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Emerging Concerns of Foodborne Pathogens

OPEN ACCESS

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Foodborne pathogens remain a serious concern for public health, as they can cause severe diseases and economic losses worldwide. Studies have shown that microorganisms such as Salmonella, *Cronobacter* and *Escherichia coli* are frequently present in various food sources, including cereals, cereal products, poultry, herbs, and spices. According to WHO, the incidence of Salmonella in Pakistan is reported to be the highest in the world, 412 cases out of 100,000 cases per annum [1]. Contamination in these foods highlights the need for ongoing monitoring and evaluation to prevent infections [2,3].

Cronobacter spp., for instance, are important foodborne pathogens that can cause meningitis, sepsis, and necrotizing enterocolitis in neonates. Analysis of diverse food products has revealed that cereals and cereal products have the highest prevalence of *Cronobacter,* whereas commercial powdered infant formula, infant food formula, vegetables, and fruits may show lower rates of contamination. Molecular methods, including 16S rDNA sequencing and PCR-RFLP, can be used to identify isolates accurately, and *Cronobacter sakazakii* is frequently the dominant isolate. The susceptibility testing of antimicrobials reveals that the majority of the strains are still susceptible to the widely used antibiotics, though some resistance has been reported against some antibiotics like ampicillin [4,5].

Similarly, *E. coli* is a commensal bacterial flora of the gastrointestinal tract of poultry, yet pathogenic serotypes may be the cause of colibacillosis, a systemic infection with significant economic consequences. Research in poultry farms has shown that *E. coli* isolates are widely resistant to different drugs. High resistance rates to tetracycline, rifampicin, and oxytetracycline have been reported, and isolates often show resistance to multiple antibiotics used in poultry production. The development of antimicrobial-resistant strains is associated with the massive usage of antibiotics in the form of feed additives, growth promotion, and disease prevention. This resistance can be transferred to the human pathogens, posing a threat to the population health [5].

Salmonella is one of the prominent food-borne pathogens which often contaminate poultry and meat products, eggs and other food products of animal origin. It causes various diseases with such mild cases as gastroenteritis and severe diseases like typhoid fever and bacteremia. Research has revealed that infections of Salmonella are widespread in such countries as Pakistan where it is one of the highest rates in the world. Surveillance and hygiene Surveillance and hygiene practices are important in the management of Salmonella outbreaks and food safety [6].

The importance of food hygiene, controlled use of antimicrobial agents, and frequent monitoring is emphasized by foodborne pathogens and antimicrobial resistant strains. Modern molecular techniques are helping in more precise and quicker identification, which assists in carrying out epidemiological studies and prevention of risks. Contamination patterns and resistance profiles are vital issues in the prevention of foodborne outbreaks and spread of resistant strains. Food safety is a microbiological, public health, and agricultural cross over that is taken seriously. The need to carry on research, monitoring and responsible practices is necessary to minimize the risks, safeguard human health and deliver safe food products across the globe.

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Review Article

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The Role of PI3K/AKT Signalling Pathway in Cancer Stem Cells: Emerging Therapeutic Targets and Resistance Mechanisms

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ABSTRACT

Cancer stem cells (CSCs) are an insignificant, however enormous population of tumor cells that display capacities of self-renewal, differentiation, and tumor initiation, consequently being the core feature in cancer progression, recurrence, and drug resistance. The phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) is one of the most critical signalling cascades regulating CSCs and controlling their stemness, survival, evasion of the immune system under stressful conditions, as well as metabolic reprogramming. This review provides an overview of the morphological features and functional aspects of the PI3K/AKT immune cascade and pathway, and how it essentially connects with both upstream and downstream effectors in CSC biology. The cross-communication of PI3K/AKT with other pathways, e.g., Wnt, Notch, and Hedgehog, is elaborated to emphasize the redundancy of the networks facilitating CSC maintenance and drug resistance. Additionally, we provide an in-depth scrutiny of the processes through which PI3K/AKT signalling leads to CSC resistance to chemotherapy, radiotherapy, and targeted therapy, as well as their plasticity, metastasis, and immune escape mechanisms. Current and future therapeutic approaches targeting the PI3K/AKT axis, such as small molecule inhibitors, combination therapy, and drug delivery nanotechnology, are also discussed. Finally, we present clinical issues and prospects for improving CSC-based therapy by using PI3K/AKT blockade to eliminate resistance and induce protracted, long-lasting cancer remission.

INTRODUCTION

Cancer stem cells (CSCs) are a particular subpopulation in the heterogeneous tumor mass with the ability of self-renewal, differentiation, and tumorigenic potential. These stem-like cells are highly similar to normal stem cells, yet they possess dysregulated signalling mechanisms that make them resistant to conventional treatments, allowing them to survive cytotoxic therapies and repopulate the tumor [1]. CSCs have been increasingly shown to play an important role in the initiation, development, metastasis, and recurrence of numerous malignancies, thus representing an important therapeutic target in contemporary oncology. The survival and function of CSCs post-treatment is emerging as a major cause of therapeutic resistance and disease recurrence, hence the urgent need to understand the molecular mechanisms that regulate CSC survival and function [2]. Among the various

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signalling cascades involved in CSC biology, the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signalling cascade stands out as one of the most critical. It regulates essential cellular processes such as proliferation, apoptosis, metabolism, and survival [3]. Aberrant elevation of PI3K/AKT signalling is characteristic of many cancers and has been linked to oncogenic transformation, tumor progression, and poor clinical outcomes. This is largely due to the pivotal role PI3K/AKT plays in sustaining the stem-like phenotype of CSCs, which contributes to resistance against chemotherapy and radiotherapy, epithelial-mesenchymal transition (EMT), and immune evasion. These findings suggest that the PI3K/AKT pathway is not only central to general tumor biology but also has a unique role in promoting CSC-associated pathological behaviours [4]. Due to this strong association between PI3K/AKT signalling and CSC maintenance, it has emerged as a highly attractive therapeutic target. In-depth understanding of its molecular interactions with CSC regulatory networks is essential for developing more effective and lasting cancer treatments. Despite recent advances, challenges such as pathway redundancy, compensatory mechanisms, and the toxicity of targeted inhibitors still hinder clinical translation [5]. The study discusses the mechanistic role of this pathway in CSC-mediated therapeutic resistance and relapse [6]. Furthermore, the study highlights promising therapeutic strategies targeting PI3K/AKT, identifies existing gaps in clinical application, and outlines future directions to improve CSC-targeted therapies. By dissecting this essential signalling cascade, we hope to contribute to ongoing efforts to enhance the effectiveness of cancer treatment through the targeted elimination of CSCs [7].

Despite extensive research on the PI3K/AKT signalling pathway in general tumor biology, its precise and context-specific role in regulating cancer stem cell (CSC) maintenance, therapeutic resistance, and immune evasion across different tumor types remains incompletely understood. Current therapeutic strategies often target bulk tumor cells without adequately addressing CSC-driven relapse and metastasis. Furthermore, pathway redundancy, complex crosstalk with Wnt, Notch, and Hedgehog signalling, and limited clinical success of PI3K/AKT inhibitors highlight a significant translational gap. A comprehensive synthesis of mechanistic insights and emerging therapeutic strategies specifically focusing on CSC-associated PI3K/AKT signalling is therefore urgently needed.

Fundamental Constituents of the PI3K/AKT Pathway

The PI3K/AKT signalling pathway is a cascade of tightly regulated proteins that orchestrate key cellular processes, including growth, proliferation, and survival. The principal molecule initiating this cascade is Class I phosphoinositide 3-kinase (PI3K), a heterodimer composed of a catalytic subunit, p110 isoforms (α, β, δ, or γ) and a regulatory subunit (p85 or p101). Among these, p110α and p110β are ubiquitously expressed and frequently mutated or overexpressed in solid tumors, whereas p110δ and p110γ are predominantly found in leukocytes and are often implicated in immune-related malignancies. Upon activation, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2), converting it into phosphatidylinositol-3,4,5-trisphosphate (PIP3), a key secondary messenger that recruits AKT to the cell membrane for activation [8]. Protein kinase B (AKT) exists in three isoforms: AKT1, AKT2, and AKT3, which share high sequence similarity but differ in function depending on tissue type and cancer context. Full activation of AKT requires phosphorylation at two critical residues: threonine 308 by phosphoinositide-dependent kinase-1 (PDK1) and serine 473 by the mechanistic target of rapamycin complex 2 (mTORC2) [9]. Once activated, AKT transmits signals downstream by phosphorylating a wide range of substrates involved in metabolism, apoptosis, and cell cycle regulation. Phosphatase and tensin homolog (PTEN) functions as a tumor suppressor by dephosphorylating PIP3 back to PIP2, thereby negatively regulating the PI3K/AKT pathway. Loss or mutation of PTEN is a frequent oncogenic event in multiple malignancies. Another key downstream component is the mechanistic target of rapamycin (mTOR), a serine/threonine kinase that exists in two distinct complexes: mTORC1 and mTORC2. These complexes regulate protein synthesis, cellular growth, and cytoskeletal organization, positioning mTOR as a critical effector of AKT signalling [10].

Upstream Controllers and Activation Machinery

External stimuli usually initiate the PI3K/AKT pathway by binding the pathway to membrane-bound receptors. EGFR, HER2, FGFR, and PDGFR are among the most characterized upstream activators, the so-called receptor tyrosine kinases (RTKs). When ligated, these receptors become auto-phosphorylated on tyrosine residues, forming binding sites on PI3K through the SH2 domain of its regulatory subunit. Equally, G-protein-coupled receptors (GPCRs) and integrins may activate PI3K directly, as well as using an adaptor protein such as IRS-1/2 and GAB1. These upstream cues lead to catalytic conversion of PIP2 into PIP3 in the inner leaflet of the plasma membrane to localize AKT and PDK1 at the membrane [1]. Basic anchoring of AKT in the plasma membrane promotes its phosphorylation and complete activation. PDK1 activates AKT by phosphorylation of the threonine residue (Thr308) and mTORC2 activates AKT by phosphorylation of the serine residue (Ser473), a two-step process being critical in its full

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activation. This controlled activation is highly temporally conditional and spatially restricted to downstream signal transduction. Interestingly, PI3K signal amplification could also be through oncogenic mutations, amplification, or loss-of-function mutation in some regulatory domains such as PTEN, which is often missing or down-regulated in many cancers [2].

Downstream Effectors and Cellular Functions

Phosphorylation Once turned on, AKT targets a wide variety of downstream proteins, affecting numerous cellular processes that can increase tumor formation and growth. mTORC1 is a key downstream effector that controls the growth of cells and protein synthesis by phosphorylating S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1), which are important controllers of mRNA translation. This causes improved anabolic conditions and biomass gain, which promotes uncontrolled cell growth in cancer [3]. The forkhead box O (FOXO) collection of transcription factors is another important category of targets. Phosphorylation of FOXO proteins by AKT leads to nuclear expulsion and inactivation of the protein, repressing the activation of the genes dealing with apoptosis, cell cycle arrest, and oxidative stress response [4]. On the same note, glycogen synthase kinase 3 beta (GSK3B) gets phosphorylated by AKT to be inhibited in a way that advances cell phase and boosts the transcription of 8-catenin, a feature that is involved in epithelial-mesenchymal transition and stemness. AKT also inactivates the pro-apoptotic protein BAD through phosphorylation and stimulates MDM2, resulting in the degradation of the tumor suppressor p53, and all these inhibit intrinsic apoptotic pathways [5]. A combination of these downstream effects also gives cancer cells an augmented rate of proliferation, liability to cell death, metabolic reconfiguration, and immune evasion consequences. Within the framework of the cancer stem cells, the above-mentioned outputs mediate the maintenance of the stem cell phenotype through self-renewal, pluripotency and conventional therapy resistance, making the PI3K/AKT pathway the master regulator of oncogenic signalling [6].

Characterization and Major Identifiers of CSCs in Cancers

Cancer stem cells (CSCs) are a subset of cancer cells which exhibit, like normal stem cells, stem-like characteristics, such as the ability to self-renew and differentiate into a heterogeneous population of tumor cells. Like normal tissue stem cells, CSCs are conceptually presumed to be at the top of a cellular hierarchy in tumors and able to initiate and perpetuate tumorigenesis. The first cases of their detection were in acute myeloid leukaemia (AML) and then in solid tumors of breast, brain, colon, prostate, pancreatic and liver cancer [7]. Specific surface markers have been able to aid in the identification and isolation of CSCs, but this is tissue and tumor-type-dependent. As an example, the CD44high/CD24low and ALDH1 positivity is a standard universal in the breast cancer CSC, and the CD133 and nestin are universal markers in glioblastoma. In colorectal cancer, CD44, CD166 and Lgr5 have been used to describe CSCs; in hepatocellular carcinoma, they utilize CD133 and EpCAM. These markers are frequently functionally involved in the regulation of stemness, signalling patterns and engagement of the tumor environment [8] (Table 1).

Table 1: Key CSC Markers and Their Expression in Various Cancer Types

Cancer Type	Common CSC Markers	Marker Frequency (%)*	References
Breast Cancer	CD44^high/CD 24^low, ALDH1	CD44^high: 15-30%, ALDH1: 20-40%	[1]
Glioblastoma	CD133, Nestin	CD133: 10-25%	[2]
Colorectal Cancer	CD44, Lgr5, Cd166	CD44: 12-28%, Lgr5: 15-35%	[3]
Pancreatic Cancer	CD133, CXCR4	CD133: 7-20%	[4]
Hepatocellular Carcinoma	CD133, EpCAM	CD133: 10-25%	[5]

Biological Properties: Self-Renewal, Differentiation and Quiescence

The distinct characteristics of CSCs are self-renewal, the capacity to generate daughter cells with the characteristics of stem cells, and differentiation, the capacity that causes the generation of daughter cells with distinctive phenotypes [9]. The process of self-renewal is highly governed by both intrinsic and extrinsic signals that are controlled by both intrinsic transcription factors (e.g., NANOG, SOX2 and OCT4) and extrinsic signalling pathways (Notch: Wnt/beta-catenin, Hedgehog, and PI3K/AKT). The presence of these networks also preserves the stem-like state as well as eliminates early differentiation. Moreover, CSCs tend to be in quiescence or grow at low rates, and this aspect enables them to avoid the chemotherapeutic drugs, which are usually effective in destroying fast-growing cells [10]. This dormancy is not limited to being only a survival strategy, but a long-term maintenance of tumor as well as a delay of relapse. Moreover, CSCs are capable of rapid transformation between quiescence and proliferative phenotypes upon environmental stimuli, making them hard to attack therapeutically. Plasticity supplies CSCs with the ability to adjust to various micro-environmental conditions, be resistant to the cytotoxic stress, and support the re-formation of the tumor despite punishing treatment protocols [11].

Therapy Resistance, Recurrence, and Metastasis of CSCs Cells

Among the most clinically relevant peculiarities of CSCs, the first thing to be mentioned here is their high resistance to classic cancer treatment methods such as chemotherapy, radiotherapy, and targeted medications. Such resistance can be due to a variety of mechanisms,

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which include: an augmented repair capacity of DNA damage, up-regulation of drug efflux transporters (ABCG2 and ABCB1), an induction of anti-apoptotic signalling and a heightened expression of reactive oxygen species (ROS) scavengers. Further, interaction with constituents of the tumor microenvironment, such as hypoxic niches, cancer-associated fibroblasts, as well as immune suppressive cells, protects CSCs against therapeutic insult. Such defence mechanisms enable CSCs to evade treatment, causing minimal residual cancer and tumor relapse [12]. CSCs also play major roles in causing metastasis. By the acquisition of higher motility and invasive capacity through epithelial-to-mesenchymal transitions (EMT), a process frequently controlled by PI3K/AKT and other signalling pathways as well, CSCs develop the capacity to be more migratory and invasive. Such migratory CSCs have the capacity of spreading to far-away organs, becoming quiescent and afterwards reviving to develop the metastatic lesions. In clinical trials, increased expression of CSC markers is associated with poor survival, a greater chance of recurrence, and a lower survival rate in different cancers. CSCs, therefore, do not just constitute a mechanistic connection to therapeutic failure but also form a major barrier in attaining long-term cancer remission [13].

Maintaining CSC and Self-Renewal

The PI3K/AKT signalling pathway is the key to maintaining the stemness and survival of cancer stem cells (CSCs). The PI3K/AKT pathway combines external signals with internal transcriptional negative signalling to adjust the essential stem cell attributes, which include self-renewal, growth, and metabolic reprogramming. The phosphorylation of pro-apoptotic agents like BAD and transcription factors FOXO occurs when AKT is activated, and existing cell death and survival are inhibited and enhanced accordingly [1]. On top of that, AKT-induced activation of mTOR promotes anabolic growth as well as protein synthesis, which is fundamental in sustaining the high functional requirements of CSCs. In CSCs, PI3K/AKT signalling also enhances the expression of pluripotency-related transcription factors NANOG, SOX2, and OCT4, which also help in maintaining the undifferentiated tumor-initiating phenotype. Blocking the PI3K/AKT axis was found to decrease tumor sphere formation, clonogenic potential and expression of CSC markers in a variety of cancer cells, attesting to its central importance in CSC modulation [14].

EMT and Phenotypic Plasticity Advertisement

Epithelial dysregulated multipotent stem cells, an example of one of the mechanisms by which PI3K/AKT enhances CSC enrichment, is the induction of epithelial-to-mesenchymal transition (EMT), a biological process during which epithelial cells lose their polarity and adhesive property and gain the mesenchymal and migratory qualities. EMT is strongly linked to the development of stem-like characteristics, and the process is often induced in CSCs when they undergo metastasis as well as therapeutic resistance [15]. The PI3K/AKT pathway also plays a role in EMT through the activation of transcription factors (Snail, Slug, Twist, and ZEB1), the expression of which downregulates E-cadherin levels and activates the remodeling of the cytoskeleton. Such a transition not only enhances invasion and metastasis but also strengthens the plasticity of cancer cells such that non-CSCs can transition to a CSC-like state during stressful conditions [16]. Moreover, AKT promotes the stability of β-catenin by inhibiting GSK3β, a Wnt inhibitor, to facilitate the translocation of nuclear β-catenin into transcription of EMT-related genes. The plasticity of tumors is supported by the ability to promote the convergence of EMT and CSC phenotypes mediated by the PI3K/AKT signalling to enable them to adapt to hostile microenvironments and resist treatment [15].

Crosstalk among Other Stemness-Related Pathways

The PI3K/AKT pathway does not work independently but is highly involved in combining with other important signalling pathways implicated in regulating CSCs, like the Wnt/ 9912-catenin, Notch, as well as the Hedgehog pathway [17]. The PI3K/AKT pathway in the Wnt signalling pathway helps stabilize and translocate into the nucleus the β-catenin, thereby increasing the transcription of Wnt target genes relating to stemness. The cleavage and activity of the Notch intracellular domains in the Notch pathway are known to have PI3K/AKT signalling, regulating downstream gene expression in the Notch pathway, regulating cell fate and stem cell renewal. Likewise, PI3K/AKT signalling is vulnerable to stimulation by the Hedgehog pathway either indirectly through Smoothened or directly through cross-regulatory points, mTOR and GLI transcription factors. This widespread crosstalk forms a strong, highly redundant network responsible for helping CSC maintain, survive and acquire therapeutic resistance, which makes it difficult to target any individual pathway alone. It is a possibility that a more efficient approach to destroying CSC populations can be found by targeting the areas of convergence with such signalling cascades [18].

PI3K/AKT4.4 -CSC Interactions Type-Specific Cancer Evidence

Regulation of CSC through the PI3K/AKT signalling pathway has been reported in various malignancies. PI3K/AKT hyperactivity in breast cancer (which in many instances is associated with PIK3CA mutation or PTEN loss) is associated with higher levels of ALDH1+ CSCs and resistance to endocrine drugs, including tamoxifen. Blocking of PI3K in these models decreases mammosphere formation and chemotherapeutic sensitivity to CSCs. Patients with glioblastoma have AKT

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constitutively activated in CD133+ CSCs, and this leads to temozolomide and radiation resistance. Prevention of AKT in these cells decreases the tumorigenicity and induces apoptosis [19]. CSCs in colorectal cancer positive for CD44 and Lgr5 also depend on the PI3K/AKT pathway for their proliferation and survival. In this case, Wnt antagonists in combination with PI3K inhibitors have demonstrated the potential in the decrease of tumor reoccurrence and metastasizing. In pancreatic cancer and prostate malignancy, as well, faulty PI3K/AKT signalling helps shield CSCs in low-oxygen or reduced feed conditions, and leads to metastasy and drug resistance (Figure 1) [20].

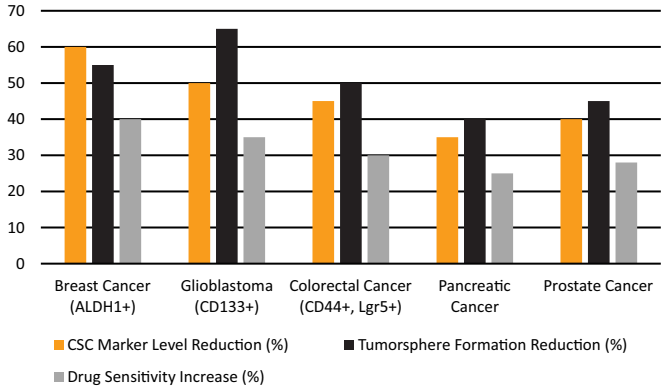

Figure 1: Effect of PI3K/AKT Inhibition on CSC Characteristics Across Cancer Types

Therapeutic Targeting of the PI3K/AKT Signalling in CSCs

Due to therapeutic resistance, targeting the PI3K/AKT signalling pathway has emerged as a promising strategy for eliminating cancer stem cells (CSCs) and overcoming resistance. Multiple inhibitor classes have been developed and are currently undergoing clinical or early-stage testing, each designed to disrupt different components of the pathway. Pan-PI3K inhibitors, including buparlisib (BKM120) and pictilisib (GDC-0941), inhibit all Class I PI3Ks to provide broad-spectrum suppression of the pathway. The study illustrates the distribution of inhibitors. These inhibitors have demonstrated efficacy in reducing CSC populations and tumor formation in various cancer models (Figure 2). However, their clinical application is often limited by toxicity resulting from systemic PI3K inhibition in normal tissues.

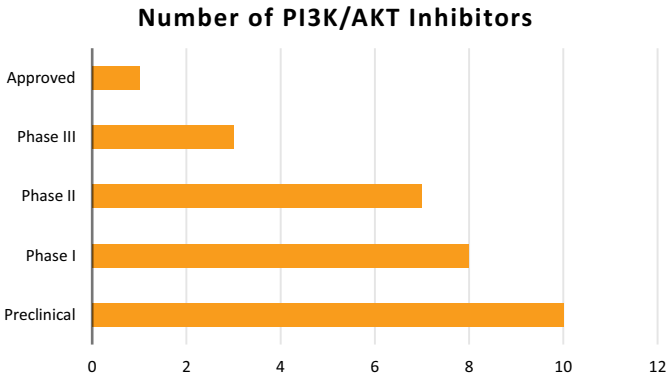

Figure 2: Distribution of Inhibitors by Clinical Trial Phase

Combination Therapy of CSCs and Bulk Tumor Cells

Deficiencies in eradicating tumor with monotherapy alone are common and are well known through the concept of pathway redundancy and compensation. As a result, combination therapy has been an appealing method of attacking CSCs and the majority of the tumor cells and cells that make up the bulk of the tumor. As an example, cytotoxic resistance of CSC is overcome in combination with PI3K/ AKT inhibitors and chemotherapy minimizing tumor recurrence. Likewise, combining PI3K/AKT inhibition with other anti-CSC pathways (e.g. Wnt, Notch or Hedgehog) has produced synergistic antitumor activity in preclinical models [21]. Furthermore, there has been a potential for the use of PI3K/AKT pathway inhibitors with immune checkpoint inhibitors (e.g. anti-PD-1/PD-L1 antibodies) to overcome the CSC-mediated immune evasion. The strategies are directed towards affecting the tumor microenvironment that is immunosuppressive, to induce strong anti-tumor immune responses. More efficacies with reduced resistance may further be achieved with personalized combination regimens that are based on tumor genomic and proteomic profiling [22].

Nanotechnology-Based Drug Delivery System and Nano-based Approaches

Improvements to drug delivery methods have allowed a more effective abrogation of the PI3K/AKT inhibitors to CSCs, reducing overall systemic toxicity. Liposomes, polymeric nanoparticles, and dendrimers are nanoparticle-based delivery systems that enable the encapsulation of PI3K/AKT inhibitors, resulting in minimal drug release, increased bioavailability, and enhanced tumor penetration [23]. These nano-carriers may be modified with ligands or antibodies that bind to surface markers expressed selectively on CSCs (e.g., CD44, CD133), and therefore, can be targeted to CSCs, leaving normal stem cells unharmed. Delivery of this type enhances the therapeutic index and off-target effects. In addition, co-packaging of PI3K/AKT inhibitors with chemotherapeutics or siRNAs against complementary pathways would also create a flexible system to administer a combination

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therapy. Into this category fall also recent developments involving stimuli-responsive nanoparticles which liberate their load in response to tumor micro-environmental factors, e.g. pH, enzymes, or redox conditions. Such intelligent delivery systems increase the concentration of drugs in CSCs micro-niches and bridge the physical barriers by the tumor stroma [24].

Clinical Challenges and Future Directions

Although it has made significant breakthroughs in the treatment of cancers through inhibition of the PI3K/AKT signalling pathway, there are still clinical issues that curtail therapeutic efficacy and expand clinical use of such drugs. The major limitation is systemic toxicity related to pathway inhibition, since PI3K/AKT signalling is involved in many normal bodily functions, including glucose metabolism, immune system regulation, and vascular homeostasis [25]. This mostly leads to undesirable side effects such as hyperglycemia, rash, gastrointestinal disturbances, and immunosuppression, which limit dosing and patient compliance. In addition, cancer cells often respond by initiating compensatory feedback signals after inhibiting PI3K/AKT and end up restoring either the upstream receptor tyrosine kinases or activating alternative survival pathways. Hence, developing resistance to drugs. Heterogeneity in tumors also adds to the problem of treatment, since cancer and cancer stem cell (CSC) subsets can utilize various signalling pathways to escape therapy. As a result, monotherapies against the pathway do not tend to produce long-lasting responses. Because of this, patient stratification and real-time monitoring of the effectiveness of the therapy have demanded the development of robust biomarkers in order to tailor patient outcomes [26]. Although molecular changes like PIK3CA mutations and PTEN loss are associated with increased activity of the pathway, they are not sufficient to forecast clinical benefit, which stresses the importance of dynamic biomarkers that take into account the activity of the pathway and the burden of CSCs. Resistance may be overcome and more effective cancer control achieved by the combination of targeted inhibitors to PI3K/AKT signalling together with other biologically relevant pathways known to be co-activated or microenvironmental substances that currently remain untargeted [27].

Limitations and Future Prospects

This review is limited by reliance on currently available preclinical and clinical data, with comparatively fewer long-term clinical trial outcomes specifically evaluating PI3K/AKT-targeted therapies in CSC populations. Tumor heterogeneity, compensatory signalling mechanisms, and systemic toxicity of pathway inhibitors remain major barriers to successful clinical translation. Additionally, standardized biomarkers for monitoring CSC burden and pathway activity are still lacking. Future research should emphasize precision oncology approaches, development of selective and less toxic inhibitors, integration of nanotechnology-based delivery systems, and rational combination therapies incorporating immunomodulatory strategies to achieve durable and personalized cancer remission.

CONCLUSION

Combining PI3K/AKT inhibitors with immunotherapy is a potential approach that would address tumor immune evasion, offering a solution to treatment durability. Pathway inhibition has preclinical evidence of modestly decreasing immunosuppressive molecules such as PD-L1 and reshaping the tumor microenvironment to drive immune-activating biology, which would render both CSCs and bulk tumor cells more vulnerable to immune destruction. The use of PI3K/AKT inhibitors in combination with immune checkpoint blockade is under ongoing clinical trials, but the best regimens and selection criteria of patients are still awaited. Moving on, precision oncology strategies integrating in-depth molecular characterization and a new generation of computational methods have the promise to make the therapy more individualized depending on the tumor or CSC characteristics. The future direction of PI3K/AKT-targeted therapies is therefore the smooth interconnection between molecular diagnostics, targeted inhibition, and immune modulation, resulting in durable, individual patient-specific therapy outcomes.

