, *E. coli* was the most prevalent bacterium in all fecal samples, with relative abundances of 100% in Groups A, B, and C. Additionally, *B. pseudolongum, E. faecium, L. amylovorus, L. ruminis*, and *L. reuteri* were consistently present across all samples.

On Day 28, *E. coli* remained the dominant species, with relative abundances of 100% in Groups A, B, and C. *E. faecalis, E. gallinarum, E. hirae, L. amylovorus, L. ruminis*, and *L.*

reuteri were also present in all samples. Notably, an increased relative abundance of *E. faecalis, E. gallinarum*, and *L. reuteri* was observed in Group B, while *B. choerinum, L. amylovorus*, and *L. ruminis* showed an increase in Group C. Furthermore, *B. porcinum* and *B. thermophilum* were detected exclusively in Group B, whereas *B. pseudolongum* was identified only in Group A.

By Day 35, *E. coli* continued to exhibit the highest relative abundance, with 100% in Groups A, B and C. *E. coli* and *B. thermophilum* were significantly higher ($p \leq 0.05$) in Group A compared to the other two groups. *L. gallinarum* and *L. xylaniliticus* were identified solely in the experimental Groups B and C, while *B. pseudolongum* was found only in Group A. *L. ruminis* and *L. reuteri* were identified in Groups A and B. Additionally, Groups B and C exhibited an increased relative abundance of *B. thermophilum, E. gallinarum*, and *L. amylovorus*. In contrast, Group A showed an increase in *B. choerinum, B. porcinum*, and *L. reuteri*.

Table 3.4. Identification and distribution of microbial species (log cfu/mL), presenting the frequency of each species in fecal samples of finishing pigs (6 in total per group) and their mean counts.

Isolated bacteria	**Group A**		**Group B**		**Group C**		**SEM**	**P**
Day 1	# samples (%)	Log_{10}	# samples (%)	Log_{10}	# samples (%)	Log_{10}		
Bifidobacterium choerinum	4 (67%)	5.66	0 (0%)	-	3 (50%)	5.93	-	-
Bifidobacterium porcinum	2 (33%)	6.24	0 (0%)	-	1 (17%)	5.26	-	-
Bifidobacterium pseudolongum	1 (17%)	5.65	2 (33%)	5.48	2 (33%)	5.83	0.077	0.217
Bifidobacterium thermophilum	1 (17%)	5.48	0 (0%)	-	1 (17%)	5.60	-	-
Enterococcus faecium	1 (17%)	5.00	2 (33%)	5.00	1 (17%)	4.60	0.100	-
Escherichia coli	6 (100%)	6.48	6 (100%)	5.43	6 (100%)	5.61	0.269	0.243
Lactobacillus amylovorus	4 (67%)	7.10	2 (33%)	7.60	3 (50%)	7.30	0.218	0.735
Lactobacillus delbrueckii	1 (17%)	6.48	0 (0%)	-	0 (0%)	-	-	-
Lactobacillus kitasatonis	2 (33%)	6.98	0 (0%)	-	0 (0%)	-	-	-
Ligilactobacillus ruminis	1 (17%)	7.70	3 (50%)	8.42	2 (33%)	7.85	0.613	0.922
Limosilactobacillus reuteri	4 (67%)	7.28	5 (83%)	7.96	5 (83%)	7.50	0.262	0.602
Day 28	# samples	Log_{10}	# samples	Log_{10}	# samples	Log_{10}		
Bifidobacterium choerinum	2 (33%)	5.37	0 (0%)	-	2 (33%)	4.98	-	-
Bifidobacterium porcinum	0 (0%)	-	1 (17%)	4.78	0 (0%)	-	-	-
Bifidobacterium pseudolongum	1 (17%)	5.23	0 (0%)	-	0 (0%)	-	-	-
Bifidobacterium thermophilum	0 (0%)	-	1 (17%)	5.30	0 (0%)	-	-	-
Enterococcus faecalis	2 (33%)	4.65	3 (50%)	4.60	1 (17%)	3.90	0.291	0.753
Enterococcus gallinarum	1 (17%)	4.48	3 (50%)	4.23	2 (33%)	4.70	0.331	0.878
Enterococcus hirae	5 (83%)	4.81	1 (17%)	4.30	4 (67%)	4.10	0.183	0.183
Escherichia coli	6 (100%)	5.55^{ab}	6 (100%)	6.17^{b}	6 (100%)	5.27^{a}	0.142	0.019
Lactobacillus amylovorus	2 (33%)	9.65	5 (83%)	9.86	5 (83%)	8.72	0.299	0.208
Ligilactobacillus ruminis	2 (33%)	10.53	1 (17%)	7.70	2 (33%)	10.13	0.608	0.232
Limosilactobacillus reuteri	3 (50%)	9.63	4 (67%)	9.18	1 (17%)	10.30	0.297	0.527
Day 35	# samples	Log_{10}	# samples	Log_{10}	# samples	Log_{10}		
Bifidobacterium choerinum	5 (83%)	5.48	3 (50%)	5.00	3 (50%)	5.43	0.149	0.430
Bifidobacterium porcinum	3 (50%)	4.84	2 (33%)	5.04	2 (33%)	4.90	0.148	0.858
Bifidobacterium pseudolongum	2 (33%)	5.80	0 (0%)	-	0 (0%)	-	-	-
Bifidobacterium thermophilum	2 (33%)	5.30^{b}	5 (83%)	4.65^{a}	5 (83%)	4.81^{a}	0.094	0.034
Enterococcus faecalis	2 (33%)	3.25	3 (50%)	4.53	1 (17%)	3.48	0.445	0.495
Enterococcus gallinarum	1 (17%)	3.95	3 (50%)	3.78	2 (33%)	0.34	0.248	0.758
Enterococcus hirae	5 (83%)	4.33	1 (17%)	4.60	4 (67%)	4.31	0.221	0.944
Escherichia coli	6 (100%)	6.18^{b}	6 (100%)	5.13^{a}	6 (100%)	4.91^{a}	0.193	0.005
Lactobacillus amylovorus	3 (50%)	8.97	6 (00%)	8.77	6 (100%)	9.14	0.297	0.877
Lactobacillus gallinarum	0 (0%)	-	1 (17%)	7.18	1 (17%)	7.00	-	-

