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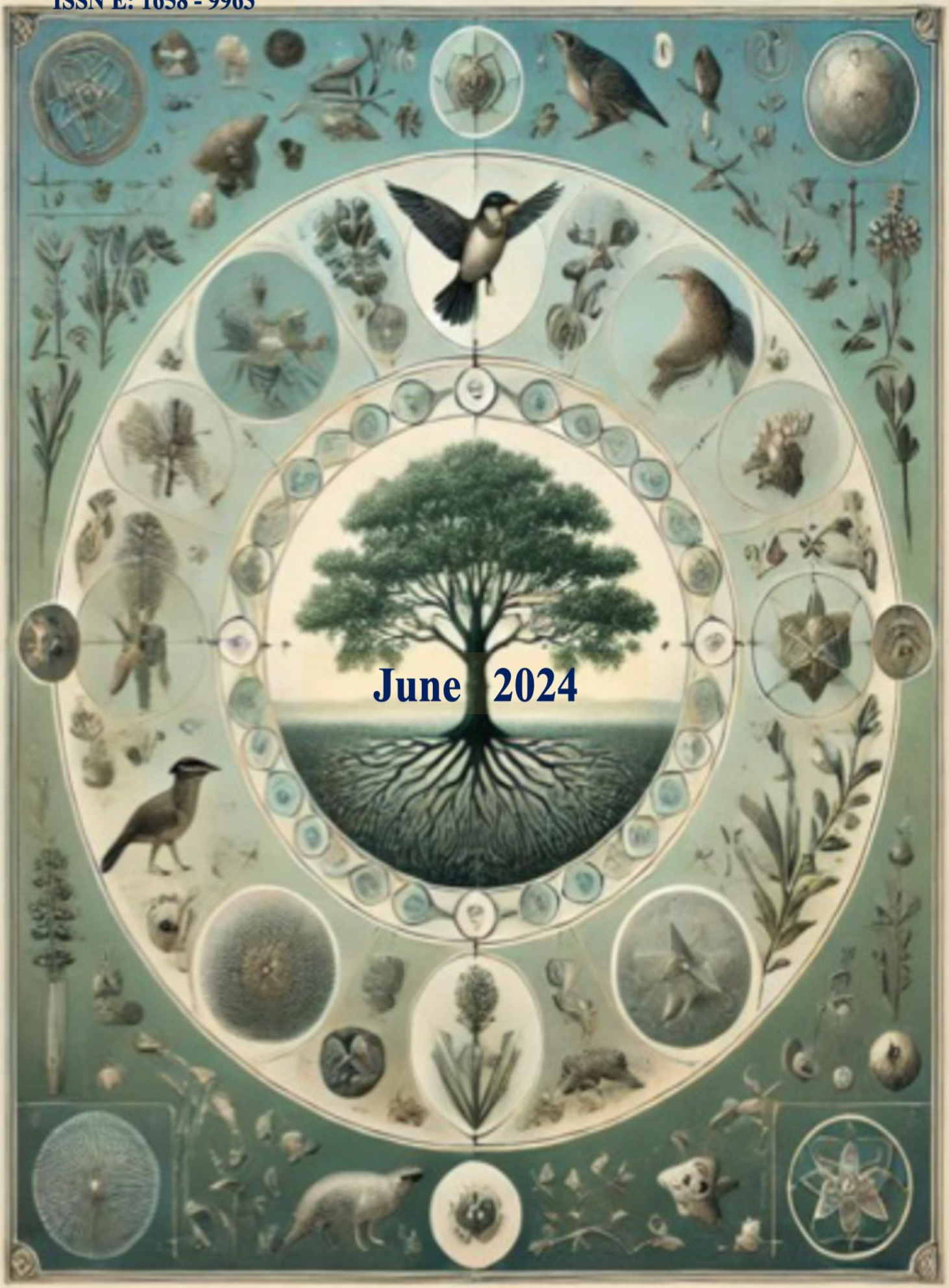

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Princess Nourah Bint Abdulrahman University
College of Science

جامعة الأميرة نورة بنت عبدالرحمن
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AEROBIC BACTERIAL MICROBIOTA OF THE UPPER RESPIRATORY TRACT (ORAL AND NASAL CAVITIES) IN SAUDI ADULTS

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Keywords

Microbiota, Oral and Nasal Cavities, Biochemical, Molecular, Bacteria

Abstract

The collective micro-community residing in the human body is called the microbiota. Studying and comparing its composition from different body sites is now a promising approach for microbiologists. The bacterial composition of the upper respiratory tract URT flora has been extensively studied, considering many factors, including health state, age, and gender. In our study, we identified the bacterial microbiota of two specific parts of the URT: mouth and nose. We isolated and identified bacteria from 40 males and 40 females using the VITEK biochemical identification method, followed by molecular identification using 16s rRNA primers. Fifteen bacterial genera were identified, making up 29 species, of which 11 were common in both genders. The two most identified genera are *Staphylococcus*, dominant in the nose but absent in the mouth, and *Streptococcus*, dominant in the mouth (p-value < 0.05). The *Staphylococcus* genus has a significantly higher "overall" number of culturable isolates (40% ($p<0.05$)), and the most abundant species of the *Staphylococcus* are *S. epidermidis* and *S. aureus* (41% and 39% of total *Staphylococcus* species). Age significantly affects the abundance and diversity of oral flora, with higher diversity in younger participants and higher abundance in the elderly, specifically oral *Streptococcus* ($F=9.09$, p-value= 0.003) young > old. Gender affects the abundance, where females have significantly higher oral bacterial density than males but no significant effect on the nasal system. Based on the unique microbial signatures retrieved from this study in each test group, elderly groups of both genders show more gram-negative pathogenic species than commensals, specifically the older females with a high abundance of *Enterobacter* cloacae complex.

INTRODUCTION

The human body is a habitat of a complex micro-community of bacteria, viruses, and fungi that outweigh the number of our body cells. The composition, proportions, and diversity of microorganisms in our body are known as the microbiota. The most populated human body sites are the skin, gut, upper respiratory tract URT, and genitourinary tract.

Dissecting and analyzing the makeup of the human microbiota is essential in microbiology due to its impact on health (Proctor and Relman 2017). Many studies compare factors that affect microfloral diversity, such as gender, age, health state, habitat, lifestyle, and diet (Cuesta-Zuluaga et al., 2019; Durack and Lynch 2019; Zhong et al., 2019). Computerized data from identified site-specific microbiomes can be used in health risk assessments and age and gender predictions (Huang et al., 2020). We chose to study the aerobic bacterial microbiota of URT, concentrating specifically on the oral and nasal sites. The oral and nasal cavities are important sites of the URT, harboring numerous commensal and pathogenic bacteria strains, which have been highly correlated with many health issues and diseases (Kumpitsch et al., 2019). Dissecting and identifying patterns in healthy individuals in these two sites will aid us in understanding and comparing them with individuals with related illnesses. For instance, the co-colonization of certain bacterial species and their interaction is essential in outgrowing pathogenic species. One example is the *S. epidermidis* biofilm formation in the nasal cavity, which inhibits the colonization of the pathogenic *S. aureus*. Generally, research on human microbiota in Saudi Arabia is lacking, specifically the effects of gender and age. The majority of the Saudi population-based microflora studies focus more on the impact of chronic diseases, i.e., diabetes (Al-Obaida et al., 2020; Amr et al., 2018), host habits such as smoking (Al Moaleem et al., 2020; Hattan et al., 2018), as well as habitat and lifestyle of the individuals such as fuel station workers (Alwakeel 2017). The only study found to compare the effect of age and gender is on skin microbiota published in 2019 by a research team at Princess Nourah bint Abdulrahman University (Shami et al., 2019). Dissecting the bacterial microflora of URT is essential to a better understanding the typical healthy diversity and abundance. The goal of our study was to identify and compare culturable URT aerobic bacterial microbiota of healthy Saudi Adults, taking into consideration age and gender.

MATERIALS AND METHODS

Ethics approval and consent to participate

The protocol of this study was approved by Princess Nourah bint Abdulrahman University IRB with IRB Registration Number KACS'I, KSA: H-01-R-059, and written informed consent from all the participants in this study was obtained.

Sample Source

Eighty Saudi adults from Riyadh were enrolled in this study and were divided into four groups according to their gender and age (older males, older females, young males, and young females). The average age in each group was 51, 62, 21, and 20, respectively. The study was conducted at Princess Nourah bint Abdulrahman University and King Saud University Research Centers in Riyadh.

Sample Collection

Sterile cotton swabs were used for sample collection. The same swab for each individual was passed against 5 oral sites (tongue, palate, buccal mucosa, gingiva, and mouth floor) for the oral cavity. A different swab was inserted into the nostrils along the nasal passage for the nasal cavity. The samples were stored at 4 °C for less than 2 hours (Shami et al. 2019).

Biochemical Identification

Swaps were inoculated and incubated overnight at 37 °C on MacConkey, blood, and tryptic Soy agar. Biomérieux VITEK® 2 system was used for the biochemical identification of grown bacterial colonies.

Molecular identification

According to the manufacturer's instructions, a QIAGEN DNeasy Blood & Tissue kit was used to extract DNA from grown bacterial colonies. PCR products using 16s rRNA universal primers (27F 5′ AGAGTTTGATCMTGGCTCAG 3′ - 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) were purified using ExoSAP-IT (Usb. Affymetrix, Inc.), then 16s rRNA sequencing was conducted (MOLECULE-ON, New Zealand).

Data analysis

The data were analyzed using R software. Descriptive statistics were performed for quantitative variables. We used one-way ANOVA to test the difference among the four groups (older males, older females, young males, and young females).

RESULTS

Forty male and forty female subjects from Saudi Arabia, each divided into two age groups (elderly and young), participated in this study. Samples were collected from each individual's oral and nasal cavity to identify the composition of aerobic bacterial flora of the upper respiratory tract, URT. Biochemical and molecular analyses were performed to identify cultivable/ culturable strains. A total of 29 bacterial species belonging to 15 different genera were identified in this study, of which only 11 species were common in both genders, as shown in Table 1. The exact number of isolates for each of the 11 common species, isolated from each of the 4 test groups, are illustrated in Table 1. *Staphylococcus haemolyticus* was only found in elder groups, and *Streptococcus pneumonia* was only found in young groups, highlighted in grey. The distribution and details of uniquely isolated bacteria species for each group are illustrated in Figure 1. 20.6 % (6 out of 29) and 17.2% (5 out of 29) of the 29 identified bacterial species belong to the *Staphylococcus* and *Streptococcus* genus, respectively (Table 2). Although both genera are equally common in terms of the number of species, shown by the black bar in Figure 2, the *Staphylococcus* genus has a significantly higher "overall" number of culturable isolates (40% ($p<0.05$)), shown by the red bar in Figure 2. Site-specific comparisons (nose vs mouth) of *Staphylococcus* and *Streptococcus* bacteria numbers were carried out using one-way ANOVA. The number of *Staphylococcus* isolates from the nasal flora is significantly higher than oral flora (F=27.8, p-value= 4.4 E-7). The most abundant species of the *Staphylococcus* genus are *S. epidermidis* and *S. aureus*, 41% (19 out of 46) and 39% (18 out of 46) respectively (Table 1).

On the other hand, *Streptococcus* is highly abundant in the oral flora and absent in the nasal flora (F=24.6, p-value= 1.8E-6). Microbial composition is influenced by various factors, such as the host's immune response, moisture, temperature, and available nutrients, contributing to the observed differences between oral and nasal floras**.** Age significantly affects the number of oral *Streptococcus*, which is higher in young participants (F=9.09, p-value= 0.003).

Comparisons of the diversity between oral and nasal sites amongst the 4 different groups are illustrated in Figure 3. Our results show that the nasal flora is slightly more diverse than the oral flora within each group except the young male group, but the difference is insignificant. The diversity of the oral microflora from our data is slightly higher in younger groups than in the elderly (Figure 3).

Table 1: Detailed numbers of bacteria isolated from the 11 common species found in both genders' oral and nasal cavities. The four groups based on gender and age are shown separately in this table. The highlighted rows indicate species isolated from different age groups: *S. haemolyticus* (elder only) and *S. pneumonia* (young only).

Total Number of The Common Isolated Bacterial Species of Oral and Nasal Cavities

Bacterial Specie Isolate	Females		Males		Total
	Elderly	Young	Elderly	Young	
Staphylococcus epidermidis	9	3	3	4	19
Staphylococcus aureus	2	7	5	4	18
Staphylococcus pseudintermedius	1	2	-	1	4
Staphylococcus haemolyticus	1	-	2	-	3
Streptococcus parasanguinis	2	4	2	2	10
Enterococcus faecalis	1	1	-	3	5
Acinetobacter baumannii complex	3	1	1	1	6
Escherichia coli	-	1	1	-	2
klebsiella pneumoniae	2	-	-	2	4
Enterobacter cloacae complex	10	-	3	1	14
Streptococcus pneumoniae	-	2	-	3	5

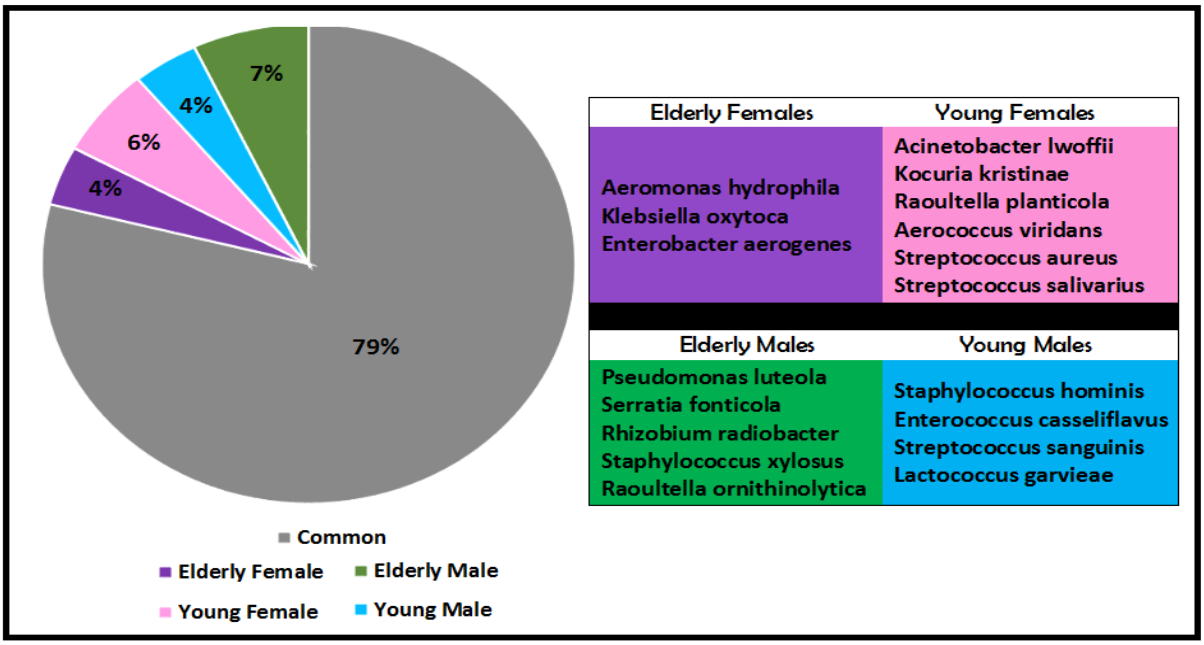

Figure 1: Distribution of Isolated Bacteria from URT Microflora. The pie chart illustrates the percentages of uniquely isolated bacteria from each test group. The table on the right presents more details of bacterial species isolated from each test group. The pie chart mentions the percentages of the number of isolates out of the 114 isolates.

Table 2: Number of species and isolates in each bacterial genus cultured from oral and nasal flora

Number of Species and Isolates in Each Bacterial Genus Cultured from Oral and Nasal Flora		
Genus	Number of Species	Number of Isolates
Staphylococcus	6	46
Streptococcus	5	19
Enterococcus	2	6
Enterobacter	2	16
Acinetobacter	2	7
Escherichia	1	2
Aeromonas	1	1
Klebsiella	2	6
Kocuria	1	1
Raoultella	2	4
Aerococcus	1	1
Lactococcus	1	1
Pseudomonas	1	2
Serratia	1	1
Rhizobium	1	1
Total		
15	29	114

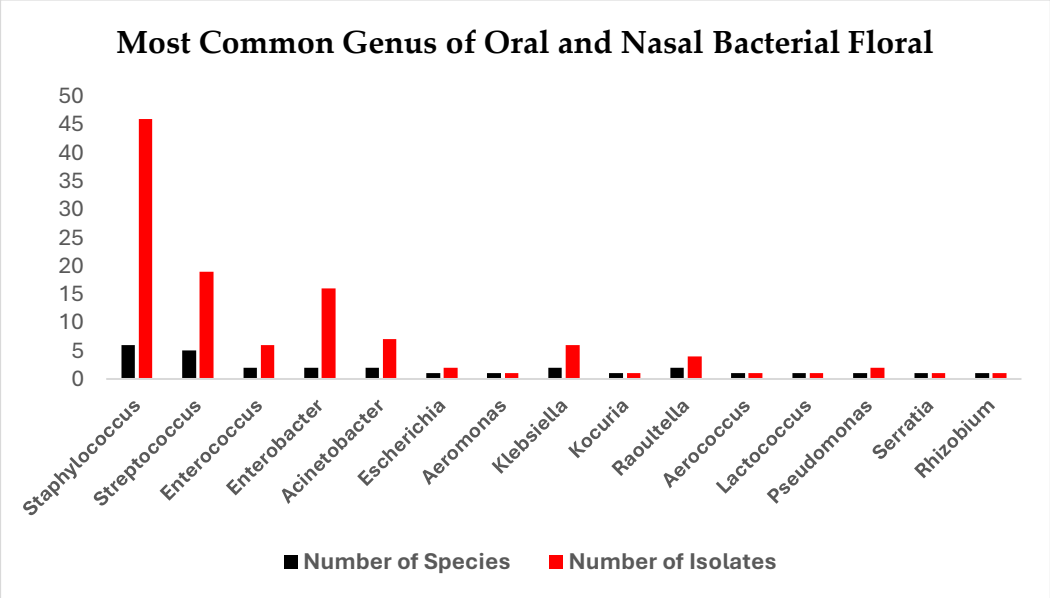

Figure 2: The number of species, black, and number of bacteria, red, for each genus isolated from the oral and nasal flora in this study.