Authors' Contribution

Conceptualization: KA
Methodology: ZN[2]
Formal analysis: ZN[2]
Writing and Drafting: KA, HI, ZA, ZN[1], AA, HMFA, FJ, ZN[2]
Review and Editing: KA, HI, ZA, ZN[1], AA, HMFA, FJ, ZN[2]

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Algae, Third-Generation Energy Source: A Comprehensive Review on Methods from Cultivation to Biodiesel Production

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ABSTRACT

An increase in population growth has elevated the energy demand, and diminished fossil fuel sources. Their combustion releases carbon dioxide and contributes to environmental pollution. This has initiated intensive research to find alternative sources for economic and environmental sustainability. Amongst all, biodiesel originating from oil crops is a biodegradable, environment-friendly substitute and has properties similar to fossil diesel. Algal sources are promising substrates that require only sunlight and water for oil production. They could fulfil global demand, reduce the use of petroleum-based diesel and have higher oil productivity than other oil-yielding crops. Therefore, the third-generation production of biodiesel through microalgae is the renewable choice to overcome the energy crisis. This review covers algal cultivation methods, including both open and closed systems, lipid-extracting techniques for taking out algal oil or lipids from microalgae, and biodiesel production by the transesterification process. This article aims to assist in selecting appropriate cultivation and extraction methods for biodiesel generation.

INTRODUCTION

Energy demand has been rising steadily over the past ten years, along with adverse environmental effects. Since fossil fuels are the main source of energy for the automobile industry, global warming and a rapid decline in the availability of natural resources are being observed. Previously, researchers have carried out research on first- and second-generation biofuel production methods, and their engine testing showed that the cultivation of the feedstocks used is unsustainable. However, a substantial reduction in the emission of nitrogen oxides was observed [1]. The growing concerns regarding climate change and the depletion of fossil fuel reserves have led to an increased focus on renewable and eco-friendly energy sources to preserve the beauty of the environment and address the depletion of natural resources [2]. One promising avenue for sustainable energy production is biofuels, which are derived from organic matter such as plants and algae. Amongst the various sources of biofuel, third-generation algal biomass has gained significant attention due to its high potential for efficient and environmentally friendly biofuel production [3]. Compared to first-generation (e.g., corn or sugarcane) and second-generation (e.g., lignocellulosic) biofuels, microalgae offer up to 58,700 L/ha/year of oil yield, significantly higher than soybean (446 L/ha) or rapeseed (1,190 L/ha) [1]. Moreover, algal cultivation can occur on non-arable land with 95%

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lower freshwater use and up to 70% reduction in GHG emissions per MJ produced, as shown in recent LCA studies. Techno-economic analyses also suggest competitive production costs with continued optimization and integration of co-products [2, 3]. Algae can grow in diverse environments, including freshwater, seawater, and wastewater. However, this adaptability is strain-dependent—e.g., *Dunaliella salina* tolerates high salinity, while *Chlorella vulgaris* thrives in freshwater. Nutrient needs differ across strains, affecting lipid yields. In wastewater systems, growth is challenged by fluctuating COD/BOD, heavy metals, and microbial contamination, which hinder biomass productivity and require careful pretreatment and monitoring to maintain stable cultures [4-6]. This versatility enables algae production without competing for arable land [7], making it an attractive option for biofuel production without compromising food production. Algal sources are regarded as sustainable feedstocks due to their rapid growth rates and potential for biodiesel production. Moreover, many algal strains contribute to wastewater remediation through mechanisms such as nutrient uptake (e.g., nitrogen, phosphorus), heavy metal sequestration via biosorption, and reduction of chemical and biological oxygen demand (COD/BOD) by assimilating organic pollutants and supporting microbial communities involved in biodegradation [8]. Furthermore, algae have a remarkable ability to photosynthesize and convert sunlight into biomass at an unparalleled rate [9]. They can produce a high yield of biomass per unit area [10] compared to traditional crops, such as corn or soybeans, making algae a highly efficient feedstock for biofuel production. After cultivation, algal biomass is harvested and processed for the extraction of oil. The oil is converted into biodiesel through a chemical process called transesterification [11]. Biodiesel is the most sought-after biofuel due to its high biodegradability and environmentally friendly, non-toxic properties. Algal species are selected depending upon the percentage of the lipid content present in algal cells and the type of oil, hydrocarbons, and lipids to be extracted [10]. Biodiesel can be produced by both macroalgae and microalgae; the common algal species examined for the production of biodiesel are *Thalassiosira pseudonana, Chlorella* sp., *Chlamydomonas reinhardtii, Phaeodactylum tricornutum, Isochrysis* sp., and *Dunaliella salina* [12]. Some species of algae have a high content of lipids as much as 60% of their total weight. Triglycerides (TAGs) are commonly found in lipids that are stored in metabolites, storage products, and components of membranes [10]. The primary storage lipids in microalgae are triglycerides (TAGs) made during times of stressful conditions such as nitrogen starvation. They contain three fatty acids that are esterified to glycerol; consequently, they make the best raw materials for use in the transesterification process as a result of their fidelity in fatty acids and limited polarity. In contrast, TAGs make it possible to achieve a high percentage conversion to biodiesel (>95%) without the formation of undesirable byproducts, as is with phospholipids. Therefore, increased accumulation of TAGs will have a direct positive effect on biodiesel productivity and quality [13]. Studies have been conducted to compare the biodiesel production between macroalgae and microalgae, and it has been found that high biodiesel production is produced by using microalgae as a substrate due to the high growth rate of microalgae, which leads to better yield. Microalgae can produce large amounts of lipids; typically, 30% lipid content is present in algal cells, which increases the quantity of extracted oil that turns into biodiesel. Strain selection of microalgal strains depends on the availability of raw materials, optimization of growth and economic viability. Hossain *et al.* [14] compared the *Oedogonium* and *Spirogyra* algal species and found *Oedogonium* as a good source of biodiesel on one hand; and *Spirogyra* to yield more residual biomass after extraction. This proves that strain-specific lipid productivity and biomass profiles directly affect the amounts of biodiesel as well as process scalability [14]. Besides being environmentally friendly, algal biodiesel production in Pakistan could significantly boost the economy by utilizing 27–28 million acres of saline land, creating rural jobs, supporting energy independence, and generating up to 195 million PKR/year per 1-ton/day plant with a 4-year payback period [15].

Despite the recognized potential of microalgae as a third-generation biofuel feedstock, large-scale commercialization remains limited due to technological, economic, and operational constraints across cultivation, harvesting, lipid extraction, and transesterification stages. Existing literature often discusses these processes independently, with limited integrated evaluation of system efficiency, scalability, and sustainability. Furthermore, variations in strain performance, reactor design, and extraction efficiency create inconsistencies in reported productivity and cost-effectiveness. Therefore, a comprehensive synthesis of cultivation strategies, extraction technologies, and downstream processing approaches is required to bridge the gap between laboratory-scale research and industrial-scale biodiesel production.

Algal Cultivation System: Open Ponds

An open cultivation system is a method of growing microalgae in an open environment, typically in shallow ponds, raceway ponds, or other open containers. This growth method allows for natural sunlight exposure and atmospheric gas exchange, resulting in the production of algal biomass. The open cultivation system is considered

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the most traditional and cost-effective approach for large-scale microalga production [1]. Open systems account for approximately 98% of overall biomass production. Due to the high growth rate of microalgae, which reaches 1.5–2.0 grams per liter per day, they can produce 15 to 20 tons of dry biomass per acre per year, with oil comprising 50 to 60% of the dry weight in high-yielding strains; thus, it is economically feasible to produce biodiesel using microalgae [16]. Natural resources like concrete and rammed earth can be used for building an open pond system. The main disadvantage of such reactors is the gradual degradation of the light-transmitting walls, among others, because of the deposition of biofilm on the inner surface. Compared to open ponds, closed ponds are made of acrylics and are more expensive [17]. Open pond systems are cost-effective but limited by environmental stress; most algae grow best between 20–30°C and light intensities below 400 µmol m^{-2} s^{-1}, while thermotolerant strains like *Chlorella* sp. GD M4 can withstand up to 49°C and high irradiance, sustaining biomass yield [18]. Accumulation of unwanted contaminants due to fungal growth and algae invasions, uneven distribution of light, and an open pond's inability to hold photosensitive dark zones (as light can only penetrate to a particular depth) are also causes for concern [1]. Phytoremediation using *Chlorella* species has proved to be successful through pilot-scale tests over the recent past. A techno-economic analysis of a tubular photo-bioreactor treating agricultural centrate wastewater with *Chlorella* sp. yielded 34.6 g/m^2/day (TSS) and removal of 70% COD, 61% TKN, and 61% phosphorus [19]. Closed systems overcome numerous disadvantages of open ponds [20]. Most algal biomass is produced in open cultivation systems; such systems are also known as raceway or circular ponds [1]. Although raceway ponds with paddle wheels overcame some limitations of the earlier designs, such as poor scaling of the system and the possibility of contamination [1, 20], mixing and yield were enhanced [17, 21].

This man-made closed system not only reduces contamination during the manufacture of costly metabolites but also prevents evaporation loss, which is a major concern in open systems. Photobioreactors are artificial systems for the continuous cultivation of microalgal strains by recirculation at optimum pH, temperature and light. Light path length is critical, as shorter paths (typically <30 mm) reduce self-shading and improve light utilization efficiency. Effective mixing regimes (e.g., airlift or mechanical agitation) enhance gas–liquid mass transfer and prevent biomass sedimentation. Mass transfer coefficients are equally important to avoid oxygen accumulation and ensure CO_2 availability, directly influencing productivity. A comparative analysis by Carvalho *et al.* highlights how reactor geometry, mixing strategy, and light penetration together impact overall reactor performance [22]. Most studies report biomass productivities in the range of 20–35 g/m^2/day under optimal conditions. However, a huge temperature rise is a major pitfall that can be easily controlled by agitation and modifying the open and closed system organization that upgrades the biomass production [1]. Future modifications of closed PBRs are based on their geometric configurations, like vertical, horizontal, helical and flat panel PBR. As compared to open systems, these PBRs have 5–10 times higher efficiency but are uneconomical [23]. Although PBRs are expensive, they also have many advantages that are given below: (1) Reduce or eliminate external algae, fungi or amoeba contamination, (2) Minimize the evaporation loss to save backup water for open ponds, (3) All the parameters e.g., nutrients and gases levels are supervised and maintained, (4) Biomass can be produced at night by using LED systems which work like natural sunlight. Although artificial lights, especially LEDs, allow biomass production to continue at night by imitating photosynthetically active radiation (PAR), they also make the cost of operation highly energy-dependent. It has been found that artificial lighting may comprise as much as 50–270% of the total energy input in closed photo-bioreactors [22]. Conversely, solar-based systems are more energy-efficient and environmentally viable, especially when evaluated using life cycle analysis [1]. Photobioreactors (PBRs), in the form of transparent glass or acrylic, take the form of tubular geometries and are adapted to sunlight exposure with different modes of operation that enhance algal productivity [23]. The types include vertical (airlift, bubble column) [16, 14], horizontal [16], helical [22], and flat-plate PBRs [22]. Stirring implies the utilization of bubbling/swirling, and PVC/PE PBRs can deteriorate rapidly [24], Figure 1.

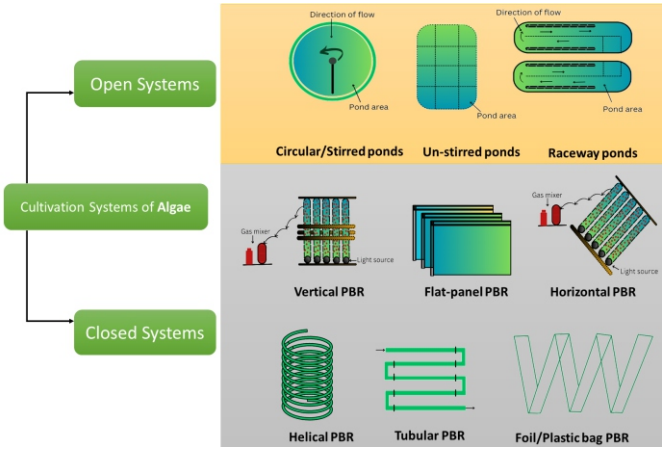

Figure 1: Algae Cultivation Systems: Open and Closed Pond Systems

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Pre-processing of Cultivated Microalgae

Microalgal biomass can be wet (70–80 wt.% water), and preprocessing is necessary to dehydrate before lipid extraction because the additional moisture adversely affects the efficiency of solvents [25]. The solvent penetration is improved by the drier, which enlarges the surface area, but the cost of drying biodiesel is increased by 35%; drying with an oven is better than drying in the sun because the drying process can be controlled [17]. Flocculation, sedimentation, centrifugation, membrane filtration, and air flotation have been identified as dewatering processes, and they are determined depending on the biomass load [26]. Centrifugation is required in small-scale because of the cost [25], and membrane filtration is motorized by pressure [21]. Flocculation and coagulation are similar phenomena of charge- or coagulant-mediated aggregation, and air flotation relies on bubbles to float particles [27].

Lipid Extraction Methods

Lipids are extracted after harvesting and lyophilizing the microalgae. The fundamental principles of lipid extraction from microalgal cells include easy scalability and minimal disturbance to lipid components. Common methods are solvent extraction and supercritical fluid extraction [28]. Technological advancements have led to many new methods of extraction that are eco-friendly (Figure 2).

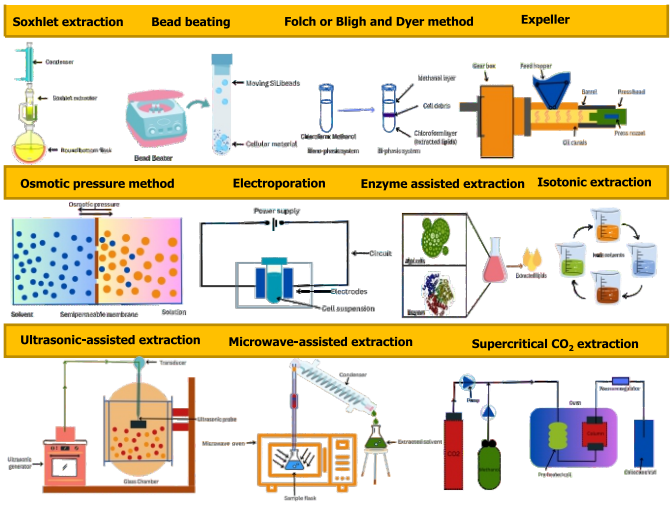

Figure 2: Different Lipid Extraction Techniques

In 1879, the first introduced method of lipid extraction was the Soxhlet method. It was used to measure the total lipid quantity in milk [29], and it has since been steadily driven in the domains of pharmaceuticals, food, and other industries. This method uses a Soxhlet extractor for extraction. In Soxhlet extraction, rather than rupturing the cells to release lipids, the process relies on diffusion across the cell wall. The choice of solvent is critical; various solvents and their combinations have been evaluated, with polarity ranging from 0.1 (petroleum ether and n-hexane) to 5.2 (ethanol) for extracting lipids from microalgae [30]. According to the data, chloroform, n-hexane, and ethanol yield high lipid contents, whereas acetone yields the least [31]. Due to its low cost, simple operations, and high yield, the Soxhlet method remains widely used in research and industry. However, it presents certain limitations, such as the use of hazardous solvents (e.g., carcinogenic benzene, less-toxic hexane), high reagent consumption, and being time-consuming [32]. The Folch and Bligh, and Dyer methods are well applied in the extraction of animal, plant, and microalgal samples. The major differences lie in the ratios of solvents: Folch is 2:1:0.7 and Bligh and Dyer 1:2:0.8 (chloroform: methanol: water) [33]. Both procedures are efficient; however, when it comes to microalgae, rigidity in cell walls is a problem that can create the risk of loss of lipids during the process of disruption [28]. Bligh and Dyer are more cash-efficient and safer. Supercritical CO_2 extraction and greener adjustments are long-established alternatives with better performance [34, 35].

Traditionally, solvent-free expelled pressing involves high-pressure hacks scaling up dried algal biomass to rupture, allowing extraction of oil: 70–75% depending on morphology and strain [36]. Nonetheless, it is expensive because it requires a lot of energy, drying (30% of the production cost), and maintenance of the equipment [26], even though it extracts high-quality oil with minimal oxidation [35]. Laboratory-scale bead beating, where vibratory action rapidly agitates the solution in the presence of small beads, is an efficient cell disruptor by grinding or collision of cells. It is economical and does not dry out, and it also maintains heat-sensitive biomolecules through cooling jackets [35]. Ultrasonication has been proposed to increase both cell lysis and release of lipids due to cavitation and acoustic streaming [37, 38], but its application is energy- and reactor-type-dependent and cell type-dependent [39, 21]. First described in 1986 [36], microwave (MW) extraction applies a variable electric field to the sample, leading to rupture of the cells via internally generated pressures and electroporation, to effect extraction in as little as 15–20 minutes [40]. Despite its effectiveness, it has issues of scalability, heating uniformity, and high maintenance [41]. Electroporation involves using electrical voltages to raise the permeability of the membranes and has advantages such as low energy requirement, reduced contaminants, preservation of membranes, and low energy [36, 25]. Osmotic pressure, as a means of rupturing the cells with hyper- or hypo-osmotic stress due to salt, scales well and has the potential to be a low-equipment process that needs additional species-specific research [21, 25]. There is an opportunity to use ionic liquids, or so-called green solvents and anion-cation pairs, which can be tuned to achieve environmentally

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friendly extraction [35, 36]. The method can be used instead of toxic solvents, and future studies are required to prove the method. The enzymatic extraction allows the removal of lipid through the rupture of the algal cell walls using special enzymes, which is precise but species-specific and depends on the composition of the lipid and low temperatures (36; 25). Supercritical CO_2 also involves the extraction of selective lipids via pressurized CO_2, but the method requires expensive, complicated hardware [35, 42]. Limitations and applications of the mentioned techniques are discussed (Table 1).

Table 1: Comparative Analysis of Different Oil Extraction Methods by Using Microalgae

Methods	Reaction conditions	Microalgae	Advantages	Limitations	References
Ultrasonic-associated extraction	Intensity of ultrasonic: 40KHz, Ultrasonic Power: 2.68 W/m^2	*Nannochloropsis, Chlorella vulgaris, Trichosporum,*	Enhances extraction rate, Reduce the time of extraction, Fewer solvents, Great penetration into algal cells	Energy loss concerning distance, Expensive approach, Difficult to scale up	[43]
Microwave-assisted extraction	T:120°C Irradiation, Power: 880W	*C.sorokiniana, N.salina, Galdieria sulphuraria*	Efficient heat and mass transfer, Higher extraction yield than conventional methods	Maintenance cost is higher, Scale-up is difficult	[44]
Organic Solvent (chloroform/ methanolextraction method)	T:20°C, Light intensity: 300 µmol m^{-2} s^{-1}	*Chlorella zofingiensis, Isochrysis galbana*	High biodiesel yield, Efficient and reliable, Easy solubility of lipids	Presence of solvent residues after extraction, Some solvents are toxic	[45]
Electroporation	Treatment Intensity: 28kWh/m^3, Appropriate culture conditions	*Nannochloropsis salina, Chlorella vulgaris*	A small amount of energy is required to use short electrical pulses, 90% lipid extraction	Intensity of field, frequency of field and geometry of electric pulses have an impact on the resulting extraction	[30]
Isotonic extraction method	T:0-140°C, Organic and inorganic ions	*Chlorella sorokiniana, Chlamydomonas reinhardtii, Botryococcus braunii*	Ionic liquids enable synthetic flexibility, tailoring the properties of the solvent like polarity, solubility and conductivity	Energy-intensive, High cost of solvents as the solvents used are synthetic, "green"	[46]
Osmotic pressure	T: 20°C, speed: 20-25rpm	*reinhardtii, Chlamydomonas reinhardtii*	Economically feasible, Cost-effective, Consumes low energy	Consumes much time Generates waste salt water	[47]
Supercritical Co_2 extraction	Pressure: 40 MPa, T: 333K, Co_2 flow rate from 0.3 – 0.5 kg h^{-1}	*Nannochloropsis oculata, Cylindrotheca closterium, Chlorella vulgaris*	Non-toxic, solvent-free lipids, consistency supports mass transfer balance.	Expensive equipments needed	[48]
Bead beating	Microscopic beads with high speed	*Nannochloropsis oculata, Chlorella zofingiensis*	Cost-effective, Better disruption of the cell, Extraction with high efficiency	Energy-intensive, Difficult to scale up	[49]
Enzyme-assisted extraction (cellulose, neutral protease, alkaline protease)	T:53°C, pH=4.4	*Nannochloropsis Sp.Chlorella vulgaris, Scenedesmus dimorphus*	Easy extraction of internal lipids, Cell disruption with minimal damage, High lipid recovery	Affected by the composition of lipid class and type, the Type and dosage of enzymes for extraction are high in cost, strongly dependent on pH	[46]
Expeller press	Dried algal biomass, High mechanical pressure to crush and extract oil	*Nannochloropsis oculata, Chlorella zofingiensis, Isochrysis galbana*	Solvent-free extraction, High-quality oil yield, Less oxidation	High cost, Heat generation and possible damage to the compounds	[50]

Transesterification Process

Transesterification is commonly adopted as a process of converting algal oil into biodiesel. It is a reversible combination of triglycerides and surplus methanol (at a 3:1 molar proportion), yielding glycerol and methyl derivatives. The stepwise reaction occurs in three phases: triglycerides transform into diglycerides and then monoglycerides, and then into methyl esters and glycerol, with the highest yield of 98% [1]. Acids, bases (e.g., NaOH, KOH), biocatalysts (lipases), and alkoxides such as sodium methoxide are catalysts used. The catalytic reaction is four thousand times quicker in bases compared to acidic conditions, and the temperature is usually maintained at 60°C under 1 atmospheric pressure, with a time of 90 minutes [51, 16]. Methanol and oil have to be dry to prevent the formation of soap. Nevertheless, the fact that methanol does not mix easily with oil causes mass transfer problems; thus, intense mixing is necessary. Biodiesel and glycerol cannot be easily purified after the reaction because these two components separate into different phases. An essential recovery of methanol, which is beneficial in terms of costs and the environment, is obtained through flash evaporation or vacuum distillation [46, 52] (Figure 3).

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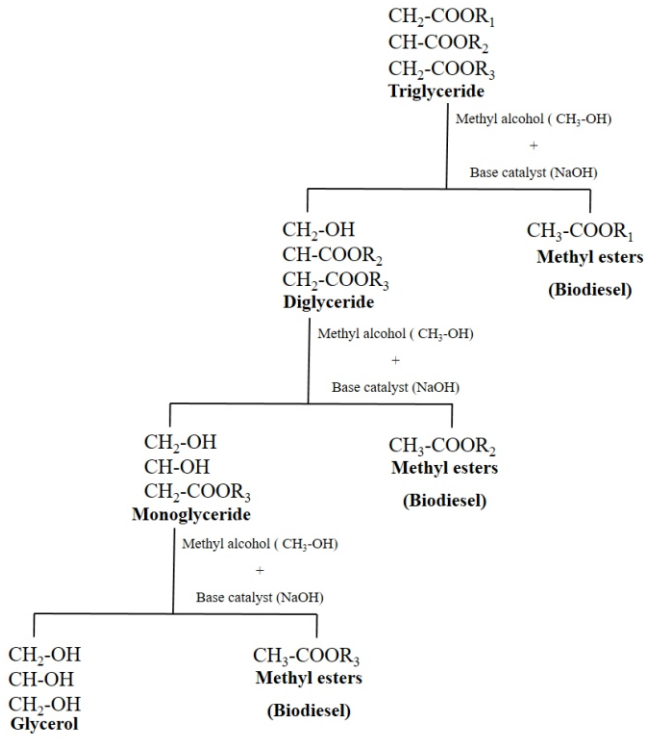

Figure 3: Transesterification Reaction for Conversion of Lipids into Biodiesel

Economic Challenges

The microalgae are bioactive products that contain several bioactive compounds, such as lipids and carbohydrates, which can be processed through processing by enzymatic and mechanical processes to produce biodiesel efficiently. This practice is dubbed green and commercially feasible as well as time-effective. Microalgae are a third-generation feedstock and, as such, can provide an environmentally friendly alternative to fossil fuels as well as other biofuels of the first and second generation. Nevertheless, commercialization, particularly the high costs involved in the process, is its major challenge, as far as cultivation, harvesting, and the extraction of lipids are concerned. Physical and biological factors light, temperature and pH, also influence large-scale production. In the base-catalyzed transesterification process, which is most often employed, the separation and purification of biodiesel and glycerol are both complex and require vigorous mixing and repetition of the washing procedures. Vacuum distillation plays an important role in the quality of the products and the sustainability of the environment in recovering methanol [52]. Although the existing extraction methods have presently not yet been perfected, multidisciplinary studies coupled with algal genomics have opened the portals to optimal lipid synthesis and an enhanced yield in biofuels of numerous strains of algae [53]. CRISPR/Cas9 and Cas12a systems have improved editing precision and multiplexing capacity for transcriptional modulation and metabolic rerouting [53].

Limitations and Future prospects

Despite the significant potential of microalgae as a third-generation biofuel source, several limitations hinder its large-scale commercialization, including high production and energy costs, contamination risks in open systems, environmental sensitivity, and challenges in downstream processing and scalability of advanced extraction techniques. Variability among algal strains and complex purification steps further reduce process efficiency. However, future prospects remain promising with advancements in genetic engineering tools such as CRISPR, development of cost-effective and hybrid cultivation systems, integration with wastewater treatment, and adoption of green extraction technologies. Additionally, a biorefinery approach and improved reactor designs can enhance overall sustainability and economic feasibility of algal biodiesel production.

CONCLUSION

Gradually increasing energy demand globally cannot be met with the usual biofuel production methods. The constant use of these sources of biofuel also changes our global carbon cycle. Algae, as an autotrophic organism, are utilized as a prospective mass production source for biofuel production. Biomolecules of algae cells, like lipids and carbohydrates, can be exploited for bioethanol and biodiesel production. In this review article, the prospects of algae as an emerging source for biofuel production for biofuel are thoroughly narrated. For research purposes, the cultivation of algae in various methods, like open and closed (photo-bioreactors) pond systems with their drawbacks, is comprehensively discussed. Solvent extraction and supercritical fluid extraction were seen as the most common methods of extraction. However, although every method has its advantages and limitation, new technological advancements lead to many new methods that will be eco-friendly, will have high efficiency, and require low maintenance costs. Still, a lot of research and development work is required for an efficient biofuel production system from algae.

Authors' Contribution

Conceptualization: NI
Methodology: NI, JI, MIR, EA, MA
Formal analysis: NI, EA, AM
Writing and Drafting: NI, AM, MA
Review and Editing: NI, AM, MA, JI, MIR, EA

All authors approved the final manuscript and take responsibility for the integrity of the work.

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Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Biofilm-Associated Infections on Biomedical Implants and Control Measures

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ABSTRACT

Biofilms are bacterial colonies that adhere to surfaces, forming protective barriers against immune responses and antibiotics, which contribute to the development of chronic infections, particularly in medical implants. This study aims to investigate the factors that influence biofilm formation on medical implants and evaluate current strategies for preventing biofilm-related infections. A review of the literature on biofilm formation mechanisms, including quorum sensing and recalcitrance, was conducted, focusing on intrinsic (e.g., quorum-sensing molecules, c-di-GMP) and extrinsic factors (e.g., temperature, surface properties). Biofilm-related infections are common in medical devices, complicating treatment and contributing to increased mortality. New strategies, including antimicrobial peptides, quorum-sensing inhibitors, and nanotechnology-based approaches, show promise in preventing biofilm formation. Surface modifications, such as antibiotic-loaded and nano-silver coatings, significantly reduce bacterial adhesion. Despite progress in biofilm prevention, further research is necessary to refine strategies for controlling implant-related infections and improving patient outcomes.