Ligilactobacillus ruminis	1 (17%)	10.30	1 (17%)	10.26	0 (0%)	-	-	-
Limosilactobacillus reuteri	5 (83%)	8.64	4 (67%)	8.15	0 (0%)	-	-	-
Lysinibacillus xylanilyticus	0 (0%)	-	1 (17%)	6.70	1 (17%)	9.95	-	-

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. [a, b] Means with different superscripts are significantly different ($p \leq 0.05$).

3.3.3. Effect on Hematological and Biochemical Parameters

Table 5 presents the impact of dietary supplementation on hematological and biochemical parameters. No significant differences ($p > 0.10$) were observed in hematological parameters among the three groups. Regarding biochemical parameters, group C tended to have higher albumin levels ($0.05 < p \leq 0.10$) compared to the other treatments. All other biochemical markers showed no significant differences ($p > 0.10$) between the groups.

Table 3.5. Effect of dietary *Tenebrio molitor* larvae meal supplementation on the blood profile of finishing pigs.

Hematological Parameters	**Group A**	**Group B**	**Group C**	**SEM**	**P**
WBC ($x10^3/mm^3$)	21.05	21.18	19.23	0.922	0.660
Lym (%)	51.58	50.64	51.96	1.520	0.944
Mon (%)	6.28	7.00	6.06	0.248	0.290
Gra (%)	42.14	42.32	41.98	1.395	0.996
RBC ($x10^6/\mu L$)	7.89	6.54	7.45	0.373	0.350
Hct (% of red blood cells)	39.40	37.04	36.42	1.309	0.653
Hb (g/dl)	15.46	13.56	13.98	0.516	0.308
THR ($x10^3$ /µL)	249.20	248.00	248.20	17.497	1.000
Biochemical Parameters					
ALT (U/L)	80.00	77.60	69.20	4.450	0.617
AST (U/L)	36.60	35.80	48.60	4.739	0.500
GLU (mg/dL)	88.00	91.20	85.20	2.900	0.730
ALB (g/dL)	3.22^x	3.24^x	3.40^y	0.038	0.093
CK (U/L)	664.40	681.20	656.20	52.251	0.983
CHOL (mg/dL)	89.20	92.40	88.40	2.007	0.721
TBIL (mg/dL)	0.10	0.10	0.16	0.014	0.117

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. [x, y] Means with different superscripts tended to differ ($0.05 < p \leq 0.10$). WBC, White Blood Cells; Lym, Lymphocytes; Mon, Monocytes; Gra, Granulocytes; RBC, Red Blood Cells; Hct, Hematocrit; Hb, Hemoglobin; THR, Thrombocytes; ALB, Albumine; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CHOL, Cholesterol; CK, Creatine kinase; GLU, Glucose; TBIL, Total bilirubin.

3.3.4. Effect on Meat Quality Parameters

Table 6 shows the effects of the two *T. molitor* meal treatments on the microbiological populations of meat from finishing pigs. In the shoulder cut, a trend ($0.05 < p \leq 0.10$) was observed for increased levels of *Staphylococcus* spp. In Groups B and C compared to Group A. In the belly cut, a similar trend ($0.05 < p \leq 0.10$) was noted for higher *Clostridium* spp. Levels in Group B compared to the other two groups. Additionally, a statistically significant increase ($p \leq 0.05$) in *Staphylococcus* spp. Was observed in Group B compared to Groups A and C. Moreover, in all shoulder and belly samples there was absence of *Salmonella* (in 25 g of sample) and *Listeria monocytogenes* (in 25 g of sample).

Table 3.6. Effect of dietary *Tenebrio molitor* larvae meal supplementation on meat microbial populations of finishing pigs.

