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Wound Healing and Skin Regeneration: Present Status and Future Directions

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ABSTRACT

Wound healing and skin regeneration involve intricate interactions between various cellular, molecular, and biochemical factors. This narrative review aims to provide an in-depth analysis of the present status of therapeutic strategies for wound healing and skin regeneration. The literature review was performed using the Google Scholar search engine with the help of relevant keywords. Selected publications were used to synthesize different sections of the narrative review. The quest for innovative therapeutic approaches to accelerate wound healing and enhance skin regeneration has led to remarkable advancements in recent years. The landscape of therapeutic approaches for wound healing and skin regeneration is evolving rapidly, driven by groundbreaking discoveries and interdisciplinary collaborations. From advanced wound dressings and growth factor therapies to stem cell-based interventions and gene editing techniques, the arsenal of tools at our disposal continues to expand. As researchers continue to unravel the intricate mechanisms underlying wound repair and regeneration, the potential for transformative therapies to revolutionize patient care remains immense. Through a combination of

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innovative technologies, personalized approaches, ethical considerations, and global accessibility, the future of wound healing holds promise for improving the lives of countless individuals worldwide. Despite significant advancements, several knowledge gaps persist in the field of wound healing and skin regeneration. Further elucidation of cellular and molecular mechanisms governing wound repair, inflammation resolution, and scar formation is warranted. Exploring the crosstalk between wound healing and the microbiome and the influence of ageing and systemic diseases will unravel new therapeutic targets and strategies. As researchers delve deeper into understanding the intricate mechanisms underlying wound repair, the development of novel therapies and their clinical translation become increasingly promising. With a multidisciplinary approach and ongoing advancements in technology, biology, and medicine, the future holds great potential for transforming the field of wound healing and skin regeneration.

1 Introduction

Wound healing is a fundamental physiological process crucial for maintaining tissue integrity and restoring normal function following injury (Gonzalez et al. 2016). Skin plays a pivotal role in protecting underlying tissues and organs from external factors (Herskovitz et al. 2016). Impaired wound healing can lead to chronic wounds, infections, and severe health complications. Over the years, various therapeutic approaches have been developed to promote wound healing and skin regeneration (Frykberg and Banks 2015). This article offers a comprehensive overview of the current landscape of wound healing therapies and envisions the potential directions for future research and development. Wound healing comprises distinct phases, including hemostasis, inflammation, proliferation, and remodelling. Each phase involves orchestrated interactions between immune cells, growth factors, cytokines, and extracellular matrix components (Gonzalez et al. 2016). Therapeutic interventions targeting specific phases aim to expedite healing and minimize scar formation. Traditional approaches, such as wound dressings, antibiotics, and surgical techniques, have paved the way for advanced therapies that harness the body's innate regenerative potential (Kolimi et al. 2022).

Modern wound dressings have evolved beyond basic wound coverage. Bioactive dressings incorporate growth factors, antimicrobial agents, and extracellular matrix components to create a favourable microenvironment for wound healing (Yu et al. 2022). Alginate, hydrocolloid, and collagen dressings have effectively enhanced wound closure and reduced infection risk (Sood et al. 2014). Furthermore, emerging smart dressings equipped with sensors and drug delivery systems enable real-time monitoring and controlled release of therapeutic agents (Pang et al. 2023). Growth factors are crucial in cell proliferation, angiogenesis, and tissue repair (Raica and Cimpean 2010). Topical application or controlled release of growth factors accelerates wound closure and enhances tissue regeneration (Kwon et al. 2006). However, challenges include short half-lives, potential side effects, and dose optimization. Combinatorial approaches and sustained-release formulations promise to overcome these limitations (Kalaydina et al. 2018).

Stem cells have garnered immense interest for their regenerative potential in wound healing (Banu et al. 2023). Mesenchymal stem cells (MSCs) exhibit paracrine effects, immunomodulation, and differentiation capabilities contributing to tissue repair (Bist et al. 2021; Peer et al. 2022; Sivanarayanan et al. 2023). Encouraging results from preclinical studies have paved the way for clinical trials exploring stem cell-based therapies (Sharun et al. 2020; Bist et al. 2021; Peer et al. 2022; Sharun et al. 2022; Sivanarayanan et al. 2023). However, standardization of protocols, ethical considerations, and long-term safety assessments remain crucial challenges. Advancements in biomaterials science have revolutionized wound healing and skin regeneration. Scaffolds, grafts, and skin substitutes composed of natural and synthetic polymers provide structural support, encourage cell adhesion, and facilitate tissue integration (Negut et al. 2020). Decellularized matrices retain native tissue architecture and signalling cues, promoting host cell infiltration and functional tissue restoration. Three-dimensional bioprinting technology enables precise deposition of cells and materials, offering personalized solutions for complex wounds (Liang et al. 2023).

This review article provides an in-depth analysis of the present status of therapeutic strategies for wound healing and skin regeneration, highlighting their mechanisms of action, applications, and limitations. Additionally, it explores emerging trends and future directions in the field, encompassing cutting-edge technologies, biomaterials, and regenerative medicine approaches.

2 Skin

Skin is the largest organ by surface area, covering the entire external surface of the body (Rodrigues et al. 2019). Healthy skin plays a pivotal role in regulating the proper haemostasis of the body. Skin performs an array of functions like protection from external damage and Infection, thermal regulation, and fluid balance of the body. Skin plays a vital role in the production of vitamin D as well as in producing immune responses. Skin also

assists in the smooth functioning of the joint by providing flexibility and rigidity wherever required (Sorg et al. 2017). The skin is an essential barrier to protecting from environmental influences like physical, chemical, and mechanical injuries (Bouwstra and Honeywell-Nguyen 2002). The epidermis forms the outermost structure of the skin, containing the keratinocyte layer and skin appendages. The keratinocyte layer forms the railing between the body and the external surface (Shaw and Martin 2009). Skin appendages in the epidermis consist of hair follicles and sebaceous glands (Takeo et al. 2015).

Histological features of the epidermis reveal 4 to 5 layers of stratified epithelium, including stratum corneum, stratum granulosum, stratum lucidum, stratum spinosum, and stratum basale (Strong et al. 2017). Stratum basale forms the basement membrane that separates the epidermis and dermis. The next layer is formed by the dermis, which is composed of fibroblasts, an extracellular matrix, nerves, lymphatics, and blood vessels (Shaw and Martin 2009). The dermis is divided into two layers: the superficial papillary layer and the deep reticular layer. Below the dermis lies the hypodermis, which contains adipose tissue and blood supply. Hypodermis acts as a thermal insulator and powerhouse of energy-supplying skin (Strong et al. 2017). The various skin components like the epidermis, skin appendages, and dermis contain stem cells essential for regulating normal haemostasis and skin regeneration (Ojeh et al. 2015).

3 Wound and types of wound

Loss of skin integrity due to illness or injury leads to cutaneous wounds (Clark et al. 2007). A wound is defined as damage or disruption in the normal skin anatomy. Irrespective of the causative agent, the wound alters the local environment surrounding the wound (Robson et al. 2001). The disruption of skin and the damage to the mucus membrane and organ/tissue is termed a wound (Young and McNaught 2011). Wound management is a critical clinical concern that significantly impacts the patient, their family, and the health care system (Witte and Barbul 1997). No standard system for wound classification exists. Based on the type of injury, the wound can be incised, sheared/de-gloving, crushed, burns, or contaminated wounds. Based on timing, the wounds are of three types: acute, early, and chronic wound. Acute wounds are less than 6 hours of age, early wounds are those that are less than 24 hours of age, and chronic wounds are those that are more than 24 hours old.

Based on depth, the wound can be either superficial or deep dermal wound. The wounds that are restricted only to the epidermis and papillary dermis fall under superficial wounds. Deep dermal wounds can be either partial-thickness or full-thickness wounds. A partial-thickness wound affects the deep layers of skin but does not involve whole skin layers. When a wound involves complete skin layers, including hypodermis, then such a wound is classified as a full-thickness wound (Percival 2002). Another important type of wound is a surgical wound. Depending on the level of gross contamination, surgical wounds can be categorized as clean, clean-contaminated, contaminated and dirty (Onyekwelu et al. 2017). The potential factors influencing the gross contamination of surgical wounds are the location of injury, presence or absence of acute inflammation, failure in aseptic technique, and Infection at the surgical site (Zinn 2012).

4 Wound healing

Wound healing is a complex process that aims to restore the injured/damaged tissue to normal. It involves the synchronized orchestration of sequential cellular and molecular events (Gonzalez et al. 2016). Cutaneous wound healing is a unique procedure involving the participation of various components like growth factors, cytokines, and many other cells. The target of physiological wound healing is to restore the integrity of damaged tissue. All wound healing procedures are mostly limited to wound repair only (Tottoli et al. 2020). Wound healing directs the activation and recruitment of various cells to the site. Therefore, any alteration in the immediate microenvironment, like the oxygen tension, chemokines, extracellular matrix, growth factor synthesis, and mechanical forces, can result in aberrant wound healing (Rodrigues et al. 2019). Wound healing has four phases: haemostasis, inflammatory, proliferative, and tissue remodelling (Figure 1) (Li et al. 2007). The inflammatory phase is the immediate phase following an injury and haemostasis that alarms to stop the damage. It involves cutaneous neurogenic inflammation and platelet haemostasis. Following an injury, the nociceptive receptors in the skin will activate and act via the peripheral nervous system to initiate vasoconstriction and inflammation.

Vasoconstriction is followed by vasodilation due to the release of factors like the substance P. This causes an increase in blood flow to the sites, increases vascular permeability, oedema, and recruitment of inflammatory white blood cells (WBCs) to the site. Ultimately, the injured area will have redness and swelling by the end of the inflammatory phase (Cañedo-Dorantes and Cañedo-Ayala 2019). The primary goal of the proliferative phase is the closure of the wound and reduction in the size of the wound. This phase lasts from 48 hours to the 14th day after injury. The various events in this phase include angiogenesis, fibroplasia, and re-epithelialization. As a result of angiogenesis, the injured site will be covered with newly formed microvasculature. Fibroplasia follows the beginning of granulation. Fibroblast proliferates and produces collagen. Wound contraction also occurs simultaneously in this phase due to the action of myofibroblasts and as a result of fibroplasia. The hedgehog signalling pathway, responsible for differentiating endothelial cells into mesenchymal components, also occurs in this phase (Gonzalez et al. 2016).

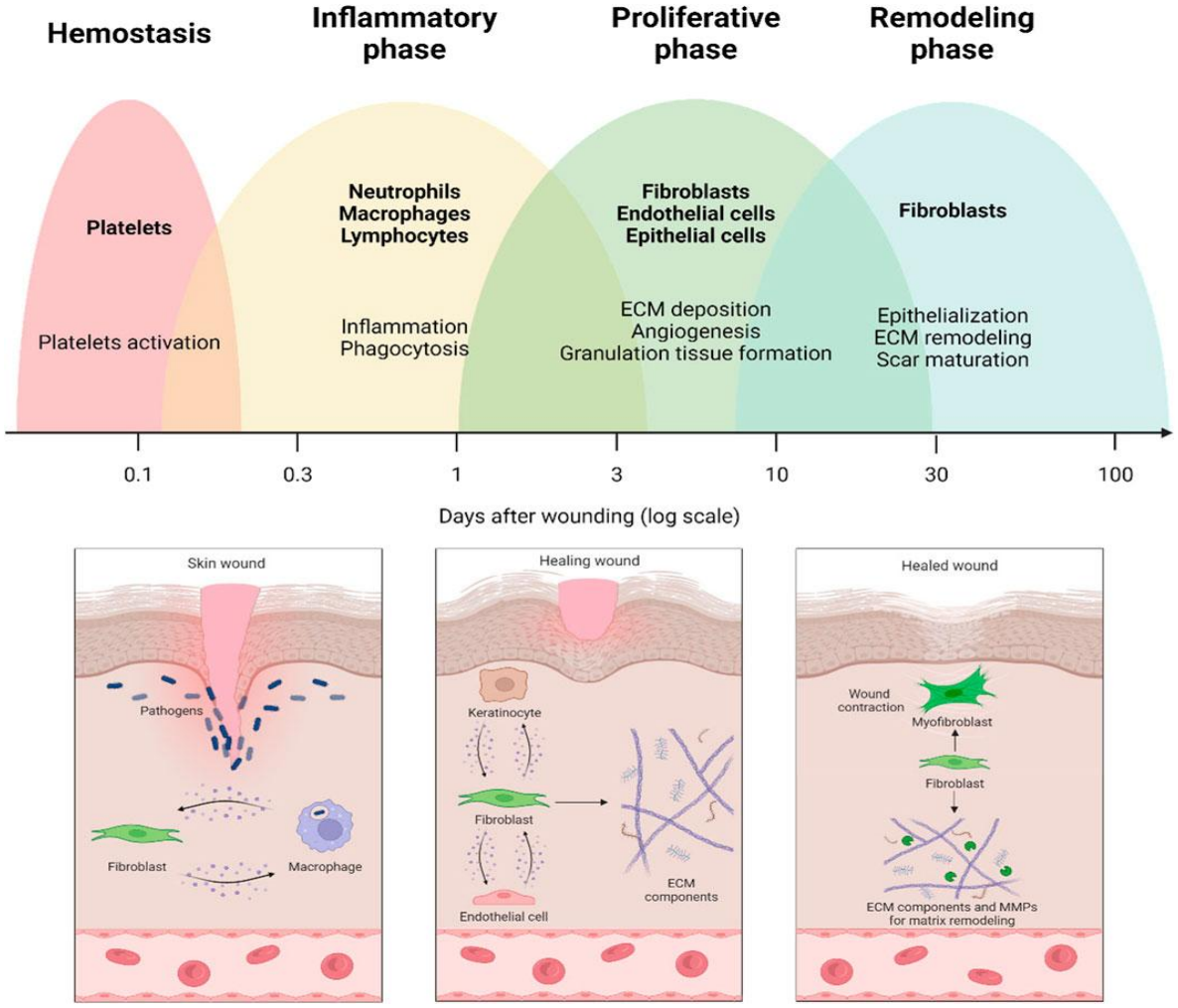

Figure 1 The wound healing process involves distinct phases, with fibroblasts playing a pivotal role. In the initial stages of wound healing, intricate communication takes place between fibroblasts and immune cells. Subsequently, fibroblasts synthesize key components of the extracellular matrix (ECM) while establishing communication channels with endothelial cells and keratinocytes. As the healing process progresses, fibroblasts contribute to the remodelling of the ECM through the secretion of matrix metalloproteinases (MMPs) and other matrix constituents. The figure was reproduced from Cialdai et al. (2022) under the Creative Commons Attribution License (CC BY) terms.

The remodelling phase aims to develop normal skin tissue and maturation of the scar. A balance between collagen synthesis and degradation accomplishes this phase. Nevertheless, the tissue achieves original strength, but approximately 80% of wound strength is achieved following long-term wound healing. Nutrition, hypoxia, chronic inflammation, Infection, and immunosuppression are important factors that influence wound healing. Therefore, a thorough understanding of the physiological events and factors affecting wound healing is necessary to reduce the morbidity and mortality of wounds (Singh et al. 2017).

5 Wound management

For achieving optimum wound healing, the foremost consideration is maintaining an aseptic state during wound care and using suitable antiseptics to lower the infection rate if the wound has been doubtful about its contamination. The chosen wound management method is also essential to minimize tissue necrosis. The wound management begins with providing adequate haemostasis, followed by further techniques like applying pressure bandaging. Finally, the traditional use of caustic and cauterization agents applied over the wound is replaced mainly with topical collagen, gelatin, thrombin, etc. (Brown and Zitelli 1993). According to Anderson (1996), there are six essential points to be considered while managing any wounds: preventing further wound contamination, debridement of necrotizing tissue, eliminating debris, foreign contaminants, and adequate wound drainage, ensuring the formation of a healthy vascular bed and selection of an effective method for closure of skin defect.

The underlying principle of wound management is to optimize the environment for wound healing. The treatment modality of wounds mainly depends on the size and type of wound. The initial procedure for wound management is decontamination, followed by debridement. These procedures allow for eliminating both infections and removing dead and devitalized tissues. The outcome of wound management aims to form a sound and viable wound bed that ensures wound closure (Anderson 1996). The wound healing rate, tissue return to normal function, final appearance, client satisfaction, etc., will ultimately depend on how the wound is managed. It also has miscellaneous topical applications of various agents like Aloe vera gel, honey, live yeast cell derivatives, etc. In addition, growth factors like epidermal growth factors and epidermal growth factors like peptide GF, PDGF, and TGF potentially enhance wound repair (Liptak 1997).

6 Factors Affecting Wound Healing

Impaired wound healing results from several factors that can participate in one or more phases of the wound repair process. Guo and DiPietro (2010) broadly classified these factors as local and systemic factors affecting wound healing.

6.1 Local factor

The local factors affecting wound healing include oxygenation, Infection at the wound site and high levels of metalloproteinases (MMPs).

6.1.1 Oxygenation

Oxygen is essential for preventing wound infection, inducing angiogenesis, keratinocyte differentiation, collagen synthesis, re-epithelization, and allowing normal wound contraction. Conversely, inadequate oxygenation induces temporary hypoxia and impairs wound healing (Rodrigues et al. 2019), and such impaired wounds result in the formation of chronic or ulcerated wounds.

6.1.2 Infection

Inflammation is the first phase in the process of normal wound healing. This process is essential to remove all the contaminating microorganisms. If there is a failure to effectively decontaminate the wound, the inflammation process will get prolonged along with improper microbial clearance. In the absence of an incomplete inflammatory process, there will be a prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. This prolongs the inflammatory phase and increases the level of matrix metalloproteases (MMPs), a group of proteases that can degrade the extracellular matrix. In relation to the increased protease content, there will be a decrease in the level of protease inhibitors that occurs and results in chronic wounds (Menke et al. 2007).

6.1.3 Metalloproteinases

Elevated levels of MMPs, a group of enzymes capable of breaking down the ECM, coincide with a reduction in natural protease inhibitors. This shift in the protease balance can hasten the degradation of growth factors present in chronic wounds (Menke et al. 2007). Infections in wounds follow a pattern akin to other infectious processes, manifesting as biofilms—complex communities of aggregated bacteria encased in a self-produced extracellular polysaccharide matrix. Fully developed biofilms create shielded microenvironments, rendering them more resilient to typical antibiotic treatments (Guo and DiPietro 2010).

6.2 Systemic factors

The systemic factors affecting wound healing include the animal's age, stress, diabetes, sex hormones, medication, obesity, nutrition, etc.

6.2.1 Age

The risk of impaired wound healing increases with age. In healthy elderly individuals, there is a delay in wound healing without altering the quality of wound healing (Gosain and DiPietro 2004). This delay is thought to be due to decreased macrophage function and reduction in the inflammatory response associated with old age (Swift et al. 2001).

6.2.2 Sex hormones

Sex hormones in aged individuals affect wound healing either positively or negatively. For example, it is observed that estrogen improves age-related impairment in wound healing, whereas androgens negatively influence cutaneous wound healing (Gilliver et al. 2007).

6.2.3 Stress

Studies suggest that there is stress-induced alteration occurs in the normal wound healing process by reducing the function of the immune system (Boyapati and Wang 2007).

6.2.4 Diabeties

The alteration of healing in diabetes individuals occurs due to hypoxia, fibroblasts and epidermal cell dysfunction, deviation of angiogenesis and neovascularization from normal, increased metalloproteases levels, reactive oxygen species induced damage, decrease in the immune resistance and neuropathy (Guo and DiPietro 2010).

6.2.5 medication

Different medications, such as glucocorticoids, NSAIDs, chemotherapeutic agents, etc., can affect the normal wound healing

process. Systemic steroids are often used as an anti-inflammatory in wounds but can contribute to incomplete granulation tissue and reduced wound contraction (Franz et al. 2007). It also increases the risk of Infection. NSAIDs are also widely used agents in the treatment of wounds. However, short-term usage of NSAIDs has a less negative impact on wound healing, whereas long-term use causes anti-proliferative effects and impairs wound healing (Guo and DiPietro, 2010). Chemotherapeutic agents inhibit DNA, RNA, and protein synthesis, reducing fibroplasia and wound neovascularisation (Franz et al. 2007).

6.2.6 Obesity

Mostly, this will alter the functioning of the immune system and lower inflammatory response, thereby impairing the normal wound repair process.

6.2.7 Alcohol and smoking

These habits are found to have a negative impact on wound healing but are the least concern in veterinary practice.

6.2.8 Nutrition

Energy, fat, proteins, amino acids, micro-nutrients, etc., greatly influences the normal wound healing process. Energy sources provide the glucose precursor required for ATP synthesis and are essential for cellular repair. Protein is the most important nutrient needed for the normal wound-healing process. Among all the nutrients, micronutrients like vitamins and minerals have more influence on wound repair. Any inadequacy in the level of these nutrients alters the normal repair mechanism. For example, vitamin C and A deficiency impair re-epithelization, normal collagen synthesis, etc., whereas an excess of vitamin E impairs healing. A lower zinc level is essential for normal wound healing, but excess levels negatively affect healing (Guo and DiPietro 2010).

7 Dressing materials in wounds

Wound dressings have evolved through extensive development in recent years (Figure 2). In ancient times, dressing material was restricted to traditional natural products covered over the wound directly. But, over the years, there were significant changes, and newer dressing materials were developed to achieve specific functions (Zahedi et al. 2010). Recent studies suggested that simple gauze dressings are unsuitable for all types of wounds because the gauze absorbs all the moisture and exudates and can dry the wound environment. This causes further damage while the wound dressing is being replaced (Kucharzewski et al. 2019). The property of an ideal wound dressing material includes the capacity to attain rapid healing, low cost, and maximum comfort to the patient. There are several ways for the classification of wound dressing materials. Based on the functions of the dressing material used, it can be classified as debridement, antibacterial, occlusive, absorbent, or adherent dressing.

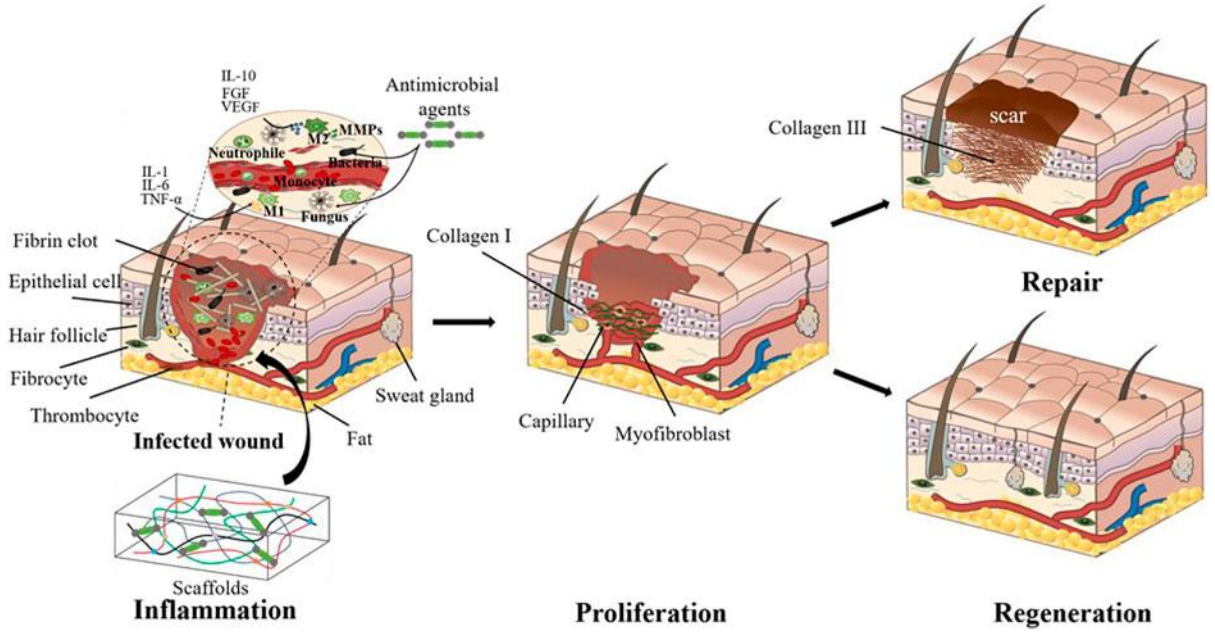

Figure 2 Tissue-engineered scaffolds have emerged as a promising approach for treating cutaneous wounds. Currently, wounds are addressed using two main types of scaffolds: conventional biological scaffolds and pro-regenerative counterparts. While both scaffold types contribute to wound healing, the pro-regenerative scaffolds exhibit a greater capacity for fostering complete and well-structured skin regeneration. In contrast, traditional biological scaffolds often result in the formation of scar tissue. The figure was reproduced from Qin et al. (2022) under the Creative Commons Attribution License (CC BY) terms.

Based on the materials used to produce the dressing they can be hydrocolloid, alginate, or collagen dressing. Depending on the physical form of the dressing material, it can be in the form of an ointment, film, foam, gel, etc. One of the important classifications of dressing is based on the physical contact that the dressing material makes with the wound. Dressings making direct contact with the wound surface are called primary dressings. Secondary dressings are those that are applied over the primary dressing. Island dressings exhibit an absorbent area at the centre of the dressing material surrounded by an adhesive region. The most commonly used dressing material classification is traditional, modern, advanced dressings, skin replacement material, and wound healing devices (Boateng et al. 2008).

The foremost function of dressing material is to protect the wound and provide the ambient environment for healing so that dressing material can contribute to cutaneous repair. There are various synthetic and natural dressing materials with satisfactory results. The natural dressing includes polysaccharides (chitin, chitosan, etc.), proteoglycans, and proteins (collagen, gelatin, eggshell membrane, etc.). The biocompatibility, biodegradability, and similarity with extracellular matrix contribute to the extensive use of natural dressing material. Synthetic dressing materials are obtained by electrospinning technique and are designed to exhibit specific and extraordinary functions. Examples of synthetic dressings include bio-mimetic micro/nanoscale fibres (Mogoşanu and Grumezescu 2014).

8 Eggshell membrane in wound healing

Many bio-materials, which are capable of managing wounds, have been developed. They help protect against microbial contamination and are also used to deliver therapeutic agents. Eggshell membrane (ESM) is a highly produced global waste that has potential applications in various fields (Banu et al. 2023). ESM is a thin layer of 100µm thickness lying between the eggshell and egg albumen. It is a double-layered membrane that has a light pink colour. ESM has a semi-permeable structure. The porous architecture is contributed by the peculiar arrangement of protein fibres (Mittal et al. 2016).

Structurally ESM is composed of 3 layers: outer ESM, inner ESM, and limiting membrane. The ESM can be separated by manual peeling, acid treatment, or dissolved air flotation (Yoo et al. 2009). ESM is composed of both organic and inorganic matrices. The organic components are proteins, including collagen (type I, V, X), osteopontin, keratin, proteoglycan, and glycoprotein (Sah and Rath 2016). The other important chemical constituents of ESM include glucosamine, chondroitin, hyaluronic acid, ovotransferrin, desmosine and isodesmosine, sialic acid, lysyl oxidase, lysozyme, β-N-acetylglucosaminidase (Ruff et al. 2009). ESM constitutes a protein network composed of collagen I, V, X, etc., which is responsible for enhanced tissue regeneration (Guha Ray et al. 2018). Tavassoli (1983) has established the biocompatibility of ESM with stromal cells. ESM exhibits properties that alter the quality and rate of wound healing (Amitha et al. 2023; Banu et al. 2023).

9 Fibrin glue

Fibrin glue is a tissue sealant that has been widely used as an adjunct to haemostasis for many years (Mooney et al. 2009). However, over decades, the significant functions of fibrin glue have been limited to being a tissue sealant and an agent for controlling bleeding. But recently, the potential of fibrin glue has been widely exploited as a drug delivery system. The use of fibrin glue as an agent for drug delivery involves effective drug-matrix interaction and controlled drug release (Spicer and Mikos 2010). The fibrin glue can be prepared either from PRP or by combining concentrated fibrinogen solutions with thrombin (Silver et al. 1995). Human fibrin glue comprises two different components contained in separate vials. This component includes freeze-dried clotting proteins (fibrinogen, fibronectin) and freeze-dried thrombin, which act as catalysts. The clotting factors are reconstituted in a solution containing aprotinin to inhibit tissue fibrinolysis. The thrombin portion is dissolved in a calcium chloride solution. These components play an inevitable role in haemostasis and wound healing.

The mechanism of action is through its capability to mimic the physiological coagulation process, serving its haemostatic and sealing action. Moreover, it evokes bio-stimulation that directs the formation of new tissue (Canonico 2003). When injected at the desired site, the two components club together in equal volumes, where the thrombin gets converted into the fibrinogen to fibrin. An enzymatic reaction mediates the conversion, and the rate of reaction is controlled by the thrombin concentration. Spotnitz et al. (1997) compared suture technique and fibrin sealant application and concluded that fibrin sealant is a suitable biological tissue glue that can effectively function as a potent adjunct to sutures. Fibrin sealant can be used alone or with sutures or tape to promote optimal wound integrity. In addition, these fibrin sealants can be used independently in wounds where sutures cannot control or aggravate bleeding. These adhesives' advantages include sealing tissue planes and eliminating potential dead spaces. It is interesting to notice that fibrin sealant has resulted in a low infection rate and has promoted healing. The effectiveness of fibrin glue dramatically depends on the intended surgical use. The fibrin glue causes a reduction in drainage and seroma formation, but these animal studies are not well predictive for clinical usage. Moreover, application methods like spraying or applying on a stream will significantly influence the effectiveness of fibrin glue. The major drawback with commercial preparation is that the constituent and outcome of fibrin glue may vary considerably (Clark 2003).

Fibrin glue has been proven to potentiate wound-healing effect on oral cavity wounds compared with traditional sutures (Yucel et al. 2003). Fibrin adhesives are highly useful for averting leakage and promoting healing in conditions like complex gastrointestinal surgeries, especially with intestinal anastomosis. The stumbling block of fibrin sealant application in such conditions is due to the high cost of these products. Fibrin glue has also been found to have osteoinductive potential (Abiraman et al. 2002). Takagi et al. (2001) established the prospects of using fibrin glue in tracheal anastomosis. Topical application of BM-MSCs and their conditioned media via fibrin glue vehicle effectively improved the quality of cutaneous wound healing in chronic excisional wounds in rats without any marked acceleration at the rate of wound closure (Mehanna et al. 2015).

10 Animal models for wound healing studies

In vitro and in vivo models have primarily contributed to the understanding and studying of the physiologic model and aberrant wound healing mechanism (Parnell and Volk 2019). The criteria for selecting an animal model include inter-species anatomical considerations and physiological attributes, differences in wound healing mechanisms among different animals, sample size, and analytical techniques applied for the study (Lindblad 2008). The other factors influencing the selection of an animal model for wound studies are availability, cost, ease of handling, and investigator familiarity. Small mammals are commonly used for wound healing and studies (Sullivan et al. 2001). Full-thickness wound models are the ones that are created by the complete removal of the epidermis, dermis, fascial planes, and even subcutaneous fat. Healing starts from the margins and progresses to the base of the wound.

The formation of the fibrin clot will be followed by the infiltration of granulation tissue and the migration of an epidermal tongue along the interface between the granulation tissue and the clot. This type of wound is created using standard equipment like a biopsy punch, scalpel, and dermatome. The major advantages of this model are a significant wound volume involving all dermal layers, epithelialization occurring from the margin of the wound, and the provision to assess chemistry, histology, and cell population within the wound site. The rate of wound healing is usually monitored through re-epithelialization rate, histological organization of connective tissue, angiogenesis, and extent of collagen or proteoglycan deposition. The major demerits of this model are excess bleeding and an increased chance of infections (Davidson 1998). Rabbits are widely used as experimental models in wound healing studies. Rabbits are loose-skinned animals where wound healing occurs primarily by contraction. The merits of this model are relatively inexpensive and highly prolificacy.

11 Assessment of wound healing

Wound healing studies are complex processes that integrate various cells and consist of various repair phases, such as inflammation, proliferation, re-epithelialization, and remodelling. The wound healing assessment can be done through non-invasive and invasive methods. The non-invasive protocols for wound healing estimation are done through wound tracing, photographic evaluation, biophysical techniques, etc. The invasive protocols require wound biopsies involving histopathological, biochemical, and immunological techniques of wound evaluation (Masson Meyers et al. 2020). Evaluation of wound healing is done generally through measurement of the size of the wound, morphological appearance of the wound, including wound bed and its relationship with tissue growth, the extent of scarring noticed, as well as vascular and pathophysiological conditions that may interfere with the healing (Falanga 2005). The use of digital photography and image analysis software helps in the assessment of healing and provides accuracy in choosing treatment protocols. This also helps monitor patients' treatment responses (Papier et al. 2000).

Histopathology of wounds is a useful tool that helps exclude malignancy, evaluate healing progress during treatment, and understand the pathophysiology of non-healing wounds (Romanelli et al. 2013). In the immunological method of wound healing estimation, signalling molecules involved in the repair process are identified and quantified with the help of several techniques like immunohistochemistry and enzyme-linked immunosorbent assay (Carlson and Longaker 2004). The biochemical assays employed for wound healing assessments are hydroxyproline, myeloperoxidase (MPO) assay, N-acetylglucosaminidase (NAG), oxidative stress profile, etc. These are very helpful to monitor the progression of wound healing. One commonly employed assay is the hydroxyproline assay, which indirectly indicates collagen content and thereby comments on wound healing. An increase in the hydroxyproline content shows that there is an increased deposition of collagen. The higher the collagen synthesis and deposition, the more enhanced wound healing will be. Techniques like flow cytometry and macrophage polarization are used for wound healing assessment (Masson Meyers et al. 2020).

12 Conclusion and prospects

The wound healing and skin regeneration field has witnessed remarkable progress, driven by interdisciplinary collaborations and innovative technologies. Advanced wound dressings, growth factors, stem cell therapies, biomaterials, and gene editing tools have transformed the treatment landscape. While challenges persist, including standardization, safety, and ethical concerns, the convergence of cutting-edge approaches holds immense potential to revolutionize wound care and improve patient outcomes in the coming decades. In conclusion, therapeutic approaches for wound

healing and skin regeneration have evolved significantly, offering innovative strategies to accelerate healing and promote tissue regeneration. As researchers delve deeper into understanding the intricate mechanisms underlying wound repair, the development of novel therapies and their clinical translation become increasingly promising. With a multidisciplinary approach and ongoing advancements in technology, biology, and medicine, the future holds great potential for transforming the field of wound healing and skin regeneration.

The era of personalized medicine is revolutionizing wound healing and skin regeneration. Advances in genomics, proteomics, and metabolomics enable the identification of patient-specific factors influencing wound healing outcomes. Biomarker profiling facilitates early diagnosis, prognosis, and treatment selection for optimal therapeutic responses. Pharmacogenomics guides drug selection and dosing, minimizing adverse effects and improving efficacy. Integrating patient-specific data with regenerative therapies empowers clinicians to tailor interventions, optimize wound healing trajectories, and enhance patient outcomes. Translating novel therapeutic approaches from bench to bedside requires rigorous preclinical studies and adherence to regulatory guidelines. Safety assessments, pharmacokinetics, and long-term outcomes must be thoroughly evaluated before embarking on clinical trials. Regulatory agencies play a critical role in ensuring the ethical and safe conduct of research, particularly in the context of gene editing, stem cell therapies, and novel biomaterials.

While wound healing and skin regeneration are poised for remarkable advancements, several challenges and ethical considerations warrant attention. Long-term safety assessments of emerging therapies are paramount to mitigate unforeseen risks and adverse effects. Standardizing protocols, outcome measures, and patient selection criteria is essential to ensure consistent and reproducible results across clinical trials. Additionally, ethical considerations surrounding gene editing, stem cell research, and human experimentation require thoughtful discourse and guidelines to balance scientific progress with societal and moral values. Wound healing therapies must be scientifically robust, economically feasible, and globally accessible. Developing nations face unique challenges in wound care, including resource limitations and healthcare disparities. Innovations in low-cost wound dressings, telemedicine, and community-based care models can bridge the gap and improve wound healing outcomes on a global scale. Collaborative efforts between researchers, clinicians, policymakers, and non-governmental organizations are pivotal in ensuring equitable access to cutting-edge wound healing technologies.

Despite significant advancements, several knowledge gaps persist in wound healing and skin regeneration. Further elucidation of cellular and molecular mechanisms governing wound repair, inflammation resolution, and scar formation is warranted. Integration of advanced imaging modalities, such as multiphoton microscopy and single-cell RNA sequencing, will provide unprecedented insights into dynamic cellular processes. Exploring the crosstalk between wound healing and the microbiome and the influence of ageing and systemic diseases will unravel new therapeutic targets and strategies.

Ethical approval

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Data statement

The authors confirm that the data supporting the findings of this study are available within the article.

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Declaration of Interest

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An overview of artificial nutrition in apiculture

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KEYWORDS

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Diet patterns

ABSTRACT

Artificial nutrition in apiculture is a long-term subject of discussion and investigation. The maintenance and boosting of bee colonies in apiculture depends on synthetic food around the globe to overcome the suppressing factors, including dearth periods. The information on types of food components and their combinations used is haphazard and hardly helpful in determining the advancements in the artificial feeding of bees. This study aimed to extract the available information on artificial feeding on honeybees and arrange it most scientifically. The information in the form of research or review articles available on every platform, *viz*., soft portals, printed journals, books and scientific proceedings, were collected and analyzed to produce a comprehensive and informative review article on the artificial nutrients in apiculture. Compilation of the available information revealed that artificial feeding of bees depends on food components and their combinations. Based on this, it can be suggested that nectar and pollen are basic foods of honey bees, and based on this, the food components were further categorized as nectar supplements and pollen supplements. These supplements were fed to bees as natural nutrients and food components. The natural nutrients include proteins, carbohydrates, vitamins, yeasts, antibiotics, amino acids, enzymes, antioxidants, etc. Meanwhile, under natural food components, cereal grains, pulses, beans, fruits, vegetables, medicinal plants, spices, condiments, and some non-traditional/ miscellaneous kinds of stuff have been included in the bee diets. On the other hand, many diet categories have been prepared using the abovementioned nutrients and food components in various forms and proportions. In general, the pollen and nectar, the main food of bees, have been supplemented under different diet combinations. These diet

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combinations used pollen and nectar substitutes or combined with other nutrition, drugs, antibiotics, etc. The present investigation provides an updated overview of the food categories and their combinations used in the artificial feeding of bees to date. These findings can help explore new food items and their effective diet combinations.

1 Introduction

The honey bee (*Apis mellifera*) is one of the most economically and ecologically significant insects providing nutritive honey, propolis, venom, wax, and pollination to the agriculturally important crops (Calderone 2012; Marcelino et al. 2022; Naz et al. 2022). The decline in bee populations may negatively affect agricultural productivity as these social insects are responsible for pollinating 3/4^{ths} of the world's angiosperm plants (Morse and Calderone 2000; Aizen and Harder 2009; Grossman 2013). The possible reasons behind this decline are the non-judicious use of unsafe agrochemicals, climate change, and the socio-economic condition of the farms. These factors, alone or in combination, may affect honeybees and their valuable products. Uncertain fluctuations in temperature, relative humidity, shortage of water, deforestation of floral plants, non-scientific apicultural practices, and pests/diseases also contribute to bee population decline and concerned production (Wakgari and Yigezu 2021).

The availability of quality food through natural resources may ensure the better survival of honeybees. However, constant and continuous natural food availability is impossible, and honeybees often face dearth periods. Apiculturists feed the bees with sugars and different artificial foodstuffs to maintain the colony and avoid losses during these food exhaustion intervals. More specifically, for an individual bee's overall development and physiological functioning, a variety of nutrients, such as carbohydrates, protein, vitamins, amino acids, minerals, lipids, etc., are needed through a single diet (Brodschneider and Crailsheim 2010). Like in other living beings, a balanced supply of nutrition plays a vital role in multiple aspects of bees as caste development (Slater et al. 2020), disease resistance (Zheng et al. 2014; Basualdo et al. 2014; Hoffman and Chen 2015; Glavinic et al. 2017), increases lifespan (Knox et al. 1971), development of hypopharyngeal glands (Keller et al. 2005a, b; Mohamed et al. 2023), behaviour and development (Ament et al. 2008, 2010; Toth et al. 2005), and neural development (Moda et al. 2013). The nutrient contents and their proportions in diets also influence bee-associated microfauna responsible for nutrient processing and boosting immunity (Hildebrandt et al. 2009; Turnbaugh et al. 2009; Ponton et al. 2013; Raymann and Moran 2018; Kešnerová et al. 2020).

In the last few decades, a wide range of edible materials alone or in combination with *viz*., cereals and pulses, fruits, vegetables, synthetic drugs and minerals, yeasts, vitamins, proteins, sugars, etc., have been tested as quality feed options for honey bees. These materials were used with different processing methods and varying provisions of care. A thorough literature survey has been done and analyzed in the next section of this article.

2 Information collection and analysis

The literature on artificial feeding in apiculture available on all the plate forms viz., papers on the internet, printed journals, thesis, and published books were studied. Literature in other languages was also used after their proper translation (languages mentioned against the references in the bibliography). It was observed that almost all the publications showed bee food in the form of diets. These diets were investigated for two broad categories: the types of food components and the combination pattern of different food components.

To analyse the types of food components used, the diet is segregated under pollen and nectar substitutes as they are the primary natural food sources for honey bees. The food components not falling under either pollen or nectar substitutes were classified under the miscellaneous category. After establishing the types of food components, all the possible patterns of their combinations were also investigated.

3 Components used in the artificial feeding of honey bees

Based on origin, two categories of food components are natural nutrients and natural food components.

3.1 Natural nutrients

3.1.1 Protein

Protein is an important nutrient essential for the growth and proper functioning of the body system, including the development of different bee glands (Keller et al. 2005b). In general, protein deficiency may result in the deformation or size reduction in the hypopharyngeal glands of worker bees (DeGrandi-Hoffman et al. 2010). In contrast, an optimum protein provision also plays a significant role in resistance development against pathogens in bees (Rowley and Powell 2007; Behmer 2009; Alaux et al. 2010; Mao et al. 2013), whereas deficiency of protein leads to susceptibility against harmful pathogens (Field et al. 2002; Li et al. 2007). Other immunity mechanisms like encapsulation, phenyl oxidase, and lysozyme activities are also protein-dependant (enzyme-based) (Lee et al. 2006). For this protein intake, honey bees depend on pollen grains and royal jelly for quality protein; however, the natural supply of pollen is not continuous throughout the year.

Table 1 Use of commercial and isolated proteins in artificial feeding of bees

S.N.	Commercial/isolated Proteins	References
1.	Albumin	Kumari and Kumar (2020), Morais et al. (2013), Sabir et al. (2000), Haydak (1967)
2.	Casein	Kumari and Kumar (2020), Mahfouz (2016), Pirk et al. (2010), Al-Eitby (2009), Herbert and Shimanuki (1979a),
3.	Crude protein	Zheng et al. (2014), Herbert and Shimanuki (1979a)
4.	Gevral protein	Al-Eitby (2009)
5.	Isolated soy protein	Sereia et al. (2013)
6.	Lactalbumin	Shimanuki and Herbert (1986)
7.	Protein Hydrolysate	Kumar and Agrawal (2014)
8.	Sodium caseinate	Malone et al. (2004)
9.	Tankage protein for animal feed	Haydak (1936)
10.	Whey protein flour (milk protein)	Mahfouz (2016), van der Steen (2007)

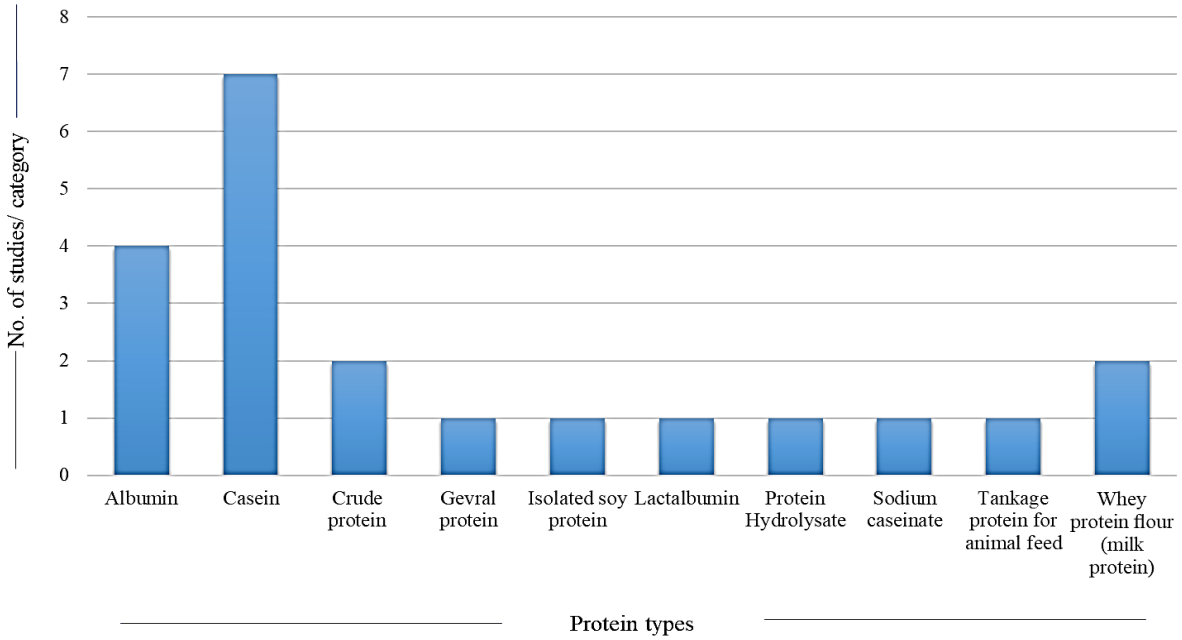

Figure 1 Comparative use of various proteins in artificial feeding of honeybees

Keeping the importance of protein in mind, researchers worldwide have used almost ten types of protein formulations, either isolated or commercial (Mahfouz 2016; Kumari and Kumar 2020). Among the reported proteins, albumin and casein have dominantly been used in the artificial feeding of bees (Table 1 & Figure 1). The economy of such a diet, however, exceeded.

3.1.2 Vitamins

A wide range of vitamins are also required for the development and physiology of bees. Along with others, water-soluble vitamins B and C are more commonly found in floral pollen grains. However, fat-soluble vitamins (A, D, E, and K) encourage more brood production (Herbert and Shimanuki 1978b; Roulston and Cane 2000). To date, overall, 14 different vitamins, *viz*., A, B, B1, B2, B6, B12, C, D, etc., have been utilized in the artificial feeding of honey bees (Akyol et al. 2006; Abd El-Wahab et al. 2016; Tawfik et al. 2020; Kumar et al. 2021) (Table 2). Among all the utilized vitamins, a mixture of multivitamins has been the most extensively used form (Figure 2).

3.1.3 Lipids

Lipids are also essential to bees, but for their supply, they depend on pollen grains (Haydak 1970). Specifically, lipids are needed during the brood stage and are considered an important precursor

Table 2 Utilization of synthetic vitamins in honey feeding

S.N.	Name of Vitamin	References
1.	Multivitamin mixtures	Kumar et al. (2021), Abd El-Wahab et al. (2016), Sihag and Gupta (2013), Amro et al. (2016)
2.	Vitamin A (Retinol)	Islam et al. (2020), Abd El-Wahab et al. (2016), Akyol et al. (2006)
3.	Vitamin B-complex	Al-Shammary and Al-Gerrawy (2017), Sabir et al. (2000),
4.	Vitamin B 1 (Thiamine)	Akyol et al. (2006), Omar (2006)
5.	Vitamin B2 (Riboflavin)	Akyol et al. (2006), Omar (2006)
6.	Vitamin B6 (Pyridoxine)	Akyol et al. (2006)
7.	Vitamin B12 (Biotin, folic acid, and the cobalamins)	Colibar et al. (2011), Akyol et al. (2006)
8.	Vitamin C (Ascorbic)	Ahmad et al. (2021), Tawfik et al. (2020), Zahra and Talal (2008), Akyol et al. (2006)
9.	Vitamin D (Calciferol)	Islam et al. (2020), Abd El-Wahab et al. (2016)
10.	Vitamin D3 (Cholecalciferol)	Akyol et al. (2006)
11.	Vitamin E (Tocopherol)	Islam et al. (2020), Abd El-Wahab et al. (2016), Akyol et al. (2006), Şahinler et al. (2005)
12.	Vitamin K3 (Menadione)	Akyol et al. (2006)
13.	Ca-d-Pantothenete	Akyol et al. (2006)
14.	Vitamix Formula- Topkim	Akyol et al. (2006)

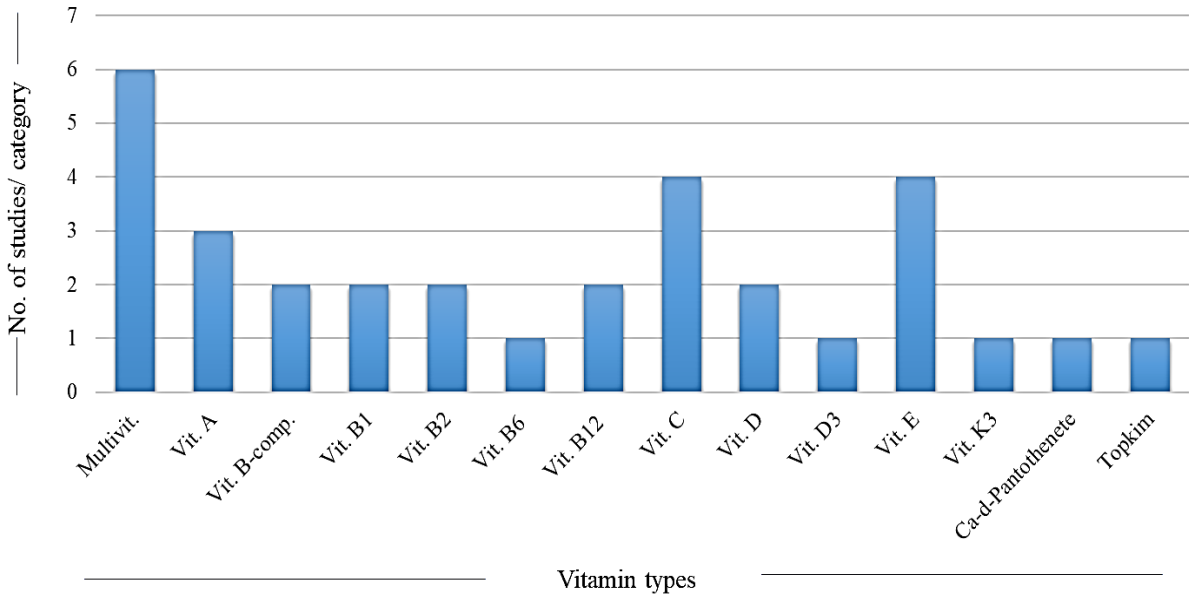

Figure 2 Comparative use of various vitamins in artificial feeding of honeybees

for further bio-molecular synthesis in honey bees (Cantrill et al. 1981). At the brood stage, the actual extraction of lipids from pollen inside the bee brood stomach increases by 2-4% (Herbert et al. 1980). When available literature was studied, no evident use of isolated lipids could be traced; however, many oilseed grains and essential oils rich in lipids have been used in bee feeding.

3.1.4 Minerals

Minerals are another important constituent of honey bee nutrition (Haydak 1970). Naturally, the main sources of minerals to bees are pollen, nectar, water, or the existing mineral pools in adult honeybees (Imdorf et al. 1998; Brodschneider and Crailsheim

2010). However, any artificial addition of minerals, *viz*., 1% pollen ash, increases brood production (Herbert and Shimanuki 1978a).

3.1.5 Carbohydrates

Carbohydrate is a universal energy source for honey bees, obtained from the flower nectar of different plants (Brodschneider and Crailsheim 2010). The needed energy is derived from carbohydrates for various physiological activities viz., physiological metabolism, immune reactions, and microbial resistance (Erler et al. 2014). Adult worker bees generally require an average of 4 mg of sugar per day for survival (Barker and Lehner 1974), and around 59.4 mg of carbohydrates are needed during their larval period (Rortais et al. 2005). Honey bees collect nectar from flowers, turn them into honey, and store them in hexagonal eyes, which are the primary source of carbohydrates, essentially required for natural growth and development and to generate energy for movement, body heat, and other functions. It has been estimated that the amount of food in the colonies should not be less than 9-12kg or the equivalent of 3-4 honey frames to keep brood rearing at a high level. Honey bee colonies are also fed with supplementary carbohydrates, *viz*., sugars in warm countries to stimulate queens to lay eggs (even if the sects have abundant honey). In the literature, no use of synthetic carbohydrates was witnessed here. However, Sugars have been used extensively in feeding.

Over 14 types of sugars in different forms have been utilized in artificial food for honey bees (Hoover et al. 2006; Rashid et al. 2018; Khan et al. 2021; Szczęsna et al. 2021). Sucrose is the most commonly used sugar as a solution, syrup, candy, and in-ground form (Gemeda et al. 2018; Kumar et al. 2021). These sugar forms are either used as a single component or in addition to other ingredients. The common sugar was followed by honey, which was utilized in

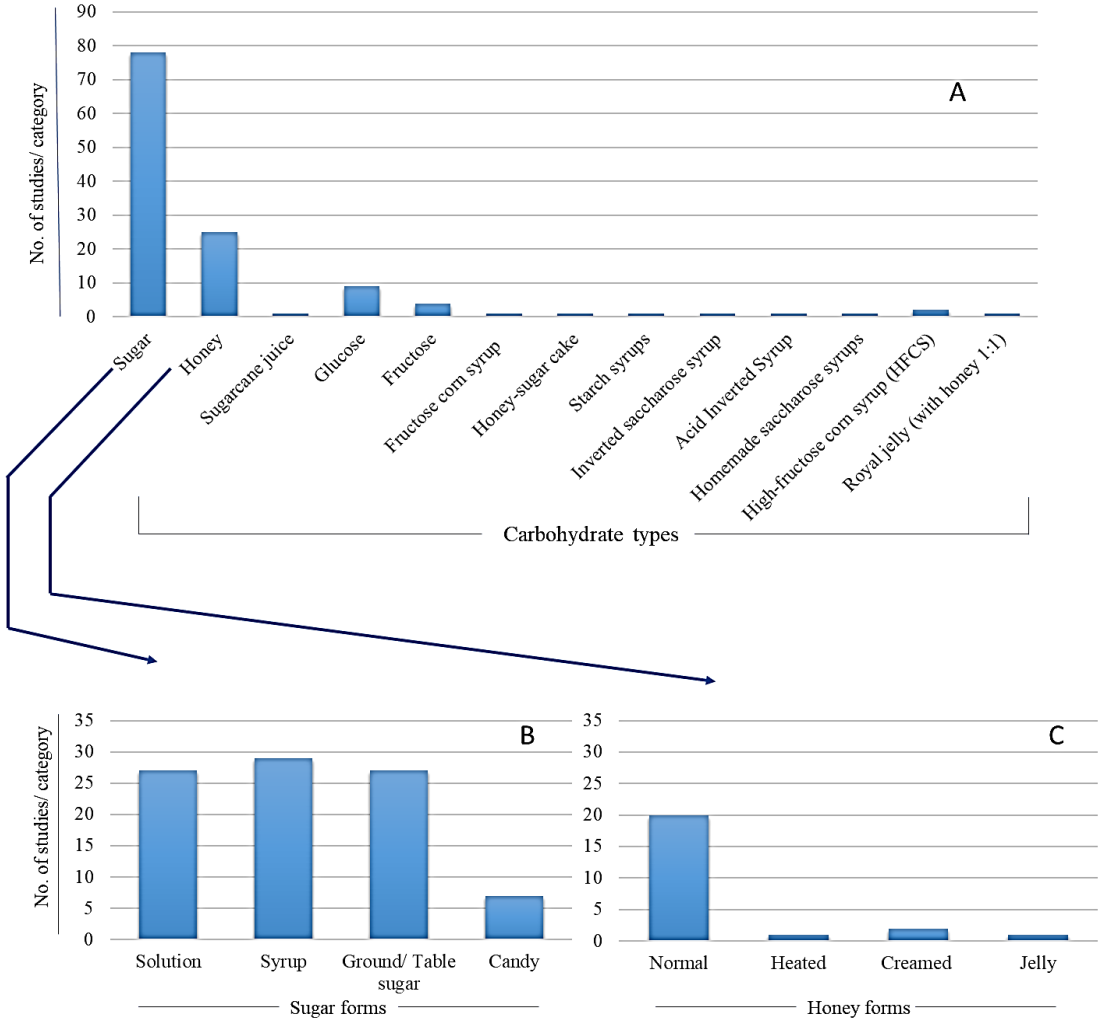

Figure 3 Comparative use of- A- various carbohydrates, B- various forms of sugar, C- various forms of honey in artificial feeding of honeybees

normal, creamed, heated, and jelly forms (Abou-Shaara 2017; Ullah et al. 2021). The third dominant sugar is glucose and fructose (DeGrandi-Hoffman et al. 2010; Guler et al. 2018) (Figure 3). In addition to this, various syrups (fructose corn, inverted saccharose, starch, acid inverted, homemade saccharose, etc.), honey-sugar cake, mixtures of sucrose and dextrose, and royal jelly have been tested for artificial feeding of honey bees (Hoover et al. 2006; Mirjanic et al. 2013; Wilde et al. 2014; Szczęsna et al. 2021) (Table 3, Figure 3).