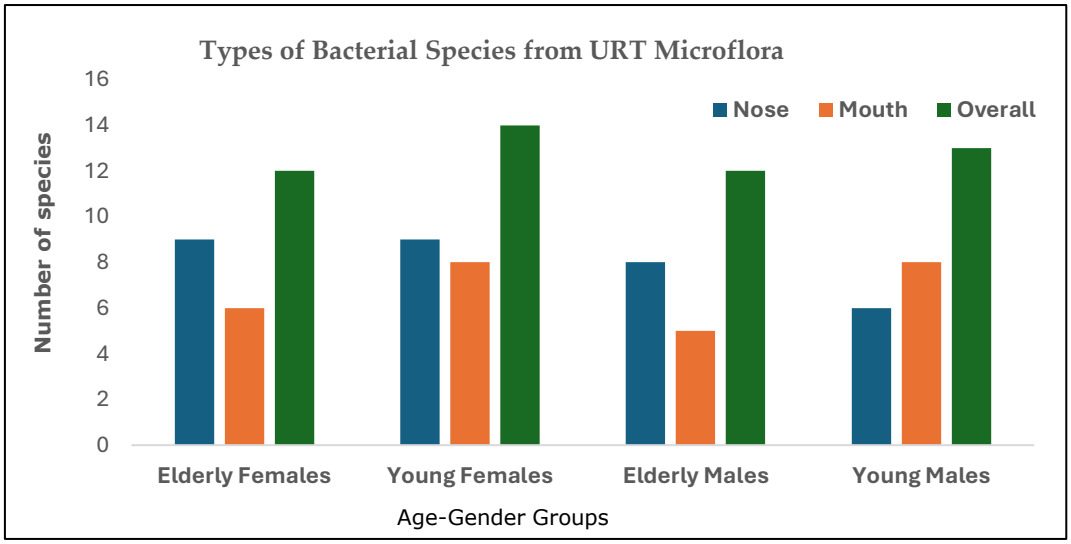

Figure 3: Site-specific demonstration of the number of types of bacterial species in each tested group.

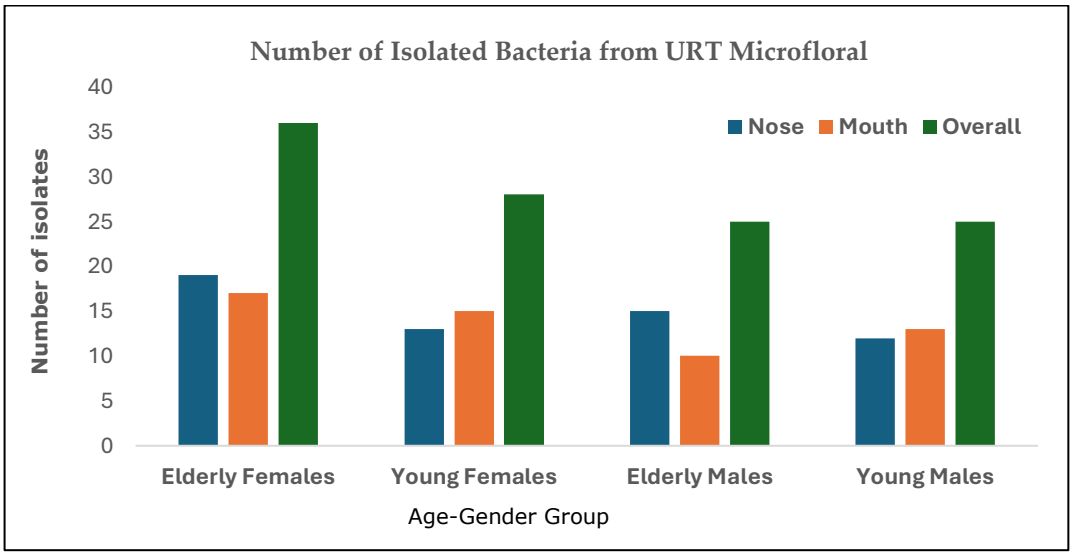

Figure 4: Site-specific demonstration of the numbers of isolated bacteria in each tested group.

The total number of isolates in each site for each group has also been compared, as illustrated in Figure 4. The number of isolates is higher in the nasal than the oral cavity amongst the elderly groups, while the younger groups showed more oral isolates than the nasal. Elderly females have the highest number of URT bacterial flora abundancy (36 isolates) shown in Figure 4, of which 53% are species of *Staphylococcus epidermidis* (9 out of 36 isolates) and *Enterobacter cloacae* complex (10 out of 36 isolates) shown in Table 1.

DISCUSSION

The distribution of aerobic oral and nasal bacterial microbiota is examined in 80 Saudi adults divided into 4 age and gender groups: elder females, young females, older males, and young males. Findings from the current study indicated *Staphylococcus* and *Streptococcus* as the two most abundant genera with significant site-specific patterns, nose and mouth, respectively. Staphylococcus was more dominant in the nose, replicating findings by Chen et al., that *Staphylococcus* makes up 9.61 % of the nasal micro-community (Chen et al., 2019). The two most common *Staphylococcus* species in our data are S. *epidermidis* and *S. aureus*, similar to findings from several studies on the nasal microbiota (Chen et al., 2019; Chen et al., 2016). A 2010 study conducted by Frank et al. showed a significant negative correlation between the nasal density of S. aureus and S. epidermidis (Frank et al., 2010). Although not statistically significant, a negative pattern is observed between the two species from the nose of each test group. Our results show that the Streptococcus genus was more dominant in the oral cavity. A recent study indicated that the Streptococcus genus was highly appendant in the oral cavity (Abranches et al., 2018). In addition, the absence of the Streptococcus genus in the nasal cavity might be due to specific conditions related to the studied individual, such as the host's immune response, moisture, temperature, and available nutrients, contributing to the observed differences between oral and nasal floras**.** Age has always impacted the diversity and the density of microbiomes in general (Sampaio-Maia and Monteiro-Silva 2014). It has been shown that the diversity decreases with host aging, but the overall density or microbial overload increases (Sampaio-Maia and Monteiro-Silva 2014). Our results match previous studies in terms of the effect of age on microfloral diversity and abundance (Sampaio-Maia and Monteiro-Silva 2014; Wu et al., 2016). In our study, the combined bacteria isolated from both sites are more diverse in younger individuals and less abundant in the elderly, specifically the oral microflora (Zawadzki et al., 2017). However, looking closely at the oral Streptococcus genus, even though it is more diverse in younger participants, it is also more abundant and does not follow the expected pattern. That could be a possible consequence of the dental conditions of our older test subjects; perhaps they have a higher rate of age-related tooth extraction and less bacterial growth, as previously shown by a periodontal microflora study in 2011 (Quirynen and Van Assche 2011).

The host aging effect on the nasal flora abundance from our study is, as expected, higher in the elderly and more diverse than in young individuals, which was not expected based on the reviewed literature (Prector and Relam 2017). Although not significant, we have highlighted in Table 1 that Streptococcus pneumonia was only present in the younger oral cavity and absent from the elderly oral cavity. However, *Streptococcus parasanguinis* was present, as was found in a 2009 Norwegian study conducted on elderly oral microflora (Preza et al., 2009). Based on the unique microbial signature for each test group (Figure 1), we can see the age-related effect on the ratio of pathogenic to commensal bacterial composition of the oral-nasal cavities. Species isolated exclusively from elderly males are mainly pathogenic, and some come from soil, contaminated meats, and water: *Pseudomonas luteola, Serratia fonticola, Rhizobium radiobacter*, *Staphylococcus xylosus, Raoultella ornithinolytica* (Turner et al., 2018; Cantas et al., 2012; Aljorayid et al., 2016; Mantadakis et al., 2015; Lia et al., 2004; Ferrocino et al., 2018; Hajjar et al., 2018).

The same is observed in elderly women but with less diversity: *Aeromonas hydrophila, Klebsiella*

oxytoca, and *Enterobacter aerogenes* (Citterio and Francesca 2015; Baker et al., 2019; Darby et al., 2014; Davin-Regli and Pages 2015). That pattern of mainly gram-negative bacillus species in the oral-nasal cavity of aged individuals corresponds with other findings in scientific literature. In contrast, species isolated only from younger groups are mostly gram-positive (Bodineau et al., 2009; Aleman and Valenzano 2019; Nagpal et al., 2018). Gender affects microflora composition, but from the data of our study, no significant gender-specific pattern was detected other than the higher abundance of Enterobacter cloacae complex in elderly females. This observation is not necessarily gender-related but could be age-related since the age factor has been well-documented in the literature on increased levels of some gram-negative bacilli, including *Enterobacter*, in older people (Bodineau et al., 2009). The other gender-associated pattern seen from our data is that a greater number of Streptococcus species were identified in younger males than females. Females in our study show significantly higher numbers of oral bacteria than males, which matches the findings of Ma and Li (2019) in their study comparing gender microbiomes from six body samples, including nares, saliva, and tonsils (Ma and Li 2019). However, in terms of nasal bacterial diversity in their study, it was significantly higher in males than females, whereas no significant differences were found between sexes in our study.

We know that the oral-nasal microbiota is much more diverse than what has been retrieved from this study. The automated biochemical identification of bacteria species using the VITEK 2 system has limitations. It can only identify strains cultivable in VITEK 2 recommended media: tryptic soy agar, MacConkey agar, and blood agar. Many oral and nasal fastidious commensals will not grow in such media (Kilian et al., 2016). Therefore, a vast majority of the bacterial microflora cannot be detected, reducing the accuracy of microbiome identification. As for the molecular approach used in this study, the oral sample collection by saliva would have retrieved more identifiable species by directly extracting DNA from the sample rather than pre-culturing samples from swaps.

Therefore, if this study were to be repeated, a more accurate molecular approach such as next-generation sequencing (NGS) methods would yield more representable results, allowing us to accurately measure alpha and beta diversity, the abundance of the microbiota, and the effects of age and gender on its composition. In conclusion, our findings indicated that the oral and the nasal cavities have more in common regarding the composition of the bacterial microflora. Age has a general effect of decreased bacterial diversity that consists mainly of pathogenic gram-negative species posing a threat to their health by increasing their risk of developing fatal infections.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Data availability

All datasets generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

National regulations for the protection of human subjects, rules and regulations of the Government of Saudi Arabia, the Princess Nourah bint Abdulrahman Institutional Review Board Policies and procedures, and the ICH Good Clinical Practice guidelines governed the techniques used in the study. Princess Nourah bint Abdulrahman University IRB approved the protocol of this study with IRB Registration Number with KACS'I, KSA: H-01-R-059, and written informed consent from all the participants in this study was obtained.

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A REVIEW OF THE PROMISING EFFECTIVENESS OF INSECTS IN WASTE MANAGEMENT

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Abstract

Waste management is an urgent global issue. Thus, developing innovative and long-term solutions is essential. Recently, there has been a growing interest in using insects for waste management as one of the main alternatives. One of the emerging applications is using insect larvae as a viable approach for breaking down hazardous organic waste and plastic waste. Aquatic insects effectively decompose water waste containing microplastics, as their decomposition rate has reached 50%. Another application uses vermicompost and insect-based bioconversion as bioeconomic waste management methods to produce pollution-free or non-toxic by-products from day-to-day waste. Insects such as black soldier flies, flesh flies, and some beetle species have been studied for their efficiency in converting various types of waste such as sewage sludge, municipal waste, food residues, restaurant and market waste, residual plant waste after oil extraction and non-organic waste into valuable resources. This article provides a comprehensive review of the promising effectiveness of insects in waste management, highlighting their ability to transform organic waste into valuable resources, minimize non-organic waste and contribute to sustainable practices.

INTRODUCTION

In a world marked by swift population growth, we face two critical challenges: the imminent threat of food shortages (Kee, et al., 2023), which poses significant risks to both human health and the environment (Siddiqui et al., 2022), and the growth in waste volumes highlighting a fundamental reality. Waste remains an underappreciated asset until we harness it for productive purposes (Mannaa et al., 2024). The practice of utilizing insects in the conversion of overlooked waste into valuable resources is where innovation thrives. Insects are highly prolific and have broad adaptability to various food sources, making them a viable solution for the ongoing waste accumulation problem (Beesigamukama, et al., 2023). These exceptional creatures pave the way by efficiently converting various organic waste materials, including food remnants, animal byproducts, and agricultural residues (Shaboon et al., 2022). Interestingly, the idea of using fly larvae for organic waste processing was first proposed a century ago (Čičková et al., 2015). In modern agricultural biogas plants, utilizing biowastes in larvae breeding farms has become more common. This dual-

purpose strategy helps us dispose of waste and yields biogas, a valuable byproduct (Czekała et al.,2020). It is a harmonious synergy of waste reduction and resource generation. Their adaptability and efficiency in converting organic waste into valuable assets demonstrate their significance (Mannaa et al., 2024). This review highlights the multifaceted role of insects while delving into the practices surrounding insect-mediated waste conversion, as they act not only as a remedy for waste management dilemmas but as champions of sustainability.

Aim of the Review

We aim to uncover innovative solutions for waste management that can mitigate environmental concerns by using insects. The current review focuses on four types of insects, as indicated in Table 1.

Table 1: The insects studied in the current review.

English name	Scientific name
Black Soldier Fly	*Hermentia illucens* (Linnaeus, 1758)
Yellow Mealworm Beetle	*Tenebrio molitor* (Fabricius 1778)
Darkling Beetle	*Zophobas morio* (Fabricius, 1776)
Flesh Fly	*Sarcophagidae sp. Sarcophaga* (Meigen, 1826)

The Role of Insects in Waste Management

Insects are widespread components of all terrestrial and freshwater food webs, although their collective biomass is minor compared to plants and microorganisms. As a result, it is frequently believed that these species contribute very little to ecosystem processes. Despite their small stature and total biomass, these creatures frequently modulate the quality and quantity of resources entering the food chain, with implications for ecosystem-level carbon and nutrient cycling (Yang et al., 2014). Multiple processes, such as direct inputs of insect biomass, detrital biomass transformation, and indirect effects of predators on herbivores and detritivores, cause these effects. Typically, the impacts of these routes on the environment transpire. Insects aid in decomposing organic waste, which brings significant environmental benefits. One of the key advantages of vermicomposting is its ability to suppress pathogens and curial greenhouse emissions. Recent studies have shown that incorporating insect-based technologies for feed, oil, and organic fertilizer production can reduce carbon dioxide emissions by 55-83% and deplete conventional energy sources like fossil fuels by 46%, indicating higher environmental sustainability and a lower ecological footprint (Van Phl et al., 2020).

Black Soldier Fly

Hermetic illucens are found in tropical and warm regions (Üstüner et al., 2003). The black soldier fly's feeding is associated with the outdoors and livestock. It is found around decaying organic matter such as rotting fruits and vegetables, animal manure, and human waste. These insects feed during the larval stage (Manyara, 2018).

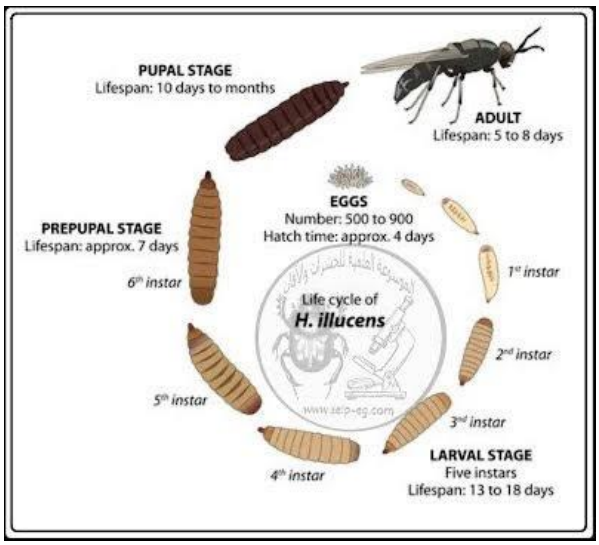

Figure 1: lifecycle of *Hermetia illucens*. (De Smet *et al.*,2018)

Once hatched, the larvae start to feed on the waste, thus achieving a dry mass volume waste reduction of ~55% (Myers et al., 2008; Newton et al., 1995; Sheppard,.1983). Their short life cycle (Figure 1) contributes to building a high-density population. The larvae have voracious appetites, and fresh material is processed extremely fast, reducing the production of bad smells to a minimum. An additional advantage of *Hermetia illucens* is its capacity to repel the oviposition of female house flies (Bradley and Sheppard,1984). It is a disease vector, especially in developing countries. Under ideal conditions (i.e. waste deposits), larvae can mature in two weeks. The food shortage and low temperatures can extend the larval period up to four months (Furman et al., 1959).

The Role of Black Soldier Fly Larvae in Waste Management

Hermetia illucens has garnered increasing interest (Makkar et al., 2014; Smetana et al., 2016; Surendra et al., 2016), particularly since its initial use in the 1990s for waste treatment as an efficient method to convert bio-waste into protein- and fat-rich biomass for animal feed. The process involves feeding fly larvae with biowaste. *Hermetia illucens* can reduce the biomass of organic waste, such as slaughterhouse waste, food waste, fruit and vegetable waste, and human feces, by 50-60% (Ojha et al., 2020).

Hermetia illucens does not risk human health when compared to other fly species. *Hermetia illucens* adults have a short lifespan and do not feed due to redundant mouthparts. They typically avoid human habitats and foods and rely on stored energy from their larval stage in their fat body. The ample fat reserves in the larvae reduce or eliminate the need for adult feeding, minimizing the potential spread of diseases (Furman et al., 1959; Sheppard et al., 1983). *Hermetia illucens* can be utilized to manage organic waste and valorise various biodegradable wastes, which has led to extensive studies in this area. (Čičková et al.,2015 ; Mertenat et al., 2019 ; Czekała et al.,2020 ; Ojha et al.,2020 ; Siddiqui et al., 2022 ; Mannaa et al.,2024) Laboratory experiments at the Asian Institute of Technology (AIT) have been conducted to evaluate the digestibility of fecal sludge by Black soldier fly larvae insects. (Alamgir et al.,2011) The experiments used soldier fly larvae from a colony raised in a small 3m x 3m greenhouse equipped with an automated water spraying system. The larvae were subjected to various ratios of waste products (0%, 25%, 50%, 80%, and 100% fecal sludge) every three days until all the larvae had completed their transformation into prepupae. The experimental results revealed that in terms of fecal sludge processing, black soldier fly larvae (BSFL) not only survived

and developed in pure fecal sludge but also significantly reduced the volume of sludge. A recent study by Purkayastha and Sarkar (2023) demonstrated that BSFL can consume human feces, though the larvae had significantly lower body weight compared to those fed on food waste. Interestingly, the degradation and bioconversion of human feces were significantly enhanced when mixed with food waste, compared to larvae reared on human feces or food waste alone. These results suggest that optimizing rearing conditions. particularly through various substrate mixtures, could enhance bioconversion and degradation even for challenging waste types like human feces, which have little food waste remnants and limited nutritional value after the absorption of nutrients in the human gut. Recognizing and extracting valuable products from human excreta is essential from a sustainability perspective. Another study tested the ability of BSFL to recycle biological waste (Abd Rahman et al., 2020). It was observed that BSFL has a remarkable ability (75%) to recycle biological waste, as 800 g of larval biomass was produced from 4 kg of waste. To improve the effectiveness of bioconversion. BSFL should be maintained under optimal environmental conditions, including humidity, nutrient composition, physical properties, temperature, and oxygen level.