INTRODUCTION

Biofilms are heterogeneous bacterial colonies that adhere to surfaces and can act as a shield against the host immune system and antibiotic treatment. The colonies are embedded in a self-secreted matrix called extracellular polymeric substance (EPS), which is primarily composed of biological macromolecules and provides structural support to nearby cells, enabling the exchange of genetic material and facilitating quorum sensing [1, 2]. Approximately 80% of all human microbial infections are caused by biofilms, thereby posing a significant risk of chronic illnesses. Biofilms on medical implants are associated with severe morbidity and mortality [3]. Artificial medical implant devices, which are inserted either partially or entirely, are used to replace damaged structures and restore body functions in patients, whose conditions would otherwise be impaired. These devices provide structural support and therapeutic benefits [4, 5]. With advancements in device technology, their demand has increased significantly; for example, in 2018, the annual growth rate of the US medical device market reached approximately $90 billion. Approximately 0.4 to 5 million devices of various types are implanted in the US each year [6]. While these devices have improved the treatment of numerous diseases and enhanced patient well-being, they remain a global health concern due to their medical and economic implications [7]. Unfortunately, implanted devices are often associated with infection problems. Nosocomial infections (NIs), defined as infections acquired after two days of hospitalization, are frequently linked to medical devices and biofilms, accounting for

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approximately 60–70% of cases. These are commonly referred to as medical device-associated infections (MDAIs). The risk of such infections is significantly higher in patients in intensive care units (ICUs), organ transplant recipients, and neonates. Various bacterial species, including both Gram-positive and Gram-negative organisms, contribute to these infections. Their capacity to form biofilms renders conventional treatment methods, such as antibiotic therapy, less effective [8, 9]. Medical device-associated infections include catheter-associated urinary tract infections (CA-UTIs), which are the most common type, accounting for 40% of nosocomial infections globally, 70% of urinary tract infections, and 20% of urinary catheter use. Venous catheters (VCs) and urinary catheters (UCs) are widely used in hospitals. The insertion of a VC can allow skin flora or environmental contaminants to reach underlying tissues, causing severe complications such as central venous catheter-related bloodstream infections (CRBSIs) in ICUs, with mortality rates ranging from 12–25% [10, 11]. After contamination of an implanted device with bacteria, biofilm formation is influenced by several factors, including the type and number of bacterial cells, which affect the rate of attachment. Additionally, fluid flow through the device, surface properties such as hydrophobicity and charge, and the duration of surface exposure before permanent attachment play important roles [12]. Once bacteria are attached and mature, factors such as flow rate, nutrient composition, temperature, and antibiotic exposure can further influence biofilm development and stability [8]. New practical approaches are being implemented to prevent biofilm infections, including the use of antimicrobial peptides and quorum-sensing inhibitors that inhibit biofilm formation [13, 14]. Additionally, surface modification of medical devices has been explored to control biofilm formation and contamination. Various modification strategies, such as antifouling, anti-adhesive coatings, and lamination, have shown promise [15]. This review aims to discuss the intrinsic and extrinsic factors affecting biofilm formation, infections caused by bacterial biofilms on medical implants, and their clinical impacts. It also examines recent control measures and strategies designed to minimize the adverse outcomes associated with these infections.

Despite significant advancements in medical implant technology, biofilm-associated infections remain a major challenge, leading to treatment failure, prolonged hospitalization, and increased mortality. While numerous studies have explored the mechanisms of biofilm formation and surface modifications to prevent bacterial adhesion, there is limited understanding of how specific intrinsic and extrinsic factors interact to influence biofilm development on different implant materials. Moreover, most preventive strategies have been evaluated in vitro, with insufficient clinical correlation. This highlights the need for integrated research to identify effective, clinically translatable interventions against implant-associated biofilms.

Biofilm Formation

Biofilm development is a complex, gradual process involving sequential stages of adhesion, aggregation, microcolony formation, maturation, and dispersal [12, 16]. During adhesion, bacteria initially attach reversibly to surfaces through electrostatic and hydrophobic interactions, followed by irreversible binding mediated by adhesins such as pili, fimbriae, and flagella, whose expression marks this stage [17]. Free-floating bacteria then aggregate via cell-to-cell adhesins and proliferate to form microcolonies, with quorum sensing activated at a threshold density to coordinate EPS production and collective behavior [18, 19]. In the maturation stage, EPS secretion, extracellular DNA release, and formation of water-filled channels support nutrient exchange and waste removal, stabilized by active gene regulation and intercellular signaling [20]. Finally, bacteria disperse from mature biofilms through proteolytic activity, alterations in intracellular signaling (e.g., c-di-GMP), or mechanical forces, enabling colonization of new surfaces [21] (Figure 1).

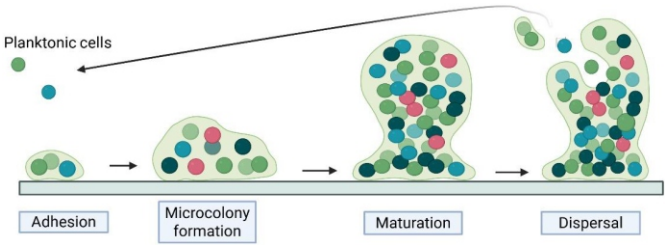

Figure 1: Various Stages of Biofilm Formation on the Surface of the Biomedical Devices

Factors Affecting the Formation of Biofilm

Various factors, both intrinsic and extrinsic, significantly affect biofilm formation, as illustrated in Figure 2. Environmental factors and the mechanism of gene expression in bacterial cells largely influence the development of biofilm. Intrinsic Quorum-sensing (QS) molecules, C-di-GMP, and efflux pumps are intrinsic factors that regulate the formation of biofilms. Quorum Sensing (QS): It is a means of communication mediated by pheromones and extracellular molecules, which make possible the structural and developmental stability of the biofilm [22]. Quorum-sensing molecules like acyl-homoserine lactones (AHLs) induce adhesion expression by binding with receptor proteins that promote attachment and stable aggregation of bacteria [23]. They regulate the production of EPS and also coordinate activities that develop microenvironments within biofilms, where functions such as nutrient acquisition, defense

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mechanisms, and active growth are carried out [24]. C-di-GMP: It is a signaling molecule having a direct relation with biofilm formation and has an inverse relation with the motility of bacteria [25]. C-di-GMP functions as a regulator. It regulates the transition from a free-floating state to a sessile lifestyle, characterized by a biofilm. Like QS molecules, it also promotes attachment and aggregation by increasing the expression of adhesion genes and other EPS matrix proteins and polysaccharide genes. A high level of c-di-GMP leads to the synthesis and secretion of EPSs, as well as the upregulation of genes involved in biofilm development and the formation of a strong matrix [26]. Efflux Pumps: As the name implies, "pump out." These pumps play a role in enhancing antibiotic resistance by effluxing antibiotics and toxic compounds [27]. Their involvement in biofilm formation lacks specific pathways; however, the long-term persistence of bacteria with reduced vulnerability to antibiotics is a potential effect of the activity and presence of efflux pumps [28]. Extrinsic Factors: Temperature, oxygen level, osmotic pressure, and hydrodynamic effects are extrinsic factors (Figure 2) that influence the physiological states of biofilm cells. Temperature: Temperature can affect both physical and physiological properties of biofilms and substrata. An optimum temperature is required for bacterial growth; any increase or decrease below this temperature has a consequential effect on the growth rate. The surface attributes of cells, such as hydrophobicity and charge, may also be influenced by incubation temperature [29]. For example, in Listeria monocytogenes, the hydrophobicity level of the cell surface increases with an increase in incubation temperature, which leads to biofilm formation. The rate of biofilm formation is independent of the temperature effect. The EPS matrix responds to stress, such as temperature, by forming a thick film and realigning polymers to avoid biofilm dissolution. Rise in temperature affects the viscosity of polysaccharides, resulting in a gel-like substance. Biofilms are favored at low temperatures where the polysaccharides are more uniform and stable [30]. Oxygen Concentration: Within a biofilm, a low level of oxygen mitigates metabolic activities and the growth of bacteria. Low oxygen cannot supply enough energy to sustain attachment, this triggers dispersal e.g., *Staphylococcus aureus* in the presence of oxygen minimizes biofilm formation through the sigB gene activity, whose lower expression promotes detachment while in *Staphylococcus epidermidis*, the high expression of the sigB gene activates operon icaADBC, which produce enzyme that results in the synthesis of adhesion polysaccharide leading to biofilm formation [26]. Osmotic Pressure: It is a pressure exerted on a membrane due to a concentration difference between the cell and its environment. A high level of osmotic pressure alters the composition of the extracellular matrix and the general structure of the biofilm. Specific genes regulate the osmotic level response and can affect biofilm formation. High osmotic pressure alters adhesion gene expression in the *Aeromonas hydrophila* cell, affecting attachment and the initiation of biofilm formation [31]. Surface/Charge: A rough surface favors the initiation of biofilm. Opposite charges on surfaces favor attachment due to electrostatic interactions. Hydrodynamic Effects: High shear rates lead to an increase in the detachment of attached bacteria, while making the biofilm thinner and denser [32] (Figure 2).

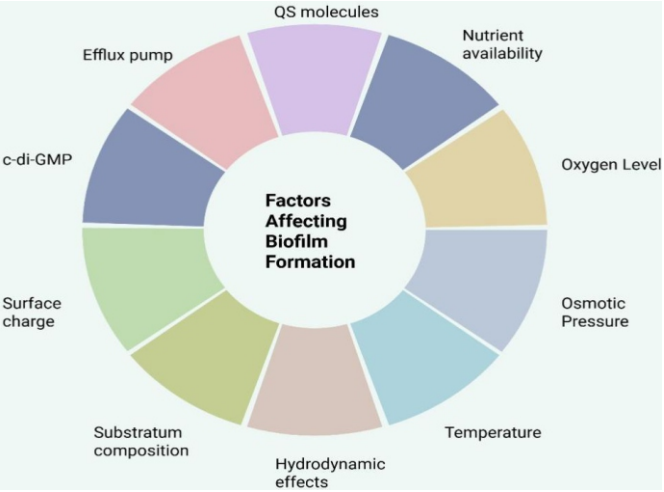

Figure 2: Intrinsic and Extrinsic Factors Affecting Biofilm Formation

Device-Related Biofilm Infections

Factors involved in device-associated infections are primarily related to the biomaterial, host, and microbial origins. Host factors include tissue damage and improper integration of tissue at the junction of biomaterial, which lead to immunity loss and inflammation, respectively. These conditions initiate an infection associated with the device [33]. Biomaterials regulate biological processes, such as cell attachment and body defense, at their surfaces. Tissues are more hydrophobic, so they do not attach to polymer surfaces. Hydrophobic cells adhere well to the hydrophobic surfaces of biomaterials [34]. Microbial factors, such as the outer surface of bacterial cells, create pathogenicity, and many cells produce a glycocalyx that facilitates attachment and colonization [35]. Intravascular Catheters: Intravascular catheters are commonly used to monitor blood circulation and the administration of nutrition, medicine, and various fluids, as shown in Table 1. As the device passes through the skin, contamination with germs can result in colonization of the inner (lumen) side of the catheter, which is the second most common cause of infections associated with these devices [36]. Reports demonstrate that 82% of 2073 hospital-acquired bacteremias are linked with intravascular catheters. In the USA, at least 120,000 cases per year of septicemia are CVC-

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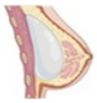
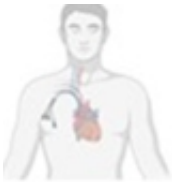
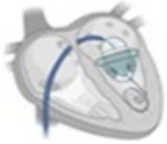
related. Prevalent pathogens associated with catheter-related infections include *S. aureus*, which is often isolated from skin contamination at the insertion site on the catheter hub. *Candida* species attach to the catheter surface. *S. epidermidis* enhances colonization [37]. *Pseudomonas* and *Xanthomonas maltophilia* are also other agents. *E. coli* and enterococci strains rarely cause catheter-associated infections [38] (Table 1).

Table 1: Biofilm and Device-Related Infections

Infection Type	Common Bacterial Species	References
 Breast implants	Staphylococci > Anaerobes	[39,40]
 Contact lenses	*Pseudomonas aeruginosa* and staphylococci	[41]
 Endotracheal tubes	*P. aeruginosa* > *S. aureus* > *Escherichia coli*	[42]
 Intravascular catheters	Coagulase-negative staphylococci > Enterococci > Gram-negative bacilli > yeasts	[43]
 Orthopedic devices	Staphylococci > Gram-negative bacilli > Anaerobes	[44]
 Valves, pacemakers, grafts	Staphylococci, Streptococci	[45,46]

Vascular Prosthesis

A prosthesis is used to replace damaged blood vessels. Vascular grafts (tubes) are used in atherosclerosis (a type of cardiovascular disease). Various agents cause infections of these grafts, such as coagulase-negative staphylococci, which are responsible for late prosthetic graft infections (Table 2) and the pseudoneurysm condition in occult graft infections [47] (Table 2).

Table 2: Infections and Pathogens Associated with Vascular Prosthesis

Infections / Conditions	Associated Pathogen (s)	References
Bacterial biofilm infections	*S. aureus*, coagulase-negative staphylococci, and Gram-negative bacteria	[48]
Late prosthetic graft infections	Coagulase-negative staphylococci (especially CoNS forming biofilms)	[49,50]
Occult graft infection	Coagulase-negative staphylococci	[51,52]
Anastomotic femoral pseudoaneurysm	Coagulase-negative staphylococci (e.g., S. epidermidis)	[53]

Orthopedic Prosthesis

Patients who receive orthopedic devices are at risk of infections, such as septic arthritis and bacteremia [54]. These infections are a significant cause of failure of such devices. The risk of infections is increased by polymethylmethacrylate cement, which is used to fix prostheses to nearby bone, as well as by the heat released during the polymerization process, which damages tissue and ultimately increases the likelihood of infections [55].

Endotracheal Tubes (ETTs)

Polyvinylchloride endotracheal tubes enable bacterial colonization more easily than those made of Teflon and polyurethane. These tubes are placed in the mucosal environment of the respiratory tract in patients who require mechanical ventilation. The colonization of the tube can cause pneumonia infection. According to a study by de Mendonça Bisneto *et al.* [56], 84% of endotracheal tubes are covered with biofilm.

Contact Lenses

Eye infections involve multiple factors, including low tear production, a compromised cornea due to inadequate oxygen exposure, and contaminated solutions used for cleaning purposes [57]. Contact lenses serve as a substrate for colonization and also as a source of infection sites, where bacteria detach and spread to other areas. *P. aeruginosa* and *S. epidermidis* are pathogens associated with these devices [58].

Intracardiac Prosthesis

Prosthetic valve endocarditis is a heart-related infection that can occur during the preoperative period or during surgery (Figure 3). Microorganisms gain entry during implantation. At early and late onset, the organisms found in prosthetic valve endocarditis are Coagulase-negative staphylococci and *S. aureus*, respectively [59]. *Streptococcus viridans* and enterococci are also found in late-onset cases. Treatment often needs the removal of the prosthetic device (Figure 3).

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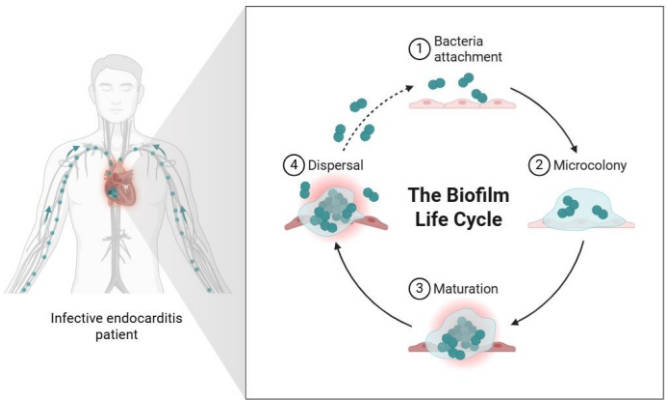

Figure 3: The Biofilm Life Cycle While Causing Infections

Breast Implant Infections

The most common problem reported in 5% to 30% of patients with breast implants is the association of biofilm with the advancement of capsular contracture [60]. *S. epidermidis* and *S. aureus,* as well as anaerobes, are frequently found in cultures of breast implants. Studies show that povidone-iodine used for irrigation of the implant was the best antiseptic for removing biofilm [61].

Control Measures

Prevention of biofilm-related infections relies on interventions that target microbial mechanisms such as adhesion, aggregation, and EPS formation. Surface modifications, including antimicrobial peptide coatings, quorum-sensing inhibitors, enzyme-based treatments, antibiotic-loaded hydroxyapatite, and nanomaterial or nano-silver coatings act by disrupting bacterial communication, attachment, and EPS synthesis, while locally delivering antibiotics to inhibit growth and reduce adhesion, thereby minimizing resistance and directly counteracting microbial colonization [62].

Antibiotic hydroxyapatite-based coatings are effective in long-term therapy because they deliver antibiotics directly to the local site, thereby controlling infections [63]. In this method, the quantity of drugs is kept low, which possibly reduces antibiotic resistance. Amoxicillin, vancomycin, cephalothin, gentamycin, and tobramycin are used in controlled-release medications. The common carrier of antibiotics is polymethylmethacrylate [64]. The controlled release of antibiotics in biodegradable carriers, such as polyglycolic acid (PGA), poly (lactic-co-glycolic acid) (PLGA), and polyethylene glycol (PEG), is highly effective in preventing infections in the long term. A biodegradable gentamycin-hydroxyapatite coating for infection prophylaxis in cementless hip prostheses, and coating PLGA with gentamycin reduces bacterial adhesion by 99%. The immersion technique is used on the surface to facilitate the absorption of antibiotics [65]. Antiseptic coatings reduce the potential for antibiotic resistance in implants [66]. Hydroxyapatite (HA), which consists of anti-septic coatings, has been proven effective in preventing infections during implant fixation development in goats [67]. Nano-silver coating effectively controls infections by killing bacteria [68]. These particles are directly applied to the surface of implants or polymer coatings, where they eliminate bacteria through their gradual release. Silver is integrated in various devices, where nearby bacteria may be significantly influenced [69]. Silver ions disrupt cell membrane functions, protein functions, and interrupt DNA [70]. Nanomaterials are very useful in the development of modern biomedical implants and their coatings. Nanofilms, nanostructured surfaces, and nanocoatings are more beneficial than usual coatings due to their controlled drug release process [71]. Inorganic Nanocoating for Drug Delivery in Implantable Sensors and Stents. Ceramic nanoparticles promote bioactivity, adhesion, and fracture toughness to the substrate without requiring high temperatures. Diamond nanoparticles (NDs) have captured widespread attention in local drug release due to their superior physical properties and biocompatibility [72] (Figure 4).

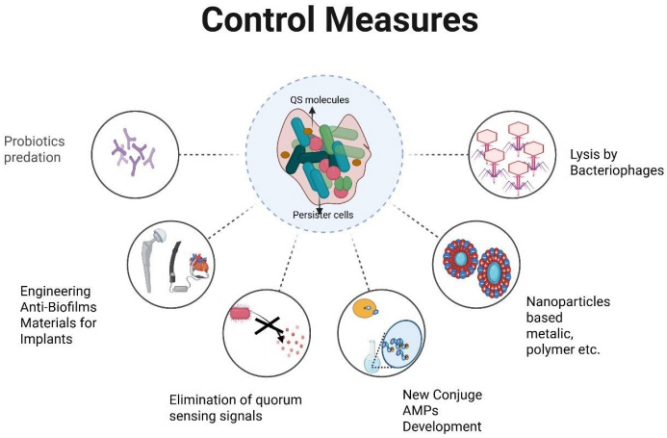

Figure 4: Some of the Control Measures for Biofilm-Associated Infections

Limitations and Future Prospects

Although current strategies, such as antimicrobial coatings, quorum-sensing inhibitors, and nanomaterial-based interventions, show promise in reducing biofilm formation, their long-term efficacy and safety in clinical settings remain uncertain. Limitations include variability in implant materials, microbial diversity, and host immune responses. Future research should focus on in vivo studies, the development of multifunctional coatings, and the integration of advanced omics technologies to better understand biofilm dynamics, ultimately guiding the design of safer and more effective biomedical implants.

CONCLUSION

The biofilm can be associated with the diagnosis and severity of medical infections related to the device. Infections related to biofilm increase the risk of disease

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and mortality because the free-floating cells can migrate from the initial infection site to the bloodstream, potentially leading to systemic complications. However, significant advancements have been made in the prevention and treatment of biofilm-related infections, which are based on effective technologies and novel compounds. Advances in antiadhesive coatings on the surface of medical implants, which consist of nanoparticles and lipids, are used to decrease the infections related to implants. Both types of coatings play a crucial role in enhancing the performance of biomedical implant devices. The molecular apparatus is becoming increasingly advanced and accessible, enabling the physiological analysis of these small and complex biofilms. The progression in omics technologies also allows us to understand the development of biofilms. Overall, this review highlights the importance of integrated strategies for preventing and managing biofilm-related implant infections. Despite the emergence of these technologies, further efforts are needed to advance knowledge of the various microbiota compositions associated with a particular device. A successful plan of action to counter biofilm-related implant infections must be preemptive, merging advanced material design, strict aseptic procedures, and timely intervention. Sustained interdisciplinary research is crucial for advancing to more effective, safe, and clinically applicable solutions that promote implant durability and improve patient outcomes.

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Authors' Contribution

Conceptualization: IL
Methodology: IL, MKS, NM
Formal analysis: IL, NM
Writing and Drafting: IL, MKS, NM
Review and Editing: IL, MKS, NM

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Next-Generation CRISPR Biotechnology for Pakistan: AI-Driven, Climate-Resilient Super Crops and the Future of Food Security

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ABSTRACT

Climate change poses a significant threat to Pakistan's agriculture, with projections indicating 10–25% yield losses in staple crops by 2050. Frequent floods, prolonged droughts, and pest infestations have already reduced wheat and rice production by up to 30%, exposing the limitations of traditional breeding and genetically modified crops. CRISPR-Cas9 genome editing, when combined with artificial intelligence (AI), offers a faster and more precise route to developing climate-resilient varieties suited to Pakistan's diverse agroecosystems. A review of recent studies highlights key advances, including AI-assisted sgRNA design, which enhances editing efficiency by 30–50%, and CRISPR-modified wheat and rice lines that show 20–30% improved stress tolerance. Yet, barriers such as complex polyploid genomes, limited genomic resources, and outdated biosafety policies hinder progress. Addressing these challenges through policy reform, capacity-building, and technology integration could transform Pakistan's agriculture, aligning directly with Sustainable Development Goals on Zero Hunger and Climate Action.

INTRODUCTION

Global food security is increasingly at risk from climate change, with the IPCC projecting that yields of staple crops could decline by 10–25% by 2050. Pakistan, ranked among the ten most climate-vulnerable countries by the Global Climate Risk Index [1], faces particularly severe challenges. Recurring floods and droughts have already reduced wheat and rice yields by 15–30% [2], while cotton production suffers annual losses of around $5 billion due to the cotton leaf curl virus. Traditional plant breeding and transgenic GM crops have proven inadequate, largely because of their long development cycles, which often take 10–15 years, as well as persistent public resistance [3]. CRISPR-Cas9 offers a promising alternative, allowing precise and non-transgenic edits within just 2–5 years [4]. More recent innovations, such as prime editing [5] and epigenome editing [6], provide additional opportunities to enhance stress tolerance and climate resilience without altering DNA sequences. Artificial intelligence tools, including the AlphaFold 3, which is used in the prediction of protein structure, and drone-assisted phenotyping technologies,

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are creating new opportunities in the improvement of crops. However, CRISPR is still not widely used in Pakistan. In addition to the persistent problem of inadequate research funding, this is mostly because of antiquated biosafety laws that continue to classify CRISPR as a GMO under policy from 2005. Climate change is one of the significant threat factors to the agriculture sector, with increased temperatures, [7], unnecessary rainfall and a noticeable rise in pest and disease ratio, ultimately reducing productivity of crops. These shifts have made Pakistan among the more susceptible countries by offering severe risks to food security as well as sustainable farming [7]. Due to a rapid increase in population and low availability of farmland, there is more pressure on water and a reduction in crop yields, leading towards food insecurity in rural areas. Conventional breeding techniques and intensive farming techniques are insufficient to overcome these problems emphasizing the need for innovative and research-oriented solutions to strengthen agricultural adaptability [8]. CRISPR-Cas9 is among the cutting-edge developments that facilitate accurate, effective and inexpensive modifications of the genetic make-up of plants. CRISPR can enhance traits including drought resistance, pest resilience and nutritional content, without inserting foreign DNA, which was required in former gene editing methods. Implementation in food crops, including maize, rice, and wheat, has evidenced substantial improvements in yield and stress resistance [9]. CRISPR is progressively considered as a technology of transformation for organic farming and a motive power for the next agricultural revolution. Artificial intelligence offers a crucial role in agricultural systems by providing targeted interventions. By the use of tools like satellite imagery, drones, sensors and machine learning, AI is being implemented to adjust irrigation, use of nutrients, insect control and prediction of yield. In combination with CRISPR, AI can play a vital role by identifying the gene targets efficiently, enhancing phenotyping and supporting the production of climate-resistant varieties suitable for the local environment [10]. This advancement is now facilitating the production of “super crops” adapted to the specific conditions of Pakistan's varied agro-ecological regions, which are eco-friendly and provide maximum yield [11], resulting in opportunities for Productive longevity and sustainable output. There are enormous efficient benefits of the integration of CRISPR biotechnology to AI for the agricultural system in Pakistan. Shortage of food has affected 11 million people, approximately, and the climate crisis is worsening [12].

Despite progress in crop improvement, Pakistan continues to face critical challenges in agricultural productivity due to climate extremes, limited access to advanced genome-editing tools, and outdated biosafety regulations. Most research focuses on global staple crops, leaving local varieties underexplored, while AI-enhanced CRISPR applications remain largely untested under Pakistan's agro-climatic conditions. This highlights a clear research gap in developing region-specific, climate-resilient crops through the integration of AI and CRISPR technologies, which can accelerate breeding cycles and improve yield stability. Review investigates the potential benefits of using AI and CRISPR together to assist Pakistan in creating climate-resilient crops. It describes current issues, real-world uses, recent scientific developments, and wider implications for securing food security in the years to come.

CRISPR-Cas9 for Climate-Resilient Crops and Food Security in Pakistan

Ahmad and Hameed highlighted that by developing crops that are resilient to environmental stress [13], CRISPR-Cas9 can solve the interconnected problems of climate change and food security in Pakistan. Their findings suggested that through precise gene editing, the resilience and yield can be increased by modifying the OsCKX gene in rice for drought tolerance and the R22M59 gene in maize for heat resistance. However, there are some research gaps in this study, including local crops negligence and risks of regional climate, including droughts and floods. The Widespread adoption is also restricted by the limited access of smallholders. To overcome these barriers, there is a demand for the development of region-specific crops via integrated climate and genetic research.

CRISPR-Cas9 Advancements in Crop Improvement

Kaur *et al.* demonstrated that stress tolerance and yield in the crops, including wheat, rice, and maize, have increased significantly by CRISPR/Cas9 with greater precision and efficiency than traditional methods [14]. But its wider implementation has been limited due to some challenges, including editing complex genomes and minimizing off-target effects and regulatory uncertainty, the absence of integration with advanced tools like AI and the limited acceptance of the public. To deploy CRISPR for the enhancement of climate resilience in vulnerable regions like Pakistan, the overcoming of these technical, scientific and policy hurdles is crucial.

AI and Machine Learning in CRISPR Design

According to Chuai *et al.* the genome editing is improved by Deep-CRISPR via a hybrid neural network for the accurate prediction of sgRNA efficiency and effects of genome-wide off-target [15]. As compared to methods used in the past, it learns from billions of data points that are unlabeled and integrates epigenetic data by automatically identifying key features without any need for manual design. This effectively resolves the common data imbalances and increases prediction accuracy across different cell types. Despite progress, challenges remain, including the lack of

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high-quality labelled data for on- and off-target effects, limited deep learning architectures, and underutilization of multiomic integration. Translating AI-based sgRNA design into key Pakistani crops, combined with field-level phenotypic and climate data, is underexplored. Addressing these gaps is essential to harnessing AI-enhanced CRISPR for developing climate-resilient super crops. Data extraction focused on CRISPR tools, AI methods, target genes, and phenotypic outcomes, followed by thematic synthesis and expert validation.

Evolution of CRISPR Tools: Key Effector Proteins

The CRISPR-Cas system has evolved into a flexible genome-editing tool over time. Because of their comparatively straightforward single-protein construction, Class 2 effectors, particularly Cas9, Cas12, and Cas13, have emerged as the most often used tools among their many classes [16]. Although RNA-guided nucleic acid cleavage is the basis for all three Cas9, Cas12, and Cas13, their classification, sequence recognition, and modes of activity vary. Cas13 is unique in that it can target RNA, while Cas9 and Cas12 primarily function as DNA-targeting nucleases. This variety has significantly broadened the CRISPR toolkit, enabling new therapeutic methods, transcriptome engineering, diagnostic tools, and genome editing [17, 18]. These effector proteins' architectures, recognition requirements (PAM/PFS), and biological uses are compared to demonstrate how CRISPR has evolved from a bacterial defensive mechanism to one of the most revolutionary developments in contemporary biotechnology, as shown in table 1.