Shoulder Meat Microbiota (Log_{10} CFU/g)	**Group A**	**Group B**	**Group C**	**SEM**	**P**
Total microbes	4.11	4.28	4.58	0.175	0.580
Escherichia coli	0.50	1.10	1.33	0.198	0.163
Clostridium spp.	0.80	0.50	1.23	0.170	0.272
Staphylococcus spp.	2.80^{x}	3.64^{y}	3.60^{y}	0.161	0.058
Staphylococcus aureus	1.88	2.06	1.66	0.142	0.563
Campylobacter jejuni	0.94	1.57	1.38	0.265	0.643
Belly Meat Microbiota (Log_{10} CFU/g)					
Total microbes	5.09	5.40	5.05	1.776	0.711
Escherichia coli	1.69	1.80	1.48	0.300	0.948
Clostridium spp.	0.82^{x}	1.79^{y}	0.92^{x}	0.188	0.054
Staphylococcus spp.	3.54^{a}	4.27^{b}	3.60^{a}	0.130	0.026
Staphylococcus aureus	2.00	1.40	1.50	0.169	0.320
Campylobacter jejuni	1.54	2.11	0.86	0.977	0.129

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. $^{a, b}$ Means with different superscripts are significantly different ($p \leq 0.05$). $^{x, y}$ Means with different superscripts tended to differ ($0.05 < p \leq 0.10$).

In the chemical composition analysis of the meat cuts presented in Table 7, a statistically significant increase ($p \leq 0.05$) in collagen was observed only in Group B compared to Group C, specifically in the belly cut.

Table 3.7. Effect of dietary *Tenebrio molitor* larvae meal supplementation on the meat chemical composition of finishing pigs.

Shoulder Meat Chemical Composition (%)	**Group A**	**Group B**	**Group C**	**SEM**	**P**
Fat	6.67	6.59	7.04	0.535	0.943
Protein	19.92	19.53	19.92	0.129	0.381
Moisture	73.19	73.59	72.82	0.473	0.698
Salt	0.85	0.82	0.88	0.038	0.797
Collagen	1.93	1.91	2.02	0.093	0.888
Belly Meat Chemical Composition (%)					
Fat	5.75	6.58	5.94	0.330	0.598
Protein	20.17	18.70	20.22	0.308	0.114
Moisture	73.86	74.10	74.10	0.346	0.956
Salt	0.82	0.71	0.75	0.051	0.725
Collagen	1.40^{ab}	1.75^{b}	1.17^{a}	0.097	0.027

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. $^{a, b}$ Means with different superscripts are significantly different ($p \leq 0.05$).

The result of the insect meal supplementation on the meat's total antioxidant capacity, oxidative stability, and phenolic content are presented in Table 8. The total phenolic content of the groups did not differ significantly ($p > 0.10$) between the three groups. On the other hand, group C's belly meat had significantly higher levels of malondialdehyde (MDA) than group B's ($p < 0.05$). Similarly, group B's belly meat tended to have higher levels of antioxidants than group C's ($0.05 < p \leq 0.1$). The oxidative stability and total phenolic content of shoulder meat did not differ significantly ($p > 0.10$) between the three groups.

Table 3.8. Effect of dietary *Tenebrio molitor* larvae meal supplementation on the total phenolic content, lipid oxidation, and total antioxidant capacity of meat of finishing pigs.

Shoulder Meat	**Group A**	**Group B**	**Group C**	**SEM**	**P**
Total phenolic content (µg GAE/g dry meat)	41.20	40.02	45.19	3.695	0.856
MDA (ng MDA/g)	4.16	5.86	8.74	1.019	0.184
Total antioxidant capacity (%)	40.29	38.81	33.49	1.398	0.106
Belly Meat					
Total phenolic content (µg GAE/g dry meat)	44.05	40.27	32.33	5.140	0.670
MDA (ng MDA/g)	6.99^{ab}	3.36^{a}	8.26^{b}	0.826	0.027
Total antioxidant capacity (%)	23.45^{xy}	25.40^{y}	20.71^{x}	0.841	0.060

Group A, control diet; Group B, diet supplemented with 6% "Conventional" *T. molitor* larvae meal; Group C, diet supplemented with 6% "Enriched" *T. molitor* larvae meal (n = 6 per treatment); SEM, standard error of the mean. $^{a, b}$ Means with different superscripts are significantly different ($p \leq 0.05$). $^{x, y}$ Means with different superscripts tended to differ ($0.05 < p \leq 0.10$). GAE, Gallic Acid Equivalents; MDA, Malondialdehyde.

3.4. Discussion

One of the biggest challenges affecting the pig farming sector remains finding sufficient sources of protein. Over the last few decades, there has been significant interest in identifying potential alternative proteins sources to soybean meal [3]. In this context, insect meal has become a promising alternative feed ingredient for livestock animals [31]. Insect meals are valued both for their important nutritional components and for their functional abilities, containing bioactive compounds such as chitin, fatty acids, and peptides [32]. However, aspects like the optimal inclusion levels, effects on gut microbiota, animal health, and meat quality characteristics still require further analysis. To the best of our knowledge, this study investigated for the first time the replacement of 50% of soybean

meal by *T. molitor* larvae meal in finishing pig diets, which can potentially have a great practical significance if this replacement could be used at commercial scale and be cost-efficient.