Figures 2 and 3 present findings from a study exploring the metabolic, nutrient functions, and taxonomic composition of intestinal bacterial communities in BSFL fed on pig and chicken manure (Mannaa et al., 2024). The study revealed that Bacteroidetes, Firmicutes, and Proteobacteria were the dominant bacterial groups in the BSFL midgut in these systems. Bacterial genes such as cellulases, proteases, and lipases, which hydrolyze starch/cellulose, proteins, and lipids, respectively, play a role in producing enzymes within the BSFL midgut, aiding in the digestion and recycling of biomass waste and nutrients (Lee et al., 2014). A study also found that adding bacterial supplements, such as *Bacillus subtilis* to chicken manure, positively impacted BSFL cultures (Yu et al., 2011).

Supplements containing arthropods and Rhodococcus in the diet of BSFL are also promising. Faster-growing, harvestable larvae and pupae save industrial BSF production costs and increase benefits. A study on Black Soldier Fly (BSF) was conducted to evaluate greenhouse gas (GHG) emissions associated with biowaste processing and compare them to conventional composting methods (Mertenat et al., 2019). The study used a life cycle approach to evaluate an Indonesian BSF processing facility's global warming potential (GWP). Previous research on the greenhouse gas emissions of BSF has often relied on data from other insects and compared BSF with other feed production or waste diversion in high-income countries. This study aims to fill this research gap by directly assessing GHG emissions from the BSF treatment process and comparing it to an Indonesian composting facility. The BSF treatment process involves using larvae to process kitchen waste into plastic bins (Dortmans et al., 2017). Treatment took 13 days, with kitchen waste added regularly according to recommended procedures. Direct GHG emissions from the BSF processing process were assessed by conducting a gas sampling campaign at the facility (Chan et al., 2011). Gas samples were taken daily in triplicate to measure methane, nitrous oxide and carbon dioxide production. The results indicated that BSF larvae could effectively reduce biowaste with an average reduction of 50% and a biomass growth of 20-25% (wet weight). Methane production was estimated at 0.4 grams per ton of treated organic household waste, and nitrous oxide production was estimated at 8.6 grams per ton of treated waste. The study found that when considering direct greenhouse gas emissions, BSF processing had lower emissions than composting. In conclusion, this study indicates that BSFL

biowaste treatment can lead to lower direct greenhouse gas emissions than traditional composting methods.

Table 2: The waste reduction and bioconversion rates by BSFL on various substrates compared to chicken feed bioconversion (Siddiqui et al.,2022).

Substrate	Waste reduction (%)	Bioconversion rate (%)	References
Individual wastes			
Abattoir waste	46.3	15.2	Lalander et al. (2019)
Canteen waste	37.9	15.3	Gold et al. (2020a)
Cow manure	12.7	3.8	Gold et al. (2020a)
Digested sludge	13.2	0.2	Lalander et al. (2019)
Food waste-1	66.7	7.7	Salomone et al. (2017)
Food waste-2*	52.3	27.9	Ermolaev et al. (2019)
Food waste-3	55.3	13.9	Lalander et al. (2019)
Fruits and vegetable	46.7	4.1	Lalander et al. (2019)
Human feces-1	73.0	NA	Lalander et al. (2013)
Human feces-2	45.8	22.9	Banks et al. (2014)
Human feces-3	47.7	11.3	Lalander et al. (2019)
Human feces-4	43.9	20.7	Gold et al. (2020a)
Municipal organic waste	68.0	11.8	Diener et al. (2011a)
Poultry manure	60.0	7.1	Lalander et al. (2019)
Poultry slaughterhouse waste	30.7	13.4	Gold et al. (2020a)
Primary sludge	63.3	2.3	Lalander et al. (2019)
Swine manure	56.0	NA	Newton et al. (2005a)
Undigested sludge	49.2	2.2	Lalander et al. (2019)
Vegetable canteen waste	58.4	22.7	Gold et al. (2020a)

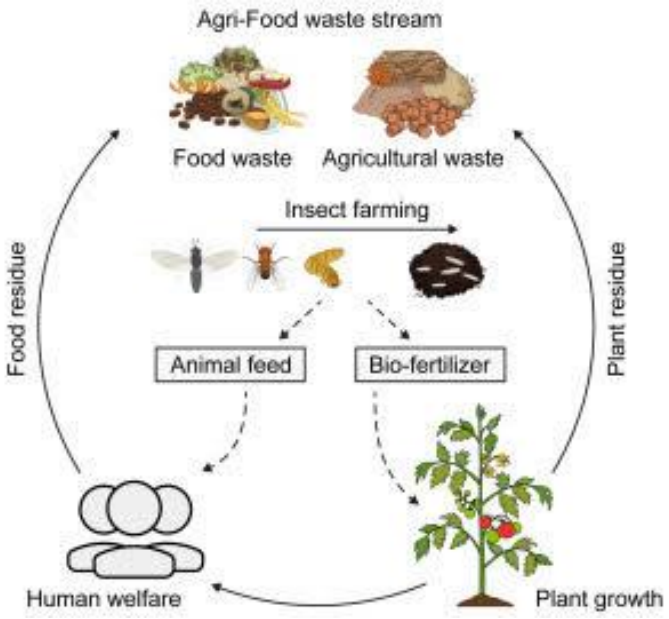

Figure 2: Black Soldier Fly Larvae Converting Waste to Value (Mannaa et al.,2024)

Production with low carbon

In regions with low- and middle-income economies, biowaste includes municipal solid waste from households' food, market, park, and food manufacturing plant residue, constituting a significant portion of waste, accounting for up to 70%. Typically, this bio-waste ends up in landfills and wastewater, contributing to approximately 90% of global waste sector emissions (Mertenat et al., 2019). Including greenhouse gasses like methane (CH4), ammonia (NH3), and nitrous oxide (N2O) during the decomposition process in landfills. To address this environmental challenge, redirecting biowaste to feed BSFL for mass production offers a sustainable alternative to landfills. It presents cost-effective raw materials for BSF protein meals and oils. This approach enhances the efficiency and sustainability of end-products. A case study in Indonesia highlights that BSF waste treatment facilities produce fewer hazardous gas emissions (CO_2, CH_4, and N_2O) and consume less energy (electricity and diesel) when compared to open-windrowing composting (Klammsteiner et al., 2020). A heat map study also reveals a positive correlation between BSFL application and various environmental factors such as temperature, pH, C/N ratio, gaseous emissions and PB population. Moreover, insect-based protein meal, according to Smetana et al., (2016), is a significantly more environmentally friendly product with environmental benefits up to 2-5 times that of commercially available alternatives. This shift toward BSFL-based waste treatment offers a promising avenue for reducing greenhouse gas emissions and improving sustainability in waste management.

Black soldier fly larvae as an animal feed

Using BSFL to convert organic wastes into larvae or prepupae is an environmentally friendly and sustainable recycling technology (Diener et al., 2011). BSFL, with its variable nutritional content depending on the substrate and age, stands out as an excellent source of animal feed due to their high crude protein and crude fat content (Kim et al., 2019; Spranghers et al., 2017). BSFL can serve as animal feed in multiple forms, including processed options like dried larvae, extracted oil/protein meal, and live larvae. Protein extracted from BSFL larvae and pupae is a viable substitute for common feed ingredients such as soybean or meat meal (Čičková et al., 2015).

Moreover, BSFL protein contains all ten essential amino acids required for animal nutrition: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Halver et al., 1957, Halver and Shanks, 1960, Shumo et al., 2019). The quality of animal feed is influenced by the amino acid composition of the proteins (Muller et al.,2017). The amino acid content in BSFL can vary depending on the substrate the BSFL is fed with, and the highest total amino acid content was observed in BSFL fed with kitchen waste.

The Utilization of Insect Frass as Agricultural Fertilizer

Waste management challenges and soil degradation pose significant environmental issues that adversely impact global food security (Pimentel et al., 1975). Additionally, issues like micronutrient deficiency, low organic matter, and soil acidity hinder the effectiveness of mineral fertilizers (Kihara et al., 2016). Organic fertilizers serve as a cost-effective and viable solution for soil enhancement. However, their adoption needs to be improved due to extended production timelines, subpar quality, and insufficient on-farm organic material sources. Therefore, exploring alternative, cost-effective, and readily available sources of high-quality fertilizers is imperative. Numerous studies have investigated the use of insect frass, such as that from BSF and Yellow Mealworms (YMW), as potential sources of organic fertilizers that provide plants with essential nutrients and beneficial microorganisms. In this regard, BSF larvae swiftly convert organic waste into stable, mature, and nutrient-rich frass fertilizer in just five weeks, a process significantly faster than conventional composting, which can take 8-24 weeks (Beesigamukama et al., 2021). Crops such as maize, barley, cowpeas, French beans, tomatoes, and chilli peppers grown using BSF or YMW frass fertilizers have higher yields and nutritional quality than those cultivated with conventional fertilizers. Furthermore, applying BSF and YMW frass fertilizers as soil amendments has improved nutrient availability, nitrogen mineralization, and soil microbial activity, reducing the presence of soil-borne pathogens, soil salinity, and acidity. These factors collectively contribute to enhanced soil quality for crop production. To provide tailored recommendations for efficiently using insect frass in soil fertility enhancement and crop yield improvement.

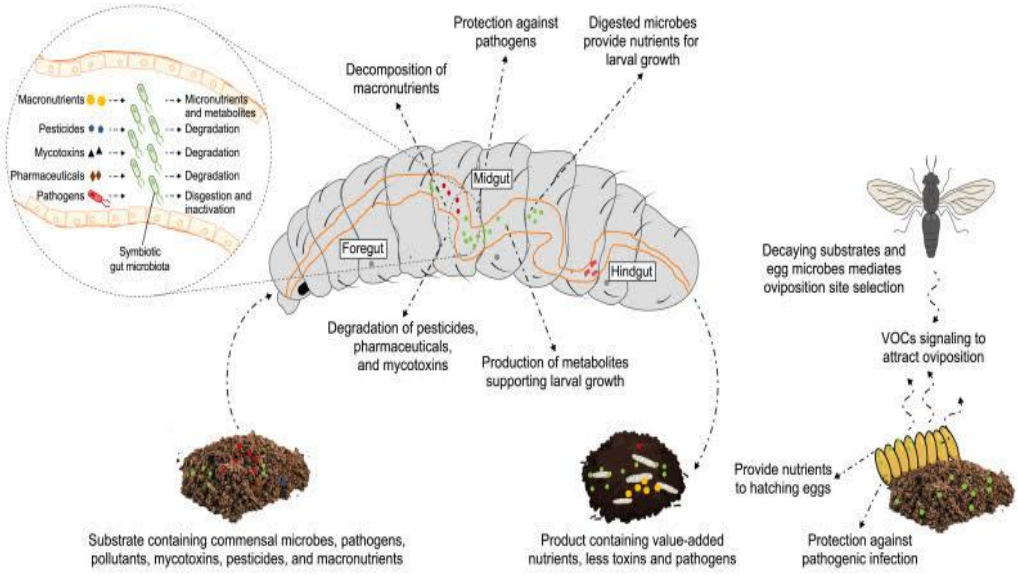

Figure 3: Illustration of the different roles of the gut-associated microbiota of the black soldier fly within the agri-food waste biorefinery system from the study conducted by (Mannaa et al., 2024).

Beetles and Their Ability in Plastic Waste Management

Beetles (Coleoptera) are a diversified and abundant species of insects that play a range of ecological functions in several sub-environments across most continents. (Marshall, 2018) Beetles in current habitats can be scavengers, saprophytes, carnivorous, herbivorous, or frugivorous. (Coleman et al., 2004) Tenebrionidae (darkling beetles) are a prevalent and diversified group of beetles spread out worldwide. It comprises soil-dwelling species in various habitats but is most closely connected with dry environments such as steppes and deserts. (Marshall, 2018) Darkling beetles undergo a complete life cycle encompassing four distinct life stages – egg, larva, pupa, and adult, each characterized by unique morphological features and behaviors. (Marshall, 2018) The larvae of certain Tenebrionidae species can be found in three primary soil textural categories: clay, loam, and sand. (Calkins & Kirk, 1975) In their natural habitats, *Zophobas morio* and *Tenebrio molitor* are considered generalist omnivores, consuming decaying plant material, fresh plant material, decaying insects, and fungi (Marshall, 2018), which makes them ideal candidates for decomposing organic waste**.** The developmental stages of *Zophobas morio* and *Tenebrio molitor* are highlighted in Figure 4.

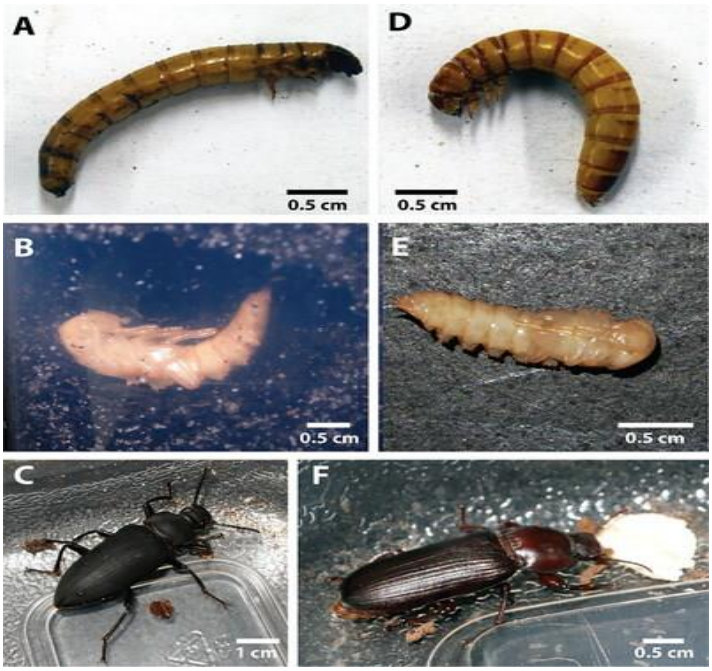

Figure 4: Developmental stages of the studied darkling beetle species. In the left column, *Zophobas morio* stages: (A) larva, (B) pupa, and (C) adult; in the right column, *Tenebrio molitor* stages: (D) larva, (E) pupa, and (F) adult (Hayden et al., 2021)

The Use of Insects in Plastic Waste Management

Traditional plastics such as polyethylene (PE), polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), and other polymer compounds are known for their prolonged degradation in various environments. (Wang et al., 2022) However, more recent research revealed many microorganisms capable of breaking down polymers in natural environments, including soil, seawater, sludge, and compost. The intriguing relationship between insects and plastics traces its origins to consumer complaints about insects damaging

chocolate-based consumable packaging (Terence, 1997). Observations of insects destroying and consuming plastic packaging materials shed light on the degradation potential of these creatures, including insects belonging to the order Coleoptera. In recent years, studies have been conducted on the larvae of beetles, including *Tenebrio molitor* and *Zophobas morio,* and their ability to reduce plastic waste by feeding.

Studies Conducted on *Zophobas morio* in Plastic Waste Management

A recent scientific study revealed the remarkable resilience of superworms, as they demonstrated the ability to grow into beetles on a strict PBS foam diet. (Jung et al., 2023) This study acquired superworm larvae (*Z. atratus*) from a local supplier, Mealworm Nara, in Yeoju, Korea. These larvae had a length ranging from 5 to 6 cm. The life cycle of superworms consists of four stages: egg, larva, pupa, and adult, with a significant portion of their lifespan spent in the larval stage. These larger-than-average larvae exhibit improved mobility, heightened food consumption, and enhanced cutting capabilities. Collectively, these attributes enhance their effectiveness in breaking down plastic materials. Before commencing the feeding experiment, the superworms underwent 48 hours of fasting. A total of 30 superworms were housed in a polypropylene container (dimensions: 103 x 78.6 mm), and they were provided with PBS foam (4 g) as their sole diet. For comparison purposes, three control groups were established: the first group was given bran, a widely used feed in the industry; the second group received PS, a common plastic studied for superworm plastic degradation; and the third group was deliberately left unfed to induce a state of starvation. The superworms were kept for four weeks at a temperature of 26 ± 1°C. Deionized water was supplied to maintain adequate moisture levels, and any deceased superworms were promptly removed. The superworms' survival rate, changes in body weight, and plastic consumption were recorded every seven days, with all treatments conducted in triplicate. Every seven days, 4 g of PBS, 2 g of PS, and 2 g of bran were replenished. After the experiment, superworms exclusively fed with PBS exhibited a survival rate of 95.2 ± 1.6%, which was on par with that of bran-fed superworms (95.6 ± 4.4%) and exceeded the survival rates of PS-fed (90.7 ± 2.7%) and starved (84.4 ± 2.9%). The average weight of super worms that consumed bran, PBS, and PS increased by 32.4 ± 2.6% (equivalent to 32.23% in dry weight; 212 mg superworm^{-1}), 5.1 ± 0.5% (equivalent to 4.77% in dry weight; 33 mg superworm−1), and 3.6 ± 5.9% (equivalent to 3.30% in dry weight; 23 mg superworm−1), respectively. In contrast, the average weight of unfed superworms decreased by 11.1 ± 2.1% (equivalent to 9.67% in dry weight; 67 mg superworm^{-1}). These results indicated that PBS-fed super worms exhibited similar survival rates to bran-fed super worms and experienced a 5.1% increase in weight during the experiment, demonstrating that super worms can derive their energy and nutrition for growth from digesting PBS. Previous studies have reported similar trends, with comparable survival rates in PS-fed superworms (ranging from 90% to 100%).