Table 3 Different sugar forms used in feeding honey bees

S.N.	Sugar	Form	References
1.	Sugar	Solution	Gemeda et al. (2018), Rashid et al. (2018), Omar et al. (2017), Carrillo et al. (2015), Rezaei et al. (2015), Zhang et al. (2015), Somerville (2014), Usha et al. (2014), Ghazala and Nowar (2013), Singh and Singh (2012), Fasasi (2011), Al-Maktary (2009), Al-Sarhi (2008), Shehata and Nafea (2006), Diemer (2005), Aupinel et al. (2005), Al-Jubouri (2005), Abd El-Wahab and Gomaa (2005), El-Sherif (2002), Al-Hammadi (2001)
		Syrup	Khan et al. (2021), Tawfik et al. (2020), Islam et al. (2020), Younis (2019), Balkanska and Salkova (2018), Guler et al. (2018), Stevanovic et al. (2018), Abou-Shaara (2017), Abd El-Wahab et al. (2016), Haleem et al. (2015), Mirjanic et al. (2013), Anđelković et al. (2011), Colibar et al. (2011), Fasasi (2011), Sihag and Gupta (2011), De-Grandi-Hoffman et al. (2010), Saffari et al. (2010), Versluijs (2010), Bodla et al. (2009), Skubida et al. (2008), Fasasi et al. (2007), Akyol et al. (2006), Omar (2006)
		Ground/ Table sugar	Mohamed et al. (2023), Kumar et al. (2021), Islam et al. (2020), Younis (2019), Abou-Shaara (2017), Al-Shammary and Al-Gerrawy (2017), Zaghloul et al. (2017), Abd El-Wahab et al. (2016), Abd El Hamid and Abou-Shaara (2016), Amro et al. (2016), Kishan and Srinivasan (2016), Wheeler and Robinson (2014), Johnson (2014), Sammataro and Weiss (2013), Ghazala and Nowar (2013), Li et al. (2012), Al-Ghamdi et al. (2011), Fasasi (2011), Al-Eitby (2009), Avni et al. (2009), Al-Maktary (2009), Dodologlu and Emsen (2007), Keller et al. (2005a, b),
		Candy	Al-Ghamdi et al. (2021), Abou-Shaara (2017), Aly et al. (2014), Anđelković et al. (2011), Skubida et al. (2008), Akyol et al. (2006), Beota et al. (2005)
2.	Honey	Normal	Ullah et al. (2021), Islam et al. (2020), Younis (2019), Stevanovic et al. (2018), Zaghloul et al. (2017), Abd El-Wahab et al. (2016), Amro et al. (2016), Kishan and Srinivasan (2016), Taha (2015), Pande and Karnatak (2014), Usha et al. (2014), Wheeler and Robinson (2014), Sihag and Gupta (2013), Mirjanic et al. (2013), Anđelković et al. (2011), Al-Eitby (2009), Skubida et al. (2008), Hoover et al. (2006), Omar (2006)
		Heated	Barker and Lehner (1978)
		Creamed	Abou-Shaara (2017), Abd Elhamid and Abou-Shaara (2016)
		Jelly	Abou-Shaara (2017)
3.	Sugar cane juice		Beota et al. (2005)
4.	Glucose		Ullah et al. (2021), Guler et al. (2018), Abd El Hamid and Abou-Shaara (2016), Kaftanoglu et al. (2011), Beota et al. (2005)
5.	Fructose		Kaftanoglu et al. (2011), Peng et al. (1992), Vandenberg and Shimanuki (1987), Rembold and Lackner (1981)
6.	Fructose corn syrup		DeGrandi-Hoffman et al. (2010)
7.	Honey-sugar cake		Wilde et al. (2014)
8.	Mixtures of sucrose and dextrose		Nabors (1996)
9.	Starch syrups		Szczęsna et al. (2021)
10.	Inverted saccharose syrup		Szczęsna et al. (2021)
11.	Acid Inverted Syrup		Mirjanic et al. (2013)
12.	Homemade saccharose syrups		Szczęsna et al. (2021)
13.	High-fructose corn syrup (HFCS)		Guler et al. (2018), Wheeler and Robinson (2014)
14.	Royal jelly (with honey 1:1)		Hoover et al. (2006)

Table 4 Use of essential/ non-essential amino acids/ enzymes/ anti-oxidants/ drugs/ antimicrobial agents in artificial feeding of bees

S.N.	Essential/ Non-essential amino acids/ Enzymes/ Anti-oxidants/ Drugs/ Antimicrobial agents	References
1.	Glysine amino acid	Sabir et al. (2000)
2.	Methionin	Sabir et al. (2000)
3.	Biotin B7	Gençer et al. (2000)
4.	Niacin	Akyol et al. (2006)
5.	Pantothenic acid	Gençer et al. (2000)
6.	Cholesterol	Srivastava (1996)
7.	Folic acid	Gençer et al. (2000)
8.	Anicotinic acid	Omar et al. (2016)
9.	Essential amino acids (EAA)	Hendriksma et al. (2019)
10.	Nonessential amino acids (NAA)	Hendriksma et al. (2019)
11.	Creon (Enzyme based drug)	Al-Eitby (2009)
12.	Enzyme Invert Syrup	Mirjanic et al. (2013)
13.	Alphacel drug	Srivastava (1996)
14.	Antioxidants	Li et al. (2012)
15.	Multivitamin drug (Becosule)	Haleem et al. (2015)
16.	Fumagillin (Antibiotic)	Akyol et al. (2006)
17.	Tetracycline (Antibiotic)	Al-Shammary and Al-Gerrawy (2017), Omar et al. (2016)

3.1.6 Use of amino acids/enzymes/anti-oxidants/drugs/ antimicrobial agents

Apart from protein, vitamins, lipids, and carbohydrates, few studies used essential and non-essential amino acids, enzymes, antioxidants, drugs, microbial formulations, and antimicrobial agents. Among the available information, it was reported that 17 essential and non-essential amino acids were used in the artificial feeding of honey bees (Table 4). Proper development, physiological functioning and immunity against different diseases of an individual bee depend on a balanced diet comprised of carbohydrates, protein, vitamins, amino acids, minerals, lipids, etc. (Brodschneider and Crailsheim 2010; Basualdo et al. 2014; Zheng et al. 2014; Hoffman and Chen 2015; Glavinic et al. 2017; Slater et al. 2020). The immunity to fight pests and diseases was probably considered in opting for the abovementioned components.

3.1.7 Microbe-based nutrition

Microbes are also an excellent source of nutrition, especially single-cell protein providing vitamins (particularly the B-complex), several bioavailable minerals *viz*., chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), zinc (Zn) etc., and dietary fiber (Pacheco et al. 1997; Jach and Serefko 2018). From 1967 to date, three microbes were used as nutritional supplements. Out of these, yeast was the more commonly utilized microbe. Over ten types of yeast nutrition based on various culturing techniques, *viz*., Brewer's, Bacto, Difco, dry, and extracts, have been utilized in diet preparation for feeding of honey bees (Vandenberg and Shimanuki 1987; Kumar et al. 2021). Powdered Brewer's yeast and Commercial Yeast have dominantly been used in the artificial feeding of honey bees (Figure 4). Generally, the yeast was supplemented with other food components as additional nutrition. Commercial baking yeast has been used by most of the authors in this field (Table 5).

3.2 Natural food components

3.2.1 Cereal grains

Cereals are a well-known source of many essential nutrients, *viz*., Gluten protein, fiber, vitamin B-complex and E, omega-3-fatty acid, folate, phosphorous, magnesium, zinc, and anti-oxidants in minor quantities (Charalampopoulos et al. 2002; Garg et al. 2021). The details of the six major cereal grains which have been commonly used in feeding bee colonies across the world are given in table 6 (Neupane and Thapa 2005; Li et al. 2012; Mahmood et al. 2013; Shehata 2016; Aly et al. 2019; Islam et al. 2020). Wheat and maize are the dominant ones among these (Figure 5).

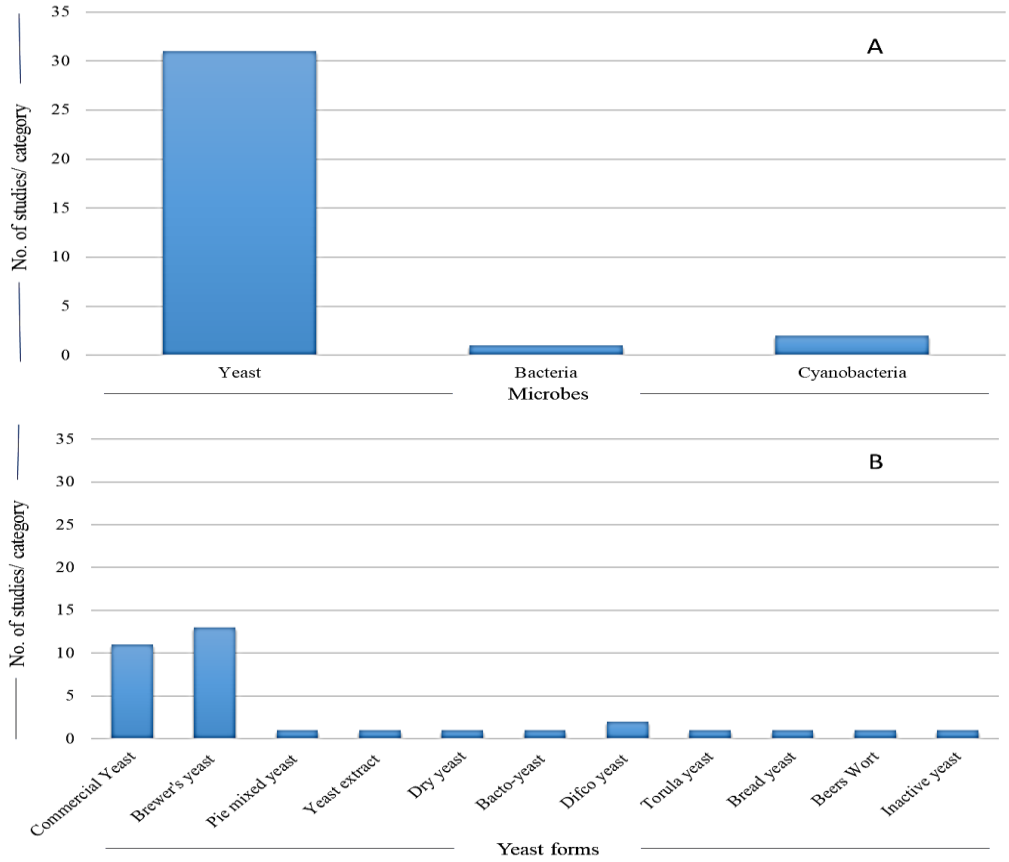

Figure 4 Comparative use of- A- microbes & B- various forms of yeast in artificial feeding of honeybees

Table 5 Utilization of microbes-based nutrition in artificial feeding honey bees

S.N.		Name of yeast	References
1.	Yeast	Commercial yeast	Kumar et al. (2021), Aqueel et al. (2017), Haleem et al. (2015), Kaftanoglu et al. (2011), Al-Ghamdi et al. (2011), Al-Sarhi (2008), Dodologlu and Emsen (2007), Shehata and Nafea (2006), Abd El-Wahab and Gomaa (2005)
		Brewer's yeast	Islam et al. (2020), Kumari and Kumar (2020), Younis (2019), Puškadija et al. (2017), Taha (2015), Pande and Karnatak (2014), Ghazala and Nowar (2013), Mirjanic et al. (2013), Morais et al. (2013), Mahmood et al. (2013)
		Pie mixed yeast	Al-Sarhi (2008)
		Yeast extract	Sihag and Gupta (2013)
		Dry yeast	Zaghloul et al. (2017)
		Bacto-yeast	Rembold and Lackner (1981)
		Difco yeast	Peng et al. (1992), Rembold and Lackner (1981)
		Torula yeast	Hanser (1983)
		Bread yeast	Irandoust and Ebadi (2013)
		Beerswort	Mirjanic et al. (2013)
		Inactive yeast	Mohamed et al. (2023)
2.	Bacteria (EM®Probiotic's live micro-organisms)	-	Tlak-Gajger et al. (2020)
3.	Cyanobacteria (Spirulina, *A. platensis*)	-	Kumar et al. (2013a, b)

Table 6 Cereal grains used in artificial feeding of honeybees

S.N.	Grain	Family	Form	References
1.	Wheat (*T. aestivum*)	Poaceae	Germinated, Gluten	Ghramh and Khan (2023), Aly et al. (2019), Younis (2019), Usha et al. (2014), Irandoust and Ebadi (2013), Omar (2006)
2.	Maize (*Zea mays* L.)	Poaceae	Flour	Ghramh and Khan (2023), Islam et al. (2020), Younis (2019), Amro et al. (2016), Usha et al. (2014), Mahmood et al. (2013), Li et al. (2012), Al-Maktary (2009), Shehata and Nafea (2006), Neupane and Thapa (2005)
			Gluten	Al-Ghamdi et al. (2011), Al-Sarhi (2008)
3.	Rice (*Oryza sativa*)	Poaceae	Grains	Aly et al. (2014)
			Bran	Neupane and Thapa (2005), Morais et al. (2013)
4.	Sorghum (*Sorghum bicolor*)	Poaceae	Flour	Ghramh and Khan (2023)
5.	Oats (*Avena sativa*)	Poaceae	Flour	Aly et al. (2014)
6.	Phalaris (*Phalaris arundinacea*)	Poaceae	Flour	Shehata (2016)

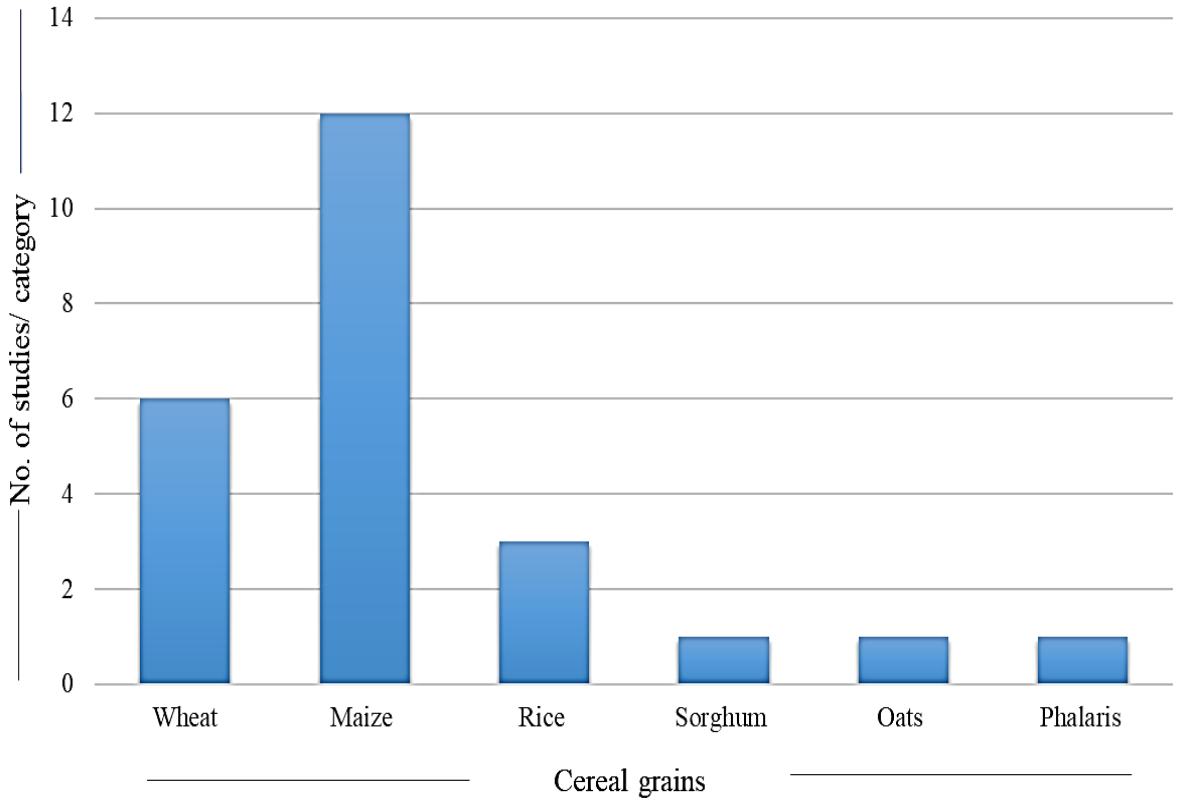

Figure 5 Comparative use of various types of cereals in artificial feeding of honeybees

3.2.2 Pulses and beans

Pulses are a novel source of protein for every living being, including bees. In addition, carbohydrates, fat, vitamins, minerals, and a broad range of essential amino acids can also be obtained from many pulses (Peas, chickpeas, lentils) and beans (Mukherjee et al. 2017; Boye and Maltais 2011). In the artificial feeding of bees, at least 14 pulses and beans in grounded form have been used in feeding honey bees to date (Puškadijaet al. 2017; Kumari and Kumar 2020; Islam et al. 2020; Ullah et al. 2021) (Table 7). Soybeans have been dominantly used as plants in two forms, *i.e.*, normal and defatted flour (Figure 6).

Table 7 Pulses and beans used in feeding of honey bees

S.N.	Grain	Family	Form	References
1.	Soybean (*Glycine max*)	Fabaceae	Flour	Ullah et al. (2021), Ahmad et al. (2021), Kumari and Kumar (2020), Islam et al. (2020), Aly et al. (2019), Younis (2019), Manning (2018), Puškadija et al. (2017), Al-Shammary and Al-Gerrawy (2017), Zaghloul et al. (2017), Abd El-Wahab et al. (2016), Shehata (2016), Mahfouz (2016), Amro et al. (2016), Rezaei et al. (2015), Taha (2015), Usha et al. (2014), Irandoust and Ebadi (2013), Kumar et al. (2013a, b), Sihag and Gupta (2013, 2011), Li et al. (2012), Al-Eitby (2009), Avni et al. (2009), Zahra and Talal (2008), Dastouri et al. (2007), Dodologlu and Emsen (2007)
			Defatted flour	Kumar and Agrawal (2014)
2.	Chickpea (*Cicer arietinum*)	Fabaceae	Powder	Ghramh and Khan (2023), Tesfaye (2019), Younis (2019), Zaghloul et al. (2017), Aly et al. (2014), Pande and Karnatak (2014), Usha et al. (2014), Mahmood et al. (2013), Sihag and Gupta (2011)
			Parched gram powder	Kumari and Kumar (2020), Singh (2003)
			Dehusked parched powder	Puškadija et al. (2017), Chhuneja et al. (1992)
3.	Pea (*Pisum sativum*)	Fabaceae	Harvested pea flour	Aly et al. (2014), Gemeda (2014), Dastouri et al. (2007)
			Germinated pea flour	Pande and Karnatak (2014)
4.	Kidney bean (*Phaseolus vulgaris*)	Fabaceae	Powder	Aly et al. (2014)
5.	Green gram (*Vigna radiata*)	Fabaceae	Powder	Tesfaye (2019), Pande and Karnatak (2014), Sihag and Gupta (2011)
6.	Hoarse gram (*Macrotyloma uniflorum*)	Fabaceae	Powder	Pande and Karnatak (2014)
7.	Mesquite pods (*Prosopis juliflora*)	Fabaceae	Powder	Amro et al. (2016)
8.	Red gram (*Cajanus cajan*)	Fabaceae	Powder	Manning (2018), Kishan and Srinivasan (2016), Sihag and Gupta (2011)
			Parched powder	Kishan and Srinivasan (2016)
9.	Winged bean (*Psophocarpus tetragonolobus*)	Fabaceae	Roasted seeds	Wijayati et al. (2019)
10.	Groundnut (*Arachis hypogea*)	Fabaceae	Powder	Ullah et al. (2021)
11.	Liquorice (*Glycrrhiza glabra*)	Fabaceae	Root extract	Al-Shammary and Al-Gerrawy (2017)
12.	Lentil (*Lens culinaris*)	Fabaceae	Powder	Irandoust and Ebadi (2013)
13.	Black gram (*Vigna mungo*)	Fabaceae	Powder	Kumar et al. (2021), Ullah et al. (2021)

3.2.3 Fruits and vegetables

The fruits and vegetables from over 12 families have been used in one or more forms for supplementary feeding of honey bees (Nowar 2011; Pande et al. 2015; Shehata 2016; Omar et al. 2017; El-Nagar et al. 2019). Among these, the Rutaceae family was most utilized in many forms, *viz*., fruit and shell juice of citrus, mandarin, and orange fruit (Table 8). Interestingly, the use of fruits dominated vegetables and other plants. In this literature, sugarcane has been kept under the fruit and vegetable category despite being a Poaceae family member.

3.2.4 Medicinal plants/Spices/Condiments

The authors probably utilised medicinal plants in bee feeding to keep the bees' immunity against various pests and diseases. Results presented in table 9 revealed that a total of 14 plants under the category of medicinal/spices/condiments have also been utilized

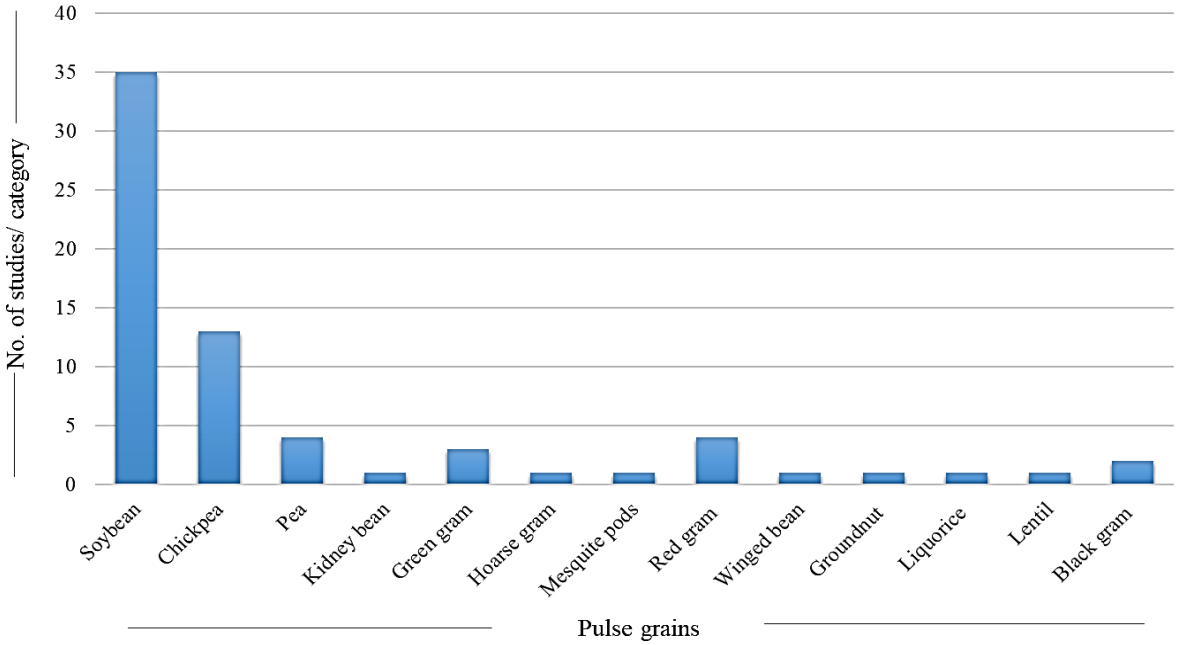

Figure 6 Comparative use of various types of pulse grains in artificial feeding of honeybees

Table 8 Utilization of different fruits & vegetables in feeding honey bees

S.N.	Name of fruit/vegetable	Plant Family	Form	References
1.	Date (*Phoenix dactylifera*)	Arecaceae	Pulp	Shehata (2016), Shehata and Nafea (2006)
2.	Papaya syrup (*Carica papaya*)	Caricaceae	Paste	Ulla et al. (2021) Amro et al. (2016)
			Pulp	Pande et al. (2015)
3.	Sweet potato (*Ipomea batatas*)	Convolvulaceae	Shell juice	Nowar (2011)
4.	Melon (*Cucumis melo*)	Cucurbitaceae		Shehata (2016), Shehata and Nafea (2006)
5.	Pumpkin (*Cucurbita pepo*)	Fagaceae	Syrup	Neupane and Thapa (2005)
6.	Chestnut (*Castanea sativa*)		Nut powder	Omar et al. (2017)
7.	Neem (*Azadirachta indica*)	Meliaceae	Fruits pulp	Singh and Singh (2012)
8.	Banana (*Musa Paradisiaca*)	Musaceae	Shell juice	Shehata and Nafea (2006)
9.	Banana (*Musa acuminate*)	Poaceae	Syrup	Pande et al. (2015), Neupane and Thapa (2005)
10.	Sugarcane (*Saccharum officinarum*)		Juice	Carrillo et al. (2015)
11.	Lemon (*Citrus limon*)	Rutaceae	Juice jelly	Ullah et al. (2021), Abou-Shaara (2017)
			Juice	El-Nagar et al. (2019)
12.	Mandarin (*Citrus reticulata*)	Sapotaceae	Shell juice	Shehata and Nafea (2006)
13.	Orange (*Citrus aurantium*)		Fruit juice	Islam et al. (2020), El-Nagar et al. (2019), Shehata (2016), Abd El-Wahab et al. (2016)
14.	Mahua (*Bassia latifolia*)		Shell juice	Shehata (2016)
			Pulp	Singh and Upadhyay (2008)
15.	Potato (*Solanum tuberosum*)	Solanaceae	Flour	Hussein (1981), Chalmers (1980)
16.	Grape (*Vitis vinifera*)	Vitaceae	Syrup	Bailey (1966)

Table 9 Use of medicinal/spices/condiments in artificial feeding of bees

S.N.	Medicinal plants/ Spices/ Condiments	Plant Family	References
1.	Coriander (*Coriandrum sativum*) seeds	Apiaceae	Aly et al. (2014)
2.	Caraway (*Carum carvi*) seeds		Mohamed et al. (2023), Aly et al. (2014)
3.	Fennel (*Foeniculum vulgare*) seeds		Aly et al. (2014)
4.	Anise (*Pimpinella anisum*) seeds		Mohamed et al. (2023), Aly et al. (2014)
5.	Chamomile (*Matricaria chamomilla*)	Asteraceae	Mohamed et al. (2023), Al-Ghamdi et al. (2021)
6.	Fenugreek (*Trigonella foenum-graecum*) Seeds	Fabaceae	Aly et al. (2014)
7.	Fenugreek (*Trigonella foenum-graecum*) seeds		Islam et al. (2020)
8.	Mint (*Mentha piperita*) oil	Lamiaceae	Al-Ghamdi et al. (2021), Abd El-Wahab et al. (2016)
9.	Thyme (*Thymus vulgaris*)		El-Nagar et al. (2019)
10.	Cinnamon (*Cinnamomum verum*)	Lauraceae	Ghramh and Khan (2023), Al-Ghamdi et al. (2021), Zaghloul et al. (2017), Shehata (2016)
11.	Laura paper (*Cinnamomumtamela*)		Mohamed et al. (2023)
12.	Turmeric (*Curcuma longa*)	Zingiberaceae	Ghramh and Khan (2023), Islam et al. (2020)
13.	Ginger (*Zingiber officinale*)		Mohamed et al. (2023)
14.	Garlic (*Allium sativum*)	Amaryllidaceae	El-Nagar et al. (2019)

from over seven plant families for supplementary feeding of honey bees (Aly et al. 2014; Zaghloul et al. 2017; Islam et al. 2020; Al-Ghamdi et al. 2021).

3.2.5 Non-traditional/miscellaneous stuff

Apart from traditionally known food materials, some nontraditional commodities were tested to explore better food options (Table 10). These include dried drone's pupa powder, fish meal, candy made with drone brood + glucose + sugar + honey, magnetized water, hexane extracts of larvae containing brood pheromone, star (Egyptian product/commercial liquid), royal jelly, milk powder, yoghurt, salt (NaCl), camphor oil, meat scrap, dried egg yolk, onion waste, calcium hydrogen phosphate (used as filler/binder in food products), eucalyptus (*Eucalyptus globule*) extract, etc. (El-Sherif 2002; Madras-Majewska et al. 2005; Dastouri et al. 2007; Irandoust and Ebadi 2013; Abd El-Wahab and Ghania 2016; Manjy and Shaher 2019; Ullah et al. 2021). However, logical reasons were insufficient to support selecting such materials in bee food.

4 Diet combinations used in the artificial feeding of honey bee

When combinations of different food components were searched in the available literature, thirteen different types of diets (combinations) were found; the detail of these combinations was given in this article's subsequent section.

4.1 Diets containing pollen substitutes alone

At least 33 types of diets based on either pollen or pollen substitutes alone or in combination with each other were noted (Table 11). In these types of combinations, the natural pollens from various plant sources alone or in combination were dominantly used in the artificial feeding of bees (Sabir et al. 2000; Puškadija et al. 2017; Ricigliano et al. 2017, 2022; Amro et al. 2020). Apart from natural pollen grains, different types of grains, *viz.*, cereals and pulses, fruits and vegetables, milk powders, meat craps, fish meals, etc., were also used as pollen substitutes for honey bees (Irandoust and Ebadi 2013; Tesfaye 2019; Ullah et al. 2021).

4.2 Diets containing pollen substitutes in combination with yeasts

Under this diet, nine combinations of pollen substitutes alone or with other similar foods were fed to honey bees with either yeast, drugs or both (Table 12). The soybean, in combination with similar supplements, was used dominantly (Alexandru et al. 1977; Mishra et al. 1979).

4.3 Diets containing pollen substitutes with vitamins/proteins/ minerals/amino acids

In this diet, at least eight pollen substitutes were combined with various vitamins, proteins, minerals, and amino acids in the artificial feeding of honeybees (Table 13). The soybean (*G. max*) and maize (*Z. mays*), as pollen supplements, were used in combination with different vitamins and other essential biochemicals (Sabir et al. 2000; Zahra and Talal 2008). Besides, the milk powder and natural pollen were also tried in a combination of vitamins and other essential biochemicals (Pirk et al. 2010).

Table 10 Non-traditional commodities utilized in feeding honey bees

S.N.	Name of component	References
1.	Dried drone's pupa powder	Beota et al. (2005)
2.	Skimmed milk powder	Ullah et al. (2021), Kumar et al. (2021), Kishan and Srinivasan (2016), Amro et al. (2016), Taha (2015), Pande and Karnatak (2014), Kumar et al. (2013a, b), Irandoust and Ebadi (2013)
3.	Fish meal	Irandoust and Ebadi (2013), Winston et al. (1983)
4.	Candy made with drone brood, glucose, sugar & honey	Madras-Majewska et al. (2005)
5.	Magnetized water	Manjy and Shaher (2019)
6.	Hexane extracts of larvae containing brood pheromone	Pankiw et al. (1998)
7.	Star (commercial liquid-Egyptian product)	Abd El-Wahab and Ghania (2016)
8.	Royal Jelly	Aqueel et al. (2017)
9.	Milk powder	Dastouri et al. (2007)
10.	Yoghurt	Nowar (2011)
11.	Salt (NaCl)	Srivastava (1996)
12.	Camphor oil	El-Sherif (2002)
13.	Meat scrap	Standifer et al. (1973)
14.	Dried egg yolk	Haydak (1945)
15.	Onion waste	Cho et al. (2021)
16.	Calcium hydrogen phosphate (used as filler/binder in food products)	Li et al. (2012)
17.	Eucalyptus (*Eucalyptus globule*) extract	Al-Maktary (2009)
18.	Parnove (*Plucheadioscoridis*)	El-Nagar et al. (2019)
19.	Pulicaria (*Pulicaria arabica*)	Al-Maktary (2009)
20.	Medicinal mushroom (*Agaricus brasiliensis*)	Stevanovic et al. (2018)
21.	White clover (*Trifolium repens*)	Omar et al. (2017)
22.	Palm oil (*Elaeisguineensis*)	Sereia et al. (2013)
23.	Cotton (*G. hirsutum*) seed	Herbert and Shimanuki (1979a), Haydak (1939)
26.	Linseed (*L. usitatissimum*)	van der Steen (2007)
27.	Crushed Panicum grass (*Panicum* sp.)	Shehata (2016)

Table 11 Diet combinations with given pollen grains and or pollen substitutes

S. N.	Diet composition Pollen/Pollen supplements		References
1.	Natural Pollen of Willow tree (*Acacia salicina*), Mustard (*Brassica tournefortii*), Land- caltrops (*Tribulus terrestris*), Prosopis (*Prosopis juliflora*), Egyptian clover (*Trifolium alexandrinum*), Broad bean (*Vicia faba*), Maize, Canola (*Brassica napus*), Fennel (*Faeniculum vulgare*), Coriander, Caper (*Brassica kaber*)		Amro et al. (2020), Puškadija et al. (2017), Ricigliano et al. (2017), Amro et al. (2015), Rezaei et al. (2015), Zheng et al. (2014), Al-Ghamdi et al. (2011), DeGrandi-Hoffman et al. (2010), Khodairy and Moustafa (2008), Al-Sarhi (2008)
2.	Maize (*Z. mays*)	Gluten	Rezaei et al. (2015), Al-Sarhi (2008)
3.		Flour	Ghramh and Khan (2023), Haydak (1936)
4.	Wheat flour (*T. aestivum*) flour		Ghramh and Khan (2023)
5.	Oat flour (*Avena. sativa*) flour		Haydak (1936)
6.	Sorghum (*S. bicolor*) flour		Ghramh and Khan (2023)
7.	Germinated wheat (*Triticum aestivum*)		Herbert and Shimanuki (1979b), Standifer et al. (1977)
8.	Pea (*Pisum sativum*) flour		Gemeda (2014), Haydak (1936)
9.	Chickpea (*Cicer arietinum*) flour		Ghramh and Khan (2023), Tesfaye (2019)
10.	Mung bean (*Vigna radiata*) flour		Tesfaye (2019)
11.	Bean (*Phaseolus vulgaris*)		Gemeda (2014)
12.	Peanut (*Arachis hypogaea*) cake		Erickson and Herbert (1980)
13.	Broad bean (*Vicia faba*) flour		Herbert and Shimanuki (1979b), Taber (1978), Barker and Lehner (1976), El-Banby and Gorgui (1970)
14.	Oats (*Avena sativa*) roasted seeds		Herbert and Shimanuki (1979a)
15.	Potato (*Solanum tuberosum*) flour		Hussein (1981), Chalmers (1980)
16.	Cotton (*Gossypium hirsutum*) seed meal		Herbert and Shimanuki (1979a)
17.	Date (*P. dactylifera*) paste		Ulla et al. (2021), Amro et al. (2016), Shehata (2016),
18.	Skimmed milk powder		Chalmers (1980)
19.	Dried whole milk		Stroikov (1966)
20.	Meat scrap		Haydak (1936)
21.	Fish meal		Irandoust and Ebadi (2013), Chalmers (1980)
22.	Hexane extracts of larvae containing brood pheromone		Pankiw et al. (1998)
23.	Soybean (*Glycine max*) flour + Pollen		Standifer et al. (1973)
24.	Soybean (*G. max*) flour + Skimmed milk		Abbas et al. (1995), Haydak (1945)
25.	Germinated wheat (*T. aestivum*) + Soybean (*G. max*)		Aly et al. (2019)
26.	Pollen of *Helianthus* sp.+ *Asparagus* sp. + *C. sativa* + *T. repens*		Omar et al. (2017)
27.	Soybean flour (*G. max*) + Wheat (*T. aestivum*)		Standifer et al. (1973)
28.	Cotton (*G. hirsutum*) seed + Skimmed milk powder		Haydak (1939)
29.	Black gram (*V. mungo*) + Skimmed milk		Abbas et al. (1995)
30.	Chestnut (*C. sativa*) + White clover (*T. repens*)		Omar et al. (2017)
31.	Pollen + Wheat (*T. aestivum*) flour		Stanger and Laidlaw (1974)
32.	Corn flour (*Zea mays*) + fish-meal + Pea (*P. sativum*)flour		Haydak (1936)
33.	Soybean (*G. max*) flour + Skimmed milk powder + Meat scrap		Standifer et al. (1973)

Table 12 Diet combinations of pollen substitutes with yeasts/drugs

S. N.	Diet composition		References
	Pollen supplements/ oils	Yeasts	
1.	Soybean (*G. max*) flour + Dried egg yolk	Brewer yeast (*S. cerevisiae*)	Haydak (1945)
2.	Soybean (*G. max*) flour + Dried skimmed milk		Haydak (1959)
3.	Soybean (*G. max*) flour + Skimmed milk powder		Mishra et al. (1979), Alexandru et al. (1977), Standifer et al. (1970), Forster (1968a, b), Hagedom and Moeller (1968)
4.	Fenugreek (*T. foenum-graecum*) + Turmeric powders (*C. longa*)		Amro et al. (2020)
5.	Chickpea (*C. arietinum*) flour + Germinated wheat (*T. aestivum*)		Amro et al. (2020)
6.	Skimmed milk powder		Haydak (1945)
7.	Fish meal		Winston et al. (1983)
8.	Dehusked parched chickpea (*C. arietinum*)+ Skimmed milk powder		Chhuneja et al. (1992)
9.	Skimmed milk powder	Yeast	Forster (1966)
10.	Wheat (*T. aestivum*) flour+ Cinnamone (*C. verum*) powder		Ghramh and Khan (2023)
11.	Wheat (*T. aestivum*) flour+ Turmmeric (*C. longa*) powder		Ghramh and Khan (2023)
12.	Wheat (*T. aestivum*) flour+ Turmeric (*C. verum*) powder + Cinnamone (*C. longa*) powder		Ghramh and Khan (2023)
13.	Maize (*Z. mays*) flour+ Cinnamone (*C. verum*) powder		Ghramh and Khan (2023)
14.	Maize (*Z. mays*) flour+ Turmmeric (*C. longa*) powder		Ghramh and Khan (2023)
15.	Maize (*Z. mays*) flour+ Turmeric (*C. verum*) powder + Cinnamone (*C. longa*) powder		Ghramh and Khan (2023)
16.	Chickpea (*C. arietinum*) flour+ Cinnamone (*C. verum*) powder		Ghramh and Khan (2023)
17.	Chickpea (*C. arietinum*) flour+ Turmmeric (*C. longa*) powder		Ghramh and Khan (2023)
18.	Chickpea (*C. arietinum*) flour+ Turmeric (*C. verum*) powder + Cinnamone (*C. longa*) powder		Ghramh and Khan (2023)
19.	Sorghum (*S. bicolor*) flour+ Cinnamone (*C. verum*) powder		Ghramh and Khan (2023)
20.	Sorghum (*S. bicolor*) flour+ Turmmeric (*C. longa*) powder		Ghramh and Khan (2023)
21.	Sorghum (*S. bicolor*) flour+ Turmeric (*C. verum*) powder + Cinnamone (*C. longa*) powder		Ghramh and Khan (2023)

Table 13 Diet combinations comprising of pollen substitutes with vitamins/ proteins/ minerals/ amino acids

S. N.	Diet composition		References
	Pollen supplements	Vitamins/proteins/minerals/amino acids	
1.	Soybean (*G. max*) flour	Vit. C, Multi-vitamins	Zahra and Talal (2008)
2.		Vit. B-Complex, Glycine	Sabir et al. (2000)
3.		Vit. B-complex, Methionine	Sabir et al. (2000)
4.	Soybean (*G. max*)flour + Egg yolk	Vit. B-Complex	Sabir et al. (2000)
5.	Maize (*Z. mays*) flour + Egg yolk		Sabir et al. (2000)
6.	Maize (*Z. mays*) flour	Vit. B-Complex, Glysine	Sabir et al. (2000)
7.	Pollen	Casein	Pirk et al. (2010)
8.	Skimmed milk	Vit. C	Zahra and Talal (2008)

4.4 Diets containing nectar substitutes alone

Around 35 nectar substitutes alone or in combination with each other were used in the artificial feeding of honeybees (Table 14). Sugar syrup has been the most dominant nectar substitute in the artificial feeding of honeybees all over the world (Ghazala and Nowar 2013; Zheng et al. 2014; Gemeda et al. 2018; Dolasevic et al. 2020; Islam et al. 2020). After sugar syrup, natural honey is the second most utilized nectar substitute in different forms, *viz*., normal creamed from cotton and clover, Jelly from cotton and candy (Abd El Hamid and Abou-Shaara 2016; Abou-Shaara 2017). The combination of two or more sugar forms viz., royal jelly + fructose, royal jelly + sugar, royal jelly + honey, honey+ sugar powder + sugar syrup, honey + sugar powder + sugar syrup, etc., was also tried in feeding honey bees (Skubida et al. 2008; Avni et al. 2009; Abd El-Wahab et al. 2016; Aqueel et al. 2017; Manjy and Shaher 2019). Besides pure sugar forms, some fruit pulp and shell juices (grapes, papaya, banana, orange, mandarin, lemon, etc.) and vegetable oils (palm and linseed) were also tried (Shehata and Nafea 2006; Nowar 2011; Pande et al. 2015; Shehata 2016; El-Nagar et al. 2019; Islam et al. 2020).

Table 14 Diet combinations comprising of nectar substitutes only

S. N.	Diet composition Nectar supplements		References
1.	Sugar syrup		Dolasevic et al. (2020), Islam et al. (2020), Gemeda et al. (2018), Gamal Eldin et al. (2018), Rashid et al. (2018), Abou-Shaara (2017), Omar et al. (2017), Mahfouz (2016), Shehata (2016), Carrillo et al. (2015), Haleem et al. (2015), Rezaei et al. (2015), Gemeda (2014), Somerville (2014), Usha et al. (2014), de Assis-Pinto (2014), Johnson (2014), Zheng et al. (2014), Ghazala and Nowar (2013), Mahmood et al. (2013), Sahinler and Kaftanoglu (2013), Sammataro and Weiss (2013), Kamandar (2012), Andelkovic et al. (2011), Fasasi (2011), DeGrandi-Hoffman et al. (2010), Sharaf El-Din (2010), Versluijs (2010), Al-Maktary (2009), Bodla et al. (2009), Al-Sarhi (2008), Skubida et al. (2008), Hammad (2007)
2.	Sugar candy		Abou-Shaara (2017), Abou-Shaara (2017), Aly et al. (2014)
3.	Inverted sugar		Carrillo et al. (2015), Atallah and Naby (1979)
4.	Honey	Creamed from cotton	Abou-Shaara (2017), Abd El Hamid and Abou-Shaara (2016).
5.		Creamed from clover	Abd El Hamid and Abou-Shaara (2016)
6.		Jelly from cotton	Abou-Shaara (2017)
7.		Candy	Abou-Shaara (2017)
8.		Normal	Abou-Shaara (2017), Barker and Lehner (1978)
9.	Royal jelly		Vandenberg and Shimanuki (1987)
10.	Sugarcane juice		Carrillo et al. (2015)
11.	Grape syrup (*Vitis vinifera*)		Barker and Lehner (1978), Bailey (1966)
12.	Papaya (*Carica papaya*) syrup		Pande et al. (2015)
13.	Banana (*M. paradisiaca*)	Fruit syrup	Pande et al. (2015), Shehata and Nafea (2006)
14.		Shell juice	
15.	Flowers extract of Mahua (*Bassia latifolia*)		Singh and Upadhyay (2008)
16.	Lemon (*Citrus limon*) juice		El-Nagar et al. (2019), Nowar (2011)
17.	Mandarin (*C. reticulata*) shell juice		Shehata and Nafea (2006)
18.	Melon shell juice (*Cucumis melo*)		Shehata (2016), Shehata and Nafea (2006)
19.	Orange (*C. aurantium*)	Fruit juice	Islam et al. (2020), El-Nagar et al. (2019), Shehata (2016), Abd El-Wahab et al. (2016)
20.		Shell juice	
21.	Corn (*Z. mays*) high fructose syrup		Sammataro and Weiss (2013), DeGrandi-Hoffman et al. (2010)
22.	Pumpkin (*Cucurbita pepo*) syrup		Neupane and Thapa (2005)

S. N.	Diet composition Nectar supplements	References
23.	Palm (*Elaeis guineensis*) oil	Sereia et al. (2013)
24.	Linseed (*Linum usitatissimum*) oil	Sereia et al. (2013)
25.	Sesame (*Sesamum indicum*) powder	Mohamed et al. (2023
26.	Sugar powder + Honey	Avni et al. (2009), Skubida et al. (2008)
27.	Royal jelly + Fructose	Aqueel et al. (2017)
28.	Royal jelly + Honey	Aqueel et al. (2017)
29.	Royal jelly + Sugar	Aqueel et al. (2017)
30.	Powdered sugar + Sugar syrup	Abd El-Wahab et al. (2016)
31.	Sucrose + magnetized water	Manjy and Shaher (2019)
32.	Honey+ Sugar powder+ Sugar syrup	Abd El-Wahab et al. (2016)
33.	Worker Jelly+ Glucose+ Fructose	Asencot and Lenky (1976)
34.	Linseed oil (*L. usitatissimum*) + Palm oil	Sereia et al. (2013)
35.	Corn Fructose-85+Corn Fructose-55+Glucose Monohydrate+ Sucrose syrup	Guler et al. (2018)

Table 15 Diet combinations comprising of nectar substitutes with yeasts/drugs

S. N.	Diet composition Nectar supplements	Yeasts/drugs	References
1.	Royal jelly, Sugar, Water	Yeast extract	Rembold and Lackner (1981)
2.	Honey, Sugar powder, Water	Dried brewer's yeast (*S. cerevisiae*)	Younis (2019)
3.		*Agaricus brasiliensis* (Fungi) extract	Stevanovic et al. (2018)
4.	Royal jelly, D-glucose, D-fructose	Solution of yeast extract	Aupinel et al. (2005)
5.		Difcobacto-yeast extract or Charcoal-treated extract	Rembold and Lackner (1981)
6.		Yeast extract	Vandenberg and Shimanuki (1987)
7.	Sugar powder	Dried brewer's yeast (*S. cerevisiae*)	Ahmed (2000)
8.	Sucrose	Yeast cake	Abd Al-Fattah et al. (2003)
9.	Sugar syrup	Torula yeast (*C. utilis*)	Peng et al. (1984)
10.		Yeast	Haleem et al. (2015), Dodologlu and Emsen (2007)
11.	Royal jelly		Aqueel et al. (2017)
12.	Sugar powder, Water	Yeast culture	Abd El-Wahab and Gomaa (2005)
13.		Brewer's yeast (*S. cerevisiae*)	
14.	Honey, Sugar syrup		Omar (2006)
15.	Sugar syrup	Fumagillin (antimicrobial)	Akyol et al. (2006)
16.	Honey, Sucrose	Torula yeast (*C. utilis*)	Lehner (1983)
17.	Glucose, Fructose, Royal jelly, Water	Yeast extract	Kaftanoglu et al. (2011)

4.5 Diets containing nectar substitutes with yeasts/drugs

Around 17 combinations were found where nectar substitutes were combined with yeasts/drugs and fed to honey bees in the past (Table 15). The nectar substitutes like royal jelly, sugar, honey, glucose, and fructose alone or mixed were used in combination with different types and forms of yeasts and drugs (Fumagillin- an antimicrobial) to feed the bees (Akyol et al. 2006; Kaftanogluet al. 2011; Stevanovic et al. 2018; Younis 2019).

Table 16 Diets containing nectar substitutes with vitamins/ proteins/ minerals/ Amino acids

S. N.	Diet composition		References
	Nectar supplements	Vitamins/proteins/minerals/amino acids	
1.	Sugar syrup	Multi-vitamin	El-Sherif (2002)
2.			Abd El-Wahab et al. (2016)
3.		Multivitamins, Microelements	Andelković et al. (2011)
4.	Sugar candy		Andelković et al. (2011)
5.	Honey	Casein	Mahfouz (2016)
6.	Honey	Whey protein concentrate	Mahfouz (2016)
7.	Royal jelly	Casein	Pirk et al. (2010)
8.	Sugar powder, Sugar syrup	Corn gluten	Gamal Eldin et al. (2018)
9.	Sugar syrup	Vit. A, E, B1, B2, B12, C, K1, Pantothenic acid, Nicotineamid, Folic acid, Biotin, K_2HPO_4	Gençer et al. (2000)
10.		Vit. A, D3, E, B1, B2, B6, B12, C, K3, Niacin, Ca-d-Pantothenate, Vitamix Formula-TOPKIM)	Akyol et al. (2006)

4.6 Diets containing nectar substitutes with vitamins/proteins/ minerals/amino acids

Nectar substitutes were also tried in a combination of vitamins, proteins, minerals, and amino acids. Among the mentioned 10 combinations in table 16, along with honey and different sugar forms, different types of vitamins (vitamin A, E, B1, B2, B6, B12, C, D3, E, K1, K3, and multivitamins), proteins (Casein, corn gluten, and whey protein concentrates), amino acids (Pantothenic acid, Nicotinamide, Folic acid, Biotin, K_2HPO_4) and minerals were tested as an alternate artificial food for honey bees (Gençer et al. 2000; El-Sherif 2002; Akyol et al. 2006; Mahfouz 2016; Abd El-Wahab et al. 2016; Gamal Eldin et al. 2018).

4.7 Diets containing pollen and nectar substitutes

Around 64 pollen and nectar diet combinations were traced from the literature (Table 17). Different forms of sugars, honey, and fruit juices were kept under the category of nectar substitutes while pollen grains (from different plants sources), commercial diets, grains of different cereals, pulses, beans, skimmed milk powder, fruit past (apple, banana, pumpkin, date, etc.), spices, condiments, etc., under pollen supplements (Omar 2006; Li et al. 2012; Usha et al. 2014; Abd El-Wahab et al. 2016; Mahfouz 2016; Omer et al. 2017; Gamal Eldin et al. 2018; Dolasevicet al. 2020). These supplements were used either alone or in mixtures of each in combination. Single grains from pulses (soybean, chickpea, pea, and

Table 17 Diet combinations of pollen and nectar substitutes

S. N.	Diet composition		References
	Pollen supplements	Nectar supplements	
1.	Pollen	Honey	de Assis-Pinto (2014), Kumar et al. (2013a, b), Al-Eitby (2009), Dodologlu and Emsen (2007)
2.	Bee-Pro®	Sugar syrup	Saffari et al. (2004)
3.	TLS Bee food®		Sena et al. (2012), Saffari et al. (2010)
4.	Feed Bee®		Dolasevic et al. (2020), Guler et al. (2018), Omar et al. (2017), Amro et al. (2016), Sena et al. (2012), Saffari et al. (2004, 2010)
5.		Honey, Sucrose solution, Water	Amro et al. (2016)
6.		Sugar powder, Water	Omar et al. (2017)
7.	High fructose corn syrup	Distilled water	DeGrandi- Hoffman et al. (2008)
8.	Spirulina (*Arthrospira platensis*)	Honey	Kumar and Agrawal (2014)
9.	Parnove (*Pluchea dioscoridis*) boiling water extract	Orange juice	El-Nagar et al. (2019)
10.	Garlic extract (*A. sativum*)	Lemon juice	

S. N.	Diet composition		References
	Pollen supplements	Nectar supplements	
11.	Apple (*M. domestica*)	Sucrose syrup	Pernal and Currie (2000)
12.	Soybean (*G. max*) extract	Honey	Mahfouz (2016), de Assis-Pinto (2014)
13.	Wheat (*T. aestivum*)	Sugar syrup	Stanger and Laidlaw (1974)
14.	Germinated Wheat	Honey, Sugar powder, Sugar syrup,	Omar (2006)
15.	EM® (commercial probiotic mix)	Sugar syrup	Tlak-Gajger et al. (2020)
16.	Soybean flour	Sugar powder	Nowar (2011)
17.		Sugar powder, Sugar syrup	Abd El-Wahab et al. (2016)
18.		Honey, Sugar syrup	Omar (2006), Usha et al. (2014)
19.			Abd El-Wahab et al. (2016)
20.		Honey, Water	Usha et al. (2014)
21.		Honey, Sucrose	Lehner (1983)
22.		Sucrose, Water	Dodologlu and Emsen (2007)
23.	Defatted soybean flour	Honey, Sugar powder, Water	Younis (2019)
24.	Milk powder	Honey, Sugar powder	Mahbobi et al. (2012)
25.	Rice bran syrup	Honey, Sugar powder	Neupane and Thapa (2005)
26.	Banana (*M. paradisiaca*) syrup	Honey, Sugar powder, Water	Neupane and Thapa (2005)
27.	Pumpkin (*Cucurbita*) syrup		
28.	Maize (*Z. mays*) syrup	Honey, Sugar powder	Neupane and Thapa (2005)
29.	Maize (*Z. mays*) flour	Honey, Water	Usha et al. (2014)
30.		Sugar powder, Eucalyptus extract-based sugar solution	Al-Maktary (2009)
31.		Honey, Sugar powder, Water	Younis (2019)
32.	Roasted winged bean (*P. tetragonolobus*) seeds	Sugar solution	Wijayati et al. (2019)
33.	Boiled winged bean seeds		
34.	Chickpea flour	Honey, Sugar powder, Water	Younis (2019)
35.	Pea flour		
36.	Germinated wheat		
37.	Liquorice (*G. glabra*) root extract	Distilled water	Al-Shammary and Al-Gerrawy (2017)
38.	Bean (*V. cowpea*) flour + Chickpea + flour + Coriander	Honey	Aly et al. (2014)
39.	Beans flour + Fennel seeds flour		
40.	Fennel (*F. vulgare*) flour + Chickpea		
41.	Pea flour + Caraway seeds (*Carum carvi*) flour		
42.	Soybean flour + Skimmed milk powder	Honey	Haydak (1937)
43.	White kidney Bean flour + Caraway seeds flour		Aly et al. (2014)

S. N.	Diet composition		References
	Pollen supplements	Nectar supplements	
44.	Sugar cane (*Saccharum officinarum*) candy + Dried drones pupa powder	Glucose	Beota et al. (2005)
45.	Cotton seed (*G. hirsutum*) + Skimmed milk powder	Honey	Haydak (1936)
46.	Linseed + Skimmed milk powder		Haydak (1937)
47.	Fenugreek flour + Sweet potato	Sugar powder	Nowar (2011)
48.	Peanut + Skimmed milk powder	Honey	Haydak (1937)
49.	Pollen/mixed pollen (*P. tanacetifolia*, *B. campestris*, *M. officinalis*, *H. annuus*; *P. banksiana*, *Asparagus* sp., *S. perfoliatum* etc.)	Honey, Sugar solution, Sugar powder, Sucrose, Fructose, Sugar candy, Water	Dolasevic et al. (2020), Gamal Eldin et al. (2018), Omer et al. (2017), Abd El-Wahab et al. (2016), Usha et al. (2014), Li et al. (2012), Al-Ghamdi et al. (2011), Saffari et al. (2010), Skubida et al. (2008)
50.	Mixed pollen + Yoghurt	Sugar powder	Nowar (2011)
51.	Defatted soybean (*G. max*) flour + Skimmed powder milk	Honey, Date (*P. dactylifera*) molasses	Taha (2015)
52.	Soybean (*G. max*) flour + Date (*P. dactylifera*) pollen grains	Powdered sugar, Sugar syrup	Abd El-Wahab et al. (2016)
53.		Sugar powder, Melon shell juice, Mandarin shell juice	Shehata and Nafea (2006)
54.	Soybean flour+Dried skimmed milk	Sugar syrup, Sucrose syrup	Sahinler and Kaftanoglu (2013)
55.	Roasted soybean flour + Mixed pollen	Honey, Sucrose	Avni et al. (2009)
56.	Pollen + Soybean (*G. max*) flour + Wheat (*T. aestivum*) flour	Sugar syrup	Standifer et al. (1973)
57.	Oats (*A. sativa*) flour + Rice (*O. sativa*) flour + Anise (*P. anisum*) flour	Honey	Aly et al. (2014)
58.	Date (*P. dactylifera*) paste + Defatted soybean flour + Skimmed milk powder		Taha (2015)
59.	White kidney beans (*P. vulgaris*) flour + Pea (*P. sativum*) flour + Coriander flour		Aly et al. (2014)
60.	Fenugreek (*T. foenum-graecum*) flour + Beans (*Vi. cowpea*) flour + Fennel flour		
61.	Chickpea flour + Fenugreek flour + Cumin (*C. cyminum*) flour		Aly et al. (2014)
62.	Rice (*O. sativa*) flour + Pea flour + Fennel flour + Fenugreek flour		Aly et al. (2014)
63.	Soybean (*G. max*) + Date (*P. dactylifera*)	Crushed Panicum grass (*Panicum* sp.) + Melon shell juice,Orange shell juice	Shehata (2016)
64.	Soybean (*G. max*) + Date (*P. dactylifera*)	Panicum grass (*Panicum* sp.) + Orange shell juice, Melon juice, Cinnamon oil	Shehata (2016)

beans) or cereals (rice, maize, and wheat) were dominantly used in combination with different forms of nectar supplements (sugars and honey) (Sahinler and Kaftanoglu 2013; Aly et al. 2014; Taha 2015; Shehata 2016; Younis 2019) (Table 17).

4.8 Diets containing pollen and nectar substitutes with yeasts/ drugs

In this category, 44 diet combinations were seen where yeasts/drugs were added to pollen and nectar substitutes (Table 18). Under yeasts/drugs, the most common yeast, *viz*., dried brewer's of yeast (*S. cerevisiae*) used in different forms, in combination with antibiotics, spirulina (*A. platensis*), and Creon have been utilized. Another form of sugars and honey, camphor (*C. camphora*) oil, shell juices (*M. paradisiaca*, *C. melo*), enzyme inverted syrup, glucose, date (*P. dactylifera*) syrup, etc., were also treated as nectar supplement. In the case of pollen supplements, pollen grains (from different plant sources), commercial diets, grains of different cereals, pulses, beans, skimmed milk powder, etc., have been used (Shehata and Nafea 2006; Al-Eitby 2009; Nowar 2011;

Table 18 Diet containingpollen substitutes and nectar substitutes with yeasts/drugs

S. N.	Diet composition			References
	Pollen supplements	Yeasts/drugs	Nectar supplements	
1.	Pollen grains	Yeast	Sugar syrup	Dodologlu and Emsen (2007)
2.	Milk powders	Antibiotics	Honey, Citrus juice, Volatile oils	Omar et al. (2016)
3.	Soybean flour	Yeast extract	Honey, NaCl salt	Sihag and Gupta (2013)
4.		Brewer's yeast	Powdered sugar, Sugar syrup	Islam et al. (2020)
5.				
6.			Honey, Sugar syrup, Sugar powder	El-Waseef (2002)
7.				Ghazala and Nowar (2013)
8.			Sugar powder	
9.		Antibiotics	Honey, Citrus juice, Volatile oils	Omar et al. (2016)
10.		Yeast extract	Honey	Sihag and Gupta (2013)
11.		Brewer's yeast	Sugar powder	Mahmood et al. (2013)
12.			Sugar powder, Water	Abd El-Wahab and Gomaa (2005)
13.	Defatted soybean flour		Enzyme inverted syrup	Puškadija et al. (2017)
14.	Defatted soybean flour	Brewer's yeast, Spirulina	Sugar powder, Glucose	Kumar and Agrawal (2014)
15.				Kumar et al. (2013a, b)
16.	Maize flour	Brewer's yeast	Powdered sugar, Sugar syrup	Islam et al. (2020)
17.			Sugar powder	Mahmood et al. (2013)
18.	Chickpea flour			
19.	Skimmed milk powder		Date syrup	Amro et al. (2020)
20.	Germinated horse gram + Skimmed milk powder		Honey	Pande and Karnatak (2014)
21.	Germinated chickpea + Skimmed milk powder			
22.	Pea + Skimmed milk powder			
23.	Germinated Green gram + Skimmed milk powder			
24.	Defatted Soybean flour + Skimmed milk powder		Sugar powder	Kumar et al. (2013a, b)
25.	Soybean + Skimmed milk			Al-Ghamdi et al. (2011)
26.	Chickpea cake + Pollen		Sugar syrup (with Camphor oil)	El-Sherif (2002)
27.	Defatted Soybean flour + Skimmed milk powder		Sugar powder	Kumar and Agrawal (2014)
28.	Defatted Soybean flour + Skimmed milk powder			
29.	Defatted soybean flour + Skimmed milk powder		Honey	Taha (2015)
30.	Soybean flour + Sweet potato	Powdered yeast	Powdered sugar	Nowar (2011)
31.	Mixed pollen +Sedge pollen	Creon	Sugar powder,Water	Al-Eitby (2009)

S. N.	Diet composition			References
	Pollen supplements	Yeasts/drugs	Nectar supplements	
32.	Chickpea parched powder + Skimmed milk powder	Brewer's yeast	Sugar Powder, Sugar syrup, Water	Singh (2003)
33.	Maize +Date paste		Shell juices *(M. paradisiaca, C. melo)*, Sugar powder	Shehata and Nafea (2006)
34.	Egyptian bean + Date paste			
35.	Skimmed milk powder + Soybean flour + Clover extract		Sugar powder	Mansour (2002)
36.	Defatted soybean flour + Parched chickpea flour		Sugar powder, Glucose	Kumar et al. (2013a, b)
37.	Skimmed soybean + Cinnamon	Dry yeast	Honey, Sugar powder	Zaghloul et al. (2017)
38.	Chickpea + Cinnamon			
39.	Yellow corn powder + Cinnamon	Yeast		
40.	Defatted soybean flour + Parched red gram	Dried brewer's yeast	Sugar powder, Glucose	Kumar and Agrawal (2014)
41.	Chickpea cake + Pollen		Sugar syrup, Camphor oil	El-Sherif (2002)
42.	Corn flour + Chickpea flour + Wheat bran		Sugar powder, water	Mansour (2002)
43.	Chamomile + Caraway + Sesame powder	Inactive yest	Powdered sugar	Mohamed et al. (2023)
44.	Anise + Laura paper and ginger		Powdered sugar	Mohamed et al. (2023)

Kumar et al. 2013 a, b; Mahmood et al. 2013; Pande and Karnatak 2014; Kumar and Agrawal 2014; Taha 2015; Omar et al. 2016; Puškadija et al. 2017; Zaghloul et al. 2017; Amro et al. 2020). The combinations were prepared as a single component with single or multiple features. Overall, 42 combinations could be seen under this category of diet combinations (Table 18).