Table 1: Key Characteristics and Comparative Overview of the Cas9, Cas12, and Cas13 CRISPR Systems

Features	Cas9	Cas12	Cas13	References
Class/Type	Class 2, Type II	Class 2, Type V	Class 2, Type VI	[16]
Origin	*Streptococcus pyogenes* (SpyCas9)	*Acidaminococcus* (AsCas12a), *Lachnospiraceae* (LbCas12a)	*Leptotrichia shahii* (LshCas13a), *Prevotella* (PspCas13b)	[17, 18]
Target	dsDNA (blunt cut)	dsDNA & ssDNA (staggered cut)	ssRNA	[18, 19]
PAM/PFS	5′-NGG-3′ PAM	5′-TTTV-3′ PAM	3′ PFS (A/U/C for Cas13a); 5′ PFS (A/U/G for Cas13b)	[20, 21]
Applications	Gene knockout, base editing	Multiplex editing, diagnostics (e.g., DETECTR)	RNA knockdown, viral detection (e.g., SHERLOCK)	[19, 22]
gRNA Requirement	crRNA + tracrRNA (sgRNA)	Self-processes pre-crRNA (no tracrRNA)	Single crRNA (no tracrRNA)	[19, 23]
Domains	HNH (cuts target strand), RuvC (cuts non-target strand)	RuvC (cleaves both strands)	Two HEPN RNase domains (ssRNA cleavage)	[21, 24]
Key Mechanism	Binds PAM → R-loop formation → HNH/RuvC cleavage	RuvC domain cleaves the non-target strand first	Binds target ssRNA → HEPN activation → collateral RNA cleavage	[24, 25]

CRISPR Success Stories in Global Agriculture

Rahim *et al.* demonstrated the potential of CRISPR/Cas9 in enhancing wheat drought tolerance by targeting the TaRPK1 gene, which regulates stress response and root development [26]. Two gRNA constructs (LR-1 and LR-2) were designed to edit conserved regions across the A, B, and D sub-genomes, introducing insertions and deletions that altered root structure. The modified wheat plants developed deeper, longer roots with increased surface area and penetration ability, enabling improved water and nutrient uptake under drought. Compared to wild types, edited lines showed a 20–30% increase in root depth and volume, reduced diameters, and steeper orientations, which collectively enhanced drought resilience. Importantly, these traits did not compromise yield; rather, grain mass and stem count improved, and although spike length slightly decreased, it was compensated for by gains in other yield components. At the policy level, regulation of genome-edited crops remains a challenge. The Cartagena Protocol on Biosafety requires member states to regulate living modified organisms [27], and Pakistan enforces this through the Biosafety Rules (2005, amended 2024) under the National Biosafety Center [28]. Current rules largely classify genome-edited organisms with foreign DNA as GMOs, while edits without foreign DNA face regulatory ambiguity. In contrast, India exempts SDN-1 and SDN-2 edits from strict GMO rules [29], and China applies a streamlined, less restrictive framework. Compared to these evolving approaches, Pakistan's rigid system may hinder the integration of CRISPR-based innovations into agriculture, despite their potential to enhance food security.

CRISPR-Enabled Biofortification: Addressing Hidden Hunger in Staple Crops

Vitamin A deficiency is a severe public health issue in regions where rice is the main staple. Introduction of (β-carotene) provitamin A in the endosperm of rice was done by the insertion of phytoene desaturase (CRTI) and phytoene synthase (PSY) genes with the help of transgenic methods. The development of Golden rice led to the initial achievements of biofortification as a result of these modifications [30]. With the carotenoid concentrations of 37 µg/g, the second-generation Golden Rice 2 (GR2) performed 23 times better than the initial prototype [31]. Although GR2 is based on transgenic methods, a faster and accurate biofortification can be achieved by CRISPR/Cas9,

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where there is no need for foreign DNA with a better and enhanced trait enrichment [30]. CRISPR has shown promising results in rice biofortification. Editing vacuolar iron transporter genes (OsVIT1/2) increased iron and zinc concentrations by 1.8- and 1.4-fold, respectively, while modifying the OsNAS promoter enhanced iron absorption 3.7-fold and zinc twofold [32]. Multi-nutrient strategies, such as in the CP105 rice line, combined edits in ferritin, OsNAS1, and PSY, producing 2.69 µg/g of β-carotene along with 1.5-fold higher iron and 1.2-fold higher zinc [30, 32]. Additionally, CRISPR-mediated silencing of the IPK1 gene reduced phytic acid by 46%, improving mineral bioavailability [33].

AI-Driven CRISPR: Synergies and Innovations in Crop Improvement

The integration of artificial intelligence (AI) with CRISPR-based genome editing is transforming agricultural biotechnology by improving accuracy, scalability, and efficiency. AI tools assist in identifying high-value gene targets, minimizing off-target effects, and expediting the development of crops with higher yields, improved nutrition, and resilience against stresses [34]. A major challenge in CRISPR-Cas9 editing is the design of single-guide RNAs (sgRNAs), which were traditionally chosen using basic parameters like GC content and PAM proximity. AI-driven approaches now leverage large datasets to predict highly efficient sgRNAs, reducing trial-and-error and broadening CRISPR's application to complex genomes. Beyond design, AI also enables the discovery of stress-associated genes and networks through analysis of multi-omics datasets, as demonstrated in the identification of ARGOS8 in maize for drought tolerance [34] and DEP1 in wheat for nitrogen-use efficiency.

AI–CRISPR synergy also supports metabolic engineering by integrating transcriptomic and metabolomic profiles to uncover genes linked to valuable secondary metabolites. For example, AI-guided CRISPR modification of the PAPI gene in tomato increased anthocyanin accumulation, stress tolerance, and nutritional value [34, 35]. These advances demonstrate how AI not only enhances precision but also enables targeted and impactful interventions across diverse crops. Ultimately, the convergence of AI and CRISPR holds promise for delivering safer, more reliable, and climate-resilient crop varieties tailored to both productivity and nutritional security. Findings are shown in table 2.

Table 2: AI Tools for CRISPR sgRNA Design

Tool (Year)	Methods	Key Features	Performance	Limitations	References
DeepCRISPR (2018)	Deep Learning (DCDNN)	Predicts on/off-target effects, Trained on 0.68B sgRNAs (13 cell lines)	AUROC = 0.804 (outperforms traditional tools)	Requires large datasets	[15]
CRISPRscan (2015)	Gradient Boosting (ML)	Incorporates sequence context, chromatin accessibility, Validated in zebrafish/human cells	High in vivo accuracy	Less effective for epigenome editing	[36]
CRISPRon (2021)	Deep Learning	Integrates multi-omics data, Accounts for Cas9 variants, repair pathways (NHEJ/MMEJ)	Superior to rule-based tools (e.g., CRISPOR)	Struggles with repair-induced indels	[37]
TIGER (2023)	CNN	Analyzes sgRNA mismatches, Tested on 200K RfCas13d gRNAs	Best at distinguishing essential vs. non-essential genes	Limited to Cas13 systems	[38]
DeepHF (2019)	Bidirectional LSTM (Bi-LSTM)	Combines RNN + biological features,Optimized for high-fidelity Cas9	Spearman R = 0.867	Sparse endogenous validation data	[38]

Case Study: CRISPR Editing for Maize Yield Improvement

Maize, a vital staple crop, faces yield limitations from drought and low soil nitrogen, with genetic trade-offs making it difficult to balance stress tolerance and productivity despite advances in conventional breeding [40]. To address this, Corteva Agriscience applied CRISPR-Cas9 and advanced data analysis to identify key genes for targeted improvement [41]. A large-scale genomic assessment of 1,671 maize genes revealed 22 candidates linked to drought tolerance, yield, and nitrogen use efficiency [42]. Among them, ARGOS8, which regulates ethylene signalling, was edited with CRISPR-Cas9 to enhance expression in maize lines [43]. Field trials showed that edited plants achieved a 5–10% yield advantage under drought and low-nitrogen stress without unintended genetic alterations, confirming both efficacy and safety [41]. In Pakistan, where maize is the third major cereal after wheat and rice, similar challenges persist. Local studies have identified drought-tolerant genotypes such as "Jalal" in Balochistan using molecular markers [44] and significant genetic diversity in inbred lines from Murree for traits relevant to stress resilience. Nutrient use efficiency is another priority, with field trials in Faisalabad demonstrating improved yields through combined nitrogen and phosphorus application [45], while nitrogen response trials in Gilgit highlighted genotype-specific adaptability, with the "Pahari" variety performing best under High-N regimes [46]. Although large-scale CRISPR applications in Pakistan are yet to be realized, these findings provide a strong foundation for integrating genome editing to enhance drought tolerance, early maturity, and NUE, thereby strengthening maize productivity under the country's climate-vulnerable and resource-limited farming systems.

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Strategies to Minimize Off-Target Effects in CRISPR

One of the major issues with the CRISPR-Cas9 is the chance of unintentional changes at non-target sites. However, due to modern advancements in computational tools, the ability to anticipate and prevent these errors has substantially improved. Models that improve the design of guide RNA (gRNA) and have been used to evaluate sequence specificity and several other general genomic features are increasingly employed. In contrast to previous techniques, platforms like DeepSpCas9 and CRISPR have decreased off-target activity by up to 90%. These were developed to utilize extensive screening datasets [47].

Key Approaches in Off-Target Control

Key approaches to controlling off-target effects in CRISPR include predictive modelling, editing optimization, and high-throughput screening. Algorithms that consider sequence homology, chromatin accessibility, and DNA methylation, along with convolutional neural networks (CNNs) for gRNA-DNA binding and recurrent neural networks (RNNs) for repair simulation, help predict off-target binding [48]. Base and prime editing allow precise single-nucleotide modifications without double-strand breaks, with cytosine deaminases refined to reduce unintended edits [49]. Additionally, large CRISPR libraries combined with computational methods facilitate high-throughput screening to identify synthetic lethal interactions and validate gRNA efficiency across different genetic backgrounds [50].

Climate Threats to Staple Crops in Pakistan: Punjab and Sindh

In Pakistan, climate extremes are threatening key crops. In Punjab, rising temperatures and heatwaves, particularly during wheat's grain-filling stage, reduce yields by up to 9%, with sudden autumn frosts cutting output by over 16% [51]. Heat-tolerant wheat varieties and improved irrigation can mitigate losses, with irrigated fields experiencing up to 70% less damage. However, climate models predict further declines in the rice–wheat system by mid-century [52]. In Sindh, floods pose a major risk to rice, with the 2022 floods inundating 2.8 million hectares and destroying 1.9 million tons of rice [53]. Prolonged flooding disrupts photosynthesis and root function, threatening both food security and export earnings.

Projected Impacts Under IPCC Scenarios

Climate projections based on IPCC's CMIP6 scenarios (SSP245 and SSP585) show mounting pressure on crop water requirements (CWR) across Pakistan's agricultural zones. By the late 21st century, Kharif (summer) rice: CWR is expected to rise by 8–14% under the high-emission SSP585 pathway, worsening water scarcity [54]. Rabi (winter) wheat: CWR may increase by 12–15% under SSP585, intensifying stress on already limited irrigation supplies. Without timely interventions, these changes could trigger steep declines in staple crop yields. Three main adaptation measures are frequently mentioned by experts: increasing the effectiveness of irrigation, expanding the use of climate-resilient crop types, and adjusting cropping calendars to better accommodate changing seasonal patterns.

Limitations and Future Directions

Despite rapid advances, several barriers limit the application of CRISPR and AI in agriculture. Wheat's complex allohexaploid genome complicates guide RNA design and raises off-target risks, while low transformation efficiency restricts commercial use [55]. AI models trained on limited datasets often fail to capture in vivo conditions or regional crop diversity, and locally curated genomic resources remain scarce in Pakistan, reducing the relevance of global models [56]. Socioeconomic constraints, such as low farmer literacy, poor digital access, and affordability, further slow adoption. Moreover, the absence of a clear regulatory framework contrasts with structured policies in China and the EU, discouraging investment and delaying innovation [57]. To harness CRISPR and AI for climate resilience and food security, Pakistan must integrate scientific, technological, and policy solutions. Priorities include establishing a science-based regulatory framework, developing national genomic databases for major crops, and strengthening rural digital infrastructure to enable AI-driven advisory systems [58]. Public engagement campaigns and farmer training programs through universities and extension services can build awareness and capacity, while targeted subsidies or financing schemes may enhance access for smallholders. International collaborations with countries such as China and EU members would also facilitate knowledge exchange and strengthen governance. These steps, combined with advances in explainable AI and CRISPR-based crop improvement, can accelerate the development of resilient, nutritionally enhanced crops, securing Pakistan's agricultural future [33, 59].

CONCLUSION

The integration of CRISPR-Cas9 and artificial intelligence (AI) offers a transformative pathway for developing climate-resilient crops in Pakistan. Early studies demonstrate significant promise: TaRPK1 editing has generated drought-tolerant wheat, while Sub1A alteration has produced flood-tolerant rice, together capable of mitigating 15–30% yield losses under stress. AI strengthens these applications by refining sgRNA design and integrating multi-omics datasets, with platforms such as Deep-CRISPR providing greater precision. Yet major barriers remain, as wheat's complex polyploid genome complicates precise editing, limited local genomic datasets weaken AI model performance, and socioeconomic constraints, such as digital illiteracy, cost,

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and regulatory ambiguity, particularly the treatment of CRISPR products as GMOs, restrict adoption. In summary, evidence indicates that AI can enhance sgRNA design while CRISPR offers tangible yield and resilience benefits, but scientific, infrastructural, and policy barriers persist. To bridge these gaps, Pakistan must modernize biosafety frameworks to distinguish gene editing from transgenics, invest in local genomic resources and digital infrastructure, and conduct pilot-scale field trials in vulnerable regions such as Punjab and Sindh. International collaborations and farmer-focused training initiatives will be essential to ensure equitable adoption. By addressing these challenges, Pakistan can accelerate the safe and effective deployment of CRISPR–AI innovations, advancing both national food security and global goals like Zero Hunger and Climate Action under the 2030 Agenda.

Authors' Contribution

Conceptualization: SSU, RI, AG, TB
Methodology: SSU, RI, AG, SAB
Formal analysis: SAB, TB, AM
Writing and Drafting: SSU, RI, AG, TB, AM
Review and Editing: SSU, RI, AG, TB, AM, SAB

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Therapeutic Interventional Probiotic Approach and the Treatment of Chronic Kidney Disease (CKD) Associated Uremia

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ABSTRACT

Chronic kidney disease is a heterogeneous disorder characterized by progressive renal malfunction triggered by low Glomerular filtration rate (GFR) with increased morbidity and associated mortality. Intestinal microbiota dysbiosis has recently emerged as an important player in progressing chronic renal disease complications by increasing uremic toxins. Recently, the interest in developing new research initiatives focusing on therapeutic modulation of the intestinal microbiome through a probiotics approach, preserving kidney functionality by maintaining the physiological balance of intestinal microbiota, decreasing uremic toxins production, and improving the kidney-gut axis functionality has been considered as a comprehensive therapeutic approach in controlling and managing chronic kidney disease and associated complications within vitro or in vivo trial analysis. This review shed light on highlighting and exploring chronic kidney disease symptomatic triggers, uremic toxins generation and utilization of strain specified probiotic therapeutically approach exploring its significant efficiency through a wide range of randomized controlled trial analysis within chronic kidney disease patients (CKD) on HD and PD therapy which significantly reported low inflammatory biomarkers and improved dysregulated intestinal microbiota, increased uremic toxin excretion (IS and PCS), improved homeostatic regulatory mechanism and quantifies health furthermore delaying the progression towards kidney failure emerging probiotic approach as a new therapeutically CKD management tool.

INTRODUCTION

Chronic Kidney Disease (CDK) is characterized by gradually progressive decrease in kidney structure and function may arises from any one or more of these underlying conditions as (1) GFR less than 60 mL/min/1.73 m2; (2) albuminuria (i.e, urine albumin ≥30 mg per 24 hours or urine albumin-to-creatinine ratio [ACR] ≥30 mg/g); [1], renal tubular disorders; or (3) history of kidney transplantation; (4) or inflammatory biomarkers of progressive renal malfunctioning such as proteinuria, erythrocyturia, or frequent malformations diagnosed through scanning and labs testing being persistent and underlying for maximum of 3 months [2], Glomerular filtration rate (GFR) presents as a substantially significant variable which diversified CKD into five stages based on GFR [3]. Chronically progressive renal malfunctioning generally advances gradually, even though the vast majority of individuals affected remain

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asymptomatic until the disease progresses and the condition worsens, with anticipated GFR progressing to 30 mL/min per 1.73 m2. Kidney structural dysfunction and impairment entail prolonged periods of time of several months and years [4].

Chronic kidney disease (CKD) remains a major global health challenge, with progressive renal dysfunction and accumulation of uremic toxins contributing to high morbidity and mortality. Although probiotics have shown potential in modulating the gut microbiome and reducing uremic toxin levels, most studies are limited to small sample sizes, short durations, or specific patient populations. There is a critical need for comprehensive investigations to evaluate strain-specific probiotic interventions and their long-term efficacy in diverse CKD patients.

Emerging Role of Probiotics

Probiotics conceptual commencement was first put forward in the year XX with Metchnikoff's investigation [5], In accordance with (ISAPP), "Probiotics are referred as biologically active microbes, that when delivered in an appropriate proportion, offered health advantages to the host and are capable to endure the digestive system (GIT), and improves or restores intestinal microbial equilibrium" Lactobacillus Acidophillus, Streptococcus thermophiles with Bifidobacteria longum considered the most frequently researched probiotics [6, 7] (Figure 1).

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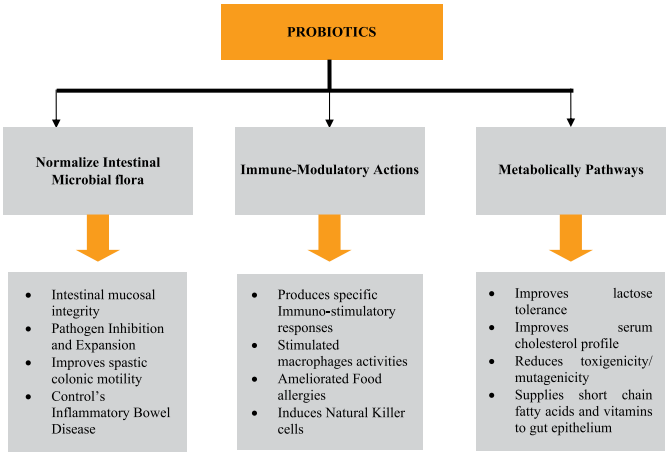

Figure 1: Probiotics Mechanism of Action on Health

Several probiotics have anti-inflammatory advantageous implications as follows: [8] Improved gut mucosa by enhancing gastric mucosal integrity, Microbicide peptidases (AMP's) and probiotic's associated defensive mechanisms, Anti-inflammation impact as well as enhanced immunological response and Competing for micronutrients and bile acids metabolically reactions [9]. There are various sources of probiotics [10] (Figure 2).

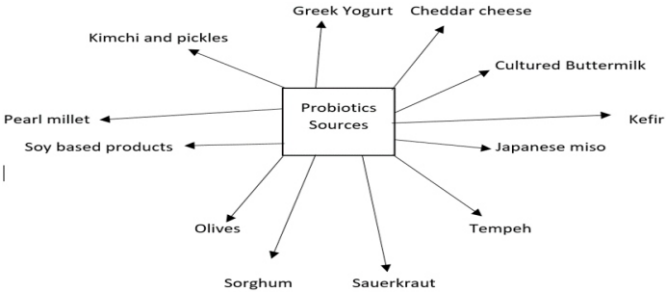

Figure 2: Sources of Probiotics

Probiotics and CKD

Probiotics administration to patients having chronic kidney disease aimed at removing URS, which is the end product of protein metabolism, and lowering the transformation of amino acids into TMAO. Probiotic supplemental therapeutic intervention resulted in p-cresol excretion, potentially reducing the plasma p-cresol levels within hemo-dialysis patients [11]. An experimental analysis demonstrated five-week probiotic therapeutic intervention reduces indoxyl glucuronidase activity in maintained hemodialytic patients (MHD) [12, 13]. Additionally, probiotics' supplementation to eight MHD patients was administered in the form of oral *L. Acidophilus* for 1 to 6 months reduces serum dimethylamine as well as nitrosodimethylamine levels, two significant potential URS that potentially increase CKD mortality [14]. Recent demonstration that probiotic supplementation probably mitigated chronic inflammatory consequences where inflammatory biomarkers negatively correlated with Kidney functioning [15]. Chou *et al.* demonstrated that therapeutic interventional treatment with probiotics within a mouse model increases short-chain fatty acids (SCFAs) within the plasma cells and protects mice from acute kidney injury (AKI) associated with ischemia reperfusion by modulating the gut macrobiotic inflammatory reactions [16]. Natarajan and his colleagues in the year 2014 noticed a decline in the C-reactive protein levels within twenty-two maintained hemodialytic patients (MHD) only after therapeutic interventional supplementation with probiotics for 50 days [17]. In view of the validation of its probable efficacy in an experimental human model, a double-blinded multi-centered trial on forty-two patients with CKD (stage III–IV) showed that the probiotic cocktail resulted in a substantial decrease in blood urea percentiles with probable reduction in plasma

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creatinine levels, which was additionally observed [18, 19]. Probiotics mechanism of cascade focuses on restoring gut motility by modifying disrupted gut microbial flora, by modulating GIT tract sensations, as well as improving motility movements associated with bowel functions within the gut tract of CKD patients [20]. Different studies were conducted on the probiotic strain against CKD (Table 1).

Table 1: Review of Clinical Experimental Analysis of Probiotic Supplementation as a Targeted Therapeutic Approach Against CKD

Probiotic Strain	Study Type	Dosages	Participants	Treatment Duration	Outcomes/Remarks	References
L.Acidophilus, B. bifidium, Prebiotic inulin fiber, Omega 3 fatty acids (B-vitamins and VitaminE)	Randomized Experimental Human Research Analysis	Nutri-health (1 capsule each day), 2.0× 1012 CFUs	Eighteen patients with CKD (Stage 1-3)	Eight Weeks	Probiotic supplementation was effective in improving glomerular filtration rate (GFR) and strengthening healthy bacterial colonies *(B. bifidium)*	[21]
L. Acidophilus, L. Casei, L. Lactis, B. bifidum, and *B.infantis*	Randomized Experimental Human Clinical Research Study	Probiotic sachets 6 × 1010 CFUs (2 times each day; morning and evening in 250ml of water taken with meals.	Hundred patients with CKD (Stage 1-3)	Twelve weeks	Probiotic supplementation is effective in dramatically reducing BUN and creatinine levels within overweight and obese individuals with high blood urea profiles, and RFTs and LFTs analysis verified no serum toxicity.	[22]
L. casei, L. cidophilus, L. bulgarigus, L. rhamnosus, B. breve, B. longum, S. thermophilus, and fructo-oligosaccharide	Randomized Experimental Clinical Research Analysis	Familact (500mg) (2 capsules each day after meal)	Sixty-six patients with CKD stage III–IV	Six weeks	Symbiotic probiotic treatment resulted in a notable reduction in mean blood urea levels in patients with CKD stage III, with no serum toxicity verified	[23]
L.acidophilus, B. bifidium, Inulin, Fructo-and galacto-oligosaccharidases	Randomized Experimental Clinical research study	(a) Initial twenty-one days' Interventional phases: (7 g of prebiotic powder + 1 probiotic tablet in the morning with a meal). (b) Last twenty-one days Interventional phases: (7 g of prebiotic powder + 1 probiotic tablet at night with meals)	Thirty-one patients with CKD	Eighteen weeks	Probiotic-mediated symbiotic approach effectively resulted in statistically noteworthy and probably potential clinical reduction in blood serum levels of Indoxyl Sulfate and p-cresyl sulphate within CKD patients	[24]
L.bulgaricus and *S. thermophilus*	Cross-sectional transverse research analysis	Probiotic yogurt (1 time each day)	Eight Hundred and Eighty-eight Patients with CKD stage (III–V)	Five years	Probiotic-supplemented yogurt markedly reduces inflammatory markers (CRP, fibrinogen, and coagulation factor VIII) within CKD patients	[25]

Probiotics' Effectiveness in Patients with Dialysis (Stage 5D)

Due to the exaggerated decline of residual renal functionality, ESKD symptomatic consequences, along with dialysis complications, could trigger dysbiotic intestinal microflora. Consequently, various investigations evaluated the implications of probiotic therapy in individuals with PD or HD. Constipation-associated dysphasia, causing irregular bowel motility, is a frequently experienced symptomatic sign within patients with HD (62.5%) or PD (28.9%) [26]. Slow transit constipation with bowel obstruction via the gastrointestinal tract causes Blind-loop syndrome (bacterial overgrowth within the stools), which plays a triggering role in the genesis of the dysbiotic gut microflora. Luo *et al.* and Hu *et al.* evaluated and correlated gut microflora genomic sequences of 16S Ribosomal ribonucleic acid within healthy subjects, CKD patients (stage I-III), CKD patients (HD and PD), among the Chinese population. Notably significant distinction within intestinal microbial diversification before and following dialysis, and conclusively figured out that patients with HD and PD are hindered metabolic, inter- and intra-cellular signalling pathways [27, 28]. The randomized control research trial analysis of probiotic therapy within HD and PD patients presented (Table 2).

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Table 2: Review of Clinical Trial Analysis of Probiotic Supplementation in Patients with Maintained Hemodialysis and Peritoneal Dialysis

Probiotic Strain	Study Type	Dosages	Participants	Treatment Duration	Outcomes/Remarks	References
L.acidophilus and *B. bifidum*	Double-blinded, placebo-controlled, randomized allocated research analysis	Probiotic supplementation forming 2×109 CFU's	Sixty-two subjects within two intervention groups (Experimental group n=Forty CKD patients on (HD); Control group n=Twenty-two healthy volunteers)	Thirty-five days	Probiotic supplemental therapy is effective in improving dysbiotic intestinal microflora, reducing fecal uremic toxicants (IS), and strengthening glomerular filtration rate (GFR) within CKD patients.	[29]
S.thermophilus, *L. acidophilus* and *B. longum*	Randomly allocated, double-blinded, prospective analysis	RenadylTM forming 30 billion CFU's (2 capsules 3 times each day with meals)	Twenty-two patients with CKD on HD therapy	Six months	Probiotic supplemental therapy efficiently displayed noticeable improvement in symptomatic gastrointestinal disturbances (IBS and IBD) and possesses a marked reduction in inflammatory bio-markers (IL-6 and IL-18) stabilizing QOL appropriately, validating its use for ESKD patients undergoing HD therapy	[30]
B. bifidum BGN4 and *B. longum BORI*	Randomly allocated, double-blinded, control analysis	Robiotic supplemental strains forming 2.0 ×1010 CFUs (2 times/day)	Twenty-two patients with CKD on HD therapy	Three months	Probiotic therapeutic regimen efficiently enhances systemic anti-inflammatory responses, with the regulatory T-cells' action potential possessing a probable decline in pro-inflammatory phagocytic leukocyte production within the CKD patients.	[31]
B.longum, *L. bulgaricus*, and *S. thermophilus*	Randomly allocated control analysis trial	Probiotic therapeutic supplementation forming 1 × 109 CFUs (2 times/day)	One hundred and sixteen patients with CKD on PD therapy	Two months	Probiotic supplementation efficiently reduces malnourishment scores, decreases inflammatory bio-markers (IL-6), subsequently lowering fecal uremic toxicants (IS) and stabilizing QOL within PD patients	[32]
L. acidophilus and *B. lactis* + prebiotic (inulin)	Multi-centered, double-blinded, placebo-controlled research analysis	Synbiotic Gel/ placebo therapy	Forty patients (2 groups of 20 patients each) with CKD on HD therapy	Two months	Synbiotic supplementation therapy efficiently improves GIT tract symptomatic disturbances, reduces inflammatory biomarkers (Plasma C-reactive proteins), and improves quality of life within CKD patients.	[33]

Limitations and Future Prospects of Probiotic Therapy

Current evidence is limited by small-scale trials, heterogeneity in probiotic strains and dosages, and short-term follow-ups, restricting the generalizability of findings. Future studies should focus on larger, multi-centered clinical trials with standardized probiotic formulations, longer intervention periods, and detailed evaluation of gut-kidney interactions. Such research could establish probiotics as a reliable adjunct therapy for improving renal function, reducing uremic toxins, and enhancing quality of life in CKD patient. Probiotics' propensity to adhere to gastrointestinal cellular epithelium and their microbicide efficiency has been analyzed within in-vitro studies showing a positive probable effect with the dosage range of 16 × 109 CFU to 2.0 × 1012 CFU within uremic rats and human trial analysis [12]. *L. plantarum* supplementation for eight weeks has been shown to considerably decrease the oxidized glutathione quantities within hyperglycemic patients having a GFR of more than >90 mL per minute and albuminuria of more than 300mg per day [34]. Probiotic treatment with *B. pasteuri* and *L. sporogenes* within a mouse model decreases significant levels of blood urea nitrogen and serum creatinine percentiles [35]. Future trends of using probiotics entailed a vast array of experimental clinical trial demonstrations owing to its markable positive health implication within CKD patients thus potentially improving intestinal integrity, modulating gut motility, increases fecal uremic toxins excretion and reduces endo-toxins levels and pro-inflammatory biomarkers further strengthening gastro-intestinal permeability, bowel motility, preserving residual kidney functionality within patients with permanent kidney disease outlined probiotic supplementation as promising therapeutically functional approach for patients with Chronic Kidney disease in near future.

