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REVIEW ARTICLE

Utilization of Fruit and Vegetable Pomace in Pet Food Formulation: A Sustainable Approach

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Abstract

Pet owners and researchers are increasingly looking for sustainable and healthy options in animal nutrition. Fruit and vegetable pomace, the solid material left after juice or puree extraction, is one promising ingredient. Pomace from apples, tomatoes, grapes, carrots, and spinach contains useful nutrients such as fiber, pectin, polyphenols, vitamins, and natural sugars. Studies show that including moderate amounts of pomace (about 2.5–10% of the diet) can improve digestion, support beneficial gut bacteria, enhance antioxidant activity, and lead to better stool quality in dogs and cats. Processing methods like drying, fermentation, and extrusion help make pomace safer, more stable, and easier to digest. At the same time, using pomace reduces food waste, lowers disposal costs, and supports environmental sustainability. Safety and regulatory guidelines from organizations such as AAFCO and FEDIAF ensure proper use in pet food. This review summarizes current knowledge about pomace composition, benefits, processing, and safety, and highlights future research needs for making pomace a reliable ingredient in sustainable pet diets.

1. Introduction

The global pet food industry has witnessed remarkable growth over the past two decades, driven by rising pet ownership, the humanization of pets, and increasing demand for premium, health-oriented products (Alexander et al., 2020). As consumers become more conscious of sustainability and nutrition, there is a growing interest in alternative ingredients that are both functional and environmentally responsible (Alexander et al., 2020). One such promising category is fruit and vegetable pomace—the fibrous, nutrient-rich by-product left after juice extraction or food processing.

Pomace typically includes peels, pulp, seeds, and stems, and is often discarded or underutilized despite its valuable nutritional profile. Fruit and vegetable waste can account for up to 30–50% of the original raw material, representing a significant loss of potential feedstock (Nagarajaiah and Prakash, 2016). In the context of pet food formulation, these by-products offer a unique opportunity to enhance dietary fiber, introduce natural antioxidants, and reduce reliance on synthetic additives—all while contributing to circular economy goals (Lu et al., 2022).

From a compositional perspective, pomace is a concentrated source of dietary fiber (including pectin and fermentable fibers), bioactive compounds such as polyphenols, flavonoids, and carotenoids, as well as residual simple sugars, vitamins, and minerals (Granucci et al., 2023, Mnisi et al., 2022). These components have been associated with improved gas-

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trointestinal health, modulation of gut microbiota, enhanced antioxidant status, and potential immune support in companion animals (Lu et al., 2022, Mnisi et al., 2022). For example, moderate inclusion of tomato pomace in dog diets has been shown to maintain digestibility and meet safety standards when appropriately processed (Lu et al., 2022).

Beyond nutritional benefits, the incorporation of pomace into pet food aligns with global sustainability frameworks such as the United Nations Sustainable Development Goals (SDGs), particularly SDG 12: Responsible Consumption and Production (Alexander et al., 2020). By valorizing agro-industrial by-products, manufacturers can reduce environmental burdens associated with waste disposal, lower feed production costs, and decrease dependence on resource-intensive raw materials (Alexander et al., 2020, Lu et al., 2022)

Despite these advantages, the use of pomace in pet food remains underexplored compared to its application in livestock and poultry nutrition (Mnisi et al., 2022). Challenges such as variability in composition, microbial stability, palatability, and compliance with regulatory standards (e.g., guidelines from the Association of American Feed Control Officials and the European Pet Food Industry Federation) must be addressed through targeted research and innovative processing techniques (Granucci et al., 2023, Lu et al., 2022).

This review synthesizes current knowledge on the nutritional composition, functional properties, processing methods, safety considerations, and regulatory frameworks related to the use of fruit and vegetable pomace in pet food. It also identifies research gaps and future directions to support the integration of pomace as a sustainable, functional ingredient in the evolving pet food market.

2. Nutritional Composition of Fruit and Vegetable Pomace

Fruit and vegetable pomace, the solid residue remaining after juice or puree extraction, is a nutrient-dense by-product containing substantial amounts of dietary fiber, residual sugars, vitamins, minerals, and bioactive compounds (Granucci et al., 2023, Nagarajaiah and Prakash, 2016). Although the primary extraction process removes most juice and soluble nutrients, the remaining pulp, skins, and seeds retain significant nutritional value that can be harnessed in pet food formulations.

Dietary fiber is the predominant component of pomace, with insoluble fractions such as cellulose, hemicellulose, and lignin contributing to fecal bulk and bowel regularity, and soluble fractions such as pectin and gums providing fermentable substrates for beneficial gut microbiota (Mnisi et al., 2022, Nagarajaiah and Prakash, 2016). Apple pomace typically contains around 30% total dietary fiber, rich in pectin, while carrot pomace can exceed 50%, dominated by cellulose and pectin (de Brito et al., 2021, Granucci et al., 2023, Sagar et al., 2018). Adequate inclusion of these fibers in pet diets can improve stool quality, promote satiety, and support colonic health (Mnisi et al., 2022, Valinezhad and Hallaj-Salahipour, 2025).

Polyphenols and antioxidants are abundant in many fruit pomaces, particularly grape, apple, and berry residues. These include flavonoids (e.g., quercetin, catechins), anthocyanins, resveratrol, and carotenoids such as lycopene in tomato pomace (Nagarajaiah and Prakash, 2016, Singh et al., 2020), Such compounds exhibit antioxidant, anti-inflammatory, and antimicrobial activities, potentially supporting immune function, skin health, and mitigating oxidative stress in companion animals (Mnisi et al., 2022, Singh et al., 2020).

Vitamins and minerals are retained in varying amounts depending on the source and processing method. Common micronutrients include vitamin C (notably in citrus and apple pomace), vitamin A precursors such as beta-carotene (in carrot and spinach pomace), B-complex vitamins, and minerals like potassium, calcium, magnesium, and iron (Lu et al., 2022, Nagarajaiah and Prakash, 2016).

Residual sugars and organic acids persist in pomace despite juice extraction. Apple pomace may contain 8–14% sugars depending on pressing method, while tomato pomace contains glucose, fructose, and malic acid (Kengoo et al., 2023, Valinezhad and Hallaj-Salahipour, 2025, Kumar et al., 2024a). These components can enhance palatability and, in moderation, serve as fermentable energy sources or prebiotics for gut microbiota (Mnisi et al., 2022).

Protein and lipids are present in modest amounts, with vegetable pomaces such as tomato containing up to 15–20% crude protein and 3–5% lipids, and grape pomace containing $\sim$10% protein and $\sim$5% lipids (Granucci et al., 2023, Singh et al., 2020). While insufficient as primary protein or fat sources, they contribute to the overall nutrient profile when combined with other ingredients (de Brito et al., 2021).

Values are approximate and may vary depending on cultivar, growing conditions, and processing methods. Fiber values represent total dietary fiber content, including both soluble and insoluble fractions. Protein and lipid contents are expressed on a dry matter basis. Functional benefits are based on reported bioactive properties and observed effects in animal nutrition studies.

Table 1
Comparative nutritional composition and functional benefits of selected fruit and vegetable pomaces (de Brito et al., 2021, Granucci et al., 2023, Mnisi et al., 2022, Sagar et al., 2018, Singh et al., 2020).

Pomace type	Fiber (%)	Key bio actives	Vitamins / Minerals	Protein (%)	Lipids (%)	Functional benefits in pets
Apple	∼30	Polyphenols, pectin	Vitamin C, potassium	∼5	<2	Gut health, antioxidant support
Carrot	>50	Beta-carotene, pectin	Vitamin A, calcium	∼6	<1	Vision support, digestive regulation
Grape	∼40	Resveratrol, tannins	Iron, magnesium	∼10	∼5	Anti-inflammatory, cardiovascular health
Tomato	∼45	Lycopene, flavonoids	Vitamin A, potassium	15–20	3–5	Antioxidant, skin and coat health
Spinach	∼35	Chlorophyll, flavonoids	Folate, iron	∼8	2–4	Blood health, immune support

3. Benefits and Challenges of Using Pomace in Pet Food

The incorporation of fruit and vegetable pomace into pet food formulations offers a diverse range of nutritional, economic, and environmental benefits, but also presents certain limitations that require careful consideration to ensure safety, palatability, and nutritional adequacy for companion animals.

3.1. Benefits

Environmental sustainability – Pomace utilization supports circular economy principles by diverting food processing waste from landfills and reducing greenhouse gas emissions. For example, apple pomace can account for up to 25% of the original fruit mass during juice production, and its disposal poses environmental challenges unless repurposed (Sagar et al., 2018).

Cost-effectiveness – Pomace is often available at low or no cost from juice and food manufacturers, making it an economical alternative to conventional fiber sources such as beet pulp or cellulose. Tomato pomace, for instance, has been incorporated into dog diets at 2.5–5% without compromising digestibility, offering a cost-effective ingredient option (Singh et al., 2020).

Digestive health – Rich in both soluble and insoluble fiber, pomace can promote gut motility, improve stool consistency, and support beneficial gut microbiota in dogs and cats (Moreno et al., 2022).

Functional properties – Bioactive compounds such as polyphenols, flavonoids, and carotenoids present in pomace exhibit antioxidant, anti-inflammatory, and antimicrobial effects. Blueberry and cranberry pomace, for example, are rich in flavonoids that may aid DNA repair and cellular health in dogs (Xu et al., 2021).

Weight management and special diets – The high fiber and low caloric density of pomace can increase satiety and reduce energy intake, making it suitable for diets targeting overweight, diabetic, or senior pets (Kumar et al., 2024a).

3.2. Challenges

Anti-nutritional factors – Some pomaces contain tannins, oxalates, or phytates that can interfere with nutrient absorption or cause gastrointestinal discomfort. High inclusion levels of certain flavonoids may also negatively affect digestibility or cause toxicity (Erinle and Adewole, 2022).

Microbial contamination – Due to high moisture content, fresh pomace is susceptible to microbial spoilage. Without proper drying or preservation, it may harbor molds or pathogens. Extrusion processing has been shown to improve microbial stability compared to raw or boiled forms (Singh et al., 2020).

Palatability issues – Certain pomaces impart bitter or sour flavors that can reduce feed acceptance, particularly in selective eaters. Flavor masking or blending with more palatable ingredients may be necessary (Marti et al., 2024).

Nutrient imbalance – Pomace alone cannot meet the complete nutritional requirements of pets and must be balanced with adequate protein, fat, and micronutrients. Substituting corn starch with tomato pomace, for example, reduced protein digestibility in dog diets (Singh et al., 2020).

Regulatory constraints – Pet food regulations in some regions restrict the use of certain by-products or require extensive safety validation. AAFCO and FEDIAF currently recognize apple and tomato pomace for pet food use, while other pomace types may require separate approval (AAFCO, 2015).

4. Review of Existing Studies and Experimental Findings

Over the past decade, the nutritional and functional potential of fruit and vegetable pomace has been investigated in a variety of animal diets, including poultry, livestock, and, more recently, companion animals. These studies provide important insights into the feasibility, benefits, and limitations of pomace inclusion in pet food formulations.

5. Studies in Companion Animals

Apple pomace in dog diets Brito et al. (2021) evaluated the inclusion of dried apple pomace at 3–9% in dry dog food. The results showed improved fecal consistency, increased fecal butyrate concentrations, and enrichment of beneficial gut bacteria (e.g., *Faecalibacterium*, *Bacteroides*, *Blautia*), without adverse effects on feed acceptance, although protein digestibility declined slightly at higher inclusion levels (de Brito et al.,

2021).

Tomato pomace in canine nutrition – Singh et al. (2020) supplemented raw, boiled, and extruded dog diets with 2.5% and 5% tomato pomace. Extruded diets showed the highest digestibility, with 2.5% inclusion performing better than 5%. No significant changes in crude protein digestibility were observed, and all diets met safety standards. Improvements in skin and coat condition were attributed to lycopene and vitamin A precursor (Singh et al., 2020).

Grape pomace potential – Although more extensively studied in poultry, grape pomace is rich in polyphenols and has demonstrated anti-inflammatory and antimicrobial effects in animals (Mnisi et al., 2022), suggesting possible immune-support benefits in pets.

Carrot and spinach pomace in treats – (Sagar et al., 2018) reported that incorporating carrot and spinach pomace into pet treats increased fiber content and antioxidant capacity. These formulations were well accepted by dogs, indicating good palatability.

6. Comparative Studies in Livestock and Poultry

While direct research on pets remains limited, findings from livestock and poultry nutrition support the potential value of pomace. Mnisi et al. (2022) reviewed the use of grape, apple, and citrus pomace in poultry diets, noting improvements in gut morphology, feed efficiency, and immune response, alongside reduced ammonia emissions and oxidative stress. Colombino et al. (2020) found that berry pomace (apple, blackcurrant, strawberry) in broiler diets increased ileum and ceca weight, enhanced digesta viscosity, boosted short-chain fatty acid production, and altered microbiota composition (Colombino et al., 2020). Granucci et al. (2023) analyzed beverage industry pomaces and confirmed their high fiber and antioxidant content, supporting their suitability for animal feed (Granucci et al., 2023).

7. Identified Research Gaps and Priority Areas for Future Investigation

Despite promising findings, several important gaps remain in the current body of research on the use of fruit and vegetable pomace in pet nutrition. There is a clear need for more species-specific trials, particularly involving cats and exotic companion animals, as most existing studies focus on dogs or extrapolate from livestock and poultry data (de Brito et al., 2021, Mnisi et al., 2022). Long-term investigations are lacking, especially those assessing the effects of pomace inclusion on metabolic health, healthy ageing, and the management of chronic conditions in pets (de Brito et al., 2021, Singh et al., 2020). Considerable variability in pomace composition between different fruit and vegetable sources, as well as across processing methods, highlights the necessity for standardization in both raw material quality and processing protocols (Granucci et al., 2023, Sagar et al., 2018). Finally, consumer acceptance and market feasibility studies are limited, yet they are essential for understanding how pet owners perceive pomace-based products and for ensuring successful commercial adoption (Kumar et al., 2024a).

8. Processing Techniques and Formulation Strategies

The safe and effective use of fruit and vegetable pomace in pet food requires processing and formulation approaches that preserve nutritional value, enhance palatability, and minimize anti-nutritional or microbial risks (Granucci et al., 2023, Sagar et al., 2018).

8.1. Processing Techniques

Drying and dehydration – Reducing moisture content is essential for extending shelf life and preventing microbial spoilage. Methods include low-cost but weather-dependent sun drying, controlled hot-air drying, and freeze-drying, which best preserves bioactive compounds but is costly (Sagar et al., 2018).

Grinding and milling – Once dried, pomace is milled into a fine powder to ensure uniform mixing in formulations. Particle size influences both digestibility and texture (Granucci et al., 2023).

Fermentation and enzymatic treatment – Fermentation with lactic acid bacteria or enzymatic hydrolysis can reduce anti-nutritional factors, improve nutrient bioavailability, and enhance flavor while supporting gut health (Mnisi et al., 2022).

Extrusion and palletization – Incorporating pomace into extruded kibble or pelleted treats improves digestibility and allows for the inclusion of complementary proteins, fats, and micronutrients (Singh et al., 2020).

8.2. Formulation Strategies

Inclusion levels – Pomace is typically included at 2.5–10% of the total diet, with lower levels (2.5–5%) suited for treats or sensitive pets, and higher levels (up to 10%) for fiber-rich or weight-control diets (Singh et al., 2020).

Complementary ingredients – To achieve complete nutrition, pomace is combined with protein sources (e.g., chicken meal, fish meal, soybean meal), fat sources (e.g., fish oil, flaxseed), carbohydrate sources (e.g., rice, oats, sweet potatoes), and vitamin–mineral premixes (Kumar et al., 2024b, Sagar et al., 2018).

Functional additives – Prebiotics (e.g., inulin), probiotics (*Lactobacillus*, *Bifidobacterium*), antioxidants (e.g., blueberry extract, citrus bioflavonoids), and flavor enhancers (e.g., natural meat flavors, yeast extracts) can be incorporated to improve gut health, oxidative stability, and palatability (Atuahene et al., 2024).

Product types – Pomace can be used in dry kibble, semi-moist treats, dental chews, and supplement powders, with processing tailored to the intended format (FEDIAF, 2021).

9. Regulatory and Labelling Considerations

In the USA, AAFCO requires clear ingredient definitions and nutritional adequacy statements, while in the EU, FEDIAF recommends accounting for rehydration factors when using dehydrated ingredients. Functional claims such as "supports digestion" or "rich in antioxidants" must be substantiated by scientific evidence and comply with labelling laws (AAFCO, 2015).

10. Example Formulations Reported in the Literature

Several studies have described complete or partial pet food formulations incorporating fruit and vegetable pomace for specific nutritional goals. For instance, De brito et al. (2021) and Singh et al. (2020) reported diets for senior dogs that included blends of apple, carrot, and spinach pomace (5–10% of the formulation) alongside high-quality protein sources, omega-3 fatty acids, and joint-support additives such as glucosamine and chondroitin. These diets aimed to enhance fiber intake, antioxidant status, and joint health while maintaining palatability and digestibility (de Brito et al., 2021, Singh et al., 2020).

11. Safety Considerations and Regulatory Frameworks

The incorporation of fruit and vegetable pomace into pet food must comply with stringent safety standards to protect the health and well-being of companion animals. While pomace offers recognized nutritional and functional benefits, its use is subject to regulatory oversight and rigorous quality control measures (Mnisi et al., 2022, Sagar et al., 2018).

From a safety perspective, fresh pomace is highly perishable due to its moisture, sugar, and organic acid content, making it susceptible to microbial contamination by molds (*Aspergillus*, *Penicillium*), yeasts, and pathogenic bacteria such as *Salmonella* and *E. coli*. Appropriate mitigation strategies include hot-air or freeze-drying, extrusion cooking, and fermentation, which can reduce microbial load and extend shelf life (Singh et al., 2020). In addition, pomace from conventionally grown produce may contain pesticide residues or heavy metals (e.g., lead, cadmium, mercury), necessitating sourcing from certified suppliers and batch-level contaminant testing (Kumar et al., 2019).

Certain pomaces also contain anti-nutritional factors such as tannins, oxalates, phytates, and saponins, which can impair nutrient absorption or cause gastrointestinal irritation. Processing methods including enzymatic hydrolysis, fermentation, and blending with protein-rich ingredients can help reduce these compounds (Mnisi et al., 2022). Furthermore, some pets may exhibit sensitivities or allergic reactions to specific fruit proteins or fibers, highlighting the importance of digestibility trials and careful monitoring during product development (de Brito et al., 2021).

Regulatory frameworks vary by region but share common principles of safety, nutritional adequacy, and truthful labelling. In the United States, the Food and Drug Administration (FDA) enforces safety under the Federal Food, Drug, and Cosmetic Act, while the Association of American Feed Control Officials (AAFCO) defines approved feed ingredients, sets nutritional standards, and requires that pomace be listed under recognized feed materials (AAFCO, 2015). In the European Union, the European Pet Food Industry Federation (FEDIAF) regulates safety, composition, labelling, and functional claims (FEDIAF, 2021).

12. Conclusion and Future Perspectives

The integration of fruit and vegetable pomace into pet food emerges as a scientifically supported and practically viable approach to advancing sustainability, nutritional innovation, and waste valorization in the pet food industry. Across the reviewed literature, pomace—particularly from apples, tomatoes, grapes, and carrots—has consistently demonstrated its value as a rich source of dietary fiber, polyphenols, vitamins, and residual sugars. When incorporated at moderate inclusion levels (typically 2.5–10%), it can improve gut health, modulate microbiota, enhance antioxidant status, and support overall well-being in companion animals, without compromising palatability or nutrient digestibility (de Brito et al., 2021, Singh et al., 2020)

Beyond its nutritional merits, pomace utilization directly addresses environmental and economic challenges. By diverting millions of tonnes of by-products from landfill and reducing disposal costs, it supports circular economy principles and lowers the carbon footprint of pet food production (Alexander et al., 2020, Kumar et al., 2019). Life cycle assessments indicate that diets incorporating such by-products can have substantially lower greenhouse gas emissions compared

to premium meat-based formulations.