4.9 Diets containing pollen and nectar substitutes with vitamins/proteins/minerals/amino acids

In this category of diet combinations, vitamins/proteins/minerals/ amino acids were added singly or more than one to pollen and nectar substitutes (Table 19). At least nine such diet combinations are known in the available literature. Here, calcium caseinate, whey protein, vitamin B-complex, casein, and several protein were used under the vitamins/proteins/minerals/amino acids section (Sabir et al. 2000; van der Steen 2007; Al-Eitby 2009). Rest, common pollen, and nectar substitutes were used (Table 19).

4.10 Diets containing pollen and nectar substitutes with yeasts/drugs and vitamins/proteins/minerals/amino acids

From the available literature, a minimum of 34 diet combinations have been noted under this category (Table 20). These diet combinations utilized yeasts/drugs, vitamins/proteins/minerals/ amino acids, and pollen and nectar substitutes. Under yeasts/drugs,

Table 19 Diet combination with pollen supplements, nectar supplements and vitamins/ proteins/ minerals/ amino acids

S. N.	Diet composition			References
	Pollen supplements	Vitamins/proteins/minerals/Amino acids	Nectar supplements	
1.	Soybean flour	Calcium caseinate, Whey protein flour (milk protein 80%)	Sucrose solution, Linseed	van der Steen (2007)
2.		Vit. B-complex	Sugar solution	Sabir et al. (2000)
3.	Maize flour			
4.	Bee-Pro	Casein,Gevral protein	Sugar powder, Water	Al-Eitby (2009)
5.	Maize flour + Egg yolk	Vitamin B-Complex	Sugar solution	Sabir et al. (2000)
6.	Mixed pollen + Bee-Pro®	Gevral protein	Sugar powder, Water	Al-Eitby (2009)
7.	Sedge pollen (*Cyperus compressus*) + Soybean (*G. max*) flour			
8.	Soybean flour + Egg yolk	Vit. B-Complex	Sugar solution	Sabir et al. (2000)

Table 20 Diet combination with pollen supplements, nectar supplements, vitamins/ proteins/ minerals/ amino acids, and yeasts/drugs

S. N.	Diet composition				References
	Pollen supplements	Yeasts/drugs/oils	Vitamins/proteins/minerals/ Amino acids	Nectar supplements	
1.	Soybean flour	Dried Brewer's yeast	Protein	Sugar syrup	El-Waseef (2002)
2.			Protein Hydrolysate	Sugar powder, Glucose	Kumar and Agrawal (2014)
3.			Protein Hydrolysate	Sugar powder, Glucose	Kumar et al. (2013a, b)
4.	Soybean flour	Yeast extract	Vitamins, Minerals	Honey	Sihag and Gupta (2013)
5.	Soybean flour		Multivitamin		Lakra (2006)
6.	Soybean				Sihag and Gupta (2011)
7.	Mung bean flour				
8.	Chickpea flour				
9.	Pigeon pea flour				
10.	Pollen + Soybean flour				
11.	Pollen + Mung bean				
12.	Pollen + Chickpea				
13.	Pollen + Pigeon pea				
15.	Mixed- pollen	Fumagillin	Multivitamins	Sugar powder, Sugar syrup	Akyol et al. (2006).
16.	Mixed pollen	Brewer's yeast	Sodium caseinate,casein	Sucrose, water	Malone et al. (2004)
17.		Brewer's yeast	Avidin (protein)	Sucrose, water	Malone et al. (2004).
18.		Brewer's yeast, Aprotinin	Sodium caseinate		
19.		Dried Brewer's yeast			
20.	Palm pollen + Sedge pollen	Creon	Casein	Sugar powder, water	Al-Eitby (2009)
21.	Defatted soybean flour + Pollen	Tetracycline	Vitamin B-complex	Honey, Sugar powder, water	Al-Shammary and Al-Gerrawy (2017)
22.	Defatted soybean flour + Pollen	Brewer's yeast	Protein Hydrolysate	Sugar powder, Glucose	Kumar and Agrawal (2014)
23.	Corn flour + Soybean + Corn gluten	Calcium hydrogen phosphate, Calcium carbonate	Antioxidant Premixes	Sucrose	Li et al. (2012)
24.	Soybean flour + Powder of Fenugreek and Turmeric	Brewer's yeast	Vit. A, D, E	Honey, Sugar syrup, Sugar powder, Orange juice	Islam et al. (2020)
25.	Maize flour + Fenugreek powder + Turmeric				
26.	Soybean flour + Fenugreek Powder + Turmeric				
27.	Maize flour + Fenugreek powder + Turmeric				
28.	Soybean flour + Turmeric + Fenugreek powder	Mint oil			Abd El-Wahab et al. (2016)
29.	Anicotinic acid + Citrus juice	Dried Brewer's yeast, Antibiotics, Volatile oils	Vit. B1, B2	Honey	Omar et al. (2016)

S. N.	Diet composition				References
	Pollen supplements	Yeasts/drugs/oils	Vitamins/proteins/minerals/ Amino acids	Nectar supplements	
30.	Mesquite pods powder + Fresh mixed pollen pellets + Dried skim milk	Brewer's yeast	Multivitamins, minerals (Centrum)	Honey, Sugar powder, water, Coriander oil	Amro et al. (2016)
31.	Date paste + Fresh mixed pollen pellets + Dried skim milk				
32.	Soybean meal + Fresh mixed pollen pellets + Dried skim milk		-	Honey, Sugar powder, water	
33.	Soybean + Liquorice root extract	Tetracycline	Vit. B-complex,	Honey, Sugar powder, water	Al-Shammary and Al-Gerrawy (2017)
34.	Citrus juice	Antibiotics, Volatile oils	-	Honey, Sugar Powder	Omar et al. (2016)

Table 21 Diets either pollen or nectar supplements with yeasts/drugs/oils and vitamins/ proteins/ minerals/ amino acids

S. N.	Diet composition				References
	Pollen supplements	Yeasts/drugs/oils	Vitamins/proteins/minerals/Amino acids	Nectar supplements	
1	Soybean flour	Dried Brewer's yeast	Protein based sugar syrup	-	Ghazala and Nowar (2013)
2	Defatted soybean flour	Brewer's yeast	Soy protein hydrolysate	-	Kumar and Agrawal (2014)
3	-	Yeast	Gluten	Sugar powder	Al-Ghamdi et al. (2011)
4	-	Cholesterol	Casein (Protein)	Sucrose, NaCl Salt	Srivastava (1996)
5	-	Cholesterol, Alphacel (polysaccharide)	Casein, Tocopherol (Vit. E)	Sucrose, Salt mixture	Srivastava (1996)
6	-	Brewer's yeast	Isolated soy protein	-	Sereia et al.(2013)

the most common brewer's yeast (*S. cerevisiae*), along with calcium hydrogen phosphate (CHP), calcium carbonate (CC), tetracycline, aprotinin, Creon, fumagillin, antibiotics, volatile oils, and mint oils were placed. In the case of vitamins/proteins/ minerals/amino acids, different proteins (sodium caseinate, casein, avidin, etc.) and protein hydrolysates, vitamins (A, B, D, E, K, etc.) & multivitamins, minerals, and antioxidant premixes were used. Further, under the nectar category, common sugar, honey, glucose, sucrose, fruit juices (orange), and coriander oil have been part of such diets (Lakra 2006; Akyol et al. 2006; Sihag and Gupta 2011; Li et al. 2012; Kumar and Agrawal 2014; Omar et al. 2016; Amro et al. 2016; Al-Shammary and Al-Gerrawy 2017; Islam et al. 2020). These diet combinations were prepared with single versus single or multiple food components (Table 20).

4.11 Combinations missing pollen or nectar substitutes with yeast/drugs and vitamins/proteins/minerals/amino acids

Further, in the artificial feeding of honey bees, two diet combinations of pollen substitutes, yeasts/drugs and vitamins/proteins/minerals/amino acids, three of yeasts/drugs, vitamins/proteins/minerals/amino acids, and nectar supplements, and another one in a combination of yeasts/drugs in vitamins/proteins/minerals/amino acids (Al-Ghamdi et al. 2011; Ghazala and Nowar 2013; Kumar and Agrawal 2014) (Table 21).

4.12 Combinations missing both pollen and nectar substitutes

In many cases, neither pollen nor nectar substitutes were used, but other contents were fed to honey bees (Table 22). These contents are yeasts, crude proteins, vitamins, multivitamins, and essential and non-essential amino acids (Zahra and Talal 2008; Sereia et al. 2013; Zheng et al. 2014; Haleem et al. 2015; Hendriksma et al. 2019). The yeasts (*Candida utilis*, *S. cerevisiae*, and fodder yeast) have been commonly fed to honey bees in this category (Chalmers 1980; Shimanuki and Herbert 1986).

5 Commercial artificial diets

In commercial bee keeping, ready-to-use diets are also available for feeding bees. These artificial diets are complete nutrition meant to

Table 22 Diets without pollen and nectar substitute

S. N.	Diet composition		References
1.	Yeast	Torula (*Candida utilis*)	Shimanuki and Herbert (1986), Doull (1977)
2.		Brewer's (*S. cerevisiae*)	Chalmers (1980), Free and Williams (1971), Stroikov (1966)
3.		Liquid	Sereia et al. (2013), Al-Sarhi (2008), Hammad (2007)
4.		Baker's (*Saccharomyces cerevisiae*)	Standifer et al. (1977), Standifer et al. (1973), Free and Williams (1971)
5.		Fodder	Free and Williams (1971)
6.	Crude protein		Zheng et al. (2014), Herbert and Shimanuki (1979a)
7.	Casein		Herbert and Shimanuki (1979a)
8.	Commercial casein mixtures		Haydak (1936)
9.	Lactalbumin		Shimanuki and Herbert (1986)
10.	Tankage protein for animal feed		Haydak (1936)
11.	Multivitamins		Haleem et al. (2015), Zahra and Talal (2008)
12.	Thiamine (vit. B)		Haleem et al. (2015)
13.	Vitamin C		Zahra and Talal (2008)
14.	Essential amino acids (EAA)		Hendriksma et al. (2019)
15.	Nonessential amino acids (NAA)		Hendriksma et al. (2019)

Table 23 Common artificial diets being used all over the world

S.N.	Commercial diets	References
1.	Royal king 4 H (German product)	Abd El-Wahab and Ghania (2016)
2.	Royal star (German product)	Abd El-Wahab and Ghania (2016)
3.	Bee-Pro	Saffari et al. (2010), Al-Eitby (2009)
4.	Bee-Pol	Huang (2010)
5.	Feed-bee	Guler et al. (2018), Omar et al. (2017), Saffari et al. (2010), Saffari et al. (2004)
6.	Ultra-bee	Ricigliano et al. (2022)
7.	Global	Ricigliano et al. (2022)
8.	Bulk-Soft	Ricigliano et al. (2022)
9.	Mega-Bee	Ricigliano et al. (2022)
10.	AP23	Ricigliano et al. (2022)
11.	Healthy-Bees	Ricigliano et al. (2022)
12.	Honey-sugar cake with Immunebee solution	Wilde et al. (2014)
13.	Honey-sugar cake with beetonic solution	Wilde et al. (2014)
14.	Honey-sugar cake with Beeodine	Wilde et al. (2014)
15.	Nektapol	Al-Ghamdi et al. (2011)
16.	TLS Bee-food	Sena et al. (2012), Saffari et al. (2010)
17.	Beltsville-bee (BBD)	Jimenez and Gilliam (1989), Herbert and Shimanuki (1983)

maintain the hive's aces and boost bees' immunity. These artificial diets have been developed commercially and are easily available in the market; however, the cost-benefit ratio is of concern. This literature survey has documented over 17 such commercial diets, tested by various authors (Saffari et al. 2010; Al-Ghamdi et al. 2011; Sena et al. 2012; Wilde et al. 2014; Abd El-Wahab and Ghania 2016; Omar et al. 2017; Guler et al. 2018) (Table 23).

Conclusion

The artificial food of honey bees can be categorized in two ways: the first one is the food components, and the second one is their combinations. The food components can further be classified into two categories: natural nutrients and natural food components. The natural nutrients have been proteins, carbohydrates, vitamins, yeasts, antibiotics, amino acids, enzymes, antioxidants, etc. In contrast, the natural food components comprise cereal grains, pulses and beans, fruits and vegetables, medicinal plants, spices, condiments, and some non-traditional/ miscellaneous kinds of stuff. In the artificial feeding of bees, the diet combinations were prepared using the abovementioned nutrients and food components in various forms and proportions. In general, the pollen and nectar, the main food of bees, have been supplemented under various diet combinations. These diet combinations either used pollen and nectar supplements or alone combined with other nutrition, drugs, antibiotics, etc. The present investigation provides an updated overview of the food types and their combinations used in the artificial feeding of bees to date. Apiculturists can use this outcome to develop new effective diet combinations. It will also help researchers explore new food items that have yet to be tested.

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Colistin the last resort drug in 21st century antibiotics to combat Multidrug resistance superbugs

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ABSTRACT

Polymyxin' E' (Colistin) is considered the last resort therapy against Multidrug resistance (MDR) bacteria, mainly *Klebsiella peumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli* and play a critical role in causing life-threatening infection, and their prevalence is increasing as a big concern globally. Apart from immunological adaptation, chromosomal mutations and plasmid-mediated genes are mostly associated with this resistance at the molecular level. Therefore, the current review extensively focused on Colistin as a drug in 21st-century antibiotics, the activities spectrum with diverse resistance mechanisms of bacteria against Colistin, and emerging approaches of Colistin from discovery to tackling MDR. In the study, we got to know about the challenges and new developments with old weapons like phage therapy as well as new approaches like Phage display and drug repurposing, in addition to the chromosomal and plasmid-mediated genes that play a role in antimicrobial resistance (AMR). The present study would provide insight into the prognostic aspect of combating MDR.

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1 Introduction

Over the last ten years, the prevalence of serious illnesses due to antibiotic-resistant gram-negative bacteria has continued to rise, and these infections now form a serious risk to global public health. Gram-negative bacteria, particularly *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, have caused a massive increase in infection. Several studies have reported polymyxins (A to E) as frequently key accessible active antibiotics agents (Carroll et al. 2019; Lima et al. 2018; Vázquez-López et al. 2020). Where polymyxins E, known as Colistin (Landman et al. 2008; Lima et al. 2018), became accessible for scientific use during the 1960s. However, it was supplanted during the 1970s with different antibiotics inferable from its lethality (Carroll et al. 2019). Use of Colistin was limited when aminoglycosides and other antipseudomonal agents, which were potentially less toxic, became available. Due to increasing reports of nephrotoxicity, intravenous formulations of Polymyxin E and polymyxin B were considerably restricted in practically all countries of the world in the early 1980s (Deris 2015; Deekshit et al. 2023). However, due to MDR (Multiple Drug Resistance), Gram-negative bacteria (GNB) involving patients with cystic fibrosis, the arterial use of Colistin for treating lung infections was routinely limited in the previous two decades (Conway et al. 1997; Mazzitelli et al. 2023). Since then, Polymyxins (Colistin) have been considered a significant therapeutic method capable of acting against Gram-negative bacteria, owing to increasing bacterial resistance to the bulk of widely available antibiotics and a lack of novel drugs. On the other hand, rates of polymyxin E resistance have been comparatively less, likely due to the rare usage; another reason could be the increased formation of colistin-resistant bacteria infection as a result of its use (Zhang et al. 2021; Zhou et al. 2022). Due to the scarcity of novel antibiotics capable of combating GNB, finding a new one will take nine to eleven years (Zhou et al. 2022), so optimizing CMS/colistin is critical.

The increased prevalence of colistin resistance in human and animal species during the past few years can be attributed mainly to the drug's widespread use in livestock and food production. *E. Coli* isolates from swine and bovine sources in Belgium and China harbour variants such as mcr-2 and mcr-3 after the identification of mcr-1 (Timmermans et al. 2021). Moreover, *S. paratyphi* B from German poultry (Borowiak et al. 2019) was found to harbour mcr-5, whereas *Salmonella* spp. and *E. coli* from pigs in Belgium, Italy, and Spain exhibited mcr-4 (Carattoli et al., 2017). Notably, *K. pneumoniae* strains from domesticated animals in China have been found to carry the carbapenemase-encoding gene blaNDM in combination with the discovery of mcr-8 (Mathy et al. 2018). This finding has caused grave concerns because plasmid-mediated mcr-1-associated genes are generally mobile, and colistin resistance may arise quickly in the human microbiome, making colistin therapy ineffective (Zaneveld et al. 2011). Additionally, it was discovered during these surveys that these adjuvants could boost the efficacy of Colistin by more than 2048 folds. This suggests that lower colistin *in-vivo* dosage against Colistin in Gram-negative microorganisms may be possible with adjuvant pairing (Wang et al. 2018; Sheng et al. 2022). This review article investigates the significance of Colistin as a last-resort antibiotic against multidrug-resistant Gram-negative bacteria. This review article also highlighted the colistin efficacy, resistance mechanisms, immunological adaptation and genetic factor development over the last ten years.

2 Colistin

In the subsequent section of this review article, a comprehensive overview of Colistin as a critical antibiotic for combating multidrug-resistant gram-negative bacterial infections and its key features were discussed.

2.1 Colistin 21st century antibiotics overview as a drug

Polymyxin B and Colistin are mostly safe in clinical use. The polymyxins class of antibiotics is dynamic against some selected GNBs, including *P. aeruginosa*, *A. baumannii*, *K. pneumonia*, and *Enterobacter* species (Van Loon et al. 2017). Colistin is the last line of antibiotics treated against multidrug-resistant gram-negative bacterial infection caused by ESKAPE pathogens. Currently, the therapeutic option against carbapenem resistance Gram-negative bacilli creates a great problem in clinical practices. Moreover, the increasing rate of drug resistance is known as 'superbugs' that give new challenges for scientists (Falagas et al. 2005; Giamarellou and Poulakou, 2009; Tan and Tatsumura 2015).

Antibiotic resistance is not only a sectional issue but a worldwide one. Typically, bacteria resist antibiotics, limiting the treatment option, raising mortality and morbidity, and increasing the hazard of antibiotics-related adverse situations. The resistance to antibiotics is termed as the ability of a microorganism to withstand the impacts of antibiotics, and it is a sort of medication opposition. Antibiotic resistance develops using natural selection via random mutation; likewise, it could be built by applying a developmental weight to a population. When such a gene is formed, the microorganisms can transfer the hereditary information horizontally (Between the individuals) by plasmid exchange. If a bacterium transmits a few resistance genes, it is called multidrug resistance or a superbug. So, Colistin has been re-surveyed as a fundamentally significant antimicrobial in people because of its efficiency against multidrug-resistant Gram-negative bacteria, specifically against *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*. In the 21st century, Colistin is probably the last combat antibiotic against gram-negative multidrug-resistant pathogens.

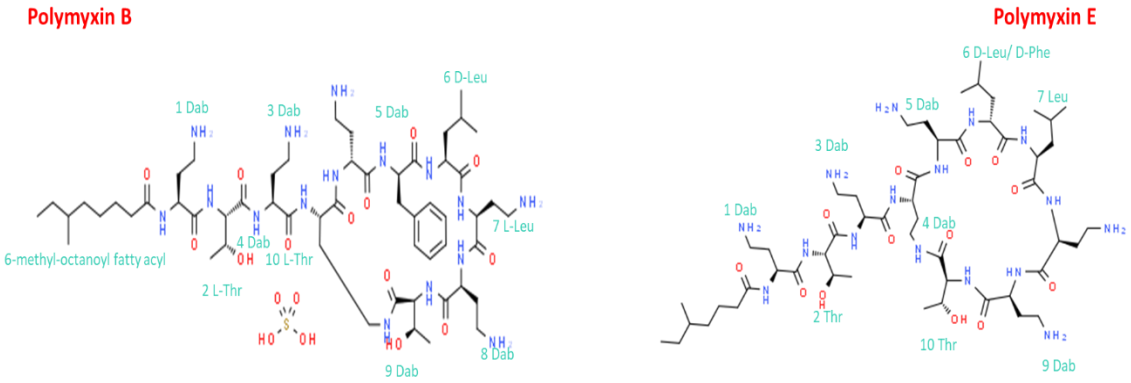

Figure 1 Structure difference between Polymixin B and Polymixin E

The closest look at antibiotic resistance will help researchers develop new antibiotics. Keeping away from the wrong use and abuse of antibiotics would hinder the spread of resistant microbes. As microorganisms dependably advance and can grow increasingly more resistant, new antibiotics are expected to fight against them (Figure 1).

3 Structure and Pharmacodynamics properties of Colistin

Colistin is a broad-spectrum antibiotic with a molecular weight of 1750 Da. Colistin sulfate is cationic, whereas Colistimethatesodium (CMS) is anionic. CMS is modified *in-vivo* to generate polymyxin E, responsible for antibacterial action. A cationic polypeptide rattles the cell membrane through a detergent-like mechanism. Colistimethate sodium is hydrolyzed in aqueous solutions, leading to a composite fusion of sulfomethylated metabolites and Colistin (Michalopoulos and Karatza 2010; Michalopoulos and Falagas 2011; Ayoub Moubareck 2020; Chiu et al. 2022)

4 Mechanism of Action

Polymyxin B and Polymyxin E possess bactericidal activity by binding to lipopolysaccharides (LPS) and phospholipids in Gram-negative microbes' outermost layer (Ayoub Moubareck 2020; Slingerland et al. 2022). Polymyxin-E's Antimicrobial action specifically targets the bacterial cell membrane (Conrad and Galanos 1989). The interaction of cationic polypeptide (Colistin) and anionic lipopolysaccharide (LPS) within the outermost layer of Gram-negative microbes disrupts the cell layer via electrostatic interactions, displacing stabilizing agents such as magnesium (Mg^{2+}) and calcium (Ca2+) from negatively charged LPS particles (Madhumanchi et al. 2020; Tiwari et al. 2022). The Microscopic studies revealed that the bacterial intracellular membrane was partially damaged, releasing cytoplasmic material through membrane gaps (Koike et al. 1969). Because Colistin disrupts membrane integrity, hydrophilic antibiotics such as carbapenem, glycopeptides, rifampicin, and tetracycline have synergistic effects. Although some reports suggest that polymyxins may act through mechanisms other than the bacterial cell layer, the precise mechanism by which Colistin eliminates bacterial cells remains unknown (Ayoub Moubareck 2020).

5 Susceptibility Breakpoint

Polymyxin E susceptibility breakpoints are determined differently in countries, notably Germany, the United Kingdom, and France. Colistin sulphate is commonly used to define sensitivity breakpoints. The French Society for Microbiology has established 2mg/L as the polymyxin E resistance breakpoint, while >2mg/L is generally considered the Enterobacteriaceae resistance breakpoint. On the other hand, the British Society for Antimicrobial Chemotherapy has set the sensitivity and resistance breakpoints at 4mg/L (Andre et al. 2010). Polymyxin E criteria for *Acinetobacter* species (four milligrammes per litre) have been adjusted by the Clinical and Laboratory Standards Institute (CLSI), differing from those specified for *P. aeruginosa* (eight milligrammes per litre) and other non-*Enterobacteriaceae* bacteria (Walsh and Amyes 2004; Karakonstantis 2021). Notably, no polymyxin breakpoints for *Enterobacteriaceae* have been established by CLSI. Colistin disc diffusion testing guidelines have been developed by the French Society of Microbiology and the British Society of Antimicrobial Chemotherapy (Li et al. 2006; Al-Bayssari et al. 2021). However, additional clinical data are required to define optimal susceptibility breakpoints, and standardization across antimicrobial susceptibility testing methods is critical for consistent and reliable results in diverse research settings.

6 Plasmid-mediated colistin resistance gene

Plasmid-mediated polymyxin E resistance emergently has been reported in numerous nations of the globe, including countries like Asia, Africa, Europe, and America (Breazeale et al. 2005; Llobet et al. 2008). The mcr1 gene (mobilized colistin resistance genes) was first identified in human isolates in 2008, and it was highlighted in *Shigella sonnei* from Vietnam (Aghapour et al. 2019). The mcr

gene is particularly concerning since it is present in plasmids, which are very small units of mobile DNA that carry genetic instructions and information from one bacterium to the next (Hassan et al. 2019). This indicates that plasmids carrying resistant mcr genes can transform other bacteria, including carbapenem-resistant *Enterobacteriaceae*, resistant to Colistin (CRE). The term plasmid-mediated polymyxin E resistance (mcr) includes the capability of a gene to survive the effects of Colistin and to spread this ability to other bacteria. These findings suggest that the mcr1 gene has long been present in Enterobacterales but has gone unnoticed (Campos et al. 2004; Biswas et al. 2012). The mcr1 gene has been found in various *Enterobacterales* species, most notably in *E. coli*, *Salmonella*, *Klebsiella*, *Shigella*, *Vibrio*, *Enterobacter*, and others (Dalmolin et al. 2018). Some examples are mcr 1.2, which is isolated from *K. pneumoniae* in Italy; mcr-1.3 and 1.4, which is separated from *E.coli* in China; mcr 1.5, which is isolated from *E.coli* in Argentina, and *Salmonella typhimurium* in China. Further, Mcr 1.7 is isolated from *E. coli* in China, mcr 1.8 from *E.coli* in Brunei and mcr.1.10 is isolated from *Moraxella spp*. in Britain (AbuOun et al. 2018).

Furthermore, mcr-1 exhibits reduced resistance to colistin or polymyxin B, with isolates bearing the mcr-1 gene frequently showing susceptibility to Colistin (Anyanwu et al. 2020). The gene was found to be truncated in an elucidating instance involving a *Shigella sonnei* isolate, elucidating the presence of mcr-1 in isolates susceptible to Colistin (Pham Thanh et al. 2016). Conjugation studies have shown that reactivating the truncated mcr-1 gene can result in the emergence of a colistin-resistant phenotype. The identification of an intact mcr-1 gene in *E. coli* isolates resistant to Colistin, on the other hand, suggests that gene truncation may not be the primary mechanism influencing polymyxin sensitivity in mcr-1-carrying isolates (Liu et al. 2016).

Previously, specific colistin resistance pathways were linked to chromosomal mutations or horizontal gene transfer. Mutations in two-component systems such as PmrA/PmrB and PhoP/PhoQ, as well as changes in the mgrB gene, which encodes a negative regulator of PhoPQ, have been linked to colistin resistance in *K. pneumonia* (Cannatelli et al. 2014). Recent findings, however, indicate the emergence of plasmid-mediated colistin resistance genes in various regions, which contribute significantly to colistin resistance. GenBank currently contains ten distinct sets of mcr genes (mcr-1 to -10) and 18 mcr-1 subtypes (mcr-1.1 to -1.18) (Doumith et al. 2016; Ara et al. 2021) (Figure 2).

The mcr3 had 94.1 % to 94.8 % amino acid sequence similarity with proteins from three Aeromonas species. Similarly, a shortened transposon element (TnAs2), exclusively seen in *Aeromonas salmonicida*, was found upstream of mcr3. These findings imply that mcr3 genes in *Enterobacteraceae* may have originated in the Aeromonas species (Yin et al. 2017). Surprisingly, the mcr4 gene is placed in a tiny, non-self-conjugative plasmid. Including an

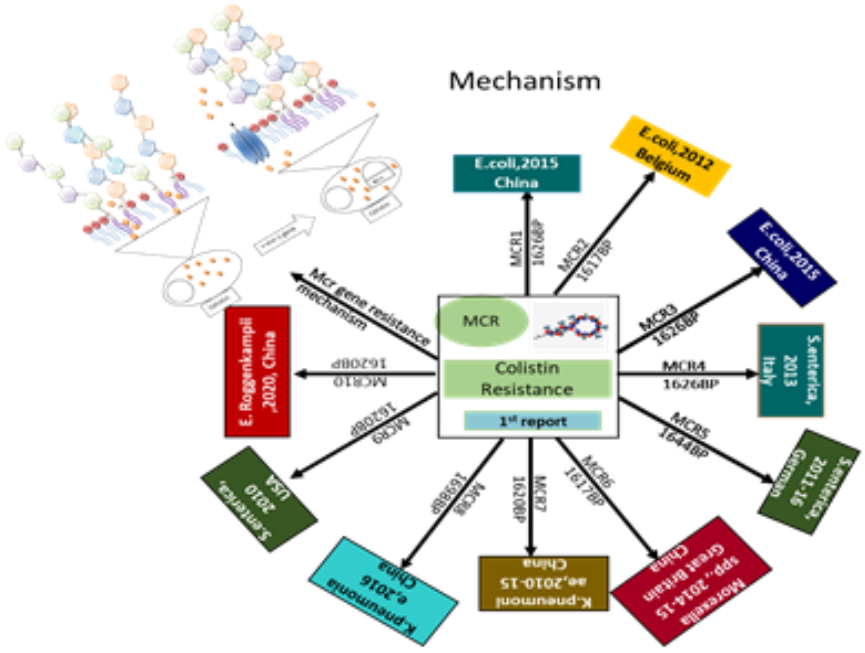

Figure 2 Specific resistance to Colistin is facilitated by the plasmid-mediated mcr-1 gene, encoding a phosphor ethanolamine transferase, which modifies lipid A with a phosphor-ethanolamine (PEP) group, preventing interaction between colistin and lipid A, along with this figure also shows details of mcr-1 to -10 genes

auxiliary plasmid, on the other hand, can increase conjugation. Leaving this variant, there are also reported 13 mcr-1 subgroups throughout the Globes. These type subgroups are only due to differences in only one nucleotide from mcr1. The mcr 1 gene is not limited to the Incl2 plasmid type. It may be found in IncX4, InHI2, IncF, IncHI1, IncY, and IncP plasmid. The MIC for Colistin of the strain with multiple mcr genes was not raised in any scenario (Chambers and Sauer 2013; Srinivasan and Rajamohan 2013) (Figure 2).

7 Phage-mediated colistin resistance

The SCKP83, a *Klebsiella pneumoniae* clinical isolate, has been found to contain colistin-resistant gene mcr-1, making it resistant to Colistin. Zhou et al. (2022) reported an mcr-1-shipping P7 phage-like plasmid of 97.4 kb; out of that, a 90.9-kb region was envisaged to be an integral phage. Even though this plasmid was not self-transmissible, the plasmids that are self-transmissible are found to contain 2600 bp long mcr-1 sequence, flanking at both ends with ISApl1, and there are evident roles of ISApl1 in mcr-1 mobilization (Peirano et al. 2014; Brennan-Krohn et al. 2018). An example of self- transmissible phage like plasmid is pSLK172-1, from a human atypical enteropathogenic *E. coli* that is found to contain phage P1-like sequences along with 12 antimicrobial resistance-encoding genes including florfenicol/chloramphenicol resistance gene (floR), beta-lactams resistance gene (blaCTX-M-14), fosfomycin resistance gene (fosA3) and florfenicol resistance gene (floR), streptomycin resistance (strA), Plasmid-Mediated Sulfamethoxazole resistance (sul2) and tetracycline (tetR).

8 Drug repurposing

Drug repurposing for Colistin is looking for a drug available for treating other diseases but not as antibacterial effects. Various drugs like antibiotics, antimycotics, antihelminth, and other drugs that are currently being used have been evaluated to combat colistin resistance (Figure 3, Table 1). Colistin was initially identified in 1950, and later, it was found to be identical to polymyxin E. Out of the 5 polymyxins (polymyxins A to E), only 2, (polymyxins B and Colistin) are being used in clinical settings. Colistin and polymyxins B differ by only one amino acid (D-phenylalanine to D-leucine) and are used as an alternative to Colistin (Ayerbe-Algaba et al. 2018). However, polymyxin B is moderately effective at clinical concentrations, and the emergence of strains resistant to polymyxin B has been reported. To overcome the resistance, the pharmacokinetic/pharmacodynamic parameters must be checked meticulously, and the average steady-state concentration of the drug should be more or equivalent to the pathogen's MIC to ensure optimal antibacterial action. The grouping of Colistin along with other antibiotics such as azithromycin, linezolid, and rifampin desensitized obtained colistin-resistant bacteria to Colistin, demonstrating that suboptimal concentrations of Colistin along with other antibiotics can produce a synergistic effect to prevent MDR bacteria. Colistin, when combined with antibiotics having protein or RNA synthesis inhibiting roles, poses synergistic effects against colistin-resistant Enterobacteriaceae, and thereby colistin resistance may be prevented by using the drugs in combination (Thangamani et al. 2015; Rajivgandhi et al. 2018) (Figure 3, Table 1).

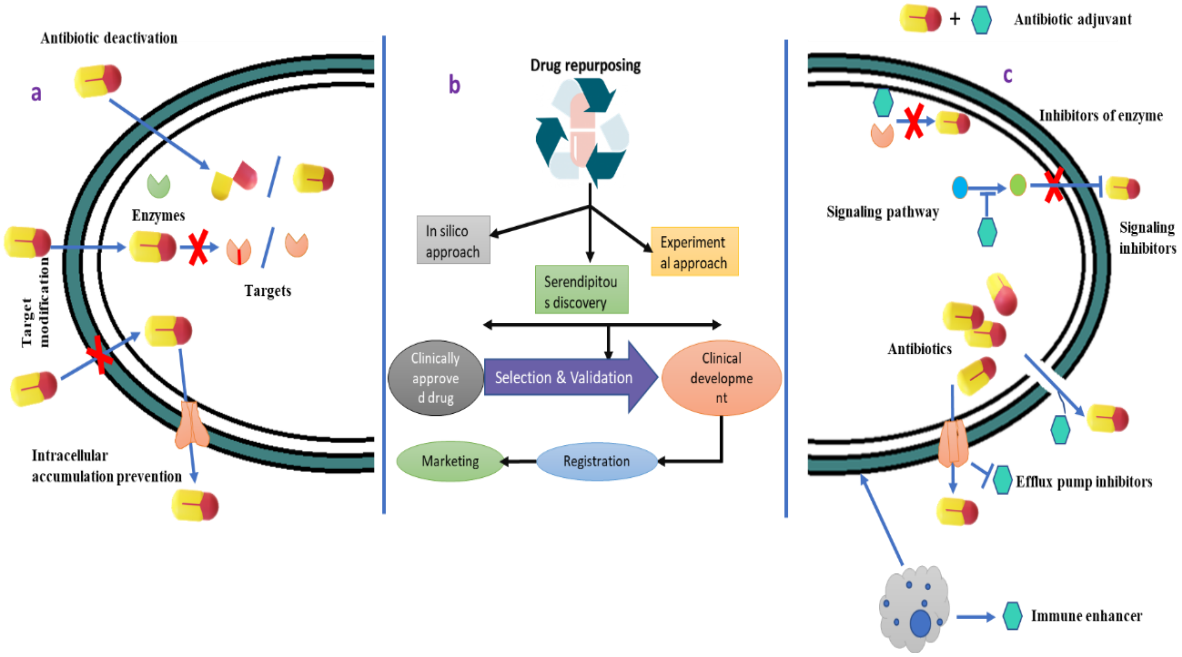

Figure 3 a) Different mechanisms of colistin resistance b) Drug repurposing flowchart c) Combination therapy against AMR

Table 1 The existing drugs of Multidrug resistance bacteria with the mode of action and adverse effects

Groups	Drugs	Mode of action	Bacteria	Important adverse effect	References
Beta-lactams	Penicillins, cephalosporins, carbapenems, monobactams, oxacillin, amoxicillin, amoxyclave, imipenem	Inhibit cell wall synthesis	*Enterobacteriaceae*, *P. aeruginosa*, *Acinetobacter spp*, *Haemophilus influenza*, *E. coli*, *Proteus* spp.	Skin, ENT, UTI, Respiratory infection	Kaur et al. 2011
Glycopeptides	Vancomycins	Inhibit cell wall synthesis	Gram-positive bacteria	MRSA, Skin, Endocarditis	Sarkar et al. 2017
Macrolides & ketolides	Azithromycin, Erythromycin, Clarithromycin	Inhibit protein synthesis (50S)	*S. pneumoniae*, *S. pyogenes*	Pneumoniae, Sinus, ENT, STIs	MacDougall 2017
Aminoglycosides	Gentamycin, Amikacin, Tobramycin, Streptomycin	Inhibit protein synthesis (30S)	*Pseudomonas aeruginosa* and other gram-negative bacilli	Bacteraemia, Abdominal infection	Durante-Mangoni et al. 2009
Tetracyclines	Tetracycline, Tegecycline	Inhibit protein synthesis		Lyme disease, PID, STIs	Abushaheen et al. 2020
Quinolones	Ciprofloxacin, levofloxacin, Moxifloxacin,	Inhibit DNA gyrase	*Campylobacter* spp	Anthrax, Bloodstream infections, Bone infection, Bronchitis, Bladder infection, Bacterial infection, prophylaxis. Campylobacter gastroectasis	Chala and Hamde 2021

9 Nonantibiotic compounds

Various nonantibiotic compounds have been reported to enhance the activity of polymyxins. In this case, antidepressants like amitriptyline, citalopram, imipramine and sertraline worked synergistically with polymyxin B to tackle the infectious bacteria. Similarly, Liu et al. (2023) also report antipsychotics belonging to the phenothiazine family and diuretics like spironolactone and statins (atorvastatin and simvastatin) have been found to act synergistically with polymyxin B to work against multiple drug bacterial infection.

10 Antiviral drugs

The antiviral drug zidovudine is found to be active against HIV (human immunodeficiency virus) and is a nucleoside reverse transcriptase inhibitor. It was the first commercial anti-retroviral for HIV therapy in 1987 (Peyclit et al. 2018). Colistin-resistant *Enterobacteriaceae* members were found to be susceptible to zidovudine with a MIC range between 0.2 – 6.25 μM (0.05 – 1.67 μg/mL). The zidovudine also influenced a clinical isolate of *K. pneumoniae* strain 853. Zidovudine is given at a dose of 600 mg/day for the treatment of HIV, indicating that the plasma concentration of a drug above the MIC can be easily achieved (Chow et al. 2009; Poirel et al. 2016).

11 Anthelmintic Drug

FDA-approved anthelmintic drug Niclosamide acts in synergy with Colistin to eliminate both the Colistin-susceptible and colistin-resistant Gram-negative bacteria isolates. Niclosamide alone has demonstrated a feeble action with a MIC of 512 μg/ml in opposition to the wild-type *P. aeruginosa* PAO1 strain. The MIC is reduced drastically to 2 μg/ml in opposition to efflux deficient PAO750 strain, while MIC remained unchanged in MexAB-OprM tripartite efflux lacking *P. aeruginosa* strain PAO200, and this strongly suggests that the niclosamide is a drug of efflux system (Abavisani et al. 2021). The potentiating effect of niclosamide with Colistin is concentrations above 1 μg/ml (3 μM). The combination of Colistin with Niclosamide has been found effective against both the Colistin susceptible (Col-S) and Colistin resistant (Col-R) *A. baumannii* and *K. pneumonia* (Snesrud et al. 2016).

12 Antineoplastic Drug

A bioactive compound has been isolated from marine endophytic actinomycete *Streptomyces coeruleorubidus* GRG 4 (KY457708) residing in marine macroalgae *Turbinaria ornate*. The active component has anticancer activity. Apart from anticancer activity, it was effective against colistin-resistant uropathogens *P. aeruginosa* and *K. pneumonia* (Bai et al. 2017).

13 Possible future strategic

AMR is the biggest mystery to solve around the globe. The review expands on infectivity due to carbapenem resistance microbes being hard to treat because of the restricted accessibility of therapeutic agents (Giannella et al. 2023). Colistin is commonly

favoured for treating cases brought about by pan-drug-resistant strains of carbapenemase producers despite its toxicity. Other than Colistin, different other anti-microbials in the polymyxin groups are dynamic against those Gram-negative microbes that include *Klebsiella* species, *Pseudomonas aeruginosa*, *Acinetobacter* species, and *Enterobacter* species. Colistin is a viable suitable antimicrobial agent for treating a wide range of multidrug-resistant Gram-negative microbes. It is a last-line drug to fight against serious diseases caused by gram-negative microorganisms (Bialvaei and SamadiKafil 2015; Liu et al. 2023). Different therapeutic management is considered to overcome the administration of last-line antibiotics. During the most recent couple of years, it's also been observed from Japan in science news, which was published in the Journal of Antimicrobial Chemotherapy, that nightmare bacteria exhibiting resistance to Colistin, which was the last resort therapy against MDR (Giurazza et al. 2021). Colistin obstruction is a significant issue because of the absence of particular antibiotics. Many known methods of investigation for AMR have fizzled, so we have to return to the planning phase and search for new ones. One promising approach is using bioinformatics in blend with systems biology and synthetic biology to recognize and deliver novel antibiotics through mining genome and metagenome sequence information for BGCs (biosynthetic gene clusters) (Navid et al. 2009). BGC generally encodes antimicrobial molecules, like non-ribosomal peptides, polypeptide antibiotics, terpenoids, alkaloids, saccharides and bacteriocins, which mostly regulate pathogenic microorganisms.

Two approaches that can create more impact in this multiple drug resistance bacterial infection management are systems biology and synthetic biology. Systems biology signifies creating novel processes to consider the usefulness of the living system as an entire (Yan et al. 2023). When contemplating bacteria, these approaches assist in comprehending how microbes evolve, acclimatize, and interrelate with other living beings. It also uncovers the outline and the dynamics of metabolites, proteins, and RNAs. It also deciphers their intracellular interactions and reveals the complex regulatory networks. Another strategy is synthetic biology, which creates artificial implements to achieve specific functions. Microbes are excellent hosts for significant purposes, such as bioconversion, bioproduction, biodegradation, and bioremediation. Predominantly, the engineered microorganisms have been broadly employed to make therapeutic proteins, chemicals, enzymes, biofuels, small molecular pharmaceuticals and other materials. Despite all these facts, the systems and synthetic biology focus on research and innovation. It carries out the goal of better-engineered research tools, which can then provide experiences to systems biology. In this unique circumstance, drug repurposing, which consists of utilizing a nonantibiotic compound to treat MDR, is supported. Using novel antibiotics like beta-lactam/beta-lactamase inhibitor-based and non-beta lactam-based agents could greatly relieve. To prevent the spread of disease and oncoming septicemia, an effective drug routine is an essential requirement. So, a treatment strategy must be commenced considering all these considerations.

Conclusion

This comprehensive review elucidates the significance of Colistin in the fight against MDR bacteria, highlights the challenges posed by resistance mechanisms, and suggests potential future directions. Combating the looming threat of antimicrobial resistance requires a multifaceted approach that includes drug repurposing, innovative biological methodologies, and robust surveillance systems.

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Declarations

No declaration.

Conflict of interest

The authors declare no conflicts of interest.

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Exploring the untargeted metabolites of *Moringa oleifera* Lam seed oil using two-dimensional gas chromatography with time of flight mass spectrometry for therapeutic application

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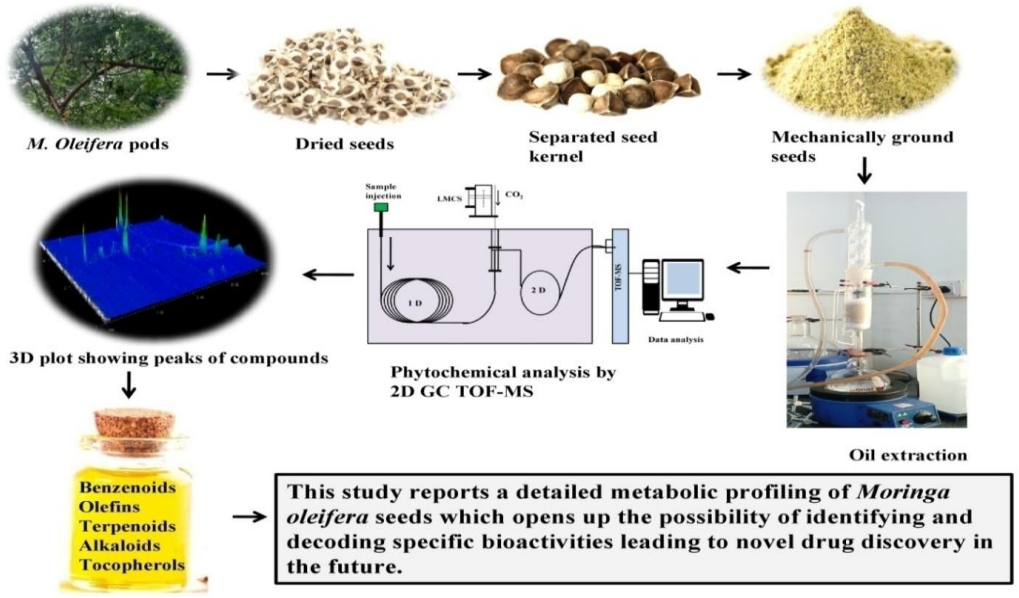
GRAPHICAL ABSTRACT



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ABSTRACT

Moringa oleifera Lam is an economically and medicinally important plant. However, its essential oil characterization has been limited to one-dimensional gas chromatography and mass spectrometry. This study identified secondary metabolite composition and variation in *M. oleifera* seed oil through two-dimensional gas chromatography with time of flight mass spectrometry and their associated bioactivity. GC×GC TOF MS analysis of *M. oleifera* seed oil was performed on an Agilent 7890 Gas chromatograph equipped with Pegasus 2D GC-TOFMS. About 1µl of the sample (dissolved in n-Hexane) was injected into the system, and the carrier gas was Helium. Identification was made using ChromaTOF software with reference to the NIST library. A total of 2000 phytoconstituents were obtained, of which 236 were identified using the NIST mass spectral values**.** Total constituents were classified into alkanes (64), alkenes (11), aldehydes (7), alcohol (10), acids (18), acid esters (70), Ketones (10), benzenoids (10), Monoterpenoids (1), olefins (6), Phenols (1), an alkaloid (1), triterpenoid (4), diterpenoid (1), sesquiterpenoid (2), tocopherol (2), and Others (18). Based on area percentage, fatty acids and their derivatives were predominant. The major constituents were Erucic acid (9.10%), trans-13-Octadecenoic acid (6.06%), Triethyl citrate (5.15%), Bis-(3,5,5-trimethylhexyl) phthalate (4.94%). This study reports a detailed metabolic profiling of *M. oleifera* seeds, which opens up the possibility of identifying and decoding specific bioactivities leading to novel drug discovery in the future.

1 Introduction

Moringa oleifera (MO) is the best-known species in the Moringaceae family, which consists of only 14 species. It is also known as a drumstick tree, with a height ranging from 5 to 10 meters (Liu et al. 2022). The plant has three pinnate compound-structured leaves and yellowish or white flowers without red streaks. The three-valved and elongated fruits contain winged seeds (Patil et al. 2022). This plant grows best at temperatures between 25 and 35 °C and can withstand 48°C. It is susceptible to weather conditions and poor soil varieties and has even been known to withstand draughts, high temperatures, and light frosts (Trigo et al. 2020). Further, it is indigenous to the sub-Himalayan regions of North-western India and is now cultivated in many other countries (Mashamaite et al. 2021).

It is also known as the Miracle Tree because of its tremendous medicinal value with no harmful side effects. Various parts of *M. oleifera* are used for the treatment of various ailments, such as stress, hypertension, depression, diabetes, anemia, blindness, malnutrition, arthritis, and kidney stone disorders, and also help in the regulation of the blood glucose levels, cardiovascular health, urinary tract infection, as well as provides anti-inflammatory, antioxidant, and anticancer activity (Meireles et al. 2020; Mohanty et al. 2021). According to research, most of the studies have been conducted on *M. oleifera* leaves compared to other parts of the plant, and they have reported beneficial effects on various chronic conditions (Islam et al. 2021). Its seeds constitute a high proportion of oil, making it an excellent source of edible and non-edible oil (Özcan 2020). Its seed oil is popularly known in the cosmetic industry for its use in perfume, hair care, and cream due to its efficacy in enhancing skin hydration, reducing skin erythema and has no side effects like skin irritation (Athikomkulchai et al. 2021). Plant seed extracts are also used for tertiary wastewater treatment through sedimentation, flocculation, coagulation, and rapid granular filtration (Andrade et al. 2021). A recent surge of interest has resulted from the discovery of potent antimicrobial, diabetic, hypertensive, antioxidant, anti-inflammatory, anticancer, and cardio-protective properties **(**Gu et al. 2020; Wang et al. 2022; Aldakheel et al. 2020; Das et al. 2023**).** The extent of effects obtained from the seeds is attributed to variability in metabolite composition. According to the previous estimation, over a hundred and thousands of phytochemicals are present in this plant (Kashyap et al. 2022). Detecting and identifying the complete phytochemical profile in a given extract is a serious challenge for plant biologists because the available platforms have limited sensitivity (i.e., limited metabolic coverage) (Fiehn 2002).

Chromatography with mass spectrometry is the preferred method for analyzing secondary metabolites from plant extracts. Using a one-dimensional GC with MS to analyze complex seed oils leads to poorly resolved metabolites and consequently limits metabolic coverage. This study identifies secondary metabolite composition and variation in *M. oleifera* seed oil through systematic untargeted chemical profiling by two-dimensional gas chromatography with time-of-flight mass spectrometry and their associated bioactivity.

2 Materials and methods

2.1 Plant materials

Fresh and matured fruits of *M. oleifera* were harvested from the Malkangiri district of Odisha, India, in December. The plant

sample was validated by Prof. Pratap Chandra Panda, Taxonomist, and a voucher specimen (2023/CBT dated 27.12.2021) was deposited at the herbarium of Centre for Biotechnology, Siksha O Anusandhan University, Odisha, India.

2.2 Preparation of seed oil

The fruits were cleaned with tap water and dried under the sun for three days. After three days, the seeds were separated from the fruits and shade-dried for two weeks until they were entirely dried. The seed kernels were separated and dried under shade for another two weeks. After the seed kernels were utterly dried, they were ground into a fine powder and stored in airtight bags. Oil extraction was done in a soxhlet apparatus by solvent distillation (Palafox et al. 2012). 100g of ground sample was put into the thimble of the soxhlet apparatus, and 250 ml of 100% ethanol was used as a solvent for oil extraction. The process continued for 8 hrs at a temperature of 30℃. The oil and solvent mixture was collected from the round bottom flask in a beaker and left in a water bath for solvent evaporation. The oil was collected in sterile bottles, appropriately sealed, and stored at 4℃ for further use.

2.3 2D GC×GC TOF MS analysis of *M. oleifera* seed oil

The phytochemical profiling of *M. oleifera* seed oil was performed on an Agilent 7890 gas chromatograph attached to a Pegasus 2D GC-TOFMS. A non-polar Rxi-5 MS column (30 m × 0.25 mm × 0.25 µm) was separated. Rxi-17Sil MS (2 m × 0.25 mm × 0.25 µm) was used as a secondary column. A volume of 1µl of the sample (dissolved in n-Hexane) was injected into the system in split mode (1:100). The carrier gas was high-purity Helium at a flow rate of 1ml/min. The initial temperature was set at 60℃, heated at a rate of 3℃ per minute gradually; the temperature was increased to 280℃, then held for 6 mins isothermally. This study used a 200℃ ion source temperature, a 250℃ interface temperature, a 70ev solvent cut time, and a linear velocity of 36.8 cm/sec for the column.

2.4 Identification of phytochemicals in the seed oil of *M.oleifera*

Data were processed using ChromaTOF software (LECO, version: 4.51.6.0 Optimized for Pegasus®). Compounds were identified by comparing their mass spectra to the mass spectral database (NIST 11). Based on PubChem and the Human Metabolome Database, compounds were classified into different chemical groups (Figure 1).