Growth performance parameters are critical in the pig industry because they significantly affect farm productivity and profitability [33]. The protein content and its sources are essential for finishing pigs' growth performance. Variations in protein levels and sources can have an influence in average daily gain, feed conversion ratio, and the overall health and productivity of the pigs [34]. In the present study, inclusion of *T. molitor* larvae meal to replace the 50% of soybean meal did not have any detrimental effects in the growth performance of finishing pigs, indicating that 6% *T. molitor* larvae inclusion was acceptable for finishing pigs. These findings are consistent some with previous studies indicating that *T. molitor* had no negative effects on growth performance in growing pig diets [1, 35]. However, other previous studies that examined the use of *T. molitor* larvae meal in swine feed have reported inconsistent results. Some studies have noted beneficial effects, such as increased body weight and bodyweight gain [11, 36]. Another research found that a 10% inclusion rate of *T. molitor* in the diet of growing pigs could negatively affect their growth performance [37]. This variability of results is likely influenced by the nutritional quality of the insect meal, and especially their amino acid composition and digestibility which varies depending to insect species, life stage (adult, larva, or pupae), the rearing substrate, and the processing methods applied to transform them to feed material [38]. Further studies are required to examine the effects of insect meal on growth performance, ADFI and FCR in finishing pigs.

Dietary modulation plays a critical role in regulating gut microbiota composition, essential for optimizing animal health and performance [39]. The gut microbiota influences intestinal structure maintenance, immune regulation, and overall health [40, 41]. Prior to dietary intervention, microbial populations showed no significant differences; however, by day 28, Enterobacteriaceae increased significantly in Group B ($p \leq 0.05$), with *E. coli* as the dominant species across all groups. This increase suggests that conventional insect meal influenced microbial composition, likely due to differences in substrate composition, physicochemical properties, and or digestibility [42]. Insect meals contain chitin, serving

as a substrate for gut microbiota, and bioactive peptides with antimicrobial, immunomodulatory, and antioxidant properties [32]. Enterobacteriaceae levels in Group B declined post-intervention, while they increased in Group A, implying a lasting suppressive effect of insect meal. Notably, Lactobacillus species were present across all groups, supporting gut health [43]. On day 28, Group B showed an increase in *E. faecalis*, *E. gallinarum*, and *L. reuteri*, while Group C exhibited higher *B. choerinum*, *L. amylovorus*, and *L. ruminis* levels, suggesting selective promotion of beneficial bacteria due to plant-derived bioactive compounds [42, 44]. MALDI-TOF MS analysis highlighted notable shifts, including an increase in *B. thermophilum* in Groups B and C, species known for probiotic properties [45], and the exclusive presence of *L. gallinarum* and *L. xylaniliticus* in insect meal-fed groups, further illustrating selective effects [46]. Fecal analysis on day 35 indicated lasting microbiota changes, with Group B showing increased total anaerobes, contributing positively to digestion and immune function [47], while reducing harmful Enterobacteriaceae [48]. The bioactive compounds in *T. molitor* meal continued exerting benefits post-administration. Overall, insect meal, particularly enriched with plant-derived components, significantly influenced pig gut microbiota, likely due to its bioactive compounds from medicinal and aromatic plants [35, 42, 49, 50]. Longer-term studies are necessary to evaluate the persistence of these microbiota changes, their effects on immune responses, and their overall impact on animal performance. Understanding whether these alterations are beneficial or detrimental in the long run will be crucial for refining insect meal supplementation strategies in pig nutrition.

Hematological traits are essential indicators of an animal's health and physiological status, providing very important information on its ability to adapt to various physiological challenges [51]. In the present study, a detailed analysis of hematological and biochemical parameters demonstrated that all measured values fell within the established physiological limits for swine. This strongly indicates that the animals were in good health and that the test diets were of sufficient quality to support their well-being. These findings are in agreement with those of Ao et al. (2019), who examined the inclusion of *T. molitor* larvae in the diets of growing pigs [52]. Additionally, recent studies on other insect-based meals, such as those derived from *H. illucens*, have similarly reported no adverse effects on the blood biochemical parameters of broilers and pigs [53-55]. Interestingly, Group C

exhibited a tendency toward higher albumin levels compared to the other groups; however, these values remained within the expected physiological ranges [56]. This increase in albumin levels could be attributed to the higher dietary protein content, as elevated protein intake stimulates the synthesis of albumin to maintain protein balance [57]. Moreover, inflammatory or stress conditions could elevate albumin levels as part of the body's acute-phase response [58].