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CONCLUSION

Chronic renal disease triggers progressive decline of renal structural and functional capabilities owing to decreased glomerular filtration rates, which causes excessive accumulation of uremic toxicants within the blood that significantly contributes to End-stage renal disease. Recent research based on in-vitro and in-vivo experimental trials with probiotic supplemental therapy markedly reduces pro-inflammatory cytokines, improves gut dysbiosis and irregular bowel movements, frequently attenuating kidney fibrosis lesions and scarring, presenting as a promising therapeutic interventional approach within the CKD populace. However, additional research investigations of various strain-specific probiotic treatment trials on a larger scale within hemodialysis and peritoneal dialysis patients with chronic kidney disease, primarily evaluating therapeutic outcomes, are further needed to comprehend the role of dysbiotic microbiota and Chronic kidney disease-related complications within HD and PD patients.

Authors' Contribution

Conceptualization: SI, MKN
Methodology: FH, MAI, NA
Formal analysis: SI, MKN
Writing and Drafting: SI, NA, ZS, SG, NFA, MKN, QAS
Review and Editing: SI, NA, ZS, SG, NFA, MKN, QAS, FH, MAI

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Molecular-Based Investigation of Methicillin-Resistant *Staphylococcus Aureus* from Bovine Mastitis in Kasur

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant threat to the dairy industry through mastitis, causing substantial economic losses. MRSA is a zoonotic pathogen that transmits between livestock and humans through direct contact, contaminated environments, or animal products. Its prevalence is further exacerbated by inadequate research and the misuse of antibiotics. **Objectives:** To find molecular-based investigation of methicillin-resistant *Staphylococcus aureus* from bovine mastitis in Kasur. **Methods:** This study characterized MRSA isolates from bovine mastitis using biochemical tests and genotypic analysis of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*), which carries the *mec*A gene that makes the bacteria resistant to β-lactam antibiotics. SCC*mec* typing distinguishes hospital-associated (HA-), community-associated (CA-), and livestock-associated (LA-) MRSA strains, the latter (notably CC398) exhibiting rising zoonotic concern. **Results:** Among 100 mastitic milk samples, 85% (85/100) carried *mec*A, confirming MRSA. Alarmingly, 78% (78/100) co-harbored the Panton-Valentine Leukocidin (PVL) gene, a key virulence determinant linked to severe infections in humans. **Conclusions:** The high co-occurrence of *mec*A (mediating multidrug resistance) and PVL (enhancing pathogenicity) in LA-MRSA isolates underscores a critical one health challenge, necessitating urgent interventions in antibiotic resistance and farm hygiene to mitigate transmission risks.

INTRODUCTION

Bovine mastitis, or a mammary gland inflammation, is a major problem affecting the dairy industry in the world because it comes with a decrease in milk production, quality, and the cost of treatment [1]. In countries like Pakistan, where the dairy sector is economically vital, mastitis causes annual losses in the billions due to milk wastage, veterinary expenses, and culling [2]. *Staphylococcus aureus*, especially methicillin-resistant strains (MRSA), is a leading mastitis pathogen, known for its antibiotic resistance and zoonotic potential. Subclinical infections, poor diagnostics, and unregulated antibiotic use have accelerated the emergence of multidrug-resistant strains on dairy farms [3]. MRSA resistance is primarily driven by the mecA gene, which encodes PBP2a, a penicillin-binding protein with low β-lactam affinity [4]. This gene resides within the SCCmec element, classified by MRSA types: HA-MRSA, CA-MRSA, and livestock-associated MRSA (LA-MRSA) [5]. Another key virulence factor is the *PVL* gene, encoding a cytotoxin that destroys immune cells and promotes tissue damage. While well-documented in human infections, *PVL*-positive MRSA is increasingly found in livestock, including bovine mastitis cases. The co-detection of *mecA* and *PVL* genes signals the emergence of highly virulent, multidrug-resistant strains,

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posing significant zoonotic and public health risks [6]. Transmission from infected cattle to humans can occur through direct contact, contaminated environments, or unpasteurized milk. In Pakistan, where veterinary infrastructure and hygiene standards are often lacking, farm workers and consumers are particularly vulnerable. Studies have shown genetic overlap between human and animal MRSA strains, emphasizing the need for One Health–based surveillance and control strategies [7]. Despite growing concerns, MRSA detection in Pakistan's dairy industry remains limited to conventional tools like culturing and CMT, which lack sensitivity and fail to identify key resistance genes. Phenotypic assays may miss genotypic resistance, leading to treatment failures and prolonged infections. In contrast, PCR-based molecular diagnostics offer rapid and precise detection of *mecA* and PVL, guiding more targeted treatments and reducing empirical antibiotic use [8]. This study aims to fill a critical gap in MRSA epidemiology by using PCR to characterize resistance and virulence in bovine mastitis isolates from Pakistan. Beyond laboratory insights, the findings can inform veterinary policies, antibiotic stewardship, and molecular diagnostic adoption. Addressing MRSA's dual threat of antibiotic resistance and virulence requires urgent, integrated responses to protect public health and sustain dairy productivity.

Despite the increasing reports of methicillin-resistant *Staphylococcus aureus* (MRSA) in dairy herds worldwide, comprehensive molecular epidemiological data from Pakistan, particularly from Kasur district, remain limited. Most local investigations rely primarily on phenotypic identification methods, with insufficient characterization of resistance determinants such as mecA and virulence factors like PVL. The coexistence of multidrug resistance and enhanced virulence in livestock-associated MRSA has not been adequately explored in the regional dairy context. This gap restricts effective surveillance, targeted antibiotic stewardship, and evidence-based One Health interventions in Pakistan's dairy sector. This study aims to find molecular-based investigation of methicillin-resistant *Staphylococcus aureus* from bovine mastitis in Kasur.

METHODS

This study utilized a descriptive cross-sectional design with a laboratory-based component. This study was conducted in the Department of Microbiology at the University of Punjab (May 2022 to April 2023), Lahore, with a focus on understanding the phenotypic and genotypic traits of MRSA in cases of bovine mastitis. The sampling technique was purposive (non-random). Samples were collected specifically from animals showing clinical signs of mastitis or positive California Mastitis Test (CMT) results to ensure the selection of infected individuals for MRSA analysis. This design was appropriate as it characterizes the prevalence, resistance patterns, and genetic profiles of MRSA in a defined population at a single point in time. The analytical methods, including descriptive statistics and correlation, align with this study design. A total of 100 raw milk samples were collected aseptically from mastitic animals in the Kasur district, comprising 65 from cows and 35 from buffaloes. The sample size of 100 was justified based on an expected high MRSA prevalence of approximately 80-90% reported in prior regional studies. This provides a precision (margin of error) of roughly ±8% at a 95% confidence level for prevalence estimates, which was deemed sufficient for this initial investigation. The collection was performed using sterile vials, and the samples were immediately. The samples were immediately transported in an insulated box maintained at 4°C, and processing was completed within 4 hours of collection to preserve bacterial viability. To isolate *Staphylococcus aureus*, each milk sample was cultured on Mannitol Salt Agar (MSA). MSA is both selective and differential: its high salt concentration inhibits the growth of most other bacteria, allowing only halotolerant organisms such as staphylococci to grow. The differential aspect lies in its ability to distinguish *S. aureus* from coagulase-negative staphylococci based on mannitol fermentation. Colonies of *S. aureus* that fermented mannitol turned the medium yellow due to acid production. Presumptive isolates were further confirmed using Gram staining, biochemical tests (catalase, coagulase, DNase), and PCR-based molecular identification targeting the mecA and PVL genes. Following initial screening, isolates were sub-cultured on Blood Agar Plates (BAPs) to assess their hemolytic activity. Hemolysis was categorized as α (partial), β (complete), or γ (no hemolysis). *S. aureus* commonly exhibits β-hemolysis, a useful identifying trait [9]. Presumptive Staphylococcus colonies were subjected to Gram staining. Under microscopic examination, Gram-positive cocci arranged in clusters were suggestive of *Staphylococcus aureus* [10]. Further biochemical confirmation was carried out through: Catalase Test: A drop of hydrogen peroxide (3%) was placed on a glass slide with the bacterial colony. Immediate bubbling indicated the presence of the catalase enzyme, confirming the staphylococcal species. Coagulase Test: A definitive test for *S. aureus*, this was done by mixing the bacterial culture with rabbit plasma. The presence of clot formation within 4 hours indicated coagulase-positive S. All tests were performed in duplicates. aureus. DNase Test: To assess the ability of isolates to produce Deoxyribonuclease, cultures were streaked on DNase agar plates and incubated. Plates were flooded with 1N HCl to precipitate DNA [11]. Clear zones around colonies confirmed DNase activity, supporting *S. aureus* identification. Laboratory Standards Institute) guidelines.

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The antibiotics used included: Oxacillin (1 µg), Cefoxitin (30 µg), Vancomycin (30 µg) [12]. The bacterial lawn was prepared on Mueller-Hinton Agar (MHA), and disks were placed aseptically. After incubation at 35℃ for 24 hours, zones of inhibition were measured. Resistance to cefoxitin and oxacillin indicated MRSA. The zone diameter interpretive criteria followed CLSI standards: for oxacillin (1 µg), a zone ≤10 mm indicates resistance; for cefoxitin (30 µg), a zone ≤21 mm indicates resistance. Vancomycin susceptibility was interpreted according to CLSI guidelines. Vancomycin-resistant isolates, if any, were noted for further molecular confirmation. Control strains included S. aureus ATCC 25923 (MSSA) and ATCC 43300 (MRSA) for validating antibiotic testing and PCR. Cefoxitin and oxacillin were used as surrogate markers for the detection of MRSA phenotypically. Isolates showing reduced susceptibility to these antibiotics were selected for genotypic confirmation of the *mecA* gene, a critical determinant of methicillin resistance. Genomic DNA was extracted using the CTAB method with a lysozyme pretreatment step to break the thick peptidoglycan layer of Gram-positive bacteria, ensuring high-quality DNA for downstream PCR. Briefly, bacterial pellets were lysed using lysozyme, followed by treatment with CTAB buffer. Proteins were precipitated using chloroform-isoamyl alcohol, and DNA was precipitated with isopropanol. The DNA was then washed with ethanol and resuspended in TE buffer. DNA quality and quantity were assessed using a NanoDrop spectrophotometer and 1% agarose gel electrophoresis. Only samples with an A260/280 ratio of 1.8–2.0 and an A260/230 ratio >1.8 were used for PCR amplification [12]. The presence of the *mecA* and Panton-Valentine Leukocidin (PVL) genes was confirmed via polymerase chain reaction (PCR) using gene-specific primers. mecA primers: Forward: 5'-AAA ATC GAT GGT AAA GGT TGG C-3', Reverse: 5'-AGT TCT GCA GTA CCG GAT TTG C-3'. PVL primers: Forward: 5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCCA-3', Reverse: 5'-GCA TCA AST GTA TTG GAT AGC AAA AGC-3'. PCR conditions: Initial denaturation at 94°C for 5 minutes. 30 cycles of: Denaturation at 94°C for 30 seconds, Annealing at 55°C (mecA) / 57°C (PVL) for 30 seconds, Extension at 72°C for 30 seconds, Final extension at 72°C for 5 minutes. PCR products were analyzed on a 1.5% agarose gel electrophoresis stained with ethidium bromide. PCR bands (mecA ~533 bp, PVL ~433 bp) were confirmed using a MRSA-positive control, and representative products were sequenced for validation. In this study, statistical analyses were carried out using IBM SPSS Statistics version 25. The prevalence of MRSA was determined by expressing the number of *mecA*-positive isolates as a percentage of the total samples. Inhibition zone diameters from the disk diffusion assays for oxacillin, cefoxitin, and vancomycin were summarized using descriptive statistics, calculating means and standard deviations to capture central tendency and dispersion. The data analysis plan was explicitly tailored to meet the study's objectives and handle the specific types of data collected. Descriptive statistics characterized the basic features of the data, the Chi-square test assessed crucial categorical associations, the independent t-test compared means between two animal species, and Pearson's correlation quantified relationships between key continuous variables, providing a comprehensive analytical approach. To evaluate the association between phenotypic resistance (disk diffusion results) and genotypic confirmation (mecA presence), a Chi-square test of independence was employed, with $p<0.05$ indicating statistical significance. Differences in inhibition zone diameters between cow- and buffalo-derived isolates were compared using a one-way ANOVA. Finally, Pearson's correlation coefficient (r) was calculated, with r = 0–0.3 considered weak, 0.3–0.6 moderate, and 0.6–1.0 strong correlation. "Finally, Pearson's correlation coefficient (r) was calculated to assess the strength and direction of the relationship between *mecA* and PVL gene carriage and between antibiotic inhibition zones. The strength of correlation was interpreted using the following thresholds: |r| = 0.00–0.30 was considered weak, |r| = 0.31–0.60 moderate, and |r| = 0.61–1.00 strong." to assess the strength and direction of the relationship between *mecA* and PVL gene carriage. All microbiological work was performed in a Biosafety Level 2 laboratory under sterile conditions. Positive and negative controls were run with each PCR batch to ensure validity. Autoclaved materials and proper aseptic techniques were employed throughout the study.

RESULTS

Although 100 milk samples were collected for MRSA analysis, these were obtained from 104 animals because some animals contributed samples from multiple udders. Mastitis was detected in 103/104 (99%) of animals. Herds were generally small-to-medium (2–5 animals; 85%), with infrequent milking (0–2 times/day) and limited udder sampling (1–3 udders/animal). Hygiene practices were poor: only 19/104 (18.3%) of samples reflected proper milker hand hygiene, and 28/104 (26.9%) were collected under hygienic milking conditions. Weak correlations between hygiene and mastitis positivity ($r \approx 0.05$–0.06) suggest that current practices are insufficient to control infection. Phenotypic resistance profiling confirmed universal oxacillin resistance (0mm, median; range 0–2mm) in all 100 isolates. Cefoxitin resistance was observed in 60% of isolates (mean inhibition zone: 15.3 ± 3.2mm; range 10–22 mm), while 51% showed vancomycin resistance (mean: 19.77 ± 5.89mm; range 12–28mm). Vancomycin showed variable susceptibility (median zone: 21 mm), contrasting with minimal inhibition for oxacillin/cefoxitin (median: 0

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mm). A moderate correlation existed between cefoxitin and vancomycin responses ($r > 0.4$), while oxacillin resistance was independent. Correlation of the surf field mastitis test with sampling parameters like herd distribution, Udder number, Frequency of lactation, Herd size, and hygiene graphs is made using pandas and Matplotlib libraries for statistical analysis (Figure 1).

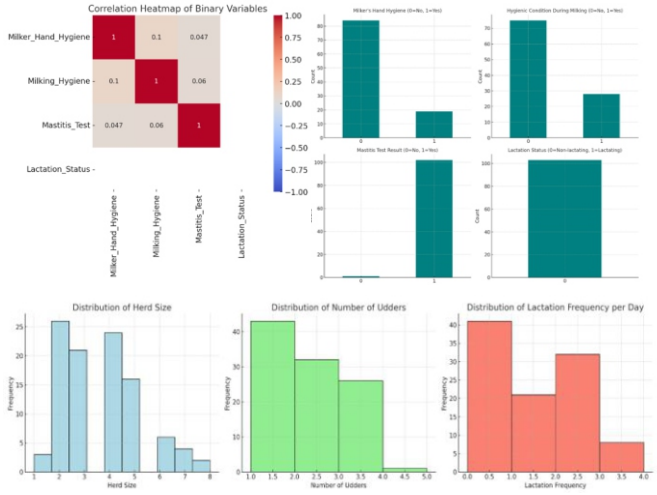

Figure 1: Correlation of Surf Field Mastitis Test, with Sampling Parameters Like Herd Distribution, Udder Number, Frequency of Lactation

Genotypic analysis detected the *mecA* gene (encoding PBP2a) in 85% (85/100) of MRSA isolates, confirming β-lactam resistance. The remaining 15% (15/100) *mecA*-negative MRSA implies alternative resistance mechanisms (e.g., modified PBPs). The virulence-associated PVL gene was prevalent in 78% (78/100) of isolates. Strain profiling revealed significant divergence: HL-SK exhibited hyper-virulence with robust mecA/PVL co-occurrence and high enzymatic activity (catalase: 27/30, DNase: 22/30, coagulase: 18/30). In contrast, HL-DS and HL-PK showed minimal enzymatic/genotypic virulence. Strains like HL-KK (high *mecA*, low PVL) suggested Biochemical heterogeneity was evident, with catalase/DNase activity ranging from high (HL-DH, HL-KK, HL-KP) to low (HL-PK, HL-DS) (Figure 2).

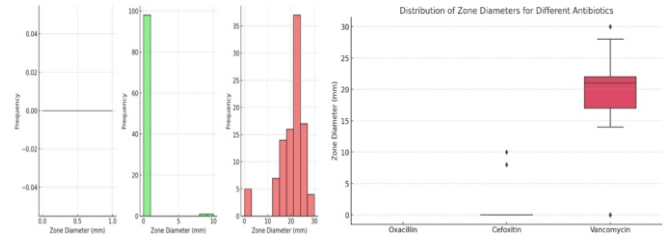

Figure 2: Correlation of Oxacillin, Cefoxitin, and Vancomycin Distribution among MRSA Strains

The convergence of widespread *mecA*-mediated resistance (85%), high PVL virulence (78%), and deficient on-farm hygiene underscores significant zoonotic risks, particularly from hyper-virulent strains like HL-SK in dairy settings. Vancomycin resistance (51%) further threatens last-line therapy efficacy (Figure 3).

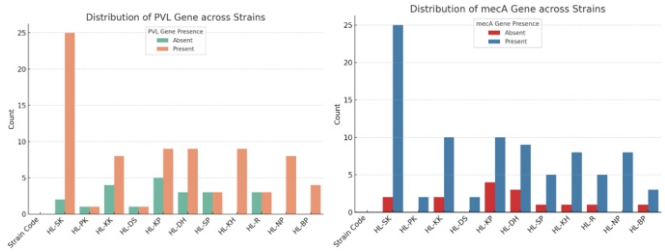

Figure 3: Distribution of mecA and PVL Gene among MRSA Samples

DISCUSSION

This study offers an in-depth analysis of antimicrobial resistance (AMR) patterns in *Staphylococcus aureus*, specifically targeting MRSA strains isolated from bovine mastitis milk samples [13, 14]. The resistance prevalence data will be revised to include 95% confidence intervals (95% CI) for all percentage estimates. For example, the prevalence of the **mecA** gene will be reported as 85% (95% CI: 76.2% - 91.1%). This addition will provide a measure of the precision and reliability of the prevalence estimates reported in the study [15, 16]. Complete resistance to oxacillin and notable resistance to cefoxitin and vancomycin reflect the growing clinical and zoonotic risks posed by these strains in livestock environments. Universal phenotypic resistance to oxacillin validates the MRSA classification of all isolates. However, only 85% harbored the *mecA* gene, indicating a possible divergence between phenotypic and genotypic resistance. This suggests the presence of alternative resistance mechanisms such as PBP mutations, efflux pumps, or epigenetic modifications that enable methicillin resistance without *mecA* involvement [17]. Alterations in native PBPs, such as PBP2, can reduce affinity for β-lactam antibiotics, leading to resistance without the presence of *mecA* [18]. The Discrepancy underlines the necessity of using combined diagnostic approaches rather than relying solely on genetic markers. Cefoxitin resistance in 60% of isolates, typically a reliable marker for *mecA* presence, supports its diagnostic value, though 40% of isolates remained susceptible despite being phenotypically resistant to oxacillin. This may reflect suppressed *mecA* expression or phenotypic heterogeneity. Vancomycin resistance in 51% isolates is especially alarming, as it compromises one of the last-resort antibiotics for MRSA [12]. Such resistance indicates the emergence of VISA/VRSA strains, presenting a direct threat to veterinary and public health. The molecular detection of virulent genes further clarifies the pathogenic profile of these isolates. The *mecA* gene, encoding the penicillin-binding protein PBP2a, was prevalent in 85% of strains, while 78% carried the PVL gene, a bicomponent leukotoxin associated with increased necrosis and immune

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system evasion (Itodo, 2023). The co-presence of mecA and PVL genes, particularly in strains like HL-SK, suggests simultaneous selection of resistance and virulence traits, potentially increasing pathogenicity and complicating treatment, although direct clinical outcome data were not assessed in this study. Biochemical testing supported the genotypic data, with HL-SK exhibiting strong enzymatic activity and maximum *mecA/PVL* expression, classifying it as a hypervirulent MRSA variant. In contrast, HL-DS and HL-PK showed low enzymatic and genotypic virulence. These observations suggest a potential role for biochemical assays in screening strain virulence and resistance levels. Epidemiological findings contextualized these results, revealing a nearly universal mastitis prevalence in herds despite partial hygiene measures. Weak correlations between hygiene practices and mastitis incidence suggest that current efforts are inadequate or inconsistently applied [19, 20]. Poor milk-hand hygiene and unsanitary environments likely contribute to persistent transmission of resistant strains. Biofilm formation was not assessed in this study, but it is recognized as a key factor in bacterial persistence and antimicrobial resistance in dairy settings. Resistance profiling through statistical and graphical analysis revealed distinct susceptibility patterns. Cefoxitin and oxacillin showed narrow, compressed inhibition zones reflecting widespread resistance. In contrast, vancomycin displayed a broader range, indicating mixed susceptibility. Heatmap correlations showed moderate linkage between cefoxitin and vancomycin resistance, implying possible shared regulatory pathways [10]. Oxacillin resistance, with minimal correlation to other antibiotics, suggests independent or unique resistance mechanisms. These insights emphasize the need for comprehensive AMR surveillance, multi-target diagnostics, and enhanced farm biosecurity to control hypervirulent MRSA in dairy settings. This study has certain limitations, including a relatively small sample size confined to a single district and the use of purposive sampling, which may limit the generalizability of findings to other regions. Whole genome sequencing (WGS) and biofilm formation assays were not performed, restricting deeper insight into resistance mechanisms and strain evolution. Additionally, clinical outcome data and direct zoonotic transmission assessment were beyond the scope of this study. Future research should incorporate multi-regional surveillance, larger sample sizes, WGS-based molecular typing, and longitudinal studies to better understand MRSA transmission dynamics. Strengthening farm biosecurity, antibiotic stewardship programs, and integrated One Health monitoring frameworks will be essential to mitigate the growing threat of hypervirulent and multidrug-resistant MRSA in Pakistan.

CONCLUSION

This study on MRSA from bovine mastitis cases in Kasur highlights a complex antimicrobial resistance profile that necessitates integrated control strategies. All 100 isolates showed phenotypic resistance to oxacillin, confirming widespread MRSA prevalence. However, only 85% carried the *mecA* gene, suggesting alternative resistance mechanisms such as beta-lactamase overproduction or mutations in penicillin-binding proteins. Cefoxitin resistance in 60% of isolates reinforces its diagnostic relevance, though the remaining 40% may involve heteroresistance or suppressed *mecA* expression. Vancomycin resistance in 51% of isolates raises serious concerns, indicating the emergence of VISA/VRSA strains in livestock. The PVL gene, detected in 78% of isolates, points to significant virulence potential, particularly in co-*mecA/PVL* positive strains like HL-SK, which also exhibited peak enzymatic activity, marking it as hypervirulent. Epidemiological data showed generally poor farm hygiene, including limited handwashing and unsanitary milking practices, which were only weakly associated with mastitis prevalence.

Authors' Contribution

Conceptualization: AQH
Methodology: HA, NJ
Formal analysis: NFT, MB
Writing and Drafting: AQH, FA, MNA
Review and Editing: AQH, FA, MNA, NFT, MB, HA, NJ

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Hospital-Associated Synanthropic Insects as Carriers of Methicillin-Resistant *Staphylococcus aureus*: Evidence from Lahore, Pakistan

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ABSTRACT

The issue of antimicrobial resistance (AMR) is significant in the world, and one of the most important types of pathogens that transmit both nosocomial and community infections is methicillin-resistant *Staphylococcus aureus* (MRSA). **Objectives:** To determine the existence of antibiotic-resistant Gram-positive bacteria in hospital-related insects, to describe the resistance mechanisms and biofilm-forming capabilities of the bacteria. **Methods:** Two hundred houseflies were taken from one hospital, which was a tertiary care hospital in Lahore. The isolates of *S. aureus* were determined by mannitol fermentation, Gram stain, and standard biochemical assays. The Kirby-Bauer disk diffusion method of performing antimicrobial susceptibility testing was conducted as per CLSI (2023) guidelines. A microtiter plate assay was used to determine biofilm formation. PCR detection of the *mecA* and *mecC* genes was done by extracting their genomic DNA. The agar well diffusion assay was employed to determine the activity of FGE as a single entity and in conjunction with the 3-lactam antibiotics. **Results:** Sixteen *S. aureus* isolates (8% of the flies) were recovered. All isolates were methicillin-resistant and carried the *mecA* gene, while the *mecC* gene was absent. Resistance was universal to oxacillin (100%) and nitrofurantoin (100%), and was high against erythromycin (87.5%) and rifampicin (75%). Most isolates (62.5%) were strong biofilm producers. **Conclusions:** Hospital-associated houseflies can serve as reservoirs and potential vectors for multidrug-resistant, biofilm-forming MRSA. The synergy observed between FGE and β-lactam antibiotics highlights the potential of plant-derived adjuncts in addressing resistant infections.

INTRODUCTION

The problem of antimicrobial resistance (AMR) is among the most acute global public-health issues of the 21st century. Recent global analyses estimate bacterial AMR was associated with millions of deaths worldwide in 2019. The overall burden continues to rise, with projections warning of a steep increase in AMR-attributable mortality and societal costs unless decisive action is taken [1-3]. Among resistant pathogens, MRSA remains a leading cause of difficult-to-treat infections in both hospital and community settings. MRSA is associated with severe outcomes, including bloodstream infections, pneumonia, surgical-site infections, and increased length of hospital stay and mortality, and it continues to rank highly in global pathogen–drug burden studies. The principal genetic determinant of methicillin resistance is the *mecA* gene, which encodes an altered penicillin-binding protein (PBP2a) with low affinity for β-lactams; a divergent homologue, mecC, has also been reported but remains less common in many regions [4-6]. The epidemiology of MRSA in Pakistan and the surrounding region demonstrates notable diversity in phenotypic resistance and the distribution of resistance genes. Molecular surveillance from Pakistan and nearby settings has reported variable frequencies of *mecA* and *mecC* among MRSA isolates, highlighting the local circulation of classic and, in some reports, *mecC*-bearing strains, underlining the need for

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molecular monitoring alongside routine susceptibility testing [7]. Environmental reservoirs and mechanical vectors have been increasingly recognized as contributors to the persistence and dissemination of antibiotic-resistant bacteria. Synanthropic insects, especially houseflies (*Musca domestica*), frequent both clinical and waste environments and can carry viable bacteria on their exoskeletons and in their digestive tracts. Experimental and field studies demonstrate that flies can acquire, transport, and deposit pathogenic bacteria, including MRSA and other multidrug-resistant organisms, making them potential, though often overlooked, vectors in hospital and peri-hospital environments. These findings support the inclusion of vector surveillance and environmental controls in AMR mitigation strategies [8]. Compounding the problem, many MRSA strains form biofilms (surface-attached, matrix-embedded communities) that markedly reduce antibiotic penetration, protect bacteria from host defenses, and facilitate persistent colonization of surfaces and medical devices. Biofilm production, therefore, contributes to treatment failure and environmental persistence, further motivating studies that pair phenotypic (biofilm) and genotypic (resistance gene) characterization of isolates from clinical and environmental sources [9]. Given the dwindling antibiotic pipeline and the high burden of resistant infections, adjunctive and alternative antimicrobial approaches are under active investigation. Natural products, particularly sulphur-containing compounds from *Allium sativum* (garlic) such as allicin, have demonstrated in vitro antibacterial activity against MRSA and other pathogens. A study conducted by Murugaiyan *et al*. highlighted that FGE or purified organosulfur compounds can act synergistically with β-lactam antibiotics, enhancing inhibition zones or lowering minimum inhibitory concentrations, suggesting a possible role as adjuvants to restore or augment antibiotic efficacy. Nonetheless, results vary by strain and method, and further targeted evaluation is required [10, 11]. Against this background, investigation of antibiotic-resistant Gram-positive bacteria isolated from synanthropic insects in hospital environments can illuminate overlooked transmission routes and local resistance gene distributions, while also providing a platform to screen potential adjunctive agents such as FGE. This study characterized Gram-positive isolates recovered from houseflies collected in a tertiary-care hospital in Lahore, performed phenotypic antibiotic susceptibility testing and biofilm assays, and conducted molecular screening for *mecA* and *mecC*. This study also evaluated fresh garlic extract's in vitro synergistic activity with representative β-lactams against methicillin-resistant isolates.