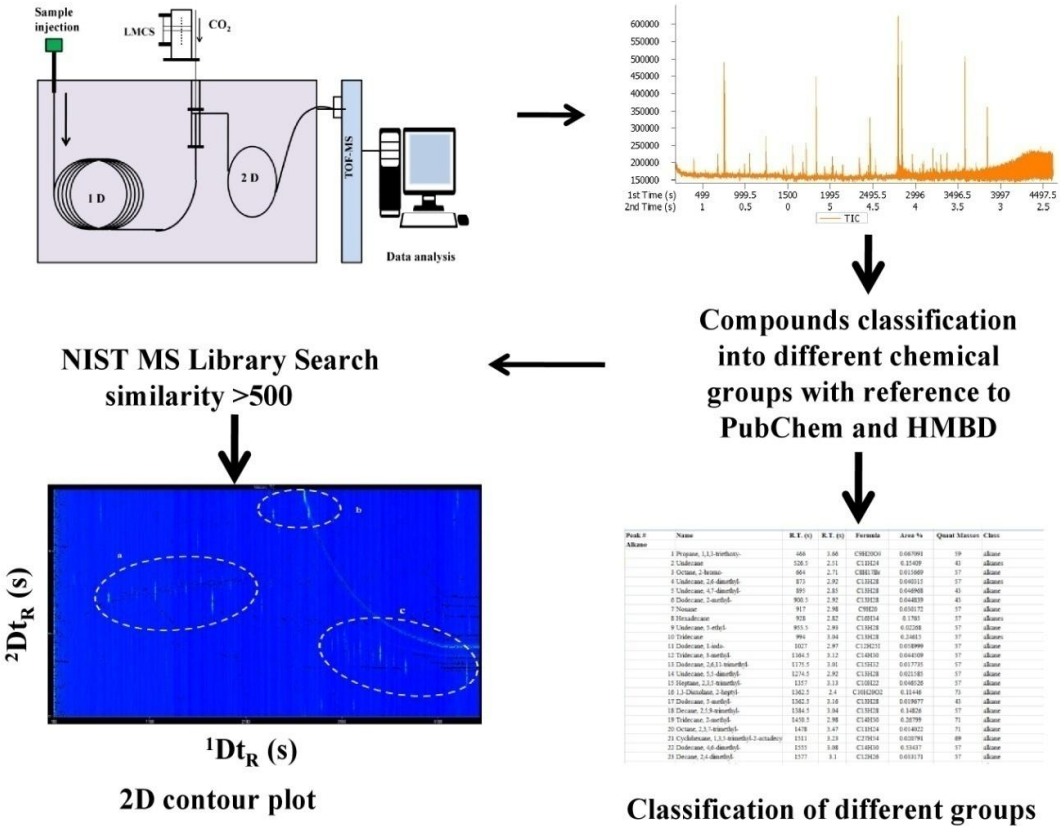

Figure 1 Schematic representation of phytochemical analysis by GC×CG-TOFMS

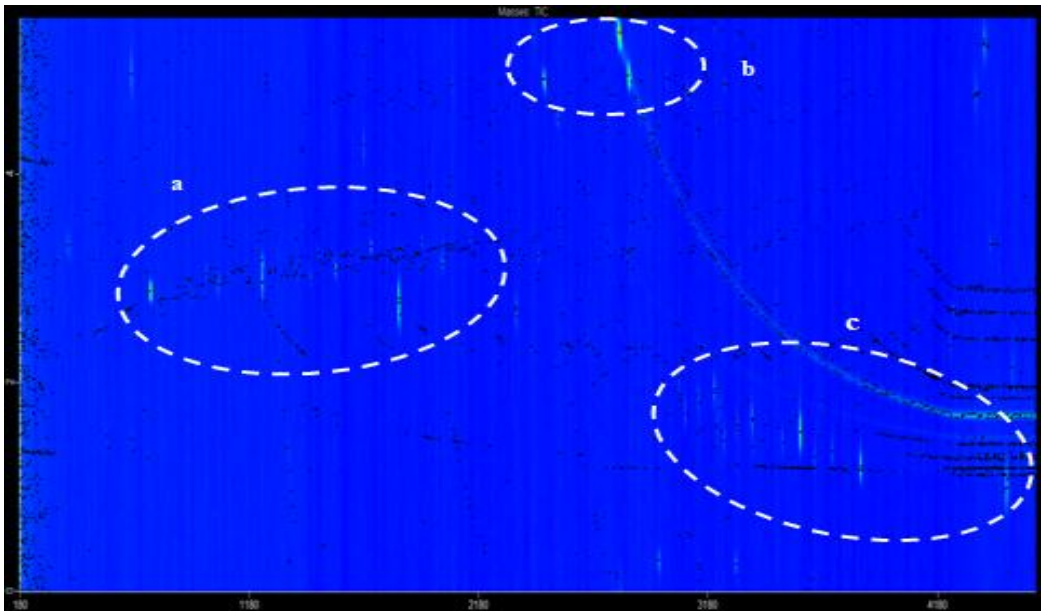

Figure 2 Contour plot showing the area acquired by the identified secondary metabolites (a) alkanes and benzenoids (b) fatty acids and esters (c) terpenes and tocopherols

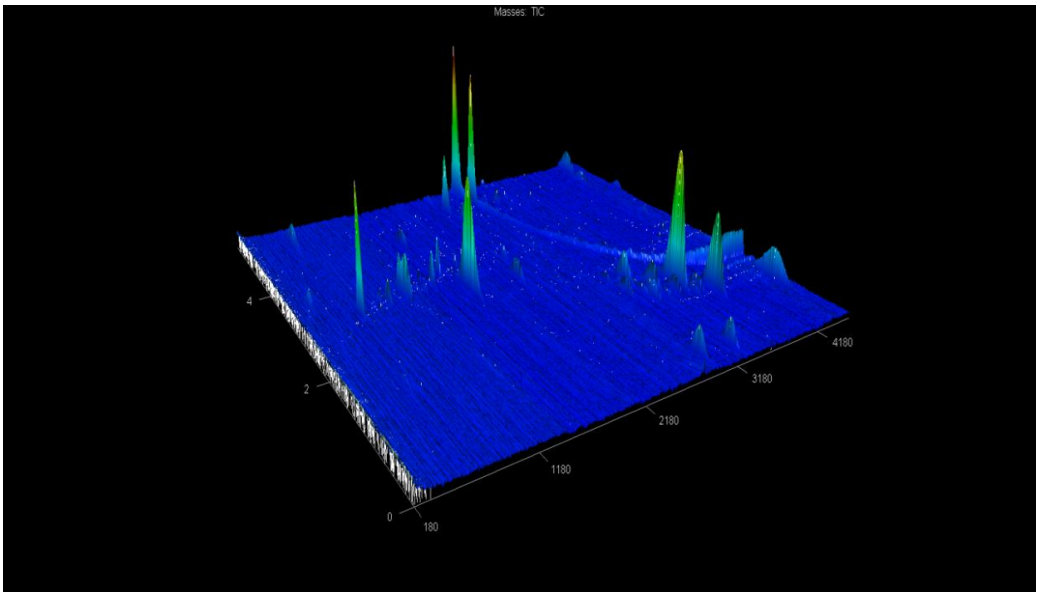

Figure 3 3-dimensional plot of phytochemicals from MOSO analyzed by GC×GC- TOF- MS

3 Results

The oil obtained from *M. oleifera* seeds was light brown, with a total oil yield of 7.2% (v/w). A detailed phytochemical characterization of the *M. oleifera* seed oil (MOSO) was obtained by the GC×CG-TOFMS approach. The GCGC-TOF-MS system achieved higher peak capacities and improved sensitivity through thermal modulation. To detect the peaks in the GC×GC chromatogram, automated peak screening of ChromaTOF software was applied (Figure 2 & 3). The only peaks considered were those with minimum signal noise (S/N) ratio thresholds more significant than 100. A total of 2000 phytoconstituents were obtained with total area percentage of 99.967, out of which 236 (total area % of 94.07) were analyzed using the NIST mass spectral values with a similarity of more than 500 considered. Based on PubChem and the Human Metabolome Database, compounds were classified into different chemical groups.: Alkanes, alkenes, Aldehydes, Alcohol, Acids, acid Esters, Ketones, benzenoids, Monoterpenoids, olefins, Phenols, alkaloids, triterpenoid, diterpenoids, sesquiterpenoid, tocopherol, and Others (Table 1) The major components found were:

Table 1 Compounds identified in *M.oleifera* seed oil analyzed using GC×CG-TOFMS

S. N.	Name	RT (s)	RT (s)2	Formula	Area %	Quant Masses
	Alkane					
1.	Tridecane	994	3.04	$C_{13}H_{28}$	0.24615	57
2.	Tridecane, 2-methyl-	1450.5	2.98	$C_{14}H_{30}$	0.26799	71
3.	Dodecane, 4,6-dimethyl-	1555	3.08	$C_{14}H_{30}$	0.53437	57
4.	Heptacosane	2022.5	3.14	$C_{27}H_{56}$	0.56746	43
5.	Isooctadecane	2143.5	3.4	$C_{18}H_{38}$	0.24582	57
6.	Heptacosane	2022.5	3.14	$C_{27}H_{56}$	0.56746	43
7.	Tetracosane	2721	3.52	$C_{24}H_{50}$	0.38289	43
8.	2,8,9-Trioxa-5-aza-1-silabicyclo(3.3.3)undecane, 1-methoxy-	2770.5	4.75	$C_7H_{15}NO_4Si$	0.19657	174
	Alkenes					
9.	4,5-Nonadiene	4294 ,	1.7	C_9H_{16}	0.54436	67
10.	5,7-Dodecadiene, (Z, Z)-	4426	1.7	$C_{12}H_{22}$	0.15768	81
	Alcohols					
11.	2-Ethyl-1-hexanol	389	3.32	$C_8H_{18}O$	1.0724	57
12.	(-)-Etafedrine	2308.5	3.65	$C_{12}H_{19}NO$	0.24139	86
	Acids					
13.	Hexadecenoic acid, Z-11-	2418.5	5.34	$C_{16}H_{30}O_2$	0.16225	55
14.	Palmitic Acid	2462.5	4.91	$C_{16}H_{32}O_2$	1.0783	60
15.	trans-13-Octadecenoic acid	2792.5	5.35	$C_{18}H_{34}O_2$	6.0679	55
16.	Oleic Acid	3089.5	4.75	$C_{18}H_{34}O_2$	0.30975	57
17.	Erucic acid	4283	1.68	$C_{22}H_{42}O_2$	9.1027	43
18.	linoleic acid	4602	1.69	$C1_8H_{32}O_2$	0.23508	67
	Acid esters					
19.	Triethyl citrate	1830	2.77	$C_{12}H_{20}O_7$	5.1576	157
20.	ethyl palmitate	2523	4.52	$C_{18}H_{36}O_2$	0.37497	88
21.	Phthalic acid, 2-methyl butyl pentyl ester	2655	2.37	$C_{18}H_{26}O_4$	0.3287	149
22.	Ethyl elaidate	2831	4.94	$C_{20}H_{38}O_2$	2.1677	55
23.	Glycol palmitate	2963	0.31	$C_{18}H_{36}O_3$	0.44578	43
24.	Phthalic acid, cyclohexyl 2-pentyl ester	3078.5	1.71	$C_{19}H_{26}O_4$	0.73616	149
25.	1,2-Benzenedicarboxylic acid, butyl octyl ester	3227	1.76	$C_{20}H_{30}O_4$	0.55539	149
26.	Phthalic acid, di(2-methyl butyl) ester	3304	1.6	$C_{18}H_{26}O_4$	0.57039	149
27.	1,2-Benzenedicarboxylic acid, butyl octyl ester	3227	1.76	$C_{20}H_{30}O_4$	0.55539	149
28.	Phthalic acid, di(2-methyl butyl) ester	3304	1.6	$C_{18}H_{26}O_4$	0.57039	149

S. N.	Name	RT (s)	RT (s)2	Formula	Area %	Quant Masses
29.	Phthalic acid, dodecyl pentyl ester	3370	1.66	$C_{25}H_{40}O_4$	1.5529	149
30.	Phthalic acid, 4-methyl pent-2-yl nonyl ester	3375.5	1.39	$C_{23}H3_6O_4$	0.32399	149
31.	Phthalic acid, bis(7-methyl octyl) ester	3502	1.43	$C_{26}H_{42}O_4$	0.61359	149
32.	Bis-(3,5,5-trimethylhexyl) phthalate	3579	1.53	$C_{26}H_{42}O_4$	4.9429	57
33.	Phthalic acid, hex-3-yl undecyl ester	3639.5	1.29	$C_{25}H_{40}O_4$	0.52037	149
34.	Didecan-2-yl phthalate	3843	1.2	$C_{28}H_{46}O_4$	2.0879	149
35.	Z-10-Tetradecen-1-ol acetate	4365.5	1.68	$C_{16}H_{30}O_2$	2.2428	55
	Ketone					
36.	3-Hexanone, 2,5-dimethyl-4-nitro-	1676	5.13	$C_8H_{15}NO_3$	0.37313	71
37.	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	2341.5	2.7	$C_{17}H_{24}O_3$	0.4964	57
38.	Diazoprogesterone	4470	0.96	$C_{21}H_{30}N_4$	0.12988	43
	Benzenoids					
39.	Diphenyl ether	1236	2.97	$C_{12}H_{10}O$	1.3847	51
40.	Diethyl Phthalate	1676	4.28	$C_{12}H_{14}O_4$	1.0292	149
	Monoterpenoids					
41.	Isoborneol	664	4.96	$C_{10}H_{18}O$	1.0472	95
	Phenol					
42.	2,4-Di-tert-butyl-phenol	1500	0.46	$C_{14}H_{22}O$	0.05232	191
	Alkaloid					
43.	Neronine, 4á,5-dihydro-	2957.5	4.21	$C_{18}H_{21}NO_6$	0.00866	73
	Titerpenoid					
44.	Squalene	3826.5	5.44	$C_{30}H_{50}$	0.075712	69
45.	Stigmastan-6,22-dien, 3,5-dedihydro-	4376.5	5.25	$C_{29}H_{46}$	0.29268	55
46.	Stigmasterol	4382	5.29	$C_{29}H_{48}O$	0.027612	131
	Diterpenoid					
47.	Andrographolide	4343.5	4.76	$C_{20}H_{30}O_5$	0.29971	43
	Sesquiterpenoids					
48.	(Z, E)-alpha-Farnesene	4497.5	1.27	$C_{15}H_{24}$	0.02049	81
	Tocopherols					
49.	ç-Tocopherol	4134.5	2.15	$C_{28}H_{48}O_2$	0.19886	151
50.	dl-à-Tocopherol	4233.5	2.36	$C_{29}H_{50}O_2$	0.4854	165

2-Ethyl-1-hexanol (1.07%), Palmitic Acid (1.07%), trans-13-Octadecenoic acid (6.06%), Erucic acid (9.10%), Triethyl citrate (5.15%), Ethyl elaidate (2.16%), Phthalic acid, dodecyl pentyl ester (1.55%), Bis-(3,5,5-trimethylhexyl) phthalate (4.94%), Didecan-2-yl phthalate (2.08%), Z-10-Tetradecen-1-ol acetate (2.24%), Diphenyl ether (1.38%), Diethyl Phthalate (1.02%), Isoborneol (1.04%), Silane, tetramethyl- (2.14). The percentage area covered by different groups is depicted in the figure 4.

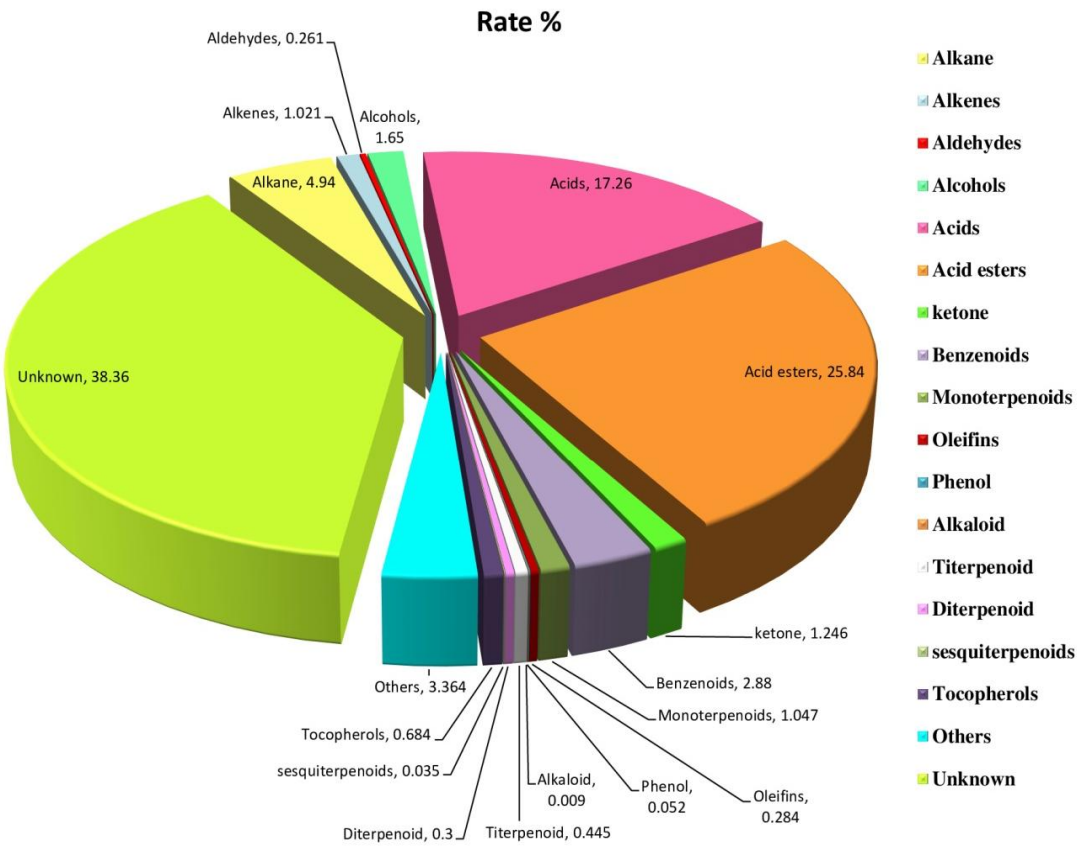

Figure 4 Pie chart depicting the areas covered by different groups of secondary metabolites

4 Discussion

A plant metabolomics analysis seeks to identify and quantify plants simultaneously and unbiasedly (Salem et al. 2020). The plant kingdom contains more than thousands of secondary metabolites (Wang et al. 2019). However, the oils require identification of the entire suite terminal and intermediate metabolites present for the associated pathways to be further interpreted for various bioactivities and potential biomarkers to study the metabolism process. Accurately and reliably identifying phyto-components in plant essential oils and achieving reasonable separation is a significant challenge. Co-eluting components contribute differently to an unresolved peak, identifying fewer compounds (Wong et al. 2015).

The most widely used method in metabolomics is one-dimensional gas chromatography-mass spectrometry, with nearly 50 years of established protocols. Being the most cost-effective one, it is the most commonly used method (Liu et al.2021). Various reports describe the chemotype of MOSO using GC-MS across the globe. According to a study conducted in Egypt, Phytochemical analysis of *M. oleifera* seed extract by GC-MS reported 50 identified phytochemical and major components were 2,5-Di-tert-butyl-1,4-benzoquinone (15.43%) and 4',6-dimethoxyisoflavone-7-O-β-D-glucopyranoside (9.25%) (Atta et al. 2019). An analysis with multidimensional gas chromatography and gas chromatography-mass spectrometer of Nigerian *M. oleifera* seed oil revealed twenty-four components, and among these Oleic acid was identified in its most concentrated form (Adegbe et al. 2016). The seed extract of *M. oleifera* from Iraq resulted in 41 bioactive compounds by GC-MS analysis, and Pentadecanoic acid 34.43% being the major one (AL-Obaidi et al. 2021). GC-MS analyzed thirty-six phyto-compounds in a study carried out in Tunisia, Cis, 6-octadecenoic acid showed the highest peak area (70.68%) (Zhou et al. 2023).

Prior work should have addressed the complexity and limitations of 1DGC, particularly when components with low abundance overlap with the major ones (Abdulhussain et al. 2021). There is an increase in demand for comprehensive metabolomics approaches for measuring plant metabolism, improving detection, global compound identification, and gaining a deeper understanding of how plants regulate biochemical processes (Raza 2020). The only existing study reported on phytochemical analysis using 2D GC-TOF-MS chemical profiling of MOSO; 250 compounds were obtained with cis-octadecenoic acid (78.62%) as the major compounds (Bassey et al.

2022). In this study, a total of 2000 phytoconstituents were obtained, out of which 236 were identified due to 2-dimensional TOF-MS analysis with Erucic acid (9.10%) as the major component. According to previous records, the total number of phytochemicals analyzed is more than double that identified in GC-MS.

The bioactivity of a plant extract is generally determined by its major phytoconstituents, but a synergistic effect between corresponding mixtures results in more significant bioactivity than individual constituents alone (Vaou et al. 2022). For instance, some studies have reported that Isoborneol (monoterpenoid) has antioxidant and antiviral properties and is a potent inhibitor of the herpes simplex virus (Kazi et al. 2023). The *M. oleifera* oil contains a high level of monounsaturated fatty acids like Palmitic Acid, Oleic Acid, Capric acid, Erucic acid, Linoelaidic acid, Hexanoic acid, Capric acid, Laevulinic acid, linoleic acid. It is related to reducing all-cause mortality, stroke, cardiovascular events, and cardiovascular mortality (Leone et al.2016). The tocopherol group is a higher source of essential vitamin E than other oils. Alfa-tocopherol has the most significant vitamin E potency (Delgado et al. 2020). Monoterpenes, diterpenes, tetraterpenes, triterpenes, sesquiterpenes,and glycoside compounds have substantial roles as anti-inflammatory, anticancer agents, antiallergic, antimicrobial, neuroprotective, antioxidant, anti-coagulation, sedative and analgesic activity (Masyita et al.2022).

This study reports a detailed metabolic profiling of secondary metabolites in the seed oil of *M.oleifera* using high-resolution GC×GC TOF-MS analysis. It provides a deeper characterization of the metabolic composition of *Moringa* when compared with conventional 1-dimensional GC-MS. Oil metabolism can be discriminated against using the metabolic profile on a contour plot. This can be further extended to chemo-taxonomical applications like metabolite fingerprinting and characterization of seed oil of *M. oleifera*. In addition to the high-resolution platform and identification procedures proposed, a wide range of metabolite profiles can be determined using untargeted phytochemical profiling of other parts of plant-derived extracts, resulting in a substantial increase in coverage of secondary metabolites. This opens up the possibility of identifying and decoding specific bioactivities in the future.

Conclusion

Many pharmaceutical uses have been reported for *Moringa* in India, mainly for its leaves. However, a recent upsurge in interest has risen to explore the pharmaceutical potential of the seeds to combat various health conditions**.** The extent of effects obtained from the seeds is attributed to variability in metabolite composition. GC×GC TOF-MS is considered over 1D GC-MS to unfold the metabolic coverage in this work. The experimental conditions were optimized to achieve high metabolic coverage, and as a result, some untargeted metabolites were discovered. The oil's fatty acid content was more similar to that of Olive oil. In addition, other groups of compounds like benzenoids, monoterpenoids, olefins, phenols, alkaloids, triterpenoids, diterpenoids, sesquiterpenoids, tocopherol are obtained, which is attributed to various pharmaceutical potency. These new untargeted metabolites can be helpful for future research after proper in-vitro and in-vivo validations.

Abbreviations

MOSO: Moringa oleifera seed oil; GC-MS: Gas chromatography Mass spectrometry; GC×GC TOF-MS: Two dimensional Gas chromatography Time of Flight Mass spectrometry

Ethics approval and consent to participate

Not applicable

Consent for publication

The authors declare no conflict of interest and have approved for publication.

Availability of data and material

This article includes all the data generated or analyzed during this study.

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Author’s contributions

Conceptualization, RB, and SKB; Validation, RB, and JNM; Original draft preparation, MD; Review and editing, RB and JNM; Supervision, RB, and SKB.

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Genomic DNA extraction from the medicinal plant *Crocus sativus* : Optimization of Standard Methods

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Cetyltrimethylammonium bromide

CTAB

SDS

Crocus sativus

ABSTRACT

High-quality genomic DNA is essential for genomic and molecular investigations such as next-generation sequencing. However, DNA extraction from medicinal plants like *Crocus sativus* can be challenging due to their high secondary metabolite content, which can interact with nucleic acids and affect the quality and yield of extraction. This study aimed to optimize the quality and yield of DNA using the cetyltrimethylammonium bromide (CTAB) extraction method from the leaves, stigma, and saffron corm. This new method is easy to use and can be performed using standard equipment and inexpensive reagents. The modifications made to the CTAB lysis buffer in this study, with the addition of SDS, resulted in a yield of 4233 ng/µl of DNA per sample of saffron corm (100 mg). This protocol is efficient and cost-effective for DNA extraction for studies with large samples and limited resources. This method is expected to be widely used for large-scale plant extraction and has a broad application in PCR-based sequencing studies.

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1 Introduction

Crocus sativus L. is a bulbous plant that belongs to the Iridaceae family. It is also known as saffron and is used as a spice and food coloring due to its expensive dried red stigmas. Saffron has gained popularity recently due to its numerous medicinal and pharmaceutical properties, such as anti-inflammatory, antifungal, antimicrobial, antibacterial, and anticancer properties (Boskabady et al. 2020; Shahrajabian et al. 2021). Saffron is mainly cultivated in Iran, India, Greece, Morocco, Spain, and Italy. Worldwide saffron production is estimated at 418 tons per year, and Morocco is the fourth-largest producer of saffron in the world after Iran, India, and Greece, with an estimated production of 5.9 tons per year (El Caid et al. 2020). However, due to the high economic value and unique properties of saffron, there is a rising demand for its production. Saffron production is limited due to its prolonged vegetative reproduction and sterility (Alavi-Siney et al. 2022). Saffron production is based only on vegetative propagation by corms, and continuous vegetative propagation leads to low genetic diversity, which limits different genetic breeding and production improvement programs. However, increasing efforts have been dedicated to boosting saffron yield production with superior product quality and conservation of genetic viability. Therefore, several scientific investigations have been conducted to improve the yield and corm production of saffron, in particular, conducted studies involving agro-morphological (Ghanbari et al. 2019a; El Caid et al., 2020), soil, and fertilization management for superior quality saffron production (Ghanbari et al. 2019b; Esmaeilian et al. 2022). Other complemented molecular studies have been extensively performed for saffron to assess genetic diversity and to evaluate the saffron ecotypes for conservation purposes (Anabat et al. 2020; Alavi-Siney et al. 2022) and used for saffron quality control by identification of the purity and detection of adulteration of saffron (Villa et al. 2016; Bansal et al. 2019; Zhao et al. 2019). To conduct molecular-based studies on saffron, high-quality genomic DNA extraction is a critical prerequisite, as it is the primary key to a successful polymerase chain reaction (PCR) assay (Aboul-Maaty and Oraby 2019). However, DNA purification can be hindered due to the high content of phenolic compounds and other secondary metabolites in *C. sativus*. DNA degradation during extraction is also a common problem (Moratalla-López et al. 2019). Further, polyphenols and polysaccharide compounds interfere with total DNA isolation. DNA degradation during extraction is another encountered problem during purification due to the endonuclease's enzymes (Rezadoost et al. 2016; Nath et al. 2022; Singh et al. 2023)

Several DNA extraction protocols based on cetyltrimethylammonium bromide (CTAB) have been widely used (Schenk et al., 2023). However, a single DNA extraction protocol is unsuitable for all plant tissue due to the variety and complexity of secondary metabolite content. Furthermore, commercial extraction kits can be expensive and inappropriate for laboratories with limited funds (Yu et al. 2019). Therefore, a simple, reliable, and cost-effective CTAB-based method has been developed in this study for extracting high-quality total DNA from *C. sativus* and other medicinal plants with high phenolic content.

2 Materials and Methods

2.1 Plant material

This study extracted DNA from frozen leaves, dried frozen leaves, stigmas, and saffron corms. The plant materials were collected from saffron plants grown in the experimental field of the Faculty of Sciences at Ibn Zohr University in Agadir, Morocco, during the growing season. The leaves were collected and stored at -20 °C, while the stigma was dried before extraction.

2.2 DNA extractions

The saffron genomic extraction was performed using Doyle's (1991) CTAB-based method with modifications. DNA saffron extraction was performed in quadruplicate for all samples.

2.2.1 Protocols 1

100mg of different parts of saffron were ground using a pestle and mortar. Then, 1ml of extraction buffer containing 2% (w/v) CTAB, 1% (w/v) PVP 400, 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM Na-EDTA (pH 8.0), and 0.3% (v/v) β-mercaptoethanol was added. The mixture was incubated at 60°C in a water bath for 1 hour with continuous gentle agitation. Next, one ml of chloroform: isoamyl alcohol [24:1, v/v] was added, and the mixture was centrifuged at 20,000g for 15 minutes. After that, the supernatant was transferred to a new microtube (1.5 ml). Then, 1/3 of 6 M NaCl and 2/3 of cold isopropanol were added to the aqueous phase and gently mixed. The mixture was incubated at -20 °C overnight to allow DNA precipitation. The microtubes were again centrifuged at 20,000g for 10 minutes, and then the DNA pellet was rinsed with 1 mL washing buffer (75% ethanol, 10 mM ice-cold ammonium acetate). After that, centrifugation was carried out at 20,000g for 5 minutes, and double washing of DNA pellets with 75% ethanol was performed. Finally, the DNA pellet was air-dried at room temperature and resuspended in 50µl of TE buffer (1 mL 1 M Tris–HCl, 20 mL 0.5 M 2Na2EDTA (pH 8.0), and 79 mL Milli-Q water).

2.2.2 Modified Protocol 2

After an initial DNA extraction method, small tubes were mixed with 2% CTAB extraction buffer and incubated for an hour. Following this, the tubes were allowed to sit at room temperature for 5 minutes and then 1 ml of phenol: chloroform [1:1] was added to them. The tubes were gently mixed and centrifuged at 20,000g for 15 minutes. The upper phase was extracted with an equal amount of chloroform: isoamyl alcohol [24:1, v/v] and centrifuged

at 20,000g for 15 minutes. The genomic DNA was then precipitated using NaCl/isopropanol and washed, and the obtained DNA pellet was resuspended in 50µl of 1% TE buffer.

2.2.3 Modified Protocol 3

100mg of various plant parts were ground in a mortar. Instead of using the standard 2% CTAB lysis buffer, a modified extraction buffer with SDS was used. To make the mixture's final concentration of 0.2% SDS, 10% SDS was added to the 2% CTAB buffer. The ground samples were then homogenized with 1 ml of the extraction buffer with SDS and incubated at 60°C for 1 hour. After this, all subsequent extraction steps followed protocol 1.

2.3 Assessing the quality and quantification of the DNA genomic extracted

The quality of the genomic DNA extracted from different parts of saffron using three different protocols was evaluated. Electrophoresis was performed to run the DNA on a 1% agarose gel at 100V for 60 minutes, using 1 × TBE as the electrophoresis buffer. The gel was then stained with ethidium bromide and analyzed under UV light. The DNA concentration was also determined spectrophotometrically at 260 nm using 5µl of DNA diluted in 1500µl of TE buffer. The purity of the samples was also assessed by checking for contamination from proteins and polysaccharides based on the A260/A280 ratio of the absorbance.

2.4 PCR amplification analysis

The PCR reaction was carried out in an Eppendorf tube. The reaction mixture consisted of 1 µL of template DNA, 1 × PCR buffer, 0.5 U Taq DNA polymerase (Bioline Meridian Bioscience Inc, USA), 0.06 µM of ITS2 primer (Jiang et al., 2014), forward primer (5' ATGCGATACTTGGTGTGAAT), and 0.06µM reverse primer (5' GACGCTTCTCCAGACTACAAT). The PCR process involved a denaturation step at 95°C for 2 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The process concluded with a final extension step at 72°C for 7 minutes. Subsequently, the 10 µL PCR product was loaded and run on an agarose gel. The result was recorded and visualized using a UV detector after the run.

3 Results

This study evaluated a straightforward and efficient method for extracting genomic DNA from various parts of the saffron plant. DNA extraction protocols such as extraction kits can be time-consuming, expensive, and inefficient, especially for plants rich in secondary phytochemicals like phenolic compounds (Yu et al., 2019).

The CTAB-based protocol is the most commonly used option for labs with limited resources and repetitive extraction. However, this protocol still needs to be optimized for plants with high levels of polysaccharides and polyphenols. In this study, the total genomic DNA of saffron was extracted from frozen and dried leaves, stigmas, and saffron corms (Figure 1) using a CTAB buffer. Three extraction protocols were evaluated, and approximately 100mg of samples were used for each extraction method. The concentration and yield of the extracted DNA varied depending on the different parts of the saffron plant and the type of protocol employed in the extraction procedure (Table 1).

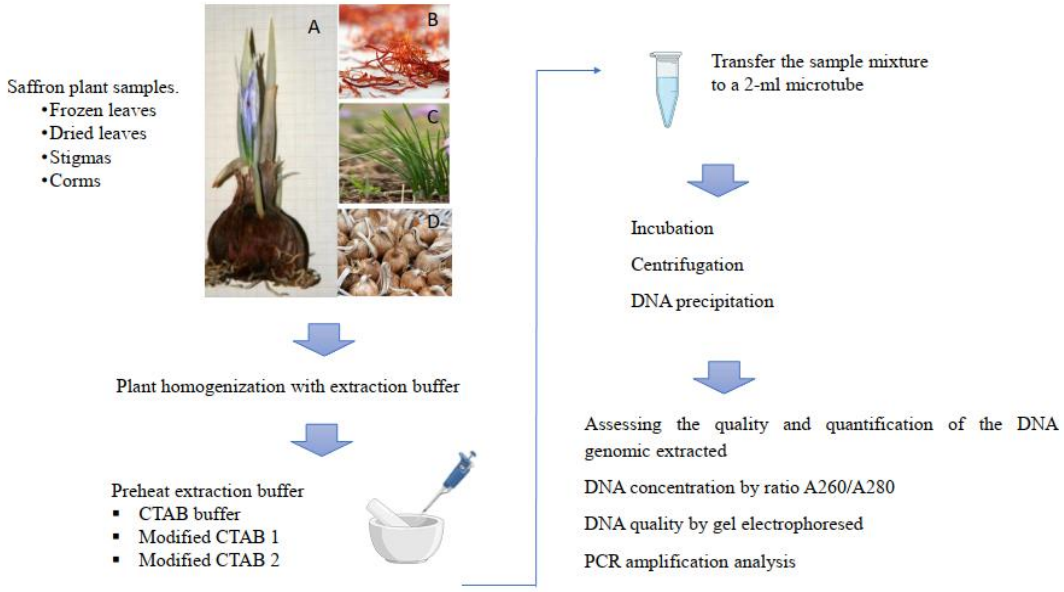

Figure 1 Flow diagrams of the three CTAB methods for saffron DNA extractions. A: *Crocus sativus* L. plant, B: stigmas, C: leaves and D: corms

Table 1 The purity and concentration of DNA extracted from saffron samples with three protocols

Samples	Protocols	A260/A280 (Purity)	DNA Concentration (ng/µl)
Frozen leaves	P1	1.58	1641
	P2	1.71	1992
	P3	1.80	2538
Dried leaves	P1	2.02	1797
	P2	1.69	723
	P3	1.02	1597
Stigmas	P1	1.77	3153
	P2	1.66	2856
	P3	1.75	3072
corms	P1	1.81	2709
	P2	1.85	3069
	P3	1.68	4233

P1: traditional CTAB, P2: Modified protocol, and P3: CTAB with 10% SDS

The study found that the DNA extracted from the leaves, stigmas, and corm using three different extraction buffers had a purity between 1.7 to 2.0 at the A260/A230 ratio. The ratio A260/A280, measured to determine DNA quality, ranged from 1.02 to 2.02. In the traditional CTAB method (protocol 1), the highest ratio of 2.02 was obtained from the dried leaves, while the DNA purity was recorded at 1.77 and 1.81 in the case of stigma and corm, respectively. In protocol 2, using phenol-chloroform, the highest purity ratio was obtained from corm, which was 1.85. Lastly, in protocol 3, where SDS was combined with CTAB buffer, the ratio A260/A280 ranged from 1.68 to 1.80 for corm and frozen leaves, respectively.

The overall DNA concentration obtained with protocol 1 based on the traditional CTAB buffer ranged from 1641ng/µl to 2709ng/µl, obtained from frozen leaves and corm. Protocol 2 successfully increased the DNA genomic yield and got 3069 ng/µl from the corm. However, modification in the CTAB buffer by adding the SDS substantially increased genomic DNA concentration to 4233ng/µl from Corms samples. Among the samples tested, the highest DNA crude purity and concentration were obtained from corm samples by protocol 3 with SDS addition. The quantitatively efficient protocol was CTAB with 10% SDS buffer to extract frozen leaves and corms.

The saffron DNA genomics extracted from various samples using multiple buffer protocols were analyzed for background compounds and potential degradation during extraction. Gel electrophoresis separation revealed some intense bands near the gel wells for DNA extracted by the CTAB buffer with SDS from frozen leaves and corms (Figure 2). Similarly, the most effective protocols for leaves were CTAB with SDS. PCR amplification

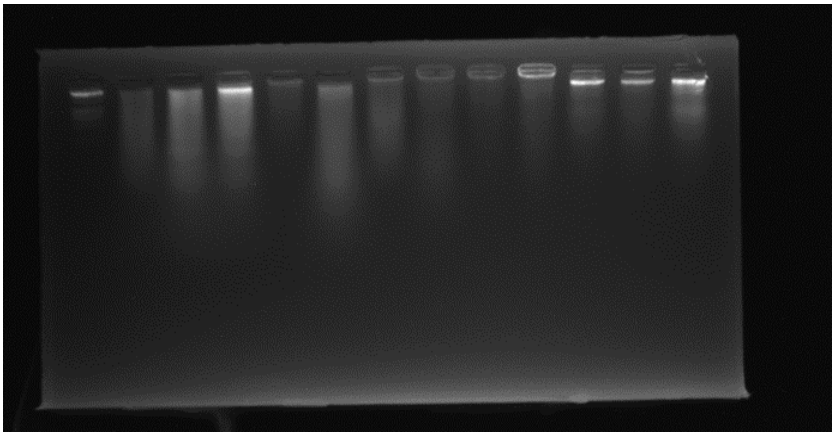

Figure 2 Electrophoretic patterns of *Crocus sativus* genomic DNA; Lanes 1 to 3: DNA extracted from frozen leaves using protocols 1, 2, and 3, respectively; Lanes 4 to 6: DNA extracted from dried leaves; lane 7 to 9: DNA extracted from stigmas, and lanes 10 to 12: DNA extracted from corm

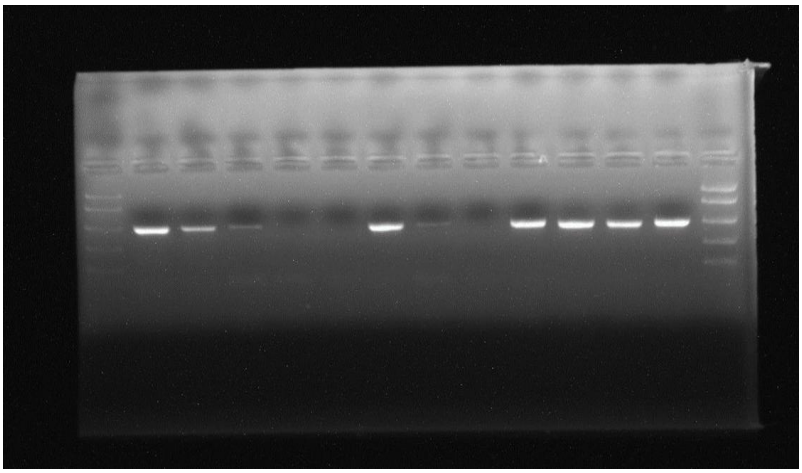

Figure 3 Agarose gel electrophoresis of ITS-PCR amplification products of *Crocus sativus* genomic DNA extracted by three protocols; Lanes 1 to 3: DNA extracted from frozen leaves using protocols 1, 2, and 3, respectively; Lanes 4 to 6: DNA extracted from dried leaves

with ITS primer showed a well-differentiated band pattern in the case of DNA extracted by modified protocols 3 for all saffron samples (Figure 3). The 560 bp length band was consistently observed in all DNA extracted from corm with the three protocols tested.

4 Discussion

Extracting high-quality genomic DNA is crucial for plant biology research, including genetic diversity, genetic improvement, and detection of adulteration through PCR-based analysis (Yu et al. 2019; Schenk et al. 2023). However, obtaining high-quality DNA can be challenging, especially for medicinal and aromatic plants with high levels of phenols and other secondary metabolites that can interfere with DNA purity. While several protocols for genomic DNA isolation are available, some are expensive or may not yield good DNA quality for certain plant species (Li et al. 2013; Yu et al. 2019). This study describes a simple and efficient method for extracting genomic DNA from saffron that can be applied to plants with high amounts of secondary metabolites. The method involves using a CTAB-SDS buffer as a lysis buffer with detergent, which proved to be more effective than other methods tested. This buffer promotes plant cell wall lysis, prevents RNAse activity and polyphenol precipitation, and consistently produces a pure and high-quality DNA yield that can be used in subsequent molecular analysis techniques. The protocol involves using a high concentration (0.3%) of 2-β-mercaptoethanol in a CTAB buffer to remove polyphenols successfully and generate a clear translucent DNA pellet (Heikrujam et al. 2020; Schenk et al. 2023). The extraction buffer also includes a chloroform-isoamyl alcohol step, which removes proteins, lipids, and cellular debris by binding with non-aqueous compounds as precipitate. Although the CTAB-based extraction buffer is the most widely used for plant DNA extraction, this study obtained better results using CTAB-SDS as a lysis buffer with detergent. The addition of SDS to the classic CTAB buffer had a significant effect on the extraction process. The CTAB/SDS buffer helps break down the plant cell wall and prevents RNAse activity and polyphenol from precipitation (Sika et al. 2015). Among the three tested protocols, the modified CTAB protocol with SDS proved more effective than the other two. This result is consistent with Mançano et al. (2022), who reported that using 20% SDS combined with CTAB yields high DNA (700ng) from Chrysobalanus icaco. Sabriu-Haxhijaha et al. (2020) used 2% SDS for soybean genomic extraction and obtained a high DNA yield. Moreover, Wang et al. (2012) obtained the highest DNA yield from soybean (25.89±3.2 µg) using the SDS-based extraction method compared to the classic CTAB method and commercial kits. The main modification for saffron DNA extraction involves adding SDS to the lysing buffer. This modification consistently produced a pure and high-quality DNA yield, which can be used in subsequent molecular analysis techniques such as PCR and sequencing. This study's simple and efficient extraction protocols are essential for assessing food safety by detecting adulteration and further biodiversity conservation. They are particularly useful for large analyses involving a large number of plant samples.

Conclusions

Research on molecular aspects of saffron plants lags behind other crops. This means that the information on saffron plants is limited and needs further exploration for crop improvement. Some studies have been conducted to examine genetic diversity, genome sequencing, and identification of markers for crop development. However, genomic DNA isolation is required for all molecular and genomic sequencing studies. Although several protocols have been developed for plant genomic DNA extraction, they are often expensive and less effective for certain plant species. The CTAB method is commonly used but still has limitations such as low quantity and purity, especially in plant species with high secondary metabolite content. This study has attempted three different

protocols to extract saffron DNA and found that adding 10% SDS to the CTAB buffer significantly increased the quantity and quality of extracted DNA. This method is simple, rapid, low-cost, and does not require special equipment. Therefore, it efficiently extracts high-quality genomic DNA from plant species with high secondary metabolite content. However, further studies are necessary to standardize this method for different plant species under varying conditions.

Authors' Contributions

SEM Conceptualization; SEM, IB, HEF, ML, methodology; SEM, IB, HEF, ML, KL, validation; SEM, IB, HEF, KH, ML, writing—original draft preparation; KL, MBEC, REL, writing—review and editing; REL, MAS supervision. All authors read and approved the final manuscript.

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Conflict of Interests

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

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Root attributes governing drought stress adaptation and the associated molecular markers in chromosome segment substitution lines in rice (*Oryza sativa* L.)

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ABSTRACT

The wild relatives of cultivated rice offer crucial resistance genes for combating stresses like drought. Developing rice varieties with drought tolerance is possible through chromosome segment substitution lines (CSSLs), which blend the genetic background of a high-yielding parent with specific chromosome segments from a donor parent. This study aimed to study the effect of drought stress on various root traits of chromosome segment substitution lines (CSSLs) and their relationship with specific molecular markers. Ninety-six genotypes, including 80 chromosome segment substitution lines (Curinga x *O. rufipogon* and Curinga x *O. meridionalis)*, 9 New Rice for Africa (NERICAs) and 7 controls were grown in Basket and PVC pipe methods for phenotyping different root traits. Under drought stress (DS), MER16, MER20, RUF10, RUF16, RUF44, NERICA1, and NERICA3 showed superior performance for most of the root traits. These evaluations were supplemented with association analysis of 17 root trait-linked simple sequence repeat (SSR) markers with root phenotypic traits. The marker RM201 is strongly associated with multiple root traits, found to be independent of three growth conditions (well-watered "WW" under Basket, WW condition and DS conditions under PVC pipe). The marker RM316 is associated with root volume, and the marker RM7424 and RM1054 show maximum root length. In conclusion, these markers can be used in marker-assisted breeding programs, and the lines carrying them can be used as parental lines in variety-development programs for drought tolerance.

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1 Introduction

Rice is consumed as a primary dietary source by 340 million people worldwide, predominantly in southern and south-eastern Asia (Rezvi et al. 2022). In a rainfed ecosystem, standing water quickly disappears once the rain stops, harming rice productivity (Ogura and Forwell 2023). Rice can be grown both in lowland and upland environments. Asia's Rainfed lowland and upland areas are 34 million hectares and 8 million hectares, respectively, for rice cultivation (Anilkumar et al. 2023). The sensitivity of rice to different climatic changes, such as an increase in temperature and an extended drought period (Yoshida et al. 2015), poses complex challenges in securing the future of global food demand. Rice production is severely hampered by drought stress (DS), especially in water-limited upland environments (Ndikuryayo et al. 2022). The situation is worse in rainfed upland areas than in lowland areas, as the crop experiences mild to severe DS between the rainfall events throughout their life cycle, and standing water is rarely available. To avoid DS, the rainfed upland rice varieties develop a more profound and thicker root system that allows the plant access to deep soil-water reserves, thereby maintaining a higher leaf-water status (Zampieri et al. 2023). Higher root volume (RV) and root length (RL) have been reported as a better combination for the selection and development of lines suitable for DS conditions (Dash et al. 2017). Previous studies have focused on rice improvement efforts to promote vigorous deep rooting and enhance yield stability for rainfed uplands (Anilkumar et al. 2023). Deep rooting helps maintain plant-water status in an upland ecology where intermittent drought is common (Sandar et al. 2022). The root traits that affect drought tolerance include root length density, root depth, root thickness, root dry weight, root penetration index, and deep root ratio (Jeyasri et al. 2021). Most current rainfed rice varieties were originally developed for irrigated ecosystems; however, these varieties fail to produce good crops under DS conditions (Venkateshwarlu et al. 2022). It is, therefore, necessary to develop high-yielding varieties with enhanced drought tolerance traits. Developing rice varieties with desirable root traits for upland ecologies by incorporating these traits from landraces into the high-yielding varieties is considered beneficial (Sabar et al. 2019). Attempts have been made to develop varieties using chromosome segment substitution lines (CSSLs) carrying the genetic background of the high-yielding recurrent parent and overlapping donor parent chromosome segments (Pinta et al. 2018; Ding et al. 2022). However, very few reports are available on the genetic improvement of known cultivated varieties for drought tolerance through introgression from wild rice species using the CSSLs approach. Genus *Oryza* has twenty-one wild relatives of the domesticated rice (Vaughan et al. 2003) that serve as a virtually untapped reservoir of genetic diversity and contain many novel resistance genes for biotic and abiotic stresses (Barik et al. 2017; Long et al. 2023). Wild rice prefers various habitats, including wetlands, drylands, and fresh or salty soils. It has a large genetic pool within Oryza that contains characteristics that confer tolerance to many different abiotic stresses. According to Atwell et al. (2014), a study employing a Geographic Information System (GIS) approach that superimposed environmental maps over georeferenced wild rice species occurrences identified many candidate species that warrant additional investigation in the quest for tolerance to cold (1 species), heat (5 species), submergence (4 species) or drought (5 species).

Around 40% of these important alleles in rice were lost from wild to cultivated during domestication. Exploring these favorable alleles of wild rice, through the development of CSSLs from crosses between wild species and cultivated varieties might be a powerful tool by identifying naturally occurring favorable alleles and to overcome yield limitations. CSSL libraries have been developed for many wild rice species, helping identify many quantitative trait loci (QTLs) of biological and economic interest (Subudhi et al. 2015; Zhao et al. 2022). These QTLs have been utilized for their important agronomic traits by effective mapping, cloning, and identification of gene interactions (Bimpong et al. 2011; Li et al., 2023). In the present investigation, 80 CSSLs having chromosome segments of wild rice *O. rufipogon* accession IRGC 105491 (RUF) and *O. meridionalis* accession OR 44(MER) in the genetic background of Curinga, the elite tropical *japonica* upland cultivar from Brazil, were studied.

O. rufipogon and *O meridionalis* possess AA genomes and are cross-compatible, and they are estimated to have diverged around two million years ago (Toulotte et al. 2022). Previous studies showed that *O. rufipogon*, originating in Southern China and presently found throughout Asia, is more genetically diverse than *O. sativa* (Toulotte et al. 2022). *O. meridionalis* is native to Australia, is more drought-tolerant, and is better adapted to arid climates than *O. sativa*. Wild species such as *O. rufipogon* Griff and *O. glaberrima* Steud were extensively used for their improved tolerance to drought stress (Chen et al. 2023).

The recurrent parent Curinga is a commercial rice variety developed by Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA, Goiania, Brazil), tolerant to drought released in 2005 (de-Morais et al. 2005). It is resistant to rice blast leaf scald and tolerant to acidic soils. Nine NERICA lines were derived by crossing *O. glaberrima (*African rice*)* and IR64. *O. glaberrima* is grown in West Africa. They are resistant to African gall midge, nematodes, rice yellow mottle virus (RYMV), DS, acidity, iron toxicity, and have strong weed competitiveness. Understanding the root system structure can promote a second green revolution focusing on crop performance under nutrient and water constraints (Anilkumar et al. 2023).

Since root traits are incredibly crucial, the function of the root under drought stress adaptation creates a hydraulic environment that permits the optimal use of water by plants to maximize the water use efficiency (WUE) under critical conditions (Abdalla et al. 2022; Fonta et al. 2022)

Genetic markers for different QTLs governing root traits relevant to drought tolerance are valuable resources to identify the lines with important QTLs. CSSLs help uncover many desired genes/alleles from wild rice species. Hence, in this study, 80 CSSLs derived from two crosses (Curinga and *O.rufipogon*-IRGC105491; Curinga and *O. meridionalis* OR44) and nine NERICA lines have been selected to characterize their root traits and identify lines with drought tolerance chosen relevant root QTLs. This study aims to identify suitable lines for drought tolerance with desired alleles and phenotypic characteristics. In the current investigation, a set of CSSLs and NERICA lines were phenotyped for root architectural traits, and an attempt was made to identify the lines with desirable root QTLs.

2 Material and Methods

2.1 Plant materials

A set of 96 genotypes, including 80 CSSLs, 9 NERICA lines, and 4 drought-tolerant genotypes collected from the Indian Council of Agricultural Research, National Rice Research Institute (ICAR-NRRI), Cuttack, Odisha, India, and three international control lines were used for this study. The 80 CSSLs included 48 developed lines from the cross between Curinga and *O. rufipogon* IRGC105491 (RUF), and 32 derived lines resulted from the cross between Curinga and *O. meridionalis*-OR44 (MER) (McCouch et al. 2007). Professor Susan McCouch developed these lines at Cornell University, USA. The nine NERICA lines were obtained by crosses between *indica* elite cultivar, IR64 and African rice *O. glaberrima* in the same lab. Four drought-tolerant genotypes, namely Mahulata (a drought-tolerant landrace), Satyabhama (newly released varieties for drought-prone upland areas), CR 143-2-2, and CR 2702, were used as local control lines in this study. The international controls included IR 64 (drought sensitive), Azucena (drought-tolerant) and Curinga (the recurrent parent). The CSSLs and NERICA lines were obtained through a collaborative project on sustainable crop production for international development (SCPRID).

2.2 Phenotyping of root traits for drought stress tolerance using the basket method

Here, we conducted two experiments utilizing the Basket and PVC pipe methods for the root phenotyping of 96 genotypes. The Basket and PVC pipe method experiments, were performed under field conditions and in rainout shelters, respectively. The Basket method approach evaluated the deep-rooted traits from previous research studies with few modifications (Subudhi et al. 2015). This field study was conducted following a complete randomized design (CRD) using two replications. In each replication, six baskets were installed per genotype. The baskets had a 2 mm mesh size and measured 8 cm in height, with top and bottom diameters of 18 cm and 9 cm, respectively. The volume of soil contained in the basket was 5100 cm^3. In each basket, four to five seeds were sown at the center of the basket. Seven days after germination, one plant was retained per basket after thinning. The baskets were buried in the field at 10 cm depth with a 20 cm gap between adjacent baskets and containing soil and sand in a 2:1 (vol:vol) ratio. In the experimental field, 10 cm depth was specified to maintain the ground level and the plants were irrigated daily. The recommended amounts of $N:P_2O_5:K_2O$ @ 80:60:60 kg ha^{-1} were followed when applying urea, DAP, and MOP fertilizers, respectively. Weeds were removed from the field manually as per requirement. During the entire crop growth season, essential plant protection techniques were implemented. Surface irrigation was provided until the completion of root harvest utilizing a jet pipe. The baskets were then soaked in a water tank for 24 hours. The next day, the roots were thoroughly washed with a fine water jet, and the number of roots emerging from the mesh of the baskets was counted. The roots protruding from the basket's sides and bottom were considered deep roots (DR) and shallow roots (SR), respectively. The entire root system present inside and outside of the basket was scanned. Three plants for a single genotype were sampled from each replication for trait evaluation. Seven lines out of 96 genotypes (RUF-35, MER-2, MER-3, MER-5, MER-8, MER-10, and MER-11) showed inferior growth under the basket and were excluded from further root phenotypic evaluation and genotypic study. The following formulae were used for calculating the total roots and ratio of deep rooting (Oyanagi et al. 1993)

$$\text{Total roots (TR)} = \text{DR+SR},$$

$$\text{Ratio of deep rooting (RDR)} = \text{DR/TR}$$

The images of the roots were captured at 400 DPI resolution using an EPSON professional scanner (Magalhães et al. 2011) and analyzed with Win Rhizo Pro 2007a (Regent Instrument Inc., Quebec, Canada) root analysis software.

2.3 Root phenotyping using Polyvinyl chloride (PVC)pipe method

Eighty-nine genotypes were evaluated for root traits in PVC pipes under two different water regimes: well-watered (WW) and drought stress (DS) in two replications for each treatment. Each pipe was filled with 10 kg of sandy clay loam soil collected from the institute field, having moderate acidic pH (4.5-5.5) and a medium organic carbon content (0.50-0.75%). The pipes were

placed in cement pits at a depth of one meter to avoid an increase in temperature due to the direct incidence of solar radiation sunlight on the surface of the pipes. The pipes were one meter long and twenty centimetres in diameter. Before sowing, the pipes were watered daily for 4-5 days till uniform compaction was attained. In each pipe, four to five seeds were sown and 7 days after germination, a single plant was retained after thinning. Thirty-day-old seedlings were exposed to DS through a 20-day stress period (20 DSP) of water restriction. Soil Moisture Content (SMC) was estimated gravimetrically at a soil depth of 50 cm. Since the interval soil sampling might cause root damage, soil sampling was done at the end of the DS. The root and shoot samplings were done at 20 DSP when the tips of the leaf started drying and were completely rolled in the sensitive variety. Under the DS condition, the SMC at a depth of 50 cm was reported as 12%. The pipes were carefully removed and immersed in a water tank for 24 hours for sampling. The roots were then cleaned, following which different root and shoot traits were measured, similar to the prior study (Toorchi et al. 2002). Different traits measured were maximum root length (MRL), shoot length (SL), root volume (RV), root dry wt. (RDW), shoot dry wt. (SDW), total plant dry wt. (TDW), root-to-shoot dry weight ratio (RDW/SDW), root-to-total plant dry weight ratio (RDW/TDW), maximum root-to-shoot length ratio (MRL/SL), specific root length (SRL), the root growth rate in depth (RGRD), the root growth rate in volume (RGRV), root length density (RLD) and the ratio of deep rooting (RDR). Four plants for a single genotype were sampled from each replication under both growth conditions for trait evaluation. Plasticity for each root trait was calculated similarly to understand the previous study (Sandhu et al. 2016).

2.4 DNA isolation followed by PCR amplification and marker visualization linked to root traits

Leaves were sampled from fifteen-day-old seedlings for genomic DNA extraction and molecular screening. The leaves were crushed in liquid nitrogen, and then the total genomic DNA was extracted using the Qiagen kit for DNA extraction (Qiagen, Germany). The extracted DNA was quantified using NanoDrop (Thermo Scientific, 1000-spectrophotometer). The DNA amplification was performed in a 10µl reaction volume containing 0.2mM of dNTP mix, 0.4 picomoles of forward and reverse primers, 30ng of genomic DNA, and one Taq polymerase unit (Mohanty et al. 2019). Amplification was done in a programmable thermal cycler (Eppendorf, USA) with pre-denaturation for 5 minutes at 94^0C, 35 cycles of denaturation for 30s at 94^0C, annealing for 1 minute at 56^0C, extension for 1 minute at 72^0C, and a final extension for 7 minutes at 72^0C. Polymerase Chain Reaction (PCR) products were stored at a temperature of 4^0C. Thirty previously reported root trait-linked SSR markers were used for PCR amplification, 17 of which were found to be polymorphic. The PCR amplification products were mixed with 3 µl of loading buffer, and from this mixture, 10µl was loaded in a 2.5% agarose gel containing 0.5µg/ml of ethidium bromide. Electrophoresis was performed in 0.5X TBE buffer (pH 8.0) at 80 volts (2.5V/cm) for four hrs and photographed using a Gel-Doc System (SynGene, UK). The amplicon size was determined using a DNA ladder of 100 bp

2.5 Genetic Diversity

For each genotype, scoring was performed on the database in the presence or absence of alleles obtained with the primers. A similarity matrix was constructed using Jacquard's coefficients. The Power Marker Ver3.25 software was used to estimate the number of alleles, gene diversity, heterozygosis, allele frequency, and polymorphic information index (PIC) (Lu et al. 2005). Using the software TASSEL 5.0, a general linear model (GLM) and mixed linear model (MLM) were used to investigate the association between SSR markers and root traits.

2.6 Statistical Analysis

The phenotypic and physiological data were calculated using Microsoft Excel. Descriptive analyses, including mean, analysis of variance (ANOVA) and standard deviation (SD) estimates, were calculated on the tested traits over two moisture regimes using CROP STAT ver 7.2. For allele scoring, data were scored according to the presence or absence of the amplified products for each genotype-primer combination. Discrete variables were entered into a binary data matrix. The Power Marker Ver3.25 program determined allele frequency, the number of alleles, gene diversity, heterozygosis, and polymorphic information index (PIC) (Lu et al. 2005). The hypothesis that the SSR markers are associated with root traits was tested by the generalized linear model (GLM) and mixed linear model (MLM) of the TASSEL 5 program (Bradbury et al. 2007).

3 Result

3.1 Phenotyping of root traits using the Basket method

A wide range of variation among the tested lines was observed for all the 14 different root and shoot traits measured after 40 days post-germination under the WW condition. The RDR of 89 genotypes ranged from 30.86% to 87.31%. Three lines, namely, RUF-16, RUF-10, and RUF-44, had a higher RDR and values in other root traits. It was seen that RUF-6 had a shallow rooting (RDR=30.86%), whereas RUF-16 had a relatively deeper rooting (RDR=87.31%). RDR of RUF-16 was significantly higher (p<0.01) than RUF-6 (Figure 1). The highest RLD value of 1.60cm cm^{-3} was recorded from Mahulata, followed by the local tolerant control CR 143-2-2 (1.57 cm cm^{-3}) and RUF-2 (1.51cm cm^{-3}) (Table 1). Among the tested lines, Mahulata, RUF-2, RUF-10, RUF-37, RUF-39, MER-18, MER-23, Azucena and CR 143-2-2 had higher values for multiple root traits under WW condition.

Figure 1a **Figure 1b**

Figure 1 Rooting pattern of contrasting CSSLs showing deep and shallow rooting pattern under well watered (WW) conditions using basket method (a) Deep rooting of RUF-16 (b) shallow rooting of RUF-6

Table 1 Mean (M), range and standard error (SE) of all the measured root traits of 89 genotypes under the basket method

Traits	M± SE	Min.	Max	Range	Best three Lines (values)
Ratio of deep rooting (RDR %)	63.84±0.013	30.86	87.31	56.45	RUF-16 (87.31), RUF-10(84.69), RUF-44(84.52)
Root length density(RLD, cm cm^{-3})	0.72± 0.034	0.16	1.60	1.44	MAHULATA (1.60), CR 143-2-2 (1.57), RUF-2 (1.51)
Maximum root length (MRL, cm)	28.15±0.730	14.15	43.85	29.70	MER-23(43.85),MER-18 (43.70), NERICA-5 (43.50)
Shoot length (SL, cm)	50.26±0.846	29.80	68.50	38.70	RUF-2(68.50), RUF-6 (66.85), RUF-47(65.15)
Root volume (RV, cc)	5.04±0.252	0.60	11.64	11.04	CR 143-2-2(11.64), RUF-2(10.38), MAHULATA (9.48)
Root dry weight (RDW, g)	0.37±0.016	0.13	0.85	0.61	MAHULATA (0.85), AZUCENA (0.74), CR 143-2-2 (0.68)
Root average diameter (RAD, mm)	0.49±0.019	0.20	0.89	0.69	RUF-10 (0.89), NERICA-3 (0.88), CR 143-2-2 (0.85)
Shoot dry weight(SDW, g)	0.94±0.043	0.11	1.80	1.69	CR 143-2-2 (1.80), RUF-10(1.75), RUF-2 (1.64)
Total dry weight (TDW, g)	1.30±0.055	0.31	2.49	2.18	CR 143-2-2 (2.49), RUF-10 (2.39), AZUCENA (2.17)
RDW/SDW (g)	0.44±0.025	0.17	1.85	1.68	RUF-37(1.85), MER-27(1.43), RUF-39(1.19)
RDW/TDW (g)	0.29±0.008	0.14	0.65	0.51	RUF-37(0.65),RUF-39 (0.54), MER-13(0.47)
MRL/SL (cm)	0.57±0.017	0.29	1.11	0.82	RUF-28 (1.11), RUF-31(1.05), MER-23(1.00)
Specific root length (SRL, cm/g)	87.43±2.788	31.73	136.59	104.86	RUF-14(136.59), RUF-4(135.39), RUF-38(134.10)
Total root length (TRL, cm)	3651.65±173.60	804.42	8122.29	7154.41	MAHULATA (8122.29), CR 143-2-2 (7958.83), MER-18 (7764.20)

Table 2 Mean (M), range and standard error (SE) of all the measured root traits of 89 genotypes under the PVC pipe method

Traits	Treatments	Mean± SE	Min.	Max	Range	Best 3 lines
MRL (cm)	WW	78.07±1.597	34.05	100.35	66.30	RUF-16(100.35), MER-26(100.20), MER-32(98.75)
	DS	59.61±1.638	24.05	90.90	66.85	RUF-16(90.90), NERICA-3(88.95), RUF-10(87.30)
SL (cm)	WW	71.65±0.572	54.65	86.05	31.40	RUF-42(86.05), NERICA-1(81.40), RUF-46(81.25).
	DS	58.52±0.644	41.60	72.90	31.30	NERICA-1 (72.90), MER-16(71.20), CR 143-2-2(68.00)
RV (cc)	WW	39.91±2.528	6.00	127.00	121.00	MER-16(127.00), RUF-44(91.50), MER-28 (89.00).
	DS	19.34±1.538	4.00	78.50	74.50	RUF-44(78.50), NERICA-1(66.50), NERICA-3(66.50).
RDW (g)	WW	3.40±0.198	0.99	14.34	13.35	MER-16(14.34), RUF-4(8.86), RUF-24(8.06).
	DS	1.73±0.084	0.45	4.82	4.37	MER-16 (4.83), RUF- 44(3.76), NERICA-4(3.38).
SDW (g)	WW	4.25±0.213	1.30	13.91	12.62	NERICA-4(13.91), NERICA-1(9.20), RUF-4(8.88).
	DS	1.88±0.118	0.37	5.98	5.61	NERICA-1(5.98), NERICA-4(5.76), RUF-23(4.98).
RDW/SDW (g)	WW	0.86±0.051	0.21	3.27	3.06	RUF-28(3.27), RUF-24(2.76), MER-16(2.71).
	DS	1.10±0.068	0.22	3.63	3.41	RUF-28(3.63), MER-20(3.39), MER-16(3.18).
TDW (g)	WW	7.68±0.346	2.73	19.63	16.90	MER-16(19.63), NERICA-4(18.35), RUF-4(17.74).
	DS	3.65±0.177	0.83	9.20	8.37	NERICA-1(9.20), NERICA-4(9.14), RUF-44(7.87).
RDW/TDW (g)	WW	0.43±0.012	0.17	0.76	0.59	RUF-28(0.76), RUF-24(0.73), MER-16(0.73).
	DS	0.49±0.012	0.18	0.78	0.60	RUF-28(0.78), MER-20(0.77), MER-16(0.76).
MRL/SL (cm)	WW	1.10±0.022	0.45	1.47	1.02	RUF-4(1.47), RUF-10(1.44), MER-32(1.44).
	DS	1.04±0.028	0.36	1.67	1.31	RUF-10(1.67), RUF-4(1.60), MER-32(1.47).
SRL (cm/g)	WW	27.28±1.153	6.39	67.80	61.41	RUF-21(67.80), RUF-47(65.85), MER-6(49.07).
	DS	39.52±1.732	17.91	96.00	78.09	RUF-18(96.00), RUF-47(88.67), MER-19(84.04).
RGRD (cm/day)	WW	1.90±0.037	0.83	2.45	1.62	RUF-16(2.45), MER-26(2.44), MER-32(2.41).
	DS	1.45±0.039	0.59	2.22	1.63	RUF-16(2.22), NERICA-3(2.17), RUF-10(2.13).
RGRV (cc/day)	WW	0.95±0.059	0.15	3.10	2.95	MER-16(3.10), RUF-44(2.23), MER-28(2.17).
	DS	0.46±0.035	0.10	1.91	1.81	RUF-44(1.91), NERICA-1(1.62), NERICA-3(1.62).