Studies Conducted on *Tenebrio* Monitoring Plastic Waste Management

An experiment in 2023 was conducted studying T. molitor larvae's ability to degrade polyurethane used in refrigerator insulation. (Zhu et al., 2023) The replacement of refrigerator insulation often leads to the disposal of significant quantities of waste refrigerator polyurethane (WRPU). While insect larvae such as mealworms have been employed in the biodegradation of pristine plastics, there is limited knowledge regarding their ability to degrade WRPU. This study conducts a comprehensive investigation into the degradation of WRPU by mealworms, examining micro-

morphological changes, composition variations, and alterations in functional groups within the WRPU, as well as characterizing the properties of the resulting frass. The findings reveal that WRPU debris in the frass indicates that mealworms both ingest and degrade WRPU. The carbon content in WRPU-based frass was lower than in WRPU, suggesting that mealworms utilized WRPU as a carbon source. Furthermore, the study shows that urethane groups in WRPU were broken down, while certain C=C and C-H bonds in the isocyanate-derived benzene rings disappeared after mealworm ingestion. Thermal analysis indicated a lower weight loss temperature for WRPU-based frass compared to WRPU, signifying reduced thermal stability in the ingested material. Carbon balance analysis confirmed increased CO2 release from ingested WRPU, suggesting partial mineralization. Interestingly, the carbon content in the mealworm biomass that ingested WRPU decreased, possibly due to insufficient nutrient supply, impurities, and odor influencing the mealworms' appetite. Additionally, the study revealed that WRPU had a considerable impact on the gut microorganisms of mealworms. These collective findings provide strong evidence of mealworms' capacity to degrade WRPU. Yellow mealworm (*Tenebrio molitor* L.) larvae have also demonstrated their ability to break down and depolymerize polyethylene (PE), polystyrene (PS), and polyvinyl chloride (PVC). In a study conducted in 2022 (Jin et al., 2023), mealworms were employed to facilitate the biodegradation of these plastic materials, which included PE, PS, and PVC. Furthermore, the research delved into the repercussions of plastic degradation on the growth and developmental aspects of yellow mealworm larvae by closely examining various physiological indicators and the nutritional constituents of the larvae following plastic degradation. The findings revealed that the degradation of plastics (specifically PS, PE, and PVC) was notably enhanced when a feeding amount of 0.50 g was utilized. It's noteworthy, however, that at this concentration, the degradation of PVC led to an increase in the mortality rate of yellow mealworms. In contrast, the degradation of a smaller quantity of PS (0.10 g) positively impacted the nutritional value, with higher levels of crude protein (45.7 ± 2.08%) and phosphorus (1.23 ± 0.04%), along with a lower larval mortality rate (7.90 ± 1.10%). This, in turn, had a limited effect on yellow mealworms' overall growth and development**.**

Comparison Between *Z. morio* and *T. molitor* Abilities in Plastic Waste Management

A comparative study was conducted (Wang et al., 2022) observing the distinct plastic ingestion preferences and efficiency variations between the superworm (*Zophobas morio*) and the yellow mealworm (*Tenebrio molitor*). The study highlighted the concurrent alterations in their gut microbiomes. The survival and plastic consumption abilities of superworms (Zophobas morio) and yellow mealworms (Tenebrio molitor) on exclusive plastic diets are compared in this study. While both species exhibited the capability to survive solely on plastic diets, a side-by-side assessment of their plastic degradation had not been previously conducted. In a 35-day experiment, superworms and yellow mealworms were fed polystyrene (PS) and polyurethane (PU) foam plastics as their sole diets, with bran as a control. Superworms displayed 100% survival rates on all diets, albeit a slight weight reduction was observed after 20 days of consuming exclusive plastic diets. In contrast, yellow mealworms had 84.67% and 62.67% survival rates when fed PS and PU diets, respectively, with both plastic diet groups showing increased weights. Cumulative plastic consumption by superworms amounted to 49.24 mg-PS per larva and 26.23 mg-PU per larva, which were 18 and 11 times that of yellow mealworms, respectively. When normalized to mg/g-larvae, superworms exhibited a higher PS consumption rate, while both species displayed similar PU consumption rates.

Notably, changes in the chemical functional groups of plastics found in their frass indicated oxidation and biodegradation processes occurring within the guts of both species. These transformations were associated with alterations in the gut microbial communities, with distinct dominant microbiomes related to the specific plastic feedstocks and the species of larvae. Superworms fed on PS showed increased relative abundances of unclassified *Enterobacteriaceae, Klebsiella, Enterococcus, Dysgonomonas*, and *Sphingobacterium*, while Hafnia was strongly associated with PS diet in yellow mealworms. *Enterococcus* and *Mangrovibacter* dominated in superworm guts for PU diets, whereas unclassified Enterobacteriaceae and Hafnia were strongly associated with PU feeding in yellow mealworms. These findings underscore both species' different plastic ingestion preferences and efficiencies linked to distinct dominant microbiomes, although similar changes in plastic chemistry were observed.

Flesh Fly

The adult is between 9 and 13 mm in size. Typically, the thorax of this fly has three black stripes and is light grayish. (Watson and Dallwitz, 2003) Males have stronger front legs and are hairier than females, which helps them when they try to copulate. Male and female flesh flies have red abdomen ends, which resemble the "tails" of many other species. The front femora are frequently light gray and somewhat bigger than those on the other legs. A characteristic black stripe with golden or yellowish edges runs between the eyes of adults. (Watson and Dallwitz, 2003) Adult flies eat a variety of liquid materials without biting. Most larvae feed on feces, carrion, and wounds. Because they feed on the eggs, nymphs, or larvae of more dangerous insects, the larvae of certain species of meat flies are advantageous. Common hosts of flesh flies include grasshopper nymphs, blow fly larvae, and smaller house fly larvae. The lateral view of *Sarcophaga dux* adult is presented in Figure 5.

Figure 5: Lateral view of adult *Sarcophaga dux*, a flesh fly Photograph by *Lazaro A. Diaz, University of Florida.*

The Role of Flesh Fly in Waste Management

In many parts of the world, *S. dux* (Diptera: Sarcophagidae), sometimes referred to as a meat fly, is a species of medicinal significance. (Cherix et al., 2012) Even though many fly species are regarded as serious pests, they also aid in the biodegradation of organic waste and are crucial to the recycling of organic matter in the environment. (Čičková et al., 2015). According to Abo Hasan and Phun (2018), the flies' larvae included biologically active substances, including lectin, chitin, and antimicrobial peptides that will aid in the composting of organic waste and help decrease manures.

This study first aimed to assess how different manures (chicken, goat, and cow) affected the development rate of the flies and their larvae's *(S. dux* and *M. domestica*) capacity to digest animal manures. Secondly, the effectiveness of manure reduction was compared in both species of flies. Third, the ideal growth of the two species of flies in three different types of manures was also determined. The flies frequently use animal manures, especially those from poultry and cattle operations, as part of their natural diets, becuase they are known to be voracious eaters, the larvae of common filth flies like *Sarcophaga dux* and *Musca domestica* can be employed to transform manures into a residue that is not contaminated. In a lab setting, one hundred freshly hatched *S. dux* and *M. domestica* larvae were each placed into 150 g of manures. After the larvae were deposited into the manures, measurements were made of the dry mass and mortality rate, and the initial wet mass and larval length were noted. Studies have shown that various types of manure significantly affect the growth of *M. domestica* and *S. dux* larvae ($p < 0.05$). Of the three manure types, cow manure produced the highest growth rate in M. domestica, while chicken manure led to significantly better development of *S. dux*, as indicated by the mean increase in wet mass and larvae length. *M. domestica* exhibited the highest mean dry mass in cow manure, while *S. dux* did so in chicken manure.

Figure 6: *Chironomus sp* Larvae. (image source: *Steve Hopkin).*

Figure 7: *Chironomus sp adult* Contributed by *Nolie Schneider*

Figure 8: *Culex sp.* Larvae and adult By *CDC; Centers for Disease Control and Prevention,cdc.gov.Hydrophilus sp.*

Figure 9: *Hydrophilus sp*. adult by *Claudio Mendez*

Figure 10: *Aeshna sp.* Nymph, by *Christine Young.*

Figure 11: *Aeshna sp.* Adult by Jim Petranka

Aquatic insect

Water wastage is seen as common because it is one of the most widespread wastes on the planet. The preservation of these ecosystems depends heavily on the aquatic species and insects that live there. With a decomposition rate of up to 50%, the Chironomus aquatic insect is one of the most efficient insects at breaking down aquatic waste, including microplastics. The eye spot, tube, abdominal plate, antenna, and abdominal tubes are the basic features that help identify Chironomus. Larvae and adults of *Chironomus* sp. are presented in Figures 6 and 7, and *Culex* sp. in Figures 8. *Hydrophilus* sp. adult is presented in Figure 9. Nymphs and adults of *Aeshna* sp. are displayed in Figures 10 and 11. *Culex pilosus* larvae have long antennae with a prominent tuft at the tips and a wide head.

An oval gill is introduced at the base of the antennae on the ventral side of the larval head (Carpenter and LaCasse, 1955). Their siphon is upcurved, and at the end of the siphon is a curved preapical spine. *The Hydrophilus* beetles have a distinctively elongated and slender body. The largest beetle in this genus can reach a maximum size of 40 mm (Cranshaw, 2010*)*.

The Role of Aquatic Insects in Water Waste Management

The low-lying desert region of EL Cola, which is roughly 14.6 km east of the city of Sohag (26° 33′ 04″ N and 31° 50′ 55″ E), is home to the wastewater project where investigations on the use of aquatic insects for wastewater management were carried out. (Khedre et al.,2023) It is situated between a plateau made of limestone and a desert. This location is made up of several asymmetrical basins that span about 4.4 km2. A single basin, spanning approximately 1.27 km2, was selected for sampling due to its perceived closure. Specimens were collected from ten sampling stations around the basin to give a representative image of MP contamination. All samples were collected in January 2021; each point was around 100 meters apart. Aquatic insects were collected from 0 to 60 cm below the water's surface using a pond net with a 200 m mesh. Ten duplicates were taken at each sampling location at the same stations to collect silt and water. Every precaution was taken to avoid upsetting or releasing aquatic insects. Ten surface water duplicates (5 L) were collected using a steel bucket from each sample site after the first collection, sorting, and taxonomic identification in the field. These replicates were then stored in clean glass containers for laboratory analysis. Vials for collecting samples were promptly sealed to stop airborne contamination. According to the present findings, the insect under examination had fewer MPs after a 24-hour period, demonstrating its exceptional capacity for digestion. After twenty-four hours, the average number of MPs per insect in *Chironomus* sp. larvae dropped dramatically, with 53% of the MPs being expelled. The egestion of MPs of several freshwater invertebrates was examined, including diploids (*Sphaerium corneum*), frogs (*Gammarus pulex*), and flies. They proposed that the form of MPs may influence an organism's capacity to digest and egest MPs. It is, therefore, a helpful technique for looking at MP pollution in freshwater environments (Akindele et al., 2020). Numerous morphological, behavioral, and physiological traits are seen in aquatic insects (Phuge et al., 2020). Additionally, they are essential to the food chain because they filter organic particles, participate in the cycling of nutrients through leaf division, hunt insects and other fish, and provide food for other vertebrates and invertebrates (Ribeiro-Brasil et al., 2021). Nevertheless, research on this insect and its application in the analysis of water waste is still lacking.

Aquatic insects' capacity to consume MPs

The quantity and kind of MP particles removed from the guts of several aquatic insects collected from the wastewater are displayed in Tables 3 and 12. With variations in the observed egestion ability, the results showed a decrease in MPs in all examined insect taxa following a 24-hour deputation period. Chironomus sp. and Culex sp. larvae showed a substantial decrease in the mean number of MPs per individual following the depuration stage ($p < 0.05$), with the egestion percentages of 53% and 40%, respectively. Aeshnidae nymphs had a 25% decrease in MP egestion ability, and adults of Hydrophilidae had the lowest percentage, only 9% of egestion. When the adult Hydrophilidae and Aeschnidae nymphs were permitted to expel their stomach contents spontaneously, the relative abundance of MPs was not substantially decreased ($p > 0.05$).

When comparing gut contents evacuated samples to non-evacuated samples, the mean lengths of MP particles were shorter in all species studied; however, these changes were not statistically significant ($p > 0.05$) (Khedre, et al., 2023).

Table 3 : Microplastics abundance in aquatic insect samples with non-evacuated and evacuated gut content (*Khedre* et al.,*2023*)

Microplastic abundance in aquatic insects' samples with non-evacuated and evacuated gut contents.

Order	Family/genus (stage)	FFG	Mean MPs particles/individual.		Percentage of ejection	Mean length of MPs fibers (μm)	
			Non-evacuated	Evacuated		Non-evacuated	Evacuated
Diptera	Chironomidae/*Chironomus* sp. (larva)	CG (2)	0.88 ± 0.1	0.41 ± 0.15	53%	507 ± 182 (321–730)	482 ± 215 (343–620)
	Culicidae/*Culex* sp. (larva)	CF	0.25 ± 0.07	0.15 ± 0.02	40%	710 ± 108 (580–815)	695 ± 103 (580–810)
Coleoptera	Hydrophilidae/*Hydrophilus* sp. (adult)	CG (1)	2.12 ± 0.62	2.01 ± 0.50	9%	1258 ± 315 (980–1513)	1153 ± 394 (980–1326)
Odonata	Aeshnidae/ *Aeshnae* sp. (nymph)	p	4 ± 1	3 ± 1	25%	1190 ± 270 (986–1490)	1040 ± 109 (986–1092)

Substantial gut clearance was assumed after aquatic insects have been kept for 24 h in dist. water. (FFG legend: P = predators; CG = collector-gatherers; CF = collector-filterers). (Mean ± SD).

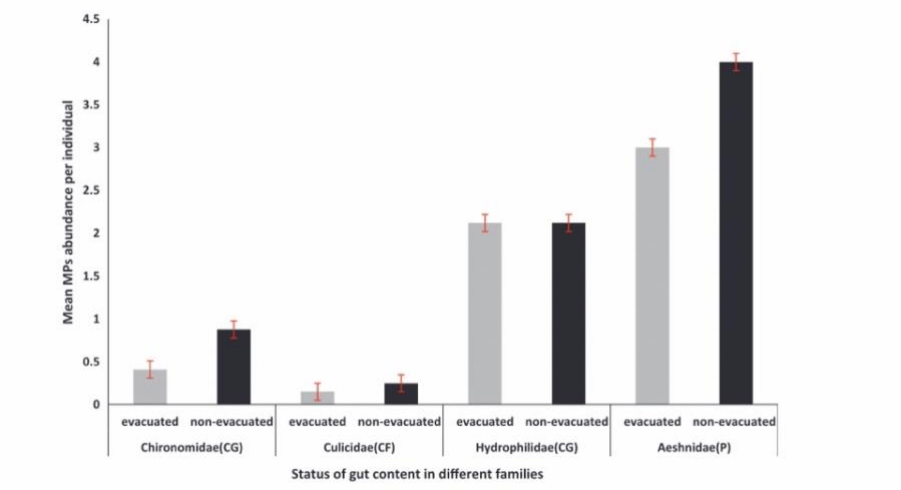

Figure 12 : Microplastics abundance in aquatic insect samples with non-evacuated and evacuated gut content (Khedre et al., 2023).

CONCLUSION

This paper concluded that insects are ideal candidates for waste management. *Hermetia illucens* has gained interest in waste management due to its ability to convert bio-waste into protein-rich biomass. *Hermetia illucens* can reduce organic waste biomass by 50-60%. Research has shown that some insects, including beetle larvae such as *Tenebrio molitor* and *Zophobas morio*, can reduce plastic waste by feeding on it. The relationship between insects and plastics arose from observations of insects destroying and consuming plastic packaging materials. *Sarcophaga dux* has been used to biodegrade

organic waste and plays an important role in material recycling, which will help reduce compost and convert organic waste into fertilizer. The aquatic *Chironomus sp* and *Culex* sp., *Hydrophilus sp, and Aeshna sp* are known for their effectiveness in breaking down aquatic waste, including microplastics.

RECOMMENDATIONS

Based on this review, we hope to see the following implemented:

- Recognize the superiority of *Hermetia illucens* among insects with an ability to manage organic waste.
- Encourage increased future research and experimentation on the potential utilization of insects in waste management and recycling.
- Expand the scope of insect-based waste recycling by exploring a variety of insect species rather than restricting it to just one.
- Promote in-depth studies on aquatic insects and their pivotal role in managing freshwater waste, given its significance as a primary water source.
- Emphasize the application of insects to reduce organic waste and offal, replacing chemical methods for sustainability. Utilizing insects in research and experiments, rather than eliminating or disposing of them, will contribute to environmental preservation.
- Investigate the impact of insect-based waste management in landfill sites.
- Integrate insect-based waste management into broader recycling and waste management systems. This entails segregating organic waste and introducing suitable insect species to convert specific biodegradable materials, resulting in more efficient waste reduction and resource recovery.
- Enhance waste containment by employing sealed containers or boxes with secure lids to prevent house flies from accessing waste and breeding. It is crucial to ensure that containers remain tightly closed, especially in areas where waste is temporarily stored prior to disposal.
- Explore the potential for waste reduction through insect-derived products, which can be harnessed from organic waste materials.

Future Aspects

This article opens the door for government bodies such as the National Centre for Waste Management, the Ministry of Environment Water and Agriculture, the Ministry of Investment, and the Ministry of Energy to promising avenues for further research on utilizing insects in waste management. One key area of future exploration is incorporating insect farms in waste management practices as a cost-friendly alternative to traditional methods. Additionally, further research on insects and their role as decomposers can reduce the overall mass of landfill waste.

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EFFECT OF ULTRAVIOLET RADIATION ON THE PHENOTYPIC AND MOLECULAR MUTATION IN *Aspergillus flavus*

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Keywords

Aspergillus flavus, Mutation, Aflatoxin, HPLC, Corn grains

Abstract

The present study aimed to investigate the effect of ultraviolet radiation induced genetic mutations (phenotypic and molecular) in *Aspergillus flavus* collected from corn grains and tested for their ability to produce aflatoxin using the high liquid chromatography technique (HPLC). A Hanau Fluotest Forte 5261 UV test lamp was used for treating *A. flavus* at a transition wavelength of 254 nm for 24 h. The results showed that UV radiation exposure at a transition wavelength of 800 has a mutagenic effect on *A. flavus* since a change in the shape and acceleration of growth was observed compared to the non-exposed sample. In addition, the second-generation conidia showed slower growth than the non-exposed sample. The aflatoxin types AfB1, AfB2, AfG1, and AfG2 differed in quality and quantity based on fungal isolates. The concentrations ranged from 35.210 to 195.75 ug /kg of grains. It was found that *A. flavus* (UV) produced AfB1 at 35.250 ug/kg of seeds. Meanwhile, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 with 45.74, 10.4, 15.24, and 135.20 ug/kg of grains, respectively.

On the other hand, the genetic variability among the two isolates was detected. The DNA genome of *A. flavus* isolates (UV and O) was tested using PCR to amplify the FIR gene. PCR amplification confirms its inter-specificity to *A. flavus*, UV, and O isolates with expected sizes 1335 and 1346bp, respectively. Translating the lR gene nucleate sequence to amino acids revealed genetic variability among the two isolates of 445 and 448, respectively. The percentage of nucleotide sequence replacement was 0.89%, while the percentage for amino acid sequence replacement was 1.57%.