However, realizing the full potential of pomace in pet nutrition requires addressing key challenges. Anti-nutritional factors, microbial contamination risks, and variability in composition must be managed through advanced processing methods such as extrusion, fermentation, and enzymatic hydrolysis. Regulatory compliance with frameworks such as AAFCO and FEDIAF remains essential to ensure safety, labelling accuracy, and consumer trust (AAFCO, 2015, FEDIAF, 2021).

Looking ahead, five priority areas stand out for future research and industry development:

1. **Species-specific trials** – Controlled, long-term studies in dogs, cats, and other companion animals to determine optimal inclusion rates and health outcomes.

2. **Functional formulations** – Targeted diets for senior pets, weight management, gastrointestinal health, and metabolic disorders, leveraging specific pomace types.

3. **Processing innovations** – Novel techniques to enhance nutrient bioavailability, reduce anti-nutritional compounds, and improve shelf life.

4. **Consumer perception** – Market research to understand pet owner attitudes toward pomace-based products and the influence of sustainability claims on purchasing decisions.

5. **Policy harmonization** – Development of unified global standards for by-product use in pet food, including inclusion in authorized feed material registries (Kita et al., 2024).

By bridging nutritional science, sustainable resource management, and product innovation, fruit and vegetable pomace can transition from an underutilized by-product to a cornerstone ingredient in next-generation pet foods. Its adoption offers a rare convergence of benefits—supporting animal health, reducing environmental impact, and enhancing economic efficiency—positioning it as a transformative element in the future of companion animal nutrition.

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ORIGINAL ARTICLE

Optimization of a Culture Protocol for Murine Bone Marrow-Derived Mesenchymal Stromal Cells

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Abstract

Background: Mesenchymal stem cells (MSCs) have attracted extensive research due to their high proliferative capacity, multipotent differentiation, and essential role in tissue regeneration. Despite their application in clinical research, there are limitations in the culture and maintenance of these cells. This study aimed to optimize a cost-effective and efficient protocol for the culture and expansion of murine MSCs (M-MSCs).

Methods: MSCs were isolated from BALB/c mouse femoral bone marrow (6–8 weeks old) and cultured in DMEM-Low Glucose with 20%-5% fetal bovine serum (FBS), penicillin/streptomycin (1%), and gentamicin (0.5%). At passage 3, cells exhibited fibroblastic morphology and were immunophenotyped for MSCs (CD73-PE, CD105-PerCP-Cy5.5) and hematopoietic markers (CD34-PE, CD45-FITC). The growth rate, post-freezing cell viability, and population doubling time were evaluated.

Results: Fibroblastic morphology was observed at 48 hours post-primary culture, with cells forming a scaffold and reaching 80% confluency by day 5. Under 5% FBS conditions, the time to reach 90% confluency decreased to <5 days by passages 2 and 3. Flow cytometry confirmed a typical MSCs profile: high CD73 (99.9%) and CD105 (98.5%) and low CD34 (5.99%) and CD45 (2.86%) expression. Results show a positive correlation between increasing passage number and enhanced MSCs growth rate, viability and functional characteristics ($p \leq 0.05$).

Conclusion: Optimization of M-MSCs culture through a FBS reduction (20% to 5%) is shown to preserve key cellular properties: fibroblastic morphology, proliferation rate, and immunophenotypic markers. Our protocol reduces microbial contamination associated with animal serum and provides an economical, and viable strategy for large-scale M-MSCs production in regenerative medicine.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal and differentiation into mesodermal lineages, including osteoblasts, chondrocytes, and adipocytes, a property designated as trilineage differentiation potential (1). This defining characteristic underlies their widespread application in regenerative medicine and tissue engineering (2). Although MSCs can be isolated from multiple tissues such as adipose tissue, umbilical cord, and dental pulp (3), bone marrow represents the most extensively charac-

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terized source in both preclinical and clinical investigations (4). MSCs serve as essential experimental models for elucidating fundamental mechanisms of cellular differentiation, tissue development, and repair (5). Furthermore, their immunomodulatory properties position them as promising candidates for cell-based therapies targeting inflammatory (6), autoimmune, cancer(7, 8) and, degenerative conditions (9).

Moreover, murine and human MSCs exhibit conserved biological properties, including comparable immunophenotypic profiles ($CD73^+/CD90^+/CD105^+/CD34^-/CD45^-$)(10), trilineage differentiation capacity, and immunomodulatory mechanisms, making murine models highly relevant for protocol development. Optimization of culture conditions in murine MSCs provides a framework that can inform and accelerate the refinement of human MSCs expansion protocols, thereby enhancing reproducibility and stability for clinical applications while reducing reliance on high serum concentrations (4).

Despite their therapeutic potential (11), technical challenges limit the isolation and expansion of bone marrow-derived MSCs in murine models, specifically BALB/c mice, are constrained by several technical challenges (5). The inherently low concentration of bone marrow cellular content, stromal microenvironment, limits isolation efficiency and low initial cell yield. This limitations, coupled with reduced proliferative capacity and phenotypic changes induced during serial passaging. Conventional protocols typically require high fetal bovine serum (FBS) supplementation ($\geq$10%). One of the aim of the present study is to reduce FBS use for MSCs culture and passaging, making the process both economical and less prone to animal-derived contamination. Alternatively, cells can be maintained without additional growth factors, relying on the secretory factors of MSCs themselves.

2. Materials and Methods

2.1. Animal

Female BALB/c mice were purchased from the Pasteur Institute of Iran. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Bu-Ali Sina University (Ethics Code: IR.BASU.REC.1404.012) and conducted. Female BALB/c mice (6–8 weeks old, 20–30 g) were housed under standardized conditions: 12-h light/dark cycle, temperature of 22 ± 1°C, 50–60% relative humidity.

2.2. Isolation and Primary Culture of Bone Marrow-Derived MSCs

BALB/c mice were euthanized under deep anesthesia (ketamine/xylazine, 90/10 mg/kg, i.p.). Femurs were aseptically harvested, cleared of adherent soft tissues, and epiphyses were excised. Bone marrow was flushed using 5 mL of DMEM(Dulbecco's minimal essential medium)-Low Glucose (DMEM-LG) supplemented with 1% penicillin/streptomycin (Pen/Strep). The cell suspension was collected in 15-mL tubes and centrifuged at 300 × g for 5 min. The supernatant was discarded, and the cell pellet was washed three times with PBS containing 5% Pen/Strep. The final pellet was resuspended in complete growth medium (DMEM-LG supplemented with 20% FBS, 1% Pen/Strep and 0.5% gentamicin) and seeded into T12.5 culture flasks. Cultures were maintained at 37°C in a humidified incubator with 5% CO2. Twenty-four hours post-seeding (passage 0), 90% of the medium was replaced to remove non-adherent hematopoietic cells, using DMEM-LG containing 10% FBS and 1% Pen/Strep and 0.5% gentamicin. Subsequent medium exchanges occurred on days 3 and 5. The culture media was gradually replaced with percentages of 80, 60, and 50 percent from passage 1 and FBS concentration was progressively decreased from 20% → 15% → 10% → 5% across three sequential passages, while maintaining 1% Pen/Strep and 0.5% gentamicin supplementation throughout.

2.3. Cell Passaging

Cells were passaged upon reaching 80% confluency (typically day 5 for P0, and <5 days for subsequent passages under 5% FBS). The culture medium was aspirated, and adherent cells were washed twice with PBS containing 5% Pen/Strep. Cells were detached using 0.25% trypsin-EDTA (3 min, 37°C), neutralized with complete medium, and transferred to 15-mL tubes. After centrifugation (300 × g, 5 min), the pellet was washed three times with PBS containing 5% Pen/Strep, resuspended in DMEM-LG containing 10% FBS and 1% Pen/Strep and 0.5% gentamicin.

2.4. Freezing and Storing

One of the major challenges in long-term storage of mesenchymal stem cells is their low resistance to freezing and damage by liquid nitrogen. For this purpose, third-passage cells were trypsinized, suspended in cryotubes containing 90% FBS and 10% dimethyl sulfoxide (DMSO) and quickly placed in liquid nitrogen.

2.5. Immunophenotypic Characterization

At passage 3, cells were harvested, washed twice with PBS, and counted using trypan blue exclusion (1×10^6 cells/sample). Cells were then incubated for 30 min at 4°C with fluorochrome-conjugated monoclonal antibodies: CD34-PE and CD45-FITC (negative markers), CD73-PE and CD105-PerCP-Cy5.5 (positive markers; all from BD Biosciences). Isotype-matched controls were included for gating validation. After two

washes with FACS buffer (PBS+2% FBS), samples were analyzed on a BD FACSCalibur flow cytometer (v10.8)(12).

2.6. Morphological Assessment and Data Analysis

Cell morphology was documented using a digital camera mounted on an inverted phase-contrast microscope (Olympus). The proliferation ratio were assessed by recording the time required to reach 80–90% confluency at each passage. Flow cytometry data were acquired on a BD FACSCalibur instrument and analyzed using FlowJo software (version 10.8). The data was statistically analyzed using GraphPad prism 8. One and Two-way ANOVA following post-hoc Tukey's test were performed ($P\text{-}value \leq 0.05$).

2.7. Growth Rate

Cell growth rate was determined by seeding cells at a specific density and monitoring cell numbers over a defined period. Cells were harvested at regular intervals, and viable cell counts were performed using a hemocytometer. The growth curve was plotted with cell number on the y-axis against time on the x-axis.

2.8. Post-Thaw Viability

Post-thaw viability was assessed by thawing cryopreserved cells according to standard procedures. Immediately after thawing, cells were stained with trypan blue, and the percentage of viable (unstained) cells was determined using a hemocytometer. Viability was calculated as: Viability (%) = (Number of viable cells / Total number of cells) × 100.

2.9. Population Doubling Time

The population doubling time (PDT) was determined from the exponential growth phase. Cells were seeded at time T1 with an initial cell number (N1), and at time T2, the cell number reached N2.

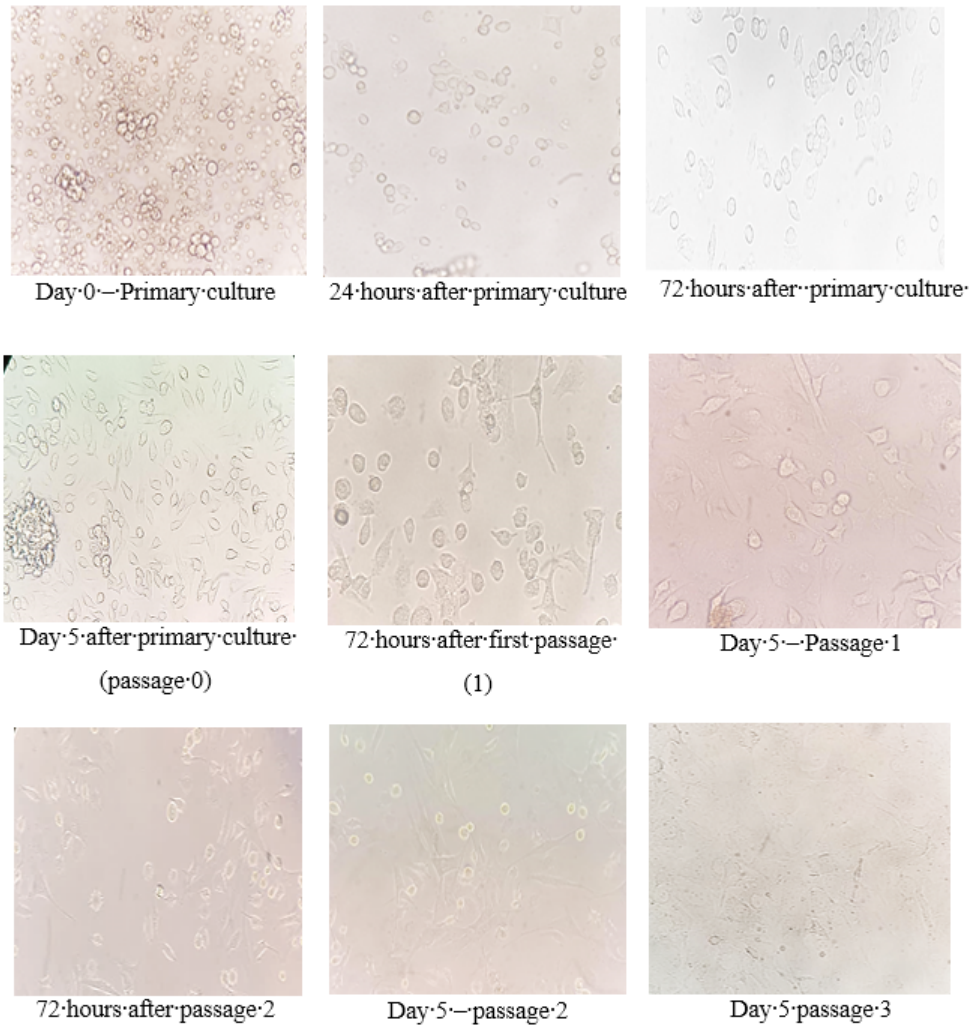

Fig. 1. Morphological changes of murine bone marrow-derived mesenchymal stem cells: from isolation to subsequent passages.

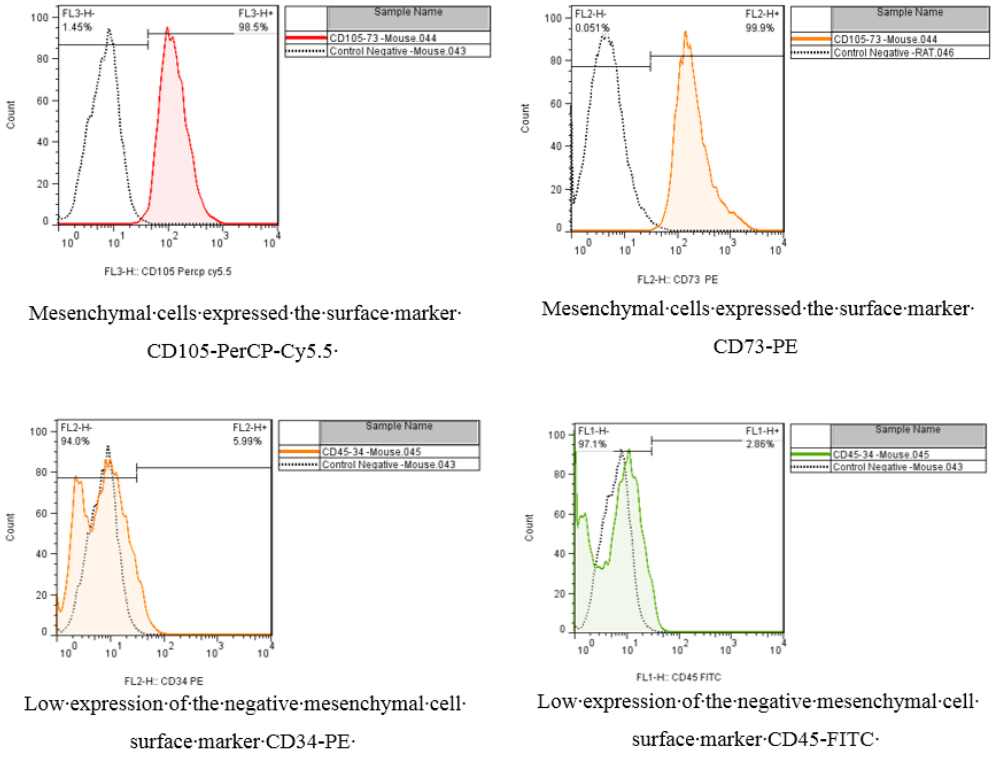

Fig. 2. Immunophenotyping of bone marrow-derived mesenchymal stem cells.

3. Result

3.1. Cell Culture and Passage

Adherent spindle-shaped fibroblastic cells were observed within 24 hours following primary culture initiation. By day 5, the monolayer reached 80% confluency and was subjected to enzymatic passaging using 0.25% trypsin-EDTA. Post-passage assessment revealed preservation of characteristic morphology without cytoskeletal disruption. Notably, cellular attachment and formation of interconnected stromal networks accelerated progressively across sequential passages under the optimized serum-reduced protocol. By gradually replacing the cell culture medium with varying percentages of serum-reduced medium, the growth factors present in the culture supernatant supported the cells. Despite the reduction in FBS, the growth of non-specific cells decreased, while the growth and proliferation rate of mesenchymal cells increased. The use of the antibiotic gentamicin was also effective in controlling contamination of the culture medium (Fig. 1).

4. Immunophenotypic Characterization

Flow cytometric analysis of surface markers confirmed high purity of the bone marrow-derived mesenchymal stem cell population, with minimal contamination by hematopoietic lineages. The cells exhibited very low expression of hematopoietic markers CD34 (5.99%) and CD45 (2.86%). Concurrently, characteristic MSCs markers were highly expressed: CD73 (99.9%) and CD105 (98.5%). This immunophenotypic profile, $CD73^{+}/CD105^{+}/CD34^{-}/CD45^{-}$, validates the efficacy of the optimized serum-reduction culture protocol in maintaining phenotype and purity throughout expansion(Fig. 2).

4.1. Cell Growth Rate

Analysis of cell growth rates across three passages revealed a consistent increase in growth rate with time. Passages 2 and 3 exhibited a higher initial growth velocity compared to passage 1, reaching maximum cell density between days 20 and 28 post-seeding. Notably, the growth rate accelerated with subsequent passages. Passage 3 cells demonstrated a 100% growth rate by day 10, while passage 2 reached this threshold by day 20. In contrast, passage 1 cells did not reach a 100% growth rate within the observed experimental period ($P \leq 0.05$) (Fig. 3).

4.2. Post-Thaw Viability

Cryopreservation and subsequent thawing of (MSCs) commonly present significant viability challenges, potentially leading to a decrease in cell survival of up to 50%. In this study, we observed a significant increase in post-thaw viability that correlated with increased passage number. Earlier passages, particularly passage 1, demonstrated diminished recovery. In contrast,

MSCs at passage 3 exhibited a significantly more functional post-thaw phenotype, characterized by substantially higher viability percentages, reflecting their more mature characteristics at this stage (P≤0.05) (Fig. 4).

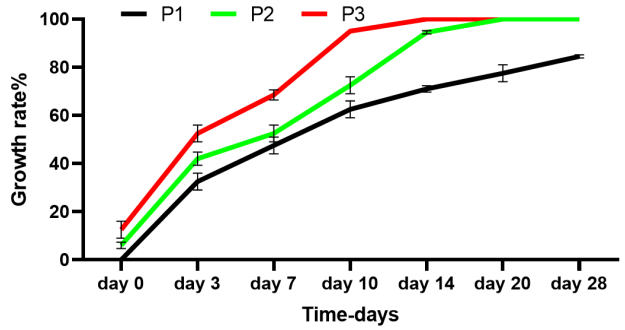

Fig. 3. Cell growth curve across different days for multiple cell passages.

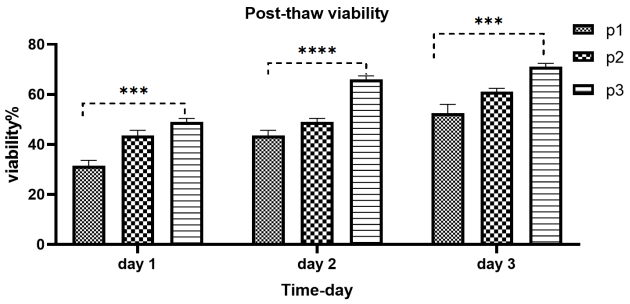

Fig. 4. Post-thaw cell viability during the initial three days.

4.3. Population Doubling Time

An increase in passage number was associated with a acceleration in cell proliferation. When cultured for an equivalent incubation period, a significant rise in total cell count was observed in higher passages. Cells at passage 3 exhibited approximately a three-fold increase in number compared with passage 1 and a two-fold increase compared with passage 2 (Fig. 5).