*WW-well watered, DS-drought stress, Max-maximum, Min-minimum

3.2 Phenotyping of root traits using PVC pipe method

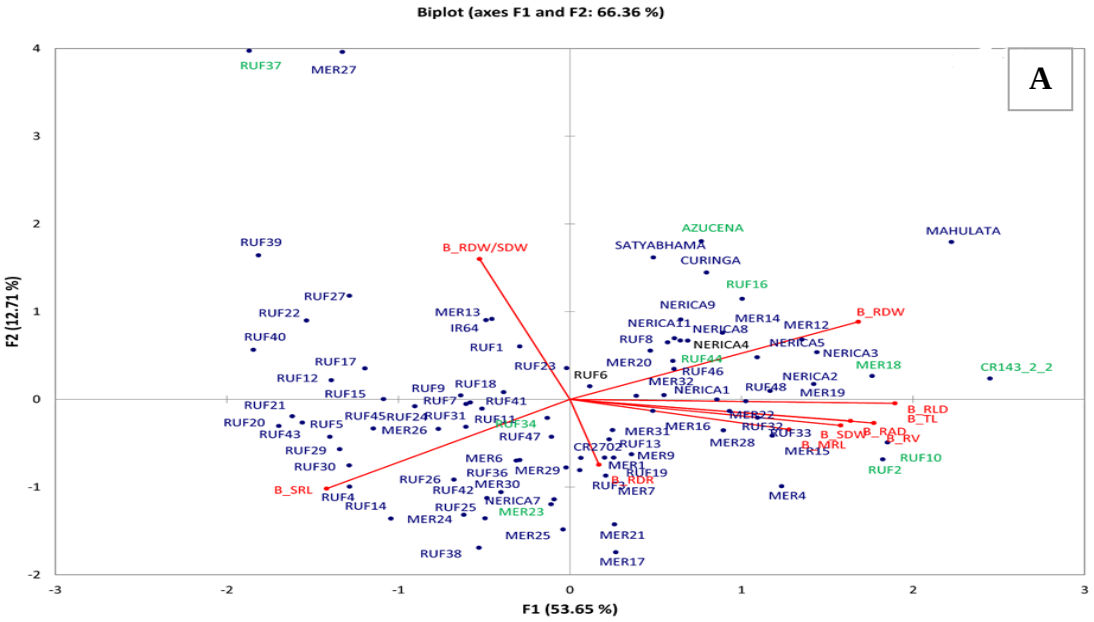
Significant differences ($p<0.05$) were found among the lines for the 12 different root traits and the two treatment conditions, i.e., WW and DS. While comparing the WW to the DS condition, the average values of RV, MRL, RDW, SL, SDW, TDW, MRL/SL, RGRD and RGRV decreased by 51.53%, 23.07%, 47.89%, 18.60%, 56.04%, 52.42%, 5.20%, 23.07% and 51.53%, respectively. However, the average values of SRL, RDW/TDW and RDW/SDW increased by 31.07%, 11.10% and 22.58%, respectively, under the WW condition compared to the DS condition. These results showed that biomass partitioning was more towards the root under DS with a high root length per unit root biomass. Roots grew vertically and horizontally under DS, enabling the plants to access water from larger soil volumes. It was observed that for multiple traits, the lines RUF-10, RUF-16, RUF-28, RUF-44, MER-16, MER-20, NERICA-3, NERICA-1, and NERICA-4 had higher values under the DS condition than the WW condition using the PVC pipe method (Table 2).

The percentage of reduction in SDW, RDW and increment in RDW/SDW were calculated for 89 tested genotypes, of which 10 lines, namely RUF-6, RUF-43, RUF-48, MER-32, RUF-44, RUF-8, RUF-27, RUF-33, RUF-16, and RUF-10 showed less than 30% reduction in SDW and RDW under DS compared to WW. However, 16 lines had a low reduction in RDW and a high reduction in SDW, 20 had a moderate reduction in both SDW and RDW, and 43 had a high reduction in SDW under DS compared to WW. The plasticity of each root trait for the individual lines were estimated by the relative change of a particular root trait under DS

compared with WW condition, and positive plasticity values were obtained for the traits such as RDW/SDW, RDW/TDW, MRL/SL, and SRL, although, most of the root traits showed negative plasticity. The lines RUF-2, RUF-10, RUF-44, and MER-16 showed positive plasticity values for multiple root traits.

3.3 Multivariate analysis and correlation among different root traits

Two different methods, namely the PVC pipe method and the Basket method, were used to construct a genotype vs trait biplot. Here, the varieties for root traits were grown under WW and DS conditions in the PVC pipe method and WW in the Basket method. To reduce noise, the biplot analysis was performed while taking seven traits (RV, MRL, RDW, RDW/SDW, SRL, RGRD and RGRV) from the PVC pipe method and ten traits (RDR, SDW, TL, RV, MRL, RDW, RDW/SDW, SRL, RAD and RLD) from the Basket method. For the PVC pipe method, the plot condensed the information into principal components, out of which the first two components (Figure 2B & C) explained 82.53% and 79.17% of the total variation of the data in WW and DS conditions, respectively. However, for the Basket method, the first two principal components



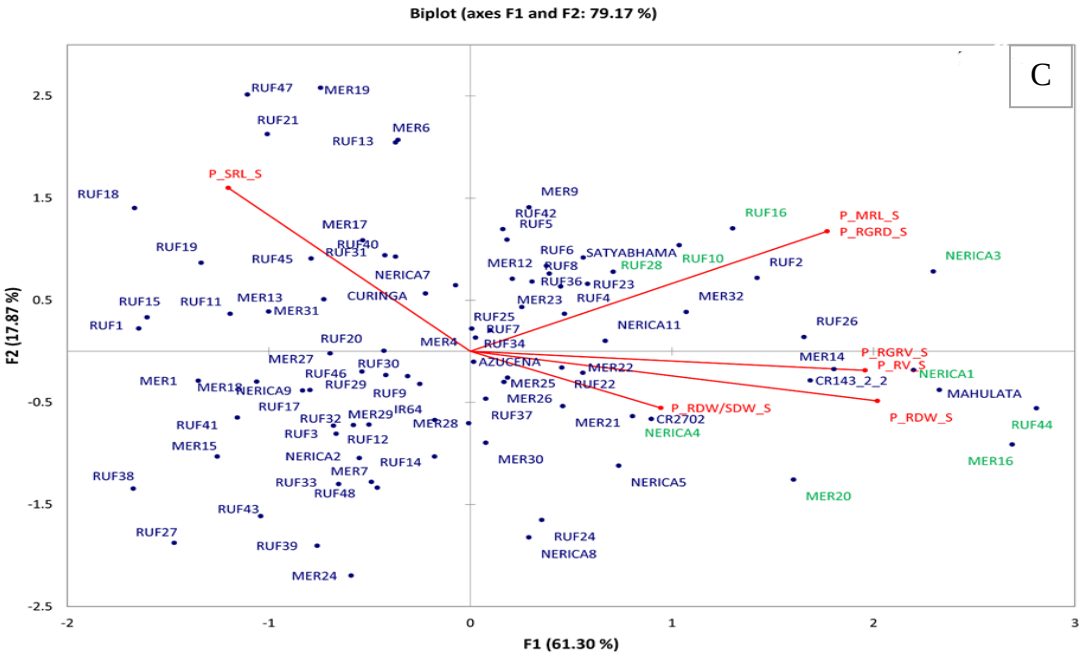


Figure 2 Biplot with two principal components representing the average root traits over two replications for 89 CSSLs under well-watered (WW) condition in basket (a), well-watered (WW) condition (b) and drought stress (DS) condition (c) in PVC pipe method. Variance of each principal component is shown as percentage of the total variance (indicated in axis legend). Black and red colour letters indicate lines and traits, respectively. Mean value was calculated taking two replications for each genotype.

Table 3 Correlation matrix of 12 different root and shoot traits under well-watered (WW) (below) and drought stress (DS) conditions (upper) grown in PVC pipes

	MRL	SL	RV	RDW	SDW	RDW/ SDW	TDW	RDW/ TDW	MRL/ SL	SRL	RGRD	RGRV
MRL		0.125	0.548**	0.666**	0.362**	0.294**	0.576**	0.271**	0.915**	-0.195	1.000**	0.608**
SL	0.112		0.111	0.049	0.198	0.061	0.263*	0.020	-0.271**	-0.150	0.125	0.352**
RV	0.608**	0.352**		0.794**	0.407**	0.277**	0.670**	0.291**	0.438**	-0.484**	0.608**	1.000**
RDW	0.456**	0.260**	0.818**		0.456**	0.456**	0.805**	0.442**	0.539**	-0.714**	0.666**	0.794**
SDW	0.320**	0.086	0.502**	0.409**		-0.462**	0.894**	-0.525**	0.276**	-0.289**	0.362**	0.407**
RDW/ SDW	0.237*	-0.013	0.427**	0.598**	-0.330**		-0.078	0.922**	0.253*	-0.345**	0.294**	0.277**
TDW	0.460**	0.081	0.781**	0.829**	0.849**	0.144		-0.127	0.454**	-0.551**	0.576**	0.670**
RDW/ TDW	0.249*	-0.013	0.421**	0.576**	-0.368**	0.917**	0.108		0.248*	-0.441**	0.271**	0.291**
MRL/SL	0.910**	-0.304**	0.480**	0.424**	0.281**	0.233*	0.417**	0.244*		-0.135	0.915**	0.438**
SRL	-0.144	-0.071	-0.564**	-0.626**	-0.265*	-0.448**	-0.524**	-0.604**	-0.118		-0.195	-0.484**
RGRD	1.000**	0.112	0.548**	0.456**	0.320**	0.237*	0.460**	0.249*	0.910**	-0.144		0.608**
RGRV	0.548**	0.111	1.000**	0.818**	0.502**	0.427**	0.781**	0.421**	0.480**	-0.564**	0.548**	

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

(Figure 2B & C) explained 66.36% of the total variation. In both the treatments, SRL showed a significant negative correlation ($p<0.01$) with all traits except MRL, RGRD in WW and DS conditions in the PVC pipe method and RDW/SDW and RDR in the Basket method.

Among the lines grown in the PVC pipe method under WW and DS conditions, MER-16, RUF-28, NERICA-1, NERICA-3, NERICA-4, RUF-44, and Mahulata had higher values for multiple root traits. Similarly, among the lines grown in the Basket method, MER-4, NERICA-3, RUF-16, RUF-2, RUF-10, MER-18,

Mahulata, Azucena, Satyabhama, Curinga, and CR 143-2-2 recorded higher values for multiple root traits (Figure 2a-c).

Correlations among different root traits for the PVC pipe method under both WW and DS conditions revealed a strong positive correlation among MRL, RV, RDW, SDW, RDW/SDW, TDW, RDW/TDW, MRL/SL, RGRD, RGRV (*p<0.01 or p<0.05 level)*. SRL showed a negative correlation with all the traits but had a significant positive association with RV, RDW, SDW, RDW/SDW, TDW, RDW/TDW, and RGRV under both treatments. SL did not significantly correlate with any of the traits except for a negative correlation with MRL/SL *(p<0.01*) in both the conditions and a positive correlation with TDW *(p<0.05*) and RGRV (*p<0.01*) under the DS condition only (Table 3).

3.4 Marker diversity analysis

Thirty root traits-specific SSR markers were used to genotype the 89 experimental lines, including 73 CSSLs, 9 NERICAs, and 7 tolerant and sensitive controls. Seventeen of the thirty markers were polymorphic, whereas the rest showed no polymorphism among the genotypes. Therefore, these 17 markers were considered for further analysis. The size of the amplicons ranged from 90 to 300 bp (Table 4). A total of 36 alleles were amplified with the 17 SSR markers. The number of alleles per marker varied from 2 to 3, averaging 2.11 per locus.RM219 and RM316 had the highest number of alleles in 89 genotypes. The mean polymorphic information content (PIC) value was 0.2580, with a minimum of 0.0777 (RM201) and a maximum of 0.4954 (RM316). The PIC value indicates the information related to the marker system. The observed heterozygosity (H_o) was zero for all the markers studied. The expected heterozygosis or gene diversity (H_e) ranged from 0.0809 (RM201) to 0.5730 (RM316), with an average of 0.3056. The major allele frequency of these root traits-specific polymorphic markers had an average of 0.7892, ranging from 0.5185 to 0.9577).

3.5 Association of molecular markers with root phenotypic traits

The marker-trait association for all the root traits studied under the Basket and PVC pipe methods was calculated using the general linear model (GLM) and mixed linear model (MLM) of TASSEL5.0 software. Table 5 lists the performance of these models and demonstrates the association between root traits and SSR markers. The squared allele frequency correlation (r^2) values using GLM analysis varied from 0.038 to 0.161, with an average reduced to 0.080, whereas, using MLM analysis, the average was

Table 4 Genetic diversity parameters and details of Simple sequence repeat (SSR) loci used for genotyping of 89 genotypes of rice

S. N.	Marker name	Chromosome number	Min. mol. wt	Max. mol. wt	No of alleles	Major allele frequency	Gene diversity	Heterozygosity	PIC value
1	RM161	1	200	210	2	0.8750	0.2188	0.0000	0.1948
2	RM212	1	180	190	2	0.9213	0.1449	0.0000	0.1344
3	RM1220	1	200	220	2	0.8438	0.2637	0.0000	0.2289
4	RM1282	1	200	210	2	0.7679	0.3565	0.0000	0.2930
5	RM1247	1	200	220	2	0.8679	0.2293	0.0000	0.2030
6	RM525	2	180	190	2	0.9474	0.0997	0.0000	0.0948
7	RM489	3	200	210	2	0.9059	0.1705	0.0000	0.1560
8	RM520	3	110	130	2	0.7978	0.3227	0.0000	0.2706
9	RM567	4	110	130	2	0.8511	0.2535	0.0000	0.2214
10	RM471	4	210	215	2	0.7209	0.4024	0.0000	0.3214
11	RM1054	5	250	300	2	0.6889	0.4286	0.0000	0.3368
12	RM160	9	150	180	2	0.7000	0.4200	0.0000	0.3318
13	RM219	9	180	220	3	0.5976	0.5559	0.0000	0.4910
14	RM7424	9	90	100	2	0.9024	0.1761	0.0000	0.1606
15	RM215	9	190	200	2	0.5185	0.4993	0.0000	0.3747
16	RM316	9	190	210	3	0.5522	0.5730	0.0000	0.4954
17	RM201	9	120	200	2	0.9577	0.0809	0.0000	0.0777
	MEAN					0.7892	0.3056	0.0000	0.2580

Table 5 Association of marker alleles with root phenotypic traits under WW condition in the basket and WW and DS conditions in PVC pipe using the GLM and MLM TASSEL analysis in 89 genotypes

Trait	Condition	Marker name	GLM			MLM		
			F value	*P* value	R^2	F value	*P* value	R^2
RDR	Basket	RM212	5.03624	0.02752	0.04902	1.87802	0.17430	0.02182
		RM489	6.86019	0.01050	0.06541	2.89027	0.09291	0.03359
		RM7424	5.06905	0.02703	0.04933	1.85975	0.17639	0.02161
		RM161	5.10657	0.02649	0.04967	3.16132	0.07911	0.03674
SL	Basket	RM316	5.12609	0.02621	0.05319	2.09271	0.15182	0.02488
		RM1247	5.97914	0.01662	0.06143	5.12266	0.02626	0.06090
		RM1220	5.60082	0.02031	0.05780	3.69655	0.05800	0.04394
		RM525	3.65065	0.05954	0.03853	4.81784	0.03099	0.05727
	Pipe-WW	RM215	5.73004	0.01896	0.05813	4.56076	0.03570	0.05290
	Pipe-DS	RM215	8.59164	0.00437	0.08503	6.00149	0.01642	0.07087
	plasticity	RM215	8.94897	0.00367	0.08803	6.29291	0.01409	0.07432
TL	Basket	RM316	12.12996	7.99E-04	0.08932	11.02210	0.00135	0.09744
MRL	Basket	RM567	7.2526	0.00858	0.06897	4.20459	0.04351	0.04923
		RM1054	5.30390	0.02381	0.05156	3.30835	0.07258	0.03873
	Pipe-WW	RM7424	4.69936	0.03307	0.05105	3.04284	0.08484	0.03588
		RM1247	5.27569	0.02418	0.05693	4.11796	0.04567	0.04856
	Pipe-DS	RM201	5.06364	0.02711	0.05654	4.31065	0.04101	0.04946
RAD	Basket	RM316	5.39851	0.02263	0.05071	4.85640	0.03035	0.05140
RDW/TDW	Basket	RM219	5.73222	0.01894	0.06224	2.22900	0.13928	0.02545
		RM567	11.96688	8.62E-04	0.12131	6.92126	0.01017	0.07903
	Pipe-WW	RM201	16.30910	1.20E-04	0.15277	10.07454	0.00212	0.11937
	Pipe-DS	RM201	17.11390	8.47E-05	0.16169	11.09713	0.00130	0.12954
	plasticity	RM201	16.77223	0.000098322	0.15909	11.04593	0.00133	0.12913
RLD	Basket	RM316	12.11299	8.05E-04	0.08906	11.00674	0.00136	0.09721
RDW	Basket	RM201	6.03276	0.01615	0.05059	3.01442	0.08628	0.03219
	Pipe-WW	RM201	15.39879	1.80E-04	0.14928	12.19985	0.00077	0.14253
	Pipe-DS	RM201	6.41538	0.01322	0.06712	6.13176	0.01533	0.07179
		RM316	6.50533	0.01261	0.06799	5.83887	0.01790	0.06836
RDW/SDW	Basket	RM567	7.06783	0.00943	0.07374	4.36842	0.03971	0.04964
	Pipe-WW	RM489	8.40763	0.00479	0.08912	5.32592	0.02353	0.06340
		RM160	4.58421	0.03524	0.05074	1.48700	0.22618	0.01770
		RM201	15.65058	1.61E-04	0.15359	6.86554	0.01047	0.08173
	Pipe-DS	RM489	5.70485	0.01922	0.06244	4.28527	0.04159	0.05117
		RM201	13.88286	3.56E-04	0.13899	9.97205	0.00222	0.11907

Trait	Condition	Marker name	GLM			MLM		
			F value	*P* value	R^2	F value	*P* value	R^2
TDW	Basket	RM1054	-	-	-	5.09776	0.02661	0.05638
	Pipe-WW	RM201	6.04963	0.01601	0.06663	5.70269	0.01924	0.06663
	Pipe-DS	RM316	6.53788	0.01240	0.06845	5.63716	0.01992	0.06723
MRL/SL	Basket	RM567	5.07825	0.02690	0.05290	2.91232	0.09169	0.03373
		RM1054	9.14598	0.00333	0.09102	3.04926	0.08452	0.03532
	Pipe-WW	RM7424	5.97067	0.01669	0.06000	3.54960	0.06310	0.03642
	Pipe-DS	RM7424	5.56146	0.02074	0.06074	2.75364	0.10085	0.03014
RGRD	Pipe-WW	RM7424	4.67313	0.03355	0.05076	3.02253	0.08587	0.03564
		RM1247	5.22311	0.02487	0.05638	4.07780	0.04672	0.04809
	Pipe-DS	RM201	5.02941	0.02762	0.05617	4.28095	0.04169	0.04910
SDW	Basket	RM567	5.49244	0.02152	0.05378	1.05683	0.30696	0.01188
		RM1054	-	-	-	5.98944	0.01653	0.06732
	Pipe-WW	RM219	4.36835	0.03971	0.04946	4.19798	0.04367	0.04946
	Pipe-DS	RM219	5.61449	0.02016	0.06000	5.36674	0.02302	0.06335
		RM201	6.85431	0.01053	0.07223	7.02003	0.00967	0.08286
RV	Basket	RM316	8.55171	0.00446	0.07720	7.31849	0.00830	0.07474
	Pipe-WW	RM160	0.07432	0.07432	0.07432	4.06693	0.04700	0.04725
		RM201	8.04847	0.00574	0.08649	6.43963	0.01305	0.07482
	Pipe-DS	RM316	15.85299	1.47E-04	0.14516	12.55963	0.00065	0.14161
	plasticity	RM316	15.88086	0.00014	0.14541	12.57342	0.00064	0.14167
RGRV	Pipe-WW	RM160	4.67210	0.03357	0.05216	4.09206	0.04634	0.04754
		RM201	8.08479	0.00563	0.08685	6.46382	0.01289	0.07510
	Pipe-DS	RM316	15.92222	0.00014	0.14569	12.61219	0.00064	0.1423
	plasticity	RM316	15.88086	0.00014	0.1454	12.5734	0.00064	0.1416

WW - well-watered condition, DS - drought stress; GLM - general linear model and MLM - mixed linear model

reduced to 0.064. Among the different comparisons between root traits and markers, 32 were significant at $p < 0.05$ and 22 were significant at p <0.01 while using the GLM analysis. Using the MLM analysis, 28 comparisons were significant at $p < 0.05$, and 13 comparisons were significant at $p < 0.01$.

GLM and MLM analysis were utilized to identify the specific robust markers for various root traits. RM1247, RM567 and RM201 were associated with MRL. RM489 and RM567 with RDW/SDW; RM316 and RM201 with RDW; RM316 with RAD; RM201 and RM160 with RV had higher F-values and lower *p*-values, indicating strong marker-trait association. Plasticity in SL and RDW/TDW were associated with RM215 and RM201, respectively, while plasticity in RV and RGRV were associated with RM316.

Figure 3 shows the Q-Q plot for the smallest log (P-values) using TASSEL 5.0. As shown in the Q-Q plots for root traits in each condition and averaged over all conditions (Figure 3.), the data points observed above the diagonal represent its significance level, and the data points that fell under the diagonal may indicate some overfitting of the model for the traits.

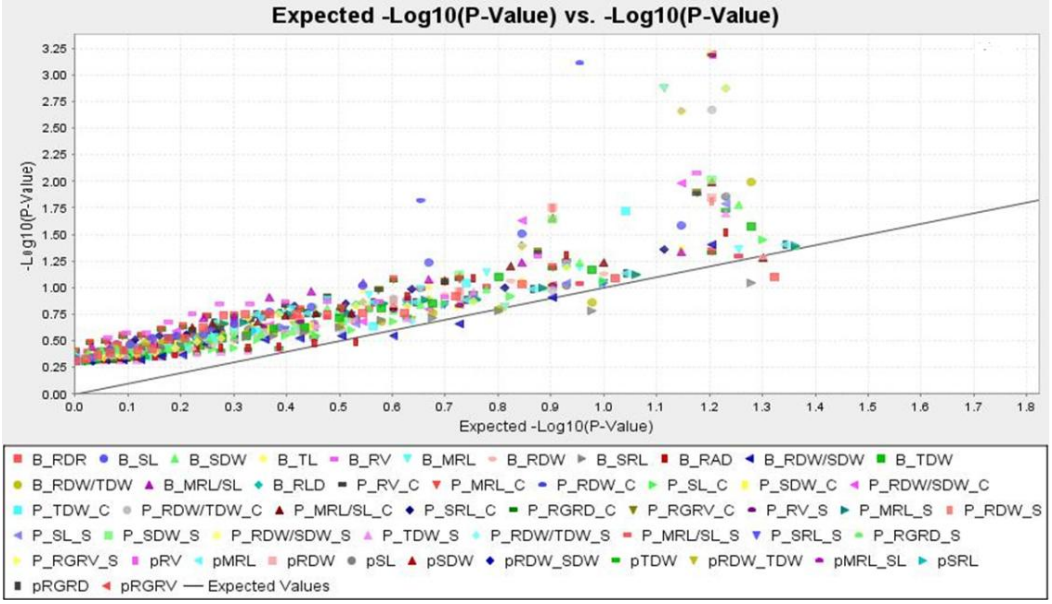


Figure 3 Quantile-Quantile (Q-Q) plot and distribution of marker-trait association from mixed linear model (MLM) analysis; B: Basket experiment under well-watered condition; PC: Pipe experiment under well-watered condition; PS: Pipe experiment under drought stress condition; *p:* plasticity

4 Discussion

The rooting pattern was studied in the Basket and PVC pipe methods in the present experiment. The reduction in SDW was more significant than that in RDW, which was shown by an increase in RDW/SDW (Voetberg and Sharp 1991). Dry matter allocation favoured root growth more than shoot growth (Matsui and Singh 2003). This trend also agreed with our result, showing a higher rate of increase in RDW/SDW. The significance ($p<0.01$) of the correlation between RDW/SDW and RDW suggested that the lines with the highest ratio tend to maintain a higher RDW (Toorchi et al. 2002). Measurement of phenotypic plasticity of root traits revealed that most lines had positive values for RDW/SDW, RDW/TDW, SRL, and MRL/SL. Previous reports showed that genotypes with plastic root traits were better adapted to stress environments (Kadam et al. 2017). RUF-2, RUF-44, RUF-10, and MER-16 had a greater plasticity for multiple root traits and could suggest a better adaptability under DS. From the biplot analysis, these lines outperformed the other lines in all three traits. Improved plant adaptability is generally evaluated by higher production of its shoot biomass under DS. Among the CSSLs, 10 lines had a <30% reduction in SDW and RDW under DS. These lines can be considered drought-tolerant due to their ability to maintain higher shoot biomass under DS since the shoot growth and overall yield production correlate to the morpho-physiological traits of roots (Ghosh and Xu 2014). From the rooting pattern of 89 genotypes studied in the Basket and PVC pipe methods, we observed that some lines with higher root plasticity did not contribute to higher shoot dry matter production under DS as observed in RUF2, RUF26, RUF28, MER16, MER9, MER14, and NERICA3. This might be due to the unfavourable linkages of undesirable traits like tallness, low yield under irrigated conditions, and the traits of interest reported in previous studies (Vikram et al. 2015). However, there is a possibility of breaking the linkage through breeding to develop drought-tolerant high-yielding cultivars (Swamy et al. 2013). Positive plasticity values indicate the trait's increased growth under stress conditions. RUF10, RFU16, and RFU44 had a lower reduction in SDW and RDW with more remarkable root plasticity under DS, which might result in a consistent performance in unpredictable environmental conditions across all seasons. RUF6 despite having a shallow rooting, had the lowest reduction in SDW and RDW under DS. The Basket method looked at the angle, and the PVC pipe method looked at the root growth at a deeper level. Both of these traits were under separate genetic control, evident from the previously studied *DRO*1 lines, which inherited steeper angles but not increased elongation at a deeper level compared to the parent Kinandang Patong (Uga et al. 2013; Singh et al. 2021).

The earlier study indicated that plants adapting to a relatively drier environment have deeper rooting to avoid DS in upland ecology than those growing in WW conditions (Uga et al. 2011; Feng et al. 2012). The TRL and RLD were also determining factors of drought tolerance in rice (Kadam et al. 2015). To meet the moisture demand in plants, RLDs ranging from 0.5 to 1 cm^{-3} were usually considered adequate (de Willigen et al. 2000). In the present study, Mahulata and CR 143-2-2 were recorded with RLD >1.0 cm^{-3}. Besides having a greater RLD and TRL, Mahulata and CR 143-2-2 were drought tolerant, possibly due to the conservative strategies imposed at the stomatal level. An alternative conservation strategy adopted by

RUF10 with more extensive root diameter and lesser RLD allows for better root penetration and exploration of less soil volume, confirmed by the previous findings (Wasson et al. 2012). An effective method to dissect the genetic basis underlying complex traits in plants is association analysis (Vallarino et al. 2023). Root trait-marker association and linkage mapping analyses have previously been reported to determine the genetic basis of certain rice traits (Henry 2013). A wide genetic diversity in root traits was identified in the present study of 89 CSSLs.

In our study, 17 root-linked SSR markers produced 36 alleles with an average gene diversity of 0.3056 and a PIC value of 0.2580. RM201, RM316, RM7424 and RM1054 were associated with 8, 7, 4 and 4 numbers of root traits, respectively. RM201 had the highest association with 8 root traits viz., RDW, TDW, RDW/SDW, SDW, RV, MRL, RDW/TDW, and RGRV. Among these, RDW has the maximum association among them. The RM201 is also associated with the QTLs for basal root thickness, resulting in larger basal root diameter and favouring better soil penetration ability, as suggested by previous findings (Meena et al. 2019). A larger root diameter may enhance the root tissue density and lower SRL, increasing the root biomass (Sandhu et al. 2016). As per the earlier studies of Meena et al. 2019, RM219 and RM316 were associated with the QTL for root number, while RM1054 and RM1247 were associated with MRL. The associated marker alleles can be employed in a molecular breeding program to improve other lines deficient in a particular trait. In the current study, the association of markers with root traits was much more precise, as the lines showed different groups of phenotypes for the root traits. The phenotypic evaluation effectively distinguished the experimental lines into various groups, indicating their heterogeneity towards tolerance to DS. This favoured the presence of linkage disequilibrium and increased the chances of detecting the marker-trait association. In the heterogeneous collection, a similar result showed the potential value of germplasm-detected marker-phenotypic trait association (Zhao et al. 2013; Nachimuthu et al. 2015). The association of markers with most of the traits studied was common between DS and WW conditions of the PVC pipe method, whereas it was uncommon under the WW condition of the Basket method. This difference might be due to the two different plant growth conditions (Basket and PVC pipe) and the duration of the growth period, which was less in the basket (40 days after germination) compared to the PVC pipe method (50 days after germination). Our results from the association of markers RM7424, RM1247, RM201, and RM316 with traits DR, MRL, RL and RV agreed with the previous findings (Uga et al. 2011; Pawar et al. 2012). Also, there is a possibility of the presence of QTLs such as QDrvc9 (root rate volume under control condition) and QMrdc9a (maximum root depth under control), as RM316 is associated with these QTLs (Yue et al. 2006). Similarly, the presence of Dro1 is possible as it is associated with RM7424 (Uga et al. 2015). Therefore, a strongly associated marker, RM201, and the markers indirectly controlling DS tolerance, such as RM7424, RM316 and RM1054, can be used in marker-assisted drought stress tolerance breeding programs.

Molecular marker data and morpho-physiological data exposed to different numerical and taxonomical techniques measure the relationship between the lines. Therefore, combining information from these datasets would be the most effective way to study genetic diversity.

CSSLs are useful in assessing the root responses (Suralta and Yamauchi 2008; Suralta et al. 2010) under transient DS. Studies using the CSSLs have made it possible to evaluate the effect of root growth on shoot dry matter under the DS condition. The finding is less confounding than studies which utilized genetically diverse varieties (Suralta and Yamauchi 2008; Suralta et al. 2010).

Conclusion

Among the 89 genotypes tested, several showed consistent tolerance to DS throughout the study. The drought-tolerant genotypes MER16, MER20, RUF10, RUF16, RUF44, NERICA1, and NERICA3 exhibited superior performance in terms of root traits, specifically in the chromosomal segments on chromosomes 1, 3, 5, 7, and 8, when compared to other genotypes. Certain CSSLs can be further examined to identify the genes responsible for QTL expression. In addition, marker association analysis identified four SSR markers (RM201, RM316, RM7424, and RM1054) that could be used in marker-assisted breeding programs to improve root traits and indirectly enhance DS tolerance. The study highlights the potential of SSR markers in association with multiple root traits for drought tolerance in rice CSSLs. Molecular marker-root trait analysis could be an alternative to linkage mapping for detecting marker-phenotype associations. The findings have significant implications for breeding programs focused on developing new rice lines with high drought tolerance.

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Author contributions

MB and EP have contributed to the research's practical work and prepared the manuscript along with analysis; PS, SKD and MJB have designed the functional problem and supervised the research work, and MB, AP, GKD, and JNM have contributed to the analysis and editing part. All have read the manuscript before publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Conflict of interest

No conflict

Consent to participate

All authors agreed

Consent to publish

All authors agreed to give consent to publishing to the publisher

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Assessing the Migration of BPA and Phthalic Acid from Take-out Food Containers: Implications for Health and Environmental Sustainability in India

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Health risks

ABSTRACT

The research investigates the escalating consumption of take-out food in India and the associated health risks stemming from the extensive use of plastic packaging. Through a comprehensive nationwide online survey, the study delved into dietary preferences, frequency of take-out food consumption, delivery service timing, and the types of packaging commonly encountered by Indian consumers. To address these concerns, the research team developed an analytical method to detect Bisphenol A (BPA) and Phthalic acid migration from food-contact materials (FCMs) into various food simulants. The investigation revealed that prolonged exposure to elevated temperatures led to increased migration of BPA and Phthalic acid, particularly in polyethylene pouches using 3% acetic acid as a food simulant, with the highest concentrations observed after 45 minutes of exposure. Additionally, a microbial bioassay demonstrated the mutagenic potential of migrated plasticizers, showcasing significant effects in mammalian systems, particularly under metabolic activation. The study underscores the substantial health risks associated with plastic packaging in take-out food, emphasizing potential implications for consumer health and calling for more extensive research and considerations regarding food packaging materials.

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1 Introduction

Fast food refers to food and drinks sold immediately after purchase, either at the food outlet or elsewhere. Take-out food consumption has increased significantly due to the popularity of mobile ordering and delivery apps. The online food delivery sector in the United States generated up to USD 17.5 billion in revenue in 2018, while in China, it generated USD 34.7 billion (Han et al. 2021). India's restaurant industry gross merchandise value reached USD 2.7 billion in 2019 from USD 300 million in 2016 (Samuel Anbu Selvan and Andrew 2021). The take-out food business is expanding quickly due to the affordability and convenience of choosing food from the comfort of home, and the age group from 15 to 34 accounts for the majority of online orders (Gallego-Schmid et al. 2019). This trend has been observed globally, and the convenience and affordability of take-out food have led to its increased popularity among consumers.

The COVID-19 pandemic has significantly impacted the food industry, with food services restricted to take-out to comply with public health and safety regulations and using personal reusable items prohibited (Molloy et al. 2022). As a result, online meal delivery services have become increasingly popular, as individuals were compelled to stay indoors to curb the spread of the virus. The pandemic has highlighted the need for contactless and hygienic food delivery options, leading to a surge in online food delivery services.

The choice of materials for take-out food containers is an essential environmental consideration in the food industry. The production and use of single-use takeaway containers, made from plastic materials and polymers like styrofoam and polyethylene, have significant environmental impacts. Plastic is the most commonly used material in take-out food containers, with polypropylene accounting for more than 60% of plastic containers. Styrofoam comprises 10 to 40% of take-out containers, while paper makes up more than 20% of the containers (Han et al. 2021). Single-use polypropylene containers have the lowest potential for recycling, contribute significantly to environmental pollution, and result in global warming. Chemical additives such as plasticizers, stabilizers, and flame retardants are commonly used in plastic and paper packaging to achieve the desired product qualities (Struzina et al. 2022). Recycled plastics and paper used to make take-out containers to reduce costs may contain harmful substances such as phthalates, plasticizers, and photoinitiators (Blanco-Zubiaguirre et al. 2021). Therefore, there is a need for more sustainable and environmentally friendly takeaway container options to minimize the adverse impact of single-use takeaway containers on the environment and human health.

Using synthetic plasticizers like bisphenol A (BPA) and phthalic acid esters (PAEs) in food and beverage packaging leads to human exposure when these plasticizers migrate from containers into the contents. Figure 1 illustrates the circumstances that result in the release of BPA and PAEs from plastic containers into foods and the potential impacts on human health. BPA is an endocrine disruptor that has a weak binding affinity for estrogen receptors (Hafezi and Abdel-Rahman 2019), while PAEs are plasticizers used for improving plasticity, flexibility, softness, and elasticity (Caldeirão et al. 2021). BPA and PAEs have been detected in food samples and are considered endocrine-disrupting chemicals with anti-androgenic properties. Even though BPA is mainly used in polypropylene and polycarbonate-based products, the occurrence of BPA and other bisphenol analogs (BPs) in polystyrene-made plastic products, such

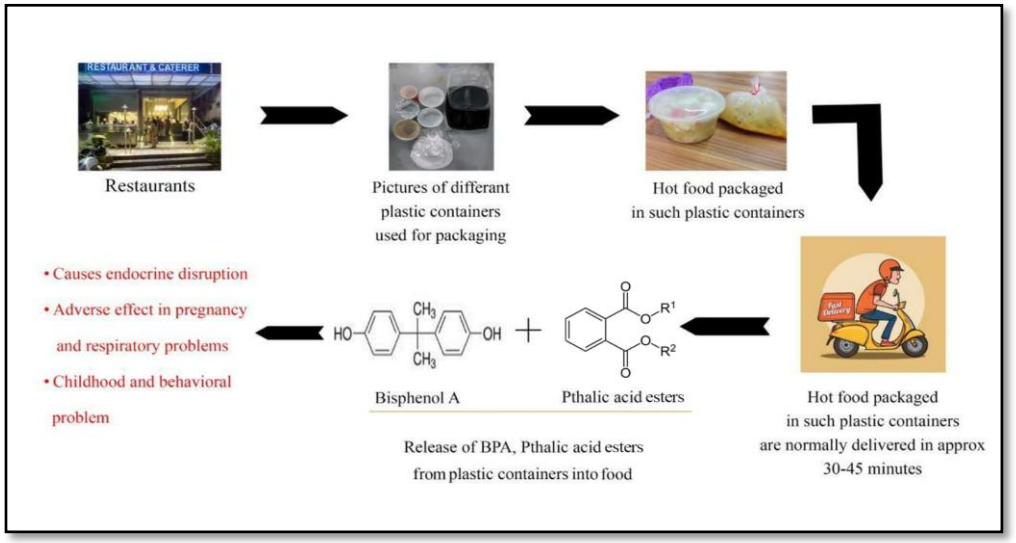


Figure 1 The series of events that result in the release of BPA and PAEs from plastic containers into foods and the potential implications on human health

as styrofoam, has also been reported (Zhao et al. 2023). Further, Wang et al. (2021) found that the migration of PAEs from packaging to food is influenced by the cooking method, with higher amounts of PAEs detected in Chinese foods cooked through methods like pan-frying, deep-frying, stir-frying, steaming, and boiling compared to simpler cooked foods consumed in US and Europe.

There is a need for more research in India on migrating BPA and PAEs from food-contact materials (FCMs) used for packaging take-out foods. Although some studies have been conducted in China regarding the migration of BPA and PAEs, it is essential to note that India, a tropical country with various hot and spicy cuisines, uses different packaging materials. Therefore, it is necessary to investigate how various packaging materials would interact with the hot and spicy foods consumed in India to understand the migration of plasticizers like BPA and PAEs from packaging materials into take-out foods. This study aims to address this research gap by using a questionnaire model to quantify BPA and Phthalic acid migration from FCMs used for packaging take-out foods commonly consumed by the Indian population. The study is the only one in India that quantifies the migration of BPA and Phthalic acid from various FCMs at different time intervals required for delivery. The research adheres to the recommended food simulants by the Bureau of Indian Standards (BIS) for migration detection. Finally, the study evaluates the potential mutagenicity of the leached plasticizers using the Ames test.

2 Materials and methods

2.1 Sampling

A nationwide online survey was conducted to understand the eating habits of people who consume takeaway food. The survey collected information about the Indian population's eating habits and used it to assess health risks to the population. The questionnaire asked general questions about the respondents, such as their gender, age, occupation, and area of residence. The age categories used were based on the USEPA classification of age groups. Participants were then asked about their regular food choices, how often they ate takeaway food, their taste preferences, average delivery times, and the packaging materials of the food. The detailed results of the survey are listed in Table 1. A total of 450 valid questionnaires were collected; most respondents were college students, company staff, workers, and freelancers. We purchased disposable containers online and selected ten containers of each type, dividing them into six main categories: polycarbonate plastics (PC), polypropylene containers (PP), polystyrene trays (PS), polyethylene pouches (PE), aluminum foils, and paper-pulp-based containers. We randomly selected three to four containers from each category for use in the experiment, resulting in 105 containers. The food container material was coded as FCM-X based on its manufacturing material. (Figure 2).

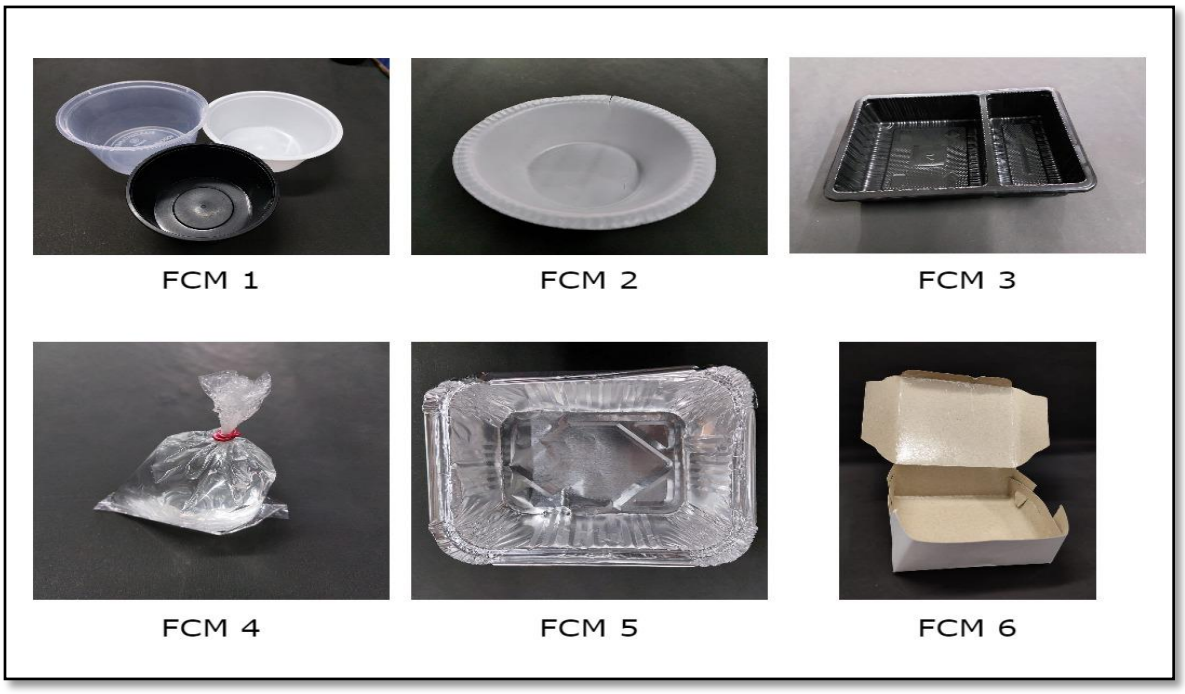


Figure 2 Design and material code FCM -X classifies six distinct food container types. These include FCM 1: rigid, transparent polycarbonate containers (2-5 mm thick), FCM 2: circular white polystyrene containers (0.3-5 mm thick), FCM 3: square polypropylene trays (1-5 mm thick), FCM 4: clear polyethylene pouches (25 and 50 µm thick), FCM 5: rectangular aluminum foil containers (30-50 µm thick), and FCM 6: degradable paper pulp containers with a high-density polyethylene inner lining (0.03 mm thick)

Table 1 The questionnaire details are presented in a table format

Characteristic	Categories	Sub total		Frequency of take-out food				Food preference				Delivery time (minutes)			
		n	%	<1/w	1/w	2/w	>2/w	PF	DF	SF	ST	<15	15-30	30-45	>45
Gender	Male	251	54.56	133 (52.98%)	89 (35.45%)	19 (7.57%)	10 (3.98%)	101 (40.23%)	62 (24.70%)	25 (9.97%)	63 (25.09%)	62 (24.70%)	81 (32.27%)	98 (39.04%)	10 (3.98%)
	Female	209	45.43	97 (46.41%)	83 (39.71%)	16 (7.65%)	13 (6.22%)	79 (37.79%)	48 (22.96%)	22 (10.52%)	60 (28.70%)	37 (17.70%)	62 (29.67%)	91 (43.54%)	19 (9.09%)
Age (years)	<17	16	3.47	11 (68.75%)	5 (31.25%)	0 (0.00%)	0 (0.00 %)	1 (6.25 %)	5 (31.25%)	5 (31.25%)	5 (31.25%)	8 (50%)	3 (18.75%)	5 (31.25%)	0 (0.00 %)
	17-22	194	42.17	131 (67.52%)	43 (22.16%)	9 (4.63%)	11 (5.67 %)	77 (39.69%)	48 (24.74%)	17 (8.76%)	52 (26.80%)	31 (15.97%)	79 (40.20%)	67 (34.53%)	17 (8.76%)
	23-28	109	23.69	71 (65.13%)	23 (21.10%)	13 (11.9%)	2 (1.83%)	52 (47.70%)	20 (18.34%)	18 (16.51%)	19 (17.43%)	21 (19.26%)	20 (18.34%)	59 (54.12%)	9 (8.25%)
	28-35	85	18.47	43 (50.58%)	22 (25.88%)	10 (11.76%)	10 (11.76%)	48 (56.47%)	19 (22.35%)	15 (17.64%)	3 (3.52%)	2 (2.35%)	27 (31.76%)	46 (54.11%)	10 (11.76%)
	35-40	11	2.39	5 (45.45%)	5 (45.45%)	1 (9.09%)	0 (0.00%)	3 (20 %)	3 (27.27%)	0 (0.00%)	5 (45.45%)	2 (18.18%)	4 (36.37%)	5 (45.45%)	0 (0.00%)
	>40	45	9.78	17 (37.37%)	23 (51.11%)	5 (11.11%)	0 (0.00%)	14 (31.11%)	10 (22.22%)	11 (24.44%)	10 (22.22%)	13 (28.89%)	28 (62.22%)	4 (8.89%)	0 (0.00%)
State of Residence	Eastern India	396	86.08	226 (57.07%)	110 (27.77%)	33 (8.33%)	27 (6.81%)	161 (40.65%)	89 (22.47%)	45 (11.36%)	101 (25.50%)	60 (15.15%)	105 (26.51%)	207 (52.27%)	24 (6.06%)
	Western India	10	2.17	5 (50%)	5 (50%)	0 (0.00%)	0 (0.00%)	6 (60%)	2 (20%)	1 (10%)	1 (10%)	4 (40%)	5 (50%)	1 (10%)	0 (0.00%)
	Northern India	47	10.21	26 (55.13%)	16 (34.04%)	5 (10.63%)	0 (0.00%)	14 (29.78%)	17 (36.17%)	10 (21.27%)	6 (12.76%)	15 (31.90%)	20 (42.56%)	12 (25.53%)	0 (0.00%)
	Southern India	7	1.52	3 (42.85%)	3 (42.85%)	1 (14.28%)	0 (0.00%)	1 (14.28%)	3 (42.85%)	0 (0.00%)	3 (42.85%)	1 (14.28%)	2 (28.57%)	5 (71.42%)	0 (0.00%)

The first number signifies quantity, while the number in brackets denotes the percentage. The frequency of take-out food consumption is denoted as <1/w (less than once a week), 1/w (once a week), 2/w (twice a week), and >2/w (more than twice a week). Food preference abbreviations are PF (pan-fried), DF (deep-fried), SF (stir-fried), and ST (steamed)

Table 2 Classification of foods and selection of simulants (Park et al., 2018).

Food stimulant	Description	Food types
Distilled water or water of equivalent quality	Aqueous, non-acidic foods (pH>5) without fat	Honey, mineral water, sugar syrups molasses, skimmed milk, sweets, etc
3% acetic acid	Aqueous, acidic foods (pH≤5) without fat	Fruit juices, squashes, vinegar, jams, jellies, carbonated beverages, processed vegetables, preparation of soups, broths, sauces etc.
10% ethanol	Alcoholic beverages having alcohol concentration less than 10 percent	Beer and some pharmaceutical syrups
50% ethanol	Alcoholic beverages having alcohol concentration above 10 percent	Wine, brandy, whiskey, arrack and other alcoholic drinks
n-heptane	Oils, fats, and processed dry foods with surface fat or volatile oil	Vegetable oils, ghee, cocoa butter, lards, biscuits, spice powder, snacks and savoury.

2.2 Reagents, standards and food simulants

BPA and Phalic acid (99% pure) were purchased from SRL (Sisco Research Laboratories), India. Methanol, ethanol, acetic acid, and n-heptane (HPLC grade) were purchased from Merck. Water (HPLC grade) for chromatography was also purchased from Merck. A stock solution of BPA and Phthalic acid was prepared by dissolving 10 mg of each substance in 100 ml of methanol and stored in dark amber bottles. Standard working solutions of five different concentrations of 0.05, 0.2, 0.5, 1, and 5 µg/ml were prepared by dilution with water. The migration was determined using five different simulants (Table 2) as per the standards laid down by BIS (IS 9845:1998) (Park et al. 2018).

2.3 Migration test

All take-out FCMs which were labeled as per the FCM-X series (FCM-1: PC, FCM-2: PS, FCM-3: PP, FCM-4: PE, FCM-5: Aluminum foil, and FCM-6: Paper-pulp) were rinsed with distilled water (25-30ºC) to remove extraneous materials before the migration analysis. The food simulants were preheated before the migration analysis to provide the general conditions for actual analysis. Water, 3% acetic acid, 10% and 50% ethanol were preheated at 70ºC and n-heptane for 25ºC (Park et al. 2018). All the containers and pouches were filled to their capacity with the preheated simulants and lids closed. In the case of pouches, air was excluded before sealing it. The containers and pouches were exposed to 70ºC maintained in a hot air oven for 15, 30, and 45 minutes, respectively. After exposure for the specified duration, the contents were transferred immediately into clean Pyrex beakers and were allowed to cool to room temperature. After cooling, the simulants were filtered through a 0.22 µm PVDF sterile syringe filter and were injected into the HPLC-UV for BPA and Phthalic acid analysis.

2.4 BPA and Phthalic acid determination

BPA and Phthalic acid in the simulants were analyzed by HPLC-UV using the protocol explained by Park et al. (2018) operating at 210 nm with a BioSuite C18 column (Table 3). The mobile phase was an isocratic elution of 30% trifluoroacetic acid (TFA) in HPLC grade water and 70% acetonitrile (ACN) at a flow rate of 1ml min^{-1}.

2.5 Ames Salmonella assay

Salmonella typhimurium strains TA 98 and TA 100 were obtained from Microbial-type culture collection (MTCC) in Chandigarh, India, to perform the top agar method of Ames *Salmonella* mutagenicity test. These histidine auxotrophic strains are typically used to detect chemical mutagens and assess their mutagenic potential (Zainol et al. 2021). *S. typhimurium* cultures (MTCC 1251 and MTCC 1252) were grown for 10 hours and used as the starter culture for the assay. 100 µl of the starter culture was mixed with 100 µl food stimulants, which showed considerable levels of

Table 3 Analytical parameters and conditions for BPA and Phthalic acid concentration determination in food simulants by HPLC-UV.

Substance detected	Parameter	Conditions
BPA and Phthalic acid	Column	Biosuite C18 PA-A (500 Å, 7 µm, 4.6 mm x 150 mm)
	Detector	UV (210nm)
	Pump	Agilent 1290 Infinity II
	Mobile phase	30% TFA in HPLC grade water + 70% ACN
	Injection volume	50 µl using Hamilton syringe
	Flow rate	1 ml min^{-1}
	Run time	8 minutes

BPA and Phthalic acid in the HPLC-UV detection method (concentration above quantification limit) along with 500 µl of S9 liver pooled fraction (for metabolic activation assays) or 500 µl of phosphate buffer (in control set for assays without metabolic activation). The solution was added to 2 ml molten top agar (0.6%) tubes seeded with histidine and then vortexed gently and poured on sterile Glucose Minimal Agar (1.5%) plates. The plates were incubated for 48 hours at 37ºC (Nepalia et al. 2018). After 48 hours, the revertant colonies on the test (with food simulants) and control plates (without food simulants) were counted. The experimental samples (food simulants) were tested with and without metabolic activation with S9 liver, pooled fraction (Sigma-Aldrich S2067) for their mutagenic potential.

2.6 Calculations

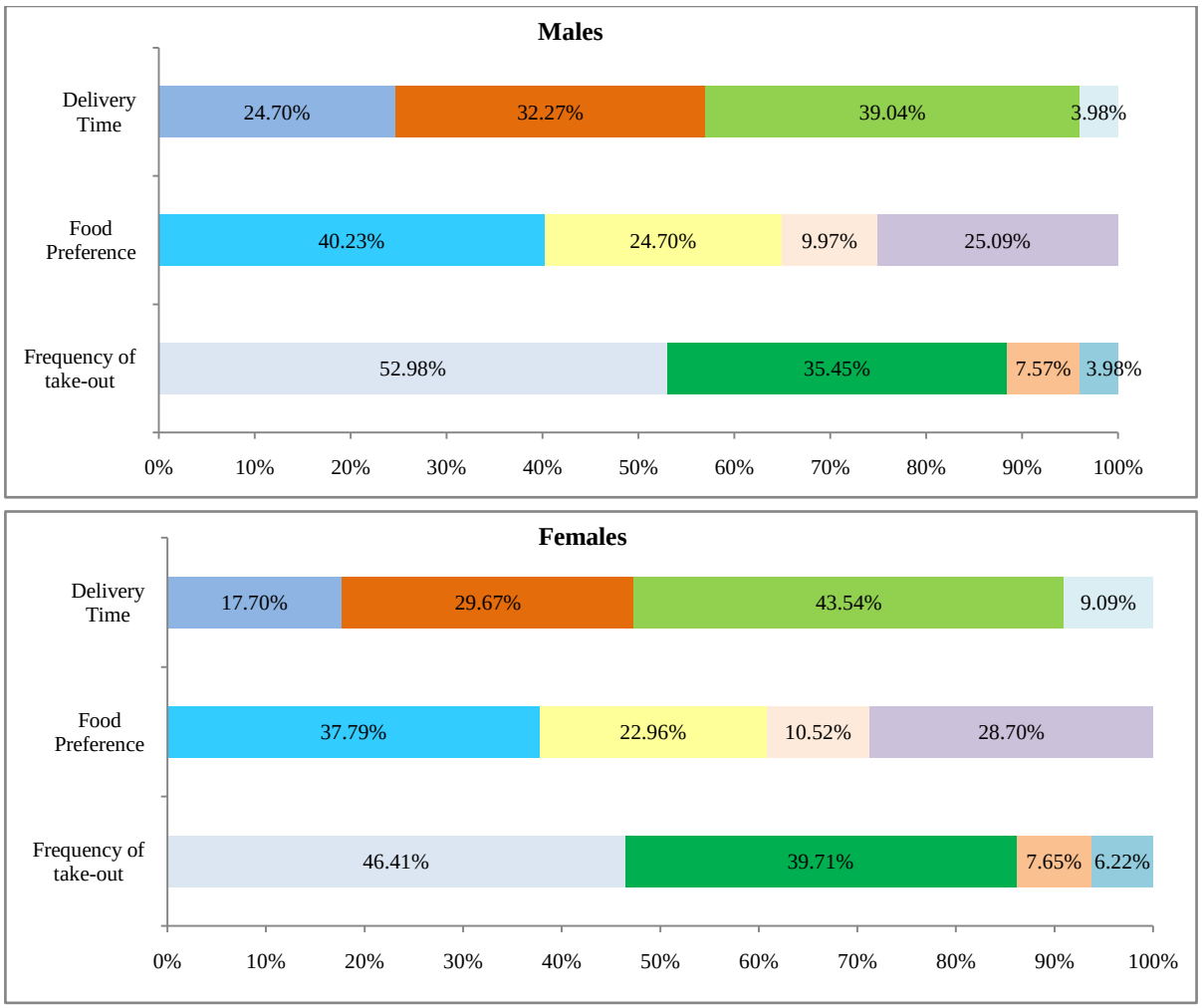
2.6.1 Non-statistical analysis

The assay results are presented in terms of mutagenicity ratio (MR) with standard deviation (Samiei et al. 2015). The MR is calculated by dividing the number of reverse mutation colonies on sample-treated plates (x) by the number of reverse mutation colonies in the negative control plate (x_0). The two-fold rule is followed, which means that if the reproductive doubling of spontaneous reversion rate (MR ≥ 2) occurs in the sample at one or two doses, it is considered a positive response (Nepalia et al. 2018).

3 Results and discussion

3.1 Results of the questionnaire

Within the sample of 450 respondents, 251 were male and 209 were female. They reported their long-term residence in various regions of India, including Eastern, Western, Northern, and Southern areas. Because the number of respondents in the Western and Southern regions was less than 20, they were excluded from the statistical analysis. Drawing upon the prevalent trends and insights gathered from questionnaire data, it is discernible that the age group from 17 to 28 represents a considerable share of online take-out food orders. As indicated in Figure 3A shows that 52.98%



Delivery Time (mins)	<15		15-30		30-45		>45	
Food Preference	PF		DF		SF		ST	
Frequency oftake-out	<1/w		1/w		2/w		>2/w	

Figure 3AThe colored bars in the graph illustrate the percentage distribution of preferences between male and female respondents about the responses they gave in the questionnaire when asked about the frequency of take-out food, food choices, and the average delivery time for take-out food.