Although MRSA is well recognized as a major nosocomial pathogen, limited data exist regarding the role of synanthropic insects as environmental reservoirs of resistant strains in Pakistani hospital settings. Most local surveillance studies focus primarily on clinical isolates, while potential mechanical vectors such as houseflies remain underexplored. Furthermore, molecular characterization of resistance determinants (mecA/mecC) and biofilm-forming capacity in insect-derived isolates has rarely been investigated in Lahore. In addition, the potential synergistic activity of plant-derived agents such as fresh garlic extract against locally circulating MRSA strains has not been systematically evaluated. Addressing these gaps is essential to better understand environmental transmission pathways and identify complementary antimicrobial strategies. This study aims to clarify the role of synanthropic insects as reservoirs of MRSA in a hospital setting, to describe the local distribution of methicillin resistance determinants, and to explore low-cost adjunctive strategies that might inform future infection-control and therapeutic approaches.

METHODS

The current cross-sectional study was carried out between June and August 2024 at a Tertiary-Care Hospital in Lahore. Synanthropic insects (houseflies, *Musca domestica*) were targeted due to their frequent presence in hospital wards, waste disposal areas, and peri-hospital surroundings. Written informed consent was taken. Sampling was designed to capture flies from diverse ecological niches within the hospital to represent patient-care and waste-handling environments. All laboratory analyses were carried out using standardized microbiological and molecular techniques at the Institute of Microbiology and Molecular Genetics (IMMG), University of the Punjab, Lahore. A total of 200 adult flies were collected using sterile entomological traps and clean sweep nets, ensuring minimal contamination. To minimize sampling bias, traps were placed according to a predetermined sampling schedule that randomized the specific collection points within the major zones (wards, waste bins, and outdoor peri-hospital sites) across different days. While a formal sample size calculation was not performed a priori, a target of 200 flies was set to provide a sufficient sample for prevalence estimation. Based on a conservative expected MRSA prevalence of 10% (from pilot data and regional studies), this sample size provides a margin of error of approximately ±4% at a 95% confidence level, which was deemed adequate for this exploratory survey. Furthermore, this sample size is consistent with similar entomological studies of AMR in the region. Traps were placed at predetermined points (wards, waste bins, and outdoor peri-hospital sites) and checked daily. The flies were caught and put straight into sterile containers and taken to the laboratory within 2 hours, and

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handled under aseptic conditions. The flies were immobilized on ice, surface-sterilized by dipping them in 70% ethanol, 30s and washing them twice in sterile phosphate-buffered saline (PBS, pH 7.4), followed by homogenizing them in 1 mL sterile PBS using a glass tissue homogenizer. Short-term centrifugation of the homogenates for 5 min at 3000 rpm was performed to remove debris, followed by the inoculation of aliquots into Luria-Bertani (LB) broth with colistin (10 2g/mL) to prevent Gram-negative bacteria. Enrichment cultures were incubated overnight at 37 o C in a shaker (150 rpm). The analysis was done through SPSS version 27.0. Data were summarized with the help of descriptive statistics: the summarization of categorical variables (like the frequency of the resistance and the type of biofilm formation) is expressed in frequencies and percentages. Continuous data (i.e., inhibition zone diameters) are expressed as mean plus standard deviation (SD). In the case of the synergy assays, the mean difference in the inhibition zone of each treatment group (antibiotic alone, FGE alone, and antibiotic/FGE) was compared using ANOVA and then using a post hoc test (Tukey) to compare all multiple results. A p-value that was less than 0.05 was treated as significant. The enriched samples were streaked on Mannitol Salt Agar and incubated at 37°C for 24h. Colonies that developed a typical yellow coloration as a result of mannitol fermentation were picked and sub-cultured in fresh MSA to acquire pure cultures. Gram staining was done to verify Gram-positive cocci in grape-like clusters. Further identification was done by catalase and coagulase tests. In further analysis, Presumptive *S. aureus* isolates were stored in tryptic soy broth (TSB) containing 15% glycerol at -80°C. The Kirby-Bauer disk diffusion procedure conducted on Mueller-Hinton agar (MHA; Oxoid, UK) was used to determine the antibiotic susceptibility according to the guidelines of Clinical and Laboratory Standards Institute. Each isolate was prepared into a standardized bacterial suspension (0.5 McFarland standard, which is about 1 × 108 CFU/mL) and inoculated onto MHA plates as a lawn. Commercial antibiotic agar plates were used, which included oxacillin (1 µg), cefoxitin (30 µg), erythromycin (15 µg), nitrofurantoin (300 µg), rifampicin (5 µg), gemifloxacin (5 µg), cephalexin (30 µg), vancomycin (30 µg), tigecycline (15 µg), and trimethoprim sulfamethox Incubation of the plates was done at 35°C, 18 to 24h. CLSI breakpoints were used to interpret zone diameters and were measured. ATCC 25923 *S. aureus* was taken as a quality control strain to ensure the accuracy of the results. Fresh garlic extract (FGE) was made by peeling and weighing the garlic cloves, followed by sterilization of the surface with the use of 70 percent ethanol, followed by swirling in sterile distilled water, then homogenized with the use of a sterile blender in a ratio of 1 g/mL. The average inhibition zone of oxacillin alone was 12.4 +/ -1.2 mm, and the average inhibition zone, when mixed with FGE, was 20.1 +/ -1.5 mm ($p<0.001$). Likewise, the average zone of the cefoxitin by itself was 14.2 + 1.0 mm, and that of the combination was 21.8 +1.7 mm ($p<0.001$). FGE on its own generated an average inhibition zone of 8.5 + 0.8 mm. The homogenate was filtered using Whatman No.1 filter paper to eliminate debris, after which it was sterilized using a 0.22 µm syringe filter. Extracts were prepared and kept at 4°C and consumed within 48 hours. The agar well diffusion assay was used to test synergy. MHA plates with isolates seeded on 0.5 McFarland suspensions had their wells (6 mm diameter) cut. Treatments were: (i) FGE alone (100 µL per well), (ii) antibiotic disc alone, and (iii) antibiotic disc with 50 µL FGE overlaid. Incubation in plates was done at 37°C for 24h, with the measurements of the inhibition in millimeters. Repeats were done on different days to guarantee that the assays were reproducible. The computations of mean zone diameters and SD were done, and a one-way ANOVA paired with the post hoc test of the Tukey test were used to examine the differences between treatments in SPSS-27. A p-value of below 0.05 was a statistically significant value. An improved activity was considered to be a discernible zone diameter change over the antibiotic disc itself. The formation of biofilms was determined by the use of the microliter plate assay [12], modified. In short, 1:100 cultures of isolates in tryptic soy broth (TSB), with 1% glucose, were left as an overnight culture. Four samples of 200 µL each were inoculated in triplicate in sterile flat-bottom 96-well polystyrene microliter plates. Plates were incubated at 37°C without shaking. Planktonic cells in the wells were removed by washing with 3 times of sterile PBS, then the biofilms were air-dried and stabilized by use of the 0.1% solution of crystal violet, followed by 15 min staining of the biofilms using 200 µL of 33% glacial acetic acid solution. The optical density (OD) at 590 nm was measured using a microplate reader (BioTek, USA). The classification of isolates was based on the OD values of isolates relative to the negative control (cut-off ODc = mean OD of control + 3 x standard deviation): non-biofilm, weak, moderate, and strong producers. The phenol chloroform isoamyl alcohol method, with slight modifications, was used to extract Genomic DNA. In brief, an overnight bacterial culture was centrifuged at 10,000 rpm for 5 min, after which it was suspended in TE buffer before being lysed with lysozyme (20 mg/mL, 37°C, 30 min) and subsequently treated with proteinase K and SDS. The extraction of the DNA was done using phenol, chloroform, and isoamyl alcohol, followed by the precipitation of the DNA using ethanol, 70% ethanol, and finally, the DNA was suspended in nuclease-free water. To validate the assay, *S. aureus* ATCC 43300 (*mecA*-positive) was used as a positive, and a *mecC*-positive control was used to confirm that the primers worked and

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that a negative result can be trusted. PCR tests were done against the *mecA* and *mecC* genes using primers that were published. The amplifications were conducted in 25 0L reaction mixtures that included 12.5 0L of 2X PCR Master Mix (Thermo Fisher Scientific, USA), 1 0L of each primer (10 0M), 2 0L template DNA (50 ng), and nuclease-free water. My ecA thermocycling conditions were: denaturation (95°C, 5-min); 35 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 45 s); and finally denaturation (72°C, 7-min). In the case of mecC, annealing was done at 58°C. PCR products were packed on ethidium bromide-stained, 1.5% agarose gels, and their position was observed under UV light with the help of a gel documentation system (Bio-Rad, USA). As a molecular size marker, a 100 bp DNA ladder was employed. *MecA* was expected to be 238 bp and mecC 304 bp in size.

RESULTS

From the 200 houseflies collected, 16 isolates (8%) were identified as *Staphylococcus aureus* based on mannitol fermentation on MSA (yellow colonies) and Gram staining, which showed Gram-positive cocci in grape-like clusters. The point prevalence of *S. aureus* carriage was 8% (95% confidence interval (CI): 4.6% to 12.7% These isolates were selected for further phenotypic and molecular characterization. Kirby–Bauer disk diffusion revealed high resistance among the 16 *S. aureus* isolates. All strains (100%) were resistant to oxacillin and nitrofurantoin. Resistance to other agents included erythromycin (87.5%), rifampicin (75%), cephalexin (68.7%), and ciprofloxacin (62.5%). In contrast, only two isolates (12.5%) showed resistance to cefoxitin, while susceptibility to tigecycline and vancomycin was relatively preserved. The mean resistance rate across ten clinically relevant antibiotics was 68.7%. Antibiotic resistance profile of Staphylococcus aureus isolates, showing the percentage of resistance observed against different tested antibiotics (Figure 1).

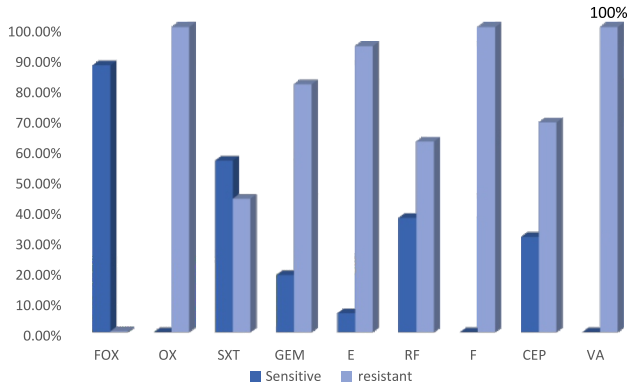

Figure 1: Antibiotic Resistance Profile of *Staphylococcus Aureus* Isolates Against Different Tested Antibiotics

FGE enhanced the activity of β-lactam antibiotics against MRSA isolates. Zones of inhibition produced by oxacillin or cefoxitin discs increased significantly when combined with FGE compared to antibiotics alone. FGE alone also produced measurable antibacterial activity, but the effect was markedly potentiated in combination. It is important to note that these findings are preliminary and represent a preclinical exploration of FGE's potential synergistic effects. Further in vivo validation and mechanistic investigations are required before any therapeutic implications can be drawn. The individual use of the garlic extract and antibiotics (OX and CTX) on the MRSA exhibited intermediate zones of inhibition, which means that the two have their antimicrobial effect independently (Figure 2).

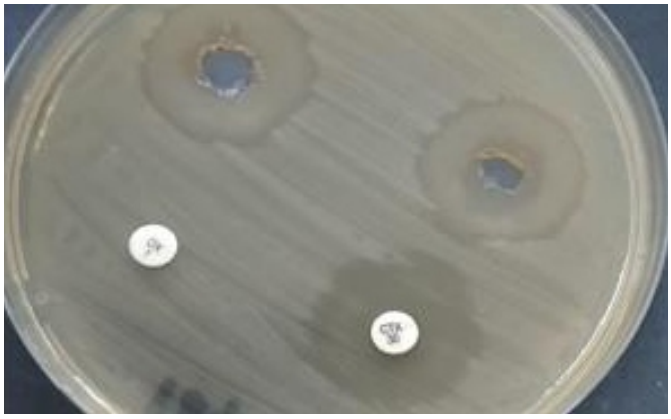

Figure 2: The Separate Application of Garlic Extract and Antibiotics Against MRSA

Conversely, there was a significantly improved zone of inhibition in the combination of garage extract using OX and CTX than when they were administered separately. This means that it has a synergistic effect that enhances the antibacterial effect against MRSA (Figure 3).

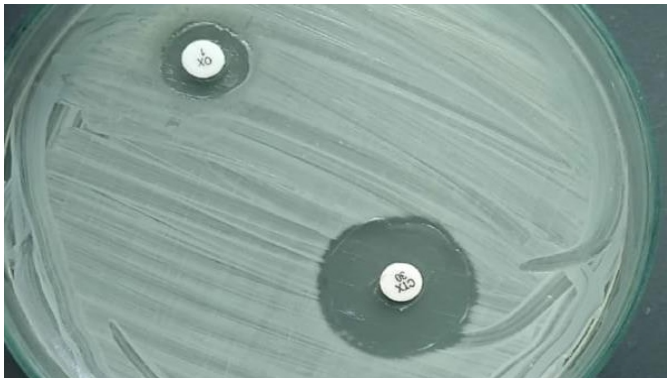

Figure 3: Concomitant Effect of Garlic Extract with OX and CTX

Quantitative biofilm assays revealed that the majority of isolates exhibited substantial biofilm-forming ability. Specifically, 10 of 16 isolates (62.5%) were categorized as strong biofilm producers, 3 isolates (18.7%) as moderate, and 2 isolates (12.5%) as weak producers, while only a single isolate (6.3%) showed no measurable biofilm formation. The predominance of strong biofilm producers suggests that most of these MRSA strains possess a considerable capacity for persistence and survival on abiotic surfaces, which may facilitate prolonged environmental

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contamination and contribute to transmission in healthcare settings(Figure 4).

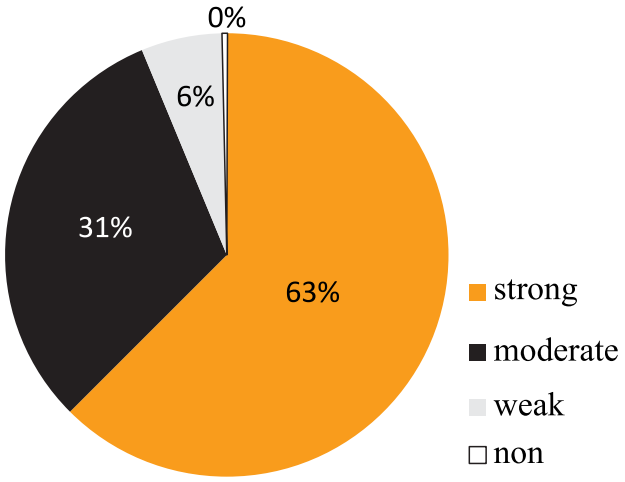

Figure 4: The Percentage Distribution of *Staphylococcus Aureus* Isolates Categorized as Biofilm Producers

PCR analysis confirmed the presence of the *mecA* gene in all 16 *S. aureus* isolates, supporting their phenotypic resistance to methicillin. Amplification consistently produced the expected 238 bp product, which was clearly visualized by gel electrophoresis under UV illumination. In contrast, none of the isolates yielded amplicons with *mecC*-specific primers, indicating the absence of this resistance determinant in the tested population. The universal detection of *mecA* highlights it as the dominant genetic mechanism of methicillin resistance among these isolates, while the lack of *mecC* is consistent with its relatively rare occurrence in South Asia compared to European settings. Summary of *Staphylococcus aureus* isolates showing their source location, antibiotic resistance profiles, biofilm strength, and *mecA* gene detection(Table 1).

Table 1: Key CSC Markers and Their Expression in Various Cancer Types

Isolate ID	Source Location	Ox	Fox	Ery	Rif	Cpx	Cep	Nit	Van	Tig	TMP-SMX	Biofilm Strength	mecA
SA01	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
SA02	Ward	R	R	R	S	R	S	R	S	S	R	Strong	+
SA03	Waste Area	R	S	R	R	R	R	R	S	S	R	Moderate	+
SA04	Ward	R	S	R	R	S	S	R	S	S	R	Strong	+
SA05	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA06	Ward	R	S	R	R	R	S	R	S	S	S	Moderate	+
SA07	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA08	Ward	R	S	R	S	S	S	R	S	S	R	Weak	+
SA09	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA10	Ward	R	S	R	R	R	S	R	S	S	R	Strong	+
SA11	Waste Area	R	S	R	R	R	S	R	S	S	R	Moderate	+
SA12	Ward	R	S	R	S	R	R	R	S	S	R	Weak	+
SA13	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
SA14	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA15	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
Sa16	Waste Area	R	S	R	S	S	S	R	S	S	S	Non-Biofilm	+

Ox = Oxacillin, Fox = Cefoxitin, Ery = Erythromycin, Rif = Rifampicin, Cpx = Ciprofloxacin, Cep = Cephalexin, Nit = Nitrofurantoin, Van = Vancomycin, Tig = Tigecycline, TMP-SMX = Trimethoprim–sulfamethoxazole; R = Resistant, S = Susceptible, + = Present.

DISCUSSION

This study recovered *Staphylococcus aureus* from houseflies collected in a tertiary-care hospital environment in Lahore, Pakistan. This represents the first systematic survey of hospital-associated flies in Lahore for MRSA carriage with both phenotypic and genotypic characterization. All 16 isolates identified as MRSA carried the *mecA* gene, 8% (95% CI: 4.6-12.7%) the *mecA* gene, showed multidrug resistance to commonly used antibiotics, and most were strong biofilm producers. These findings are consistent with regional molecular surveillance showing a dominance of *mecA* among MRSA isolates in Pakistan and variable but generally low prevalence (68.7%) of *mecC* [13, 14]. A recent molecular survey from the region reported *mecA* as the principal determinant in MRSA isolates, with *mecC* present in a minority of strains, reinforcing the prominence of *mecA* in this setting [15, 16]. The recovery of MRSA from synanthropic insects echoes a growing body of evidence that insect pests (particularly houseflies) can act as mechanical carriers and environmental reservoirs of antimicrobial-resistant bacteria in healthcare settings. Multisite surveillance studies and reviews have repeatedly identified flies in hospital and peri-hospital environments as vectors that harbor diverse bacterial species, including

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S. aureus, many carry genes encoding resistance to clinically important antibiotics [17]. Although such studies generally do not prove direct transmission to patients, they highlight an under-recognized environmental route by which resistant organisms can persist and potentially contaminate surfaces, food, or wounds. Therefore, our observation of MRSA in flies aligns with recent international surveillance data that call for including insect control and environmental monitoring within broader AMR containment strategies [18]. The high frequency of strong biofilm producers among our isolates (≈62.5%) is of particular concern because biofilms markedly increase bacterial persistence on abiotic surfaces and medical devices and decrease susceptibility to antibiotics. This high biofilm prevalence among insect-derived MRSA isolates represents a novel observation for this setting. Comparable studies from Pakistan and neighboring regions report similarly high proportions of MRSA isolates with substantial biofilm formation. This supports the idea that biofilm production is widespread among clinically relevant MRSA lineages in this geography. The confluence of multidrug resistance, presence of *mecA*, and robust biofilm formation in isolates found on flies underscores the potential for environmental persistence and the added challenge such strains pose to routine cleaning and disinfection in hospitals [19]. In vitro findings that FGE potentiated the activity of β-lactam antibiotics against MRSA is supported by experimental data describing the antimicrobial and synergistic effects of garlic-derived organosulfur compounds such as allicin. Several laboratory studies and reviews have demonstrated that crude garlic extracts and purified allicin exhibit antibacterial and antibiofilm activity against MRSA and can act synergistically with conventional antibiotics under controlled conditions. However, these findings should be interpreted as preliminary and preclinical, reflecting laboratory-based observations only. While these results are promising and suggest low-cost adjunctive options for resource-limited settings, they remain in vitro and exploratory in nature, and translation to clinical application would require rigorous pharmacological, toxicological, and in vivo efficacy testing [20].

This study was limited by its single-center design and relatively small number of MRSA isolates, which may restrict the generalizability of the findings to other healthcare settings. Whole genome sequencing and detailed molecular typing were not performed, limiting deeper insights into clonal relationships and transmission dynamics. Additionally, the synergistic effects of fresh garlic extract were evaluated only through in vitro assays, without in vivo validation or mechanistic analysis. Future research should include multi-center surveillance, genomic characterization, and longitudinal environmental monitoring to clarify transmission pathways. Further pharmacological and toxicological investigations are also required to determine the clinical feasibility of plant-derived adjunct therapies against multidrug-resistant MRSA.

CONCLUSION

Hospital-associated flies in Lahore can harbor mecA-positive, multidrug-resistant, and biofilm-forming MRSA strains. Finding mecA-positive, multidrug-resistant, biofilm-forming S. aureus on hospital-associated flies emphasizes the need to broaden infection-control measures to include vector surveillance and control in hospital settings, particularly in tropical and resource-constrained environments with abundant synanthropic insects. Integrating environmental surveillance of insects into AMR monitoring programs could provide early indicators of circulating resistance genes in the hospital ecosystem. Additionally, the observed in vitro synergy between FGE and β-lactams suggests a potential role for natural product research in identifying adjunctive agents that could restore or enhance antibiotic activity.

Authors' Contribution

Conceptualization: SR
Methodology: TZ, HAR, MQ
Formal analysis: HAR, M
Writing and Drafting: HAR, MQ, M
Review and Editing: HAR, MQ, M, TZ, SR

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Awareness and Public Perception of Dyslexia in Urban Pakistan: An Analytical Cross-Sectional Study

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ABSTRACT

Dyslexia is a neurocognitive disorder of an individual of fluent reading, writing, and memorize difficult spellings or words. **Objective:** To critically evaluate the Knowledge, Attitudes, and Practices (KAP) along with awareness of learning disability (Dyslexia) disorder in urban Pakistan. **Methods:** This analytical cross-sectional study was conducted by the Sindh Biotechnologist Association (SBA) in collaboration with a tertiary care hospital of Gomal Medical College, MTI, D.I. Khan, Pakistan. A total of 270 participants were enrolled using a convenience sampling technique. KAP of the general audience regarding Dyslexia was conducted by using an e-survey (i.e., Google document). SPSS Version 25.0 was used to analyze and correlate the data, where $p<0.05$ was considered a statistically significant response. **Results:** Out of 270 respondents, a majority of respondents (i.e., 57.8%, f=156) did not even know the signs and symptoms of dyslexia. While 37.8% participants were still in the denial mode about their closed ones' Dyslexia. Moreover, 94.8% (f=256) believed that this survey helped them to know more about Dyslexia. The study found a strong correlation between respondents' knowledge and practices about dyslexia, with positive awareness being 6.25 times more likely to engage in positive dyslexia-related behaviours. **Conclusions:** Dyslexia significantly impacts individuals' performance in routine jobs, including reading, numeracy, memory, and verbal communication. It also affects record keeping, case load management, and medicine administration. Peer support plays a significant role in receiving support, but fear of retaliation may limit public understanding.

INTRODUCTION

The census of 1998 in Pakistan identified disabilities in over 3.0 million of its people. Out of which 27.333% (i.e., n=820,000) accounts for disabilities in children of school-going age [1]. It is generally hard to identify individuals with learning impairments because most of the time the study misses those individuals who are recently diagnosed or are still unaware of his/her medical ailment [2]. "Dyslexia" is a Greek term that means facing difficulty in understanding words, characters, instructions, or sentences [3]. Dyslexia is a neurocognitive disorder of an individual of fluent reading, writing, and memorize difficult spellings or words. Hence, it is referred to as "Specific Learning Disability" (SLD). The Intelligence Quotient (IQ) of a dyslexic individual is generally either average or above average [4]. In developing countries like Pakistan, approximately 15-20% population has been suffering from this issue, which means that 12 million individuals (most of which comprises children or youngsters) are in dire need of professional help

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[5]. The criteria to analyse whether a person is Dyslexic or not vary from case to case. The perception and ideology of every investigator are unique in identifying a dyslexic individual. According to Vellutino, a dyslexic person should not always lack the extrinsic factors such as IQ>=90, disabled peripheral functioning, i.e., sense of sight and hearing, absent-mindedness, socioeconomic imbalances, etc. However, most of the time, they experience one of the pre-defined circumstances [6]. All in all, they always lack the basic opportunity of learning and find it difficult to acquire alternative literacy management techniques. Moreover, it has been observed that dyslexic persons have anger issues and low confidence or self-esteem because they think that they cannot keep pace with society [7]. There are two types of Dyslexia. Either developmental or acquired. In developmental dyslexia, the child or adult encounters the reading difficulty, whereas in Acquired Dyslexia, the brain has been affected either due to some trauma or because of some serious brain injury [8]. In developmental Dyslexia, the most common disability is learning. In earlier times, the diagnosis of Dyslexia seemed difficult as people were unable to recognize the disease in its early stages. However, now its diagnosis and analysis are relatively easy [9]. Slow and imprecise word recognition is a hallmark of the neurodevelopmental condition dyslexia [10]. There have been reports of dyslexia in every culture examined, and growing evidence highlights the commonality of its neurological and cognitive underpinnings across language boundaries. Over the past five years, significant advancements have been achieved in a variety of study fields that span the behavioural, neuropsychological, neurobiological, and causal levels of analysis [11]. Though phonological issues also interact with other cognitive risk factors, the phonological theory is still the most convincing from a neuropsychological standpoint. According to research, dyslexia is neurobiologically characterised by disruption of the healthy language network in the left hemisphere, as well as aberrant white matter development [12]. Understanding dyslexia in Pakistan is a step in developing an equitable, inclusive educational system that acknowledges and meets the various requirements of all students. In the end, it will support national development by lowering stigma, raising literacy rates, and enabling people to realize their full potential.

Despite the increasing global recognition of dyslexia as a significant neurodevelopmental disorder, public awareness and structured epidemiological data in Pakistan remain limited and fragmented. Most existing local studies primarily focus on teachers or clinical populations, with minimal exploration of the general public's knowledge, attitudes, and practices (KAP) regarding dyslexia. Furthermore, misconceptions surrounding intelligence, causes, and management continue to contribute to stigma, delayed diagnosis, and inadequate support systems. Therefore, a clear research gap exists in systematically assessing community-level awareness and behavioral responses toward dyslexia in urban Pakistan to inform evidence-based policy and awareness strategies. This study aims to evaluate the awareness and public perception of Dyslexia in urban Pakistan.

METHODS

A descriptive and analytical cross-sectional study was conducted by the Sindh Biotechnologist Association (SBA) in collaboration with the Gomal Medical College, MTI, D.I. Khan, Pakistan, from August 2022 to September 2022 after taking ethical approval from the Ethical Review Board (ERB) of Gomal Medical College, MTI, D.I. Khan (ERB No. 423(2)/E2/ME). All participants provided informed consent before completing the online survey. This study primarily targeted individuals aged 16–30 years, as this group is more likely to exhibit dyslexia-related signs. However, participants up to 60 years were not excluded, and responses from older participants were included in the analysis for completeness. People aged more than 60 years were not included in this study, as Dyslexia is least prevalent in this age group. A Google document (e-survey) was circulated in different parts of Pakistan, which comprised a well-structured and well-characterized set of questions, divided into two main sections. The questionnaire was pilot-tested for clarity and reviewed by experts for content validity. Formal reliability analysis was not performed. A convenient sampling technique was used. The internal consistency and reliability of the survey instrument were assessed using Cronbach's alpha. The calculated coefficients for the Knowledge, Attitude, and Practice domains were 0.78, 0.71, and 0.75, respectively, all of which indicate acceptable internal consistency ($\alpha \geq 0.70$). The first segment included demographic data, while the other segment consisted of questions that critically evaluated the Knowledge, Attitudes, and Practices (KAP) of the general audience regarding Dyslexia, which simultaneously assessed their awareness of this important health problem in Pakistan. A total of 270 participants have taken part in this study. The sample size was calculated as 270 participants using the formula for cross-sectional studies (Daniel & Cross, 2013; Naing et al., 2006). This calculation was based on a 95% confidence level (Z-score = 1.96), a 5% margin of error (d = 0.05), and an expected population proportion (p) of 20% population proportion as per the Ministry of Federal Education and Professional Training [13]. The statistical tool (IBM SPSS Version 25.0 analysis) was used to analyze and correlate the data. Data were analyzed using IBM SPSS Statistics Version 25.0. Descriptive statistics were computed for all variables.