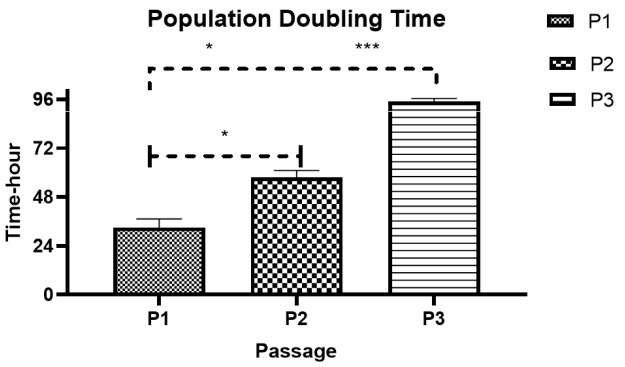

Fig. 5. Population doubling time of cells at different time points and across various passages.

4.4. Discussion

A principal limitation in the therapeutic application of MSCs is the difficulty to achieving optimal *in vitro* expansion while maintaining phenotypic stability and functional potency (13). The present study demonstrates that an optimized culture protocol, specifically adapted to the biological characteristics of bone marrow-derived MSCs from BALB/c mice, a strain known for low cellular yield, significantly improves both proliferation rates and phenotypic consistency. Key modifications in this study included: (i) gradual reduction of fetal bovine serum (FBS) concentration with medium adaptation while preventing microbial contamination; (ii) elimination of enzymatic digestion and filtration during primary isolation; (iii) accelerated growth following passaging; and (iv) efficient cryopreservation with high post-thaw viability. By passage 3 (P3), these adjustments produced a highly purified MSCs population without the use of exogenous growth factors, with a marked reduction in hematopoietic and non-mesenchymal contaminants and improved proliferative capacity and phenotypic stability. Phenotypic stability was maintained through p3, with cells reaching 80% confluence within 5–6 days after primary seeding. Flow cytometric analysis confirmed strong expression of CD105 and CD73 (>95% positive), and low expression of hematopoietic markers CD34 and CD45 (<6% positive) (Fig. 2). These immunophenotypic characteristics confirm the mesenchymal identity and high purity of the cultured cell population. The selection of an appropriate culture flask is critically important for successful monolayer expansion of MSCs (13). In this study, we utilized of T12.5 culture flasks, which have a smaller growth surface area compared with conventional T25 or T75 flasks. The reduced surface area in primary culture promoted higher effective cell density, thereby enhancing cell–cell interactions and supporting the formation of more scaffold-like cellular networks. Moreover, whereas standard protocols typically require 6–7 days to reach confluence for the first passage(5), our optimized approach achieved within 5–6 days. Accumulating evidence indicates that refined culture conditions profoundly influence the secretome of MSCs, upregulating trophic and immunomodulatory factors that are essential for self-renewal, differentiation potential, and *in vivo* functionality, thereby contributing to sustained phenotypic stability(14). Although batch-to-batch variability in culture medium components and reagents can also affect MSCs culture quality(15), this parameter warrants careful consideration in experimental design and protocol standardization. A defining characteristic of MSCs is their adherence to plastic culture surfaces(16), a property recognized as a fundamental criterion for MSCs identification and routinely used for their isolation and purification through plastic adherence-based selection(17).

Although multiple isolation and expansion methodologies have been established for MSCs, including serial medium replacement, immunomagnetic selection (positive/negative), filtration(18), and enzymatic digestion(19), each technique presents limitations regarding yield, purity, stability, or cost-effectiveness. Thus, the development of simplified protocols that minimize technical complexity while maximizing cellular quality are necessary. Preservation of optimal culture parameters to ensure long-term phenotypic stability and replicative capacity is essential for both fundamental investigations and applications including the advancement of MSCs-based regenerative medicine(20). The proliferative capacity and post-cryopreservation resilience of (MSCs) are demonstrably influenced by their *in vitro* passage number, as evidenced by the presented data. A consistent trend of accelerated growth kinetics was observed with increasing passage numbers. Specifically, passages 2 and 3 exhibited a more vigorous initial growth rate than passage 1, achieving peak cell densities between days 20 and 28 post-seeding. This enhanced proliferative potential is further supported by the finding that passage 3 cells reached 100% growth by day 10 within the experimental duration. This observations suggests that serial passaging potentiates the intrinsic proliferative capacity of MSCs, likely through adaptive mechanisms acquired during *in vitro* expansion. Consistent with this, post-thaw viability assessments revealed a significant positive correlation between passage number and cell recovery following cryopreservation. Given that cryopreservation protocols can reduce cell survival by up to 50%, the enhanced resilience observed in higher-passage MSCs is of considerable relevance. Passage 3 cells exhibited a markedly more post-thaw phenotype and higher viability compared to earlier passages, particularly passage 1, which showed reduced recovery. This improved cryosurvival at later passages may reflect the acquisition of more stable cellular characteristics and enhanced membrane integrity with progressive *in vitro* maturation. Concurrently, population doubling time analysis indicated a significant increase in total cell yield for higher passages under equivalent incubation periods, with passage 3 cells producing approximately a three-fold increase relative to passage 1. These findings highlight a critical role of passage number that optimizes both proliferative and cryopreservation tolerance, providing valuable guidance for the strategic expansion and potential clinical application of MSCs.

Although the culture protocol described here was optimized for the isolation and expansion of MSCs from BALB/c mice, several limitations should be mentioned. Notably, all animals shared the same genetic background, sex, and age range. Biological variables, including sex hormones and age-related alterations in the bone marrow microenvironment are known to significantly influence MSCs yield, proliferative capacity, immunophenotype, and differentiation potential(21). Therefore the generalizability of these findings to other murine strains, sexes, or age groups requires further investigation. In addition, although surface marker profiling ($CD73^+/CD105^+/CD34^-/CD45^-$) confirmed phenotypic purity, functional validation of multipotency through tri-lineage differentiation assays (osteogenic, adipogenic, and chondrogenic) was not performed. Phenotypic characterization alone is insufficient to definitively confirm preservation of differentiation capacity. Accordingly, future studies applying this protocol should incorporate comprehensive trilineage differentiation assays complemented by molecular analyses of lineage-specific gene expression.

4.5. Conclusion

The development of optimized culture protocols that preserve MSCs identity while reducing dependence on animal-derived sera remains a critical priority. This study presents a step-by-step serum-reduction strategy for the isolation and expansion of bone marrow-derived MSCs from BALB/c mice. By progressively decreasing FBS concentration from 20% to 5% across sequential passages, combined with controlled medium replacement, the protocol yields high-density cultures that maintain characteristic spindle-shaped morphology, exhibit enhanced proliferative rate, and retain a stable immunophenotype ($CD73^+/CD105^+/CD34^-/CD45^-$). This approach significantly reduces culture-associated costs and biosafety concerns related to xenogeneic components, while providing a reproducible and stable methodology for preclinical MSCs production.

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Authors' contributions

Study concept and design: S. H.
Analysis and interpretation of data and drafting of the manuscript: S. H. and H. R
Statistical analysis: S. H
Experimental studies: M.H. P
All authors approved the manuscript.

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Conflict of Interest

We declare that there is no conflict of interest.

Data Availability

Data for this finding are available on request from the corresponding author.

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REVIEW ARTICLE

A Comprehensive Review of Fixatives Used in Diagnostic Parasitology: Morphological Preservation, Technical Performance, and Modern Applications

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Abstract

Background: The selection of an appropriate stool fixative is pivotal for preserving parasite morphology and ensuring diagnostic accuracy in intestinal parasitology. Each fixative exhibits distinct advantages and limitations with respect to biosafety, compatibility with staining techniques, and applicability to molecular diagnostics. This review provides a systematic comparative evaluation of commonly used fixatives and offers evidence-based guidance to support their optimal selection in contemporary diagnostic laboratories.
Methods: A comprehensive literature search was performed across PubMed, Scopus, Web of Science, and Google Scholar to identify studies investigating fecal fixatives used in the diagnosis of intestinal parasites.
Results: 10% formalin remains the gold standard for helminth preservation but is unsuitable for delicate trophozoites and molecular assays. PVA and Schaudinn's fixatives provide excellent protozoan morphology yet are limited by mercury-related safety concerns. Mercury-free alternatives such as SAF, modified PVA, and single-vial systems offer safer and more versatile options, although with certain trade-offs in staining quality and consistency.
Conclusion: No single fixative meets all diagnostic needs. Choice should consider parasite type, laboratory setting, downstream applications, and safety regulations.

1. Introduction

Accurate laboratory diagnosis of intestinal parasitic infections remains a cornerstone of clinical parasitology and public health, particularly in low– and middle-income countries where these infections continue to impose a substantial disease burden [1-5]. Despite significant advances in molecular and immunological diagnostics, conventional stool microscopy remains the primary diagnostic approach in many routine laboratories worldwide [6].Within this context, the selection of an appropriate stool fixative is critical, as it directly influences parasite morphology preservation, diagnostic sensitivity, and the feasibility of applying complementary techniques such as concentration methods, permanent staining, and immunoassays.

Stool fixatives have been developed to address diverse diagnostic objectives, including the preservation of helminth eggs and larvae, protozoan cysts and trophozoites, long-term specimen storage, and safe sample transport. However, no single fixative is universally optimal for all diagnostic purposes. Each for-

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mulation exhibits distinct advantages and limitations related to parasite type, compatibility with staining procedures, suitability for concentration techniques, and interference with advanced diagnostic modalities, particularly nucleic acid–based assays. In addition, growing concerns regarding laboratory safety, environmental toxicity, regulatory restrictions, and cost-effectiveness have increasingly influenced fixative selection in modern diagnostic settings [7].

Historically, fixatives such as 10% formalin, polyvinyl alcohol (PVA), and Schaudinn's fixative have been regarded as reference standards due to their reliable preservation of specific parasitic stages [8]. Nevertheless, their limitations—including poor preservation of delicate protozoan trophozoites, incompatibility with molecular diagnostics, and the use of toxic mercury-containing compounds—have prompted the development of alternative mercury-free formulations. These include sodium acetate–acetic acid–formalin (SAF), modified PVA fixatives, merthiolate–iodine–formaldehyde (MIF), and more recently, one-vial commercial systems designed to integrate fixation, concentration, and permanent smear preparation within a single platform.

The present review provides a comprehensive and comparative evaluation of the most commonly used stool fixatives in diagnostic parasitology, including 10% formalin, MIF, low-viscosity PVA, SAF, Schaudinn's fixative, modified PVA formulations, and one-vial fixative systems. The advantages, limitations, and diagnostic applicability of each fixative are critically discussed with respect to morphological preservation, compatibility with staining and concentration techniques, safety considerations, and suitability for contemporary diagnostic workflows. This review aims to offer practical, evidence-based guidance to assist clinical laboratories and researchers in selecting the most appropriate fixative according to specific diagnostic requirements and operational constraints [9,10].

2. Methods

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines.

2.1. Search Strategy

A systematic literature search was performed to identify relevant studies evaluating stool fixatives used in the diagnosis of intestinal parasitic infections. The following electronic databases were searched: PubMed/MEDLINE, Scopus, Web of Science, and Google Scholar. The search covered all publications from database inception to the final search date.

A combination of controlled vocabulary (MeSH terms) and free-text keywords was used. Search terms included: *stool fixative*, *fecal preservative*, *intestinal parasites*, *parasitological diagnosis*, *formalin*, *polyvinyl alcohol (PVA)*, *SAF*, *MIF*, *Schaudinn's fixative*, *modified PVA*, *one-vial fixatives*, *permanent staining*, *concentration techniques*, and *diagnostic microscopy*. Boolean operators ("AND", "OR") were applied to refine the search strategy. In addition, the reference lists of eligible articles and relevant review papers were manually screened to identify further pertinent studies.

2.2. Eligibility Criteria

Studies were included if they met the following criteria:

1. Original research articles, comparative studies, validation studies, or authoritative guidelines addressing stool fixatives used in human intestinal parasitology;
2. Evaluated one or more fixatives with respect to parasite morphology preservation, staining quality, concentration methods, diagnostic performance, or laboratory applicability;
3. Published in peer-reviewed journals;
4. Available in the English language.

2.3. Exclusion Criteria Were

1. Studies focusing exclusively on non-intestinal parasites or non-fecal specimens;
2. Conference abstracts, editorials, letters, and non–peer-reviewed sources lacking sufficient methodological detail;
3. Articles addressing fixatives not applicable to routine stool examination.

2.4. Study Selection

All retrieved records were imported into a reference management software, and duplicate entries were removed. Titles and abstracts were independently screened for relevance, followed by full-text assessment of potentially eligible articles. Discrepancies during the selection process were resolved through discussion and consensus. The study selection process is summarized using a PRISMA flow diagram.

2.5. Data Extraction

Data were systematically extracted from all included studies by the authors using a standardized data extraction form. Extracted variables included:

1. type and composition of fixative;
2. target parasite stages preserved;
3. compatibility with concentration techniques and staining methods;

4. suitability for immunological and molecular diagnostics;
5. safety, environmental, and operational considerations;
6. reported advantages and limitations.

2.6. Quality Assessment

Methodological quality and risk of bias were assessed based on study design and reporting clarity. Due to heterogeneity in study types and outcome measures, formal quantitative risk-of-bias tools were not uniformly applicable. Nevertheless, priority was given to well-designed comparative studies, standardized laboratory evaluations, and widely cited references from reputable journals. Studies with insufficient methodological transparency were interpreted cautiously.

2.7. Data Synthesis

Given the substantial heterogeneity across study designs, diagnostic techniques, and evaluated outcomes, a meta-analysis was not feasible. Therefore, results were synthesized using a qualitative narrative approach. Fixatives were categorized based on chemical composition and diagnostic application, and their performance was compared across key domains relevant to routine and advanced parasitological diagnostics.

2.8. Results

The systematic review included studies evaluating a range of stool fixatives commonly used in diagnostic parasitology. Overall, the evidence demonstrates that no single fixative is universally optimal for all diagnostic purposes; each exhibits specific advantages and limitations depending on the parasite type, the target life stage, compatibility with staining and concentration methods, suitability for molecular or immunological assays, and practical considerations such as preparation complexity, safety, and storage stability. The findings are therefore presented by fixative type, summarizing their chemical properties, diagnostic performance, and operational utility in both routine laboratories and field settings.

2.9. 10% Formalin

10% formalin is easily prepared without complex equipment, making it widely accessible. It offers excellent stability for long-term storage and effectively preserves the morphology of robust parasites like helminth eggs and protozoan cysts for reliable microscopic identification (Fig. 1). It is fully compatible with common concentration methods (e.g., formalin-ethyl acetate) and important staining techniques (e.g., acid-fast, chromotrope), enhancing diagnostic sensitivity and range. Furthermore, formalin-fixed specimens are suitable for immunoassays and UV fluorescence microscopy, supporting diverse diagnostic approaches [10-12]. 10% formalin is unsuitable for preserving delicate protozoan trophozoites, limiting diagnosis of infections like Entamoeba histolytica. It can also interfere with trichrome staining, reducing the quality of permanent stained smears. A major drawback is its negative impact on molecular assays, as it cross-links nucleic acids, making samples unsuitable for PCR and other DNA-based methods [11,12]. Despite these limitations, it remains a widely used fixative, but its use must be chosen carefully based on diagnostic needs, while it is considered the gold standard for preserving helminth eggs and larvae.

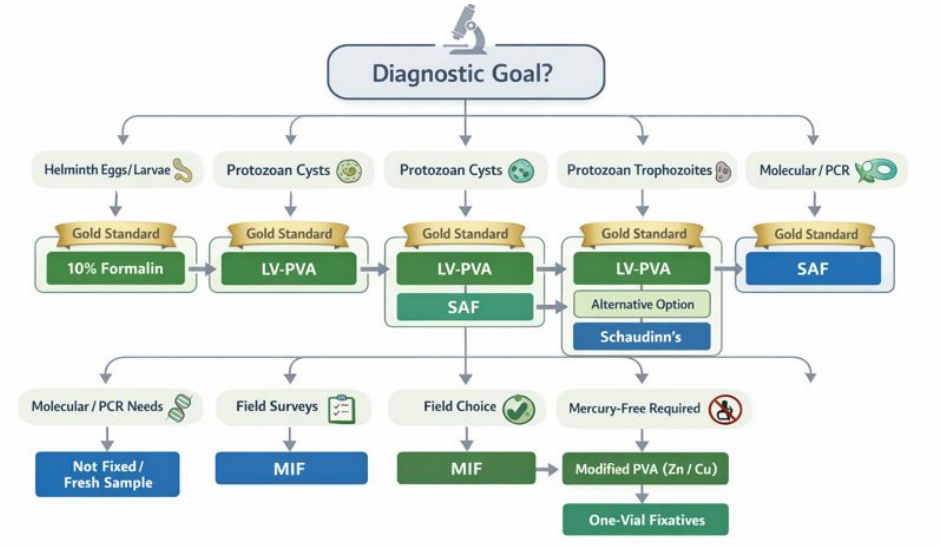

Fig. 1. Decision-making algorithm for the selection of fecal fixatives based on diagnostic objectives and environmental constraints.

3. Merthiolate–Iodine–Formaldehyde (MIF)

Merthiolate–Iodine–Formaldehyde (MIF) is a unique fixative valued in field and resource-limited settings, combining fixation and preliminary staining in one reagent due to its composition of a disinfectant, a stain, and a preservative, which distinguishes it and supports its widespread use in routine parasitology [13]. MIF is advantageous due to its simple preparation requiring only basic resources and its long chemical stability, ensuring cost-effectiveness and consistent availability. It is exceptionally useful in field surveys, as its combined fixative-and-stain function allows immediate specimen stabilization and preliminary visualization without additional reagents, aided by iodine for basic contrast. Furthermore, MIF remains compatible with common concentration methods like sedimentation, enhancing diagnostic sensitivity for detecting helminths and protozoan cysts even with low parasite burdens (Fig. 1). This makes it practical for both immediate field use and subsequent laboratory examination. A major limitation of MIF is its iodine-induced distortion of protozoan morphology, complicating species differentiation and making it unsuitable for high-fidelity morphological identification (Table 1). MIF-preserved samples are incompatible with essential techniques like trichrome staining and UV fluorescence microscopy due to poor coloration and interference. Furthermore, MIF is ineffective at preserving fragile protozoan trophozoites, limiting its diagnostic use for infections reliant on trophozoite morphology. Despite these limitations, MIF remains a practical and widely used fixative due to its ease of preparation, stability, and suitability for field work. Consequently, its limitations must be considered in routine clinical diagnostics, and alternative fixatives are preferred for detailed protozoan identification or advanced microscopy [13-16].

4. Low-Viscosity Polyvinyl Alcohol (LV-PVA)

Low-Viscosity Polyvinyl Alcohol (LV-PVA) is a benchmark fixative in parasitology, renowned for its excellent preservation of protozoan morphology and its ability to facilitate high-quality permanent stained slides, particularly for delicate trophozoites and cysts, maintaining its status despite newer mercury-free alternatives [10,17].

LV-PVA excels at preserving delicate protozoan trophozoites and cysts with minimal distortion, which is crucial for accurate microscopic identification (Fig. 1). It is highly compatible with permanent staining methods like trichrome, producing high-quality slides with sharp nuclear detail and consistent coloration, establishing it as the old gold standard for protozoan diagnosis. Additionally, LV-PVA provides exceptional long-term stability, allowing specimens to be stored for months without significant deterioration, making it reliable for reference labs, teaching, and quality assurance.LV-PVA is ineffective for preserving helminth eggs, larvae, coccidian oocysts, and microsporidian spores, often distorting their structural details. Its formulation contains toxic mercuric chloride, posing significant health, environmental, and costly disposal challenges, and requires complex, precise preparation. Furthermore, it is incompatible with modern diagnostic methods like immunoassays, concentration techniques, and key stains such as acid-fast and chromotrope, severely limiting its diagnostic utility but it still retains its status as a gold standard for protozoan morphology [1,17,18].

5. Sodium Acetate–Acetic Acid–Formalin (SAF)

Sodium Acetate–Acetic Acid–Formalin (SAF) is a mercury-free fixative developed for safety and practicality. Its key advantages are its versatility for both concentration techniques and permanent smears, a long shelf life with easy preparation, and compatibility with a wide range of staining methods including acid-fast, safranin, chromotrope, and immunoassays, making it valuable for routine diagnostics. The limitations of SAF include the frequent requirement for adhesives like albumin-glycerin to prepare permanent smears, complicating slide preparation, and its permanent stains are of slightly lower quality compared to PVA-based fixatives, potentially affecting detailed morphological observation (Table 1). Therefore, while it is a safe, versatile, and practical mercury-free fixative suitable for routine diagnostics, it does not fully replace specialized fixatives for all advanced applications [1,19,20].