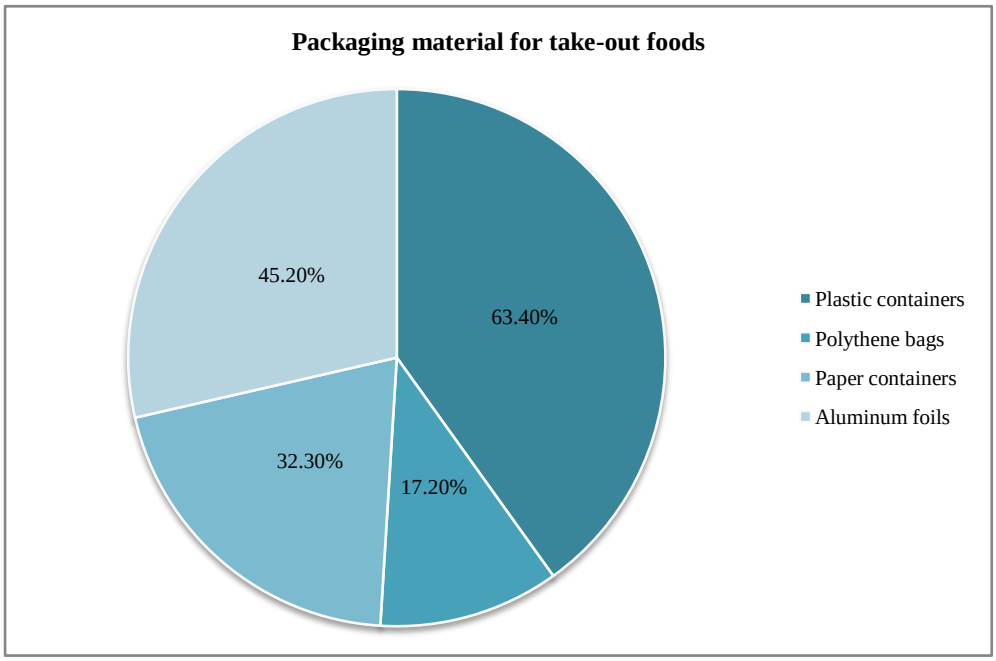


Figure 3B The pie chart displays the percentage breakdown of packaging materials used for take-out food based on survey respondents

of male respondents reported ordering take-out food less than once a week, while 35.45% ordered once a week, 7.57% ordered twice a week, and 3.98% ordered more than twice a week. Furthermore, the majority of male respondents preferred their take-out food to be pan-fried (40.23%), followed by steamed (25.09%), deep-fried (24.70%), and stir-fried (9.97%). Similar results were observed among female respondents, with 46.41% ordering take-out food less than once a week, 39.71% once a week, 7.65% twice a week, and only 6.22% more than twice a week. Female respondents also demonstrated a preference for pan-fried (37.79%) and steamed (28.70%) take-out food, followed by deep-fried (22.96%) and stir-fried (10.52%) options.

As many take-out foods are typically packaged in various containers, the questionnaire included inquiries regarding the take-out food packaging and the average delivery duration. Findings in Figure 3B shows that the most commonly used packaging method for take-out food was directly packed into plastic containers, followed by aluminum foils, paper-pulp containers, and PE pouches. In addition, most respondents reported that the average delivery time for their take-out food ranged from 30 to 45 minutes.

3.2 HPLC-UV method validation

The analytical methodology employed for detecting BPA and Phthalic acid has been rigorously validated using various parameters, including linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision (AOAC 2016). Linearity was assessed by determining the correlation coefficient (R^2) obtained from the calibration curve, constructed using five standard concentrations (0.05, 0.2, 0.5, 1, and 5 µg/ml) for both BPA and Phthalic acid. R^2 for BPA was > 0.99, and for Phthalic acid R^2 > 0.994, emphasizing the strong linearity in the calibration curves for these analytes. The LOD and LOQ were calculated employing the formulas 3.3 σ/S and 10 σ/S, respectively, where σ represents the standard deviation of the response, and S signifies the slope of the calibration curve. As reported in Table 4, the validation parameters LOD and LOQ values for BPA were determined to be 0.475 and 1.44 µg/ml, respectively, while for Phthalic acid, the corresponding values were 0.251 and 0.763 µg/ml. In order to assess recovery, experiments were conducted by introducing standard solutions into samples in triplicate. The average recovery percentages for BPA and Phthalic acid ranged from 74.98% to 125.39% and from 78.60% to 123.57%,

Table 4 Recovery and precision detection of BPA and Phthalic acid in spiked food simulants

Substance	Spiked level (mg/L)	Water		3% acetic acid		10% ethanol		50% ethanol		n-heptane	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
BPA	0.05	96.35	3.60	74.98	1.78	110.67	5.22	123.37	1.82	87.94	0.90
	0.5	118.88	2.57	88.89	2.88	99.60	0.76	115.74	4.10	104.39	4.77
	5	125.39	6.45	117.01	1.30	103.76	2.01	117.64	4.33	97.70	1.58
Phthalic acid	0.05	101.31	6.72	82.90	0.78	78.60	2.71	92.13	3.01	123.57	5.26
	0.5	97.60	2.55	100.69	8.93	102.22	3.35	98.90	6.25	100.10	3.76
	5	102.67	4.89	105.75	4.78	87.98	2.18	83.92	1.01	111.07	7.10

respectively. Precision was gauged by calculating the relative standard deviations (RSD %) of the obtained recovery values.

3.3 Migration of BPA

Maximum BPA was leached from packaging containers when 3% acetic acid was used as a food stimulant. The level of BPA migration increased when the exposure time was increased from 15, 30 and 45 minutes. Maximum BPA migration was observed for PE pouches (47.07 µg/ml) followed by aluminum foils (46.67 µg/ml), PP trays (45.98 µg/ml), and PS containers (45.59 µg/ml) when preheated food simulants were exposed in them for 45 minutes. When 50% ethanol was used as a food stimulant, PE pouches (3.36 µg/ml) and aluminum foils (1.74 µg/ml) showed increased BPA leaching only at 45 minutes of exposure time. Similarly, Cao et al. (2021) reported BPA migration from PC samples into aqueous or fatty foods when food simulants such as 4% acetic acid (12-113 µg/kg), 10% ethanol (14-407 µg/kg), and olive oil (1-30 µg/kg) was used. Park et al. (2018) observed BPA migration from different PC samples when heated with varying simulants of food such as water (83.7 µg/L), 4% acetic acid (40.9 µg/L), 50% ethanol (54.3 µg/L), and n-heptane (142 µg/L). Interestingly, paper-pulp-based containers showed BPA migration (1.66 µg/ml) for 10% ethanol when exposed for 45 minutes, which is attributed to the presence of hydrophobic inner plastic film lining, mostly made of PE and sometimes co-polymer alternatives. Similar results were reported by (Ranjan et al. 2021), where they observed that disposable paper cups commonly used to consume hot beverages (85-90ºC) are normally laminated with hydrophobic high-density polyethylene (HDPE) lining. At such a high temperature, when exposed for 15 minutes, it causes deterioration of the hydrophobic film. It causes the leaching of microplastics ($102 + 21.1 \times 10^6$ microplastic particles/ml) and ions like fluoride, chloride, sulfate, and nitrate into the liquid. However, no leaching of BPA from packaging containers was observed when water, 10% ethanol, and n-heptane were used as food simulants. The concentrations shown in Table 5 are positive mean values for each packaging sample and food stimulant. According to these results, the choice of food stimulant is an essential factor for BPA migration, as migration levels vary depending on the choice. Other than the choice of food stimulant, the exposure time of the preheated food simulants with different packaging materials also plays a vital role in BPA migration. The greater the time of exposure, the greater the level of migration.

3.4 Migration of Phthalic acid

Concentrations of migrated Phthalic acid from different FCMs are shown in Table 6. When water and n-heptane were used as food stimulants, none of the simulants extracted from different FCMs showed Phthalic acid concentration above the LOQ level (0.763 µg/ml). After exposure to 3% acetic acid for 15, 30, and 45 minutes, Phthalic acid was detected in all food contact materials stimulants. The highest Phthalic acid concentration was recorded for PE pouches (50.75 µg/ml), closely followed by paper-pulp containers (50.31 µg/ml) and PP trays (49.59 µg/ml) after 45 minutes of exposure. With 50% ethanol migrated, Phthalic acid was detected only in paper-pulp containers when exposed for 45 minutes. Phthalic acid was detected in food simulants extracted from PE pouches, paper-pulp containers, aluminum foils, and PC containers after 45 minutes of exposure to 10% ethanol.

3.5 Risk impact assessment

The Ames assay was performed to assess the mutagenicity of the migration of plasticizers into food simulants from different FCMs commonly used in packaging. As indicated in Table 7, the results show that few simulants extracted were significantly mutagenic. Thus, a risk impact assessment becomes pertinent as this is a matter of great concern, as mutagenesis is a critical component of carcinogenesis. When 3% acetic acid was used as a simulant (without S9 exposure), most of the tested FCMs demonstrated mutagenicity ratios below 2, while only a few samples demonstrated mutagenicity ratios greater than 2, mainly after 45 minutes of exposure. PS-based FCMs were the most mutagenic, exhibiting mutagenicity ratios greater than 2 for both *S. typhimurium* strains (TA 98 and TA 100) tested after 30 and 45 minutes of exposure. This was followed by aluminum foils, which

Table 5 Concentration (µg/ml) of BPA in different food simulants extracted from different food-contact materials and analyzed by HPLC-UV

Samples	Water			3% acetic acid			10% ethanol			50% ethanol			n-heptane		
	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins
PC containers	ND	ND	ND	18.67±0.05	30.76±0.04	35.53±0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND
PS containers	ND	ND	ND	33.14±0.06	40.71±0.05	45.59±0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP trays	ND	ND	ND	28.98±0.06	34.79±0.04	45.98±0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND
PE pouches	ND	ND	ND	29.59±0.06	32.34±0.05	47.07±0.04	ND	ND	ND	ND	ND	3.36±0.05	ND	ND	ND
Aluminium foils	ND	ND	ND	29.48±0.06	39.09±0.05	46.67±0.04	ND	ND	ND	ND	ND	1.74±0.05	ND	ND	ND
Paper-pulp containers	ND	ND	ND	30.86±0.05	34.04±0.05	38.96±0.04	ND	ND	1.66±0.05	ND	ND	ND	ND	ND	ND

ND: Not Detected or below LOQ (1.44 µg/ml)

Table 6 Concentration (µg/ml) of Phthalic acid in different food simulants extracted from different food-contact materials and analyzed by HPLC-UV

Samples	Water			3% acetic acid			10% ethanol			50% ethanol			n-heptane		
	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins	15 mins	30 mins	45 mins
PC containers	ND	ND	ND	19.89±0.06	33.02±0.05	38.21±0.06	ND	ND	1.08±0.05	ND	ND	ND	ND	ND	ND
PS containers	ND	ND	ND	35.62±0.05	43.84±0.05	49.14±0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP trays	ND	ND	ND	31.09±0.05	37.40±0.06	49.59±0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND
PE pouches	ND	ND	ND	31.75±0.06	34.74±0.04	50.75±0.05	ND	ND	3.25±0.04	ND	ND	ND	ND	ND	ND
Aluminium foils	ND	ND	ND	33.13±0.06	36.59±0.05	41.94±0.04	ND	ND	1.53±0.05	ND	ND	ND	ND	ND	ND
Paper-pulp containers	ND	ND	ND	31.64±0.06	42.07±0.05	50.31±0.05	ND	ND	1.66±0.05	ND	ND	1.40±0.04	ND	ND	ND

ND: Not Detected or below LOQ (0.763 µg/ml)

Table 7 Mutagenicity ratios (MR) for *Salmonella typhimurium* strains TA98 and TA100, both with and without S9 metabolic activation

Food-contact materials	Exposure time (mins)	Mutagenicity ratio TA98		Mutagenicity ratio TA100	
		Without S9	With S9	Without S9	With S9
Food simulant used: 3% acetic acid					
PC containers	15	1.13±0.06	2.00±0.30	1.57±0.03	2.33±0.46
	30	1.31±0.11	3.42±0.30	1.78±0.09	4.83±0.93
	45	1.63±0.12	4.14±0.51	3.67±0.17	6.42±0.06
PS trays	15	1.36±0.06	2.42±0.20	1.71±0.04	3.67±0.47
	30	1.81±0.09	5.14±0.09	2.07±0.22	5.75±0.30
	45	3.85±0.18	8.09±0.33	3.92±0.04	7.91±0.58
PP containers	15	1.09±0.10	2.42±0.90	1.42±0.21	2.40±0.19
	30	1.22±0.10	4.28±0.21	1.57±0.05	4.67±0.24
	45	1.63±0.09	4.71±0.38	1.92±0.05	8.83±0.07
PE pouches	15	1.04±0.08	1.42±0.09	1.85±0.22	4.33±0.25
	30	1.18±0.15	2.57±0.09	2.42±0.25	5.21±0.32
	45	1.45±0.07	3.28±0.20	2.71±0.07	5.98±0.20
Aluminium foils	15	1.22±0.35	2.39±0.41	1.35±0.04	3.00±0.47
	30	1.90±0.46	3.07±0.03	1.71±0.28	4.67±0.47
	45	2.04±0.11	7.85±0.64	2.03±0.05	4.83±0.21
Paper-pulp containers	15	1.09±0.01	1.14±0.07	1.35±0.04	2.33±0.93
	30	1.13±0.09	2.28±0.11	2.07±0.25	4.50±0.70
	45	1.95±0.10	4.59±0.25	2.28±0.19	7.34±0.47
Food simulant used: 50% ethanol					
PE pouches	45	1.40±0.09	2.76±0.16	1.08±0.11	2.17±0.09
Aluminium foils	45	1.13±0.16	3.67±0.33	1.43±0.08	2.04±0.21
Paper-pulp containers	45	1.03±0.12	1.85±0.10	1.29±0.08	1.96±0.15
Food simulant used: 10% ethanol					
PC container	45	1.10±0.14	2.08±0.16	1.42±0.21	2.28±.11
PE pouches	45	1.58±0.08	2.56±0.09	1.35±0.04	1.98±0.30
Aluminium foils	45	1.70±0.16	2.66±0.10	1.57±0.09	2.42±0.90
Paper-pulp containers	45	1.04±0.06	1.54±0.09	1.18±0.11	1.95±0.01

Values showing mutagenicity ratio ≥ 2 indicates significant genotoxicity.

showed mutagenicity ratios greater than 2 for both strains only after 45 minutes of exposure. PE pouches and Paper-pulp containers showed mutagenicity ratios greater than 2 for TA 100 strain only after 30 and 45 minutes of exposure. PC containers demonstrated mutagenicity ratios more significant than two only after 45 minutes of exposure. When food simulants were used for 50% and 10% ethanol (without S9 exposure), no tested FCMs demonstrated a mutagenicity ratio greater than 2. The addition of the S9 mix increased the number of induced revertants, thereby increasing the mutagenicity ratio. Among the tested FCMs, when 3% acetic acid was used as a simulant, the highest mutagenicity ratio recorded with the S9 mix for TA 98 strain was for PS containers after 45 minutes of exposure, and for TA 100 was for PP containers after 45 minutes of exposure. When using 50% ethanol and 10% ethanol with an S9 mix, the highest mutagenicity ratio was noted for PE pouches and aluminum foils after 45 minutes of exposure, respectively.

Conclusion

The research findings underscore the prevalent preference for hot and spicy pan-fried take-out foods among the Indian population, particularly among individuals aged 17-28, consumed at least once a week. These foods, commonly packaged in non-biodegradable plastic containers, are associated with increased migration of harmful substances like BPA and Phthalic acid, exacerbated by high-flame cooking methods and acetic acid infusion. The study, involving HPLC-UV analysis on various FCMs, highlighted higher migration levels of these substances in 3% acetic acid food simulants, particularly within delivery durations of 15 to 45 minutes. The Ames test indicated significant mutagenicity in specific samples, with effects escalating upon metabolic activation, suggesting adverse impacts on mammalian systems. These outcomes provide crucial insights for safety regulators and risk evaluators to assess health risks associated with these food containers, prompting further consideration for in vivo assays to deepen the understanding of plasticizers' effects on mammalian health.

Disclosure statement

No potential conflicts of interest are reported by the authors.

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Effect of probiotics on the histomorphometry characteristics of *Mus musculus* Jejunum infected by *Salmonella gallinarum*

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ABSTRACT

Salmonellosis is a disease caused by *Salmonella gallinarum*, which can cause digestive tract infections. Probiotics are good microorganisms for the host because they can increase the normal bacteria flora in the digestive tract. They can maintain the intestinal mucosal barrier and prevent bacterial adhesion. This study aimed to determine the histomorphometric characteristics of the jejunum from the intestines of mice (*Mus musculus*) after being infected with *S. gallinarum*. A total of 20 mice, 4-6 weeks, were divided into four research groups: P1 (probiotics and *S. gallinarum* infection), P2 (probiotic administration), P3 (*S. gallinarum* infection), and P4 (control). The probiotics used contain microorganisms such as *Lactobacillus casei*, *Saccharomyces cerevisiae*, and *Rhodopseudomonas palustris*, dissolved in distilled water in a ratio of 1:1000. Probiotics were given orally at 0.5 ml for 7 days. *S. gallinarum* infection was given orally, with a volume of 0.5 ml (1.5×10^8 CFU/ml). The results showed that the mean score of intestinal lesions differed between groups. The width of the villi, the thickness of the mucosa, and the depth of the intestinal crypts were significantly different. The best result of histology findings was in the group of mice that were induced with probiotics (P2).

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1 Introduction

The digestive system is a channel that plays a role in food digestion, absorption of nutrients, and elimination of food waste. Pathogenic bacteria in the intestines are toxic because they can cause damage to the intestinal mucosa by adhesion, invasion, multiplication, and then releasing toxins to cause various digestive tract diseases (Ersawati et al. 2018). According to Joni and Abrar (2018), bacteria that play a role in damaging the intestinal mucosa include *Salmonella* sp., *Escherichia coli*, *Staphylococcus aureus*, *Shigella sp*., *Vibrio cholerae* and *Vibrio parahaemolyticus*.

Salmonella is a type of harmful bacteria that can be found in the gut of some hosts. Different species of *Salmonella* can attach to the mucosa of the intestine and form colonies on epithelial cells. This attachment is influenced by proteins on the surface of the bacteria, known as fimbriae and fimbrial adhesins (outer membrane proteins) (Wresdiyati et al. 2013). Once attached, *Salmonella* produces cytolethal distending toxins (CDT) that can cause inflammation, ulcers, abscesses, and even cell death in the intestinal tissue. These effects are caused by the disruption of water and electrolyte secretion and the triggering of inflammatory reactions. Therefore, it is dangerous for host cells to be infected with this bacterium (Darmawan 2017).

Increasing the intestinal mucosal barrier can help defend against pathogenic bacteria like *Salmonella*. This can be done using live beneficial microbes, also known as probiotics. Probiotic products can help balance the intestinal microflora and boost immunity. This helps restore the balance between pathogenic and non-pathogenic bacteria in the intestine (Kusuma et al. 2012). To be effective, probiotics derived from the lactic acid bacteria (LAB) group must meet certain requirements, including being non-pathogenic, active and able to grow in the digestive system, resistant to bile salts, having high viability, being anaerobic, and capable of quickly adhering to and killing or inhibiting bacterial pathogens from growing (Joni and Abrar 2018).

Several studies have shown that probiotics can increase the number of lactic acid bacteria (LAB) in the intestine and reduce the number of harmful bacteria. LAB can also reduce damage caused by pathogenic bacteria, such as *Salmonella*, by adhering to the intestinal epithelium and producing antimicrobial compounds that denature membrane proteins, change cell membrane properties and inhibit the replication of *Salmonella* (Gupta et al. 2018). Additionally, LAB can improve immunity by increasing phagocytosis and modifying the production of pro-inflammatory cytokines IFN-γ and TNF-α (Castillo et al. 2011). This study aims to evaluate the effect of probiotics on the histomorphometry characteristics of the Jejunum of Mus musculus infected by *S. gallinarum*.

2 Material and Methods

This study used 20 male mice (*Mus musculus*) aged 4-6 weeks and weighing 18-30 grams. The research steps included preparing *S. gallinarum* and probiotic suspensions, inducing them in mice, preparing histology, and analyzing the data.

2.1 Bacteria and probiotic preparation

S. gallinarum bacteria were grown on *Salmonella-Shigella Agar (SSA)* media. The *S. gallinarum* colonies were cultured in a nutrient broth medium and incubated aerobically at 37°C for 24 hours. This study used commercial probiotics containing *Lactobacillus casei*, *Rhodopseudomonas palustris*, and *Saccharomyces cerevisiae*. The probiotic solution used was 1% and was given orally to mice, with each mouse receiving 0.5 ml of the solution daily for seven days.

2.2 Induction to mice

The study involved 20 mice that were divided into four groups: P1 (probiotics and *S. gallinarum* infection), P2 (probiotic administration), P3 (*S. gallinarum* infection), and P4 (control). The probiotics were administered orally at a dose of 0.5 ml for seven consecutive days, while *S. gallinarum* was induced orally for seven days with a concentration of 1.5×10^8 CFU/ml at a dose of 0.5 ml each day. The Brawijaya University Research Ethics Commission has granted ethical permission for this study under the reference number 109-KEP-UB-2020.

2.3 Histology preparation

After the animal was euthanized by cervical dislocation, a sample was taken from the jejunum and placed in 10% neutral formalin buffer (NBF). The sample was then prepared for histopathological examination through steps, including dehydration in graded alcohol, clearing with xylene, embedding, sectioning, and staining with hematoxylin-eosin (HE).

2.4 Data Analysis

The data from the study were analyzed using lesion score, villi width, mucosal thickness, and crypt depth of the jejunum among the groups. The lesion scoring was determined based on the method of Erben et al. (2014), with some modifications required (Table 1). Statistical analysis of the data was carried out in this study using one-way ANOVA (analysis of variance) to determine the differences in each treatment of the morphometric analysis of the intestines of mice.

3 Results and Discussion

The histomorphometry of the jejunum in mice was measured using the Image-J application at a microscope magnification of 400X.

Table 1 Determination of lesion scoring of jejunum

Abnormality	Score	Description
Inflammatory cells	0	None
	1	Focal
	2	Multifocal
	3	Diffuse
Epithelial damage	0	None
	1	Epithelial cells of villi are damaged at several ends
	2	Micro erosion in some villi
	3	Severe erosion of epithelial
Mucosal edema	0	None
	1	<50% of the diameter of the intestinal wall
	2	50%-80% of the diameter of the intestinal wall
	3	>80% of the diameter of the intestinal wall

The scoring is based on the method of Erben et al. (2014)

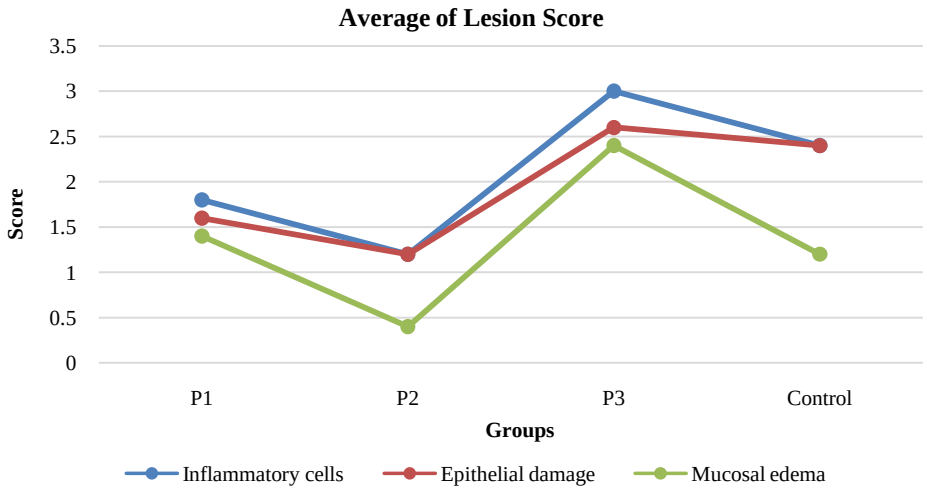

Figure 1 Average of Jejunum Lesion Score; P1 (probiotics and *S. gallinarum* infection); P2 (Only probiotic); P3 (*S. gallinarum* infection); P4 (control)

The jejunum's histomorphometric observations were conducted with four fields of view for each treatment group. The observed parameters included lesion scoring, villi width, mucosal thickness, and crypt depth. The analysis of the jejunum histopathological lesion scores results, as shown in Figure 1, revealed a significant difference in each treatment group in terms of inflammatory cells, epithelial damage, and jejunal submucosal edema of mice that were given probiotics and infected with *S. gallinarum*. Based on the average score, the induction treatment group of *S. gallinarum* had the highest levels of inflammatory cells and epithelial damage, followed by the control group, probiotics and S. gallinarum combination, and the least effective group was probiotics. The presence of inflammatory cell infiltration can be caused by infection with *S. gallinarum*, which then triggers an inflammatory reaction. The accumulation of inflammatory cells is a response to inflammation. The inflammatory reaction is a self-defence reaction in response to injury as a vascular reaction (Saraswati et al. 2015).

Epithelial damage can occur due to the penetration of *Salmonella* bacteria into the intestinal mucosa. The bacteria can stay intracellularly and proliferate before reaching the epithelium in the lamina propria. This can cause brush border degeneration. These findings support Cita's (2011) statement that *Salmonella* bacteria can penetrate the intestinal epithelial mucosa and damage the epithelial lining by multiplying in the lamina propria. Pathogenic bacterial infections can also cause submucosal edema. This

Table 2 Effect of various treatments on the mucosa thickness, crypt depth, and villi width

Groups	Average ± SD		
	Mucosal Thickness	Crypt Depth	Villi Width
P1 (probiotic + *S. gallinarum*)	347.97 ± 2.46^{ab}	114.46 ± 0.88^{c}	79.81 ± 0.72^{a}
P2 (probiotic)	369.92 ± 4.06^{a}	143.23 ± 1.99^{a}	85.13 ± 0.59^{a}
P3 (*S. gallinarum*)	280.07 ± 2.30^{b}	110.55 ± 0.76^{c}	58.46 ± 0.88^{b}
P4 (control)	295.50 ± 2.53^{b}	120.78 ± 2.22^{b}	67.20 ± 1.18^{b}

Data are mean of five replicates; ± Standard Deviation of mean; Different notations indicate significant differences between treatment groups ($p<0.05$).

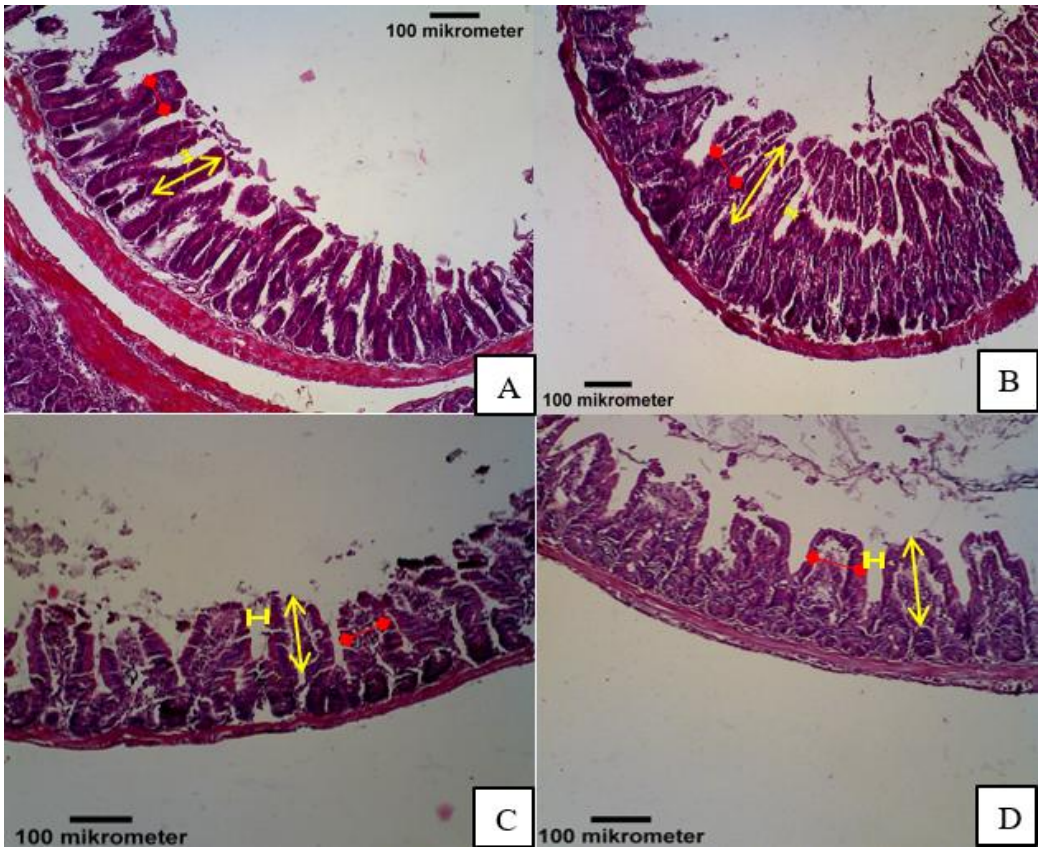

Figure 2 Histopathology of Mucosal Thickness, Crypt Depth, Villi Width (magnification of 400X); (a) probiotics and *S. gallinarum* infection; (b) Only probiotic; (c) *S. gallinarum* infection; (d) control; Yellow line represent crypt depth; red line represented villi width.

happens due to an inflammatory reaction that results in the widening of the submucosal space. Inflammatory mediators increase inflammatory proteins and substances that cause nearby blood vessels to dilate and become more permeable. This makes it easier for plasma proteins to release, causing edema (Saraswati et al. 2015).

Based on the histopathological measurements presented in Table 2 and Figure 2, it was observed that the group administered with probiotics had the highest average values for mucosal thickness, crypt depth, and villi width. The mucosal thickness of the control group was not significantly different from that of the other treatment groups. However, the group treated with probiotics differed significantly from the P1 and P3 groups. In the case of crypt depth, the group given probiotics and *S. gallinarum* was not significantly different from the other groups. However, the group given probiotics had a significant difference from the P3 and P4 groups. In contrast, no significant difference was observed in the measurement of villi width between groups P1, P2, P3, and P4. However, groups P1 and P2 significantly differed significantly from the control group and those induced by *S. gallinarum*.

An increase in the thickness of the mucosal layer and depth of the crypts in the small intestine is associated with an increase in the height of the villi. The ratio of the height of the villi to the depth of the crypts indicates a broader area for the absorption of nutrients. This is supported by Wresdiyati et al. (2013) statement, which states that the increase in the height of the villi in the jejunum is consistent with an increase in digestive and absorption functions due to the expansion of the absorption area. This is a smooth expression of the nutrient transport system throughout the body, which benefits the host. On the other hand, a decrease in mucosal thickness and crypt depth can be caused by damage to the epithelial layer due to the administration of *S. gallinarum*. According to Arya et al. (2012), *Salmonella* species can enter the small intestine by invading the intestinal tissue and persisting in intestinal cells. This can result in damage to the connecting surfaces that unite epithelial cells when penetrating the epithelial barrier.

According to research by Khan and Chousalkar (2020), providing probiotics alone as a preventive measure may not effectively inhibit the colonization of *S. gallinarum*. Administering probiotics can still cause damage to the jejunum mucosa of mice (*Mus musculus*) challenged by *S. gallinarum*. This is because probiotics' diverse and abundant microbial diversity is insufficient to reduce the number of *S. gallinarum*. Additionally, inadequate concentrations of probiotic bacteria in the intestinal tract and the short distance between the time of probiotic administration and the challenge of *S. gallinarum* could also contribute to this damage. Andino et al. (2014) also stated that the immune system may not have enough time to activate and provide sufficient protection against infection.

There was no significant difference in the width of the jejunal villi between the P1 (Probiotic+*S. gallinarum*) and P2 (Probiotic) groups. This suggests that the administration of probiotics has a significant impact on the width of the villi and that this effect was increased when probiotics were given along with *S. gallinarum*. The P1 group showed better histomorphometry than the P3 group (*S. gallinarum* only). This indicates that treatment with probiotics can reduce the damage caused by *Salmonella* by stimulating humoral and cellular immunity. The stimulation of immunity leads to an increase in the population and proliferation of lymphocytes, the maintenance of pro-inflammatory cytokines such as IFN-γ, TNF-α, and the increase of IL-12, IL-10, Immunoglobulin IgA, IgE IgG, IgM. The lactic acid bacteria found in probiotics adhere to the intestinal epithelium, stimulating macrophage activity, activating phagocytosis, and maintaining the mechanism of protection of the villi against *Salmonella*. This is achieved by maintaining the immune response (Gupta et al., 2018; Astawan et al., 2011). Probiotics in the intestines can reduce the proliferation of *Salmonella* by adhering to epithelial cells, competing with *Salmonella*, producing bacteriocin antimicrobial compounds, and interacting with the cell membrane of *Salmonella*. This results in the denaturation of proteins on the cell membrane. Probiotics also have lactic acid products that lower the pH, which disturbs the growth of *Salmonella* species (Castillo et al. 2011; Adetoye et al. 2018).

The P2 group, which was given probiotics, showed better results than the P4 group (control). The width of the villi in the P2 (Probiotic) group increased, which is influenced by short-chain fatty acids. Through fermentation, probiotic bacteria can produce short-chain fatty acids, a constituent component of intestinal epithelial cells. The more short-chain fatty acids produced, the more the multiplication of intestinal epithelial cells will be stimulated (Izzuddiyn et al. 2018). An increase in the length and width of the villi will expand the area for absorption of food and nutrients, which can improve the performance of the intestines in digesting food (Matur and Eraslan 2012). According to Dong (2019), *Salmonella* infections can significantly decrease the length and width of villi in the intestine. This is because *Salmonella* triggers inflammation, which leads to degeneration and damage of the villous epithelium. As a result, the villi become shorter and narrower, negatively affecting the intestine's ability to digest and absorb food.

Conclusions

The study's findings suggest that probiotics could repair damage to the jejunum caused by *S. gallinarum*. Moreover, the group that was given only probiotics displayed the most significant improvements in mucosal thickness, crypt depth, and villi width. They also showed minimal damage to the jejunum, as evidenced by lower histopathological lesion scores.

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THE EFFECT OF CHLOROGENIC ACID IN ROBUSTA LAMPUNG GREEN COFFEE EXTRACT (*Coffee canephora var Robusta*) AS AN ANTIOXIDANT IN LAYER CHICKEN INFECTED WITH *Salmonella enteritidis* BACTERIA

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KEYWORDS

Poultry

Bacteria

Infection

Coffee Extract

Antioxidant

Free radical

ABSTRACT

The poultry industry is crucial in meeting the population's nutritional needs worldwide. However, various bacterial infections have been reported in poultry chickens, decreasing their production rate. Farmers have been using synthetic antibiotic agents to manage these infections, which are costly and have several side effects. One of the most commonly reported bacterial pathogens is *Salmonella enteritidis*, which causes high mortality and low poultry production. Mortality and illness rates are associated with gastrointestinal diseases caused by suboptimal absorption. This study was conducted to evaluate the anti-inflammatory and antioxidant effect of Chlorogenic acid (CGA) from robusta lampung green coffee extract on *S. enteritidis* infected chickens. ISA brown day-old layer chickens were used in this study. A total of sixty chickens were divided into five groups, with each group having 12 replications. The formulated groups were C+ (chicken infected by *S. enteritidis* 10^8 CFU/ml), C- (healthy chicken), T1 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 500 mg/kg BW (bodyweight) coffee extract), T2 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 1000 mg/kg BW coffee extract), T3 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 1500 mg/kg BW coffee extract). The Nrf2, HO-1, and SOD levels were measured using BD cell quest ProT programmed through the flow cytometry method. Data of total cells were calculated for their average value and obtained data were statistically analyzed by One Way ANOVA ($p<0,5$). The results of the study showed that giving coffee extract at a dosage of 500 mg/kg BW to *S. enteritidis* infected chickens increased the Nrf2 and SOD levels but decreased HO levels. This research showed that Lampung robusta coffee extract could potentially be an anti-inflammatory compound and antioxidant for poultry industries.

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1 Introduction

The demand for poultry products increases yearly due to human population growth, and the poultry industry plays a crucial role in meeting the need for animal protein (Nataamijaya 2017). However, the industry faces various challenges, including controlling diseases that affect the chickens (Regar et al. 2013). In particular, two *Salmonella* species, *S. enteritidis* and *S. typhimurium*, can cause higher infection and mortality in young chickens (Wiryawan et al., 2005). *Salmonella* infected chickens may also spread the bacteria to healthy ones, leading to rapid outbreaks. Infection of *Salmonella* may also cause diarrhea due to damage to the intestinal microflora (Wiryawan et al. 2005). The bacteria can also survive for a long time in the environment, including poultry cages (Mshelbwala et al. 2017).

Salmonella infection is associated with oxidoreduction in infected chickens. Nuclear factor erythroid-derived 2-related factor-2 (Nrf2) is an elevated antioxidant and antitoxic gene that protects against oxidative stress by activating detoxification and antioxidant genes (Dong et al. 2008). The activation of Nrf2 triggers the formation of HO-1 (heme oxygenase-1) and SOD antioxidants, which help in reducing inflammation in body cells (Habtemariam 2019).

Indonesia has many plants that could be used as food or feed supplements with significant medicinal properties. Coffee (*Coffea canephora*) is one of them, and it contains several bioactive ingredients, including Kahweol and cafestol, which have antioxidant effects (Tribudi et al. 2020). These compounds activate HO-1 via the Nrf2 pathway, promoting cytoprotection against oxidative injury (Hwang and Jeong 2008). Coffee extract also contains other antioxidant compounds, such as phenol or phenolic acid, one of which is Chlorogenic acid (CGA). CGA has an immunomodulatory effect by inhibiting inflammation and the production of prostaglandins (PGE2) (Johnston et al. 2003). It also inhibits the synthesis of other mediators, such as IL-1β, interferon-γ, monocyte chemotactic protein-1, and macrophages inflammatory protein-1α (Bagdas et al. 2020). CGA is a flavonoid that can increase endogenous antioxidants by stimulating the expression of SOD (Liang and Kitts 2015). This enzyme defends tissues from oxidative stress by scavenging superoxide anions, one of the primary reactive oxygen species (ROS) produced from molecular oxygen in cells. Excessive accumulation of ROS can cause oxidative damage to major cellular macromolecules (proteins, lipids, and DNA) and cause necrosis (Hwang et al. 2020; Mazur-Bialy and Pocheć 2021).

SOD catalyzes the dismutation of anion O_2, reactive oxygen into hydrogen peroxide (H_2O_2), and oxygen (O_2) inside mitochondria. This reaction can block inflammatory cytokines and stimulate anti-inflammatory cytokines like IL-10 to neutralize oxidative reactions caused by *Salmonella* bacteria (Hutabarat et al. 2020). The poultry industry uses many feed additives, such as antibiotics, prebiotics, probiotics, synbiotics, short-chain fatty acids, and vaccines, to lower the mortality of livestock caused by *Salmonella*. However, disease outbreaks can still occur even after using these feed additives (Van Immerseel et al. 2002). Therefore, this study aims to determine the effect of Lampung robusta coffee extract as an anti-inflammation supplement to increase the chicken's immune system against *S. enteritidis* infection by assessing Nrf2, HO-1, and SOD levels.

2 Materials and Methods

This study used ISA Brown day-old chickens as an animal model and obtained ethical approval from Brawijaya University with the number 1142-KEP-UB.

2.1 Lampung Robusta Coffee Extraction

Creating Lampung robusta coffee extract involves dissolving 414 grams of Lampung robusta coffee in a bottle of 1500 ml of 90% ethanol. Next, the solution is homogenized using a centrifuge at 50 rpm. The solution is then filtered and evaporated at 60°C, using a rotatory evaporator until all the ethanol has evaporated (Bahrin et al. 2018). The final result is a brown-colored liquid (Qosimah et al. 2020).

2.2 Preparation of Animal Model

This study utilized a type of chicken feed called Charoen Pokphand 511-bravo (PT. Charoen Pokphan Indonesia Tbk), which contains approximately 21-23% rough protein, 5% fat, 3-5% rough fiber, and 4-7% ash (Qosimah et al. 2020). The research involved ISA Brown layer chickens divided into five treatment groups with 12 replications. The treatment groups were as follows: C- group (healthy chickens), C+ group (chickens infected with *S. enteritidis* bacteria at a concentration of 10^8 CFU/ml), T1 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 500 mg/kg BW coffee extract), T2 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 1000 mg/kg BW coffee extract), and T3 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 1500 mg/kg BW coffee extract). The coffee extract was administered as a preventative measure before the chickens were infected with *S. enteritidis*. On the first day of the research, the chickens were fed with vita stress, which contains vitamins and electrolytes (Medion, Indonesia), to prevent stress. On the fourth day, the chickens were injected with an inactivated emulsion ND (Newcastle disease)-IB (Infectious bronchitis) vaccine (Medion, Indonesia). In contrast, ND G7B vaccine (Medion, Bandung, Indonesia) and AI (Avian influenza) H5N1 vaccine (Medion, Bandung, Indonesia) were administered simultaneously on day 10 to prevent virus infection (Qosimah et al., 2020. From day 3 to day 16, Lampung robusta coffee extract was given orally at 1 ml per chicken.

2.3 Preparation of Bacterial Suspension

The *S. enteritidis* bacteria used in the study were obtained from the Microbiology and Immunology Laboratory at the Faculty of Veterinary Medicine, Universitas Brawijaya in Indonesia. The bacterial testing was conducted using Rappaport Vasiliadis Medium, Xylose Lysine Deoxycholate Agar (XLD agar), Triple sugar Iron Agar (TSIA), and LIA (Lysine Iron Agar), and supported by The Microbact ™ Gram-negative system from Oxoid. The bacteria were cultured in nutrient broth and incubated at 37°C for 24 hours. Bacterial growth was observed and stained using Gram staining and then analyzed using the biochemistry method to confirm the characteristics of the bacteria. The bacteria concentration was estimated by observing the spectrophotometer at an absorption wavelength of 580 nm to be 10^8 CFU/ml (optical density = 0.6). Each chicken was given 0.5 ml of bacterial suspension per oral, resulting in every chicken receiving a $5x10^7$ CFU/ml (Qosimah et al. 2020).

2.4 The Analysis of HO-1, Nrf2, SOD-1

A chicken necropsy was conducted on day 18, and the spleen was collected. The spleen was washed with PBS (phosphate buffer saline) and homogenized in a petri dish using a syringe. Cell suspension obtained from the spleen was washed with PBS, then mashed by a syringe and put inside a polypropylene bottle. 10 mL PBS was added to the tubes and centrifuged at 2500 rpm at 10°C for 5 minutes. The supernatant was removed from the pellets, and the pellets were resuspended in 1 ml of sterile PBS. From this, 50 µL splenic cell suspensions were separated into a 1.5 mL microtube containing 400 µL of PBS and centrifuged as previously mentioned (2500 rpm, 10°C, 5 minutes). Afterwards, 10 µL of anti-HO-1 FITC, anti-Nrf2 FITC, and anti-SOD-1 reagents (BioLegend, USA) were added, and the mixture was incubated for 30 minutes. After incubation, the mixture was rinsed with the wash-perm solution and centrifuged again as per the initial condition (2500 rpm, 10°C, 5 minutes), then incubated for 20 minutes and resuspended with PBS. After that, the sample was analyzed using a flow cytometer (BD FACS Calibur, USA) (Hermanto et al. 2020).

2.5 Statistical Analysis

The HO-1, Nrf2, and SOD-1 levels were analyzed using the BD CellQuest ProT program on the FACS Calibur TM device from BD Biosciences, San Diego, CA, USA. The statistical analysis was performed using One-way ANOVA ($p<0.5$). If a significant difference was observed between the treatments, the analysis proceeded to the post-hoc Tukey test using SPSS Statistic 21 software.

3 Results

3.1 The Nrf2

There is a significant difference in the Nrf2 level between the positive control group (C+) and the negative control group (C-). The Nrf2 level in the negative control group (11.49%) is higher than that of the positive control group (7.36%). The normal control group (C-) did not show any significant difference from the treatment groups T1 and T2 but a significant difference from treatment group T3. The cell counts of the negative control (11.49%), T1 (10.54%), and T2 (11.2%) are lower than those of T3 (15.23%). The T3 group has a higher cell count than the entire treatment group (Figure 1).

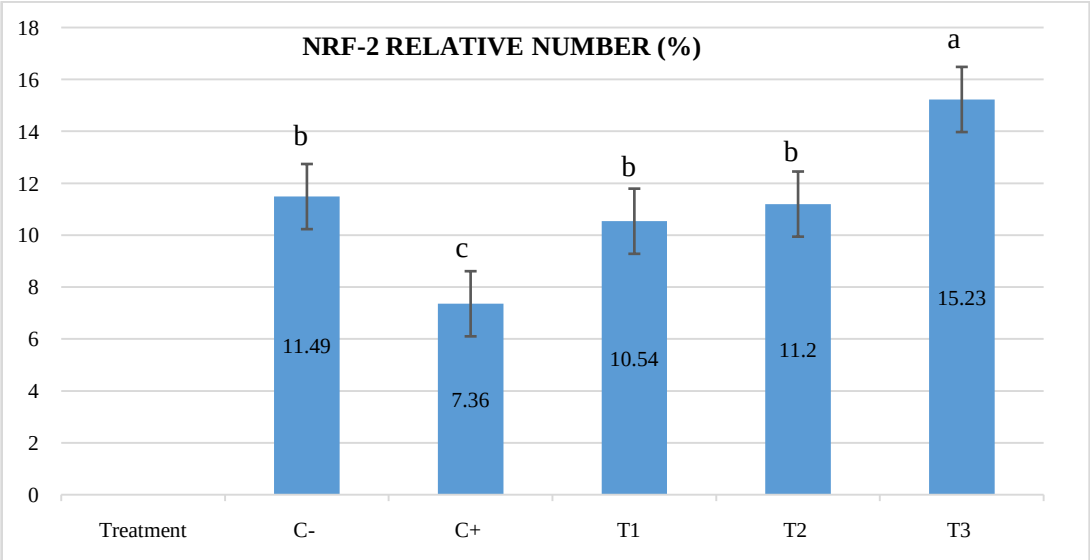

Figure 1 Nrf-2 level in chicken receiving treatments; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

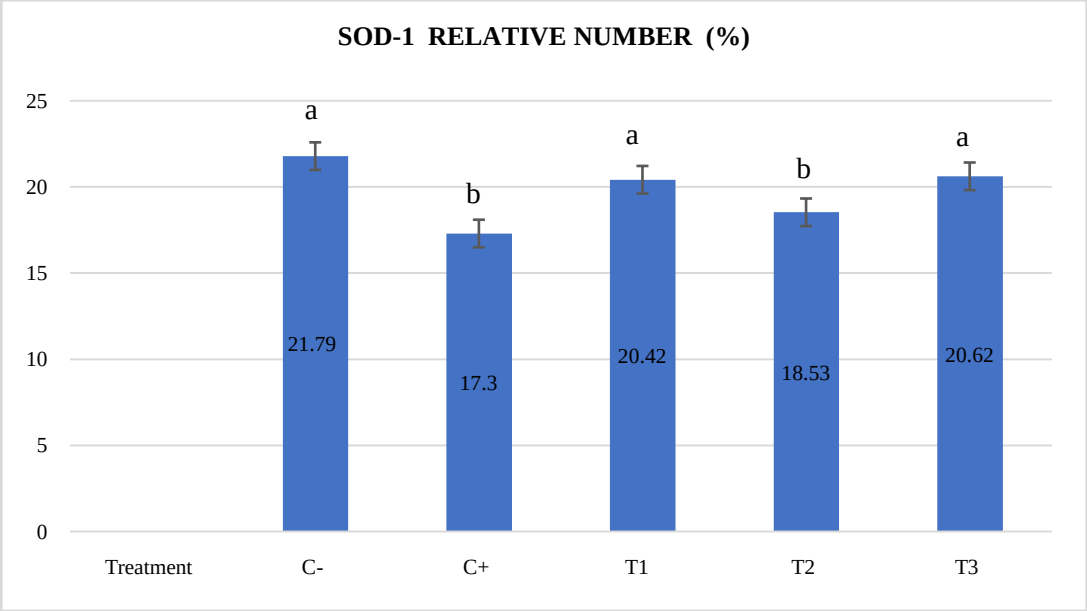

Figure 2 SOD-1 level in chicken receiving treatment; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

3.2 The SOD-1 level

The research conducted showed that the level of SOD-1 varied significantly between the positive (C+) and negative (C-) control group. Among the tested treatments, the negative control had the highest SOD-1 level at 21.79%, followed by treatment groups T1 (20.42%), T3 (20.62%), and T2 (18.53%). These values were significantly higher than the positive controls (17.3%), as shown in Figure 2.

3.3 The HO-1 level

The HO-1 level was tested alongside two other parameters and significantly differed between the groups. The highest HO-1 level was observed in the positive control C+ at 26.56%. The levels were followed by group C- (20.36%) and group T1 (19.16%). The treatment groups T2 and T3 had even lower levels at 17.6% and 15.85%, respectively. This indicates that Lampung robusta coffee significantly affects HO-1 levels, as shown in Figure 3.

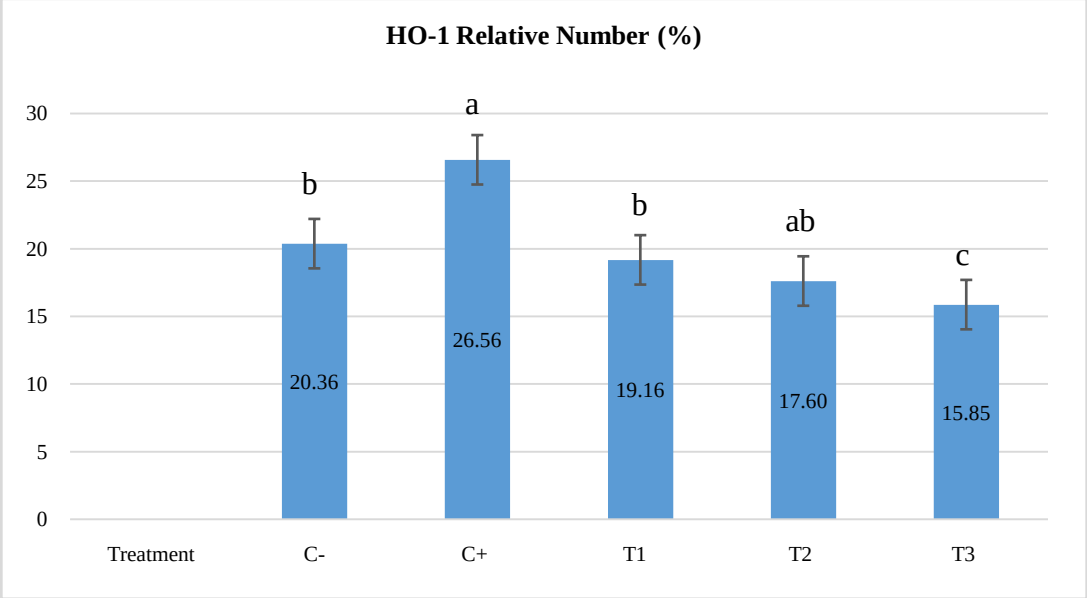

Figure 3 HO-1 levels in chickens receiving treatment; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

4 Discussions and Conclusion

Various chemical compounds, including caffeine, chlorogenic acid, trigonelline, carbohydrates, volatile flavor, and minerals, have been found in coffee. Chlorogenic acid (CGA), which protects coffee from microorganisms and insects, also exhibits antioxidant, immunostimulatory, anti-inflammatory, antibacterial, and hepatoprotective effects. Robusta coffee beans contain higher levels of chlorogenic acid (6.1-11.3 mg/gram) than other coffee beans (Farhaty and Muchtaridi 2016; Qosimah et al. 2020). Nrf2 is responsible for regulating many antioxidant genes or proteins, such as glutamate-cysteine ligase, glutathione peroxidase 1 (GPX1), thioredoxin reductase 1 (Txnrd1), NAD (P), H-quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST), SOD, catalase, peroxiredoxin (PRDX1), ferritin, and oxygenase-1 (HMOX1, HO-1). Additionally, the Nrf2 protein can stimulate HO-1 (Habtemariam 2019). Results of the study suggest that the Nrf2 level is higher in the negative group (C-) than in the positive group (C+), which can induce the SOD level. Under healthy conditions, a higher level of endogenous antioxidant SOD is present in the body, which neutralizes free radicals. When *Salmonella* bacteria are orally administered, they penetrate and invade the intestinal mucosa to proliferate and replicate intracellularly in chickens. Upon lysis or death of host intestinal cells, *Salmonella* releases LPS endotoxin inside the intestine, which can enter the lymph and blood circulation to spread into all organs and cause septicemia. This allows the bacteria to interact with phagocyte cells, macrophage, and monocyte receptors, which could stimulate the expression of IL-1, TNF, and other inflammatory cytokines (Frost et al. 2002), leading to an inflammatory response. Chickens infected with *S. enteritidis* bacteria (positive control) showed clinical symptoms of diarrhea.

The inflammation caused by *S. enteritidis* infection binds cytokine receptors to the surface of the intestine, leading to the downregulation of nuclear factor-2 (Nrf2) and SOD levels (Mshelbwala et al. 2017). SOD, or superoxide dismutase, is an antioxidant enzyme that comes in three forms: SOD1, SOD2, and SOD3, and among these, SOD1 makes up about 70% of SOD expression (Hwang et al. 2020). The mechanism of antioxidant activity involves decreasing the production of reactive oxygen species (ROS) and scavenging them (Mazur-Bialy and Pocheć 2021). SOD-1 can protect the mucosa, balance the immune system, and inhibit inflammation by blocking the entry of immune cells by regulating the intestinal immune response (Hwang et al. 2020). Antioxidants play a role in decreasing inflammation caused by *S. enteritidis* infection. Additionally, SOD catalyzes the conversion of anion superoxide radical into hydrogen peroxide (H_2O_2), which is toxic to bacteria (Hwang et al. 2020). A high concentration of *S. enteritidis* causes an increased inflammation response, but coffee can induce SOD to neutralize free radicals. All the treatment groups have chlorogenic acid (CGA) as an active compound of coffee, which is why these groups significantly increase SOD-1 levels compared to the positive control (Liang and Kitts 2015). SOD-1 blocks macrophage activation and inflammatory molecules by stopping excessive ROS generation. ROS is a product produced by macrophages and is essential in removing pathogens. However, excessive accumulation of ROS can cause oxidative damage to major cellular macromolecules such as proteins, lipids, and DNA, leading to cell necrosis (Mazur-Bialy and Pocheć 2021).

The study observed higher HO-1 molecules in the positive control group than in the other treatment groups. This may be due to the oxidative stress caused by *Salmonella* infection that upregulated the antioxidant HO-1. The expression of HO-1 is mainly found in the spleen and chicken tissue. Apart from the spleen, the expression of HO-1 is more profound in other reticuloendothelial systems, such as the liver and bone marrow (Ryter et al. 2006). HO-1 antioxidant expression responds to oxidative stress caused by intracellular bacteria. HO-1 is an enzyme that catalyzes heme degradation, which produces biliverdin, iron ions, and carbon monoxide. The degradation of heme releases Fe2+, CO, and biliverdin. Intracellular pathogens use heme as a source of iron for replicating, which directly increases their survival and growth in their host cells, including phagocytes. Due to tissue damage, CO (carbon monoxide) and biliverdin can activate the immune response to control pathogen replication and pathogenesis regulation (Costa et al. 2020). Biliverdin reductase releases bilirubin, the primary intracellular source of iron and carbon monoxide (Habtemariam 2019). The antioxidant effect of HO-1 is related to its ability to produce bilirubin, which eliminates ROS, including OH, singlet oxygen, and O2. The emergence of HO-1 also protects cells from oxidative disorders by regulating the degradation of heme and increasing biliverdin, which has strong antioxidant properties. CO and other heme products function as anti-apoptosis (Turkseven et al. 2005; Ryter et al. 2006). Other treatments (T1, T2, and T3) also produced antioxidant HO-1, but the level is lower than the positive control. Those treatment groups showed the dynamic content of coffee extracts can bind free radicals and lipopolysaccharides to achieve similar conditions with the negative control (Kim et al. 2017). HO-1 expression levels in healthy individuals under normal conditions act as natural, free radical prevention and have very low HO-1 expression levels but still respond to various transcriptional chemical activation and physical stimulation (Ryter et al. 2006). The active compound of coffee is enough to trigger HO-1, but its concentration is very low. Other compounds in coffee, such as tannin, alkaloid, and saponin, also have antibacterial properties (Pratita 2017). Saponin and tannin disturb bacteria's permeability and metabolism, inhibiting bacterial activity and growth. Alkaloids disrupt the formation of peptidoglycan in the cell (Khan et al. 2018). No difference was observed in chicken performance between all groups. Weight gain

in chicken is not significantly different between all treatment groups. However, a high dose of coffee extract might irritate the gut and trigger the increased expression of Nrf2 and SOD-1 but not HO-1. In this case, although Nrf2 was known to trigger the formation of HO-1, the level of HO-1 still decreased. The green coffee extract could reduce inflammation by increasing the level of the antioxidant enzyme SOD but not the HO-1 level. The Lampung robusta green coffee extract could potentially be used as an antioxidant against *S. enteritidis* infection in the 500 mg/kg BW dosage, as shown in the treatment group T1, by increasing the Nrf2 and SOD but decreasing the HO-1 level.

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Effect of *Psidium guajava* Juice on The Seminiferous Tubules Diameter and Epithelium Thickness in *Rattus norvegicus* Exposed by Lead Acetate

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Rats

Seminiferous tubules

White guava

ABSTRACT

Lead is one of humans and animals' most common and hazardous heavy metals. This study aimed to investigate the effect of white guava (*Psidium guajava*) fruit juice on the seminiferous tubule diameter and epithelium thickness in rats (*Rattus norvegicus*) exposed to lead acetate. The research design was a completely randomized design (CRD). A total of 25 male rats with an average weight of 200 grams were used for the study, divided into 5 treatment groups, each consisting of 5 rats. The treatments were as follows: the control group (C) was given distilled water orally; the T0 group was induced with lead acetate (50 mg/Kg BW) orally; the T1, T2, T3 groups were induced with lead acetate (50 mg/Kg BW) and then given 25%, 50%, and 100% concentration of white guava fruit juice, respectively. All the treatments were conducted for 14 days. The histopathology slides of the testis were made with HE staining, and the seminiferous tubule diameter and epithelium thickness were measured. The data were analyzed using One Way ANOVA and Duncan test ($p<0.05$). The results showed that the control group (C) which was given distilled water only had a seminiferous tubule diameter and epithelium thickness of 336.24±23.32 µm and 66.46±4.39 µm, respectively. The T0 group which was induced with lead acetate only showed a reduction in the diameter and epithelium thickness of seminiferous tubules (243.38±49.35 µm and 44.08±14.45 µm). The members of the T1, T2 and T3 groups showed positive effects on the diameter (323.49±22.82 µm; 314.41±13.04 µm; 325.04±16.88 µm, respectively) and epithelium thickness (56.36±3.36 µm; 60.50±3.81 µm; 66.744±9.50 µm, respectively). There was no significant difference reported between each group. The administration of guava juice to rats induced with lead acetate can positively affect the diameter and epithelium thickness of seminiferous tubules.

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1 Introduction

Heavy metal pollution is an unavoidable problem due to the rapid growth of the population (Yang et al. 2018). This issue is crucial because heavy metals are toxic, persistent in the environment, and bioaccumulative (Ali et al. 2019). Among the heavy metals, lead (Pb) is one of the most common pollutants found in the environment (Hansda et al. 2014). Lead (Pb) is known for its widespread presence and is considered one of the most hazardous heavy metals for human and animal health (Hansda et al. 2014; Song and Li 2015; Assi et al. 2016).