INTRODUCTION

Ultraviolet (UV)-irradiation, a type of ionized radiation, cleans and eliminates microscopic neighborhoods at wavelengths between 210-3800 nm.It is used to sterilize hospitals and eliminate different types of bacteria and fungi and toxin-producing *Aspergillus* sp. private, *Fusarium* sp., and *Botrytis* sp. types of mushrooms (Amit et al., 2003). The X-rays are of short wavelengths and have high permeability in living matter, causing aggregation of protein material within the pathogen,

which leads to the rapid killing of these neighborhoods. Recent studies have concluded that the missing food ratio because of fungus reached 30-25% or more, which represents a real threat to public health and a burden on the national economy. However, the use of irradiation technology (X-rays) increased the level of national production by eliminating 95% of the microbes without impacting the environment. Some states succeeded in conserving food using radiation; nearly two hundred thousand tons of rice, wheat, and barley were treated with X-rays, placed in high-specification stores, and used for human consumption without any registered symptoms. The irradiation process is quick but causes damage to humans and animals. Without raising the temperature, the DNA within the cells can be damaged. At specific doses, mutations can occur (Suttle, 2004) due to damage to the nucleic acids and proteins (Caasi-Lit et al., 1997). An induced mutation can occur because of the strong absorption at different wavelengths, especially between 600-890 nm, causing photochemical dimerization acidosis to adjacent nucleic acids and damaging a series of DNA (Vreugdenhil 2004). UV irradiation can cause damage to skin cells and DNA molecules, resulting in abnormal covalent bonds between cytosine bases and productive bilateral units. Therefore, the DNA polymerase will read the bilateral unit as "AA." It will not read the original "CC," ultimately causing structural changes to the DNA, adding thymine during transcription, and distorting the shape of the DNA spiral. This also hinders the repair process, preventing integration; consequently, a mutation may appear. This study aims to induce and evaluate genetic mutations (phenotypic and molecular) of *Aspergillus flavus* using ultraviolet radiation.

MATERIALS AND METHODS

Isolation of *Aspergillus flavus*

According to the standard methodology described below, the potato dextrose agar medium (PDA) was used to isolate *A. flavus* from corn grains. One hundred micrograms per mL of streptomycin sulfate was used as an antibiotic to prevent the growth of undesirable bacteria. The corn grains were sterilized with 1% sodium hypochlorite solution for 2 min, washed with distilled water several times, and dried between two layers of sterile filter paper. Five grains were inoculated in a Petri dish containing PDA medium and incubated for 4 days at 25± 1ºC. The petri dishes were investigated to screen for a single colony of *A. flavus*. The colonies showed different colors and morphological characters; hence, the colony was sub-cultured separately on agar media until a pure culture was obtained by hyphal tip or the signal spore techniques. Each pure fungus was cultured on a PDA slant and used for further identification.

Identification of *Aspergillus flavus*

The slant of purified fungi was examined first by the naked eye at different magnifications of the stereomicroscope. A colony of fungus can be easily distinguished when viewed under high magnification. To identify *A. flavus* a specific Czapek agar media was used. The colony characteristics and the morphological features were recorded. The morphological structure of *A. flavus* depends on the head, stripe, vesicle, medullae, and conidia. The identification was carried out at the Plant Pathology Department, National Research Center, Cairo, Egypt, according to Maren and John (1983) and Mathur and Olga (2003).

Ultraviolet treatment

A. flavus collected from corn grains were tested under UV radiation using a Hanau Fluotest Forte 5261 UV test lamp for treatment at a transition wavelength of 254 nm for 24 h. Under sterilization conditions, a small amount of growth mycelium was collected by a needle and transferred to small glass tubes (10 mL) containing 5 mL of sterile distilled water. The tubes were exposed to the palm source radiation facing UV for 30 min. After exposure, the fungus was transferred to PDA dishes and incubated at a temperature of 25 ± 1 C at a rate of four occurrences of check-fits. The morphological structure for *A. flavus* was detected using a microscope equipped with a camera. Samples were transferred to a glass slide containing lacto phenol and imaged using the supplied Blamer Date virtual form of the light microscope.

Determination of aflatoxins

Maize grains were artificially infected with two isolates of *A. flavus* after being moistened with 18% sterile water. UV is indicating treated isolates and O original, then incubated at 25^{o}C for 15 days for aflatoxin production (Filtenborg et al., 1983).

Preparation of standard solution aflatoxins

Diluted portions of stock solution (0.5 μg/mL) in solvent solution benzene: acetonitrile (98:2, v/v) were used to prepare concentrations of aflatoxin standards B1, B2, G1, and G2. The standards' purity criteria were determined using chromatographic purity and molar absorption. The absorbance close to 350 nm was determined, and concentration was calculated, although it was not necessary if standards were obtained from a recognized source (Hustchins and Hagler 1983).

Extraction of aflatoxins

Fifty grams of corn grains infected with two isolates of *A. flavus* were blended. The powder was transferred into a 500 ml flask, and 25 ml of water, 25 g of diatomaceous earth (DE) and 250 ml of chloroform were added to the mixture. The flask was tightly covered u masking tape and shaken for 30 min to extract the toxins. The contents were then transferred to a Buchner funnel pre-coated with a layer of (DE) about 5 mm thick and filtered using a light vacuum in case of slow filtration. The first 50 mL of filtrate was collected for further analysis.

Determination of aflatoxins quantity by High-Performance Liquid Chromatography (HPLC)

Preparation of column chromatography

A ball of glass wool was loosely placed in the bottom of a 22 x 300 mm chromatographic column, and 5 grams of sodium sulfate anhydrous was added to the silica gel. The glass column was filled with ca. 40-50 ml of chloroform, and 10 g of activated silica gel was added. Then, a 15 g of sodium sulphate anhydrous was added to the surface of the column. Finally, 50 ml of the sample extract was applied to the column. One hundred fifty ml of n-hexane was used for de-fating, followed by 150 mL diethyl ether for de-pigmentation at a 5 mL/min flow rate. One hundred fifty ml of chloroform: methanol (97:3) was used to eluate the aflatoxins from the column at a 5 mL/min flow rate. The elution was concentrated using a rotary evaporator to ca. 1 ml and directly transferred to a vial. Finally, the solvent was evaporated and re-dissolved in a known volume of chloroform (0.2-1.0 ml) and kept in a vial for quantification (Shephard et al., 1990).

Derivatization

The derivatives of positive samples and standards were done as described in (AOAC 2000).

HPLC Conditions

The HPLC instrument (U.S.A) used for aflatoxins determination was a waters (474) system equipped with a quaternary pump fluorescence detector set system at 360 nm excitation and 440 nm emission wavelengths. Data were collected and integrated using Totalchrom Navigator Chromatography Manager Software (AOAC 2000).

Isolation of total DNA

The pellets were grown in Czapek broth medium followed by 5 days at 25°C with shaking at 200 rpm. After incubation, the mycelium mass was harvested by centrifugation at 6000 rpm for 10 min. The pellets were washed twice in 135 ml of buffer solution (145 Mm NaCl; 100 mM Na_2HPO_4; pH 7.5). Total genomic DNA was isolated using Chang et al.'s method (1995).

Amplification of aflR gene DNA

The aflR gene was amplified by polymerase chain reaction (PCR) using a DNA thermal cycler (COT thermo cycler model 1105). The aflR gene was amplified using primer sets (table-1) which were designed for *A. flavus* according to the standard methodology. The amplification was performed on a total volume of 25 µL containing: 10X PCR buffer 1.5 µL, 50 mM $MgCl_2$ 1.0µL; 200 mM dNTP 1.0µL; 20 pM daflR-R primer 1.0µL; 20 pM aflR-f primer 1.0 µL; 2.5 unit Taq DNA polymerase 0.25 µL; 20 mg DNA Template 5.0 µl; and Millipore H_2O. PCR reactions were carried out in a thermocycler following the cycling program, including the initial denaturation cycle at 94°C for 5 min., the 35 cycles, each cycle including the denaturation step at 94ºC for 30 sec., annealing step at 55 for 60 sec, extension step at 72°C for 90 sec and a final extension step at 72ºC for 7 min. The temperature and salt conditions were optimized for low inputs of template DNA. The amplification products were purified on contricon 100 columns (Amican) followed by ethanol precipitation.

Table 1: Primer sets sequences designed for *aflR* gene DNA condition's reaction.

Primer pain	Gene	Primer sequence (5 -3)	Optimal annealing temp	PCR size
Omtl-F	aflP	GCCTTGCAAACACACTTTCA	55^0 C	1490 bp
Omtl-R		AGTTGTTGAACGCCCCAGT		

Agarose gel electrophoresis

The two *A. flavus* isolate PCR products (50 µl) were detected using 1.5% agarose gel electrophoresis in 1 X TAE buffer and stained with ethidium bromide. The amplified DNA bonds were visualized under a UV light-trans-eliminator, and the size of expected DNA fragments was estimated based on a DNA ladder of 100 to 2000 bp (manufactured by Bioran).

DNA sequencing

PCR products of *A. flavus* isolates were purified using the QIA prep. spin miniprep kit protocol (QIAGEN) and purified DNA was sequenced using an automated DNA sequencer.

The results were aligned to the DNA sequences of *A. flavus* isolates using Bio Edit version 7 software (www.Mbio-NCUs.Edu/bio. Edit). The nucleotide sequence of *A. flavus* isolates was compared to accessions of *Aspergillus* sp. available in the NCBI database using BLAST- algorithm to identify

closely related sequences (http/WWW.NCbI.Nih.Gov). Dendrograms were constructed using the unweighted pair Group method with Arithmetic (UPGMA).

RESULTS

Pictorial identification of isolated *A. flavus*

The *A. flavus* from the incubated corn grains was observed using a stereomicroscope and was photographed in Figure 1. The growth of fungi was covering the infected part of the grains. These characters will help the analyst to identify the isolated fungi. Photographs from different magnifications support the descriptions. The growth of *A. flavus* on disinfected corn grains was characterized by immature and white mature heads with other shades ranging from yellowish cream to green. Conidiophores were bearded with clear heads when the growth was white. They were long and hyaline, terminating in the form of bulbous heads. Conidia were globose to subglobose, usually rough, yellowish green in colour.

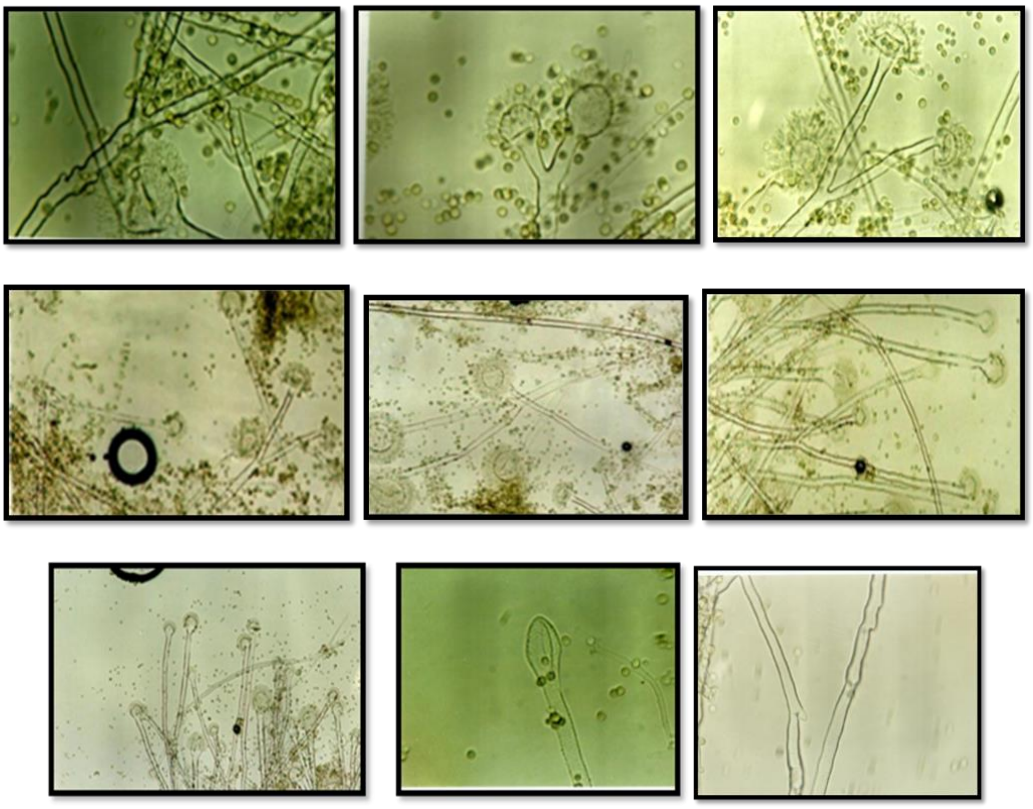

Figure 1: Photographs showing *A. flavus* treated (UV) and original (O) isolates infected corn seeds. Photographs illustrate conidiophores, conidial heads, and conidia detected at X400 magnification.

Mycotoxins production

The two *A. flavus* isolates (UV treated) and (O original) inoculated on corn grains were tested for aflatoxin production by high liquid chromatography technique (HPLC). Aflatoxin was produced by the toxigenic fungi, *A. flavus* (UV) and (O) isolates at 35.210 and 195.75 ug /kg of grains, respectively.

The aflatoxin types AfB1, AfB2, AfG1, and AfG2 produced by toxigenic fungi differed in quality and quantity based on fungal isolates. It was found that *A. flavus* (UV) produced AfB1 at 35.250 ug/kg of seeds. In comparison, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 at 45.74, 10.4, 15.24, and 135.20 ug/kg of grains (Table 2).

Table 2: The quantitative and qualitative analysis of the production of aflatoxins and fumonisins the toxigenic fungi on corn grains.

Aflatoxins		Standard	*A. flavus* (UV)	*A. flavus* (O)
	RT	9.633	9.617	9.678
	Area	66292517	214577	445546
AfB1	Height	2695050	9409	18570
	Amount		35.250	45.75
	Units			
	RT	19.009	-	19.000
	Area	31624309	-	
AfB 2	Height	1020489	-	
	Amount		-	10.40
	Units		-	
	RT	7.401	-	7.420
AfG$_1$	Area	35157208	-	112227
	Height	1833710	-	6167
	Amount		-	15.35
	Units		-	
	RT	13.265	-	13.506
	Area	21254335	-	
AfG2	Height	925554	-	
	Amount		-	124.250
	Units		-	-

Af =Aflatoxin types B1, B2, G1, G2.

Molecular characters of aflR gene

The integrity and quantity of purified total DNA of the *A. flavus* isolates (UV) and (O) were confirmed by agarose electrophoreses and visualised using a UV spectrophotometer at an absorption ratio of A_{260}/A_{280} (1.8 and 1.5), respectively. The concentration of DNA was 80 and 95 ug/5 g mycelium, respectively. These results indicated the high yield and purity of the isolated DNA. The aflatoxin DNA gene (aflR gene) from the two *A. flavus* isolates were amplified using PCR and specific primer sets. The amplified aflR gene size was analyzed using agarose gel electrophoresis and compared to the standard DNA leader. The amplified fragment of the aflatoxin gene PCR product had an expected size of ≈ 1350 bp for two *A. flavus* isolates (Figure 2).

Nucleotide sequence analysis

The PCR products of aflRgene were eluted from agarose gel using the gel DNA extraction kit. Amplicons forming the PCR were allowed for sequencing reactions through the cycle sequencing method. The sequencing was done from the forward direction at Mocrogen 3730X l6-1518-009

Korea. Primers in either the forward or reverse direction were easily identified in each segment of the sequence and were easily used to assemble the individual sequence. The sequences obtained for each primer for each isolate had sufficient overlap between the bands used to form a single continuous sequence (contig). The nucleotide sequences similarity among two *A. flavus* isolates were detected, and a phylogenetic tree revealed an 85% (moderate) degree of similarity among them (Figure 3). The partial nucleotide sequences (1335 and 1345 bp) from the DNA of the two *A. flavus* (UV and O) isolates were determined to identify any relationship with other recommended aflR gene *Aspergillus* strains registered in GenBank (Figure 4).

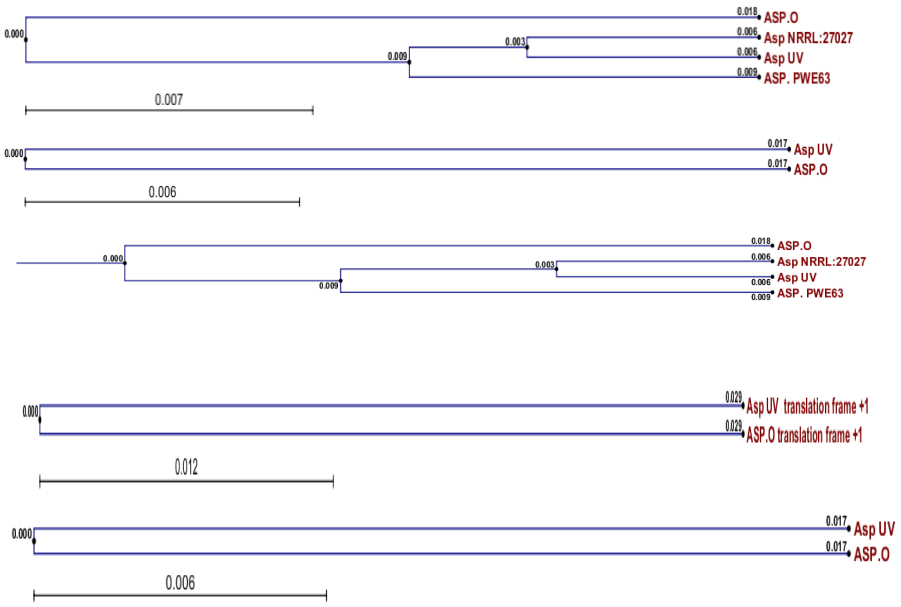

Figure 2 and 3 Phylogenic tree of aflatoxin gene for two *A. flavus* (Asp. UV and Asp. O) infected corn seeds based on the nucleotide sequences

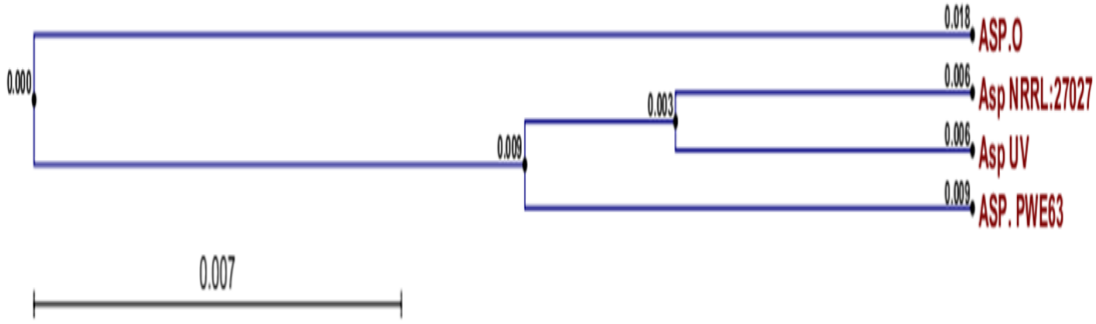

Figure 4: Phylogenetic tree of aflatoxin gene DNA for two *A. flavus* (Asp. UV and Asp. O) with corresponding two *Aspergillus* sp. isolates registered in GenBank.