Schaudinn's fixative is a historical diagnostic standard for protozoa, valued for its excellent preservation of fine morphological details in both trophozoites and cysts, and its ability to produce high-quality permanent stained smears for long-term reference and detailed analysis (Fig. 1).

6. Schaudinn's Fixative

Schaudinn's fixative contains toxic mercuric chloride, posing serious health and environmental risks, and is ineffective for preserving helminth eggs, larvae, coccidia, and microsporidia. It also has weak adhesion for mucoid samples and is unsuitable for concentration procedures. Therefore, while excellent for protozoan morphology and high-quality permanent smears, its toxicity and limited scope restrict its broader use, leading modern labs to prefer safer, more versatile alternatives [21,22].

Table 1
Comparative characteristics of commonly used stool fixatives in parasitological diagnosis.

Fixative	Main Advantages	Major Limitations
10% Formalin	• All-purpose fixative	Not suitable for some permanent smears stained with
	• Easy to prepare	• Trichrome
	• Long shelf life	•Inadequate preservation of morphology of protozoan trophozoites
	• Good preservation of morphology of helminth eggs, larvae, protozoan cysts, and coccidia	• Can interfere with PCR, especially after extended fixation time
	• Suitable for concentration procedures and UV fluorescence microscopy	
	• Suitable for acid-fast, safranin, and chromotrope stains	
	• Compatible with immunoassay kits and UV fluorescence microscopy	
MIF (merthiolate-iodine-formaldehyde)	• Components both fix and stain organisms	• Not suitable for some permanent smears stained with trichrome
	• Easy to prepare	• Inadequate preservation of protozoan trophozoites
	• Long shelf life	• Iodine interferes with other stains and fluorescence
	• Useful for field surveys	Iodine may cause distortion of protozoa
	• Suitable for concentration procedures	
LV-PVA (low polyvinyl-alcohol)	• Good preservation of morphology of protozoan trophozoites and cysts	• Inadequate preservation of morphology of helminth eggs and larvae, coccidia, and microsporidia
	• Easy preparation of permanent smears stained with such as trichrome (solution both preserves organisms and makes them adhere to slides)	• Contains mercuric chloride
	• Preserved samples remain stable for several months	• Difficult and expensive to dispose of
		• Difficult to prepare in the laboratory
		• Not suitable for concentration procedures
		• Cannot be used
		• Not suitable for acid-fast, safranin and chromotrope stains

SAF (sodium acetate-acetic acid-formalin)	• Suitable for both concentration procedures and preparation of permanent stained smears	• Requires additive (e.g., albumin-glycerin) for adhesion of specimens to slides
	• Easy to prepare	• Permanent stains not as good as with PVA or Schaudinn's fixative
	• Long shelf life	
	• Suitable for acid-fast, safranin, and chromotrope stains	
	• Compatible with s immunoassay kits	
Schaudinn's Fixative	• Good preservation of of morphology of protozoan trophozoites and cysts	• Less suitable for concentration procedures
	• Easy preparation of permanent stained smears	• Contains mercuric mercuric chloride
		• Inadequate preservation of larvae, coccidia, and microsporidia larvae, coccidia, and microsporidia
		• Poor adhesion of liquid or mucoid specimens to slides
Modified PVAc opper or zinc	• Permanent smears can be made stained with trichrome	• Staining not consistent
	Zinc is preferred over copper	• Organism morphology may be poor
	No mercuric chloride	• Copper-morphology of cysts and trophozoites is poor
		• Zinc-better morphology but not comparable to LV-PVA
One-Vial Fixatives (such as Ecofix, Parasafe, Unifix, Proto-fix, STF, and others that may be available	• Concentrate and permanent smear can be made made out of one vial	• Certain one-vial fixatives must use certain stains
	Immunoassays can be done on most	• Color difference of stain
	No mercuric chloride	• Staining not always consistent
		• Sometimes more expensive than formalin and LV-PVA

7. Modified PVA (Copper- or Zinc-Based)

Modified PVA fixatives are mercury-free alternatives developed to comply with environmental and regulatory restrictions. They are primarily used for preparing trichrome-stained permanent smears, with zinc-based formulas offering superior morphological preservation over copper-based ones for clearer visualization of protozoa, all while ensuring greater safety and environmental friendliness compared to traditional mercury-containing PVA. The key limitations of modified PVA fixatives are staining inconsistencies and morphological preservation inferior to LV-PVA, with copper-based

formulas performing most poorly (Table 1). As a safe, mercury-free alternative, they are suitable for routine diagnostics under regulatory restrictions, with zinc-based variants preferred for better clarity, though they do not match the standard smear quality of traditional PVA [1].

8. One-Vial Fixatives (Ecofix, Parasafe, Unifix, Proto-fix, STF)

One-vial fixatives are versatile, mercury-free systems that allow both concentration and permanent smear preparation from a single vial, streamlining workflow and reducing errors. They are compatible with most immunoassays, enhancing their diagnostic utility while improving laboratory safety and regulatory compliance.

One-vial fixatives often require specific staining protocols that may not align with all traditional methods, can lead to inconsistent staining outcomes, and are generally more costly, potentially limiting adoption in resource-constrained labs. Despite these drawbacks, they provide a modern, mercury-free solution for preparing both concentrated specimens and permanent smears from a single vial, streamline workflow, and maintain compatibility with most immunoassays, making them a safe and efficient choice for diagnostic laboratories prioritizing safety and modern workflow efficiency [1].

9. Discussion

The selection of an appropriate fixative is critical in stool parasitology as it directly impacts morphological preservation, diagnostic accuracy, and compatibility with complementary techniques such as permanent staining, immunoassays, and molecular methods. As illustrated in Table 1, no single fixative meets all diagnostic requirements; each exhibits inherent advantages and limitations dictated by its chemical composition and intended purpose.

10% Formalin remains widely used as a general-purpose fixative due to its long shelf life and effective preservation of helminth eggs, larvae, protozoan cysts, and coccidia. Its mechanism, involving cross-linking of proteins, stabilizes overall cellular architecture but often fails to maintain delicate protozoan trophozoites, leading to cytoplasmic shrinkage and nuclear detail loss, consistent with previous reports [10-12]. Furthermore, prolonged formalin fixation can interfere with nucleic acid extraction and downstream PCR-based diagnostics, limiting its utility for molecular applications.

In contrast, Merthiolate–Iodine–Formaldehyde (MIF) is particularly suitable for field surveys and rapid screening because it combines fixation and initial staining within a single solution, facilitating early detection of eggs and cysts, even in low-burden samples [13]. However, iodine content may induce osmotic distortion and denaturation of protozoan structures, rendering MIF unsuitable for permanent trichrome-stained smears or fluorescence microscopy. These limitations confine its use primarily to epidemiological surveys rather than high-resolution laboratory analyses.

According to the National Committee for Clinical Laboratory Standards, Low Viscosity Polyvinyl Alcohol (LV-PVA) and Schaudinn's fixative are considered gold standards for permanent protozoan smears due to superior preservation of nuclear and cytoplasmic details [21]. The effectiveness arises from adhesive properties that stabilize delicate protozoan structures and maintain their morphology on slides. However, both fixatives exhibit poor preservation of helminth eggs and larvae and are incompatible with concentration procedures, highlighting their niche applications. Additionally, the inclusion of mercuric chloride raises environmental and safety concerns, as disposal is complex and costly.

Mercury-free modified PVA formulations using copper or zinc attempt to address safety concerns. While eliminating toxic mercury, these formulations often compromise staining consistency and fine morphological preservation. Copper-based formulations particularly reduce the integrity of cyst and trophozoite morphology, whereas zinc provides better preservation but still falls short of LV-PVA quality [23].

Sodium Acetate–Acetic Acid–Formalin (SAF) presents a versatile, mercury-free alternative compatible with both concentration methods and permanent stains. Its chemical composition supports acid-fast, safranin, and chromotrope staining, as well as immunoassay compatibility. Nonetheless, permanent staining quality is inferior to PVA or Schaudinn, and adhesive additives like albumin-glycerin are required to maintain slide adherence, making it a practical but not ideal fixative for detailed morphologic studies [10,17].

Finally, one-vial fixatives represent modern solutions aimed at simplifying workflow, reducing handling errors, and maintaining immunoassay compatibility. By integrating concentration and permanent smear preparation in a single vial, these fixatives improve laboratory efficiency and standardization. Despite these advantages, reliance on proprietary stains, occasional inconsistencies in staining quality, and limited preservation of delicate protozoan trophozoites indicate that one-vial fixatives are not yet complete replacements for classical methods.

Overall, the literature supports a purpose-driven selection of fixatives based on target parasite type, diagnostic goals, laboratory resources, and downstream applications. In many cases, a combination of fixatives or targeted use of different fixatives for specific workflow steps (e.g., formalin for concentration, PVA

for permanent protozoan smears) remains the most effective strategy to maximize diagnostic sensitivity and specificity.

10. Conclusion

No single fixative can meet all diagnostic needs in intestinal parasitology. While traditional fixatives like 10% formalin and LV-PVA remain standards for specific applications, newer alternatives such as SAF, modified PVA, and one-vial systems provide safer and more versatile options. Optimal fixative selection depends on parasite type, target life stage, diagnostic method, and operational context. Future research should focus on developing integrated fixatives that combine morphological preservation, molecular compatibility, safety, and environmental sustainability, facilitating both routine diagnostics and advanced research applications.

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REVIEW ARTICLE

Evaluating the Impact of Fusariotoxins on Poultry Feed Safety

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Abstract

This study provides new insights into the prevalence and toxicological impacts of Fusariotoxins in poultry feeds, highlighting the urgent need for integrated management strategies to protect poultry health and improve food safety. These toxins negatively affect poultry by inhibiting growth and increasing their susceptibility to diseases. increased research efforts and enhanced agricultural practices are crucial for mitigating toxin levels in poultry feed. One significant aspect of poultry management and welfare practices is the prevalence of Fusarium species poultry feed. These species are commonly found and produce mycotoxins that pose significant risks to both poultry and human health. Fusarium fungi are well-known contaminants of agricultural commodities, particularly grains and poultry feeds, due to their hazardous mycotoxins, which threaten poultry health and food safety. The contamination of poultry feeds with Fusarium species and their mycotoxins represents a critical issue due to the detrimental effects on poultry health and productivity. Health challenges associated with mycotoxins produced by Fusarium species are of great concern. This survey compiles findings from various studies regarding the occurrence of Fusarium species in poultry feeds and the associated toxicological effects. The examination of Fusarium species in poultry feeds has garnered attention within the veterinary and agricultural sciences due to concerns about poultry health and food safety. As this survey will illustrate, there is a pressing need for microbiological, biochemical, and agricultural expertise within the veterinary science domain to understand and mitigate the impact of these fungi on poultry feeds.

1. Introduction

These mycotoxins contaminate feed used in poultry production, leading to serious health implications across different poultry species, affecting growth, feed efficiency, immune function, and overall marketability of poultry products (Weaver *et al.*, 2020; (Ghaemmaghami, 2024; Ghany *et al.*, 2021). Fusarium species are commonly isolated from poultry feeds, with studies indicating that they often co-occur with other fungal genera (Ghaemmaghami *et al.*, 2016). For instance, Greco *et al.* illustrate that mycotoxins produced by

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Fusarium are prevalent in poultry diets, emphasizing that 67.5% of tested feeds were contaminated with these fungi (Greco *et al.*, 2014). Moreover, Nwiyi *et al.* reported that Fusarium species contribute significantly to mycotoxin contamination, including harmful compounds like deoxynivalenol (DON), fumonisins, and zearalenone (Nwiyi *et al.*, 2019). This highlights the potential public health concerns linked to the consumption of affected poultry products. Fusarium species are largely recognized for their capacity to produce mycotoxins, which are toxic secondary metabolites that pose severe health risks to both livestock and, consequently, humans through the food chain (Krnjaja *et al.*, 2021). The contamination of animal feed by Fusarium species occurs due to various factors, including environmental conditions, agricultural practices, and storage methods, which create conducive conditions for fungal growth (Antonissen *et al*, 2014). Mycotoxin contamination of feed is a significant concern in the poultry industry, where these toxins can lead to a variety of health issues and economic losses. Chronic exposure to these mycotoxins from Fusarium can impair the immune response, reduce growth performance, and increase vulnerability to infectious diseases in birds (Girgis and Smith, 2010; (Awad *et al.*, 2013). The pervasive nature of Fusarium species within feedstuffs, particularly grains, underscores the need for vigilant monitoring and management practices to mitigate their impact (Omori *et al.*, 2019;). Fusarium species, especially those that produce mycotoxins such as deoxynivalenol, zearalenone, and fumonisins, are well-known for negatively affecting the health and productivity of poultry (Fig. 1). Fusarium species are known to produce a variety of mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN), and fumonisins, which can adversely affect the health and productivity of poultry (Fig. 2). This section maps on impact of fusarium species toxins on poultry health and productivity, including growth rates and disease susceptibility, and strategies to mitigate these toxins in poultry feeds through feed additives and agricultural practices. This comparative key research questions on growth performance, immune function, mitigation strategies, disease susceptibility, and agricultural contamination providing a comprehensive understanding to inform effective management practices.

2. Statement of Purpose

Fusariotoxins are secondary metabolites produced by toxigenic fungi of the genus Fusarium. The important and commonly encountered fusariotoxins are trichothecenes, fumonisins, and zearalenone. Research consistently shows that various animal feeds, including those for pigs, ruminants, and poultry, are susceptible to contamination by Fusarium species. Analyses of feeds indicate that Fusarium, alongside other fungal genera like Aspergillus and Penicillium, dominates the fungal contamination landscape in animal feedstuffs (Krnjaja *et al.*, 2021; Hamasalim *et al.*, 2021; Witaszak *et al.*, 2019). Notably, Fusarium species are linked to the production of mycotoxins such as deoxynivalenol (DON), zearalenone (ZEN), and fumonisins, which have been associated with various adverse health outcomes in livestock, including immunosuppression, reproductive failures, and feed refusal (Gherbawy *et al*, 2024); (Pietsch *et al*, 2013); (Dorn *et al*, 2011); (Zhang *et al*, 2018). Understanding the impact of Fusarium mycotoxins on livestock entails exploring their prevalence, effects on animal health, and strategies for mitigation.

Fig. 1. Fusariutoxins affecting the human health and animal.

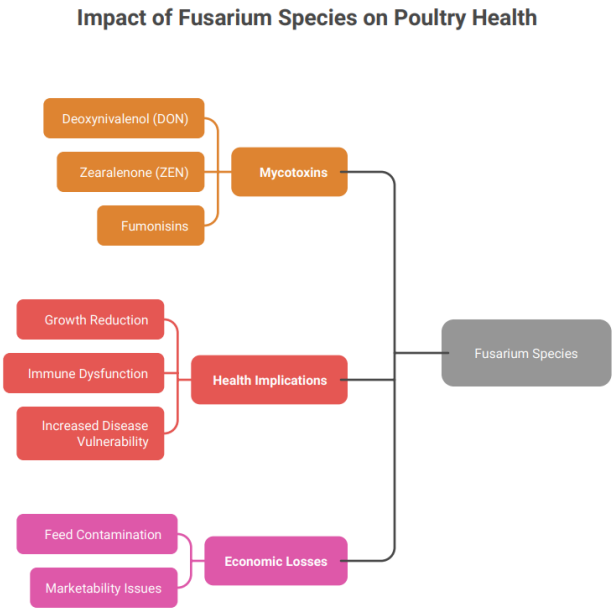

Fig. 2. Impact of fusarium species on poultry industry.

This response synthesizes the relevant literature to provide a detailed examination of the implications

of Fusarium fungi in livestock feed. The mycotoxins produced by Fusarium, notably fumonisins and zearalenone, have been well-documented to impair animal health. Fumonisins, particularly those produced by Fusarium verticillioides and Fusarium proliferatum, are known to induce various pathological conditions in poultry (Deepthi *et al.*, 2017), Mansur *et al.*, 2014). Monge *et al.* found that a significant percentage of raw materials and poultry feeds contained notable amounts of these mycotoxins, with fumonisins frequently exceeding safety thresholds (Monge *et al.*, 2012). This potential for toxicity underlines the necessity for rigorous quality control in feed production. The impact of Fusarium contamination extends beyond health implications to include economic concerns for poultry producers. As stated by Gherbawy *et al.*, the frequent detection of Fusarium species in stored poultry feeds can lead to reduced feed quality and subsequently affect meat production (Gherbawy *et al.*, 2024). Additionally, Kulcsár *et al.* demonstrated that acute exposure to Fusarium mycotoxins could lead to oxidative stress and negatively affect liver metabolism in laying hens (Kulcsár *et al.*, 2021). This indicates a direct relationship between mycotoxin contamination and poultry welfare, potentially leading to compromised economic returns on poultry farming. In geographical studies, variations in the prevalence of Fusarium species in poultry feeds have been observed, reflecting local agricultural practices and environmental conditions. For example, in a survey conducted by Krnjaja *et al.*, Fusarium species were identified in over half of the investigated feeds in Serbia, with conditions conducive to fungal growth identified (Krnjaja *et al.*, 2011). Similarly, Oliveira *et al.* noted that in Brazil, Fusarium was one of the most frequently encountered genera in poultry feeds, confirming the widespread nature of these fungi (Gherbawy *et al.*, 2024).

3. Fusarium Species in Animal Feed

Research has shown that multiple Fusarium species are prevalent in different types of animal feed. For instance, a study revealed that Fusarium spp. constitutes up to 36.1% of the fungal population found in swine feed samples, emphasizing their significant presence in feeds for livestock (MM, 2018, Nowrozi, *et al.*, 2018). Other studies corroborate the widespread occurrence of Fusarium species in ruminant feeds and poultry diets, wherein Fusarium spp., along with Aspergillus spp. and Penicillium spp., emerged as the primary genera identified (Gherbawy *et al.*, 2024; Hamasalim *et al.*, 2021). Specifically, Hamasalim *et al.* noted the dominance of Fusarium species in various feed matrices, shedding light on their systemic prevalence across species and geographic regions (Witaszak *et al.*, 2019). Fusarium mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN), and fumonisins, are among the most concerning for livestock producers. These mycotoxins can exert a range of toxic effects on animals, including reduced growth rates, reproductive issues, and increased susceptibility to diseases (Fig. 2). For instance, studies have shown that broiler chickens fed diets contaminated with Fusarium mycotoxins exhibited suppressed immune functions, evidenced by decreased organ sizes such as the bursa of Fabricius, a key component of the immune system (Cheng *et al.*, 2018; Dolenšek *et al.*, 2021). Furthermore, the mycotoxin ZEN is known for its estrogenic effects, which can lead to reproductive dysfunctions in animals, particularly in pigs that are highly susceptible to its effects (Fig. 3) (Chang *et al.*, 2017; Antonissen *et al.*, 2014).

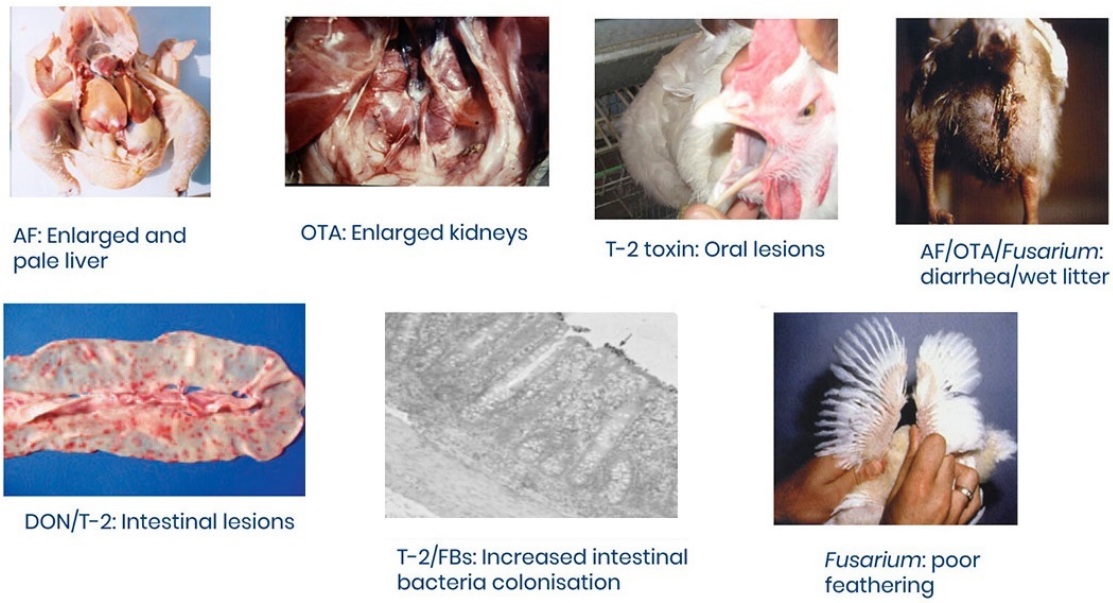

Fig. 3. Poultry health issues related to the mycotoxins produced by Fusarium species.