In recent years, studies have reported a decline in male fertility worldwide (Xu et al. 2019). This decline has been observed in humans and animals vulnerable to lead exposure (Assi et al. 2016). Lead has been found to have spermicidal potential in both humans and animals, as reported by Wang et al. (2013). Previous research has shown that exposure to lead in male rats can damage the reproductive system, such as changes in cell morphology, increased apoptosis, hormonal disruption, and impaired semen quality parameters (Lovaković 2020). Additionally, lead exposure in male reproduction causes the overproduction of Reactive Oxygen Species (ROS), which leads to the inhibition and alleviation of the activity of antioxidant enzymes such as alkaline phosphatase, superoxide dismutase, glutathione peroxidase, and catalase (El-Magd et al. 2017). This high ROS level caused by lead toxicity can lead to oxidative stress, which in turn can lead to testicular impairment (Sudjarwo et al. 2019).

Oxidative stress can damage cell membranes and disrupt hormone receptors that play a role in spermatogenesis, reducing germ cells (Al-Olayan et al. 2014). This decrease in spermatogenic and Sertoli cells can result in a thinning of the seminiferous epithelium and a reduction in the diameter of the seminiferous tubule (Tripathi et al. 2015). The diameter and thickness of the seminiferous tubules are often used as indicators of testicular toxicity (Vidal and Whitney 2014).

It has been found that antioxidants can protect the seminiferous tubules of testes from lead-induced toxicity by inhibiting free radicals (Abarikwu et al. 2020). Animal studies have demonstrated that herbal plant extracts containing various antioxidants can help to reduce oxidative stress induced by lead and thus protect against lead-induced damage (Diana et al. 2017).

Due to its delicious taste and flesh, white guava (*Psidium guajava*) is a popular fruit in Indonesia. It contains various bioactive compounds such as flavonoids, carotenoids, terpenoids, triterpenes, and more, which give it medicinal properties (Zhang et al. 2020). White guava is rich in antioxidants such as quercetin, lycopene, vitamin C, vitamin E, and vitamin A (Naseer et al. 2018), which help balance the antioxidant and oxidant levels in the body. This makes it beneficial for infertile males as it can improve sperm production (Naseer et al. 2018). This study focuses on the effect of white guava (*P. guajava*) fruit juice with different doses on testicular seminiferous tubules' diameter and epithelium thickness in rats exposed to Lead acetate.

2 Materials and Methods

2.1 Experimental Animals

The research utilized a completely randomized design (CRD). Twenty-five male rats (*R. norvegicus*) aged 10-12 weeks and weighing 200 grams were housed in the Faculty of Veterinary Medicine experimental animal laboratory at Universitas Airlangga in Indonesia.

2.2 Lead Acetate Preparation

Lead acetate ($Pb(CH_3COO)_2$) suspension was made from lead acetate powder diluted in distilled water. The dosage that was used in this research was 50 mg/KgBW.

2.3 Guava Juice Preparation

Around two kilograms of fresh white guava were used to make the juice. First, the guava was washed with clean water and then directly ground without any added solvent. The resulting juice was filtered with a fruit juice filter and stored in a container. For 100% concentration of white guava juice, no solvent was added. For 50 and 25% concentrations, distilled water was added to dilute the juice to the required dosage (Chin et al. 2020). White guava juice was prepared every two days and stored in a refrigerator at a temperature of about 5^{o}C. The oral dosages of white guava juice for rats in this study were 100%, 50%, and 25% concentration.

The rats were given lead acetate to induce toxicity, and after four hours, they were administered guava juice for 14 days. The treatment groups were as follows: distilled water only (C), rats induced with 50 mg/kg BW lead acetate (T0), rats induced with 50 mg/kg BW lead acetate and 25% concentration of white guava juice (T1), rats induced with 50 mg/kg BW lead acetate and 50% concentration of white guava juice (T2), and rats induced with 50 mg/kg BW lead acetate and 100% concentration of white guava juice (T3).

2.4 Testis Histology Sample Preparation

After 14 days of treatment, all the rats were anaesthetized with a combination of ketamine (100mg/kg BW) and xylazine (10mg/kg BW) given intraperitoneally. They were then humanely sacrificed by cervical dislocation, following the guidelines set out by Flecknell (2015). The rats' abdomen was surgically opened to collect their testes, which were subsequently stored in a container filled with 10% formalin solution.

Table 1 Effect of the various imposed treatments on the diameter and the epithelium thickness of seminiferous tubules in rats

Treatment Groups	Seminiferous Tubules Diameter (µm)	Epithelium Thickness of Seminiferous Tubules (µm)
C	$336.24^{b} \pm 23.32$	$66.46^{b} \pm 4.39$
T0	$243.38^{a} \pm 49.35$	$44.08^{a} \pm 14.45$
T1	$323.49^{b} \pm 22.82$	$56.36^{b} \pm 3.36$
T2	$314.41^{b} \pm 13.04$	$60.50^{b} \pm 3.81$
T3	$325.04^{b} \pm 16.88$	$66.74^{b} \pm 9.50$

Data are mean of five replicates; ± Standard Deviation of the mean; Values without common letters differ significantly at LSD P<0.05

2.5 Examination and Data Analysis

The diameter of seminiferous tubules and the thickness of the epithelium will be measured by examining testis histology slides stained with hematoxylin-eosin (HE) using a microscope with 200X magnification. The resulting data will be analyzed using One-way ANOVA, followed by Duncan's test for multiple comparisons to compare each group respectively. A statistically significant difference will be considered if $P < 0.05$. The statistical analysis will be performed using the Statistics Product and Service Solution (SPSS) 20.0 software (Al-Arif 2018).

3 Results

Results presented in Table 1 revealed the effect of various imposed treatments on the seminiferous tubule diameter and epithelium thickness. The rats given lead acetate (T0) showed a reduction in the mean value diameter of seminiferous tubules (243.38 ± 49.35 µm), while this value was reported 336.24 ± 23.32 µm for the control group (C). The group that was administered with 25% concentration (T1) of white guava juice shows a tubule diameter of 323.49 ± 22.82 µm, while it was reported 314.40 ± 13.04 µm and 325.04 ± 16.88 µm mean value in case of 50% (T2) and 100% (T3) concentration of white guava juice respectively and this was significantly ($P<0.05$) higher than T0 group but no significant differences was reported in the various treatment groups.

Similar findings have been reported regarding the mean value of the seminiferous tubule epithelium thickness (Table 1). Among the imposed treatments, the lowest mean value was reported in the T0 group (45.79 ± 15.65 µm), while the highest mean value (66.45 ± 4.38 µm) among all groups was reported from the only water control treatment. In the case of guava juice treatments, seminiferous tubules epithelium thickness is increased with the increasing concentration of guava juice and the highest thickness was reported from the treatment group T3 (64.72 ± 12.18 µm), and it was followed by the mean value of T2 (60.50 ±3.81 µm) and T1 (59.21 ± 4.63 µm) groups but this difference was not significantly different ($P<0.05$).

4 Discussion

The study's findings showed that treating a group of rats with lead acetate (T0) decreased the mean value of their seminiferous tubule diameter and epithelium thickness. These results align with the findings of Dorostghoal et al. (2020) and Widawati et al. (2017), demonstrating that lead toxicity can reduce the seminiferous tubule diameter and epithelium thickness in rats' testes. Lead exposure can cause oxidative stress at the testicular level due to increased reactive oxygen species (ROS). This, in turn, triggers the process of germ cell apoptosis (Nurkarimah et al. 2017). When there is an imbalance between ROS and antioxidants in the body, the mitochondrial pores become oxidized. As a result, the mitochondrial membrane permeability is disrupted, allowing free radicals and cytochrome c to leak into the cytosol (Abdrabou et al. 2019). Once cytochrome c enters the cytosol, it binds to another protein, activating the caspase cascade and initiating the mitochondria-mediated apoptosis pathway (Selvakumar et al. 2013). Lead exposure also alters cytochrome c release and Bcl-2/Bax signaling, ultimately resulting in caspase-3-dependent death (Kiran Kumar et al. 2009; Corsetti et al. 2017).

According to recent research, exposure to lead may cause reproductive toxicity, leading to a higher rate of cell apoptosis. Lead exposure also induces oxidative stress, which results in the peroxidation of membrane lipids, causing a loss in membrane integrity and a decrease in membrane potential. This results in ATP depletion and DNA fragmentation, ultimately leading to cell necrosis (Zachary and McGavin 2012). Additionally, lead exposure causes a decrease in endogenous antioxidants such as catalase (CAT), glutathione peroxide (GPx), and glutathione (GSH), leading to a high level of ROS and oxidative stress (Vigeh et al. 2011). Moreover, lead toxicity can cause hypothalamic-pituitary-testicular axis disorders, which can reduce the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Apriliani et al. 2013). This reduction in FSH and LH leads to a disruption of spermatogenesis, causing a decrease in spermatogenic cells and a reduction in the epithelium's thickness and the seminiferous tubules' diameter (Ramu and Jeyendran 2013).

The results presented in Figure 1 indicate that the T1 group of rats had a higher epithelium thickness than the T0 group. Similar results were observed for the T2 and T3 treatment groups. Additionally, significant differences were observed between the control group and the T0, T1, T2, and T3 groups. The results

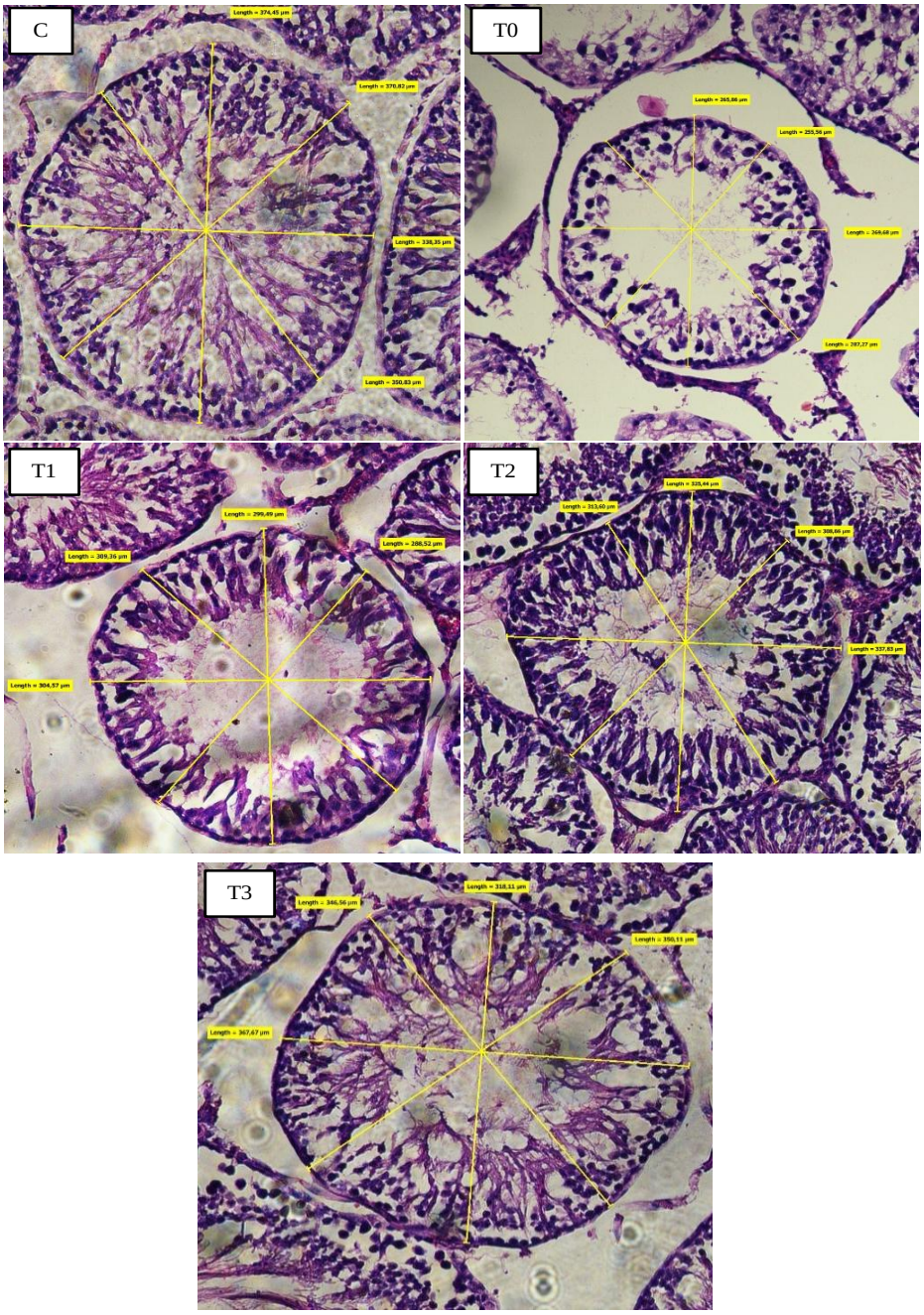

Figure 1 Histopathology and diameter of seminiferous tubules in groups C, T0, T1, T2, and T3 (200x, hematoxylin-eosing staining)

suggest that a 25-100% concentration range produced positive results and maintained normal diameter and epithelium thickness values similar to the control group (C).

Antioxidants are compounds that eliminate and scavenge the formation of ROS. Various studies have evaluated the effect of antioxidants on male fertility (Susanti et al., 2020). Many phytonutrients, such as lycopene, quercetin, vitamin C, and vitamin A, act as antioxidants and are found in white guava (Naseer et al. 2018). In cases of lead toxicity, quercetin blocks oxidative stress to protect male reproductive health and inhibits apoptosis by acting as a natural antioxidant and metal chelator (Meles et al. 2021). Several studies have shown that lycopene protects testes by reducing apoptosis and enhancing the scavenging of ROS. It also increases Bcl-2 expression, which means that lycopene is crucial in modulating and reducing the apoptotic process (Antonuccio et al. 2020; Trejo-Solis et al. 2013). Vitamin C, another antioxidant found in white guava, maintains the oxidation cycle of vitamin E.

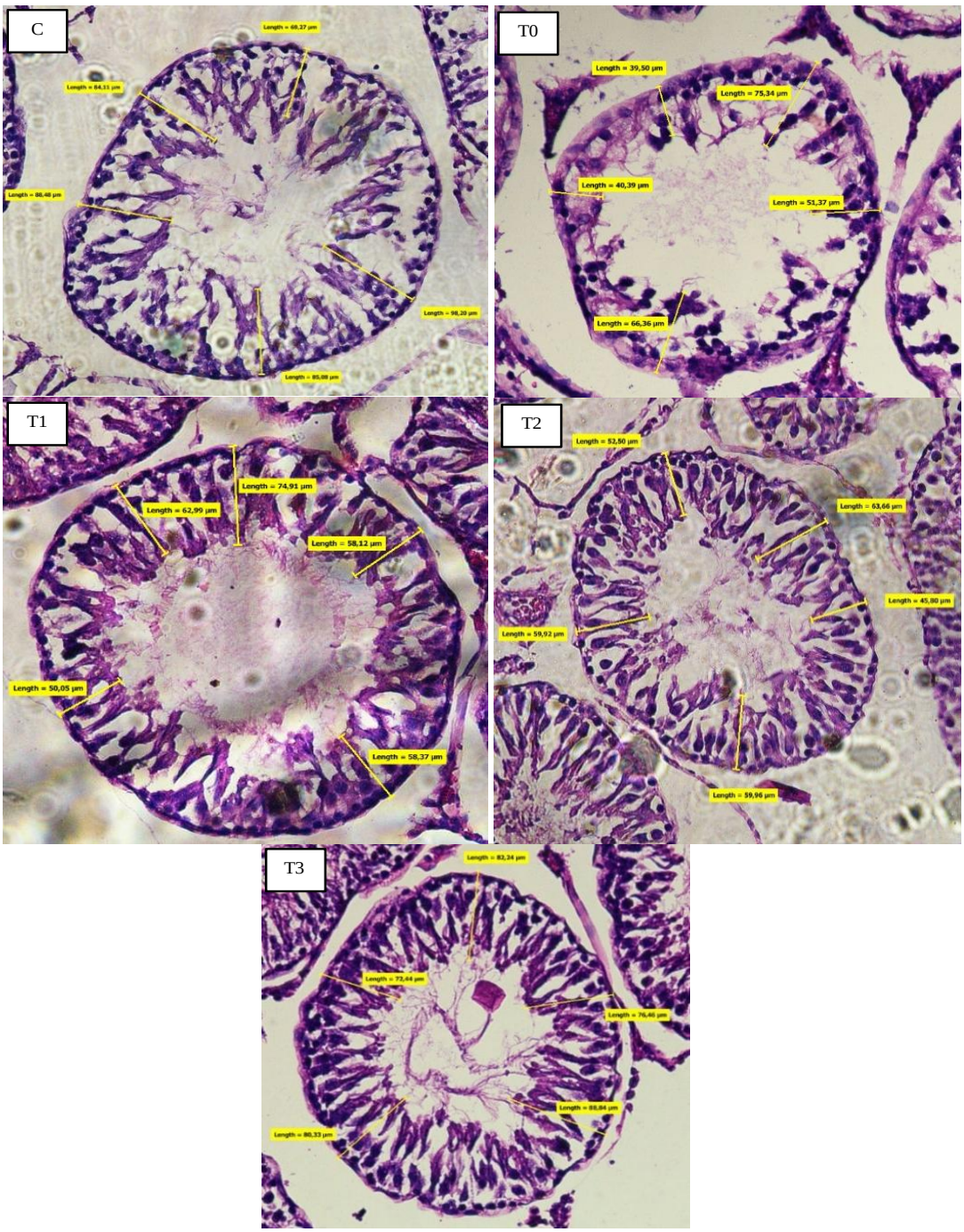

Figure 2 Histopathology and epithelium thickness of seminiferous tubules in groups C, T0, T1, T2, and T3 (200X, hematoxylin-eosing staining)

It neutralizes ROS and helps protect spermatogenic cells from oxidative damage (Freitas and de Oliveira 2018). Vitamin A, also present in white guava, can counteract free radicals (Hogarth and Grisworld 2010). Due to the presence of vitamins A and E, white guava fruit could positively affect the diameter of the seminiferous tubules in rats' testes induced with lead acetate.

In this study, no significant difference was found in the mean value of the diameter of seminiferous tubules between the negative control group (T0) and groups T1, T2 and T3 (Figure 2). These results are consistent with the findings of Wardani et al. (2019), where a higher concentration of extract led to a greater effect. Guava fruit juice contains a significant amount of moisture (water),

crude fiber, protein, fat, ash, and carbohydrates such as phytonutrients, which have an antioxidant effect (Dakappa et al., 2013). However, the composition of white guava fruit juice can lead to poor bioavailability (McClements et al., 2015), and the blood-tissue barriers actively increase the impermeability of phytonutrients from the white guava juice (Mao et al. 2020). As a result, the potential benefits of many of the antioxidant phytonutrients may not be optimally effective.

Conclusions

The study's results suggest that different white guava fruit juice concentrations significantly affect rats exposed to lead acetate. The fruit juice plays an essential role in maintaining the diameter and epithelium thickness of the seminiferous tubules. While no concentration-dependent effect on the diameter and epithelium thickness of the seminiferous tubules in rats induced with lead acetate was reported, the value of these two parameters increased with the increasing concentration of white guava juice.

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Ovine pulmonary adenocarcinoma (OPA) in sheep: an update on epidemiology, pathogenesis and diagnosis

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ABSTRACT

Ovine pulmonary adenocarcinoma (OPA) is a spontaneous lung tumor in sheep caused by Jaagsiekte sheep retrovirus (JSRV) belonging to the *Retroviridae*. The primary aim of this review work is to give brief insights into the epidemiological aspects of OPA based on a meta-analysis of available research work. This review article also discussed pathogenesis, diagnostic tests and control strategies available for OPA in Sheep. This will help in developing future strategies for disease-free status in India. This disease is endemic in Europe, Africa, Asia, and American continents, causing significant economic losses due to chronic respiratory illness and persistent infections in flocks. The virus is unique among retroviruses with selective affinity to lungs and is the only virus known to cause spontaneous lung tumors in sheep. The incubation time ranges for sheep with naturally occurring OPA ranged from one to four years. There are two pathological forms of the disease: classical and atypical. At an early stage, OPA is difficult to detect in sheep due to a lack of preclinical diagnostic methods, as JSRV is poorly immunogenic and doesn't induce an immune response. PCR, histopathology, and immunohistochemistry are recommended methods for OIE diagnosis. To become a JSRV-free country, mandatory surveillance, detection, and removal of positive animals are required, as OPA is difficult to control due to a lack of vaccines and preclinical diagnostic tests. Due to its similar histological and molecular pathogenesis to that of human lung cancer, OPA is considered an ideal large animal model of human lung adenocarcinoma.

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1 Introduction

An infectious and spontaneous lung tumor illness known as ovine pulmonary adenocarcinoma (OPA), which affects sheep and infrequently goats, is caused by Jaagsiekte sheep retrovirus (JSRV), which is a member of the *Retroviridae* family (Griffiths et al. 2010; Toma et al. 2020). The disease has similar histomorphological features and tumorigenic pathways to human lung adenocarcinomas, making it a vital prototype for understanding and studying human pulmonary cancers (Youssef et al. 2015; Gray et al. 2019a*)*. JSRV is the only known virus to cause spontaneous lung tumors by inducing the expression of the JSRV envelope protein (Env) through the binding of major receptor Hyl2 and the activation of the phosphatidylinositol 3-kinase/Akt-signalling pathway. This makes JSRV unique among other retroviruses (Palmarini and Fan 2001). Due to the identical envelope glycoproteins, JSRV and Enzootic Nasal Tumor Virus (ENTV) of sheep and goat nasal adenocarcinomas can cause cell transformation and malignancy (Monot et al. 2015). The occurrence of co-morbidities has been suggested by the frequent reports of JSRV-induced OPA in sheep flocks together with MVV and CAEV in recent years (Rosato et al. 2023). In a similar study in India, MVV, JSRV, and mycoplasma co-infection were reported in sheep and goats (Valecha et al. 2023).

OPA, also known as driving sickness, pulmonary adenomatosis of sheep, or sheep pulmonary adenocarcinoma (SPA), was initially identified as Jaagsiekte in South Africa during the 1800s. The name is taken from the Afrikaans word for "chasing sickness." (Jaagsiekte) (York and Querat 2003). In 1930, an outbreak of OPA occurred in sheep flocks in Iceland, and the disease was successfully eradicated by mass slaughter and culling of affected animals during the 1950s. OPA causes roughly 70% of lung tumors in sheep and is endemic in a few countries (Peru, Scotland, the UK, South Africa, and Spain). Although this disease posed substantial economic and animal welfare complications, particularly in sheep-farming countries, countries like Australia, New Zealand, the Frankland Islands, and Iceland are free from OPA (Sharp and DeMartini 2003). Iceland successfully eradicated OPA through a rigorous slaughter policy during the 1950s (Sanna et al. 2001). JSRV is a highly host-specific virus infecting sheep, occasionally goats and mouflon (ancestor of sheep) but not to other livestock species or humans (Sanna et al. 2001; De Las Heras et al. 2003; Wootton et al. 2006).

The first genetically modified livestock, Dolly, the cloned sheep, was also diagnosed with tumorous lung growth caused by JSRV and euthanized in 2003 at Roslin Institute. JSRV-infected sheep show symptoms like anorexia, progressive dyspnea, and debilitating condition. In severe cases, sheep may die due to respiratory failure or due to the secretion of massive amounts of fluid from proliferating Type II pneumocytes in lung tumors. The JSRV incubation period varies from six months to four years. However, it is shorter when newborn lambs are infected. Although OPA infects sheep of any age group, it is most frequently reported in adult sheep of 2 and 4 years of age (Fenner et al. 2015). The lack of T-cell responses and circulating JSRV-specific antibodies in sheep with naturally occurring or experimentally produced infection makes preclinical detection of JSRV challenging. However, JSRV capsid protein-specific antibodies were detected after the administration of recombinant JSRV proteins (Griffiths et al. 2010). For the epidemiological investigation and diagnosis of OPA, OIE has recommended PCR and RT-PCR methods targeting the LTR region of JSRV, particularly for the samples from lungs, mediastinal lymph nodes, bronchoalveolar lavage fluid, and PBMCs.

This review briefly discussed OPA and its causative agent, pathology and pathogenesis, epidemiology, and control aspects. We also gave brief insights into different diagnostic methods employed for the early detection of JSRV in Sheep. This will help in understanding JSRV and future strategies to be implemented for disease-free status in India.

2 Causative agent and genome organization

The mature virions are enveloped with glycoprotein spikes on the surface. The size of the virus ranges from 80-100 nm in diameter and has a three-layered structure. The genome has helical symmetry enclosed in an icosahedral capsid with a nucleoprotein complex (Figure 1). These glycoproteins (peplomers) have surface and transmembrane domains (SU and TM) vital in virus attachment to the host cell.

The retroviral genome is diploid with two RNA copies, linear positive-sense, single-stranded RNA. The four main genes that comprise the roughly 7.5 kb long JSRV genome are gag, pro, pol, and env. These genes encode several proteins with varied roles in viral replication (Figure 2; Table 1). The U3 nucleotide sequence of the LTR region varies between exogenous JSRV, endogenous retrovirus of sheep (enJSRV), and ovine enzootic nasal tumor virus (ENTV). Additionally, there are two types of exogenous retroviruses based on the U3 sequence and the presence of ScaI restriction sites in the viral genome: type I exJSRVs (isolates from South Africa and Kenya) and type II exJSRVs (isolates from Wyoming, USA and UK) (Cousens et al. 2009; Griffiths et al. 2010).

3 Epidemiology of OPA

3.1 Disease Transmission and Host Susceptibility

OPA is mainly spread via aerosols or droplets, according to epidemiological research. Feeding the colostrum and milk of OPA-infected sheep is another way the infection spreads spontaneously.

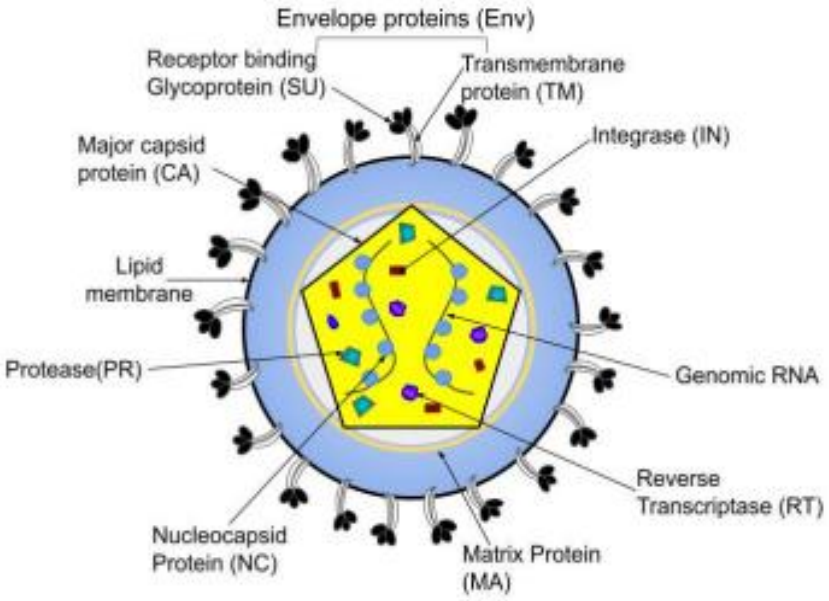


Figure 1 Structure of a mature JSRV.

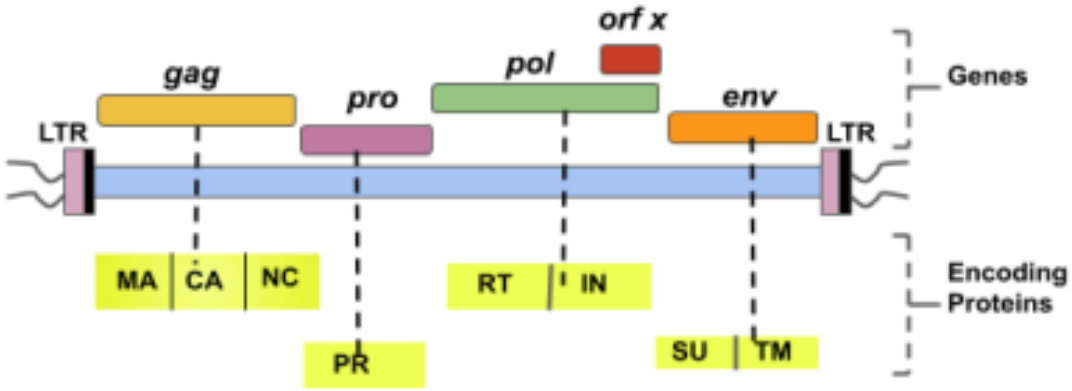


Figure 2 JSRV genome structure.

When a mother's milk is contaminated, JSRV proviral DNA can infect newborn lambs by crossing their gut epithelial barrier and infecting macrophages and somatic cells that have already experienced OPA (Voigt et al. 2007). The JSRV provirus was also found in the blood of lambs fed milk and colostrum from ewes infected with the virus (Grego et al. 2008). In spontaneous infections, the incubation time of OPA can take months or even years. However, under experimentally induced infection, particularly in lambs, it is as short as 2-6 weeks (De Las Heras et al. 2003). Sheep 2-3 years of age are more susceptible than older animals 4– 7 years. However, OPA-induced lung tumors were diagnosed in sheep up to 11 years of age at slaughter with no apparent clinical disease. Breeding flocks are more prone to disease persistence at the farm level, and few breeds are highly susceptible to OPA; for example, OPA afflicted 10% of Adalbol sheep, while the Gottorp breed in Iceland demonstrated greater susceptibility, with some farms losing nearly 90% of their Sheep (Palmarini et al.1996). Based on earlier reports, during the winter season, there are more occurrences of disease events (Griffiths et al. 2010).

In European countries, the classical form is common, and more than 50% of animals in affected flocks die due to respiratory failure, causing considerable economic losses (Ortega et al. 2023). The atypical type of OPA is less common than the classical version and less contagious. It has been mostly described in Spain, Peru, Iran, and India (Garcia-Goti et al. 2000; Azizi et al. 2014; Mishra et al. 2021). In endemic countries, the mortality rate ranges between 1-5%. However, it may reach up to 50% in case of outbreaks in newly infected flocks (Griffiths et al. 2010; Toma et al. 2020). As OPA is not included in a list of notifiable diseases that require reporting, there are few epidemiological investigations of the disease. Hence, only a few countries have data on disease

outbreaks, prevalence, diagnostic and control aspects based on clinical, pathological, and molecular diagnosis. Recently, more attempts have been made to develop and employ sensitive and rapid preclinical tests based on genomic detection to diagnose OPA (Ortin et al. 2019).

A meta-analysis using a meta package in R-Software was carried out based on a systematic review of research conducted on OPA for the past 30 years (1988-2022). The prevalence of OPA was estimated from 1% (1-2%) with the common effect model and 14% (5-27%) with the random effects model (CI- 95%, p<0.01) (Table 2).

Table 1 JSRV genes, along with encoded proteins and their functions

Gene	Size	Proteins encoded	Functions
gag	1838 bp (263..2101)	Nucleocapsid (NC), matrix (MA), and capsid (CA)	Encapsulation of viral RNA and creation of viral core
pol	2333 bp (3108..5441)	Reverse transcriptase (RT) and integrase (IN)	viral life cycle
pro	869 bp (1993..2862)	protease	Assembly of virions and budding
Env	1847 bp (5350..7197)	surface (SU) and transmembrane (TM)	Virus-host receptor attachment to HlyA on the host cell membrane
orf-x	500 bp (4606..5106)	nonfunctional ORF X protein	Unknown function

Information is generated based on the Griffiths et al. (2010), Armezzani et al. (2014), Fenner et al. (2015)

Table 2 The epidemiological studies of JSRV/OPA in sheep across the world

STUDY	% Positivity	Total samples	Country
Sarkar et al. (1988)	3	1872	India
Garcia-Goti et al. (2000)	16	16	Spain
Sanna et al. (2001)	4	4	Italy
Gonzalez et al. (2001)	10	10	Spain
Morozov et al. (2004)	19	64	Africa
Uzal et al. (2004)	8	40	Argentina
De las Heras et al. (2005)	31	104	Scotland
Maeda et al. (2011)	0	40	Japan
Sayyari and Mohamadian (2012)	4	3985	Iran
Amini and Mostafa (2013)	132	5200	Iran
Azizi et al. (2014)	9	1000	Iran
Kumar et al. (2014a)	44	903	India
Cousens et al. (2015)	31	3385	UK
Sonawane et al. (2016)	6	75	India
Oda and Youssef (2011)	7	550	Egypt
Bahari et al. (2016)	26	99	Iran
Jassim et al. (2017)	10	180	Iraq
Mishra et al. (2018)	23	800	India
Singh et al. (2018)	31	1350	India
Lee et al. (2019)	30	1911	Ireland
Mansour et al. (2019)	25	250	Iraq
Samatha et al. (2019)	22	150	India
Al-Husseiny et al. (2020)	22	50	Iraq
Toma et al. (2020)	34	2693	Romania
Shi et al. (2021)	0	1372	China
Abd-Abass and Khudhair (2022)	21	195	Iraq

3.2 JSRV Prevalence in Indian Sheep

In India, for the first time, Damodaran (1960) reported OPA in Karnataka in a flock of sheep that had chronic pneumonia, and the diagnosis was made as Onderstepoort; the illness resembled Jaagsiekte in South Africa. From 1972 to 1977, the prevalence of OPA was up to 2.46% in Sheep of Andhra Pradesh (Devi et al. 2014). Based on the histopathological evaluation, pneumonic lesions were found in 204 out of 1872 sick sheep lungs in West Bengal, and three cases among them were identified as pulmonary adenomatosis (Sharp et al.1983). Similarly, out of 203 ovine lungs with various diseases, 21.68% of cases were detected as pulmonary adenocarcinoma (Kumar et al. 2014b). Further, based on the histological diagnosis, a 4.87% prevalence of OPA (44/903) in sheep was documented in the Southern parts of India (Kumar et al. 2014b). Sonawane et al. (2016) reported OPA during the necropsy examination of 75 sheep that had died naturally, and six cases had typical adenocarcinoma-like lung lesions positive for JSRV by PCR. In another study, 1350 lungs were examined in the Indian states of Delhi, Andhra Pradesh, and Uttar Pradesh, with 31 (2.29%) animals testing positive for JSRV (sheep-3.49%, 31/888; goats -0.00%, 0/462). Capsid antigen of JSRV was demonstrated by employing immunohistochemistry in alveolar macrophages, type II pneumocytes, lymphocytes, plasma cells, and in a few normal bronchiolar epithelial cells in lung tumours in sheep. PCR tests targeting the U3 and gag region of JSRV revealed the presence of JSRV DNA in sheep lung tumors (Singh et al. 2018). In a similar study, 150 sheep lungs suspected of having OPA in Andhra Pradesh were screened by pathological lesions and U3-hnPCR, and 22 samples (14.7%) were positive for JSRV (Samatha et al. 2019).

4 JSRV viral pathogenesis

The JSRV virus replicates particularly in type II pneumocytes and bronchiolar Clara cells of the lungs (Toma et al. 2020). These cells express a specific receptor called glycosyl phosphatidyl inositol-anchored hyaluronidase-2 (Hyal2) and have been identified as the major receptor for JSRV. Major determinants of JSRV expression in hosts and cell tropism include the LTR and the JSRV envelope regions. The surface glycoprotein (SU) of JSRV attaches to the Hyal2 receptor and enters the cell via the endocytic pathway. The virion is penetrated by Env-Hyal2, which then causes the reverse transcriptase (RT) to transcribe the ssRNA and create a dsDNA. When the virus enters the nucleus during mitosis, viral integrase finds numerous places in the cell's nucleus to incorporate the viral DNA. A provirus enters the host genome and emerges from the cell as an immature virion. During this process, due to replication and infection of JSRV in lung epithelial cells, a large quantity of lung surfactant is produced from tumor cells and discharged from nostrils in sheep.

5 Clinical Pathology of OPA

An increased number of deaths in sheep flocks due to chronic pneumonia not responding to antibiotics can be a viable sign for suspecting OPA. When tumor progression and lesions are fully established, after a prolonged incubation period, affected sheep exhibit clinical symptoms such as debility, weight loss, and dyspnea. Later, as time passed, symptoms progressed to abdominal breathing, orthopneic posture, dilated nostrils, and open-mouth breathing (York and Querat 2003). The disease progression is usually acute in lambs, which frequently die within a few days,

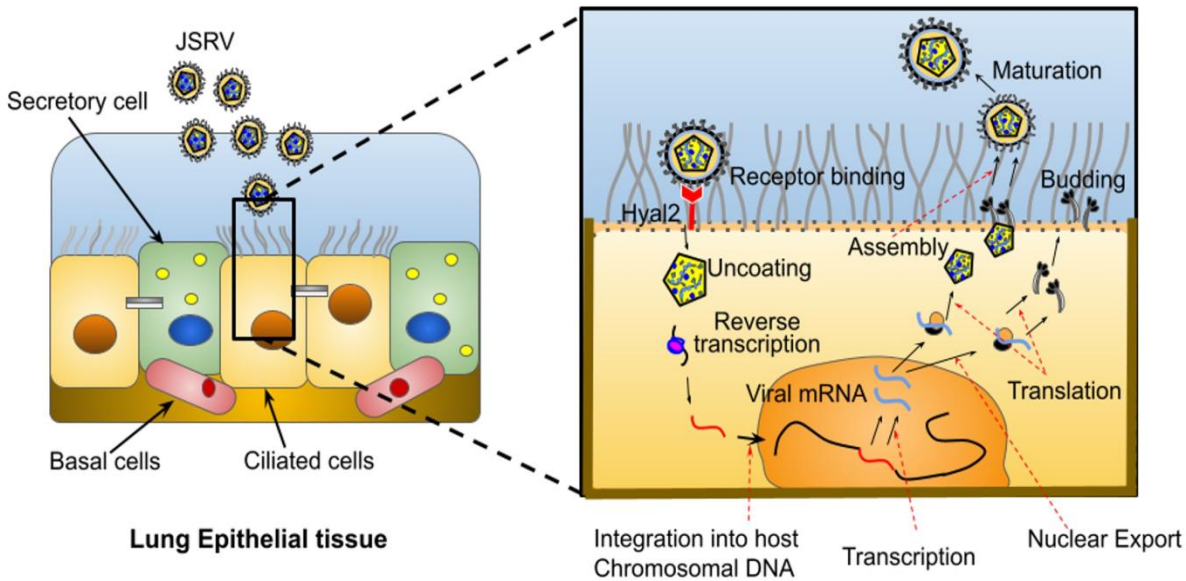


Figure 3 JSRV viral replication and pathogenesis in lungs

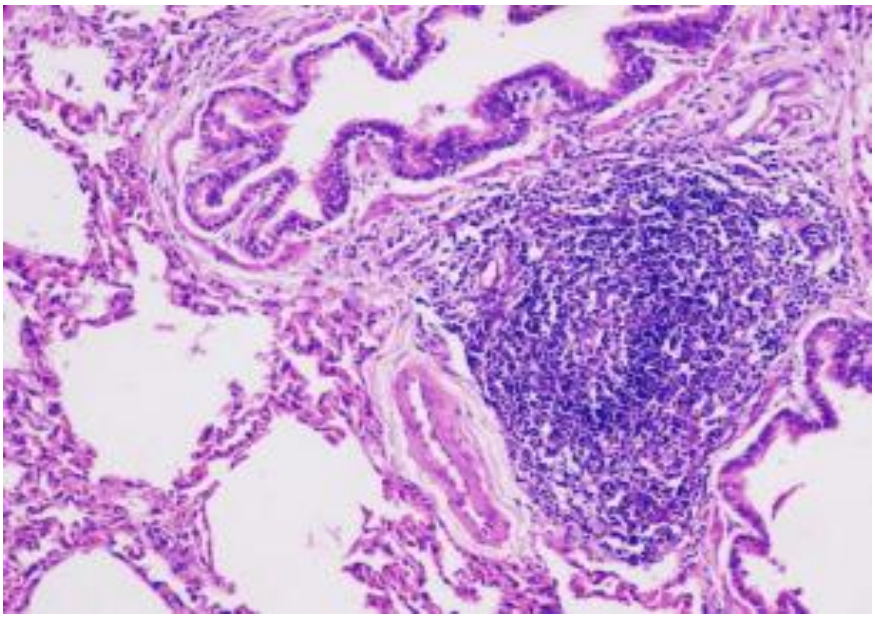
whereas in adult animals, it is slowly progressive, with clinical symptoms appearing over weeks or months before the animal dies (De las Heras et al. 2005). The clinical indications of the affected sheep are typical and include clear, foamy exudate coming from the nasal passages. The infected animal can produce frothy exudate up to 300-500 ml per day and can be collected by lifting the rear legs and lowering the head of the sheep. This is one of the practical diagnostic tests in OPA called the Wheelbarrow test (Cousens et al. 2009). When inoculated to healthy sheep, this fluid contains a large amount of JSRV viral particles, which induces disease conditions. Despite the characteristic clinical signs in affected animals, in some cases, the lung fluid is absent; therefore, diagnosis is based on gross and histopathological lesions and the detection of the JSRV genome by PCR in lung lesions (Griffiths et al. 2010).

6 Gross and Histopathology of OPA

Post-mortem examination of the advanced stage of the naturally infected animal carcass appears to be a debilitating type with frothy exudate-filled trachea and nasal passages. When the thoracic cavity opens, there will be asymmetrically enlarged lung lobes, which fail to collapse and look dark, edematous, and bulky in weight. Upon palpation of the lungs, consolidated nodules/tumorous mass in cranioventral surfaces and other lobes will be distinctly visible. Incision of affected lobes shows a clear demarcation between the tumor and normal pink lung parenchyma. The consolidated nodule shows a grey, solid, granular surface of the tumor with frothy exudate oozing out of the lesions (Griffiths et al. 2010). The slashed surface of the tumor is moist, causing the bronchioles in the afflicted regions to discharge foamy exudates. This fluid also accumulates in the upper trachea and discharges from the nostrils. All these lesions represent a classical type of OPA (Garcia-Goti et al. 2000). A single or more white, spherical, tiny nodule of 3-5cm, buried in the diaphragmatic lobe may represent the appearance of a lung tumor in some OPA cases. These nodules seem hard, gritty, and stellate upon incision; the surface is dry cut, and there is no frothy discharge from the bronchi. This form is atypical OPA (De las Heras et al. 1992). Enlargement and edema of mediastinal lymph nodes with or without metastatic tumor lesions are consistent with both forms of OPA (Garcia-Goti et al. 2000).

Based on histomorphological lung lesions, OPA were classified into two distinct pathological forms: classical and atypical (Garcia-Goti et al. 2000; De Las Heras et al. 2003; Mishra et al. 2021). In classical form, the affected lung parenchyma shows mixed adenocarcinoma with papillary and acinar growth patterns with alveolar epithelial cells converted to cuboidal or columnar cells. There will be a clear demarcation between neoplastic foci and unaffected lung parenchyma in classical OPA rather than atypical form. A pathognomonic lesion of classical OPA is the localized infiltration of macrophages around a neoplastic foci that resembles acinar and papillary patterns and arises from the alveolar and bronchiolar epithelia (De Las Heras et al. 2003). The primary proliferating tumor cell type, representing over 80% of cell populations, is the Type II alveolar epithelial cells (Pneumocytes). This is followed by Clara cells (8–10%) and other undifferentiated cells (9–10%) (Platt et al. 2002). In malignant tumors, pleomorphic cells with high mitotic index form into solid masses of necrosis foci. The infiltration of macrophages and lymphocytes in the fibrovascular connective tissue scaffold around the tumor can also

A



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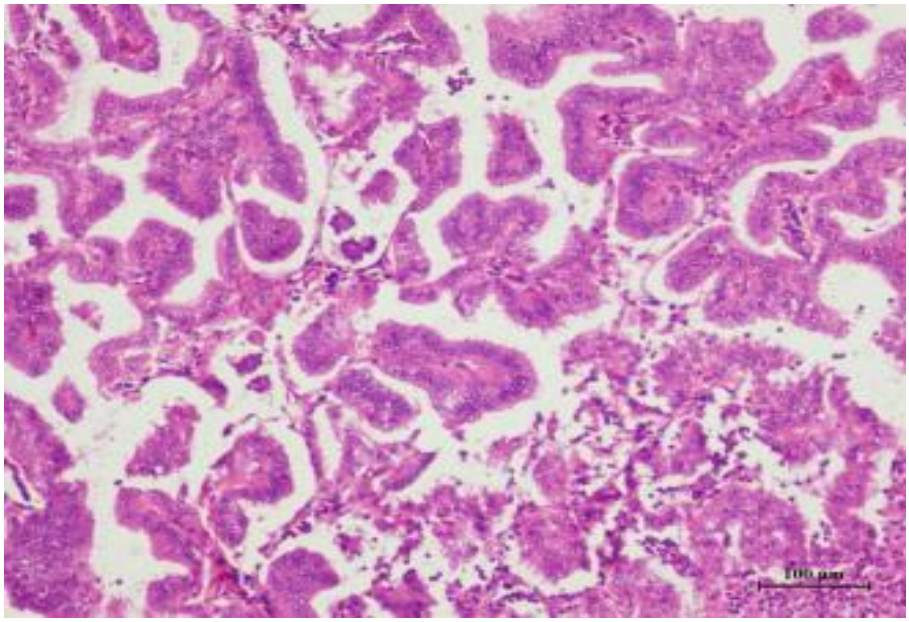

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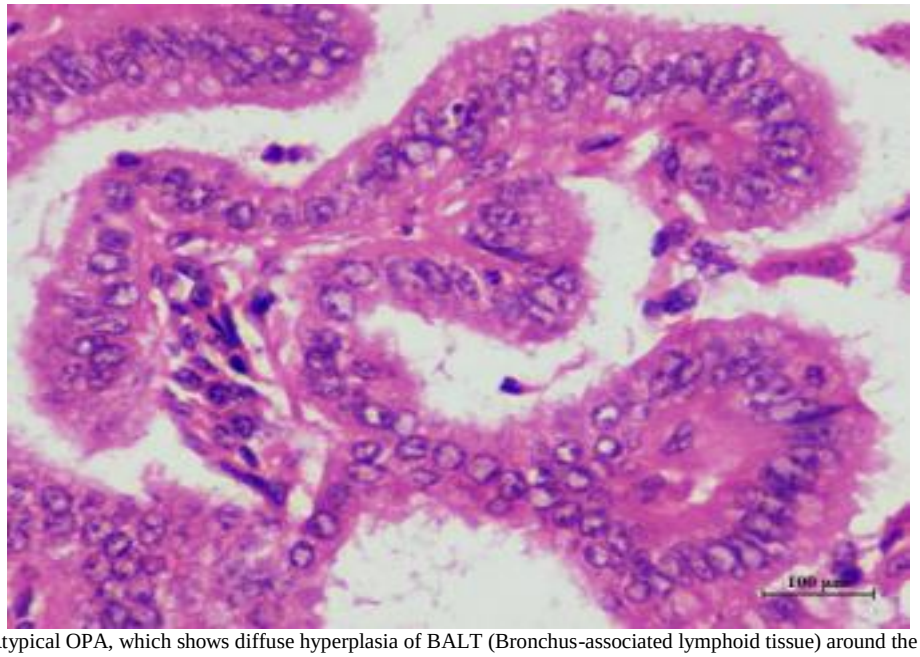

Figure 4A - Atypical OPA, which shows diffuse hyperplasia of BALT (Bronchus-associated lymphoid tissue) around the bronchiole in OPA-affected sheep (H&E, X 100); 4B - Classical OPA in which Lung parenchyma showing extensive hyperplasia and acinar type projections of adenocarcinomatous lesions (H&E, x100); 4C. Acinar and papillary growth patterns of alveolar epithelial cells converted to cuboidal or columnar cells (H&E. x400)

be observed. Mediastinal lymph nodes show metastatic neoplastic cells in acinar or papillary forms similar to lung lesions. However, there is a lower frequency of metastasis in the atypical form of OPA (De Las Heras et al. 2003). As with classical OPA, the histological alterations in atypical OPA are strikingly similar. In the abnormal variant, the lungs get invaded with connective tissue fibers, lymphocytes, and plasma cells. There is no clear-cut demarcation of tumor foci with surrounding lung parenchyma in an atypical form. The excessive proliferation of Bronchus-associated lymphoid tissue (BALT) can be observed around the neoplastic areas (Figure 4). Due to the limited tumor progression and the absence of lung fluid, the atypical form of OPA appears only as a subclinical form and is usually diagnosed in slaughterhouse specimens. Few studies also documented the peribronchial, peribronchiolar, and perivascular infiltration of mononuclear cells in atypical OPA (Sonawane et al. 2016).

7 Diagnosis of OPA/JSRV

7.1 Molecular Diagnosis of OPA by PCR and Sequencing

The U3 region in the long terminal region of JSRV is the target for single-step and hemi-nested PCRs, widely employed for epidemiological research in sheep flocks (Palmarini et al.1999). LTR-PCR is an instrumental test to detect JSRV proviral DNA specifically in peripheral blood mononuclear cells (PBMCs) in both clinical and experimentally induced infection in Sheep (De las Heras et al. 2005; Lewis et al. 2011), bronchoalveolar lavage fluid in sheep (Voigt et al. 2007), lung tumor lesions, lymphoid tissues (Singh et al. 2018; De las Heras et al. 2021), milk, and colostrum (Quintas et al. 2021). PCR testing in bronchoalveolar lavage proved more sensitive than the blood PCR (Cousens et al. 2009).

In sheep and goats, the endogenous Jaagsiekte Sheep Retrovirus (enJSRV) is present in at least 15–20 copies of the genome. Exogenous (exJSRV) and endogenous (enJSRV) retroviruses can be distinguished from one another by the presence of the ScaI restriction site in the Gag gene and the "LHMKYXXM" motif in the envelope protein. However, the ScaI restriction site is found in oncogenic JSRVs but absent in endogenous retroviruses in sheep, and the "LHMKYXXM" pattern is found in all exJSRVs but not in enJSRVs (Shi et al. 2021). The whole genome sequencing analysis of JSRV from India revealed a close relationship with China and USA isolates (Mishra et al. 2018). The hemi-nested PCR and partial gene sequencing of the U3 region of JSRV have shown 96–100% homology to the UK strain and 88–93% homology to the South African strain in South India.

7.2 Immunohistochemistry of JSRV in OPA lungs

A crucial diagnostic tool for OPA is detecting sheep lung cells exhibiting JSRV antigens. JSRV has been widely demonstrated in alveolar Type II pneumocytes, bronchiolar epithelial cells, macrophages, lymphocytes, and cytoplasm using monoclonal and polyclonal antibodies produced in rabbits against JSRV capsid proteins and envelope proteins (Murgia et al. 2011; De las Heras et al. 2014; Mishra et al. 2021). In some studies, SP-A, SP-B and SP-C (Surfactant Protein), Proliferating cell nuclear antigen (PCNA) and clara cell secretory protein (CCSP) have also been shown to be present in Type II Pneumocytes in the lungs, mediastinal lymph nodes, heart, kidneys, liver, diaphragm, gut, skeletal muscle, spleen, skin, and adrenal glands (Leroux et al. 2007; Beytut et al. 2009). Mishra et al. (2018) demonstrated tumor biomarkers such as PCNA, MYC, MMP2, FOXO3a, MHC I and MHC II in alveolar pneumocytes, bronchiolar epithelium and mononuclear cells lungs in addition to JSRV-capsid antigen in natural infection in sheep. The details of characteristic histological features, target proteins for immunohistochemistry and target genes for genomic detection of JSRV in lungs and other organs are given in Table 3.

Table 3 Target organs, histopathological changes, target proteins for immunohistochemistry, and target genes for PCR detection of JSRV in Sheep

Study	Sample/organs	Histopathological changes	Target proteins (IHC)	Target Gene (PCR/hnPCR/ RT-PCR)
Palmarini et al. (1999)	Lungs, PBLs	Papillary adenocarcinoma with proliferations of cuboidal cells, interstitial myxoid or fibrotic nodules	JSRV- CA protein	U3 -LTR JSRV
Garcia-Goti et al. (2000)	lungs, MLN, mammary gland, mammary LN, spleen, brain and kidney	Infiltration of lymphocytes and macrophages in connective tissue fibres and hyperplasia of BALT	JSRV-CA protein in neoplastic alveolar cells	U3 LTR (U3-hn-PCR)
Gonzalez et al. (2001)	Lungs, PBLs, kidney, spleen, mammary gland, mammary lymph node, MLN	Classical and atypical OPA lesions in the lungs	-	U3 region of exogenous JSRV (U3-hn-PCR)
Salvatori et al. (2004)	PBLs, lungs, MLNs, Lung fluid	The papillary and acinar adenocarcinoma of the alveoli and hyperplasia of BALT	JSRV-CA protein and JSRV-SU in the cytoplasm of neoplastic alveolar cells	JSRV-U3 region
Uzal et al. (2004)	Lungs	Cuboidal cell proliferation and infiltration of macrophages in the lungs	JSRV-CA protein	U3 LTR-JSRV
Caporale et al. (2006)	Lungs	Papillary and acinar nodules	JSRV-Env	JSRV-Env gene
Leroux et al. (2007)	Lungs	Bronchioloalveolar, acinar and papillary form of adenocarcinoma in lungs	SP-A (Surfactant Protein A)	-

Study	Sample/organs	Histopathological changes	Target proteins (IHC)	Target Gene (PCR/hnPCR/ RT-PCR)
Beytut et al. (2009)	Lungs and MLNs and bronchial LNs	Adenocarcinoma-type lesions in lungs	CA, Surfactant protein-A (SP-A), SP-B and SP-C, Clara cell secretory protein, proliferating cell nuclear antigen	
Griffiths et al. (2010)	Lungs	Tumors of the alveolar and bronchiolar epithelium into the acinar and papillary type and nonencapsulated tumors	JSRV Env proteins in type II pneumocytes using an anti-Env (SU) monoclonal antibody.	U3 LTR
Minguijón et al. (2013)	Lungs, MLNs, heart, Kidneys, liver, diaphragm, intestine, skeletal muscle, spleen, skin and adrenal glands	Metastatic nonencapsulated acinar and papillary type of adenocarcinoma with infiltration of macrophages, neutrophils, lymphocytes	JSRV env and surfactant protein C (SPC) in all tissues	JSRV-LTR
Azizi et al. (2014)	Lungs	Atypical form OPA, lymphoid infiltration	-	LTR
Singh et al. (2018)	Lungs	Papillary and acinar patterns of neoplastic cells in alveoli with infiltration of lymphocytes and alveolar macrophages	JSRV capsid antigen	Gag, U3 LTR
Mishra et al. (2018)	Lungs	Classical form-Proliferation of alveolar pneumocytes into papillary type with infiltration of neutrophils, macrophages Atypical form- bronchiolar hyperplasia fibroplasia, infiltration of MNCs	JSRV-CA in pneumocytes, bronchiolar epithelium, and MNCs Tumor biomarkers- PCNA, MHC I, MHC II MYC, MMP2, FOXO3a in lungs	JSRV-LTR
Samatha et al. (2019)	Lungs	Nonencapsulated alveolar papillary and acinar projections and bronchoalveolar growth pattern in the lungs	-	U3 LTR by U3-hn PCR
Mansour et al. (2019)	Blood, lungs, LNs	Glandular neoplastic cells bronchiolar hyperplasia lymphocytic necrosis	-	Env of JSRV by RT-PCR
Lee et al. (2019)	Lungs	Clear-cut demarcated areas of acinar-type adenocarcinoma supported by a fibrous stroma and infiltration of macrophages	Envelope protein of JSRV	RT-PCR of JSRV
Toma et al. (2020)	Lungs	Tubular, papillary and myxoid-type lesions in classical form. In atypical form, fibroblast proliferation and infiltration of lymphocytes and macrophages in stroma	JSRV-MA, multi-cytokeratin vimentin (Vim), alpha smooth-muscle actin (alpha-SMA), desmin, S100 protein, Ki67 and Thyroid Transcription Factor 1 (TTF-1)	JSRV-LTR
Belalmi et al. (2020)	Lungs	Papillary, acinar or glandular patterns with infiltration of lymphocytes and macrophages	JSRV-Env protein	-
Abd Abass and Khudhair (2022)	Nasal secretions, lungs, MLN	Glandular transformation and hyperplasia of alveolar cells	-	RT PCR of JSRV-Env

7.3 Imaging techniques in OPA diagnosis

In the preclinical form of OPA, imaging diagnostic techniques such as computed tomography, ultrasonography, and X-rays can be valuable techniques to confirm the disease. These techniques can identify any pulmonary lesions, including tumorous growths. Transthoracic ultrasonography may detect small nodular tumors of 1-2 cm in diameter even before the clinical form of OPA. Further, the computerized three-dimensional color CT scan is handy for studying the pathogenesis of OPA (Quintas et al. 2021).

8 JSRV-induced OPA as a model for human lung cancer

Sheep are good models for researching human diseases such as malignancies, respiratory syncytial virus infection, cystic fibrosis, chronic obstructive pulmonary disease, and asthma (Gray et al. 2019b). Retroviruses are an excellent model for investigating the causes of carcinogenesis because of their capacity to integrate into the host cell's genome and interfere with its regulatory mechanisms (Palmarini et al. 1997). Regarding histomorphological features, there are many similarities between OPA and the human lung adenocarcinoma; therefore, OPA is regarded as a crucial big animal model for comprehending the mechanisms behind retroviral oncogenesis (Mornex et al. 2003). Although JSRV was shown in human lung neoplastic epithelial cells, its significance in human carcinogenesis remains unclear (Miller et al. 2017). Type II pneumocytes are transformed by JSRV by insertional mutagenesis (Palmarini et al. 1997). The membrane receptor Hyal2 mediates the interaction between oncogenic JSRV envelope proteins and target cells. This causes cellular change, the activation of the PI3K/Akt and MAPK pathways, and telomerase activity (Leroux et al. 2007). Human lung alveolar cells were shown to contain the JSRV-receptor Hyal2. However, human adenocarcinoma did not have any JSRV DNA. There is no indication of JSRV transfer from sheep to humans. Additionally, there is proof that JSRV envelope protein may occasionally cause human cell neoplastic transformation. DNA sequences linked to JSRV were found in a small number of African American lung cancer cases. OPA is currently categorized as a mixed adenocarcinoma exhibiting acinar, papillary, and bronchioloalveolar development patterns by the World Health Organization's (WHO) revised classification system for human lung malignancies (Travis et al. 2012).

9 Prevention and control of OPA

OPA is an incurable disease, and death in clinical cases is inevitable due to the progression of lung lesions. Vaccines and therapies to cure OPA are not currently available. The stringent quarantine, cleaning, and disinfection of contaminated farms and equipment and removing diseased animals and their lambs from flocks continue to be the most effective methods for managing the disease. Control and prevention of JSRV are the same as that of the eradication programs in Maedi-visna. In breeding flocks, mainly JSRV transmission is considered through feeding of milk/colostrum and therefore, newborn lambs may be immediately separated from ewes and fed with heated colostrum or milk (Voigt et al. 2007). Eliminating JSRV-positive sheep from flocks as soon as possible, conducting routine epidemiological surveillance, and early diagnosis are all effective control methods. A combination of diagnostic techniques such as clinical, pathological, immunohistochemistry, PCR, and sequencing should be chosen so that the complete absence of the infection may be achieved in a sheep population.