Translation of aflatoxin gene DNA sequences

The aflatoxin gene of *A. flavus* isolates (UV and O) translation product was predicted from the universal code of aflatoxin gene nucleotide sequences: 445 (UV) and 448 (O) amino acids starting with methionine (M) for both *A. flavus* isolates (UV and O).

The maximum open reading frame (ORF) starts at AA position 1 (maybe DNA position 1 of the aflatoxin gene for two *A. flavus* isolates). The molecular weight differed among the sequences at 47.502 and 47.625 K. Da for aflatoxin gene sequences of UV and O isolates, respectively. The partial aflatoxin gene amino acid sequences for two *A. flavus* isolates were aligned using the DNAMAN program (Wisconsin, Madison, USA). The alignment revealed the similarity among the two isolates. Seven sites differed between *A. flavus* isolates (Fig. 5).

Figure 5: Phylogenic tree of aflatoxin gene translated amino acids of two *A. flavus* (Asp. UV and Asp. O) infected corn seeds based on the nucleotide sequences**.**

The similarity index of the two *A. flavus* isolates (445and 448 amino acids of *aflR* gene DNA) were done to determine the relationship with other recommended *aflR* gene *Aspergillus* strains registered in GenBank (Fig.6). They were presented in a dendrogram and homology matrix. A phylogenetic tree of amino acid sequences for two isolates revealed 92% similarity (high degree).

Figure 6: Multiple sequence alignment of aflatoxin gene translated into amino acids for the two *A. flavus* (Asp. UV and Asp. O) and two *Aspergillus* sp isolates published in GenBank based on the nucleotide sequences.

Nucleotide and amino acids diversity

Nucleotide and amino acid sequence of aflatoxin gene revealed replacement bases among (UV) and (O) *A. flavus* isolates (fig.,7 and 8). The replacements were found at 39 sites, with 2.7% of nucleotide sequence dissimilarity among the two *A. flavus* isolates (UV and O). Amino acid site replacements were 7 with a percent dissimilarity of 1.50 % of amino acid sequences among two isolates (UV and O).

Figure 7: Alignment and replacement sites of nucleotide bases (39 bp) in nucleotide sequences of aflatoxin gene for *A. flavus* isolates.

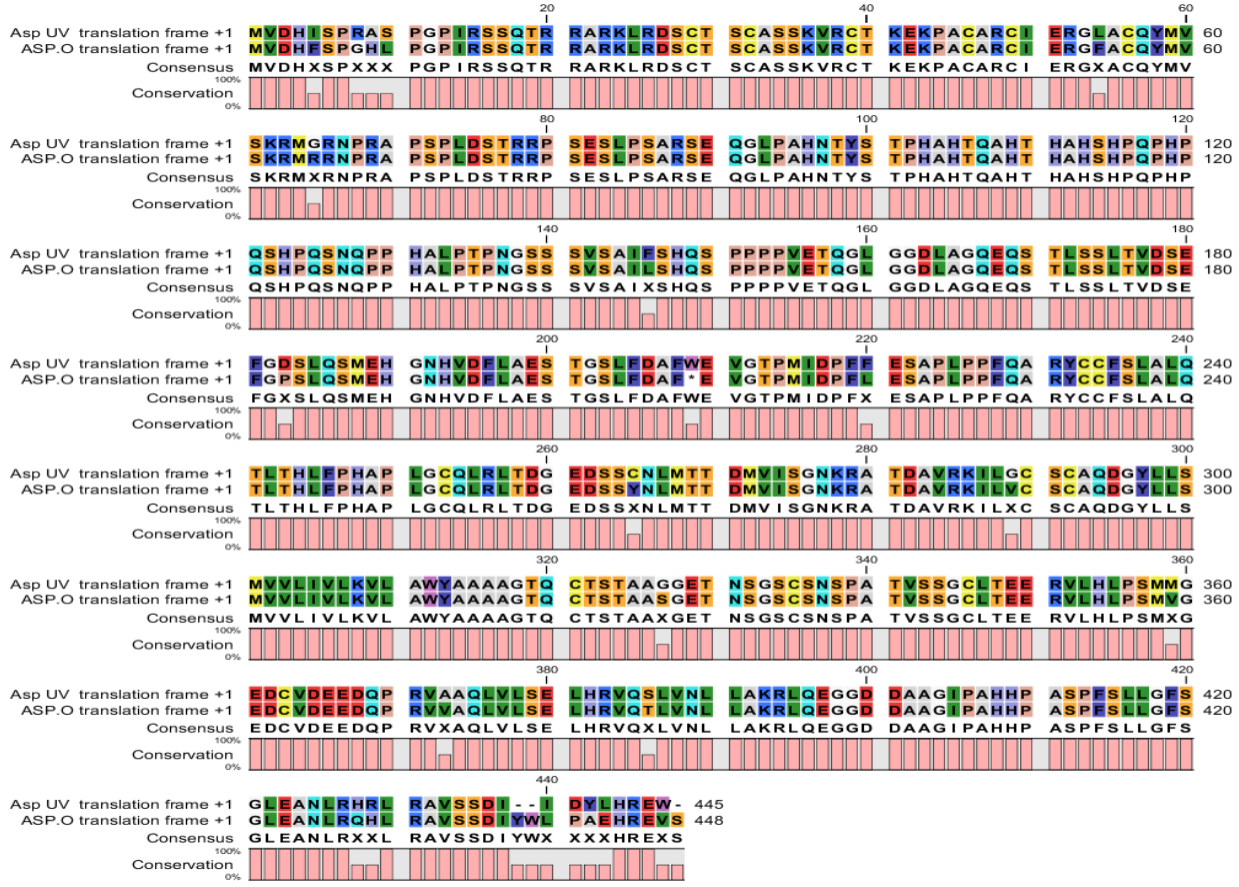

Figure 8: Alignment and replacement sites in protein sequences (7 amino acids) of aflatoxin gene for *A. flavus* isolates.

DISCUSSION

The total number of fungi isolated on PDA media was lower in sterilized maize grains than in non-sterilized maize grains. Similar results were reported by El-Nagerabi et al. (2000), who found that *Aspergillus* sp. was the most common isolated species, followed by *Rhizopus*, *Alternaria*, *Fusarium*, *Emericella*, *Drechslera*, *Cladosporium*, *Pencillium*, and *Pythium*. Many fungal species regularly associated with corn grains can infect developing seeds and still attach to the parent plant. This was demonstrated by isolating fungi from grains collected before maize grains were mature.

The identification process was carried out first by a manual method based on the naked eye at different magnifications using a stereomicroscope. To identify each species of *Aspergillus*, isolates were grown in a specific Czapek agar medium. The morphological structure of the genus *Aspergillus* depends on the head, stipe, vesicle, metulate, and conidia, according to Booth (1972) and Maren and John (1988), respectively. Colonies of *Aspergillus* consist of heads in different colors; in a few cases, some colonies showed the same color, but when viewed under the microscope, the distinction was seen in the type of head.

The two *A. flavus* isolates inoculated on corn grains were tested for their ability to produce aflatoxin by high liquid chromatography technique (HPLC). Aflatoxin concentrations were produced in UV (treated isolate) and O (original isolates) at 35.210 and 195.75 ug/kg of grains, respectively. The aflatoxin types AfB1, AfB2, AfG1, and AfG2 differed in quality and quantity based on the fungal isolate. It was found that, *A. flavus* (UV) produced AfB1 (35.250 ug/ kg seeds). In comparison, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 with 45.74, 10.4, 15.24, and 135.20 ug/kg grains, respectively (Boubakar 2013). Not all strains of *A. flavus* and *A. parasiticus* produce aflatoxin; *A. parasitica* strains generally produced aflatoxin B1, B2, G1 and G2, while *A. flavus* produce B1 and B2 aflatoxin (Diener et al., 1987). In the current study, the total concentration of aflatoxin B1, B2, G1, and G2 was 205.75 ug /kg of seeds. It is well-documented that aflatoxin has a carcinogenic effect. The DNA genomes of A. *flavus* isolates (UV and O) was used as a PCR template to amplify the FIR gene. The IR gene, with an expected size of 1335 bp, could be synthesized using the internal primer sets (FG1 and rp2) to confirm its mutual specificity with *A. flavus*. Genetic variation among isolates was also detected. The substitution of nucleotide and amino acid sequences was 0.89% and 1.57% for UV and O types, respectively. Most of the 25 identified genes clustered within a specific 70-kb region of the fungal genome, they were shown to be involved in aflatoxin synthesis (Bhatnagar et al., 2003; Yabe and Nakajima, 2004). Among them, the aflR gene encodes a key transcriptional regulator of the aflatoxin synthesis gene (Curry et al., 2000). AflR is a regulatory gene for aflatoxin synthesis. It is called aflR-2 in A. flavus, which encodes a regulatory factor (AFLR protein). In the current study, PCR was used to identify the molecular diversity among the *A. flavus* isolates by amplifying and sequencing the aflR gene, producing an aflR gene PCR product of 130 bp. The nucleotide sequence, the number of amino acids, and the amino acid sequence differed among the isolates. The DNAs of *A. flavus* isolates appeared to exhibit low levels of similarity to aflR gene since low amounts of PCR or no PCR products.

Increased expression of aflatoxin biosynthetic genus in the transformant containing an additional copy of aflR might result from an elevated basal level of AFIR, allowing it to overcome nitrate

inhibition of aflatoxin; in addition, aflR gene is involved in the regulation of multiple parts of the aflatoxin biosynthesis pathway (Chang et al., 1995).

On aflR gene mutation in *A. flavus,* all the aflatoxin genes' expression was turned off or significantly reduced (Woloshuk et al., 1994). In addition, Flaherty and Payne (1997) demonstrated that altered and elevated transcription of aflR leads to clavated and altered gene expression pathways and higher levels of aflatoxin production. Sequence analysis of the aflR gene from *A. flavus* revealed evolutionary sequence differences between isolates. These results indicated that some metabolites could be useful in distinguishing species efficiently and accurately. In addition, we measured the isolates sequence similarity by amplifying and sequencing the aflR gene.

Lee et al. (2006) demonstrated that the aflR gene cannot be amplified from some strains of *A. oryzae* and *A. sojae*. In strains of *A. oryzae* from which the aflR gene can be amplified, the aflR gene showed differences from the aflR of toxigenic *A. flavus*. On the other hand, Hua et al. (2009) mentioned that the aflatoxin cluster genes aflR, aflJ, pks, and OmtB were expressed at higher levels in afla toxigenic isolates in comparison to strains that did not produce aflatoxins. In recent years, significant advances have been made in molecular diagnosis technology, especially in developing rapid and sensitive methods for detecting plant pathogenic fungi.

PCR, is widely used as a powerful molecular tool for identifying plant microorganisms. Due to its high sensitivity, this technique is used to identify the DNA of organisms, which are difficult to cultivate or identify biologically. The ability to amplify DNA from crude mycelia preparations is essential in identifying fungi from the plant material. The primary advantage of using PCR is that it requires only small amounts of test material, and the technique is applicable even to partially degraded materials of poor quality. Therefore, we need to develop detection procedures using PCR and detect sequences in the Target DNA region to design specific primers. By using PCR technique, strains, pathotypes, species or higher classes of microorganisms can be identified, provided that specific oligonucleotide primers are available for these strains. This method depends on the alignment of sequences from target and non-target organisms and identifying primers that do not match non-target organisms and have sufficient matching to prepare and amplify all target organisms effectively.

Detecting fungal pathogens from seeds involves culturing organisms from the infected seeds, extracting the DNA from the culture, and subjecting it to PCR. Despite the feasibility of the PCR method, it is yet to be commonly used in seed health testing. More research must be done before the technique can be adapted and routinely used in seed health testing. There are several variables within PCR itself, which must be optimized for each primer-species combination. From the quarantine point of view, it is also essential to know whether the pathogen detected in the seed by PCR method is viable since PCR is a highly sensitive technique; it detects even dead fungi and spores, which are of no pathological importance. Thus, PCR has many potential uses in analyzing seed-borne fungal pathogens. Identifying the type and species of pathogens and detecting fungal pathogens that are difficult to culture or identify is essential. The technique will significantly help monitor seed health in the international seed trade (quarantine), domestic seed certification programs (seed testing laboratories), and research laboratories. There are already many examples of PCR-based assays developed for detection of fungi in plant pathology, but the reports on their

use in specific detection of seed borne fungi are limited (Mahthur and Olga, 2003, Caceres et al.,2020). Finally, it could be recommended that sterilization methods using ultraviolet rays be reviewed to detect the quality of stored seeds, and their impact on human and animal health must be further investigated.

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Conflict of interest statement

We declare that we have no conflict of interest.

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EXPLORING INTESTINAL PARASITES AMONG FALCONS IN RIYADH, SAUDI ARABIA

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Keywords

Falcon, Saudi Arabia, Fecal, intestinal parasites.

Abstract

Intestinal parasites are a major threat to the health of hosts, impacting their productivity. Falcons are considered a high-value innate wealth in various parts of the world, particularly in Saudi Arabia. The current study aimed to explore the prevalence of intestinal parasites in falcons by examining 125 stool samples from different types of falcons using the direct fecal smear method. The examination revealed 87.2% of the samples were infected with intestinal parasites. The overall parasite prevalence was as follows: *Serratospiculum seurati* 47.2%, *Caryospora spp*. 46.4%, *Strigea falconis* 6.4%, *Capillaria columbae* 1.6%, *Cladotaenia globifera* 5.6% *Ascaridia spp*. 0.8%, and *Giardia* 0.8%. Six infected samples were re-examined using the direct fecal stain and centrifugal flotation methods to compare the efficacy of these techniques. The results indicated that the direct fecal smear is one of the most effective methods for detecting parasitic infections. Chi-square tests of independence confirmed that there is no association between infection rate and the falcon's gender ,age, and species and no association between falcon species and its susceptibility to infection with a particular parasite. Falcons are highly sensitive to environmental changes and can be easily affected by parasites, which can cause serious health issues and even death Therefore, ensuring that the environment and food provided to falcons are safe and free from parasites is crucial.

INTRODUCTION

Raptors are essential to ecosystems and are seen as biological markers of environmental contamination (Rossi et al., 2021). Falcons are one of the most common raptors. They belong to the order *Falconiformes* in the family *Falconidae* (Wilcox et al., 2019) and can be found on every continent except Antarctica (Pyzik et al., 2021). These medium-sized birds breed in the Arabian Peninsula, notably in the United Arab Emirates, and in the deserts of North Africa from Eastern Libya and Egypt to Jordan, extending south-eastward along the beaches of the Red Sea and Arabian Gulf to southwestern Pakistan (Javed et al., 2012). Falcons are regarded as a highly valuable natural resource in Saudi Arabia. There are around 9,092 captive falcons in Saudi Arabia, and this number is likely to increase due to the rising interest and investment in the sport of

falconry. Falconry in Saudi Arabia holds profound cultural and historical significance, reflecting the deep-rooted traditions and values associated with the practice (Binothman, 2016). However, this sport may increase the risk of parasite transmission to humans and poultry (Bertran et al., 2012). Research on raptor pathogens offers valuable information for monitoring ecosystem health, assessing the population's health, and determining the potential role these birds may have in dispersing serious pathogens like bacteria and parasites (Rossi et al., 2021). Endoparasites are common in captive and wild raptors and can seriously threaten the birds' health (Alfaleh et al., 2020). Some protozoa and helminth species can affect a raptor's ability to fly and hunt, making them more vulnerable to secondary injuries. Additionally, bacterial infections often exacerbate lesions caused by endoparasites (Rossi et al., 2021). The most common protozoan intestinal parasites that infect falcons are *Caryospora* (Santana-Sánchez et al., 2015) and *Trichomonas gallinae* (Alrefaei et al., 2022). The most common helminth parasites are *Capillaria, Serratospiculum,* and *Strigea* (Santoro et al., 2010). Falcons are both ecologically and socially significant, but many aspects of their biology remain unknown, including the variety of parasites that infect them in the wild and captivity (Alrefaei et al., 2022). This study aims to detect intestinal parasites in falcons in the Riyadh region and identify the most effective detection methods for birds. Data has been collected and analyzed to estimate the prevalence of parasites and to observe any association between the prevalence of parasites and different groups (age, sex, and falcon species). Additionally, it recommends precautions for falconers to limit the spread of these parasites.

MATERIALS AND METHODS

Study area

This study was conducted in the Bandar Al-Daraa Falcon Care Center (24°51'54.3"N 46°50'50.5" E), Riyadh City, Kingdom of Saudi Arabia. It is the first falcon care facility in the Kingdom of Saudi Arabia. It is a specialist facility that offers falcons expert veterinary care. Its main objective is to provide exceptional veterinary care to ensure these magnificent birds' optimum health and well-being.

Sample collection

From Dec 2022 to Feb 2023, 125 fecal samples were collected from sick falcons at the Falcon Clinic Laboratory. Samples were fresh and stored. Stored samples were kept in dry, hygienic, cool, and airtight containers. Samples were from 16 male falcons and 109 female falcons. The age of falcons was among 105 adults and 20 juveniles from *F. cherrug* (*n*=69), *F. rasticolus* (*n*=28), *F. peregrinus* (*n=15*), *F. cherrug* × *F. rasticolus hybrid* (*n*=5), *and F. rasticolus*× *F. peregrinus hybrid* (*n=8*).

Processing of samples

The veterinarian determined the falcon's species. One hundred twenty-five samples were tested using the direct fecal smear technique. Six samples were re-examined using direct fecal stain, centrifugal floatation, and direct fecal smear methods.

Direct fecal smear

The samples were combined with 0.9% normal saline solution (NaCl), covered by a coverslip, and examined at×10 magnification under a light microscope to identify the parasite genus (Broussard, 2003).

Centrifugal floatation method

Six samples (4 fresh and 2 stored) were subjected to the centrifugal floatation method by adding 3-5 grams of feces mixed with 3 ml of normal saline (0.9% NaCl) in a 5-ml-tube, then placed in the centrifuge at 2500 rpm for 5 minutes. After taking it out and filling it with normal saline, the coverslip was placed on the top of the tube for 10 minutes and examined under the light microscope at ×10-×40 magnification (Maria Pyziel-Serafin et al., 2022).

Fecal stain method

The 6 samples were prepared the same way as in the direct fecal smear. After drying the samples in the incubator for 3-4 minutes, it was fixed with 70% ethanol for 30 seconds and stained with methylene blue for 1 and a half minutes. Finally, the samples were washed, and a drop of oil was placed on the slide to be examined at ×100 magnification under the light microscope. Another method used to stain the fecal smear samples was the Ziehl-Neelsen stain. The fixation step was conducted by adding one drop of methanol for 3 min. After staining with carbol fuchsin for 10 min, the samples were washed. In the decolorization step, one drop of ethanol was added for 3 min and then washed. In the last step, the slide was covered with methylene blue for 1.5 min, then washed and left to dry (Alqarni et al., 2022).