The growing demand for safer food production with minimal reliance on chemical additives, has generated growing interest in alternative control methods for foodborne pathogens [59]. Research by Chen et al. (2022) study have shown a strong correlation between gut microbiota and meat quality, highlighting the impact of an animal's diet on microbial populations, bacterial metabolites, and overall meat characteristics [60]. One promising solution is the use of insect-derived feed ingredients, which are naturally rich in antimicrobial peptides [32]. While muscle tissue in live animals is typically sterile, commercial processing—such as slaughter, cutting, and storage—introduces the risk of contamination. In our study, all measured microbial contamination levels remained within safe and acceptable limits. Additionally, no harmful bacteria, including *Salmonella spp.* or *Listeria monocytogenes*, were detected in any of the analyzed 25 g meat samples. However, we observed elevated levels of *Staphylococcus spp.* in the shoulder and belly meat cuts of Group B, along with a higher concentration of *Clostridium spp.* specifically in the belly meat of the same group, although the values were within the expected limits. Although in the European Union there are regulatory limits, based on E.C. Regulation 1441/2007, for microbial indication for pork meat, these limits refer only to the fresh carcass after slaughter and cleansing of the carcass and sampling of the carcass in the slaughterhouse, prior to refrigeration and storage of meat. However, under commercial refrigeration conditions it is not possible to totally avoid their appearance in the pig meat. Moreover, in the present study samples their counts were low and do not raise any health concerns for meat that will be cooked. Furthermore, the present findings align with our previous study [35], which suggests that the inclusion of *T. molitor* in growing pig diets may lead to increased populations of *Clostridium spp.* in belly meat cuts.

Meat quality has a direct impact on shelf life and processing potential of meat, making it a crucial factor of the pig industry's economic profitability [61]. However, there is contrary literature regarding how different production systems affect the chemical composition of pig meat. Physical activity tends to influence meat quality characteristics such as muscle tissue metabolism and pH drop after slaughter more than its chemical composition [62]. When changes in composition occur, they are most commonly related to management factors like diet formulation, feed consumption, and metabolizable energy required for maintenance [63]. Some studies have been directed more towards the effects of various production systems on the chemical composition of meat [64]. The present study found that the addition of *T. molitor* to pig diets had no significant effect on the levels of important chemical composition indicators such as fat, protein and moisture, although it was noted that the cut of belly meat had higher collagen levels. Collagen is among the most critical factors determining pork meat quality, as it has a direct impact on its tenderness [65]. There are numerous factors that affect collagen content in meat tissue, but undoubtedly one of the most crucial is the animal's nutrition [66]. Our research indicates that *T. molitor* supplementation has no detrimental effects on overall meat quality. The potential of insect-based feed ingredients is highlighted by these results, which also highlight the need for additional study, especially regarding their role in mineral deposition in muscle tissue.

The modern pork industry is dedicated to enhancing meat quality by improving key properties, for example nutrient profile [67]. Dietary components are some of the most influential parameters that affect meat quality. A key quality marker is lipid oxidation, which can degrade the nutritional value and lead to the synthesis of dangerous substances like MDA [59]. Known for their antioxidant properties, polyphenols aid in the protection of the immune system, the inhibition of the activity of oxidative enzymes, and the neutralization of free radicals [68]. Measuring antioxidant capacity is essential for evaluating the oxidative stability of meat from animals fed different diets [69]. In addition, pre-slaughter conditions also play a critical role in determining meat quality. Research by Sardi et al. (2020) highlights how factors such as transport duration, ambient temperature, and improper handling can negatively affect the fresh meat quality characteristics of pigs, such as pH, color, drip loss, and shear force [29]. In our study, incorporating *T. molitor* larvae meal at a 6% inclusion level reduced lipid oxidation and increased total antioxidant

capacity in belly meat. Similarly, Navarro Del Hierro et al. (2020) highlighted the antioxidant characteristics of larval proteins derived from *T. molitor* [70]. Yu et al. (2019) found that supplementing pig diets with *H. illucens* meal positively influenced the mRNA expression of acetyl-CoA carboxylase and lipoprotein lipase [8]. Our findings align with a previous study, which showed that incorporating *T. molitor* into growing pig diets effectively reduced lipid oxidation in the produced meat [35, 71]. These findings suggest that *T. molitor* supplementation may protect finishing pigs from oxidative stress by enhancing their antioxidant defense mechanisms.

3.5. Conclusions

This research project is the first to compare the utilization of two different *T. molitor* larvae meals, reared on distinct substrates, as feed components for finishing pigs in order to replace protein rich feed materials. The findings of the experimental trial indicate that 50% of the soybean meal can be replaced in finishing pig diets by *T. molitor* larvae meals with comparable growth performance results and good animal health. Furthermore, there were noticeable improvements in important meat quality parameters like oxidative stability. Larvae meal from insects that consumed the enriched substrates appeared to offer superior microbiota modulation and antioxidant benefits. Despite these advantages, limited availability and costly production of insect-based feeds continues to restrict its industrial use. Future studies should focus on the comparison of different kinds of insect feed material, their ideal inclusion levels in pig diets, and their long-term effect on animal health. Moreover, it is important to enhance public understanding of sustainable animal nutrition by leveraging highly transparent and communication channels such as social media.

3.6. References

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Chapter 4: Summary, limitations and future perspectives

4.1. Summary of the Study

This PhD dissertation explored the use of *Tenebrio molitor* (mealworm) insect meal as a novel and sustainable ingredient in swine diets. Aiming to go beyond traditional feed approaches, the research examined how different rearing substrates, particularly those enriched with Mediterranean herbs such as oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and sage (*Salvia fruticosa*), could enhance the nutritional value and functional properties of the insect larvae. These herbs were chosen for their high content of essential oils, polyphenols, and antioxidants, which are known to support animal health and feed efficiency [1, 2]. The goal was not only to improve the nutrient composition of *T. molitor* but also to explore whether these bioactive compounds could benefit gut health and immune response in pigs during both the early-growing and finishing phases.