4. Consern by Fusarium

The dangers posed by these fungi are not limited to their mere presence; rather, they are associated with the production of mycotoxins, which can have devastating effects on animal health and productivity (Antonissen *et al.*, 2014). Mycotoxins, such as Deoxynivalenol (DON) and Zearalenone (ZEA), produced by Fusarium species, have been widely documented to impair reproductive performance, reduce feed intake, and induce toxic effects in various animal species (Pietsch *et al.*, 2013). For example, ZEA is an estrogenic mycotoxin linked to reproductive disorders in livestock, while DON is recognized for its effects in hampering feed consumption, often leading to what is termed "feed refusal" syndrome (Tan *et al.*, 2011). In a focused examination of ZEA, it was highlighted that its occurrence was significant across animal feeds, representing a critical area for further inspection and monitoring (Chang *et al.*, 2017). Reports indicate that Fusarium species are frequently encountered in crucial feed grains like maize and barley, which serve as staple components in livestock diets. The impact of climate change, particularly increasing humidity and temperature, could exacerbate Fusarium infections within grain crops, thereby elevating the risk of mycotoxin contamination in stored feeds (Stanciu *et al.*, 2015). Indeed, the intricate relationship between environmental conditions and Fusarium prevalence is underscored by findings that highlight how improper storage and handling practices increase susceptibility to fungal infestations (Jaimez *et al.*, 2004). The mechanism by which Fusarium mycotoxins exert their detrimental effects is multifaceted. Mycotoxins such as DON and ZEN primarily affect the gastrointestinal health of poultry, disrupting nutrient absorption and leading to inflammatory responses (Awad *et al.*, 2013). These toxins can lead to a range of clinical manifestations including decreased feed intake, poor weight gain, and ultimately, economic losses for producers (Wasan *et al.*, 2022; Antonissen *et al.*, 2014). The immunotoxic effects of these compounds exacerbate issues, rendering poultry more susceptible to secondary infections like necrotic enteritis and coccidiosis, thereby compounding health challenges within flocks (Girish and Smith, 2008; Antonissen *et al.*, 2014). Moreover, Fusarium-contaminated feeds can influence the metabolic processes in poultry, leading not only to physical health issues but also to altered behavioral responses due to discomfort or illness. When exposed to contaminated feed, birds often show signs of reduced activity and increased stress levels, further compromising their overall welfare and productivity (Girgis and Smith, 2010; Wingfield *et al.*, 2021) This stress response is critical as it can lead to decreased egg production in layers and lower growth rates in broilers, ultimately resulting in substantial economic repercussions across the poultry industry (Onajobı *et al.*, 2023).

The impact of Fusarium mycotoxins on livestock health is profound and multifaceted. In particular, zearalenone is known to cause estrogenic effects, leading to reproductive issues in animals, while fumonisins have been linked to neurotoxicity and carcinogenic effects (Zhang *et al.*, 2018; ENI *et al.*, 2018; Chang *et al.*, 2017). Additionally, chronic exposure to lower levels of these mycotoxins can lead to cumulative effects, ultimately compromising animal health and productivity (Aguín *et al.*, 2013; Wu *et al.*, 2025). Studies of dietary impact have demonstrated that even moderate levels of fusariotoxins can disrupt growth and immunity in various animal species, emphasizing the imperative for stringent control measures in feed production (Wagacha *et al.*, 2010). Young broiler chickens are particularly sensitive to Fusarium mycotoxins, which can result in poor intestinal health and compromised immune function. Research has shown that exposure to chronic levels of feed contaminated with these mycotoxins can lead to reduced height of intestinal villi, impacting nutrient absorption and overall health (Weaver *et al.*, 2020; Fraeyman *et al.*, 2018). It is also established that contaminated feed correlates with an increase in gastrointestinal infections, further exacerbating health issues in young poultry (Weaver *et al.*, 2020; (Ghaemmaghami *et al.*, 2018). Furthermore, the consumption of mycotoxin-laden feed is linked to adverse performance outcomes, including reduced weight gain and poor feed conversion ratios in affected birds, significantly hampering economic viability for poultry producers (Nwiyi *et al.*, 2019; Gherbawy *et al.*, 2024; Alders *et al.*, 2018). In addition to acute effects, chronic exposure to Fusarium mycotoxins can lead to long-term health issues. The combined effects of ZEN and other trichothecenes have synergistic impacts on poultry, exacerbating immune suppression and further increasing susceptibility to infectious diseases (Wu *et al.*, 2021; (Ghaemmaghami *et al.*, 2018; Liu & Applegate, 2020). Research indicates that prolonged exposure to subclinical levels of mycotoxins can impair growth performance and immunological responses, potentially leading to increased mortality rates in affected populations (Danbappa *et al.*, 2018; Meena *et al.*, 2020; Polák and Paszczyk, 2021). Therefore, understanding the mechanisms of Fusarium toxicity is critical for developing effective prevention and intervention strategies within the poultry industry. Moreover, mycotoxin interactions with other microbial infections present further challenges. For example, the coccidiosis caused by Eimeria species can be aggravated by the immunosuppressive effects of Fusarium toxins, leading to compounded economic losses within the poultry sector (Gómez-Osorio *et al.*, 2024; Yang *et al.*, 2019). Addressing mycotoxin contamination should involve multi-faceted approaches integrating nutrition, management practices, and biosecurity mea-

sures aimed at reducing pathogen load and alleviating stressors on poultry (El-Sharkawy *et al.*, 2023).

5. Methods for Assessing the Prevalence of Fusarium

Analytically, numerous methods for assessing the prevalence of Fusarium and their mycotoxins in animal feed have been employed, ranging from traditional microbiological culturing techniques to advanced molecular diagnostics. For instance, the use of real-time PCR has emerged as a powerful tool to detect and quantify specific mycotoxin-producing Fusarium strains within feed samples, thereby enhancing the ability to monitor and manage these contaminants effectively (Sohlberg *et al.*, 2022). Moreover, there is a growing emphasis on the need for consistent monitoring practices and regulatory frameworks to oversee mycotoxin levels in animal feeds, particularly in regions where agricultural practices may enable high contamination rates (Latham *et al.*, 2023). In response to these challenges, various strategies have been proposed to mitigate the impact of Fusarium and their mycotoxins on animal feeds. Among these are biocontrol measures, such as the employment of specific microbial strains that can reduce fungal biomass and the associated toxin production in contaminated feeds (Valiullin *et al.*, 2023). Moreover, developing robust pre- and post-harvest strategies can aid in minimizing Fusarium infection rates, including implementing crop rotation practices and using resistant plant varieties (Simões *et al.*, 2023). The environmental conditions under which feeds are stored and processed significantly influence the likelihood of Fusarium contamination. High moisture content and fluctuating temperature conditions are particularly conducive to the growth of Fusarium species and subsequent mycotoxin production (Tan *et al.*, 2011; Pietsch *et al.*, 2013). Studies show clear correlations between poor storage conditions and increased levels of mycotoxins in animal feeds, suggesting that improving storage practices could mitigate these risks (Deepthi *et al.*, 2017; Herkert *et al.*, 2019; Krnjaja *et al.*, 2011). Furthermore, specific agricultural practices, such as the use of susceptible crop varieties or inadequate crop rotation, can exacerbate the prevalence of Fusarium infections in feed crops, thus elevating the risk of contamination in the feed supply chain (Pfordt *et al.*, 2020; Dolenšek *et al.*, 2021). Fusarium species, such as *F. graminearum* and *F. culmorum*, are well documented for their mycotoxin production capabilities. Their prevalence in grains used for animal feed highlights the necessity for regular monitoring and assessment strategies to ensure feed safety (Wang *et al.*, 2022; Dorn *et al.*, 2011; Krnjaja *et al.*, 2011). Research also indicates that animal feed containing higher levels of contaminated cereals potentially harbors mycotoxins exceeding permissible limits set by health authorities, thereby increasing the risk of mycotoxicosis in affected animals (Noman, 2022; Chang *et al.*, 2017). For example, zearalenone and fumonisins have been observed at concerning concentrations in commercial pet food and fish feed, illuminating the widespread nature of Fusarium contamination (Gherbawy *et al.*, 2024; Pietsch *et al.*, 2013).

A critical aspect of managing Fusarium contamination involves the implementation of effective mitigation strategies in feed processing and storage. These may include the use of mycotoxin-binding agents and probiotics that can reduce the bioavailability of mycotoxins, thus mitigating their harmful effects on animal health (Hussein & Saadullah, 2023; Gkiorsos *et al.*, 2022; Ostrý *et al.*, 2010). Advances in molecular biology and biochemistry have facilitated the development of detection methods that allow for the rapid identification of toxigenic Fusarium species and their associated mycotoxins in feed samples (Villafana *et al.*, 2019; Brown *et al.*, 2012). Such methodologies are essential for the proactive management of feed safety risks related to Fusarium contamination (Fig. 4)

Fig. 4. Management of poultry feed safety risks related to fusariotoxins.

6. Economic Consequences of Fusarium

The economic repercussions of Fusarium contamination are also noteworthy. The financial costs associated with degraded animal health and productivity directly correlate to mycotoxin levels present in feeds, ultimately translating into reduced economic returns for livestock producers (Antonissen *et al.*, 2014). It is estimated that over 2% of grains harvested each year may become economically unviable due to severe contamination with mycotoxins, underscoring the need for proactive measures in agricultural management (Wu *et al.*, 2021). Furthermore, regular surveillance and public health initiatives focused on monitoring mycotoxin levels in animal feeds are essential for ensuring food safety and protecting human health (Latham *et al.*, 2023). A thorough understanding of the epidemiology of Fusarium species across agricultural settings can illuminate the complex interactions between fungi, the products involved in animal feed, and their broader ecological implications. Continuing advances in genomic technologies and phylogenetics are pivotal for providing deeper insights into the pathogenicity and diversity of Fusarium species (Mitterbauer *et al.*, 2003), which could enable more targeted management practices in animal nutrition and health. The presence of mycotoxins such as fumonisins and ZEN can also interfere with endocrine function in poultry (Al-Gabr *et al.*, 2018). Zearalenone, in particular, acts as a non-steroidal estrogenic compound, which can disrupt reproductive processes and lead to significant implications for egg production and hatching rates (Al-Gabr *et al.*, 2018) Purnamasari & Purnomo, 2022). Such hormonal disruptions could foster increased infertility and developmental problems in offspring, thereby affecting future production capacities of poultry flocks (Al-Gabr *et al.*, 2018).

In addition to health implications for livestock, the economic burden of Fusarium mycotoxin contamination is significant. Losses occur both from reduced feed quality and the potential for decreased animal performance (Tardieu *et al.*, 2019; Stanciu *et al.*, 2015). This reality underscores the importance of not only preventive measures at the agricultural level but also regulatory oversight ensuring that feed products meet safety standards for mycotoxin levels (Hudu *et al.*, 2024). Research continues to evolve, with emphasis placed on understanding the complex interactions between environmental factors and the pathogenicity of Fusarium species, which will assist in developing improved strategies for managing mycotoxin risks in animal feeds (Sadiq *et al.*, 2019; Watanabe *et al.*, 2011; Simões *et al.*, 2022). The economic ramifications tied to Fusarium mycotoxins in livestock are considerable. The direct health impacts on livestock can lead to decreased feed efficiency and growth rates, which, in turn, can have far-reaching consequences for production systems. Losses attributed to poor livestock growth, veterinary expenses for untreated illnesses, and reduced reproductive success can accumulate swiftly into significant economic burdens for livestock producers (Griessler *et al.*, 2010; Vandicke *et al.*, 2021; Bryła *et al.*, 2022). For instance, the co-occurrence of mycotoxins, where different toxins are present simultaneously in feed, can compound these effects, further challenging the maintenance of livestock health (Fig. 5) (Stanciu *et al.*, 2015).

7. Methods for Reducing Fusarium

To address the challenges posed by Fusarium mycotoxins, several mitigation strategies are recommended. These include stringent feed management practices that involve proper storage conditions to prevent mold growth, the inclusion of mycotoxin binders in feed formulations, and regular monitoring of feed for contamination (Wingfield *et al.*, 2021; Nwiyi *et al.*, 2019).

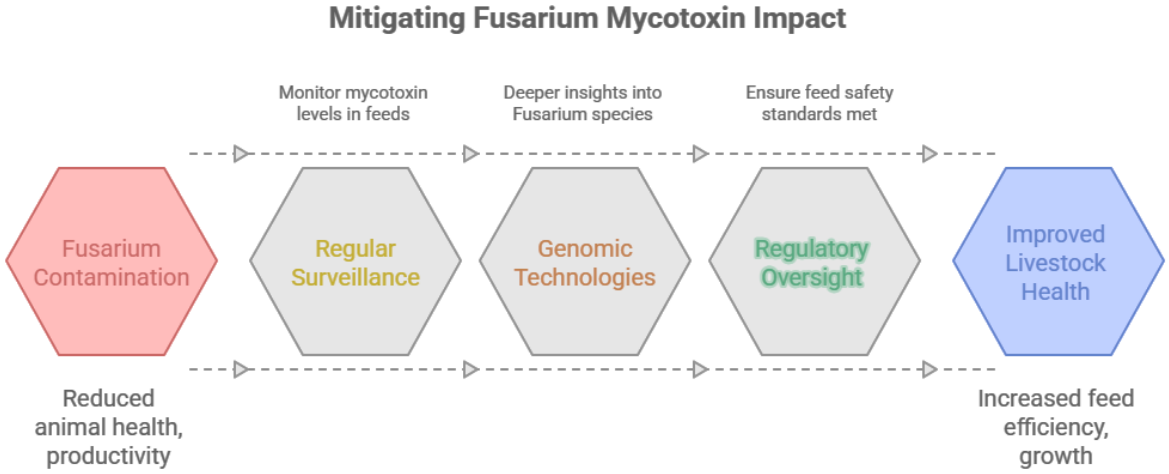

Fig. 5. Diagrams for mitigating fusariotoxins.

Additionally, the introduction of probiotics and other dietary supplements is being explored as a means to bolster immune responses and mitigate the adverse effects of mycotoxin exposure (Yang *et al.*, 2019; Ahmad *et al.*, 2022). These strategies are essential to reducing the prevalence of mycotoxin exposure and preserving both poultry health and economic viability. The mitigation of Fusarium contamination in poultry feeds is imperative. Recent studies exploring the effects of treatments, such as gamma irradiation, have shown promise in reducing mycotoxin levels, thereby improving feed safety (Deepthi *et al.*, 2017). Understanding the dynamics of Fusarium contamination can aid in developing effective feed management strategies, which may include rigorous monitoring, genetic selection of feed crops resistant to pathogen colonization, and the application of mycotoxin binders or inhibitors to negate the harmful effects of mycotoxins. The impact of Fusarium mycotoxins extends beyond immediate health risks, linking to broader concerns over food safety and public health. Continuous research efforts are necessary to elucidate the long-term implications of low-level mycotoxin exposure within animal populations, providing a comprehensive understanding of potential carryover effects into the human food chain. The occurrence of Fusarium mycotoxins necessitates a robust and multi-faceted approach to address both the immediate and long-term challenges posed to livestock health and productivity. By integrating good agricultural practices, vigilant feed monitoring, and innovative mitigation strategies, stakeholders in the agriculture sector can better manage the risks associated with Fusarium contamination in livestock feed (Fig. 6).

Fig. 6. Control of fusariotoxin strategies in poultry feed.

Regulatory entities worldwide recognize the relevance of Fusarium and strive to create frameworks for monitoring and managing mycotoxin levels in the food and feed supply chains. Various nations have implemented guidelines for acceptable limits of specific mycotoxins, including zearalenone and fumonisins, acknowledging their detrimental effects on both human and animal health (Valiullin *et al.*, 2023; Greeff-Laubscher *et al.*, 2019; Salem *et al.*, 2015). This highlights the global challenge of securing safe agricultural products in light of environmental conditions favorable to fungus propagation and toxin production. The process by which Fusarium mycotoxins contaminate livestock feed begins in the field, where environmental conditions such as high humidity and improper storage practices facilitate fungal growth and subsequent toxin production. For example, the presence of Fusarium species has been recorded in various forage types, indicating a potential risk as these samples are frequently used as livestock feed (Krnjaja *et al.*, 2021; Ekwomadu *et al.*, 2021). Factors influencing mycotoxin accumulation include agricultural practices, such as crop rotation and the use of resistant plant cultivars, although challenges persist due to environmental variability (Wegulo, 2012; Tola and Kebede, 2016). Consequently, the contamination of livestock feed with Fusarium mycotoxins is not only a direct result of fungal presence but is exacerbated by inadequate management and storage conditions. To mitigate the health risks associated with Fusarium mycotoxin contamination, various strategies have been proposed. Some include the implementation of effective feed processing techniques, the use of mycotoxin adsorbents in animal diets, and the dietary inclusion of natural feed additives (Kulcsár *et al.*, 2021; Salazar *et al.*, 2020; Latham *et al.*, 2023). Recent advancements highlight the role of certain microorganisms and enzymes in degrading mycotoxins, thus providing a biological avenue for detoxifying contaminated feed materials (Gari, 2024; Zhao *et al.*, 2023). Furthermore, strict monitoring and adherence to established regulatory limits for mycotoxin concentrations in animal feed can help limit exposure and the associated risks to both livestock and human consumers of animal products (Jensen *et al.*, 2019; Deepthi *et al.*, 2017; Brodal *et al.*, 2020). In addition to interventions at the feed level, it is critical to bolster research into genetic approaches and breeding programs to develop crops that are resistant to Fusarium infections (Wegulo, 2012; Tola & Kebede, 2016). Such advances could minimize initial contamination rates at the source, which remain pivotal in ensuring the safety and quality of animal feed. Fusarium species are prolific in various environments, particularly under incorrect storage conditions, which fosters their growth and toxin production (Onajobı *et al.*, 2023; (El-Sharkawy *et al.*, 2023). The presence of Fusarium species in feed signals not only contamination but also poses risks for

public health authorities due to potential residues in animal products (El-Sharkawy *et al.*, 2023). To mitigate these risks, appropriate management strategies must be employed, such as regular monitoring of feed for mycotoxin levels and the implementation of feed additives that can bind mycotoxins (Weaver *et al.*, 2020; Ghany *et al.*, 2021). Probiotics and yeast products have also been suggested as helpful adjuncts in dietary formulations, as they can enhance gut health and reduce the negative impacts of mycotoxin ingestion by promoting a healthier gut microbiome (Wingfield *et al.*, 2021).