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Categorical variables, including demographic data and responses to individual knowledge, attitude, and practice (KAP) questions, are presented as frequencies and percentages (n, %). For inferential analysis, composite scores were calculated for the KAP domains. Knowledge, Attitude, and Practice scores were derived by summing correct or favorable responses to their respective questions. The association between overall knowledge and practice was assessed using Fisher's Exact Test due to the categorical nature of the variables and the distribution of the data. The strength of this association was quantified by calculating the Odds Ratio (OR) with a 95% Confidence Interval (CI). A p-value of ≤0.050 was considered statistically significant throughout the analysis.

RESULTS

This survey was attempted by 270 participants from all over Pakistan, out of which 218 (80.7%) were female and the remaining 52 (19.3) were male. Among these, the maximum number of participants belonged to the 16-30 years' age group (f=232, 85.9%), while 7.4% were 31-45 years of age, and 5.9% were 46-60 years of age. Other demographic variables included relationship status, which showed maximum number of respondents were married (f=210, 77.77%)(Table 1).

Table 1: Demographic Status of the Surveyed Participants (n=270)

Variables	Characteristics	Frequency (%)
Gender	Female	218 (80.7%)
	Male	52 (19.3%)
	Others	0 (0%)
Age	0-15 Years	2 (0.7%)
	16-30 Years	232 (85.9%)
	31-45 Years	20 (7.4%)
	46-60 Years	16 (5.9%)
Relationship Status	Married	210 (77.77%)
	Single	48 (17.8%)
	Engaged	4 (1.48%)
	Others (Divorced, widowed)	8 (2.96%)
City of Residence	Karachi	83 (30.7%)
	Islamabad	45 (16.66%)
	Peshawar	23 (8.51%)
	Lahore	54 (20%)
	Hyderabad	14 (5.18%)
	Larkana	7 (2.59%)
	Sukkur	22 (8.14%)
	D.I Khan	20 (7.40%)
	Quetta	2 (0.82%)

The process of disclosure of Dyslexia is very selective, and many people feel shy to share this with others, even with their close ones, such as parents, guardians, or caretakers. A majority of respondents of this survey (i.e., 57.8%, f=156, item no. 16) did not even know the signs and symptoms of dyslexia, such as slow reading, spending long periods of time on writing exercises, Trouble completing math problems, inability to understand jokes or expressions, etc. This might be due to the reason that people in Pakistan are not very well aware of the term 'Dyslexia' and what its consequences are. When the surveyed participants were asked whether they had known Dyslexia or not (Item No.7), 94 (34.8%) participants had no prior understanding of the mental condition. As a result of this, many parents are in denial mode when their children shared that they have been constantly facing difficulty in reading, writing and spelling words, resulting in the untreated and undiagnosed patterns as suggested by Item No 22 of this survey (n=102, 37.8%). However, questions 1-6 suggested that approximately 1/4th of the participants could've encountered learning difficulties in the early phase of elementary education. This can lead to the aggravation of negative feelings such as low levels of confidence, inferiority complexes, and low self-esteem in those who are not Dyslexic. This often gives rise to frustration, anger, and trust issues in Dyslexic individuals, as indicated by item number 20th of the questionnaire, in which 108 (40%) of the participants have a firm belief in aggravation of negative feelings in dyslexic individuals. Some items, such as Q5, showed low affirmative responses, possibly due to limited awareness or ambiguity in question wording (Table 2).

Table 2: Frequency Distribution of the Surveyed Participants (n=270)

S. No.	Questionnaire	Yes	No	May Be Yes	May Be No
		F (%)			
1	Do you ever feel difficulty in reading?	64 (23.7%)	206 (76.3%)	–	–
2	Do you ever feel difficulty in remembering words or sentences?	126 (46.7%)	144 (53.3%)	–	–
3	Do you ever feel difficulty to learn or read in elementary school?	44 (16.3%)	226 (83.7%)	–	–
4	Do you ever feel difficulty in reversing the order of letters or numbers?	74 (27.4%)	196 (72.6%)	–	–
5	Do you ever feel difficulty in learning letters or the names of colours?	14 (12.6%)	236 (87.4%)	–	–
6	Do you struggle time to time in school due to homework or class work?	22 (8.1%)	60 (22.22%)	114 (42.2%)	74 (27.4%)
7	Have you heard about Dyslexia?	176 (65.2%)	94 (34.8%)	–	–
8	Do you know Dyslexia is a learning disorder that affects your ability to read, spell, write, and speak?	192 (71.1%)	78 (28.9%)	–	–
9	Do you Know dyslexia affects areas of the brain that process language?	144 (53.3%)	126 (46.7%)	–	–

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10	Do you know dyslexia is caused by dysfunction within a neural circuit that supports reading?	118 (43.7%)	152 (56.3%)	–	–
11	Do you know dyslexia is a genetic disorder, which means you are more likely to have dyslexia if your parents or siblings are dyslexic?	92 (34.1%)	178 (65.9%)	–	–
12	Do you know there's a greater risk for dyslexia in individuals who were born prematurely or had a low birth weight?	46 (17.0%)	224 (83.0%)	–	–
13	Do you know that being exposed to alcohol, drugs, or infections while in the womb can also raise the risk of dyslexia?	64 (23.7%)	206 (76.3%)	–	–
14	Do you know that late talking, difficulty learning, remembering letters, and mispronouncing words are the early signs of dyslexia in non-school-age children?	108 (40.0%)	162 (60%)	–	–
15	Do you know that difficulty reading, inability to remember sequences, trouble spelling or sounding out words are the signs of dyslexia in school age children?	132 (48.9%)	138 (51.1%)	–	–
16	Do you know slow reading, spending long periods of time on writing exercises, Trouble completing math problems, inability to understand jokes or expressions are the major symptoms of dyslexic adults?	114 (42.2%)	156 (57.8%)	–	–
17	Do you know that early diagnosis of dyslexia reduces specific learning issue?	69 (51.1%)	132 (48.9%)	–	–
18	Do you know that symptoms of dyslexia can be improved by medications?	100 (37.0%)	170 (63.0%)	–	–
19	Do you know that a dyslexic child needs special teaching assistance, unlike a normal child?	168 (62.2%)	102 (37.8%)	–	–
20	Do you know that an untreated dyslexic person finds difficulty growing professionally because of low self-esteem issues?	162 (60.0%)	108 (40.0%)	–	–
21	Do you know that the prevalence rate of dyslexia is 15-20% among children in Pakistan?	34 (12.6%)	236 (87.4%)	–	–
22	Do you know that many dyslexic children in Pakistan remain undiagnosed/untreated due to parents' denial?	168 (62.2%)	102 (37.8%)	–	–
23	Do you know that many dyslexic children suffer in academics due to a non-dyslexic-friendly environment in educational institutions?	150 (55.6%)	120 (44.4%)	–	–
24	Do you know that dyslexia occurs regardless of a person's intellectual level?	120 (44.4%)	150 (55.6%)	–	–
25	Did this survey help you to know about dyslexia?	256 (94.8%)	14 (5.2%)	–	–

A remedial therapist in an awareness seminar in Islamabad, Pakistan in 2017 had presented some amazing facts about prevalence of Dyslexia in Pakistan according to which approximately 12 million children (15-20%) need professional and experiential help due to Dyslexia and majority of the participants of this survey (i.e., f=236, 87.4%) had no clue about the prevalence rate of dyslexia of the country. In Pakistan, there is a strong need for dyslexia-friendly educational institutes. Because the teachers, parents, and therapists have the strongest impact or influence on children's mindset and if they make the learning institute friendly for them, the dyslexic children will have to suffer comparatively less in academics, as indicated by item number 23 (f=150, 55.6%). Despite genetics, the problems of dyslexia can be lessened since it's a lifelong condition. Continuous support from people around can overcome these learning weaknesses and strengthen their mental capabilities. This assessment also critically evaluated the knowledge of participants about the risks of dyslexia in individuals. Items 12 and 13 showed majority of respondents (~75%) were unaware of the risks of this learning disability, including premature deliveries with low birth weights, exposure to alcohol, narcotics, and drugs, despite the genetic impairments. Therefore, this survey proved to be a milestone in spreading awareness on Dyslexia, as indicated by Item 25th of the survey in which 256 (94.8%) participants believed that this survey helped them to explore more about different aspects of dyslexia and in critically evaluating the Knowledge, Attitudes, and Practices of the General audience regarding Dyslexia in Pakistan (Table 3).

Table 3: Relationship Between Knowledge, Attitudes, and Practices Themes and Corresponding Survey Questions

Theme	Description	Related Questions	Objective
Knowledge	This section gauges how much participants know about learning disabilities, including their causes, signs, and general information.	Q9, Q10, Q11, Q12, Q13, Q14, Q15, Q16, Q20, Q21, Q24	To measure the factual awareness and understanding that respondents have about learning disabilities.
Attitudes	This part explores how people feel about individuals with learning disabilities, covering perceptions, beliefs, and emotional responses.	Q3, Q4, Q5, Q7, Q8	To assess how supportive or biased individuals are toward those with learning disabilities.
Practices	Focuses on what individuals actually do in real-life situations when dealing with or supporting people with learning disabilities.	Q6, Q17, Q18, Q19, Q22, Q23, Q25	To determine if positive knowledge and attitudes lead to inclusive or supportive behaviour.
Dyslexia	Addresses specific knowledge and perceptions regarding dyslexia as one type of learning disability.	Q5, Q7, Q9	–

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Demographic Information	Includes basic personal details used to analyze trends across age and gender.	Q1 (Age), Q2 (Gender)	–

The respondents' knowledge and Practices about dyslexia appeared to be strongly and statistically significantly correlated, according to the results of Fisher's Exact Test. The data indicated that respondents with positive awareness about dyslexia were 6.25 times more likely than those with negative information to engage in positive dyslexia-related behaviors. This association was quantified by an odds ratio of 6.25 (95% CI: 3.53 - 11.07). The p-value of 1.35×10^{-10} indicated that there was room for improvement in the management and support of dyslexic patients in Pakistani society as a result of increased awareness about the condition. It has also been noted that the majority had no Dyslexia awareness, in which 230 (86.5%) had a negative attitude toward it (p-value=0.021). Positive behaviors might be encouraged by focused interventions like awareness campaigns and training initiatives. The results might also advocate for modifications to laws and programs that inform the public about dyslexia and help those who have the condition live in more acceptable and supportive environments (Table 4).

Table 4: Association of Knowledge and Attitude with Practice Regarding Dyslexia

Variables	Practice Positive	Practice Negative	Total	p-Value	Odds Ratio (95% CI)
Knowledge					
Positive	50	30	80	<0.001*	6.25 (3.53-11.07)
Negative	40	150	190		
Total	90	180	270		
Attitude					
Positive	60	40	100	0.021*	2.50 (1.15-5.45)
Negative	30	140	170		
Total	90	180	270		

DISCUSSION

The present survey-based study highlights a substantial lack of awareness regarding dyslexia among the general public in Pakistan. A significant proportion of respondents demonstrated limited comprehension of the characteristics and challenges associated with dyslexia, particularly within the educational sector, where teachers often lack the necessary training to recognize and support dyslexic individuals. This gap aligns with previous studies reporting insufficient attention to learning disabilities, such as dyslexia, autism, and attention-deficit hyperactivity disorder (ADHD), in educational and healthcare settings [16]. Compared to global prevalence estimates of dyslexia (~5%), our findings suggest that in Pakistan, approximately 1 in 10 individuals may experience developmental or acquired dyslexia [15], indicating a higher local burden likely compounded by low awareness and inadequate diagnostic frameworks. Our analysis of knowledge, attitudes, and practices (KAP) demonstrates critical gaps. While dyslexia are often perceived as a childhood condition, evidence suggests adults are equally susceptible, with environmental, occupational, and psychosocial factors serving as primary triggers [17-19]. Despite this, more than half of the participants (55.6%) were unaware that dyslexic individuals may exhibit average or above-average intelligence, highlighting widespread misconceptions regarding cognitive capacity. These results underscore the need for public education campaigns to correct misunderstandings and emphasize that dyslexia do not equate to low intelligence. Attitudinally, cultural and societal norms appear to strongly influence perceptions of dyslexia in Pakistan [20]. The study identified pervasive stigma and negative stereotypes, which may limit opportunities and reduce the self-esteem of individuals with learning disabilities [21, 22]. Such findings are consistent with global literature, where societal attitudes significantly impact access to educational support and social integration. Notably, while Khalid and Anjum identified the positive role of elementary teachers in fostering awareness, our findings suggest that teacher knowledge remains insufficient, perpetuating misconceptions and limiting early intervention efforts [23]. This indicates a persistent gap in translating evidence into practice, despite decades of research and policy recommendations. Practices surrounding dyslexia support in Pakistan remain inadequate. Resources and specialist educational interventions tailored to the needs of dyslexic children are scarce, and evidence-based therapies are seldom implemented [24]. The KAP framework in this context demonstrates a clear cycle: limited knowledge fosters negative attitudes, which in turn constrain practical interventions. To disrupt this cycle, it is imperative to integrate targeted teacher training, parental workshops, and community awareness programs into national educational strategies. Moreover, the survey identified that 98.5% of participants were unaware of the basic concepts of dyslexia, reflecting the urgent need for public health campaigns, media engagement, and school-based initiatives to promote awareness and reduce stigma. Comparisons with international literature reinforce that adult dyslexia are frequently overlooked, underscoring the importance of lifelong awareness and support systems [25]. The use of convenience sampling may limit the generalizability of these findings to the broader population of Pakistan. Our analysis of composite KAP scores revealed a clear pattern: while attitudes towards dyslexia were generally positive, the level of factual knowledge was only moderate, and this translated into notably poor practice scores. This suggests a concerning "know-do" gap, where

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positive intentions are not being realized in supportive actions, likely due to a lack of deep understanding and resources Furthermore, the use of convenience sampling, while practical for data collection, limits the generalizability of these findings to the broader population of Pakistan, as the sample may not be fully representative of the entire societal demographic.

This study has certain limitations, including the use of convenience sampling and an online survey approach, which may limit the generalizability of findings to the broader Pakistani population. The predominance of young and female participants may also introduce demographic bias. Additionally, reliance on self-reported responses may be subject to recall and social desirability bias. Future research should employ larger, randomized, and nationally representative samples, incorporate rural populations, and utilize mixed-method designs for deeper insight. Long-term interventional studies and structured public awareness programs are also recommended to evaluate their effectiveness in improving dyslexia-related knowledge, attitudes, and supportive practices across Pakistan.

CONCLUSION

This study reveals a critical gap in dyslexia awareness in Pakistan, with 86.5% of participants demonstrating poor awareness. A significant association was found between knowledge and practice, as individuals with good awareness were 6.25 times more likely to exhibit supportive behaviors. The condition profoundly impacts essential skills like reading, writing, and memory, often exacerbating professional challenges and self-esteem due to societal stigma and a lack of supportive environments. These findings underscore the urgent need for nationwide awareness campaigns and the development of inclusive educational and workplace policies to support individuals with dyslexia.

Authors' Contribution

Conceptualization: AA, AB
Methodology: AA, MH, AB, MA, HJ, M
Formal analysis: AA, HJ
Writing and Drafting: HK
Review and Editing: HK, AA, MH, AB, MA, HJ, M

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Green Synthesis of Silver Nanoparticles Using *Nigella sativa* Seeds and Apple Peel Extracts and Their Antimicrobial Activity Against *Escherichia coli*

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ABSTRACT

In nanotechnology, synthesizing silver nanoparticles (AgNPs) with plant-based extracts has emerged as an eco-friendly and sustainable method. **Objectives:** To focus on the green synthesis and characterization of AgNPs using extracts from *Nigella sativa* seeds (black seed) and *Malus domestica* (apple) peels, both rich in bioactive phytochemicals that serve as natural reducing and stabilizing agents. **Methods:** The synthesis process was verified by UV-Vis spectroscopy using typical surface plasmon resonance (SPR) peaks (~410 nm), which means that the AgNPs were formed successfully. Dynamic light scattering (DLS) analysis was used to determine the hydrodynamic size (117 nm) and uniformity of the AgNPs, and the zeta potential analysis showed the low negative surface charges because of capping using plant biomolecules. The antimicrobial activity of the synthesized AgNPs was tested against *Escherichia coli*, a common pathogenic bacterium. **Results:** Results showed significant antibacterial effects, with a zone of inhibition of 27 mm. The previously stated mechanisms, such as ROS generation and apoptosis-like responses, were removed, as they were not experimentally verified. The use of *N. sativa* and apple peel extracts provided a cost-effective and environmentally benign synthesis route, enhancing nanoparticle stability and bioactivity. **Conclusions:** These findings highlight the potential of green-synthesized silver nanoparticles as effective antimicrobial agents specifically against E. coli, without extending claims to untested biomedical or environmental applications.

INTRODUCTION

Escherichia coli (*E. coli*) is a Gram-negative bacterium and is a member of the family Enterobacteriaceae [1]. *E. coli* is also a pathogen that causes several common bacterial infections in humans and animals [2]. It is a leading cause of broad-spectrum infection, urinary tract infection (UTIs), enteritis, septicemia, as well as other clinical infections such as neonatal meningitis [3]. *E. coli* is worth studying since it is a component of the intestinal microbiota and may become pathogenic as well [4]. *E. coli* strains are either classified as pathogenic or non-pathogenic. The pathogenic strains can further be categorized in terms of virulence factors and related diseases, and the non-pathogenic strains, like *E. coli* K-12, are extensively utilized in laboratory research. The *E. coli* O157:H7 can cause severe disease that causes approximately 63,000 incidences of hemorrhagic colitis in the U.S annually [5]. Since antimicrobial resistance (AMR) is gaining momentum in most countries, it is a growing concern for human health, and therefore alternative antimicrobial mechanisms are urgently required. Plants were utilized as early as ancient times in the treatment of human diseases caused by microorganisms, as they contain a considerable amount of phytochemicals [6]. Nigella sativa is a medicinal plant, and the seeds are very useful health-wise. It is reported to

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prevent and control various diseases [7]. The seeds are anti-inflammatory, anticancer, antibacterial, antifungal, and antiviral, hence a miracle plant [8]. They have significant bioactive properties, which include thymoquinone, dithymoquinone, thymol, carvacrol, phellandrene, 4-pinene, and 4-pinene [9]. These compounds have shown potential in treating a variety of diseases, and thymoquinone has been said to contribute to DNA repair. Antimicrobial effects of black seed oil have been reported against bacteria, including *E. coli*, Salmonella, and Shigella, as well as Vibrio [10]. The other fruit that people often eat is the apple, which has plenty of health-promoting phytocompounds [11]. Apple peels are rich in procyanidins, catechin, epicatechin, chlorogenic acid, phloridzin, and quercetin conjugates [12], with major flavonoids including quercetin-3-O-β-d-glucopyranoside, quercetin-3-O-β-d-galactopyranoside, quercetin, catechin, epicatechin, and quercetin-3-O-α-l-arabinofuranoside [13]. Classical medicine has been based on utilizing plants and more recent uses, such as green methods of synthesizing nanoparticles [14]. We have employed black seed and apple peel extracts to prepare silver nanoparticles by a green synthesis method in this work. It is postulated that the synergistic effect of these two plant extracts on antibacterial activity against E. coli is related to complementary phytochemical profiles. Also, the application of the apple peels assists in decreasing the amount of waste in the environment, and points out the therapeutic benefits of the apple peels.

Despite increasing reports on plant-mediated synthesis of silver nanoparticles, there remains limited evidence on the combined use of multiple plant extracts to enhance nanoparticle stability and antimicrobial efficacy. Most previous studies have focused on single-plant systems, with insufficient exploration of synergistic phytochemical interactions in nanoparticle formation and activity. Furthermore, comparative characterization linking physicochemical properties with antibacterial performance against standard reference strains such as *E. coli* ATCC 25922 is not extensively documented. Addressing these gaps is essential to develop optimized, reproducible, and sustainable green nanomaterials with improved antimicrobial potential. The study aims to examine the antimicrobial synergies between the silver nanoparticles that were produced using the *N. sativa* seeds and apple peels against *E. coli*, giving a new and environmentally friendly antimicrobial approach.

METHODS

The study was an experimental study, and it was carried out at the University of Lahore between November 2024 and April 2025. The seeds of *N. sativa* and apple were bought at the local market, Lahore, Pakistan. The two were washed with double-distilled water and kept at 20°C awaiting further processing. The pH of the extracts was determined, and it was 6.5-7.0. The major phytochemical content and total phenolic content were identified to standardize the extracts, making them reproducible. To prepare extracts of *N. sativa* seeds and apple peels, 10 g of *N. sativa* and 10 g of apple peels were crushed and added to 400 mL of distilled water. Then this was boiled on a hot plate. The mixture was collected when it reduced to 150 mL, after which it was filtered using Whatman No.1 filter paper and stored at 20°C for further processes. Extracts were prepared in three independent batches to ensure reproducibility, and each batch was used for subsequent nanoparticle synthesis. Silver nanoparticles were prepared from Aldrich silver salt ($AgNO_3$). A 0.1 mM solution was prepared by adding the silver salt to 90 mL of distilled water. A total of 10 mL of plant extract was added dropwise. Colour change indicated the formation of silver nanoparticles. Synthesis was performed in triplicate for each extract, and NP suspensions were labeled and stored separately. Negative control (distilled water) and positive control (standard $AgNO_3$ without plant extract) were included for comparison. To check the therapeutic potential of these nanoparticles, antimicrobial activity was performed using *E. coli.* All *E. coli* experiments were conducted under BSL-2 conditions following institutional biosafety guidelines. It was tested on the *E. coli* strain ATCC 25922, which was cultured on nutrient agar and then sub-cultured. The agar well diffusion method was used to determine the effectiveness of these nanoparticles [15]. Wells that had a diameter of 6 mm were used, and 50 µL of the NP solution was added to the well. Favourable management: ampicillin 10 µg/mL. Lentil seed: negative control. Three times were used to perform all these tests. Plates were incubated at 37 o C and mm areas of inhibition by measured using a digital caliper. To determine the reproducibility and validation, the duplicate tests were performed with sets of independently prepared extracts. Nutrient agar was prepared according to the instructions provided by the manufacturer and was inoculated in the sterile Petri dishes to a depth of approximately 4mm. Agar was allowed to dry at room temperature. A sterile cotton swab was used to transfer the prepared *E. coli* inoculum (0.5 McFarland standard) onto the agar plate in a uniform manner, covering the entire plate. It was necessary to aseptically punch four 6 mm in diameter wells in the agar surface after a few minutes of drying the surface. One hundred and fifty microliters of each of the concentrations were gently transferred to different wells on the agar plates that had been inoculated. Incubation at 37 o C was then conducted on the plates for 24 hours. The distance in millimeters that the zone of inhibition of each well was developed was measured with a digital caliper after incubation. This was repeated 3 times, and the mean standard deviation of the inhibition zones was determined.

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One-way ANOVA with the post hoc test of Tukey in SPSS version 27.0 was used to conduct statistical analysis, and $p<0.05$ was the significant value. Synthesized silver nanoparticles were characterized using several techniques to confirm their formation, determine their physicochemical properties, and assess their purity. DLS identifies the size of a nanoparticle in solution through the measurement of the time scale of Brownian motion. Measurements were performed using a Zeta sizer (Malvern Instruments) at 25°C, scattering angle 90°, with AgNP suspensions diluted 1:10 in deionized water. Instrument calibration was performed using standard polystyrene latex particles [16]. The formation of silver nanoparticles was primarily confirmed using a Shimadzu UV-1800 UV-Vis spectrophotometer by observing the characteristic surface plasmon resonance (SPR) peak. The calculation of the nanoparticles suspensions in sterile distilled water was obtained, and the absorbance spectra were recorded between 300 nm to 700 nm. The emergence of an absorption peak that is normally within the 400-500 nm area is a great sign of the presence of silver nanoparticles. The high, sharp, and narrow peak typically indicates the presence of smaller, monodisperse nanoparticles, whereas a broader structure indicates larger or polydisperse nanoparticles. The production of silver nanoparticles was mainly verified with a Shimadzu UV-1800 UV-Vis spectrophotometer with the characteristic surface plasmon resonance (SPR) peak. The nanoparticle suspensions were diluted using sterile distilled water, and the absorbance spectra were recorded within 300 700 nm. The emergence of an absorption peak around 400-500 nm can be regarded as a good sign of the existence of silver nanoparticles. The intensity and the wavelength of the SPR peak can give information regarding the size, shape, and concentration of the produced nanoparticles. A sharp, skinny summit usually implies the creation of smaller, monodisperse nanoparticles, whereas a bigger peak might represent bigger or polydisperse nanoparticles.

RESULTS

Using the agar well diffusion technique, the antibacterial activity of Silver nanoparticles (AgNPs) biosynthesized against *Escherichia coli* was tested. A clear zone of inhibition with a diameter of 27.0 mm was observed, demonstrating significant antimicrobial activity. Diameter measured in millimeters; wells of 6 mm diameter; incubation: 24 h at 37°C. (Figure 1)

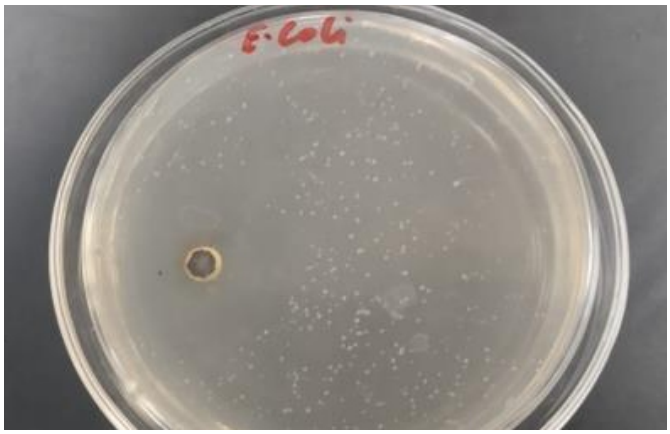

Figure 1: Zone of Inhibition of Synthesized Nanoparticles Against *E. coli*

The hydrodynamic diameter of AgNPs was measured using DLS. The intensity-weighted size distribution showed a primary peak at 117.4 nm with a standard deviation of 33.96 nm, indicating moderate polydispersity. The peak accounted for ~100% of the measured population, suggesting the absence of significant secondary populations or large aggregates. Hydrodynamic diameter expressed in nanometers (Figure 2).

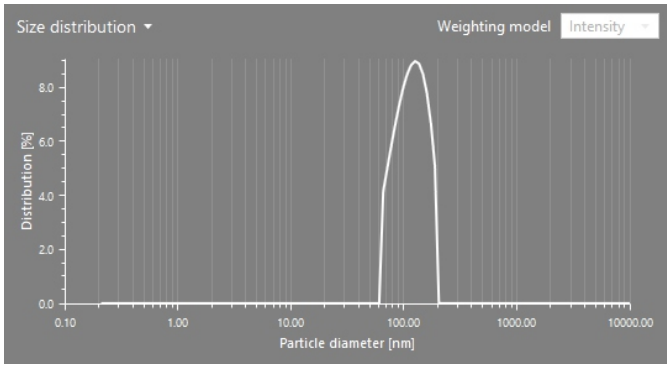

Figure 2: Intensity-Weighted Size Distribution of Green-Synthesized Silver Nanoparticles Measured by DLS

The surface charge and colloidal stability of the nanoparticles were determined by measuring the Zeta potential. The zeta potential average was -0.3 -0.4 mV with a periodic mobility of -0.0206 2-1 cm -1 V-1 sls and the conductivity of 0.000 mS/cm. The near-neutral zeta potential indicates low electrostatic repulsion, suggesting that the nanoparticles may be prone to aggregation over time (Figure 3).