As a further step, the study tested a combined dietary strategy by supplementing the insect-based feed with chitosan—a natural biopolymer derived from chitin. Chitosan has been widely recognized for its antimicrobial, immune-supporting, and gut-health-promoting properties [3-7]. When used together with the *T. molitor* meal, this combination aimed to produce a synergistic effect that could enhance overall pig performance while reducing the need for antibiotics. The effectiveness of this feed formulation was evaluated in early-growing pigs.

The overall objective of this research was to assess how these feed innovations influenced key parameters of swine production. Specifically, the study examined their effects on growth performance, blood biomarkers, gut microbial populations, and meat quality characteristics.

Firstly, a total of 60 weaned pigs (34 days old) were randomly allocated into five groups: Group A (control), Group B (10% *T. molitor* meal from standard substrate), Group C (10% *T. molitor* meal from enriched substrate), Group D (0.5g/kg high-purity chitosan powder), and Group E (combined use of 10% *T. molitor* meal from standard substrate and 0.5g/kg high-purity chitosan powder). The whole experimental trial lasted 42 days. The main conclusions obtained from this trial (**Chapter 2**) were:

1. The combined supplementation of *T. molitor* meal and chitosan significantly improved overall growth performance ($p < 0.05$).
2. Both insect-based diets and the combined use of *T. molitor* meal with chitosan notably affected the gut microbial composition, with notable modifications in fecal microflora balance ($p < 0.10$).
3. Blood biochemical analysis showed a significant increase in cholesterol levels in Group C ($p < 0.001$), whereas all other hematological and biochemical parameters remained unaffected.
4. Meat quality parameters were positively influenced, showing higher total phenolic content ($p < 0.10$), improved oxidative stability ($p < 0.10$), a more favorable balance of microbial populations on meat cuts ($p < 0.10$), and an enhanced fatty acid profile ($p < 0.10$).

Secondly, an experimental trial was conducted using eighteen finishing pigs (135 days old) which were randomly allocated to three groups: Group A (control), Group B (6% *T. molitor* from a standard substrate), and Group C (6% *T. molitor* from an enriched substrate). The feeding trial lasted 35 days, with the experimental diets administered during the first 28 days, followed by a return to the control diet for all groups during the final 7 days. The main conclusions obtained from this trial (**Chapter 3**) were:

1. Insect meal supplementation did not significantly affect overall growth performance ($p > 0.10$).
2. Significant changes were observed in fecal microbiota composition, with a reduction in Enterobacteriaceae counts ($p < 0.05$) on days 28 and 35 in Groups B and C, and an increase in total anaerobic bacteria ($p < 0.05$) in Group B on day 35.
3. Blood biochemical analysis indicated an increase in albumin levels in Group C ($p < 0.10$), while all other hematological and biochemical markers remained unchanged.
4. The insect-based diets had a notable effect on meat quality parameters, decreasing oxidative stability ($p < 0.05$) in the belly meat cut of Group B and increasing total antioxidant capacity ($p < 0.10$) in the same cut.

5. Meat composition analysis showed a significant increase in collagen content ($p < 0.05$) in the belly cut of Group B, suggesting potential improvements in meat texture and overall quality.

4.2. Future Research Directions

Based on the findings of this dissertation, which investigated the use of *T. molitor* insect meal, produced on both standard and enriched substrates, and its combination with chitosan in pig diets, several key areas for future research were identified. These suggestions were based on the results of this study and support current efforts to make pig farming more sustainable, healthier, and less dependent on antibiotics.

Although improvements in growth performance and health parameters were observed, the specific phytochemical compounds responsible for these effects are still unknown. To better understand this, future research should focus on analyzing the insect meals using metabolomic and lipidomic techniques. This would identify how key bioactive substances, like thymol and carvacrol, are transferred from the herbs, through the insects, and finally into the pigs. These analyses would support a clearer understanding of the mechanisms underlying immune modulation and antimicrobial action.

Since both *T. molitor* larvae meals and chitosan can influence gut bacteria, future research should use next-generation sequencing to study how the gut microbiome changes with these diets. It would also be helpful to measure short-chain fatty acids, which play important roles in digestion and immune function. This kind of analysis would give a clearer picture of how these feeds affect the relationship between the gut and the immune system, especially during sensitive stages like the post-weaning period.

According to current literature, the results are based only on early-growing and finishing pigs. Future research should also include breeding sows, pregnant and lactating females, and newborn piglets. This would help determine how these dietary strategies affect reproductive success, immune transfer from mother to piglet, early gut development, and overall litter growth.

Additionally, while this study used fixed inclusion levels for insect meal and chitosan, future trials should test different doses to identify optimal levels at various growth stages.