8. Future Research

Given the inherent complexity in the interaction between Fusarium species and animal feeds, future research must prioritize integrative approaches that encompass agricultural science, animal health, and food safety. Collaborative efforts across fields can lead to enhanced methodologies for the detection, prevention, and remediation of Fusarium mycotoxin contamination, thereby safeguarding both animal welfare and public health outcomes. Ultimately, the persistence of Fusarium species in animal feeds accentuates an ongoing challenge that necessitates a comprehensive understanding of fungal biology, agricultural practices, and the implications of mycotoxin exposure on health and food safety systems. This understanding can inform evidence-based policies and management practices aimed at reducing the prevalence of these mycotoxins in food production systems globally, thus ensuring sustainable agricultural practices and enhancing animal and human health. Research has also highlighted the importance of storage conditions in preventing mycotoxin production (Onajob1 *et al.*, 2023; Hof, 2020). Implementing biosecurity measures and decreasing the microbial load in poultry houses can significantly reduce the scavenging opportunities for Fusarium spp. These fungi thrive in warm, moist conditions that are typical in poorly ventilated poultry housing, thus focusing on improved airflow and moisture control can be beneficial (Sokolovic *et al.*, 2022; Grace *et al.*, 2024). Hence, addressing these environmental factors must be part of an integrated response to reducing the adverse impacts of Fusarium on poultry health.

Current research underscores the necessity for systematic assessment of mycotoxin levels in relation to poultry feed production, storage, and feeding practices. This can include routine microbiological evaluations to detect Fusarium species and assess mycotoxin levels, thus enabling proactive management to ensure poultry wellbeing and reduce the risk of economic loss due to health-related performance declines (Omori *et al.*, 2019; Krnjaja *et al.*, 2019; Cooper *et al.*, 2025). Regulatory standards for permissible mycotoxin levels in feed should be strictly enforced to minimize poultry exposure and subsequent health complications (Sohail, 2020).

9. Conclusion

In conclusion, Fusarium significantly impacts poultry health primarily through the production of harmful mycotoxins. The implications of mycotoxin exposure necessitate comprehensive monitoring and management strategies that incorporate nutritional interventions and improved biosecurity practices to safeguard poultry health and productivity. Collectively, these efforts not only promote the welfare of poultry but also serve to protect public health by ensuring the safety of poultry-derived products. The ongoing survey of Fusarium species in animal feeds reveals their widespread presence and significant role in agricultural systems. Efforts to characterize Fusarium's impact on feed quality, animal health, and economic viability continue to be paramount as we seek innovative solutions to mitigate the challenges posed by these mycotoxins. Understanding the interactions between Fusarium species, environmental conditions, and agricultural practices will be critical for improving animal feed safety and health outcomes moving forward. Also, the health of poultry is significantly impacted by Fusarium species through the production of various mycotoxins. The multifaceted effects of these contaminants not only hinder growth and productivity but also compromise the immune systems of affected birds, increasing their susceptibility to infections. Acknowledging and addressing these challenges through improved management practices and further research into mitigation strategies is essential for sustainable poultry production. Continued vigilance and innovation are required to safeguard poultry health against the ever-present risks posed by Fusarium mycotoxins.

And finally, it can be stated that the presence of Fusarium species and their mycotoxins in poultry feeds poses a multifaceted threat to poultry health, productivity, and safety. Continuous surveillance and improved agricultural practices are vital in addressing this issue, as are advancements in feed processing technologies aimed at reducing mycotoxin prevalence. Also, employing experts in the field of animal feed hygiene can greatly assist the producer in this regard.

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ORIGINAL ARTICLE

The Effects of Saccharin and Hydroalcoholic Extract of *Stevia Rebaudiana* on the Reproductive System in Adult Male Rats

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Abstract

The increasing consumption of artificial sweeteners such as saccharin has raised concerns regarding their potential adverse effects on male reproductive health. Natural alternatives like Stevia rebaudiana may offer safer options. This study aimed to investigate the effects of saccharin and hydroalcoholic extract of Stevia rebaudiana, both individually and in combination, on reproductive parameters in adult male rats. Twenty-four adult male Wistar rats (200-250 g) were randomly divided into four groups (n=6): control (distilled water), saccharin (5 mg/kg body weight), stevia (400 mg/kg body weight), and saccharin+ stevia (5 mg/kg + 400 mg/kg body weight) administered via gavage for 48 days. Following treatment, testicular biometric parameters, sperm parameters (count, viability, motility, DNA damage, morphology), histomorphometry (tubular differentiation index, repopulation index, spermiogenesis index, seminiferous tubule diameter, germinal epithelium height, testicular capsule diameter), and malondialdehyde (MDA) levels were evaluated. Saccharin treatment significantly decreased sperm count, viability, and motility ($p<0.05$), increased DNA damage and abnormal sperm percentage ($p<0.05$), reduced tubular differentiation index, repopulation index, and spermiogenesis index ($p<0.05$), decreased seminiferous tubule diameter and germinal epithelium height ($p<0.05$), and elevated MDA levels ($p<0.05$) compared to the control group. Co-administration of stevia with saccharin ameliorated these adverse effects, with parameters approaching control group levels. Saccharin induced oxidative stress and detrimental effects on male reproductive parameters in rats. Stevia rebaudiana extract demonstrated protective properties, potentially through antioxidant mechanisms and improved testicular blood flow, suggesting its suitability as a safer alternative sweetener with reproductive health benefits.

1. Introduction

In the last decade, there has been a substantial increase in the consumption of food products containing calorie-free sweeteners to address health problems associated with obesity and diabetes. Numerous research studies have focused on sweetener consumption in obese and diabetic patients, primarily aiming to reduce calorie

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intake while maintaining dietary satisfaction (Gardner *et al.*, 2012).

The growing prevalence of chronic diseases such as diabetes and obesity has amplified the importance of sugar substitutes as alternatives to sucrose. Sugar substitutes are food additives that replicate or exceed the sweetness of sucrose with minimal or no caloric content. They are broadly classified into artificial sweeteners—including sucralose, saccharin, aspartame, and cyclamate—and natural sweeteners such as stevia (Tandel, 2011). The Food and Drug Administration (FDA) has approved five artificial sweeteners: saccharin, acesulfame, aspartame, neotame, and sucralose. Additionally, stevia has been recognized as a low-calorie natural sweetener (Mats and Popkin, 2009).

The human body responds to artificial sweeteners in complex ways. Non-nutritive sweeteners possess sweetness intensities significantly greater than sucrose or high-fructose corn syrup. Frequent consumption of these artificial sweeteners can overstimulate sugar receptors, potentially reducing tolerance for naturally sweet foods such as fruits and non-sweet foods like vegetables (Farid *et al.*, 2020).

Animal studies have demonstrated addictive properties associated with artificial sweeteners (Yang, 2010). Lenoir *et al.* (2007) reported that the intense sweetness of saccharin could exert more powerful rewarding effects than cocaine, even in drug-sensitized and addicted rats (Lenoir *et al.*, 2007). Mathor *et al.* (2020) found that individuals with type 2 diabetes who consumed artificial sweeteners exhibited greater insulin resistance compared to non-consumers (Mathor *et al.*, 2020). Furthermore, research has indicated that artificial sweeteners, depending on dosage and duration of consumption, can induce pro-inflammatory effects alongside digestive disorders, correlating with alterations in digestive hormones that regulate bowel motility (Bueno-Hernández *et al.*, 2019). Emamat *et al.* (2020) further emphasized that artificial sweeteners play a significant role in altering gut microbiota and inducing dysbiosis (Emamat *et al.*, 2020).

Male reproductive health has experienced significant deterioration in recent years, with the quality and function of male reproductive cells declining substantially. Male infertility rates have increased by approximately 50% (Esmaelzadeh *et al.*, 2007). Male reproductive health is influenced by numerous environmental and genetic factors, with nearly every aspect of modern lifestyle potentially affecting male fertility. Among the most critical lifestyle factors is nutrition, which serves as a determining factor in normal reproductive function. Substantial evidence suggests a direct relationship between poor nutrition and reproductive problems.

Over the past decade, the escalating use of food additives has become a growing concern. Although many food additives are generally recognized as safe (GRAS) for human consumption, considerable concerns remain regarding their potential health effects. Multiple studies have documented adverse effects of food additive consumption on the reproductive system, making it essential to investigate these compounds' effects on male reproductive function for preserving male fertility.

Several hypotheses have been proposed regarding the effects of non-caloric sweeteners. One hypothesis suggests that low-energy sweet foods help prevent weight gain, while alternative theories propose that consuming non-caloric sweetened beverages increases appetite and subsequent food intake. Numerous studies provide conflicting results regarding the effects of artificial sweeteners on food and beverage consumption, body mass increases, and blood glucose levels. However, limited research has examined the effects of artificial sweeteners on the male reproductive system and testicular morphology.

Therefore, our objective is to investigate the effects of saccharin on the male reproductive system and evaluate its potential replacement with a natural sweetener such as stevia, while adhering to ethical principles and animal rights.

2. Materials and Methods

2.1. Animals

In this study, 24 adults male Wistar rats weighing 200-250 g were obtained from the Laboratory Animal Center of the Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, Iran. All rats were transferred to the animal facility one week prior to experimental commencement for environmental adaptation. Animals were housed individually in standard cages with ad libitum access to food and water, maintained at a temperature of 22-26°C, relative humidity of 40-70%, and a 12-hour light/12-hour dark photoperiod. Rats were randomly allocated into four groups, with six animals per group (Zeynab Saberi *et al.*, 2021).

2.2. Experimental Groups

Doses were determined based on acceptable daily intake guidelines for saccharin and the hydroalcoholic extract of Stevia rebaudiana.

Group 1 (Control): Received 3 ml of distilled water via oral gavage daily for 48 days.

Group 2 (Saccharin): Received 5 mg/kg body weight of saccharin via oral gavage daily for 48 days.

Group 3 (Stevia): Received 400 mg/kg body weight of Stevia rebaudiana hydroalcoholic extract via oral gavage daily for 48 days.

Group 4 (Saccharin+ stevia): Received 5 mg/kg body weight of saccharin combined with 400 mg/kg body weight of Stevia rebaudiana hydroalcoholic extract via

oral gavage daily for 48 days (Zeynab Saberi *et al.*, 2021).

2.3. Sampling Procedures

One day after the end of the treatment period, the rats were weighed again. After anesthesia with ketamine (100 mg/kgBW) and xylazine (10 mg/kgBW) and euthanasia by cervical dislocation, the abdominal cavity was opened and blood samples were collected directly from right ventricle. To examine the morphometric indices of the testes, after opening the abdominal cavity with a scalpel, forceps, and scissors, and retracting the intestines to visualize the testes, the left and right testes, along with the epididymis and vas deferens, were removed from the body, washed in physiological saline, and their weights were measured and recorded using a digital balance with 0.01 sensitivity (Babaei *et al.*, 2015).

By determining their weight, the ratio of testicular weight to body weight (GSI x 100) was determined. Also, the thickness, length, and width of the testes were measured, determined, and recorded with a digital caliper.

Investigation of sperm parameters: In order to study the number, viability, motility and morphology of sperm, epididymal tails were cut and transferred to a microtube containing 1 ml of pre-heated HTF medium. Sperm suspension was used to analyze different sperm parameters. The carried-out investigations include counting the mean number of sperms per unit volume and constant dilution using hemocytometer slides, determining the percentage of sperm motility, the percentage of sperm viability (Eosin-Nigrosin staining), the amount of DNA damage (using acridine orange staining method) and nucleus maturation rate (using aniline blue staining method). **Histological evaluations**: Testicular tissue samples fixed in 10% buffered formalin and after tissue passage and preparation of tissue sections (7 μm thickness), hematoxylin-eosin astaining was obtained, and all samples were evaluated in terms of tissue and morphometric changes in multiple magnifications (400x and 1000x) (20). The mean seminiferous tubules diameter (STsD), seminiferous tubules lumen diameter (STsLD), germinal epithelium height (GEH), testicular capsule diameter (TCD) in micrometers were analyzed by Dino-Lite microscope and DinoCapture software (version 2). In order to check the tubular differentiation index (TDI), the percentage of seminiferous tubules with more than three layers of differentiated germ cells from type A spermatogonia were counted and TDI was considered positive. Moreover, to calculate the sperm repopulation index (RI), the ratio of active spermatogonia (type B spermatogonia with dark nuclei) to inactive spermatogonia (type A spermatogonia with light nuclei) was calculated in seminiferous tubules. In order to determine the spermatic index (SI), the percentage of seminiferous tubules with normal spermatogenesis was considered as positive SI. In addition, the tubular degeneration percentage (TDP) was calculated in different experimental groups (Babaei *et al.*, 2018).

Oxidative parameter: Reactive Oxygen Species (ROS) attack polyunsaturated fatty acids present in cell membranes, leading to lipid peroxidation. To determine the extent of lipid peroxidation, MDA production levels were measured in serum samples. Malondialdehyde measurement will be based on reaction with thiobarbituric acid, extraction with normal butanol, spectrophotometric absorption measurement, and comparison of absorption with a standard curve. (Babaei *et al.*, 2018)

Data analysis: The results of this study were statistically evaluated using SPSS version 19 and the results were expressed as mean±standard deviation. To compare between groups, one-way analysis of variance followed by Tukey's supplementary test was used and $p<0.05$ was considered significant.

2.4. Ethical Considerations

The ethical approval for this study was issued from the Committee on Research Ethics of Bu-Ali Sina University, based on the Ethical Guidelines of Research and in accordance with institutional and national guidelines for the care and use of laboratory animals. All animal experiments complied with ARRIVE guidelines (https://arriveguidelines.org) concerning the use of animals in research.

3. Results

3.1. Biometric Parameters

Comparison of body weight, left testicular weight, gonadosomatic index (GSI), and left testicular dimensions (length, width, diameter) among control and experimental groups revealed no significant differences ($p>0.05$) (Table 1).

3.2. Sperm Parameters

Data analysis demonstrated that sperm count in the saccharin-treated group significantly decreased compared to the control and other treatment groups ($p<0.05$). Sperm viability in the saccharin-receiving group significantly decreased compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Sperm motility in the saccharin-receiving group exhibited a significant decrease compared to the control and other experimental groups ($p<0.05$). Although sperm motility showed an increasing trend in the stevia group compared to the control and saccharin+ stevia groups, this increase was not statistically significant ($p>0.05$) (Table 2).

Table 1
Comparison of mean testicular biometric parameters in experimental groups.

	BW (gr)	Left Testis W (gr)	GSI (%)	Left testis length (mm)	Left testis wide (mm)	Left testis diameter (mm)
Control	$210/8 \pm 33/43^a$	$1/39 \pm 0/09^a$	$0/006 \pm 0/001^a$	$19/2 \pm 1/82^a$	$11/34 \pm 1/03^a$	$10/51 \pm 0/53^a$
Saccharin	$241 \pm 6/55^a$	$1/39 \pm 0/04^a$	$0/006 \pm 0^a$	$20/66 \pm 0/18^a$	$11/69 \pm 0/34^a$	$10/33 \pm 0/98^a$
Stevia	$201/32 \pm 32/05^a$	$1/2 \pm 0/17^a$	$0/006 \pm 0/0006^a$	$19/08 \pm 1/76^a$	$10/84 \pm 0/97^a$	$10/04 \pm 1/08^a$
Saccharin+ stevia	$222/33 \pm 17/21^a$	$1/34 \pm 0/18^a$	$0/005 \pm 0/0006^a$	$19/6 \pm 1/41^a$	$11/77 \pm 0/35^a$	$10/08 \pm 0/22^a$

Non-similar English letters indicate significant differences in each column ($p<0.05$). **BW**: Body Weight; **W**: Weight; **GSI**: Gonadosomatic Index.

Table 2
Comparison of mean sperm parameters in experimental groups.

	Count (10^6 ml)	Viability (%)	Motility (%)	Maturity (%)	DNA Damage (%)	Abnormality (%)
Control	$62/6 \pm 3/21^a$	$83/2 \pm 3/42^a$	$79/6 \pm 4/83^a$	$99/2 \pm 0/84^a$	$5/6 \pm 2/7^a$	$4/6 \pm 2/7^a$
Saccharin	$54 \pm 4/47^b$	$62 \pm 5/34^b$	$55/8 \pm 2/77^b$	$96/2 \pm 1/78^b$	$12 \pm 2/55^b$	$10/4 \pm 1/14^b$
Stevia	$64/4 \pm 3/19^a$	$83/2 \pm 3/96^a$	$81/6 \pm 6/43^a$	$98/8 \pm 1/3^{ab}$	$6/8 \pm 3/27^a$	$5/4 \pm 2/75^a$
Saccharin+ stevia	$63 \pm 2/91^a$	$84/2 \pm 4/15^a$	$79/6 \pm 5/13^a$	$98/4 \pm 1/82^{ab}$	$8 \pm 3/32^{ab}$	$7/8 \pm 3/11^{ab}$

Non-similar English letters indicate significant differences in each column ($p<0.05$).

Table 3
Comparison of mean histomorphometric parameters in experimental groups.

	TDI (%)	RI (%)	SI (%)	STsD (μm)	GEH (μm)	TCD (μm)	DTP (μm)
Control	$88/8 \pm 1/35^a$	$86/82 \pm 4/78^a$	$92/5 \pm 2/78^a$	$365/83 \pm 8/25^a$	$128/57 \pm 10/65^a$	$19/68 \pm 2/84^a$	$5/2 \pm 2/39^a$
Sakharin	$70 \pm 3/57^b$	$67 \pm 4/59^b$	$70/8 \pm 3/58^b$	$303/75 \pm 11/39^b$	$99/59 \pm 8/99^b$	$28/61 \pm 2/95^b$	$10/4 \pm 1/14^b$
Stevia	$86/2 \pm 6/22^a$	$82/9 \pm 2/88^a$	$85/2 \pm 4/98^a$	$371/25 \pm 11/45^a$	$132/41 \pm 11/86^a$	$20/84 \pm 1/95^a$	$6/8 \pm 1/92^a$
Sakharin+ Stevia	$83/3 \pm 3/95^a$	$83/2 \pm 2/77^a$	$86/5 \pm 4/97^a$	$365/18 \pm 13/63^a$	$137/75 \pm 7/7^a$	$21/11 \pm 0/84^a$	$7/6 \pm 3/64^{ab}$

Non-similar English letters indicate significant differences in each column ($p<0.05$). **TDI**: Tubular Differentiation Index; **RI**: Repopulation Index; **SI**: Spermiogenesis Index; STsD: Seminiferous Tubule Diameter; **GEH**: Germinal Epithelium Height; **TCD**: Testicular Capsule Diameter; **DTP**: Degenerated Tubule Percentage.

Evaluation of sperm nuclear maturity percentage revealed a significant decrease in the saccharin-receiving group compared to the control group, indicating a higher proportion of immature sperm in the saccharin group ($P<0.05$). However, no significant differences were observed with other treatment groups ($P>0.05$). Assessment of sperm DNA damage demonstrated a significant increase in the saccharin-receiving group compared to the control and stevia groups ($p<0.05$). Conversely, the level of sperm with damaged DNA in the saccharin+ stevia group showed a decrease compared to the saccharin group, though this difference was not statistically significant ($p>0.05$) (Table 2).

Measurement of abnormal sperm count revealed a significant increase in the saccharin-receiving group compared to the control and stevia-receiving groups ($p<0.05$). The level of abnormal sperm in the saccharin+ stevia group decreased compared to the saccharin group, but this difference was not statistically significant ($p>0.05$) (Table 2).

3.3. Histomorphometry

Comparison of tubular differentiation index (TDI), repopulation index (RI), and spermiogenesis index (SI) among different experimental groups revealed that saccharin administration caused a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$) (Table 3).

Seminiferous tubule diameter values in the saccharin-treated group exhibited a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Germinal epithelium height in the saccharin-treated group demonstrated a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Testicular capsule thickness in the saccharin-receiving group significantly increased compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$) (Table 3).

Measurement of degenerated tubule percentage revealed a significant increase in the saccharin-receiving group compared to the control and stevia-receiving groups ($p<0.05$). The percentage of degenerated

tubules in the saccharin+ stevia group was lower than in the saccharin group, though this difference was not statistically significant ($p>0.05$) (Table 3).

3.4. Histological Examination

Histological evaluation of tissue sections from different experimental groups revealed distinct patterns. In the control group, cellular structures appeared normal with spermatids appropriately positioned within seminiferous tubule lumens. Germ cell layers exhibited normal architecture (Fig. 2A). In the saccharin group, germinal epithelium height within seminiferous tubules was markedly reduced, accompanied by an increased number of TDI-negative tubules, indicating impaired spermatogenesis (Fig. 2B). In the stevia group, cellular structures maintained normal morphology with spermatids properly distributed within seminiferous tubule lumens. Germ cell layers displayed normal architecture (Fig. 2C). In the saccharin+ stevia group, cellular structure showed improvement with fewer TDI-negative tubules compared to the saccharin group. Germinal epithelium height was greater than in the saccharin group, approaching control group values (Fig. 2D).