Statistical tests

We used the Chi-square test to determine if there is an association between the prevalence of intestinal parasites and different factors (age, sex, and species). Additionally, we employed the Chi-square test to see whether there is a link between the falcon species and the frequency of particular parasite species. We used Excel to run the Chi-square test with a significance threshold of 0.05 to determine if the observed differences were statistically significant. Illustrated figures were generated using Excel.

RESULTS

In our research, 125 fecal samples from different types of falcons were examined by direct fecal smear. The parasitic infection was detected in different aged falcons of both sexes, as shown in Table 1.

Table 1: The prevalence of parasitic infection in both sexes at different ages.

		No. tested	No. positive	Prevalence%
Age	Juvenile ≤1 year	20	18	90
	Adult > 1 year	105	91	86.6
Sex	Female	109	93	85.3
	Male	16	16	100
Total infection	-	125	109	87.2

We conducted chi-square tests to determine whether there are significant associations between age groups (juvenile vs. adult) and infection status, as well as between sex (female vs. male) and infection status. The chi-square test for the association between age and infection status was insignificant, χ^2 (1, N = 125) = 0.167, p = 0.683.

The chi-square test for the association between sex and infection status was also not significant, χ^2 (1, N = 125) = 2.693, p = 0.101. These findings suggest no significant association between either age or sex and infection status in this sample. Thus, the likelihood of infection is independent of age and sex. A total of 109 (87.2%) samples were infected: *Serratospiculum seurati* (47.2%), *Caryospora spp* (46.4%) (Figure 1a), *Strigea falconis* (Figure 1b,c), *Cladotaenia globifera, Capillaria columbae* (Figure 1d), *Ascaridia porrocecum* (Figure 1e), and *Giardia spp*. As shown in Table 2 (Figure 2), *Ascaridia porrocecum* and *Giardia spp* were the least prevalent parasitic infections (0.8%). Samples were acquired from *F. cherrug*, *F. rusticolus*, *F. peregrinus*, *F. cherrug* × *F. rasticolus* hybrid, *F. rasticolus*×, *and F. peregrinus* hybrid, and were infected with the mentioned parasites. Compared to other falcon species, *Caryospora spp* was the most prevalent intestinal parasite observed in *F. cherrug* (62.5%), and a single instance of *Ascaridia* was detected in a sample of a falcon from the same species.

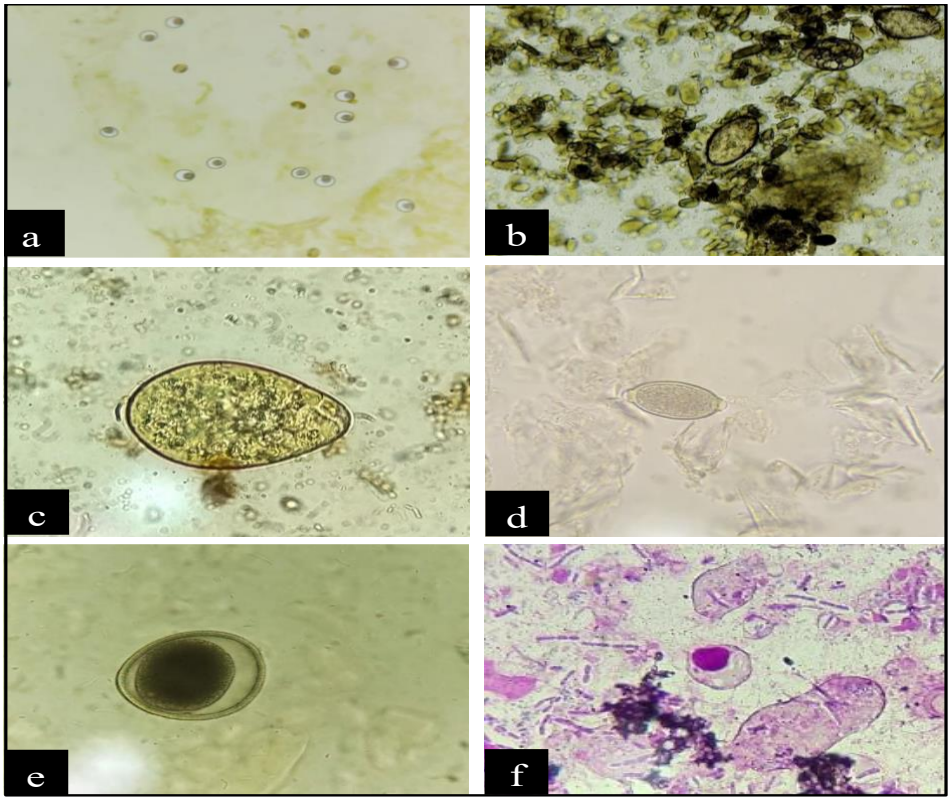

Figure 1: (a) *Caryospora* spp. was isolated from a female Gyrfalcon fecal sample by direct fecal smear (x100). (b) Sporulated ovum of *Strigea falconis* from a female saker detected by direct fecal smear (X400). (c) An unsporulated egg of *Strigea falconis* from a female saker falcon by direct fecal smear(x400). (d) An egg of *Capillaria columbae* from a male peregrine falcon (x400). (e) An egg of nematode *Ascaridia* spp. from a juvenile female saker falcon (x 400). (f) An egg of *Caryospora spp*. from a fresh female gyrfalcon sample using the Ziehl–Neelsen staining method (x1000).

Table 2: The prevalence of parasitic infection in falcons.

	No. infected	The prevalence (%)
Serratospiculum seurati	59	47.2
Caryospora spp	58	46.4
Strigea falconis	8	6.4
Capillaria columbae	3	2.4
Cladotaenia globifera	7	5.6
Ascaridia porrocecum	1	0.8
Giardia spp	1	0.8

The approximate prevalence of *S. seurati* and *Caryospora spp.* in *F. rusticolus* was close to that in *F. cherrug*. In contrast, the prevalence of *Cladotaenia globifera* was nearly similar to that of *F. peregrinus*. The greatest percentage of *S. seurati* (64.2%), *Strigea falconis* (14.2%), *C. columbae* (14.2%), and *Cladotaenia globifera* (14.2%) were found in *F. peregrinus*. *Caryospora* and *Serratospiculum* were the only parasites identified in the two-hybrid falcons, with no detection of other parasites except *Giardia spp* in one sample from *F. rasticolus*× and *F. peregrinus hybrid.* The rest of the prevalence of the parasite in each falcon species is present in Table 3.

Table 3: The prevalence of various parasites in different falcon species

Species	No. tested	No. positive / %	*Serratospiculum seurati* / %	*Caryospora Spp* / %	*Strigea falconis* / %	*Capillaria columbae* /%	*Cladotaenia globifera* /%	*Ascaridia porrocecum* /%	*Giardia spp* /%
F. cherrug	69	56 / 81.1	31/55.3	35/ 62.5	3/ 5.3	1/ 1.7	2/ 3.5	1/ 1.7	-
F. rusticolus	28	22 / 78.5	11/50	13/ 59	2/ 9	-	3/ 13.6	-	-
F. peregrinus	15	14 / 93.3	9/64.2	6/ 42.8	2/ 14.2	2/ 14.2	2/ 14.2	-	-
F. rasticolus× F. peregrinus hybrid	8	8 / 100	4/50	3/ 37.5	-	-	-	-	1/ 12.5
F. cherrug × F. rasticolus hybrid	5	5 / 100	2/40	3/ 60	-	-	-	-	-

The chi-square test for the association between bird species and infection rate was not significant, χ^2 (4, N = 125) = 4.477, p = 0.345. This result indicates no significant association between the species of the birds and their infection rate in this sample. Also, The chi-square test examining the relationship between falcon species and the presence of particular parasites was not significant, χ^2(24, N = 137) = 32.894, p = 0.106. Therefore, there is no significant association between the bird species and the presence of the parasites listed in the table. Besides direct fecal smear, 6/125 (4 fresh and 2 stored) were examined using two other techniques: centrifugal floatation and direct fecal staining. In fresh samples, 3/4 show no detection of parasitic infection in the centrifugal floatation ,while 2/4 of the stained samples gave negative results, the other half of the stained samples were positive (Figure 1f) contrary to the direct fecal smear where the infection was detected in all samples as shown in Table 4. All stored samples in direct fecal smear (Figure 2a, b) and staining methods (Figure 2c, d) showed positive results, unlike the centrifugal method

Table 4: Detection of parasitic infection in the six samples using various techniques.

Sample No.	Sample type	Direct smear method	Centrifugal floatation	Staining method
1	Fresh	*Caryospora* spp.	-	-
2	Fresh	*Strigea falconis*	-	+
3	Fresh	*Caryospora* spp.	+	+
4	Fresh	*Caryospora* spp.	-	-
5	Stored	*Caryospora* spp.	-	+
6	Stored	*Strigea falconis* & *Serratospiculum seurati*	-	+

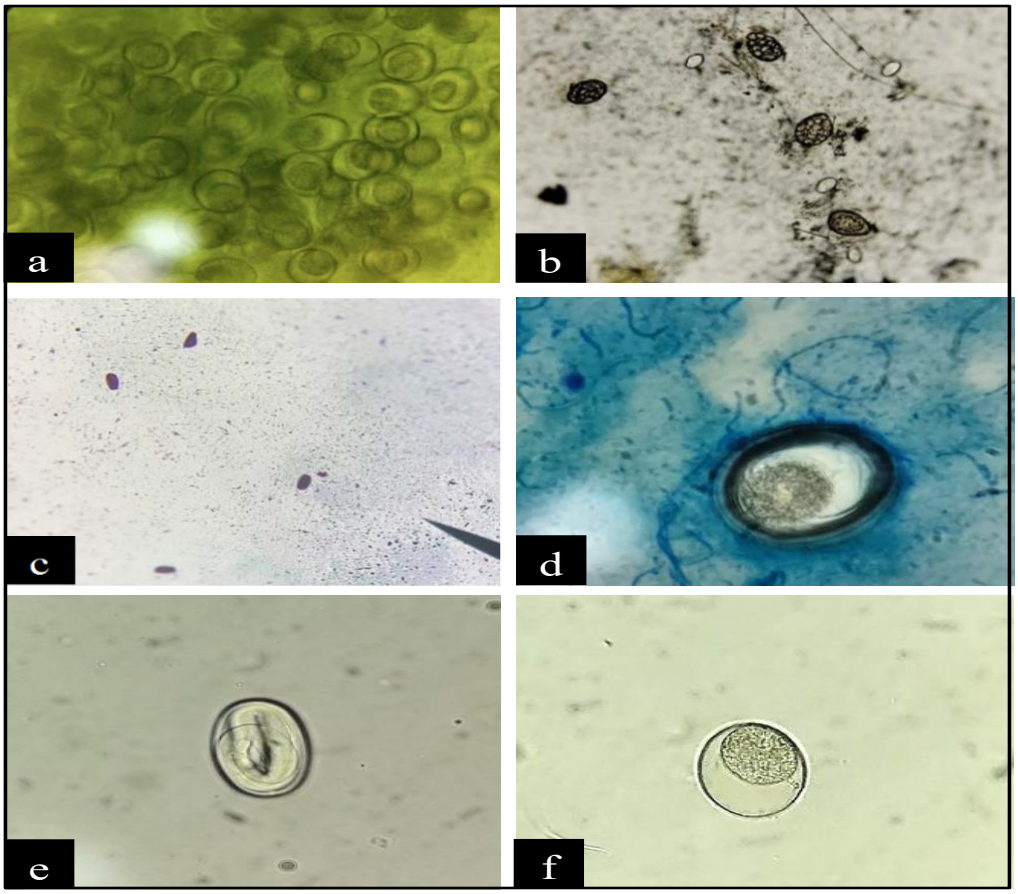

Figure 2: (a) Intensive infestation of *Caryospora spp.* In a stored male saker falcon sample (x400).(b) Co-infection with *S. seurati* and sporulated and unsporulated S. *falconis* investigated in a stored female saker falcon sample examined by direct fecal smear (X100). (c) *S. seurati* isolated from a stored female saker falcon sample using *the Ziehl–Neelsen staining method(x100).* (d) Unsporulated oocyst of *Caryospora spp.* from a stored male saker falcon sample using a direct fecal stain technique (× 1000). (e) Embryonated egg of *S. seurati* from a stored female saker falcon sample (x400). (f) *Caryospora spp.* oocyst from a stored male captive saker falcon obtained from the middle of the centrifugal floatation tube (x 400).

DISCUSSION

Despite the appearance of a convergence in the prevalence of infection between adults and juvenile brids, as the Chi-square test revealed, the infection rate is higher as the bird's age increases, as reported by Juárez et al. (2020). The most common parasite was *Serratospiculum seurati* (47.2%). Ibarra et al. (2019) and Tarello (2006) also determined that it was the most prevalent parasite in falcons of the Middle East. We found *Caryospora spp* (46.4%) to be a common parasite in falcons, as was previously identified by Alfaleh et al. (2020) and Santana et al. (2015). Similarly to other published results, we found no zoonotic parasites except Giardia. *Giardia duodenalis* (also known as *Giardia lamblia* or *Giardia intestinalis*) can infect a wide range of hosts, including humans, and cause the gastrointestinal disease called giardiasis (Maestrini et al., 2022) (Shu et al., 2022). Furthermore, our results indicate a variation in the effectiveness of the three identification methods (direct fecal smear, staining, and centrifugal floatation method) used to detect the presence of parasites in the samples, where it was concluded that the direct fecal smear method is the most efficient in detecting parasites compared to other methods.

In the staining method, 2/4 of the fresh samples gave negative results due to the breakage of the parasite oocyst at one of the staining steps. In the centrifugal floatation method, *Strigea falconis* did not appear in any of the two samples (1 fresh & 1 stored) due to its high specific gravity density, which causes it not to float, so it is preferable to detect it by sedimentation method as discussed by Inês et al. (2016). We tried a simple flotation method in one of *Strigae's* samples (fresh) without waiting for 10 minutes and received a positive result. In the second sample (stored), we found *Strigea falconis* with *S. seurati* in the direct fecal smear, but *Strigea* did not show up for the reasons discussed earlier. At the same time, *S. seurati* appeared after taking part of the floatation solution from the middle of the tube to ensure that the *S. seurati* eggs were not broken due to the solution used (Fig.3e). Hence, the negative result in Table 4 can be explained. *Caryospora* is one of the protozoans easily detected in the centrifugal floatation method due to its low density compared to the floatation solution. As claimed by Inês et al. (2016), it appeared insignificantly in the examined samples (2 fresh & 1 stored), which might be due to several reasons, one of which is the low amount of parasites in the fresh sample. The second explanation could be due to the increase in the specific gravity of *Caryospora* occurring during its storage. To ensure that the *Caryospora* was not broken, we took part of the floatation solution from the bottom of the tube as we did with *the S. seurati* sample, and the parasite was found in a healthy condition (Fig.3f).

CONCLUSION

Wildlife plays a significant role in Saudi Arabia's Vision 2030, aligning with the goals of sustainable development and the objective of achieving a diverse and sustainable economy. Our study has revealed that falcons, an important species in the Kingdom, have an alarming prevalence of intestinal parasites, with an overwhelming 87.2% of the sampled birds being affected. The most common parasites identified in the study were *Serratospiculum seurati* and *Caryospora* spp., which can be attributed to their life cycles, environmental exposure, and specific host interactions. The study recommends using the direct fecal smear method to detect bird parasitic infections due to its effectiveness, simplicity, cost-efficiency, and time-saving benefits. Maintaining and improving the hygiene of areas where falcons reside is crucial, as some parasite species' oocysts thrive in unsanitary environments. Ensuring the safety and nutritional quality of the falcons' food is essential, including incorporating anticoccidial agents to prevent specific parasitic infections. The

establishment of multiple care facilities is necessary to ensure the health of raptors and protect them from infections and the risk of extinction. Given the popularity of falconry in the Middle East, conducting routine examinations frequently is vital to ensure the continued health and parasite-free status of these birds.

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Princess Nourah Bint Abdulrahman University
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PREVALENCE OF DIFFERENT GASTROINTESTINAL PARASITES IN HORSES IN RIYADH, SAUDI ARABIA

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Keywords

Gastrointestinal parasites, Prevalence study, Horse Health, Helminths, Fecal examination.

Abstract

Gastrointestinal parasites are a significant health concern for horses, affecting their overall health and performance. Detecting intestinal parasites in horses is crucial for maintaining their health and preventing disease outbreaks, which can lead to significant economic and performance-related losses. This research provides essential data that can inform better management practices and parasite control strategies, ultimately enhancing horse health and productivity. This study was conducted to estimate the prevalence of different gastrointestinal parasites in horses in Riyadh, Saudi Arabia, and to investigate the relationship between infection rates and the horses' age, sex, and species. A total of 113 fecal samples from horses were gathered and examined using NaCl flotation and direct fecal smear techniques to detect gastrointestinal parasites. The results showed that among the 113 samples examined, 44 (38.93%) were found positive for various gastrointestinal parasites. The detected parasites included *Entrobius* spp. (10.6%), *Eimeria* spp. (6.19%), *Anoplocephala* spp. (2.65%), *Parascaris equorum* (1.76%), *Ascaris* spp. (1.76%), and *Gastrodiscus* spp. (0.88%). Additionally, 29.2% of the infections were attributed to unidentified oocysts, larvae, or eggs. These findings suggest that gastrointestinal parasites are common in horses in Riyadh, Saudi Arabia, and underscore the need for targeted parasite management and control strategies to improve equine health and welfare in the region.

INTRODUCTION

Equine companionship with humans' spans epochs, and horses have taken on important responsibilities beyond ordinary utility. Horses are valued for their strength, agility, and historical significance, which are intertwined with the fabric of human society. According to the Food and Agriculture Organisation of the United Nations (FAO), there are an estimated over 60 million horses worldwide. Despite their vast numbers, it is crucial to recognize that not all horses roam freely; a substantial portion of them, approximately 70%, are tamed and domesticated by humans (FAO, 2022). This close interaction between humans and horses highlights the long-standing

relationship developed over millennia. From serving as indispensable companions in agriculture, transportation, and sport to becoming loyal partners in therapy and leisure, horses have seamlessly integrated into human civilization. As we explore the intricate roles and connections between horses and humanity, it becomes evident that their impact extends far beyond their physical presence, reaching into the realms of culture, art, and emotional well-being (Birke & Hockenhull, 2015). In Saudi Arabia, the horse population is estimated to be more than 33,000, and more than 500 horses are imported annually from different countries such as the United Arab Emirates, the United States, and Europe to be used for various purposes, including husbandry activities, transportation, racing, showing, and breeding (Anazi et al., 2011).