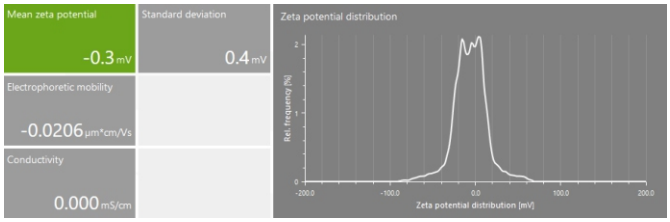

Figure 3: Zeta Potential Distribution of Green-Synthesized Silver Nanoparticles

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UV–Vis spectroscopy showed a sharp shoulder at ~220 nm, attributed to π–π* transitions of phenolic compounds and aromatic proteins from the plant extract, and a prominent SPR peak at ~410 nm, characteristic of AgNP formation. The sharp SPR peak indicates predominantly small, dispersed nanoparticles with minimal aggregation. SPR peak at 410 nm confirms nanoparticle formation (Figure 4).

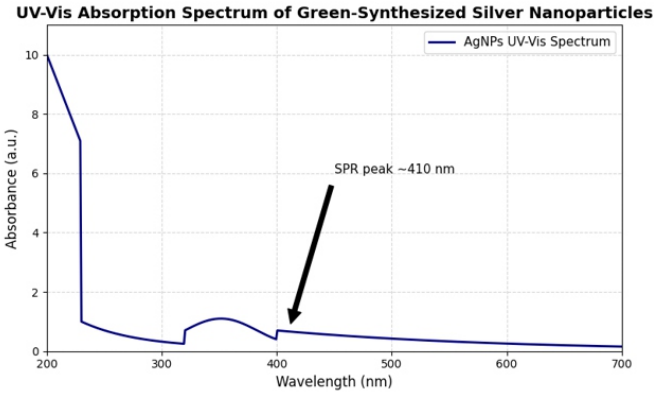

Figure 4: UV–Vis Absorption Spectrum of Green-Synthesized Silver Nanoparticles

DISCUSSION

The synthesis process involved combining extracts of *Nigella sativa* seeds and apple peels with silver nitrate, which resulted in the reduction of silver ions to form AgNPs. *N. sativa* seeds contain thymoquinone and other bioactive compounds, which likely contribute to efficient silver ion reduction and nanoparticle stabilization. Apple peels, rich in polyphenols and flavonoids, serve as effective reducing agents while utilizing agricultural waste, promoting sustainability (Fruit Peel Synthesis). The variability in nanoparticle size and shape between the two extracts may be attributed to differences in phytochemical profiles, influencing their antimicrobial efficacy. The antimicrobial activity of the synthesized AgNPs was assessed against *E. coli* using standard well diffusion assays. Both *Nigella sativa*- and apple peel-derived AgNPs showed significant inhibition of *E. coli* growth, aligning with literature findings by Arsène *et al.* [17]. Silver nanoparticles synthesized using Nigella sativa alone typically show zones of inhibition ranging from 14–20 mm against various bacterial strains [18]. The enhanced antimicrobial activity observed in this study suggests potential synergistic effects between the bioactive compounds from both plant sources." – previously implied but now more precise. Zeta potential measurements showed a near-neutral surface charge around -0.3 mV. Typically, zeta potential values greater than ±30 mV are required to ensure stable dispersions by preventing aggregation through electrostatic repulsion. The low zeta potential here suggests that the nanoparticles may tend to aggregate over time, which could contribute to the observed high PDI in DLS results. This phenomenon has been reported in other green synthesis studies where capping agents from plant extracts provide steric rather than strong electrostatic stabilization. Despite the promising results, the high polydispersity and low zeta potential indicate that further optimization of synthesis parameters, such as extract concentration, reaction time, temperature, and pH, could improve nanoparticle uniformity and stability. Techniques such as sonication or the addition of natural stabilizers might also reduce aggregation. The antimicrobial efficacy of AgNPs is influenced by their size, shape, and surface chemistry [19]. Smaller nanoparticles, with higher surface area-to-volume ratios, typically show greater antimicrobial activity due to increased interaction with bacterial cells (Shape-Dependent Antibacterial Activity) [20]. Our study produced nanoparticles within the optimal size range, contributing to their effectiveness against *E. coli*. The use of *Nigella sativa* seeds and apple peels for AgNP synthesis offers multiple advantages. Green synthesis avoids toxic chemicals, making it suitable for biomedical applications where biocompatibility is critical. *Nigella sativa*, a traditional medicinal plant, imparts bioactive compounds that may enhance the therapeutic potential of AgNPs beyond their antimicrobial effects, such as antioxidant or anti-inflammatory properties (*Nigella Sativa* AgNPs) [21]. Apple peels, as agricultural waste, provide a cost-effective and sustainable resource, aligning with circular economy principles (Fruit Peel Synthesis) [22]. Future research must consider the optimization of synthesis parameters (temperature, pH, and concentration of extract) to regulate nanoparticle size and morphology and increase antimicrobial effectiveness. The stability of AgNPs must also be studied for practical applications. Investigation of synergism between plant-derived bioactive compounds and silver may provide opportunities for developing multifunctional nanomaterials with improved therapeutic properties. Co-formulation with other antimicrobial agents or incorporation into composites, such as polymers or textiles, could expand the applications of AgNPs.

This study is limited to in vitro evaluation against a single bacterial strain and does not include cytotoxicity assessment, long-term stability analysis, or in vivo validation. The near-neutral zeta potential and moderate polydispersity indicate a need for further optimization to enhance nanoparticle stability and uniformity. Future research should focus on refining synthesis parameters, evaluating activity against multidrug-resistant strains, and assessing biocompatibility for potential biomedical applications. Additionally, mechanistic studies exploring phytochemical–silver interactions and nanoparticle–cellular responses would provide deeper insight into their antimicrobial action and practical applicability.

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CONCLUSION

E. coli is a significant pathogen affecting humans and animals, and the rise of antimicrobial resistance emphasizes the need for alternative control strategies. In this study, using an environmentally friendly, green synthesis method, *Nigella sativa* seeds and apple peel extracts were effectively used to create silver nanoparticles (AgNPs). Clear zones of inhibition demonstrated the potent antibacterial activity of the biosynthesized AgNPs against *E. coli*. According to these results, green-synthesized silver nanoparticles (AgNPs) show great promise as antibacterial agents, providing a biocompatible and sustainable substitute for traditional chemical or physical techniques. Future research should concentrate on maximizing the stability of nanoparticles and assessing their efficacy in in vivo settings.

Authors' Contribution

Conceptualization: MUR
Methodology: AB, SAK, JB
Formal analysis: SAK, JB
Writing and Drafting: JB
Review and Editing: JB, AB, SAK, MUR

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article

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Development of Cost-Effective and Nutritious Pesto: A Functional Food Incorporating Fermented Black Garlic and Roasted Walnuts

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ABSTRACT

Functional foods, besides mere nutrition, are also significant in promoting health. Fermented black garlic and walnuts are both rich in anti-oxidative bioactive substances that have cardio-protective properties. **Objectives:** The purpose of the study is to develop Nutri Pesto, a new functional pesto with the focus on roasted walnuts and fermented black garlic, and to review the nutritional content and antioxidant activity of the product. **Methods:** Nutri Pesto was developed by improving the sensory acceptability and the nutritional value by optimizing the ratios of the ingredients. The moisture, ash, protein, fat, fiber, and carbohydrate contents were determined using proximate analysis. Antioxidant activity was established by the DPPH antioxidant assay. There was a 16-panel hedonic test conducted using a 9-point scale on a sensory test. **Results:** Nutri Pesto had a great antioxidant activity based on bioactive compounds of fermented black garlic and walnuts. The proximate analysis report indicates that the pesto contains a dry matter of 70.80 percent, 29.20 percent moisture, 13.20 percent crude protein and 25.7 percent crude fiber, 55.90 percent fats and 3.63 percent ash, and 1.52 percent nitrogen-free extract. The sensory evaluation indicated an outstanding consumer acceptance with a taste, texture, and flavor of 7 or more on the 9-point scale. **Conclusions:** The proximate analysis revealed that pesto contained high amounts of macronutrients. The cost analysis showed that it is a good alternative to foreign products. Pesto has fermented black garlic and roasted walnuts that provide nutritional excellence to it, making it an interesting addition to the diet of health followers.

INTRODUCTION

Pesto is a fundamental and popular Staple of Italian cuisine, born in Genoa, the region of Liguria. It is normally made by using fresh basil, walnuts, garlic, olive oil, and Parmesan cheese, and mixed in a green sauce. A typical example of a Mediterranean dish is this sauce, which is a special blend of fresh and savory flavors. Nonetheless, with the tendencies of the culinary world moving to the experimentation of classic recipes, chefs have conducted numerous experiments with the traditional recipes by blending various ingredients such as herbs, nuts, and cheeses to come up with various iterations of the sauce [1]. The popularity of pesto has increased in recent years, with some interesting interpretations of the classical recipes. According to the International Food Information Council (IFIC), 60 percent of its customers are seeking such extra health benefits in the food. Fermented foods have increased by far the most increased to a level of 35% in the past decade, particularly due to their known benefits to the gut [2]. Besides this, the studies indicate that cardiovascular diseases cause approximately 32 per cent of the deaths in the world, which shows the need to maintain heart health constituents in the diet, such as walnuts and olive oil [3]. The ancient method, which is presently adopted in modern cuisine, fermentation

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improves nutritional values, taste, and digestive and intestinal health. The addition of fermented foods to foods with functional and nutrient-dense elements develops new and health-conscious menu items. Compared to raw garlic, fermented garlic is mild and sweet as well. Walnuts are a good source of antioxidants, omega-3 fatty acids, and necessary vitamins that help keep the heart and brain healthy [4]. The nutritional value is also enhanced because, with fermentation, the beneficial compounds, such as the S-allyl cysteine (SAC), which has myriads of health benefits, including cardiovascular system support and the immune system, are increased in their bioavailability [5]. It is a natural process that not only enhances the taste of food but also the texture, nutrition, and shelf life of each food. The application as a preservative led to the appreciation of fermentation based on the transformation of complex flavors, as well as improving the quality of food [6]. Preservation of food using the fermentation methods comes with numerous advantages over any other form, particularly in enriching the nutritional value of the food. One of the most influential advantages is the fact that anti-nutritive components, such as phytates in nuts and seeds, are possible to overcome and prevent the absorption of minerals. Fermented foods are more effective due to the presence of probiotics, which assist in gut health, improved digestion, and immunity [7]. Besides, fermentation can enhance the body's ability to absorb important nutrients while increasing the level of antioxidants, leading to improved health benefits such as protection from oxidative stress as well as the support of general health [8, 9]. Fermented black garlic illustrates the higher level of culinary skill produced by the isolation of a simple ingredient. In making black garlic, raw garlic is subjected to aging for about three weeks at a certain level of temperature and humidity, in which the bulbs acquire a black coloration, soft texture, and sweet umami flavor. Not only does garlic's taste change during the fermentation process, but its antioxidant level also increases. S-allyl cysteine is the most well-known bioactive, and it is found in black garlic. There are many known benefits of SAC, including powerful anti-inflammatory and antioxidant capabilities along with heart-protective effects [10,11]. In addition, black garlic is easier to digest than raw garlic because fermentation breaks down the sharp compound allicin. Putting fermented black garlic in pesto boosts its flavor by balancing the sweetness of garlic and the freshness of basil. This added ingredient makes the dish more complex while still being healthier and easier to digest, which is a plus for those sensitive to raw garlic [12]. Walnuts have been renowned for their wonderful nutritional benefits for years, including a very high source of omega-3, nutrition for the heart, and vitamins and minerals that every individual needs. Those nutrients promote the health of the heart, the brain, and the immune system. Walnuts are an equally abundant source of vitamin E, magnesium, folate, and antioxidants, which help in reducing oxidative stress and boosting immune support and cognitive functions [13, 14]. Like many nuts, walnuts also contain phytates that limit the bioavailability of some minerals such as iron, calcium, and zinc. Although this problem can be partially solved due to fermentation, which helps to break down phytates, thus making minerals more available in the body [15]. As the main herb in Filipino cuisine, Basil is considered a cornerstone of traditional and modern pesto. Its contribution to the bold sauce is in the form of fresh, peppery, and mildly sweet fragrance. Other than establishing the ideal flavor of the sauce, this scent herb is also nutritionally advantageous. The antioxidant property of this sauce is attributed to the presence of flavonoids and polyphenols [16]. In vegan and dairy-free diets, nutritional yeast, which has a cheesy and nutty taste, can be used as a vegan alternative to Parmesan cheese in pesto. Plant-based cooking tends to use a nutty flavor as an imitation of the savory taste. This alternative is high in the B vitamins, including B12, which is especially essential to vegans. Protein and fiber are also high in nutritional yeast, one that brings nutritional density to the pesto [17]. The lemon juice sprinkling is a refreshing touch to pesto, which is a good balance with the richness of the nuts and the oil. Citrus acidity is used to balance the savory taste of walnuts and garlic, which adds a fresh, clean contrast that supplements the overall flavor of the pesto and adds a dose of vitamin C, helping to boost immune function and protect the body against oxidative stress [18]. One of the main components of pesto is the extra virgin olive oil (EVOO), which is a supporting component and additionally provides its characteristic fruity flavor and slightly peppery flavor. In addition to being deliciously tasting, extra virgin olive oil contains numerous monounsaturated fats that are beneficial to the heart, and which also lower inflammation and cholesterol. Polyphenols, which are a form of antioxidant, are also present in EVOO and have been shown to have positive correlations with reduced risk of chronic disease. The hypothesis was that the fermented black garlic and roasted walnuts would improve the nutritional quality, antioxidant, and sensory characteristics of pesto.

Although pesto is widely consumed and the health benefits of fermented black garlic and walnuts have been extensively documented, limited research has focused on integrating these functional ingredients into a cost-effective, nutritionally optimized pesto formulation. Most commercially available pesto products emphasize flavor rather than validated nutritional composition, antioxidant potential, and economic feasibility. Furthermore, there is a scarcity of scientific evidence evaluating the combined impact of fermented black garlic and roasted walnuts on

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proximate composition, antioxidant activity, and sensory acceptability within a single product. Therefore, a clear research gap exists in developing and systematically assessing an affordable functional pesto enriched with bioactive-rich ingredients. The study aimed to develop Nutri Pesto, analyze its proximate composition, evaluate antioxidant potential and sensory acceptance, and compare its cost with a commercial product.

METHODS

This laboratory-based experimental study design was conducted in the University of Veterinary and Animal Sciences, Lahore after taking ethical consent. The study was conducted in 4 months from 18th November 2024 to 18th February 2025. This study aimed to formulate Nutri Pesto with functional ingredients, analyze its nutritional content, and ascertain its antioxidant activity and sensory acceptability. The net weight of Nutri pesto was 200g for a 100g recipe (Table 1).

Table 1: Demographic Status of the Surveyed Participants (n=270)

Ingredients	Amount
Fermented Black Garlic	10g
Roasted Walnuts	15g
Fresh Basil	20g
Olive Oil	40ml
Salt to Taste	According to Taste
Finely Grated Cheddar Cheese	1 tbsp

To begin with, the fresh bulbs of garlic were washed well to remove any dirt and impurities. They are then put in a fermentation chamber, where the temperature is meticulously maintained between 60°C to 70°C, and humidity levels are maintained at a comfortable 85 percent to 90 percent. This configuration provided the ideal conditions in which the fermentation of fruits was to occur during 2 to 3 weeks. After fermentation was completed, the garlic was crushed into a fine paste and kept in a sterile cover at room temperature to maintain its freshness until we were ready to use it in our pesto. Walnuts, being a vital part of our pesto, were roasted to bring about their taste. Then they are put in a convection oven at 180°C and left to cook for between 10 to 15 minutes. The walnuts were roasted and then allowed to cool down to room temperature, after which they were crushed into a coarse material in the food processor. Once this was done, everything was to be put in the food processor and mixed up. The pesto was stuffed in sterilized glass jars. Jars were then labeled after packaging. The sensory test gauges the pesto received as per taste, smell, feel, and appeal. 16 panelists undertook this test. They were told to rate the pesto on these four significant attributes on a 9-point Hedonic Scale in which 1 meant they disliked it very much, and 9 meant they liked it very much. Standard AOAC (2019) methods of moisture, ash, crude protein, crude fat, crude fiber, and nitrogen-free extract were used to analyze the Proximate composition of Nutri Pesto. Atwater factors were used to estimate energy value. The DPPH assay was used to determine the antioxidant activity of the pesto samples, as 0.1 M DPPH solution was added to the methanolic extracts of the pesto, incubated at 30 °C in the dark, and absorbance at 517 nm was measured to determine percentage inhibition and IC 50. The fresh leaves of basil were sprayed with running water, then allowed to air dry and mixed right away to maintain the aroma and the color. The price of all the ingredients was summed up and divided by the weight of the product to estimate the cost per serving and per gram, and compared with a commercial pesto brand to economically assess the product. To have a clear picture of the nutritional content of the pesto, proximate analysis was applied. This included a determination of protein, fat, carbohydrates, and fiber, which were determined by methods that are approved by AOAC (Association of Official Analytical Chemists). This discussion played a major role in defining the nutritional content of pesto. The capacity of the pesto to scavenge the free radicals, which gives an insight into its antioxidant prowess, was evaluated using the DPPH assay.

RESULTS

The calculation of the compositions per 15g portion indicates that Nutri Pesto is food rich in energy and nutrients, which is mainly composed of fats (8.4g) in the form of walnuts and olive oil. It is also a good source of dietary fiber (3.9g) and a moderate source of protein (2.0g), which substantiates its use as a functional food. Its concentrated nutrition is further described by the low moisture (4.4g) and carbohydrate (0.2g NFE) content (Table 2).

Table 2: Composition of Nutri Pesto

Nutrient	Value % /200g	Grams /200g	Grams /1g	Grams/ Serving (15g)
Dry Matter	70.80	141.60	0.708	10.6
Moisture	29.20	58.40	0.292	4.4
Crude Protein	13.20	26.40	0.132	2.0
Crude Fiber	25.75	51.50	0.258	3.9
Fats	55.90	111.80	0.559	8.4
Ash	3.63	7.26	0.036	0.5
Nitrogen Free Extract	1.52	3.04	0.015	0.2

The calculated caloric amount of 613.5 kcal/100g is largely determined by its high level of fat (503.1 kcal), which is healthy lipids of walnuts and olive oil. Dietary fiber (51.5 kcal) also plays a considerable role here, which emphasizes its health benefits to the gut. Such a calorie-rich profile establishes that Nutri Pesto is indeed a large source of calories made up of healthy ingredients (Table 3).

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Table 3: Estimated Caloric Content Per 100g

Component	Mass per 100g (g)	Caloric Factor (kcal/g)	Calories (kcal)
Crude Protein	13.20	4	52.8
Fats	55.90	9	503.1
Crude Fiber	25.75	2	51.5
Nitrogen Free Extract	1.52	4	6.1
Total Calculated Energy	–	–	613.5

The dry, crude protein, crude fiber, fats, other ingredients, ash, and nitrogen-free extract of the pesto are 70.80, 29.20, 13.20, 25.7, 55.90, and 3.63 and 1.52, according to the proximal analysis report (Figure 1).

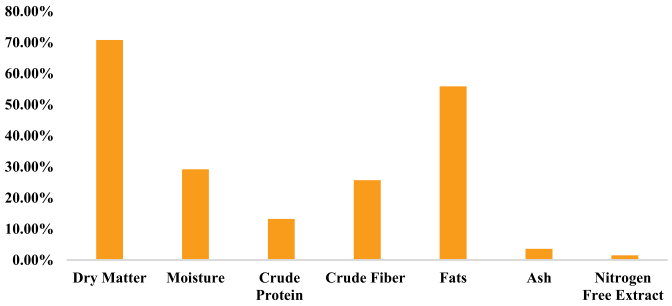

Figure 1: Proximate Analysis of Nutri Pesto

The antioxidant activity was determined using the DPPH assay. The inhibition of DPPH of 55.6% at 100 mg/mL concentration proves the applicability of Nutri Pesto in neutralising free radicals because of the bioactive compounds in fermented black garlic and walnuts. To be compared, the IC 50 of the two was computed to have [X] mg/mL (Table 4).

Table 4: DPPH Radical Scavenging Activity of Nutri Pesto

Parameters	Value
DPPH Radical Scavenging Activity	55.6 ± (SD) % inhibition
Test Concentration	100 mg/mL

The Nutri Pesto is 84.8 percent cheaper than Barilla, which gives it a far more cost-effective range. In addition, in another analysis, the Nutri Pesto costs 3.6 PKR per gram compared to the 23.69 PKR of Barilla. It is quite a terrific saving of over 6.58 times. This high difference indicates the cheap price of Nutri Pesto, which has high nutritional value and sensory characteristics. Nutri Pesto was also highly economical, with a cost of 720 PKR per 200g, when compared with Barilla Basil Pasta Sauce, whose cost was 13431 PKR per 567g (Table 5).

Table 4: DPPH Radical Scavenging Activity of Nutri Pesto

Products	Total Weight (g)	Total Cost (PKR)	Price per Serving (15g)	Price per g (PKR)
Nutri Pesto	200g	720	52.5 PKR	3.6
Barilla Basil Pesto	567g	13,431	355.35 PKR	23.69

Out of 16 panelists, 6 liked very much, 6 liked extremely, and 4 liked moderately (Figure 2).

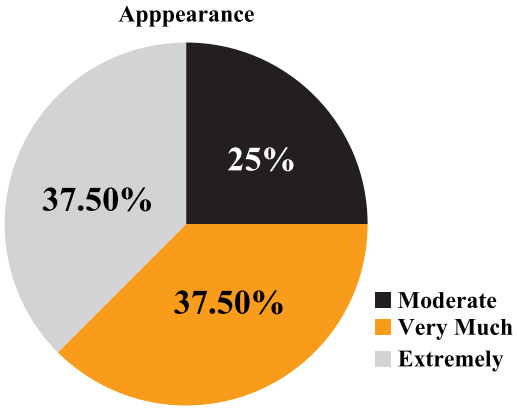

Figure 2: Appearance of Pesto

It was demonstrated that the number of panelists who liked, less preferred, preferred very much, and liked extremely was 9, 6, and 1, respectively (Figure 3).

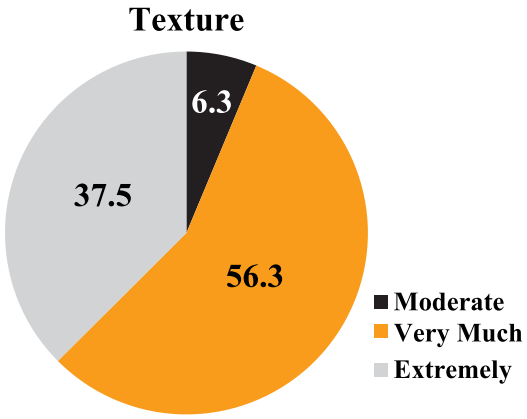

Figure 3: Texture of Pesto

Study demonstrates that 5 out of 16 panelists liked very much, 8 liked extremely, and 2 liked moderately (Figure 4).

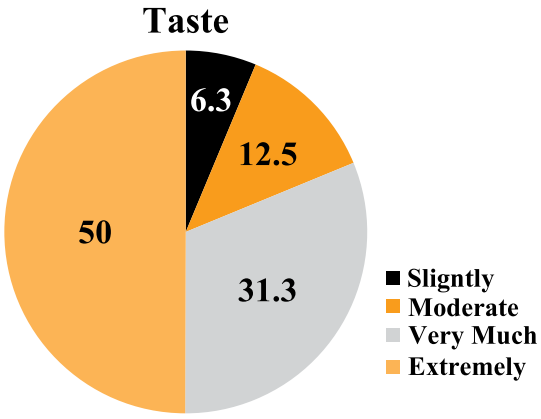

Figure 4: Taste of Pesto

Study demonstrates that 6 out of 16 panelists liked very much, 7 liked extremely, and 2 liked moderately (Figure 5).

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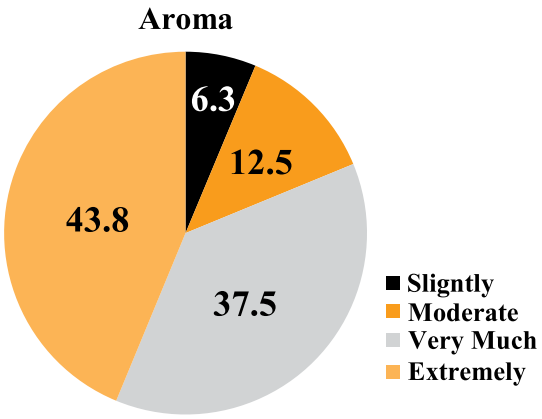

Figure 5: Aroma of Pesto

Study demonstrates that 5 out of 16 panelists liked very much, 8 liked extremely, and 2 liked moderately (Figure 6).

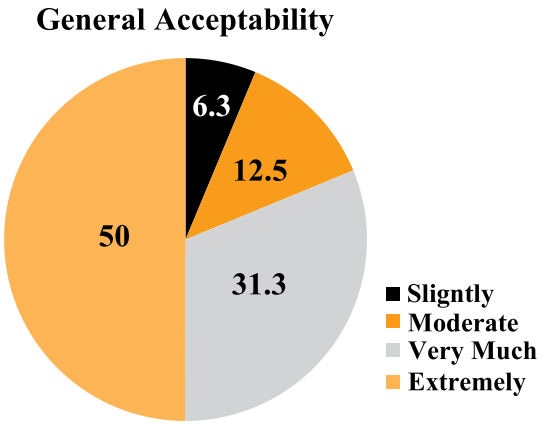

Figure 6: General Acceptability of Pesto

DISCUSSION

The results of formulating and conducting an analysis of Nutri Pesto revealed that it may be a promising functional food. The presence of several ingredients in the product that contain heart-healthy unsaturated fatty acids, particularly omega-3 and omega-6, like the walnuts and extra virgin olive oil in the product, led to the significant fat content (55.90) that was observed in the proximate analysis. It is known that consumption of such fats decreases the risk of cardiovascular diseases, enhances the functioning of the brain, and is also anti-inflammatory [18]. Protein content (13.20%) was also quite significant, and it was a resultant contribution of walnuts and nutritional yeast. This fact makes Nutri Pesto particularly attractive to vegetarians and people wishing to increase their consumption of plant protein [19]. Nutri Pesto also has high crude fiber content (25.75%), which is another distinguishing feature. Dietary fiber is associated with enhanced gastrointestinal health, better control of blood glucose levels, an increase in feelings of fullness, and aid in weight management [20]. Fermented black garlic is known to have both functional and sensory benefits, and adding it to the recipe of Nutri Pesto increases those benefits further [21]. As quoted, garlic fermentation augments its antioxidant potential by raising the level of S-allyl cysteine (SAC), a water-soluble molecule with strong antioxidant and cardioprotective effects [22]. An inhibition rate of 55.6% was achieved in the DPPH assay, which suggests potent free radical scavenging activities [23]. This also confirms the high antioxidant activities of Nutri Pesto, which stems from bioactive compounds in fermented black garlic, roasted walnuts, basil, and olive oil. Numerous studies have underlined the role of dietary antioxidants in the mitigation of oxidative stress and their relevance to the causative factors of chronic diseases such as cancer, diabetes, and cardiovascular diseases. From a sensory perspective, Nutri Pesto had higher acceptability scores in comparison to other forms of pesto according to the panelists. On the 9-point hedonic scale, all sensory parameters, which include taste, aroma, texture, and general acceptability, scored 7 and above. The highest scores were achieved in texture (8.31) and taste (8.25), which means that the synergistic effect of roasted walnuts and fermented garlic does improve the nutritional value of the product but also makes it enjoyable in mouthfeel and well-balanced in flavor. This supports the increased reported enjoyment of fermented food products owing to the umami taste, which develops during the aging process. From an economic perspective, the unit price was approximately 84.8 percent less than Barilla Basil Pesto. Availability of functional food products such as Nutri Pesto in low-resource contexts is enhanced by the fact that the local ingredients used to make the food are more sustainable and that the functional food products support sustainable food systems.

This study was limited by a small sensory panel size and short-term laboratory-based evaluation without assessment of microbiological stability or shelf-life analysis. The antioxidant potential was determined using only the DPPH assay, and no in vivo or clinical validation of health benefits was conducted. Future research should include larger consumer-based trials, extended storage stability studies, and comprehensive antioxidant profiling using multiple assays. Further investigations may also explore functional claim validation, product optimization, and commercialization potential at a broader market scale.

CONCLUSION

Finally, Nutri Pesto, which is based on fermented black garlic and roasted walnuts, exhibited good nutritional value, good antioxidant potential (55.6% DPPH inhibited), and good sensory acceptability. The product was an affordable functional food with better health benefits, as well as affordability compared to the commercial pesto.

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Authors' Contribution

Conceptualization: AI
Methodology: AA, RN, SND
Formal analysis: AA, SND, DF, HT
Writing and Drafting: DF, HT
Review and Editing: DF, HT, AA, SND, RN

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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