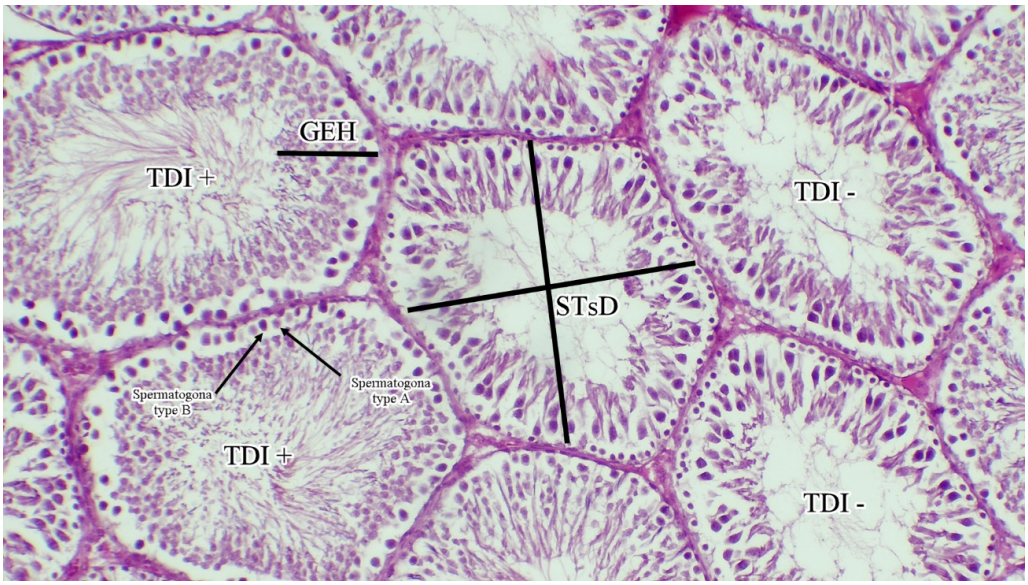

Fig. 1. Histomorphometric parameters in testis. The mean seminiferous tubules diameter (STsD), germinal epithelium height (GEH), tubular differentiation index (TDI), (H&E staining, ×400 magnification).

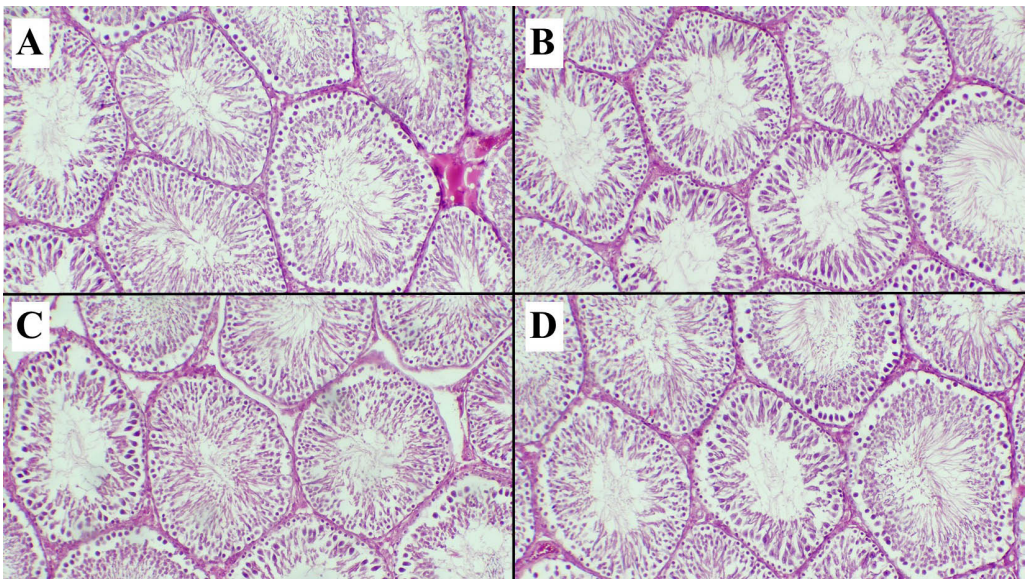

Fig. 2. Histological sections of testis in different experimental groups. A: Control group, B: Saccharin group, C: Stevia group, D: Stevia+Saccharin group. (H&E staining, ×400 magnification).

3.5. Malondialdehyde (MDA) Levels

Investigation of MDA levels in serum across different groups revealed that saccharin administration caused a significant increase in MDA concentration compared to all other experimental groups ($p<0.05$). MDA levels in the stevia+saccharin group were significantly lower than in the saccharin group and significantly higher than in the control and stevia groups ($p<0.05$) (Fig. 3).

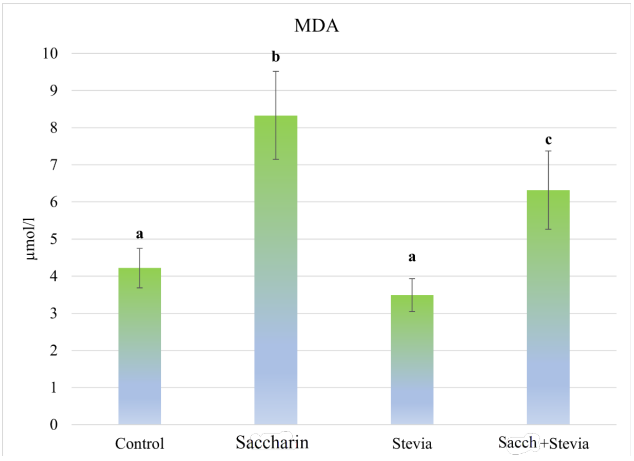

Fig. 3. MDA values in different experimental groups. Non-similar English letters indicate significant differences ($p<0.05$).

3.6. Discussion

Infertility and related reproductive issues are recognized as significant problems affecting quality of life. In recent years, considerable concerns have emerged regarding declining male fertility potential and reproductive problems, particularly in industrialized nations. One of the primary causes of male infertility is compromised sperm quality and men's inability to produce healthy, motile sperm. Multiple factors can adversely affect sperm production and contribute to infertility. The most common cause of male infertility is insufficient production of healthy, active sperm. Sperm fertility depends on various parameters including count, motility, and morphology (Esmaelzadeh *et al.*, 2007).

Stevia rebaudiana, as a natural, carbohydrate-free sweetener, was investigated in this study to compare its effects both individually and in combination with saccharin, an artificial sweetener. Results obtained in this study demonstrated that hydroalcoholic extract of Stevia rebaudiana can ameliorate adverse effects induced by sodium saccharin on multiple parameters related to the male reproductive system.

The sweetening properties of stevia derive from compounds such as Rebaudioside A and steviol (Ashwell, 2015). Numerous therapeutic properties have been documented for stevia, including antioxidant, anti-diabetic, vasodilatory, anti-hypertensive, diuretic, immunomodulatory, antimicrobial, memory-enhancing, cardioprotective, weight-reducing, and antidepressant effects (Momtazi-Borojeni *et al.*, 2017; Ashwell, 2015; Gupta *et al.*, 2014; Shahi and Mohammadyfar, 2017).

Previous studies have shown that stevia increased testosterone and estrogen levels (Juan *et al.*, 2008), while other research demonstrated that stevia extract significantly reduced weights of testes, seminal vesicles, and cauda epididymis (Melis, 1999). Additionally, Rebaudioside A administration in F0 and F1 generations had no adverse effects on reproductive parameters including mating, fertility, pregnancy duration, estrous cycle, sperm motility, and morphology (Curry *et al.*, 2008). In contrast, the present study demonstrated that stevia improved sperm quality in saccharin-receiving rats.

Multiple findings have shown that stevia induces vasodilation and increases blood flow (Melis, 1996), which may enhance blood perfusion to testicular vessels. By increasing blood supply to testicular tissue, stevia may improve reproductive function in male rats.

Examination of sperm abnormalities across control and experimental groups revealed that abnormal sperm count in the stevia group did not differ significantly from the control group. The saccharin+ stevia group also showed no significant difference from the control group. Zhang *et al.* (2017) investigated toxicity of hydroalcoholic extract of Stevia rebaudiana Bertoni leaves on sperm abnormalities in mice, reporting no adverse effects on sperm morphology (Zhang *et al.*, 2017), consistent with the present study's findings. In this study, stevia not only exhibited no adverse effects but also, by potentially increasing testicular tissue blood supply, ameliorated saccharin's adverse effects on sperm parameters to an acceptable degree.

One study demonstrated that ethanolic extract of Stevia rebaudiana exerts superior antioxidant properties and anti-proliferative effects in tumor cells compared to its diterpene glycoside stevioside (Lopez *et al.*, 2016). Interestingly, stevioside and Rebaudioside A, the primary sweetening metabolites in stevia leaves, showed lower oxygen radical absorbance capacity compared to whole plant extracts (Bender *et al.*, 2015). This activity for stevioside likely stems from the absence of phenyl aromatic rings with free radical scavenging capacity in steviol glycosides, indicating that stevia's antioxidant activity depends on other compound types such as polyphenols. Particularly, chlorogenic acids (CGA), well-known for their efficient antioxidant activities, accumulate primarily in ethanolic extract of Stevia rebaudiana Bertoni leaves (Shivanna *et al.*, 2013). These findings support stevia's promising role as a natural antioxidant source with multiple applications in food supplements and pharmaceutical industries (Lopez *et al.*, 2016).

In the present study, no significant difference in

serum antioxidant status was observed between stevia and control groups. However, antioxidant status in the stevia+saccharin group improved compared to the saccharin group, attributable to the antioxidant properties of hydroalcoholic extract of Stevia rebaudiana.

Ghaheri *et al.* (2018) demonstrated in diabetic rats that stevia consumption improved reproductive behaviors, reduced blood glucose, and increased testosterone levels and Leydig cell count (Ghaheri *et al.*, 2018). In the present study, stevia effectively counteracted adverse effects induced by saccharin. Sperm motility, viability, count, DNA integrity, and nuclear maturity were significantly compromised in the saccharin group compared to other groups. Stevia partially mitigated saccharin's adverse effects through its antioxidant properties and enhanced testicular tissue blood supply. Comparison of spermatogenesis indices (TDI, RI, SI) among groups revealed stevia's capacity to improve these parameters.

In the present study, body and testicular weights decreased in the stevia-receiving group, though not significantly, while the gonadosomatic index remained comparable to the control group. Stevia may beneficially contribute to weight management by reducing carbohydrate intake and through its antioxidant properties. Helal *et al.* (2019) found that sodium saccharin adversely affected physiological parameters in rats including body weight and insulin levels (Helal *et al.*, 2019). The present study obtained similar results, with body weight in the saccharin-receiving group showing the highest values, consistent with Helal *et al.*'s findings.

Abdi *et al.* (2023) demonstrated that diabetes caused decreased body and testicular weights and increased fasting blood glucose. Stevia consumption significantly increased body and testicular weights and decreased serum fasting blood glucose compared to the diabetic group. Furthermore, stevia significantly elevated blood testosterone levels compared to the diabetic group. Sperm parameters were significantly improved with stevia treatment compared to the diabetic group, and stevia administration significantly increased in vitro fertilization success rates and in vitro development of fertilized oocytes compared to the diabetic group (Abdi *et al.*, 2023). While body and testicular weights decreased in rats receiving stevia in the present study—inconsistent with Abdi *et al.*'s study—the improvement in sperm parameter quality and spermatogenesis parameters by stevia in the saccharin+ stevia group was consistent with Abdi *et al.*'s findings.

Ganjiani *et al.* (2020) demonstrated that stevia improved adverse effects of testicular torsion including elevated MDA levels and decreased glutathione peroxidase and superoxide dismutase activities, effectively ameliorating adverse effects of ischemic injury in testis (Ganjiani *et al.*, 2020). In the present study, MDA level examination across control and experimental groups showed that MDA levels in the saccharin-receiving group exceeded other groups. Simultaneous stevia consumption with saccharin reduced MDA levels and improved antioxidant status.

Stevia's antioxidant properties have been documented in numerous studies, demonstrating that stevia's antioxidant effects can reduce endothelial damage and protect Leydig cell integrity (Hurtado de Catalfo and De Gómez Dumm, 2017; Wankeu-Nya *et al.*, 2014). Research has shown that stevia prevents Leydig cell degradation in treated diabetic mice compared to untreated diabetic mice. As a calorie-free, anti-diabetic, vasodilatory, antioxidant sweetener, stevia can effectively treat fertility disorders by reducing blood glucose and through its aphrodisiac properties (Ghaheri *et al.*, 2018).

Oxidative stress is a condition caused by increased cellular damage due to oxygen and oxygen-derived free radicals, known as reactive oxygen species (ROS). Reactive oxygen species (ROS), also known as free radicals, have at least one unpaired electron, which makes them highly reactive molecules due to the unpaired electron in their outer shell (Miranda-Vilela *et al.*, 2010; Henkel, 2011). ROS represent a broad collection of free radicals (e.g., hydroxyl ion (OH), superoxide ion (O2), nitric oxide (NO), peroxyl (RO2), lipid peroxyl (LOO), and thiol (RS)) and non-radical molecules (e.g., singlet oxygen (1O2), hydrogen peroxide (H2O2), hypochlorous acid (HOCL), lipid peroxide (LOOH), and ozone (O3)) (Bansal and Bilaspuri, 2011). In a healthy body, pro-oxidants and antioxidants are in balance. Pathological problems occur when ROS overcome antioxidant defense systems and disrupt the complex balance between ROS and the antioxidant system. Depending on the nature, number, and duration of ROS dominance, significant damage occurs in biomolecules such as lipids, proteins, nucleic acids, and carbohydrates (Agarwal and Prabakaran, 2005). Sperm are equipped with antioxidant defense mechanisms and can neutralize ROS. Therefore, germ cells and mature sperm are protected against oxidative stress. However, under disease conditions, ROS production exceeds the capacity of seminal plasma, and oxidative stress occurs (Henkel, 2011; Trussell 2013).

Sperm were the first cell type to show high sensitivity to oxidative stress. Sperm are unable to repair damage caused by oxidative stress because they lack the necessary enzymatic-cytoplasmic repair systems. This is one of the characteristics that leads to increased sensitivity of sperm to oxidative stress (Saleh *et al.*, 2002). On the other hand, the cell membrane of sperm is rich in polyunsaturated fatty acids (PUFAs), making them highly susceptible to oxygen-induced damage and subsequent lipid peroxidation. Subsequently, the rapid loss of intracellular ATP due to lipid peroxidation causes neurological damage, reduced sperm viability, and an increase in morphological defects in the

midpiece of sperm, all of which lead to reduced sperm motility (Bansal and Bilaspuri, 2011; Gharagozloo and Aitken, 2011). With the progression of peroxidation in sperm, approximately 60% of the membrane fatty acids are lost. Hence, sperm function is affected by reduced fluidity, increased non-specific ion permeability, and inactivation of membrane receptors and enzymes (Tremellen, 2008). Oxidative stress is considered a major cause of male infertility. Furthermore, there is ample evidence that increased oxidative stress significantly impairs sperm function (Agarwal *et al.*, 2012).

Semen parameters such as sperm count, motility, and morphology are commonly used to determine the fertilizing potential of sperm. Although these parameters provide an overview of sperm quality, they do not provide information regarding DNA, which is one of the main components of reproduction. Single- or double-strand DNA breaks can be the source of differences in fertility potential between fertile and infertile men. It has been reported that chromatin in the sperm nucleus is vulnerable to oxidative damage, leading to DNA fragmentation (Zribi *et al.*, 2011).

The results of sperm parameter analysis showed a significant decrease in sperm count, motility, and viability in the saccharin group compared to the control group. Also, the number of immature and morphologically abnormal sperm increased in the saccharin group mice compared to the control group. Our data showed that the extent of DNA damage in the saccharin group was significantly higher than in the control group.

Studying spermatogenesis indices is a suitable criterion for evaluating the activity of testicular tissue. Among these, some indices can be used to properly estimate the activity of cells involved in spermatogenesis. Three indices: tubular differentiation index (TDI), spermiogenesis index (SI), and repopulation index (RI) can be useful in understanding the activity of testicular tissue (Shetty *et al.*, 2000). The tubular differentiation index (TDI) indicates the rate of cell division and the ability of cells to increase in number and differentiate. A decrease in this index indicates a decrease in the cell population of the seminiferous tubule wall.As a result of the decrease in the population of germ cell lines in the wall of the seminiferous tubules, structural changes will also be observable, among the most important of which are the reduction in the height of the germinal epithelium and the disruption of the order and arrangement of intercellular junctions. The disruption of intercellular junctions can cause cells to be released into the lumen of the seminiferous tubule, which will lead to a decrease in the tubular differentiation index. Therefore, a decrease in this index can indicate a disorder in intercellular junctions. In the process of normal spermatogenesis, cells are usually drawn in a row from the basal part and seminiferous tubules towards the lumen of the tubules. This indicates the normalcy of the cell division process (Meistrich *et al.*, 2003). Any disruption in the cell division process, such as the elimination and loss of germ cell lines, can reduce the tubular differentiation index. Therefore, a decrease in the mentioned index can be due to a decrease in cell population caused by abnormal elimination in germ cell lines (Shetty *et al.*, 2000; Meistrich *et al.*, 2003). A decrease in the tubular differentiation index is directly related to a decrease in the population of germ cells. Therefore, a decrease in this index can indicate a decrease in the population of germ cell lines, especially spermatocytes. In this regard, it should be noted that a decrease in cell population can be due to a decrease in division power or an increase in cell loss. The spermiogenesis index (SI) indicates the capability and ability of spermatid cells to differentiate into spermatozoa cells. The repopulation index (RI) indicates the number of active spermatogonia cells in the wall of the seminiferous tubules of the testicular tissue. An increase in the number of active spermatogonia cells can lead to an increase in the population of spermatogenesis cell lines (Shetty *et al.*, 2000). A drop in the IR index can be one of the reasons for a decrease in the TDI index. In the present study, saccharin had adverse effects on spermatogenesis parameters, but the consumption of stevia simultaneously with saccharin improved these parameters compared to the control. Stevia, due to its antioxidant effects, can enhance the spermatogenesis process and prevent oxidative damage. Also, given that stevia can act as a vasodilator, by increasing blood supply, it can improve the adverse effects of saccharin.

From the results obtained in this study, it can be concluded that stevia, as an energy-free sweetener that also has antioxidant properties, can be a suitable substitute for the artificial and industrial sweetener sodium saccharin, which, due to its adverse effects on the male reproductive system, is recommended not to be used in industry, and stevia, due to its availability and low cost, can be used as a sweetener and antioxidant.

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Conflict of Interest

The authors declare no conflicts of interest related to this work.

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ORIGINAL ARTICLE

Assessment of Chemical Composition and Mineral Contents of Iranian Kilka Fish Meal

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Abstract

This experiment was performed to investigate the chemical composition and mineral contents of Iranian Kilka fish meal (KFM). A total of 6 composed samples of KFM were prepared during one month sampling period from rendering units of two commercial fish meal plants in Gilan province. The results of proximate analysis indicated that the average dry matter (DM), ether extract (EE), crude protein (CP), crude fiber (CF) and ash contents of the KFM samples were 94.76, 23.11, 59.72, 0.67, and 13.05 percent, respectively. The average gross energy (GE) value for the KFM samples was 5592 kcal kg^{-1}. The average values of major elements including Ca, P, Na, K, Cl, Mg, and S were 3.92, 2.58, 0.87, 0.56, 0.69, 0.29, and 0.43 percent, in order. The average values for trace elements including Fe, Cu, Mn, Zn, and Se were 223.8, 5.9, 3.9, 79.6, and 1.64 mg kg^{-1}, respectively. There were significant negative correlation coefficients ($P<0.05$) between ash and EE, CP and GE contents of the KFM samples so that the EE, CP and GE values of the KFM samples decreased as ash content increased. Therefore, it seems that the ash content is a good indicator for prediction of other chemical composition and GE content of KFM.