Conclusion and Future Prospects

Due to intensive and migratory sheep farming practices in India, there is a considerable risk of OPA infections in sheep flocks. There is an urgent need to relook into the slow viral diseases, including OPA in sheep, for differential diagnosis of pneumonia, as OPA often goes unnoticed in farms. As no serodiagnostic tests are available due to poor immune reaction in JSRV-affected sheep, in recent years, more efforts have been focused on developing molecular tests for early detection of the disease to eradicate OPA in sheep effectively. Future control strategies should be directed to establish low-risk status for OPA in sheep herds by employing genomic methods such as RT-PCR and sequencing to detect JSRV at early stages. The only proven method to eradicate OPA from sheep was a rigorous slaughter policy involving massive culling of affected flocks. But, under Indian conditions, it is not a practically viable solution. Hence, early detection, quarantine, and good husbandry practices are to be followed to control OPA in sheep effectively.

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EFFECT OF APPLYING BIO-INPUTS ON PRODUCTION OF HIGH BUSH BLUEBERRY (*Vaccinium corymbosum L.*) cv. biloxi IN BRAZIL'S FEDERAL DISTRICT

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ABSTRACT

Blueberry production is increasing in Brazil, and growers are turning to bio-inputs or biostimulants to be used on their growth. This has been due to the growing concern about sustainability in the food production chain and the necessity to increase the yield. The current experiment aimed at evaluating the effects of Samurai King, EM-1 and Brutal Plus (Minhofértil) biostimulants on the cultivation of Southern Highbush blueberries (*V. corymbosum* L.), cultivar 'Biloxi'. The parameters evaluated were plant height (cm), diameter of the main stem (mm), number of shoots, chlorophyll content, total number of leaves, leaf length and width. The total mass, number of fruits, average mass per fruit, the transversal and longitudinal diameters, and the total sugars (°Brix) were also measured. In 2020, the treatment of Samurai King + EM-1 showed the highest efficiency for the studied parameters related to fruits and yield but with no significant difference as compared to the other treatments. Regarding the plant growth, treatments 1 (Brutal Plus) and 5 (Brutal Plus + EM-1) were the most efficient. In 2021, treatment 7 (Brutal Plus + Samurai King + EM-1) had the highest yields, except for average mass per fruit and total sugars. In the two years of evaluation, although treatments obtained lower averages than the control, the effect observed was generally positive, revealing the efficiency of products containing microorganisms for the growth of blueberry plants. In conclusion, these bioproducts could remarkably affect plant biomass, production and fruit quality, resulting in better yields.

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1 Introduction

The global production of blueberries, which belongs to the *Ericaceae* family, has grown annually to match the increase in consumption. It produces small blue-black fruits and influences health conditions by improving vision, functioning as an anti-inflammatory and antioxidant. This genus, *Vaccinium spp*., contains around 450 species from Southeast Asia and the Americas.

Peru did not export blueberries in 2014, but it became the largest exporter in Latin America in 2019. It exported over one billion dollars (Ghezzi and Stein 2021). Looking at Brazil, close to 4 tons of blueberries were exported in 2002, earning Brazilian producers about 24,000 dollars (Cantuarias-Avilés et al. 2014). Since 2010, with the introduction of low-chill varieties from research at the University of Florida, the planted area has increased even more. In 2011, a total area of 142 ha produced about 59 tons of blueberries (Cantuarias-Avilés et al. 2014). Such production levels can be reached using Bio-inputs substances or microorganisms applied to plants to increase the efficiency of nutrition, tolerance to abiotic stresses and/or crop quality traits, regardless of their nutrient content (Aung et al. 2014; Halpern et al. 2015; Koza et al. 2022). Bio-inputs can also act as catalysts for nutrient uptake by accelerating natural processes and may enhance plant protection against pests and diseases through systemic induction of resistance (Olowe et al. 2020; Kumar et al. 2022). For example, the use of EM-1 at a dose of 1mL/100mL provided an increase in vegetative growth of four apricot (*Prunus armeniaca*) cultivars when compared to control treatment and organic fertilizer application (Al-Janabi et al. 2016). In fact, using bio-inputs in agriculture is a growing practice worldwide, which has also increased their market. The global bio-input market was valued at $3.2 billion in 2021, with an expected annual growth of 12.1%, and is forecasted to be valued at $5.6 billion in 2026 (Market Research Report 2022). Their increased use in agriculture is mainly due to concerns among consumers and producers regarding factors affecting the sustainability of the food production chain, such as global warming and soil degradation (Halpern et al. 2015). The bio-inputs are therefore employed to up production to meet demand.

In an experiment in Nanjing - China, the application of different species and strains of the genus *Bacillus* showed significant gains in blueberry production (Yu et al. 2020). The blueberries of the variety 'Lanmei No.1' presented an increase in chlorophyll content and photosynthetic rate, growth and productivity, as well as an increase in the amount of ammonia, nitrogen and organic matter in the soil (Yu et al. 2020). However, in the blueberry plantation, experiments with biological treatments, especially with the inoculation of microorganisms, are still scarce, leaving an information gap. The study, therefore, aimed to evaluate the effects of bio-inputs on the growth and production of blueberries.

2 Materials and Methods

The present experimental work was carried out between March 2020 and April 2022 at the Biology Experimental Station, University of Brasília, Federal District, Brazil. According to the Köppen-Geiger criterion, the local weather is classified as tropical and type Aw, with dry winter and rainy summer (Cardoso et al. 2014). Three bio-inputs were used: Samurai King, EM-1 and Brutal Plus (Minhofertil). Samurai King and EM-1 bio-inputs are produced industrially by combining selected strains of efficient microorganisms. The biological composition of Samurai King® and EM1®, respectively, can be seen in Table 1 and 2, with appropriate adaptations (AL-Janabi et al. 2016). In contrast, Brutal Plus, currently registered as an organic fertilizer (Minho Fertil Co.,

Table 1 Microbiological characteristics of Samurai King® Brasília-DF, 2020

Bacteria	Quantification
*Bacillus cereus**	1.44 X 10^7
*Aeromicrobium kwangyangen sis***	2.8 X 10^7
*Bacillus subtilis***	1.4 X 10^7
*Aeromicrobium sp. **	1.4 X 10^7
*Bradyrhizobium japonicum***	3.2 X 10^7
*Acidovorax sp. **	8.8 X 10^7
*Stenotrophomonas sp.***	2.2 X 10^7
*Sphingomonas fennica **	2.2 X 10^7
*Acinetobacter modestus***	2.2 X 10^7
*Methylobacterium adhaesivum***	1.8 X 10^7

*Spike metagenomics (%); **serial dilution (CFU/(ml/L or g))

Table 2 Composition of the EM1®

Photosynthetic Bacteria	*Rhodopseudomonas plustris* *Rhodobacter sphacerodes*
Lacticacid bacteria	*Lactobacillus plantarum* *Lactobacillus casei* *Streptococcus lactis*
Yeast	*Saccharomyces cerevisiae*
Fungi	*Aspergillus spp.* *Penicilium spp.*

Source: AL-JANABI et al. (2016)

Table 3 Contents of the natural rice husk substrate Brasília -DF, 2020

DD	TP	pH	EC	OM	Ca	Mg	Fe
kg m^{-3}		-	mS cm^{-1}	mg L^{-1}			
131.71	85.46	5.56	0.418	224.4	47.4	1.98	0.79

Source: de-LIMA et al. 2020; here Dry density (DD); total porosity (TP); pH; electrical conductivity (EC); organic matter (OM) and calcium (Ca); magnesium (Mg) and iron (Fe)

Brazil), is produced more artisanally; thus, the microbial composition might range according to the material used. The blueberry plants evaluated were in their third and fourth year of production. One plant in each black polyethylene pot contained 60 dm^3 of substrate (Table 3). The plants were covered with mesh to protect against birds, allowing solar incidence of 80% to penetrate (20% covering mesh). The pots containing plants were distributed in rows, with 0.4 m between plants and 1.5 m between rows. Substrate was covered with raw rice husk, without any burning process.

The soils were amended by fertigation, using ever-flow systems with four holes at the soil level, with a total flow rate of 4 L h^{-1} and one emitter per plant, totalling approximately 3 L of solution per plant per day. The quantities of nutrients were controlled, providing the following amounts on an annual basis: N - 250kg ha^{-1}, P_2O_5 - 160kg ha^{-1}, K_2O - 300kg ha^{-1}, CaO - 250kg ha^{-1}, MgO - 150kg ha^{-1}, and SiO_2 - 200kg ha^{-1}.

The experiment was organized in randomized blocks containing seven treatments: (i) Brutal Plus, (ii) Samurai King®, (iii) EM-1®, (iv) Brutal Plus + Samurai King®, (v) Brutal Plus + EM-1®, (vi) Samurai King ®+ EM-1®, (vii) Brutal Plus + Samurai King ®+ EM-1®. There was also a control treatment without application, considered treatment 0. The plants were arranged in three blocks, each containing the seven treatments and control, randomly arranged, with five plants from each treatment per block, totalling 40 plants per block and 120 in the complete experiment. The doses of bio-input applied were 3 mL L^{-1} for Brutal Plus and EM-1® and 4 mL L^{-1} for Samurai King® every 15 days throughout the study, ceasing only in December 2020, just before the plants were pruned in January 2021. No pesticides were applied during the analyses to control pests or plant diseases. Ten to twelve branches of each plant were chosen to evaluate the production and quality.

In the plants, the parameters evaluated were (i) plant height (cm) (Pl. Hgt), measured by the height of the previously marked main branch, (ii) diameter of the main stem (mm) (St. Diam), (iii) number of sprouts (NS), (iv) chlorophyll, using the ATLeaf equipment (Cl), (v) total number of leaves per plant (NL), (vi) leaf length (L. Leng), and (vii) leaf width (L. Wdt), and (viii) Yield. The date of the first harvest of that year was July 13, 2020, and the weekly measurements of total fruit mass, number of fruits and average mass per fruit were taken until November 4, 2020. From August to October of the same year, the transversal and longitudinal diameters of the fruits were measured monthly, and the total sugars in the pulp (°Brix) were also measured during this period.

In 2021, the aforementioned plant parameters were evaluated monthly between March and September. On the other hand, the fruit evaluations occurred from July 13, 2021, the date of the first harvest of that year. The fruit diameter and ºBrix assessment occurred every two weeks, while the data on the other variables were collected weekly.

The Gaging model Origin Caldigital caliper with a precision of 0.01 mm was used to measure the diameter of the main stem and the transversal and longitudinal diameter of fruits. Plant height was measured with a measuring tape, and the branch was marked with coloured tape for identification. Leaf width and length were measured using a 30 cm-long millimetre-marked ruler. The chlorophyll content was measured using an ATLeaf CH Plus chlorophyll meter. The sugar content in the fruit was measured using a digital refractometer.

The parameters related to photosynthesis rate and stomatal conductance in the leaves were quantified using an evaluation with infrared gas analyzer (IRGA) equipment, which was performed on June 23, 2021. The measurements were taken twice daily (at 8 am

and 12:00 pm). The IRGA equipment was used to measure photosynthetic activity, stomatal conductance, and transpiration rate, among other gas exchanges.

The data related to the different variables was run through variance analysis and Tukey's mean comparison test at the 5% probability level using SISVAR software (Ferreira 2011).

3 Results

For the analyses performed for 2020, the variables related to the fruits presented no significant difference between the treatments at the Tukey test of means at 5% probability (Table 4). Treatments 0 (control) and 6 (Samurai King + EM1) obtained the highest absolute values, with differences of 24% and 28.2%, respectively, in comparison to the least productive one (EM1) in the variable average mass of fruit per week (FMW). For the varying number of fruits per week (NFW), the highest values were obtained with the control and Samurai King ®+ EM-1® treatments, while the treatment EM1 showed the lowest yield. Although some differences were detected between them, they are not statistically significant.

The average mass per fruit (AMF) also varied little among the treatments, with an outstanding value for treatments 5 (Brutal Plus + EM1) and 6 (Samurai King + EM1), while the lowest one was detected in the treatment 4 (Brutal Plus + Samurai King). The total soluble sugars presented low variation among the treatments, with the highest values observed in treatments 0 and 5. The concentration of soluble solids was directly related to the cover used for cultivation, with a decrease in the value of °Brix when covering was done with a shading net compared to the use of polyethene cover and the control without any cover.

In 2021, the treatments showed greater differentiation and were significant at 5% probability in Tukey's Test for the mass of fruits per week (FMW) and number of fruits per week (NFW), with p-values of 0.0002 and 0.003, respectively. In the case of NFW, treatment 7 (Brutal Plus + Samurai King + EM1) showed the highest mass of 91.27g, and this was 67.4% higher than that presented by treatment 4 (Brutal Plus + Samurai King), which is 54.51g and found the least productive. The situation is repeated for NFS, with treatment 7 standing out, with an average value higher than 84 fruits per week. In contrast, treatment 5 (Brutal Plus + EM1) stood out for low production, showing average values of approximately 48 fruits per week.

There were no significant differences for AMF and BRIX, with very close values among the treatments (Table 4). The average mass per fruit was lower in 2021 compared to the previous year's values in most treatments. In compensation, total soluble sugars were higher in all treatments in 2021, showing that the products may have exerted some effect during the regular and ongoing application.

The graphs of the average mass of fruits per week (FMW) and the number of fruits per week (NFW) (Figures 1 and 2) for both years revealed a production peak between August and September. In the 2020 graph, a shorter peak can be observed, concentrated from the middle to the end of August, while in 2021, this higher production remained more stable from the beginning of August until the middle of September before falling again. Production after the peak felt in 2020 and did not recover until the end of the harvest at the end of November, remaining at a steady low. However, in 2021, after the peak production dropped, it rose again, revealing another production peak, even higher than the first, starting in the second half of October (Figure 1).

Table 4 Characteristics of High bush blueberry fruits in 2020 and 2021

	2020				2021			
Treatment	FMW	NFW	AMF	BRIX	FMW	NFW	AMF	BRIX
0	83.20^{a}	63.74^{a}	1.38^{a}	10.58^{a}	72.82^{ab}	70.33^{ab}	1.22^{a}	12.70^{a}
1	71.14^{b}	56.81^{a}	1.34^{a}	10.36^{a}	71.90^{ab}	64.92^{ab}	1.21^{a}	12.72^{a}
2	76.66^{ab}	55.93^{a}	1.39^{a}	9.99^{a}	73.99^{ab}	67.46^{ab}	1.26^{a}	12.52^{a}
3	67.06^{c}	52.40^{a}	1.32^{a}	9.27^{a}	69.27^{ab}	65.50^{ab}	1.26^{a}	12.50^{a}
4	70.75^{b}	56.12^{a}	1.24^{a}	9.90^{a}	54.51^{b}	49.91^{b}	1.31^{a}	12.88^{a}
5	77.86^{ab}	61.02^{a}	1.39^{a}	10.79^{a}	59.43^{b}	57.00^{ab}	1.22^{a}	12.80^{a}
6	85.99^{a}	63.19^{a}	1.40^{a}	9.82^{a}	55.44^{b}	47.98^{b}	1.27^{a}	12.94^{a}
7	69.84^{b}	53.17^{a}	1.33^{a}	10.45^{a}	91.27^{a}	84.62^{a}	1.25^{a}	12.75^{a}

Treatments 0 – Control; 1 Brutal Plus; 2 Samurai King®; 3 EM-1®; 4 Brutal Plus + Samurai King®; 5 Brutal Plus + EM-1®; 6 Samurai King ®+ EM-1®; 7 Brutal Plus + Samurai King ®+ EM-1®; Average mass of fruit per week (FMW), Number of fruits per week (NFW), Average mass per fruit (AMF) and brix degrees (BRIX); Values followed by the same letters in the columns do not differ by the Tukey test at 5% probability level

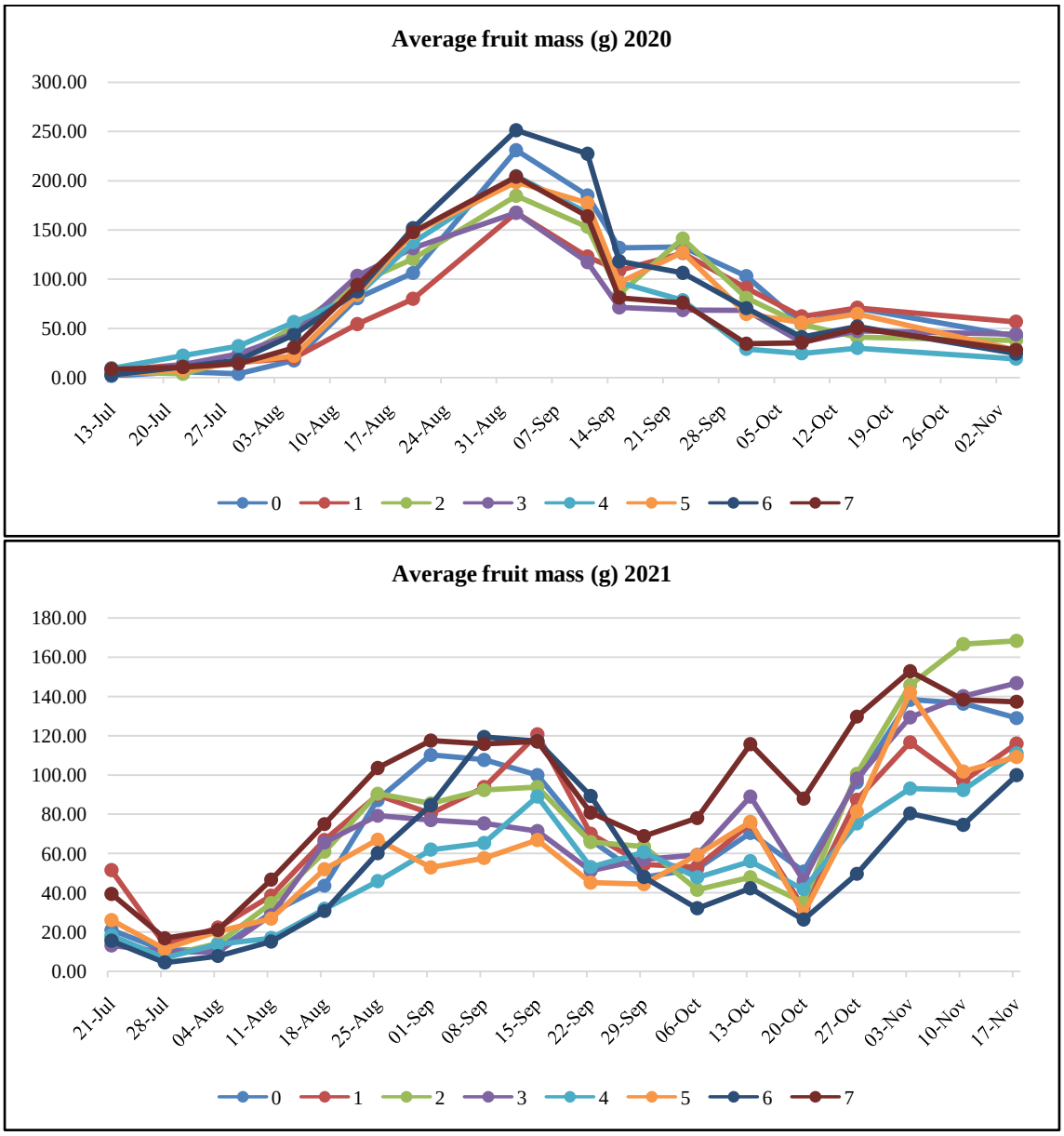

Figure 1 Trends in the average fruit mass per week (FMW) for the different treatments in the years 2020 and 2021 (treatments: 0 – Control; 1 Brutal Plus; 2 Samurai King®; 3 EM-1®; 4 Brutal Plus + Samurai King®; 5 Brutal Plus + EM-1®; 6 Samurai King ®+ EM-1®; 7 Brutal Plus + Samurai King ®+ EM-1®)

The average fruit mass per week (FMW) also increased between August and September of 2020, dropping afterwards and maintaining stability until the end of the year. In 2021, the graph revealed a slight decrease in the same months, maintaining its level until the middle of October. Here, value began to rise, and treatment 2 (Samurai King®) reached the highest value on November 17, with an average of 197.7 fruits harvested per block. The highest average fruit mass per week (FMW) were observed in treatment 2 (Samurai King®) in 2021 (163.67 g) and in treatment 6 (Samurai King ®+ EM-1®) in 2020 (250 g).

For the parameters related to plant characteristics, in 2020, a statistical difference was observed in all variables evaluated except for plant height (Pl. Hgt) and yield per plant (Yield). The yield in 2020 returned a p-value of 0.7551 for the treatments and 0.8611 for the blocks, evidencing no difference between the blocks and treatments. The most productive treatment in 2020 was treatment 6 (Samurai King ®+ EM-1®), with an average yield of 401.27 g per plant. The least effective treatment was treatment 3 (EM-1®), with a yield of 312.96 g, a difference of 28.2% compared to the most productive treatment. In 2021, the yield showed a P-value of

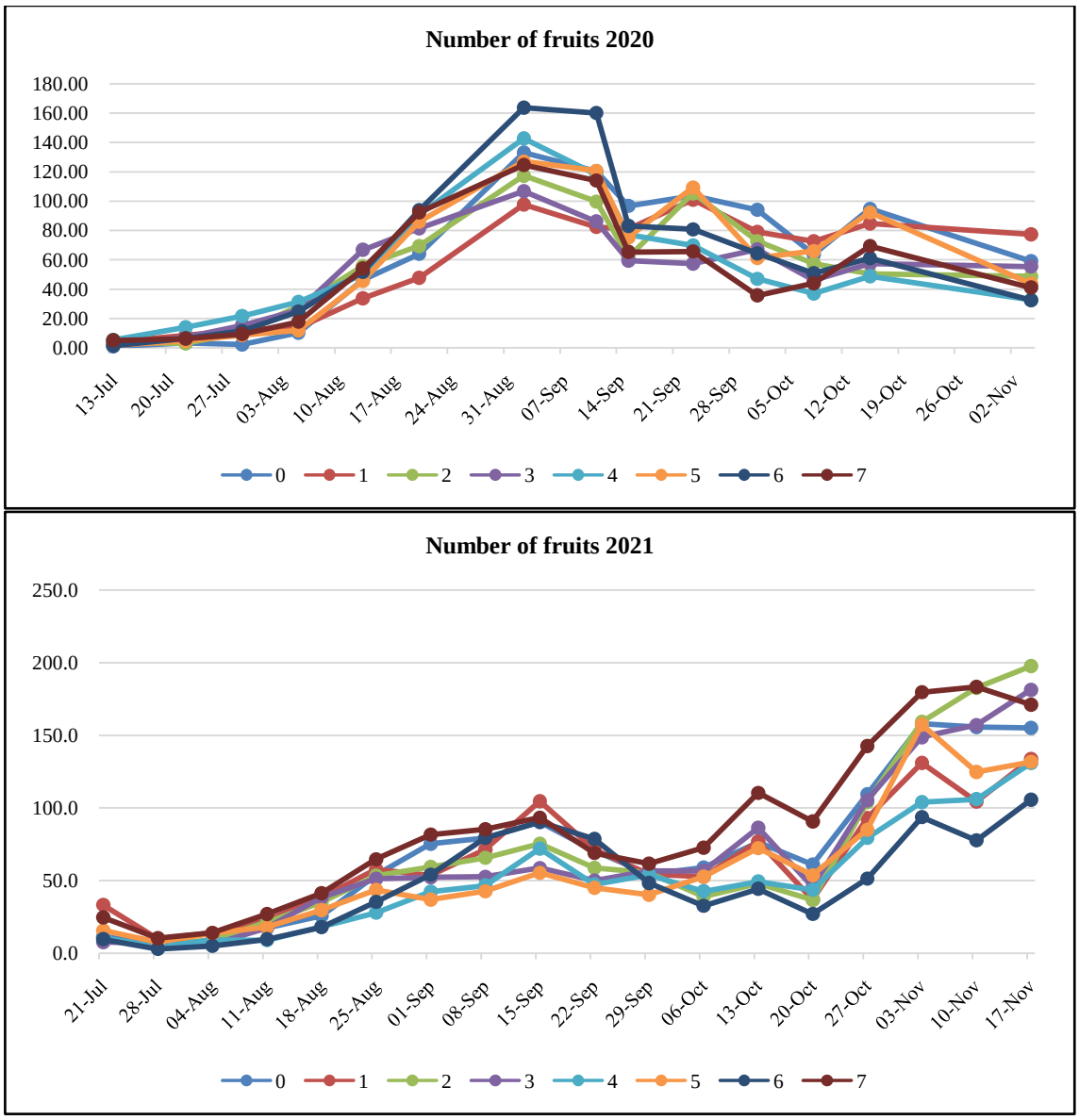

Figure 2 Trend of the Number of fruits per week (NFW) for the different treatments in the years 2020 and 2021 (treatments: 0 – Control; 1 Brutal Plus; 2 Samurai King®; 3 EM-1®; 4 Brutal Plus + Samurai King®; 5 Brutal Plus + EM-1®; 6 Samurai King ®+ EM-1®; 7 Brutal Plus + Samurai King ®+ EM-1®)

0.0498 for the different treatments at the 5% probability level. The highest yield was reported in treatment 7 (Brutal Plus + Samurai King ®+ EM-1®), with an average yield of 328.59 g per plant, and this was 67.4% higher than the average of the least effective treatment (Brutal Plus + Samurai King®). The analysis of variance also showed a significant difference between the blocks, with a P-value of 0.0031. In the spacing adopted, the productivity per hectare was 5.47 tons, with 16.67 plants. Although yield was lower in this case than that reported by large blueberry producers, this mode of cultivation is cheaper because it eliminates the use of chemical pesticides, which can add substantially to the cost of production.

In the second year of evaluation, no significant differences were observed for the variables number of shoots (NS), number of leaves per plant (NL) and chlorophyll in leaves (Cl). For all other variables, ANOVA demonstrated a difference between treatments. The chlorophyll index in leaves was higher for treatments 6 (Samurai King ®+ EM-1®) and 5 (Brutal Plus + EM-1®) in 2020 and 2021, respectively. The difference between treatments was observed only in 2020, when treatment 6 returned average values of 63.21, 12.1% higher than treatment 1 (Control), which was the least productive treatment in that year (56.38) (Table 5).

Table 5 High bush blueberry growth parameters evaluated in 2020 and 2021

Treatment	Pl Hgt	St Diam	NS	NL	Cl	L Leng	L Wdt	Yield
2020								
0	29.82^{a}	7.87^{ab}	10.67^{a}	458.33^{bc}	60.97^{ab}	4.28^{b}	2.48^{b}	388.28^{a}
1	29.81^{a}	6.55^{c}	11.19^{a}	548.67^{a}	56.38^{b}	4.83^{a}	2.84^{a}	332.00^{a}
2	30.55^{a}	6.58^{c}	8.26^{b}	344.56^{e}	62.67^{a}	4.56^{ab}	2.61^{ab}	357.75^{a}
3	34.63^{a}	7.09^{bc}	9.81^{ab}	432.67^{bcd}	61.41^{ab}	4.50^{ab}	2.50^{b}	312.96^{a}
4	30.47^{a}	6.35^{c}	9.22 ^{ab}	387.56^{cde}	62.93^{a}	4.71^{ab}	2.53^{b}	330.18^{a}
5	32.72^{a}	8.35^{a}	8.19^{b}	442.00^{bcd}	59.61^{ab}	4.75^{ab}	2.48^{b}	363.35^{a}
6	32.57^{a}	6.97^{bc}	8.19^{b}	360.33^{e}	63.21^{a}	4.50^{ab}	2.49^{b}	401.27^{a}
7	29.28^{a}	7.94^{ab}	9.96^{ab}	502.78^{ab}	60.29^{ab}	4.67^{ab}	2.60^{ab}	325.91^{a}
2021								
0	65.32^{ab}	6.01^{ab}	15.10^{a}	739.33^{a}	60.51^{a}	6.28 ^{a}	3.59^{a}	262.18^{ab}
1	74.66^{a}	6.68^{a}	13.90^{a}	633.44^{a}	62.55^{a}	6.39^{a}	3.61^{a}	258.85^{ab}
2	63.61^{b}	5.66^{b}	14.94^{a}	730.06^{a}	62.27^{a}	5.73^{b}	3.16^{b}	266.40^{ab}
3	68.52^{ab}	5.60^{b}	15.94^{a}	744.11^{a}	61.90^{a}	6.23^{a}	3.64^{a}	249.39^{ab}
4	65.53^{ab}	5.51^{b}	14.25^{a}	665.33^{a}	59.10^{a}	6.23^{a}	3.59^{a}	196.25^{b}
5	62.23^{b}	5.47^{b}	15.33^{a}	725.22^{a}	63.34^{a}	6.28^{a}	3.63^{a}	213.98^{ab}
6	60.87^{b}	5.21^{b}	16.25^{a}	760.89^{a}	62.84^{a}	6.07^{ab}	3.41^{ab}	199.59^{ab}
7	66.72^{ab}	5.75^{b}	16.04^{a}	805.33^{a}	62.91^{a}	6.14^{ab}	3.63^{a}	328.59^{a}

Treatments: 0 – Control; 1 Brutal Plus; 2 Samurai King®; 3 EM-1®; 4 Brutal Plus + Samurai King®; 5 Brutal Plus + EM-1®; 6 Samurai King ®+ EM-1®; 7 Brutal Plus + Samurai King ®+ EM-1®; Plant height (Pl. Hgt); Main stem diameter (St. Diam.); Number of sprouts (NS); Number of leaves per plant (NL); Chlorophyll content (Cl); Leaf length (L. Leng); Leaf width (L. Wdt); and Yield. Values followed by the same letters in the columns do not differ by the Tukey test at 5% probability level

The main stem diameter (St Diam) showed similar results in both years, decreasing in the second year. In 2020, the treatment that presented the largest diameter was treatment 5 (Brutal Plus + EM-1®), with an average of 8.35 mm, while in 2021, the best performance occurred for treatment 1 (Control), with an average diameter of 6.39 mm. The height of plants was measured in the first year, counting from the second growth flush (first secondary shoots). In 2021, the measurement was made from the first shoots after pruning, which is why there is a difference in values between the two years. The difference between treatments was observed only in the second year, in which treatment 1 (Control) was the fastest growing, with an average height of 74.66 cm.

For the number of sprouts (NS), the treatments differed in 2020, and among the tested treatments, treatment 1 (Control) presented more primary shoots from the crown of the plant. There was no significant difference in 2021, but the absolute values increased compared to the previous year, with treatment 6 (Samurai King ®+ EM-1®) producing an average of 16.25 shoots per plant. The control treatment also stood out in the number of leaves (NL) per plant in 2020, with an average of 548.67 leaves. In 2021, this parameter was observed for treatment 7 (Brutal Plus + Samurai King ®+ EM-1®), with a value 46.7% higher than the best treatment in 2020. For the number of shoots (NS), leaf length (L. leng) and leaf width (L. Wdt), the absolute values increased from 2020 to 2021. In both years, treatment 1 showed higher leaf sizes, except for leaf width in 2021, when it was exceeded by treatments 3 (EM-1®), 5 (Brutal Plus + EM-1®) and 7 (Brutal Plus + Samurai King ®+ EM-1®), which showed values of 3.64 cm, 3.63 cm and 3.63 cm respectively (Table 5).

In the photosynthesis and stomatal conductance analyses performed using the IRGA equipment, no differences were observed between the treatments for any of the parameters evaluated at either of the two evaluation seasons.

4 Discussion

The application of biostimulants on crops has shown varied results, depending on the crop, the composition of the input, and other factors. In the present study, fruit biomass showed no effects of the applied treatments, but previous studies demonstrated the

efficiency of foliar or seed application of similar bioproducts, especially plant hormones, on soybean production (Bertolin et al. 2010). Foliar application proved effective, especially in the crop's vegetative phase (Bertolin et al. 2010). On the other hand, apples in Italy received bio-inputs based on algae, amino acids and humic and fulvic acids, but no gains were observed in the harvest, the fruit quality, and did not show any observable difference in the photosynthetic rate of the crop (Thalheimer and Paoli 2001). The use of efficient microorganisms, such as bacteria and mycorrhiza, has already revealed an increase in crop plant yields and has a unique role in abiotic stress tolerance

A previous study showed increased harvest weight in conventional and organic carrot crops. Bio-inputs applied were based on bacteria such as *Bacillus subtilis*, *Lactobacillus*, *Saccharomyces cerevisiae* yeast, humic and fulvic acids, among other compounds common to the formulations used in this study's experiments, and administered at low doses of 2L/ha. Besides the increase in carrot weight, a greater accumulation of monosaccharides, carotenoids, and phenols and increased antioxidant activity were also observed (Gavelienė et al. 2021).

The total soluble sugars and soluble solids showed low variation among the treatments in the present study. It is also noteworthy that in the case of higher temperatures, the shade should be removed to allow for better fruit development (Pereira et al. 2021). According to these authors, a shading mesh could reduce the harvest period of the crop.

As regards the fruit mass and the number of fruits per week, different types of biostimulants showed different actions, and their combination may prove effective in some cases. The main variations in bio-inputs are associated with the origin of the material used to manufacture the product, how this material is processed, and the mixtures of other substances to make the commercial product. In addition to the increase in yield, the use of biological inputs can increase the resistance of the fruits to cracking, as observed by Rodrigues et al. (2020) in pomegranate crops. Little or no relationship has been reported between applying efficient microorganisms and total soluble solids (Auriga et al. 2018). In grapes, total soluble solids, total titratable acidity, and the amount of polyphenols present in the fruit were not influenced by the application of MS, with the amount of polyphenols altered only when a different cultivar was used and when the number of buds per branch increased (Auriga et al. 2018).

In the present study, the production peak occurred in August and September. According to Tuell and Isaacs (2010) climate is a determining factor in the production of blueberries, and colder climates prove most suitable for higher production. Colder climates also directly influence the presence of pollinating insects essential for increased crop production (Tuell and Isaacs 2010). Covering the crop and the absence of such insects may influence an earlier start for the harvest period. In an experiment with different covers for blueberry production in Portugal, an earlier harvest was observed using polyethene cover, and a later harvest was reported using shade netting cover (Pereira et al. 2021). In the case of this experiment, covering the crops with shade netting may have prevented the entry of birds, protecting the crop, but it probably also decreased the entry of pollinating insects. Various species of pollinators play a significant role in blueberry yields. In Vermont, United States, various wild bee species provided 12% increases in fruit mass and fruit set compared to treatment with hand pollination alone (Nicholson and Ricketts 2019).

An essential factor in the setting of flowers is the correct management of pruning to maintain the entry of light through the crown of the plant. The production of floral buds increases with more radiation, concentrated mainly in the first 60 cm from the crown, where light penetrates with greater intensity. In an experiment with the Chile cultivar' Choice', 63.7% of the floral clusters were present in the first 60 cm, indicating that light intensity caused a considerable increase in flower production and fruit production (Yáñez et al. 2009). The increase in productivity with soil microorganisms is linked to several factors, such as the production of auxins, one of the most important hormones for plant development, which are present in them in larger quantities. Further, biosynthesis of indol-acetic acid (IAA) can be performed by bacteria and fungi. Some of the said organisms were present in the products used here, such as *Bradyrhizobium* and can even be stimulated by the exudation of secondary compounds from the plants themselves (Theunis et al. 2004; Ortíz-Castro et al. 2012).

The use of microorganisms to increase the chlorophyll indexes and photosynthetic rate of plants has already been studied. In an experiment, Li et al. (2020) reported that the application of biological fertilizer composed of a mixture of microorganisms and biochar on tobacco plants has a positive difference of approximately 3.47% to 69% on the SPAD index and 8 to 107% on the rate of photosynthesis. This was in addition to a significant increase in stomatal conductance and transpiration rate, indicating a beneficial effect of fertilizer application on the rate of photosynthesis and other relevant characteristics (Li et al. 2020). The photosynthesis rate is also influenced by the water potential in the leaf, which is directly related to the correct irrigation of the plants (Rho et al. 2012). Improved plant development and stem enlargement may be related to the increased resistance of plants to different environmental and weather conditions. Through suppression of oxidative stress with increased antioxidant levels, the application of corn and propolis extracts increased the resistance of fava bean plants (*Vicia faba* L.) to water, salt, and cadmium (Cd^{2+}) stress. These extracts have been reported to increase the efficiency of photosynthesis and gas exchange, as well

as plant growth and yield under the mentioned stresses and optimal conditions (Desoky et al. 2021; Koza et al. 2022).

De Silva et al. (2000) reported increased stem diameter and leaf area with soil inoculation of a *Pseudomonas fluorescens* isolate. Furthermore, this bacterium also increased plant growth and nutrition and functioned as a biocontrol agents (Lally et al. 2017; Sah et al. 2021). Further, the application of magnesium also helps in nutrient uptake and makes chlorophyll development more efficient, which helps increase plant yield (Awad and El-Ghamry 2007). Increased yield in wheat was studied in a long-term experiment in which the continued action of microorganisms improved organic fertilizer uptake efficiency (Hu and Qi 2013).

Photosynthesis and stomatal conductance showed no effect arising from the treatments. In contrast, the use of MS, besides increasing indices of stomatal conductance, intercellular CO_2 concentration, transpiration rate, and photosynthetic rate, can also increase, which is associated with the induction of pathogen resistance (Hamid et al. 2021; Li et al. 2020) and are also indirectly affecting plant photosynthesis. Leaf damage caused by pathogens such as *Septoria albopuncata* has greatly decreased CO_2 assimilation and transport rates, resulting in decreased photosynthesis (Roloff et al. 2004). Thus, resistance to pathogens is crucial in improved production.

Conclusions

The analyses revealed significant differences between treatments for some parameters in the two years of evaluation of Blueberry plants, particularly in 2020, when Samurai King + EM-1 showed a remarkable effect on plant growth. Additionally, in 2021, the Brutal Plus + Samurai King + EM-1 treatment was the most productive and showed better yields in the variables related to fruit, except in AMF and BRIX. In both years, the effect was generally positive, revealing the efficiency of applying bio-inputs to develop blueberry plants. It is recommended that these products use be continued, as their efficiency in improving fruit production in the long term was demonstrated.

Authors contributions

Conceptualization: Yamanishi OK. Data curation: Pinho GP, Lima FN. Formal analysis: Data curation: Pinho GP, Lima FN. Funding acquisition: Yamanishi OK. Investigation: Murakami K. Methodology: Pinho GP, Lima FN. Project administration: Lima FN. Resources: Yamanishi OK. Supervision: Lima FN. Writing-original draft: Murakami K., Cruz AF. Writing-review & editing: Cruz AF.

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Seed characteristics and the influence of scarification treatments on the germination of *Pterocarpus angolensis* in Botswana

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Scarification methods

Seed dormancy

Seed germination

Seed size

ABSTRACT

Pterocarpus angolensis, a vital timber tree species of the Miombo and other woodlands, is native to eastern and southern Africa. Gemination studies were carried out at the Botswana University of Agriculture and Natural Resources to ascertain the seeds' features and find the most effective scarification treatments that yield the fastest, highest, and most consistent germination of *P. angolensis*. Ten seed pretreatments were included in the completely randomized design of the experiments. These treatments included nicking, immersion in boiling water for one, three, and five minutes, concentrated sulphuric acid for fifteen, thirty, forty-five, and sixty minutes, and hot water left to cool overnight. Treated seeds were allowed to germinate at room temperature (25 °C) for 30 days. An analysis of variance (ANOVA) was performed on the raw data. The findings demonstrated that nicking and sulfuric acid treatments significantly increased seed germination (p=0.0001) compared to untreated seeds. The germination rate of the seeds emersed in boiling water for one, three, and five minutes was noticeably lower than that of untreated seeds. Because of their tough seed coat, *P. angolensis* seeds must be pretreated before sown. This study found that nicking and sulphuric acid treatment were the best techniques for seed germination of *P. Angolensis.*

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1 Introduction

Dryland forests found in arid and semiarid regions have deteriorated due to human activities such as deforestation, droughts, climate change, and numerous other issues. Various arid and semiarid woody species are constantly harvested at frightening rates for their wood, fuel, and other purposes (Bertuzzi et al. 1997). Numerous studies have indicated that the loss of biodiversity occurring globally is partly caused by deforestation and forest degradation (Angelsen and Kaimowitz 1999; Blakesley et al. 2002; FAO 2020). According to estimates, between 1990 and 2020, deforestation would have destroyed 420 million hectares of land (FAO 2020). Plant species extinctions are happening at an alarming rate due to ongoing deforestation. In arid and semiarid regions, arborists and other tree planters have prioritized planting exotic trees and shrub species for many years, giving indigenous species little attention.

Indigenous woody trees can be used successfully in afforestation and reforestation projects to repair or replenish the depleted forest resources in tropical regions. However, their involvement in planting programmes is extremely low because foresters and other tree planters do not understand their propagation and management protocols. The scarcity of high-quality seeds and seedlings is another significant barrier to planting indigenous tree species in Botswana and elsewhere. Though not at the planned rate, efforts to produce and manage indigenous woody species have increased globally in recent decades (Shono et al. 2007; Raman et al. 2009).

Pterocarpus angolensis DC (mukwa), a member of the legume family, can grow to a height of 20 meters (Venter and Venter 2012). Its spreading, flat-topped crown is supported by a straight, cylindrical stem (Palgrave 2002; Therrell et al. 2007; Venter and Venter 2012). *P. Angolensis* grows naturally in east and southern Africa (Caro et al. 2005; Chisha-Kasumu et al. 2007; Barstow and Timberlake 2018) and is widespread in dry evergreen and dry deciduous forests (Sawe et al. 2014). It thrives in grasslands and woodlands between 300 and 1,550 meters above sea level (Fichtler et al. 2004). It is distinguished by a circular, indehiscent pod that is 3 cm wide and has a membranous wing (van der Riet et al. 1998). A hard bristle covers the inner fruit with one or two reddish brown seeds inside (van der Riet et al. 1998). Mukwa produces quality timber used in the furniture and veneer industries (Storrs 1995; Pagrave 2002; Takawira-Nyenya 2005). Some parts of the mukwa tree are used as medicine by local people to treat different diseases (Mulofwa et al. 1994; Storrs 1995; Schwartz et al. 2002; Graz 2004). Traditionally, the sap is used to stop bleeding from the nose (Palgrave 2002), kill ringworms, and cure ulcers (Van der Reit et al. 1998). Furthermore, the sap treats black water fever, eye cataracts, malaria, and skin inflammation (Watt and Breyer-Brandwijk 1962; Palgrave 2002) as well as urinary schistosomiasis (Ndaba et al. 1994; Nyazema et al. 1994).

Low levels of *P. angolensis* natural regeneration have been reported in forest inventories conducted in Botswana (BUAN Consult and Botswana Tourism Organisation 2021; BUAN Consult 2021a, b) and Namibia (Cauwer et al. 2018). Due to timber exploitation (Sadiki et al. 2018), the species' population has declined (Barstow and Timberlake 2018). Mukwa is threatened by human activities (Shackleton 2002), wildfires, and elephants (Holdo 2007; Mmolotsi et al. 2012). The inability of seedlings to establish and mature into trees after seeds have germinated threatens the species (Shackleton 2002). In addition, elephants debark the tree to relieve their thirst, increasing its wildfire vulnerability. Elephant debarking exposes the cambium, which is why the mukwa trees are killed by wildfires. Even in a controlled laboratory setting, the species has been shown to have low seed germination rates (Boaler 1966; Van Daalen 1991; Von Breitenbach 1973). Munyanziza (1994) estimated that only 5% of seedlings that germinate survive past their first year.

Most forest trees are propagated sexually using seeds. However, sound seeds of many species fail to germinate even if placed under normal environmental conditions that are favourable for germination (Bewley 1997; Gilani et al. 2019). Such seeds are dormant, which is a common phenomenon in many tropical woody species. Dormant seeds can be described as viable seeds that fail to germinate (Finch-Savage and Leubner-Metzger 2006), establish, and grow into plants (García-Gusano et al. 2004) when sown or planted in an environment suitable for germination. Further, dormant seeds will only germinate when environmental conditions are conducive to the growth and survival of plants (Baskin and Baskin 1998; Olmez et al. 2009).

Pre-sowing treatments are used to break the impermeable seed coats that are common in many tropical leguminous woody plants to achieve rapid and homogenous germination. Nicking, chemical, cold, and hot water techniques are frequently used to disrupt the impermeable seed coat and accelerate germination (Kildisheva et al. 2013; Botumile et al. 2020). Furthermore, seed dormancy, a plastic characteristic of plants that influences germination and the recruitment of seedlings, is seed size (Souza and Fagundes 2014). It is regarded as a crucial evolutionary characteristic that affects how many plant species reproduce (Cordazzo 2002). It influences seedling establishment, growth, and survival, as well as germination time and percentage, emergence rate, dispersal, seed water relations, and vigour (Sanderson et al. 2002; Mölken et al. 2005; Yanlong et al. 2007; Silveira et al. 2012).

Indigenous woody species in arid and semiarid regions need to be included in planting initiatives aimed at restoring and rehabilitating native forests and woodlands to avoid their extinction. On the other hand, little is known about how to manage and reproduce native woody species. The main objective of the present study was to assess optimal presowing treatments

that result in uniformly fast and high *P. angolensis* germination. The study's specific goals were to (a) measure the weight and size of the seeds, including their mass and weight of one thousand seeds, and (b) assess the effects of various seed treatment techniques, such as nicking, sulfuric acid, boiling, and hot water, on the overall germination percentage of *P. angolensis*.

2 Materials and Methods

2.1 Study Site

The study was conducted at the Botswana University of Agriculture and Natural Resources (BUAN) in Sebele, Gaborone, between November and December 2021. BUAN is located at latitude 24˚35' S and longitude 25˚56' E with an altitude of 994m. It is situated 10 km north of Gaborone, along the North-South highway. The climate of Sebele is semiarid, characterized by hot summers and mild winters.

2.2 Seed Collection

In September 2020, mature *P. angolensis* fruits were harvested from trees in Kazuma Forest Reserve in the Chobe District, northern Botswana. The fruits were gathered and brought to BUAN in paper bags. After the seeds were removed from the fruits, they were placed in clear plastic zip bags, sealed, and kept in a refrigerator at 4 °C until the experiments started.

2.3 Number of seeds within a fruit

Seeds in each fruit were counted using five replications, each with ten fruits. The seeds were then categorized into dead/eaten, aborted, and sound.

2.4 Seed weight and size

Five replications of ten seeds each were measured with a digital calliper to assess their length, width, and breadth. The weight of five replicates of ten seeds was measured using a digital balance and was used to calculate the weight of a single seed. To find the weight of 1,000 seeds, five replications of 100 seeds each were weighed.

2.5 Seed germination treatments

Four experiments comprising ten treatment levels, including untreated seeds as the control, were conducted for the study. These treatments included nicking, immersion in boiling water for one, three, and five minutes, concentrated sulphuric acid for fifteen, thirty, forty-five, and sixty minutes, and hot water left to cool overnight. A complete randomized design was used to set up the experiments. Each treatment contained 25 seeds and was replicated four times. The seeds were submerged in cold water before the experiments began, and only the ones that settled at the bottom of the beaker were used. The seeds that floated were considered non-viable and were thrown away. For nicking, seed coats were scarified by removing a small part of the testa on the slightly rounded edge opposite the embryo's location using a nail cutter with care to minimize damage or embryo removal.

In the acid treatments, seeds were exposed to sulphuric acid for four different durations: 15, 30, 45, and 60 minutes using Teketay's (1996) approach. Seeds were placed inside four 100-ml non-corrosive, heat-resistant glass beakers. Then, sulphuric acid was added, ensuring it covered all the seeds. The seeds were stirred every five minutes throughout the designated soaking periods to expose them to the acid uniformly. The chemical was poured into a fresh beaker at the end of each acid-soaking time. Seeds were removed from the chemical using an acid-resistant filter. We thoroughly cleaned the seeds and rinsed them under a running tap and distilled water to remove any remaining acid.

Three distinct boiling water exposure times (one, three, and five minutes) were applied to the seeds in the boiling water treatments. The seeds were placed inside four distinct paper coffee filters and immersed in boiling water in a cooking kettle for each exposure period. Following removal from the heat source, the seeds were placed in a beaker with cold to cool them. The hot water treatment seeds were placed in paper coffee filters and put inside beakers. After that, boiling water was poured, and the seeds were allowed to cool for a full day. Four replicates of untreated seeds were used as the control for all the treatments.

Closed 90 mm petri dishes were used for the germination of the seeds. The petri dishes were lined with cotton wool and watered whenever necessary to prevent seeds from drying. Seeds were considered germinated when the radicle reached a length of 1-2 mm. Seeds were observed daily for 30 days to record those that had germinated. To prevent double counting, germinated and counted seeds were taken out of petri dishes daily.

2.6 Analysis of Data

The germination percentage (GP) was computed using data gathered on germinated seeds. The formula used to determine the final germination was GP = (TG/TS) ×100, where TG stands for the total number of germinated seeds and TS for the total number of sown seeds. Using Statistix Software, Version 10 (Statistix 10, 1984-2003), the collected data were subjected to one-way analysis of variance (ANOVA) as well as descriptive statistics. The germination percentage data were arcsine transformed before the ANOVA to ensure they met the normalcy requirement (Zar 1996). Tukey's Honestly Significant Difference (HSD) Test was used to test significant differences in means at a significance level of $P < 0.05$.

Table 1 Number of seeds pod^{-1} and their status

Seed condition	Seed number	Range
Intact	0.65 ± 0.11	0 – 1
Dead/eaten	0.0 ± 0.0	0 – 1
Aborted	0.35 ± 0.11	0 – 1

The ''±'' shows the standard error of the mean.

Table 2 Seed characteristic of *P. angolensis*

Dimension of seeds	Size of seeds (mm)	Range (mm)
Length	11.44±0.25	10.76-12.37
Width	7.37±0.14	6.70-8.26
Breadth	4.63±0.81	4.38-5.32

The ''±'' shows the standard error of the mean.

3 Results

3.1 Seed characteristics

The average number of seeds in each fruit was 1. The mean number of intact and aborted seeds were 0.65 ± 0.11 and 0.35 ± 0.1 (Table 1). There were no signs of dead/eaten seeds, indicating they are well protected from damaging agents by the hard, bristly fruit cover.

According to Table 2, the average seed dimensions were 11.44 ± 0.25 mm for length, 7.37 ± 0.14 mm for width, and 4.63 ± 0.81 mm for breadth. The single seeds mass ranged between of 0.25 - 2.31 grammes with a mean of 0.66 ± 0.4 grammes. Likewise, the weight of the thousand seeds varied between 184 and 231 grammes, with an average of 205.5 ± 5.12 grammes.

3.2 Seed germination

The findings showed that the mean germination percentages of nicked seeds (89%) and those soaked in sulphuric acid (86-99%) were significantly higher than their untreated counterparts [One Way ANOVA: (F (9, 30) =45.78, P = 0.00001] (Table 3). The maximum germination percentage (99.0%) was recorded in seeds immersed in sulphuric acid for thirty minutes. This was followed by 45 (92%) and 15 (91%) minutes of sulphuric acid treatment, nicking (89%), and 60 minutes of sulphuric acid (86%) (Table 3). The percentage of germination seeds differed significantly between those treated with hot water left to cool overnight and the control or untreated seeds. The lowest mean germination percentages were seen in seeds that were boiled for one (32%), three (10%), and five (4%) minutes, respectively (Table 3).

4 Discussion

4.1 Seed characteristics

Seed size, expressed as weight, volume, length, breadth, and thickness, is essential to many facets of plant ecology (Pothasin et al. 2022). It is a significant characteristic that may affect a plant's

Table 3 Means and ranges of *P. angolensis* seed germination.

Treatment	Germination Percentage	Range
Control	79.0±6.6^{b}	60-88
Nicking	89.0±2.5^{ab}	84-96
Sulfuric acid (15 minutes)	91.0±3.7^{ab}	80-96
Sulfuric acid (30 minutes)	99.0±1.0a	96-100
Sulfuric acid (45 minutes)	92.0±2.8^{ab}	88-100
Sulfuric acid (60 minutes)	86.0±5.0^{ab}	72-96
Boiling water (1 minute)	32.0±6.9^{c}	12-44
Boiling water (3 minutes	10.0±3.4^{cd}	4-20
Boiling water (5 minutes)	4.0±2.8^{d}	0-12
Hot water (allowed to cool overnight)	72.0±4.8^{b}	64-84

The ''±'' shows the standard error of the mean. Means within columns that are separated by the same letters do not differ significantly.

ability to germinate, emerge, grow, and perform in the field (Singh et al. 2021). Larger seeds store more food reserves in the embryo and endosperm than their smaller counterparts. This phenomenon influences the continued existence and development of seedlings growing in the wild. Large seeds contain nutrient-rich food reserves, which offer advantages for establishing seedlings, especially on nutrient-poor soils (Vaughton and Ramsey 2001). Plants from large seeds are expected to have bigger and heavier leaves (Elliott et al. 2007) than those from small seeds. Plants from large seeds have increased survival and establishment rates compared to those from small seeds (Stock et al. 1990). However, to make more offspring, smaller seeds can be produced in more significant quantities per canopy area than large seeds, and some seeds have a higher chance of dispersing into an appropriate habitat (Westoby et al. 1996).

These results indicated that *P. angolensis* produced a single seed per pod. This is consistent with Vermeulem's (1990) observation that the species seldom yields more than one seed per pod, though this may vary depending on the geographic location. About 65% of the seeds that were recorded in this study were intact and showed no signs of damage, whereas 35% showed indications of abortion. According to Hines and Eckman (1993) and Takawira-Nyenya (2005) only 50% of *P. angolensis* fruits have seeds, and the remaining fruits are considered barren. The dimensions of the seeds were similar to those recorded for *Senegalia erubescens* (Welw. Ex Oliv.) Kyal. & Boatwr (Kahaka et al. 2018), *Vachellia robusta* (Burch.) Kyal. & Boatwr (Botumile et al. 2020), and *V.erioloba* (E.Mey.) P.J.H. Hunter (Odirile et al. 2019). Compared to *S. galpinii* (Botumile et al. 2020) and *V. rehmanniana* Schinz (Mojeremane et al. 2017), *P. angolensis* seeds were shorter, less comprehensive, and broader. Compared to *Dichrostachys cineria* (L.) Wight & Arn and *V. nilotica* (L.) Delile, the seeds were longer, wider, and broader (Kahaka et al. 2018).

In comparison to *S. galpinii*, *V. robusta* (Botumile et al. 2020), *D. cinerea*, *S. erubescens*, and *V. nilotica* (Kahaka et al. 2018), *P. angolensis* had a higher single seed mass. *P. angolensis* had a thousand seed weight that was higher than that of *V. rehmanniana* (Mojeremane et al. 2017), *D. cinerea*, *S. erubescens*, and *V. nilotica* (Kahaka et al. 2018), but similar to that of *V. robusta* and lighter than *S. galpini* (Botumile et al. 2020).

4.2 Seed germination

Numerous leguminous woody plants in dryland environments produce seeds resistant to embryo growth due to physical dormancy or impermeable, thick seed coats (Amoakoh et al. 2017). The primary obstacle in establishing native forests using leguminous woody species is thought to be impermeable, and the presence of thick seed coats that hinders natural seed germination (Opoku et al. 2018). Therefore, before sowing, seeds need pre-sowing treatment to break the hard seed coat and promote quick, uniform, and high germination (Mojeremane et al. 2017). The propagation of seedlings of many indigenous leguminous woody species in tree nurseries is negatively affected by impermeable and hard seed coats, which prevent inhibition. Consequently, various authors have proposed seed dormancy-breaking techniques to improve the germination process and raise the germination rate (Rees 1996; Teketay 1996; Nadjafi et al. 2006; Tiwari et al. 2018; Maiguru et al. 2020; Mojeremane et al. 2020; Mmulotsi et al. 2020). For example, Tiwari et al. (2018) discovered that scarification using sandpaper was the most successful method for breaking dormancy in *Abelmoschus moschatus* Medik., *Bixa orellana* L., *Cassia angustifolia* Vahl., and *Psoralea corylifolia* L. According to Mmulotsi et al. (2020), nicking, boiling and hot water, and scarification with sulphuric acid all improve the germination of *Vachellia karroo* (Hayne) Banfi & Galasso seeds. *Burkea africana* Hook seeds treated with mechanical scarification, sulphuric acid, and boiling water treatment showed increased germination compared to untreated seeds (Mojeremane et al. 2020).

According to the result of the current study, the germination percentage of nicked seeds and their counterparts immersed in sulphuric acid was statistically higher than that of the control.

The findings align with previous studies carried out on different woody species in Botswana (Mmulotsi et al. 2020; Mojeremane et al. 2020) and other locations (Salim Azad et al. 2010; Gilani et al. 2019; Magray et al. 2023). In addition, similar results have been obtained from research on the seeds of different woody species that were nicked (Danthu et al. 1995, Diallo et al. 1996) and treated with sulphuric acid (Baes et al. 2002; Righini et al. 2004; Finch-Savage and Leubner-Metzger 2006; Naim 2015; Fredrick et al. 2017; Krishan et al. 2022; De Jesús-Velázquez et al. 2023; Mahajan et al. 2023). According to studies by Krishan et al. (2022) and Magray et al. (2023), nicking and sulphuric acid helped to overcome dormancy and enhance germination in *Phytolacca acinosa* Roxb. After mechanical scarification, Mahajan et al. (2023) reported that over 85% of the seeds of *Gloriosa superba L.* germinated. According to research by De Jesús-Velázquez et al. (2023), mechanical scarification proved to be the most successful technique for preparing the seeds of eight native woody species for replanting in tropical deciduous forests, as it yielded 100% germination. Research on seeds of *Albizia versicolor* Welw. ex Oliv and *Faidherbia albida* (Delile) A. Chev revealed that mechanically and sulphuric acid-treated seeds yielded higher germination than the control seeds (Mojeremane et al. 2021). Since sulphuric acid and nicking treatments significantly increase germination by breaking the seed coat and allowing the uptake of water and oxygen, it can be concluded that the impermeable seed testa prevents *P. angolenisis* from germinating.

Compared to untreated seeds, the germination of seeds immersed in boiling water for one, three, and five minutes was noticeably lower. This could be explained by the high heat that the boiling water transmitted through the seed coat to the interior parts, which most likely killed the embryo (Kahaka et al. 2018; Rampart et al. 2021b). The study's findings are in line with earlier investigations on other native tree species in Botswana (Kahaka et al. 2018; Mojeremane et al. 2018; Odirile et al. 2019; Botumile et al. 2020; Rampart et al. 2021a, 2021b), which revealed that the seeds soaked in boiling water had the lowest rate of germination.

Conclusions

The results showed that nicking and sulphuric acid treatment increased the germination percentages of seeds compared to untreated seeds. This implies that *P. angolensis* seeds possess the physical dormancy or thick, impermeable seed coat found in many other leguminous woody plants in the Miombo and other woodlands. The hard seed coat inhibits water absorption and gas exchange, which is essential for germination. Consequently, *P. angolensis* seeds must be treated before being sown.

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Statement of Conflicting Interests

The authors have no conflicting interests.

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