It is also important to investigate how these ingredients interact with other innovative feed additives, whether they have synergistic effects, and how their performance may be influenced by different farm management practices.

Some meat quality characteristics were improved in this study, but further investigation is needed into aspects such as carcass yield, intramuscular fat composition, and oxidative stability. Research should also explore sensory traits and consumer acceptance of pork from pigs fed insect-based diets to ensure market viability.

From an economic perspective, future studies should evaluate the use of both standard and enriched insect meals, as well as their combination with chitosan, to assess the total cost of feed production and final pork output. This would help determine whether these strategies are financially sustainable for commercial pig farms compared to conventional feeds.

Finally, to better understand the environmental advantages of insect-based feed, life cycle assessments should compare these diets with traditional protein sources like soy and fishmeal. These analyses could provide valuable data on greenhouse gas emissions, land use, and water consumption. At the same time, economic models should examine how cost-effective insect rearing can be when based on agricultural by-products and local plant waste, especially within regional production systems.

4.3. Final Conclusions

This PhD dissertation provides a comprehensive evaluation of the nutritional, physiological, and functional potential of *T. molitor* meal and chitosan as innovative ingredients in pig nutrition. Across two experimental trials, the research systematically examined their effects during both early-growing and finishing phases, generating new evidence on their capacity to support sustainable, efficient, and health-promoting feeding strategies. The results of the experiments demonstrate that insect-based feeds, when properly designed and enriched, can offer impressive benefits at multiple levels, animal performance, gut microbiota, immune modulation, meat quality, and the environmental footprint of pork production.

A key contribution of this dissertation is the demonstration that *T. molitor* meal can successfully replace conventional protein sources, such as fishmeal and soybean meal,

without reducing growth performance in pigs. Beyond its nutritional role, enriched insect meal showed clear functional advantages, improving antioxidant capacity, modifying the fatty acid composition, and influencing specific gut microbial populations in both young and finishing pigs. These findings show that *T. molitor* composition can be strategically enhanced through substrate manipulation, enabling the production of insect meals with improved nutritional and functional properties.

Chitosan supplementation provided additional, complementary advantages. Its role in modulating gut microbiota, enhancing immune parameters, and improving meat oxidative stability highlights its potential as a prebiotic additive suited to the demands of modern antibiotic-free farming systems. Particularly, important is the observation that chitosan and *T. molitor* act synergistically in early-growing pigs, producing combined benefits not evident when each ingredient was used independently. This synergy supports that functional feeds should be designed not just by adding single ingredients, but by considering how different bioactive components work together to positively affect animal physiology.

In the finishing phase, where research remains limited at international level, this dissertation provides the first evidence that *T. molitor* meals reared on enriched substrates can influence finishing pig performance and the quality of the produced meat. The increases in oxidative stability, and phenolic content indicate that insect-based ingredients could be used to improve meat quality, meeting consumer demand for healthier and more naturally enriched products. Such findings are particularly relevant in an era where meat quality is criticized not only for sensory attributes but also for health-promoting characteristics.

The implications of this study go beyond the experimental results. Using insects as feed supports a circular bioeconomy by turning agricultural by-products into high-value protein with low environmental impact. The findings support the evidence that insect farming can provide a sustainable source of animal protein while reducing reliance on imported soybean meal and fishmeal, which are linked to deforestation, overfishing, and price fluctuations. In this way, the dissertation aligns with EU strategies to promote sustainable agriculture, reduce waste, and diversify protein sources.

While this research highlights a number of promising opportunities, it also shows that using insect meals and chitosan in commercial pig production will require further scientific, economic, and regulatory advances. Nevertheless, the findings of this dissertation provide a strong foundation for future optimization and outline clear pathways for practical use in both early-growing and finishing pig diets.

Overall, this PhD dissertation shows that *T. molitor* meal, especially from larvae reared on enriched substrates, together with chitosan supplementation, offers a promising approach to pig nutrition. These ingredients can support animal health, improve meat quality, and promote more sustainable production. Moreover, the innovative method of enriching insect substrates with bioactive plant residues creates opportunities to design functional feeds with enhanced nutritional profiles. Combined with the benefits of chitosan, this approach has strong potential to shape the next generation of sustainable animal diets.

In conclusion, the present study advances our understanding of insect-based feeds and functional dietary additives while providing practical guidance for their use in modern pig industry. By combining nutritional benefits with environmental responsibility, the dissertation contributes to the development of sustainable animal feeding strategies and provides a foundation for further progress in the field.

4.4. References

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Chapter 5: Scientific outputs

This doctoral thesis resulted in:

- Two (2) published manuscripts in peer-reviewed scientific journals

- Six (6) oral presentations in national and international congresses

- Three (3) poster presentations in national and international congresses.