1. Introduction

Fish meal can be produced from whole fish or wastes from the use of fish prepared for human consumption (Ponce and Gernat, 2002; Ween *et al.*, 2017; Adibrata *et al.*, 2022), but currently fish meal is mostly produced from smaller oily fish caught specifically for fish meal production (Leeson and Summers, 2001, 2005). High quality fish meal has excellent amino acid balance (Ponce and Gernat, 2002; Khatoon *et al.*, 2006) and is rich in protein and amino acids (specially methionine, lysine, arginine and tryptophan), calcium, phosphorus, iron, vitamin B_{12}, choline, niacin, pantothenic acid and riboflavin (Miller, 1970; Waldroup and Adams, 1994; NRC, 1994; Ponce and Gernat, 2002). In addition, fish meal is a good source of energy (Ponce and Gernat, 2002) and omega-3 fatty acids (Hulan *et al.*, 1989; Burke *et al.*, 1997) specially Eicosapentaenoic acid and Docosahexaenoic acid (Shahmohammadpour Askari *et al.*, 2025). Fish meal is used as a source of protein and unidentified growth factors in diets of monogastric animals like pig (Kim and Easter, 2001), Poultry (Sibbald and Wolynetz, 1984; Wu *et al.*, 1984; Ponce and Gernat, 2002; Khatoon *et al.*, 2006), Pet Animals (Folador *et al.*, 2006; Ween *et al.*, 2017) and aquatic animals (Anderson *et al.*, 1993; Steffens, 1994; Ahmad and Ibrahim, 2016; Rahim *et al.*, 2017; Zamani *et al.*, 2017; Rathika and Pugazhendy, 2018; Hodar, 2020; Al-Noor *et al.*, 2023; Majluf *et al.*, 2024; Shahmohammadpour Askari *et al.*, 2025) and also extensively used as a ruminally undegradable protein source in ruminant diets (Petit and Flipot, 1992; Petit and Castonguay, 1994; Streeter and Mathis, 1995; Burke *et al.*, 1997; Walz *et al.*, 1998). The chemical composi-

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tion and mineral contents of fish meal can vary greatly depending on the species of fish used (Wu *et al.*, 1984; Ponce and Gernat, 2002; Khan *et al.*, 2012), amount of residual oil (Anderson *et al.*, 1993), processing method (Bayrakli and Duyar, 2021), drying method and temperature (Anderson *et al.*, 1993; Rosselot *et al.*, 1996), and whether the meal is made from whole fish or the waste from some other processing operation (Anderson *et al.*, 1993), thus the chemical composition and mineral contents of fish meal is highly variable (Khan *et al.*, 2012) and needs to be evaluated continuously.

Determination of chemical composition of fish meal is important in estimating its metabolizable energy content (Sibbald and Wolynetz, 1984; NRC, 1994) and measurement of its mineral content especially calcium and phosphorus is of significance to include fish meal in the balanced diets (NRC, 1994; Leeson and Summers, 2001, 2005).

Kilka fish belongs to kingdom of *Animalia*, phylum of *Chordata*, class of *Actinopterygii*, subclass of *Teleostei*, order of *Clupeiformes*, family of *Clupeidae*, subfamily of *Ehiravinae*, genus of *Clupeonella* and species of *Engrauliformis* in animal systematic classification system (Abtahi *et al.*, 2004; Keramat Amir Kalayi and Karimzadeh, 2013). In Iran, Kilka fish with the scientific name of *Clupeonella engrauliformis* is obtained from the Caspian Sea in Golestan, Mazandaran and Gilan provinces. The most part of the catch is processed into fish meal by different processing methods. Now, there are 9 rendering plants in Iran that produce Kilka fish meal by processing whole Kilka fish, which is mostly used in poultry and cold water fish diets. Although several studies have been conducted on chemical composition and mineral contents of fish meal in other countries (Schumaier and McGinnis, 1969; Miller, 1970; Sibbald and Wolynetz, 1984; Anderson *et al.*, 1993; Ponce and Gernat, 2002), but the chemical composition and mineral contents of Kilka fish meal produced in Iran has not been described quantitatively. Therefore, the current study was conducted to determine the chemical composition and mineral contents of Iranian Kilka fish meal.

2. Materials and Methods

The Kilka fish meal (KFM) samples were provided during one month sampling period from rendering units of two industrial fish meal plants in Gilan province. Each sample was randomly taken from different parts of a batch. The samples collected in each five days were mixed together and 6 composed samples were prepared so that samples #1, #2 and #3 were taken from plant A and samples #4, #5 and #6 were taken from plant B. The raw material source of all KFM samples was whole Kilka fish. After grinding and mixing the samples, all samples were stored at -20°C until further analysis.

The dry matter (DM) content of each KFM sample was determined by oven drying method at 105°C, ether extract (EE) by Soxhlet's method, crude protein (CP) by Kjeldahl's method, crude fiber (CF) by boiling the sample in 0.255 N sulfuric acid for 30 minutes and then boiling the sample residuals in 0.313 N sodium hydroxide for 30 minutes, and ash by burning the samples in electrical furnace at 450°C for overnight as described by AOAC (1984) procedures. The gross energy (GE) content for each KFM sample was measured by burning the sample in pure oxygen in bomb calorimeter apparatus (Parr 6300, Parr instruments, Chicago, Illinois, USA). In order to measure the mineral contents of the KFM sample, firstly each sample was turned to ash in a porcelain crucible at 450°C and then 30 ml of 2N hydrochloric acid solution was added to each porcelain crucible and placed on a hot plate at 350°C for 2 hours. The porcelain crucible contents were filtered thereafter by Wattman® No. 42 filter paper and transferred to a 100 ml volumetric flask and its volume was reached to 100 ml by adding distilled water. The solution in volumetric flask was used for measurement of mineral content. Calcium, magnesium and all trace elements including iron, copper, manganese, zinc, and selenium were determined by atomic absorption, phosphorus by spectrophotometry, sodium and potassium by flame photometry, chlorine by titration, and sulfur by turbidometry methods (AOAC, 1984). Each sample was examined in six replicates. The data were analyzed in a completely randomized design using the GLM procedure of SAS (SAS, 1999). Comparison of means was conducted by Duncan's multiple range test. Comparison of the average of chemical composition and mineral contents of the KFM samples with values reported by NRC (1994) was conducted using two-sided t-test (Zar, 1996). Univariate and Corr procedures of SAS (SAS, 1999) were used for determination of descriptive statistical parameters and correlation coefficients between chemical composition and gross energy contents of the KFM samples.

3. Results and Discussion

The chemical composition and GE values of the KFM samples are shown in Table 1. The average DM, EE, CP, CF, and ash were 94.76, 23.11, 59.72, 0.67, and 13.05 percent, respectively. The average GE was 5592 Kcal Kg^{-1}. The coefficient of variation of EE, CF and ash were 8.18, 17.91 and 7.59 percent, respectively that were higher, as compared with the coefficient of variation of other chemical composition of the KFM samples. The DM content of the KFM samples varied from 93.88 to 95.73 percent. Al-Noor *et al.* (2023) reported the DM contents of four fish meal samples in the range from 93.82 to 94.89 percent. The average

DM of the KFM samples (94.76%) was significantly higher (P<0.01) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Ponce and Gernat (2002) also reported a value of 94.60 percent for the DM of one tilapia by-product meal sample, whereas Anderson *et al.* (1993) reported a value of 95.45 percent for the average DM of six fish meal samples which are significantly higher than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. The EE content of the KFM samples varied from 20.61 to 25.68 percent. Khan *et al.* (2012) reported a variation in EE content from 9.90 to 29.52 percent for 19 fish meal samples. Also, the average EE of the KFM samples (23.11%) was significantly higher (P<0.01) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals, because fat is not removed from final product in rendering units producing KFM in Iran. The high fat content may be the cause of the relatively higher DM in our KFM samples as compared with those reported by NRC (1994). Although the presence of high fat content can be beneficial in providing energy for animal, however it may facilitates the deterioration of product and reduces its nutritional value. The CP content of the KFM samples varied from 56.95 to 61.81 percent. Al-Noor *et al.* (2023) reported the CP contents of four fish meal samples in the range from 58.43 to 70.60 percent, whereas Khan *et al.* (2012) reported a variation in CP content from 37.49 to 66.57 percent for 19 fish meal samples. The average CP content of the KFM samples (59.72%) was significantly lower (P<0.01) than those reported by NRC (1994) for Anchovy and Herring fish meals but not from Menhaden fish meal. Miller (1970) reported a value of 64.48 percent for the average CP of eight fish meal samples, whereas Ponce and Gernat (2002) reported a value of 63.50 percent for the CP of one tilapia by-product meal sample. The CF content of the KFM samples varied from 0.54 to 0.79 percent. Khan *et al.* (2012) reported the CF content of 19 fish meal samples in the range from 0.12 to 12.67 percent. The average CF content of the KFM samples (0.67%) was significantly lower (P<0.01) than that reported by NRC (1994) for Anchovy fish meal but not from those of Herring and Menhaden fish meals. Similarly, Ponce and Gernat (2002) reported a value of 0.58 percent for the CF of one tilapia by-product meal sample, whereas Sibbald and Wolynetz (1984) reported a value of 0.72 percent for the average CF content of 15 Menhaden fish meal samples. The ash content of the KFM samples varied from 12.01 to 14.21 percent. Al-Noor *et al.* (2023) reported the ash content of four fish meal samples from 12.54 to 20.71 percent, whereas Khan *et al.* (2012) reported a variation in ash content from 12.74 to 28.23 percent for 19 fish meal samples. The average ash content of the KFM samples was 13.05 percent. Anderson *et al.* (1993) reported a similar value of 12.85 percent for the average ash content of six fish meal samples. The GE content of the KFM samples varied from 5394 to 5899 Kcal Kg^{-1}. Khan *et al.* (2012) reported the GE content of 19 samples of fish meal in the range from 4012 to 4776 Kcal Kg^{-1}. The average GE value of the KFM samples was 5592 Kcal Kg^{-1}. Sibbald and Wolynetz (1984) reported a value of 4667 Kcal Kg^{-1} for the average GE of 15 Menhaden fish meal samples and this value is significantly lower than that obtained in the present study. The lower GE values reported by Sibbald and Wolynetz (1984) as compared with our values may be due to the lower EE and higher ash contents of samples analyzed by Sibbald and Wolynetz (1984).

As shown in Table 2, the EE, CP and GE values of the KFM samples decreased as ash content increased. Khatoon *et al.* (2006) also reported adverse relationships between ash and CP and EE contents of 184 fish meal samples produced in Pakistan which are in agreement with the results obtained in the present study. It seems that the ash content is a good indicator for prediction of other chemical composition and gross energy content of the final product.

Table 1
The chemical composition (%) and GE (kcal kg^{-1}) contents of the KFM Samples (as is).

Sample No.	1	2	3	4	5	6	Average	SD[1]	CV[2]	NRC Data for Anchovy Fish Meal	NRC Data for Herring Fish Meal	NRC Data for Menhaden Fish Meal
Dry Matter	95.73	93.88	94.57	94.97	94.09	95.32	94.76	0.78	0.82	92**	93**	92**
Ether Extract	20.61	25.68	23.47	22.54	24.61	21.75	23.11	1.89	8.18	5**	10**	9.4**
Crude Protein	57.98	61.81	61.24	59.36	60.97	56.95	59.72	2.01	3.37	64.2**	72.3**	60.05
Crude Fiber	0.79	0.54	0.61	0.74	0.57	0.77	0.67	0.12	17.91	1**	0.7	0.7
Ash	14.21	12.01	12.65	13.25	12.15	14.03	13.05	0.99	7.59	—	—	—
Gross Energy	5394	5899	5557	5531	5695	5476	5592	194.3	3.48	—	—	—

** Indicating a highly significant difference (P<0.01) between the average nutrient value in KFM samples and that of reported by NRC (1994).
[1]SD = Standard Deviation.
[2]CV = Coefficient of Variation.

Table 2
The correlation coefficients between chemical composition and GE of KFM samples.

	Ether Extract	Crude Protein	Ash	Gross Energy
Ether Extract	1	0.885	-0.973	0.962
		(0.0193)*	(0.0011)	(0.0021)
Crude Protein		1	-0.951	0.798
			(0.0035)	(0.0570)
Ash			1	-0.892
				(0.0170)
Gross Energy				1

* The values shown in parenthesis under the correlation coefficients are significance levels.

The major element contents of the KFM samples are shown in Table 3. The calcium content of the KFM samples varied from 3.56 to 4.27 percent. The average calcium content of the KFM samples (3.92%) was not significantly different from that reported by NRC (1994) for Anchovy fish meal, but was significantly higher ($P<0.01$) than that of Herring fish meal and lower ($P<0.01$) than that of Menhaden fish meal value. Waldroup *et al.* (1965) reported a variation in calcium content from 3.57 to 5.00 with the average value of 4.27 percent for four fish meal samples, whereas Miller (1970) reported an average value of 4.50 with the range from 4.18 to 5.05 percent for calcium content of eight fish meal samples. The phosphorus content of the KFM samples varied from 2.42 to 2.78 percent. The average phosphorus content of the KFM samples (2.58%) was significantly higher ($P<0.01$) than those reported by NRC (1994) for Anchovy and Herring fish meals but was significantly lower ($P<0.01$) than that of Menhaden fish meal. Miller (1970) also reported an average value of 2.56 with the range from 2.33 to 2.88 percent for the phosphorus content of eight fish meal samples, whereas this value was reported 2.57 as an average with the range from 2.29 to 2.88 percent by Waldroup *et al.* (1965) for four fish meal samples, which are higher than those reported by NRC (1994) for Anchovy and Herring fish meals but lower than that of Menhaden fish meal. The sodium content of the KFM samples varied from 0.78 to 0.99 percent. The average sodium content of the KFM samples (0.87%) was significantly higher ($P<0.01$) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Anderson *et al.* (1993) also reported a value of 0.89 percent for the average sodium content of six fish meal samples which is higher than those reported by NRC (1994). The potassium content of the KFM samples varied from 0.49 to 0.64 percent. The average potassium content of the KFM samples (0.56%) was significantly lower ($P<0.01$) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Anderson *et al.* (1993) reported a value of 0.81 percent for the average potassium content of six fish meal samples which is higher than those reported by NRC (1994) for Anchovy and Menhaden fish meals but lower than that of Herring fish meal value. The chlorine content of the KFM samples varied from 0.57 to 0.78 percent. The average chlorine content of examined samples (0.69%) was not significantly different from those reported by NRC (1994) for Anchovy and Menhaden fish meals but was significantly lower ($P<0.01$) than that of Herring fish meal. The magnesium content of the KFM samples varied from 0.24 to 0.35 percent. The average magnesium content of the KFM samples (0.29%) was not significantly different from that reported by NRC (1994) for Anchovy fish meal but was significantly higher ($P<0.01$) than those of Herring and Menhaden fish meals. Anderson *et al.* (1993) also reported a value of 0.23 percent for the average magnesium content of six fish meal samples. The sulfur content of the KFM samples varied from 0.32 to 0.52 percent. The average sulfur content of the KFM samples (0.43%) was significantly lower ($P<0.01$) than NRC (1994) values for Anchovy and Herring fish meals but was not significantly different from that of Menhaden fish meal.

The trace element contents of the KFM samples are shown in Table 4. The iron content of the KFM samples varied from 188 to 267 mg Kg^{-1}. The average iron content of our samples (223.8 mg Kg^{-1}) was not significantly different from that reported by NRC (1994) for Anchovy fish meal but was significantly higher ($P<0.01$) than Herring fish meal value and lower ($P<0.01$) than that of Menhaden fish meal. Anderson *et al.* (1993) reported a value of 164.5 mg Kg^{-1} for the average iron content of six fish meal samples which is higher than that reported by NRC (1994) for Herring fish meal but is lower than those of Anchovy and Menhaden fish meals. The copper content of examined KFM samples varied from 4.4 to 7.6 mg Kg^{-1}. The average copper content of the KFM samples (5.9 mg Kg^{-1}) was significantly lower ($P<0.01$) than NRC (1994) values for Anchovy and Menhaden fish meals but was not significantly different from that of Herring fish meal.

Table 3
The major element (%) contents of the KFM Samples (as is).

Sample No.	1	2	3	4	5	6	Average	SD[1]	CV[2]	NRC Data for Anchovy Fish Meal	NRC Data for Herring Fish Meal	NRC Data for Menhaden Fish Meal
Calcium	4.27	3.56	3.81	4.02	3.63	4.21	3.92	0.31	7.91	3.73	2.29**	5.11**
Phosphorus	2.78	2.45	2.49	2.61	2.42	2.75	2.58	0.16	6.20	2.43**	1.70**	2.88**
Sodium	0.99	0.78	0.79	0.91	0.81	0.94	0.87	0.10	11.49	0.65**	0.61**	0.65**
Potassium	0.64	0.49	0.53	0.59	0.51	0.62	0.56	0.08	14.29	0.69**	1.09**	0.65**
Chlorine	0.78	0.57	0.71	0.73	0.59	0.76	0.69	0.10	14.49	0.60	0.90**	0.60
Magnesium	0.35	0.26	0.24	0.31	0.27	0.33	0.29	0.04	13.79	0.24	0.15**	0.16**
Sulfur	0.39	0.52	0.48	0.41	0.44	0.32	0.43	0.08	18.61	0.54**	0.69**	0.45

** Indicating a highly significant difference (P<0.01) between the average nutrient value in KFM samples and that of reported by NRC (1994).
[1]SD = Standard Deviation.
[2]CV = Coefficient of Variation.

Table 4
The trace element (mg kg^{-1}) contents of the KFM Samples (as is).

Sample No.	1	2	3	4	5	6	Average	SD[1]	CV[2]	NRC Data for Anchovy Fish Meal	NRC Data for Herring Fish Meal	NRC Data for Menhaden Fish Meal
Iron	267	188	206	234	201	247	223.8	29.57	13.21	220	140**	440**
Copper	7.6	4.4	5.4	6.5	4.6	7.1	5.9	1.32	22.37	9**	6	11**
Manganese	4.6	3.1	3.7	3.9	3.6	4.5	3.9	0.58	14.87	10**	5**	33**
Zinc	95.5	62.3	77.6	81.5	71.8	88.9	79.6	11.23	14.11	103**	132**	147**
Selenium	1.85	1.38	1.68	1.71	1.47	1.77	1.64	0.19	11.59	1.36**	1.93**	2.10**

** Indicating a highly significant difference (P<0.01) between the average nutrient value in KFM samples and that of reported by NRC (1994).
[1]SD = Standard Deviation.
[2]CV = Coefficient of Variation.

Anderson *et al.* (1993) reported a value of 5.1 mg Kg^{-1} for the average copper content of six fish meal samples which is lower than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. The manganese content of the KFM samples varied from 3.1 to 4.6 mg Kg^{-1}. The average manganese content of our studied samples (3.9 mg Kg^{-1})was significantly lower (P<0.01) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Anderson *et al.* (1993) reported a value of 11.2 mg Kg^{-1} for the average manganese content of six fish meal samples which is also different from NRC (1994) values. The zinc content of the KFM samples varied from 62.3 to 95.5 mg Kg^{-1}. The average zinc content of the KFM samples (79.6 mg Kg^{-1}) was significantly lower (P<0.01) than those reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Similarly, Anderson *et al.* (1993) reported a value of 93 mg Kg^{-1} for the average zinc content of six fish meal samples which is lower than those of NRC (1994) values. The selenium content of the KFM samples varied from 1.38 to 1.85 mg Kg^{-1}. The average selenium content of the KFM samples (1.64 mg Kg^{-1}) was significantly higher (P<0.01) than that of NRC (1994) value for Anchovy fish meal but was significantly lower (P<0.01) than those for Herring and Menhaden fish meals.

Generally, the results of the current study showed significant variation in chemical composition and mineral contents among the KFM samples. There were significant differences between the average of each chemical composition and mineral contents of the KFM samples with the values reported by NRC (1994) for Anchovy, Herring and Menhaden fish meals. Therefore, the chemical composition and mineral contents of KFM must be determined firstly before its utilization in livestock, poultry and aquatic diets and the values reported by NRC (1994) can't be used for estimation of chemical composition, mineral contents and thus the nutritional value of KFM.

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