Like any living being, horses are susceptible to various diseases caused by viruses, bacteria, and parasitic microorganisms that can impact their health and well-being. Equine diseases range from common infections to more severe conditions such as colic and laminitis. A comprehensive understanding of these illnesses is crucial for effective prevention and management in equine healthcare (Kolk & Kroeze, 2022). By staying informed about prevalent diseases and their symptoms, horse owners and caretakers can enhance these remarkable animals' overall health and longevity. Colic is a common horse illness that causes gastrointestinal pain and a high mortality rate. In 2015, it caused 31% of deaths in horses aged one to twenty years, making it the most common cause of equine death in the United States (Maskato et al., 2020). The gastrointestinal tract nematodes are the most frequently reported parasitic infections in horses, often causing colic. Animals with infections lose their appetite, leading to severe underweight. Parasitic infections, which seriously impair animal health, remain a significant concern (Panova et al., 2023). Protozoa, trematodes, cestodes, and nematodes can be found in a horse's digestive system (Denizhan & Karakuş, 2023).

Globally, gastrointestinal parasite infections are acknowledged as one of the biggest risks to horses' health, resulting in significant economic losses due to their deteriorating condition and reduced ability to grow physically and compete effectively in sports. (Carminatti et al., 2023). *Strongyle*, *Cyathostomes*, *Triodontophorus* species, *Strongyloides westeri*, *Parascaris equorum*, *Dictyocaulus arnfieldi*, *Oxyuris equi*, *Gastrodiscus*, and *Fasciola* species are the most often recognized gastrointestinal helminths of equines in various regions (Mathewos et al., 2021). Equine bloodworms, or *Strongylus vulgaris*, are known as "the horse killer" and are the most dangerous gastrointestinal parasite (Nielsen et al., 2021). Peritonitis is the most common symptom of the clinical sickness produced by this parasite, and studies have demonstrated that this condition is linked to *S. vulgaris* positivity, confirming that this parasite can kill animals (Pihl et al., 2018). *S. vulgaris* is the most well-known due to its ability to induce significant health issues, including potentially fatal colic, because of its intricate life cycle, which involves migrating through the arteries and producing thrombosis and damage that can result in necrotic lesions in the intestinal wall (Halvarsson, 2024).

Cyathostomins infect nearly all horses, and infections with 10 or more species per horse are common. Despite possible species bias in disease development and anthelmintic resistance, species-specific knowledge is scarce (Bellaw et al., 2020). Cyathostomins form in the lumen and wall of the large intestine, and horses seldom acquire substantial protective immunity. As a result, substantial Cyathostomin loads are possible in animals of all ages.

Cyathostomins cause various clinical symptoms, including impaired performance, slower development rates, weight loss, coarse hair coat, asthenia, diarrhea, and various forms of colic (Pergrine et al., 2014).

In this study, we assess the prevalence of various gastrointestinal parasites (GIPs) in horses within the Riyadh region of Saudi Arabia. Our approach combines the NaCl flotation and direct fecal smear techniques, providing a comprehensive assessment of parasitic infections. This dual-method approach is noteworthy because it increases the detection sensitivity for a broad spectrum of parasites, addressing limitations in existing diagnostic methods that often rely on a single technique. Using these complementary techniques, our method improves the accuracy and reliability of parasite detection and provides a clearer understanding of the parasite load and diversity within the equine population. This research contributes to better-informed strategies for parasite management and control, ultimately enhancing horse health and performance in the region. This study is particularly significant due to the cultural importance of horses in Saudi Arabia. Despite the prominence of equines in the region, there is a notable lack of contemporary research on gastrointestinal parasites affecting these animals. By addressing this gap, our study aims to provide critical insights and practical solutions to support the health and well-being of horses, thereby preserving their valued role in Saudi society.

MATERIALS AND METHODS

Study area

The study area includes eleven stables in the Kingdom of Saudi Arabia, Riyadh region, and its provinces. Samples were taken between March and April 2023. Seven Stables are in eastern Riyadh, one is in western Riyadh, two are in Al-Muzahimiyah, and one is in Al-Diriyah, Riyadh.

Animal samples

With the owners' permission, 113 fecal samples were collected from randomly selected horses of varying sexes (65 males and 48 females), ages (below 1 year, 1 to 5 years, 5 to 10 years, and above 10 years), and breeds (80 Arabian, 28 English, 2 French, 1 Spanish, 1 British, and 1 Hybrid).

Samples collection

The study was conducted using a simple random sampling method to examine the prevalence of infected horses. Fecal samples were collected from each selected animal from freshly dropped feces using a disposable plastic specimen container, and each sample was labeled with a description of the animal, which includes species, age, sex, and body condition. Management was recorded on a paper equivalent to the label to exclude repetition of sample collection of the same animal. Then samples were transported to Princess Nourah Bint Abdulrahman University- College of Science's laboratory for analysis. All samples were either processed immediately or kept in the refrigerator at 4°C and processed within 48 hours.

Examination methods

In the laboratory, fecal samples were processed and examined microscopically as per standard procedure by fecal floatation and direct smear for any presence of parasites (Englar & Dial, 2022). Parasite identification was made based on their morphology (Bawmann, 2009).

RESULTS

Coprological examination of 113 horses' fecal samples revealed that 44 were positive for some gastrointestinal parasites, indicating that the overall prevalence was 38.94%. (Table 2) Shows that horses were infected with *Entrobius* spp. (10.6%); *Eimeria* spp. (6.19%); *Anoplocephala* spp. (2.65%); *Parascaris equorum* (1.76%); *Ascaris* spp. (1.76%); and very low prevalence (0.88%) for *Gastrodiscus* sp. and *Trichuris* sp.

Table 1: Detection of (GIPs) and associated with risk factors among horses in Riyadh.

	No. of examined Horses	No. of Positives (%)
Species		
Arabian	80	27 (23.89)
English	28	14 (12.38)
French	2	1 (0.88)
British	1	1 (0.88)
Spanish	1	1 (0.88)
Mixed	1	None
Sex		
Male	65	23 (20.35)
Female	48	21 (18.58)
Age		
>1 year	7	5 (4.42)
1 - 5	48	15 (13.27)
5 - 10	44	19 (16.8)
< 10 years	14	5 (4.42)
Housing		
Organized	91	30 (26.54)
Unorganized	22	14 (21.38)
Body conditioning		
Good	93	39 (34.5)
Moderate	9	1 (0.88)
Poor	11	4 (3.53)
Region		
Eastern Riyadh	57	29 (25.66)
Western Riyadh	12	6 (5.3)
Al-Muzahmimiyah	38	8 (7)
Al-Diriyah	6	1 (0.88)

Table 2: Comparison of parasitological techniques for the diagnosis of (GIPs) in horses feces

Parasites	Infection No.	%
Parascaris equorum.	2	**1.76**
Eimeria spp.	7	**6.19**
Entrobius spp.	12	**10.6**
Ascaris spp.	2	**1.76**
Anoplocephala spp.	3	**2.65**
Gastrodiscus sp.	1	**0.88**
Mixed infection	33	**29.2**
Total	44	**38.93**

Table 3: Prevalence types of (GIPs) in horses.

Detection technique	Samples No.	%
Flotation	6	**5.3**
Direct fecal smear	40	**35.39**

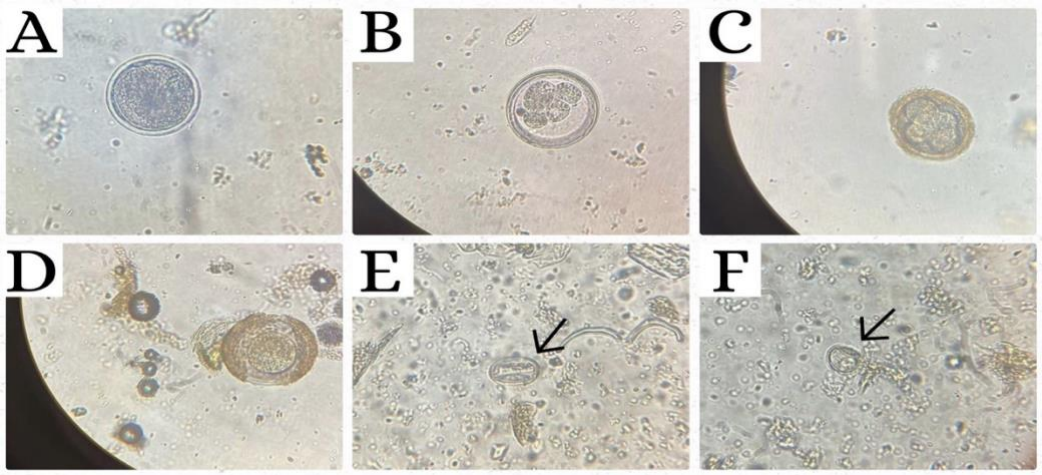

Figure 1: Microscopic view X40 of different parasites, A) Unsporulated oocyst of *Eimeria* spp. B) Sporulated oocyst of *Eimeria* spp. C) *Ascaris* spp. D) *Parascaris equorum*. E) *Entrobius* spp. F) *Anoplocephala* spp

DISCUSSION

The results of the current study indicate that parasitic infections are prevalent among horses in the Riyadh region of Saudi Arabia. However, compared with a previous report, the prevalence in horses observed in the current study (38.93%) is far lower than that reported earlier (86.6%) by Anazi et al. (2011) despite being the same study area, which can be due to the increased awareness in horse health in recent years. Authors also relied on conventional parasitologic examinations and serologic assays. DNA based technologies, including PCR, have been widely used in the diagnosis of infections in horses, camels, and cattle, given their sensitivity and specificity in detecting all stages of parasitic infection, which we did not use in our study and only depended on standard coprological tests-direct and floatation methods due to its accessibility and availability for students.

In line with our study, Al Qudari et al. (2015) reported a 30.46% prevalence of parasitic infections in horses from the Eastern Province of Saudi Arabia. Comparing these results to other regions in the world, studies by Scala et al. (2020), Papazahariadou et al. (2009), Valibasha et al. (2019), and Nagar et al. (2022) showed an overall prevalence of 40%, 34.5%, 31.80%, 30.85%, in Italy, Central and Northern Greece, India, respectively which is almost in agreement with the present findings. Moreover, Wannas et al. (2012), Elmajdoub et al. (2022), Khanum et al. (2021), Roba & Hiko (2022), Pandit et al. (2008), Oli et al. (2018), and Adeppa et al. (2016) reported parasitic infection prevalence to be 100%, 98.0%, 97.92%, 96.6%, 93.26%, 84.76%, 84.0 % in Iraq, Libya, Bangladesh, central

Ethiopia, Kashmir valley of India, Nepal, Shimoga region of India respectively, which identifies higher rates of infestation as compared with our study.

The detection of intestinal parasites among horses' based on their sex was 23/113 (20.35%) for males, while the prevalence of females was 21/113 (18.58%). Males had a higher incidence than females, which agrees with Umar et al. (2013), who reported a prevalence of 80.9% for males and 38.5% for female horses in Nigeria. However, Oli & Subedi (2018), Khanum et al. (2021), Romero et al. (2020), Kompi et al. (2021) and Ogbein et al. (2022) reports did not agree with our findings where there was a high incidence in females when compared to males with a prevalence of 92.30% and 82.27%, 100% and 95.45%, 25.23% and 22.02%, 92.2% and 85.3%, 79.5% and 68% in Nepal, Bangladesh, Central Mexico, Lesotho, Nigeria respectively.

In this study, a relatively higher prevalence of gastrointestinal parasites (16.8%) was recorded in adult horses aged between 5-10 years, which is in stark contrast with the previous findings of Elmajdoub et al. (2022), who reported a higher prevalence in young horses between 1-4 years. Moreover, Beley et al. (2016) study reported that horses above 10 years are more vulnerable to (GIPs). The parasites detected in horses' feces belonged to *Parascaris equorum*, *Eimeria* spp., *Ascaris* spp., *Anoplocephala* spp., *and Gastrodiscus* sp., which is in agreement with reports by Umar *et al*. (2013), Ola et al. (2019), Sinaga et al. (2022), Roba & Hiko. (2022), Oli et al. (2018). *Gastrodiscus* spp., infection rates were low (0.88%) and akin to the low rate reported by Mathewos *et al*. (2021) (5.6%), which was obtained around Hawassa in Ethiopia. Adeppa et al. (2016) also reported that *Gastrodiscus* spp. in Karnataka were similar (4.76%).
Furthermore, the infection prevalence *was relatively low for Parascaris equorum* (1.7%) and *Anoplocephala* spp. (2.65%) and similar to previous studies by Belay et al. (2016), Mirian et al. (2019), and Belete et al. (2015) for *P. equorum*, 3.1%, 4.42%, and 1.8%, respectively, and 3.1%, 6%, and 2% respectively for Anoplocephala spp. We encountered negligible rates of infections with *Strogylus* spp., which aligns with reports by Elmajdoub et al. (2022). However, several other studies by Fikru et al. (2005), Ioniță et al. (2013), Liu et al. (2022), Pandit et al. (2008), and Umar et al. (2013) have found it to be a highly prevalent infection in horses (92.8%, 87.97%, 82.26%, 81.19%, and 68.8% respectively). The lower prevalence in the present study could be explained by the fact that all the tested horses were less exposed to contaminated food/water and underwent regular deworming treatment. Another reason might be because *Strongylus* spp. cannot tolerate and survive hot and dry environments (Cargi wood, 2020).

CONCLUSION

The current study revealed the presence of more than six genera of gastrointestinal parasites (GIPs) in horses in Riyadh, observing a prevalence of 38.93% of infected horses. These findings emphasize the vulnerability of horses to a spectrum of significant health challenges that may include gastrointestinal disorders, nutritional deficiencies, compromised immune function, decreased performance, and economic burdens associated with treatment costs. Furthermore, it is essential to explore the complex interplay of factors, including species, age, sex, housing conditions, and geographical location, which may influence susceptibility to gastrointestinal parasite infections in

equine populations. By addressing these challenges and advancing our understanding of parasite epidemiology, we can develop more robust strategies for effective parasite control and safeguard equine health and welfare. However, it is important to acknowledge the limitations inherent in our study. The study's scope was constrained by the limited availability of diagnostic tests, with only fecal direct smear and flotation tests accessible within the research environment. Additionally, time constraints restricted using more advanced diagnostic techniques, such as polymerase chain recognition (PCR). Consequently, a portion of parasitic eggs remained unidentified, potentially impacting the comprehensiveness of the findings.

RECOMMENDATIONS

It is imperative to combine more advanced techniques, such as Polymerase Chain Recognition (PCR), Next-Generation Sequencing (NGS), and Fecal Egg Count Reduction Test (FECRT) That offer higher sensitivity and precision alongside conventional methods like direct smear and flotation tests, enhance equine parasite diagnostics. Expanding sample sizes and diversity will enhance the reliability and applicability of findings, necessitating the inclusion of horses from various geographical regions and management systems. Longitudinal studies are vital for monitoring parasite dynamics and informing targeted intervention strategies. Collaborative efforts among horse owners, researchers, veterinarians, and industry stakeholders are crucial for developing tailored parasite control strategies. Upholding rigorous quality assurance measures in diagnostic procedures is essential for maintaining the integrity of results. Ultimately, research outcomes should inform evidence-based policies and guidelines, guiding equitable parasite control practices for the welfare of equines worldwide.

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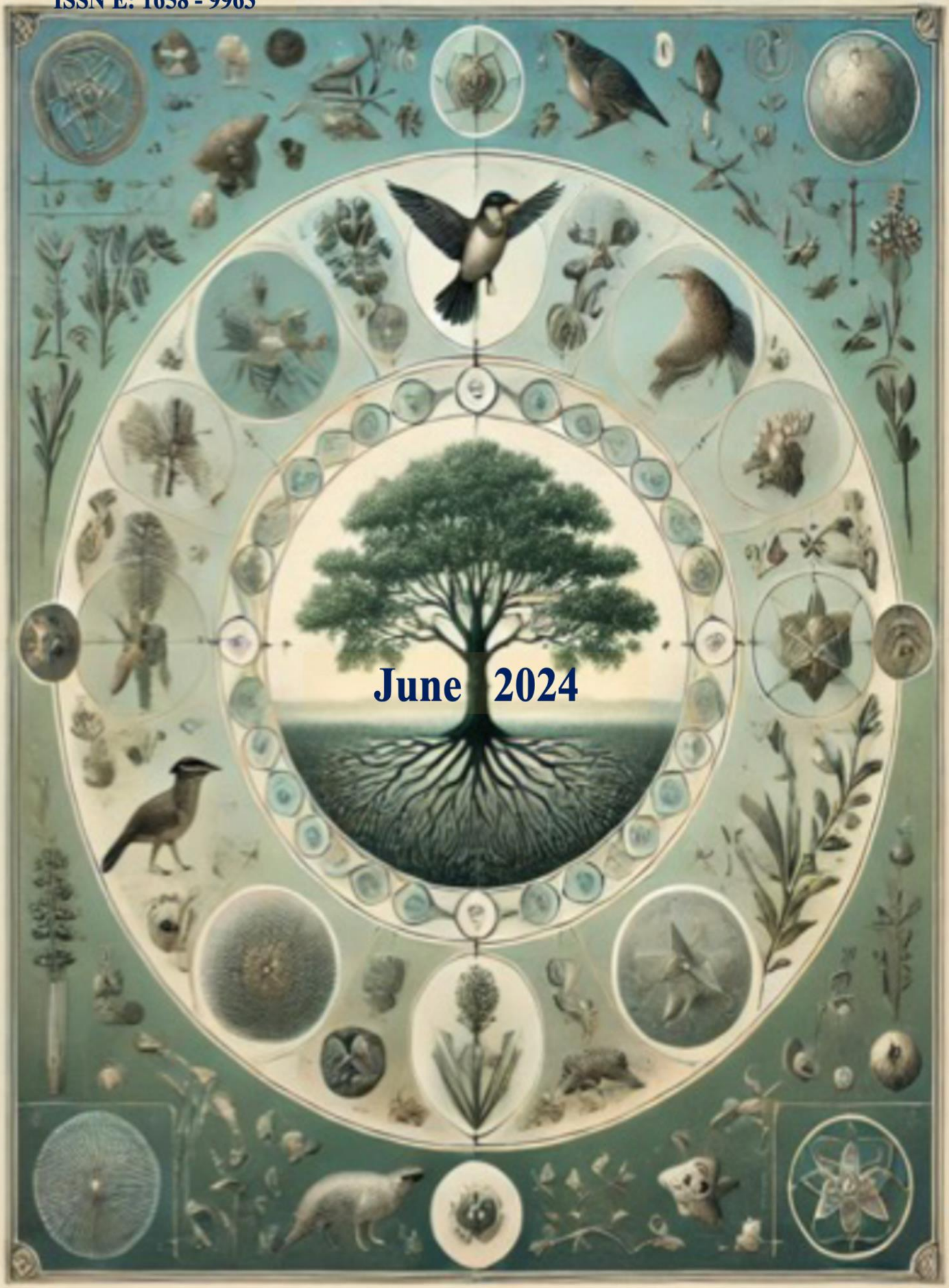

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