5.1. Published manuscripts

1. **Zacharis, C.**, Bonos, E., Giannenas, I., Skoufos, I., Tzora, A., Voidarou, C., Tsinas, A., Fotou, K., Papadopoulos, G., Mitsagga, C., Athanassiou, C., Antonopoulou, E., & Grigoriadou, K. (2023). Utilization of *Tenebrio molitor* Larvae Reared with Different Substrates as Feed Ingredients in Growing Pigs. *Veterinary Sciences*, *10*(6), 393. https://doi.org/10.3390/vetsci10060393
2. **Zacharis, C.**, Bonos, E., Voidarou, C., Magklaras, G., Fotou, K., Giannenas, I., Giavasis, I., Mitsagga, C., Athanassiou, C., Antonopoulou, E., Grigoriadou, K., Tzora, A., & Skoufos, I. (2024). Combined Dietary Supplementation of *Tenebrio molitor* Larvae and Chitosan in Growing Pigs: A Pilot Study. *Veterinary Sciences*, *11*(2), 73. https://doi.org/10.3390/vetsci11020073

5.2. Oral presentations

1. Bonos, E., Giannenas, I., **Zacharis, C.**, Tzora, A., & Skoufos, I. (2021). Use of insect meals in monogastric animals' nutrition. In 16th International Symposium of Animal Biology and Nutrition, Balotesti, Romania, 30 September - 1 October 2021, book of abstracts p. 9.
2. **Zacharis, C.**, Magklaras, G., Giavasis, I., Giannenas, I., Antonopoulou, E., Tsinas, A., Grigoriadou, A., Andreadis, S., Athanasiou, C., Tzora, A., Skoufos, I., & Bonos, E. (2023). Study of the use of innovative insect meals in weaned piglets' diets and their effects on the chemical composition, microbial analysis, and oxidative stability of the produced meat. In 7th Hellenic Congress Meat & Products Thereof, Thessaloniki, 3 – 5 February 2023, book of abstracts p. 83-85.

3. **Zacharis, C.**, Bonos, E., Tzora, A., Skoufos, I., Magklaras, G., Giavasis, I., Giannenas, I., Antonopoulou, E., Athanassiou, C., & Tsinas, A. (2023). Effects of dietary *Tenebrio molitor* meal and chitosan on health and meat quality of weaned piglets. In 74th EAAP Annual Meeting, Lyon, France, 26th August – 1st September 2023, book of abstracts p. 220.
4. **Zacharis, C.,** Bonos, E., Magklaras, G., Nelli, A., Fotou, K., Nikolaou, K., Giannenas, H., Antonopoulou, E., Andreadis, S., Athanasiou, C., Tzora, A., & Skoufos, I. (2023). Partial replacement of soybean meal with innovative insect meals in finishing pig diets. In 37th Annual Scientific Conference of the Hellenic Society of Animal Science, Nea Orestiada, Greece, 3 – 5 September 2023, book of abstracts p. 58.
5. **Zacharis, C.,** Bonos, E., Magklaras, G., Nelli, A., Fotou, K., Nikolaou, K., Voidarou, C., Tsinas, A., Giannenas, I., Antonopoulou, E., Andreadis, S., Athanassiou, C., Tzora, A., & Skoufos, I. (2023). Effect of two different *Tenebrio molitor* insect meals on performance of finishing pigs. In 17th International Symposium of Animal Biology and Nutrition, Balotesti, Romania, 29 September 2023, book of abstracts p. 16.
6. **Zacharis, C.,** Magklaras, G., Voidarou, C., Giavasis, I., Giannenas, H., Antonopoulou, E., Grigoriadou, A., Andreadis, S., Athanasiou, C., Tzora, A., Skoufos, I., & Bonos, E. (2025). Use of innovative insect meals in the diets of finishing pigs and their effects on performance parameters and the quality characteristics of the produced meat. In 8th Hellenic Meat Congress, Thessaloniki, Greece, 31 January – 2 February 2025, book of abstracts p. 82-83.

5.3. Poster presentations

1. **Zacharis, C.**, Nelli, A., Tzora, A., Fotou, K., Magklaras, G., Giannenas, I., Antonopoulou, E., Tsinas, A., Grigoriadou, A., Andreadis, S., Athanasiou, C., Skoufos, I., & Bonos, E. (2022). Use of innovative insect meals in weaned piglet diets and their effects on growth performance and gut microbiota. In 15th Panhellenic Veterinary Conference, Athens, Greece, 4 – 6 November 2022, book of abstracts p. 115-116.

2. **Zacharis, C.**, Fotou, K., Nelli, A., Magklaras, G., Giannenas, I., Antonopoulou, E., Tsinas, A., Grigoriadou, A., Skoulakis, G., Athanasiou, C., Tzora, A., Skoufos, I., & Bonos, E. (2022). Investigation of the combined use of insect meals and chitosan in weaned piglets' diets and their effects on performance parameters and intestinal microbiota. In 15th Panhellenic Veterinary Conference, Athens, Greece, 4 – 6 November 2022, book of abstracts p. 117-118.
3. **Zacharis, C.**, Magklaras, G., Giavasis, I., Giannenas, I., Antonopoulou, E., Tsinas, A., Athanassiou, C., Tzora, A., Skoufos, I., & Bonos, E. (2023). Investigation of the suitability of two *Tenebrio molitor* meals on weaned piglets' diets. In 74th EAAP Annual Meeting, Lyon, France, 26th August – 1st September 2023, book of abstracts p. 230.