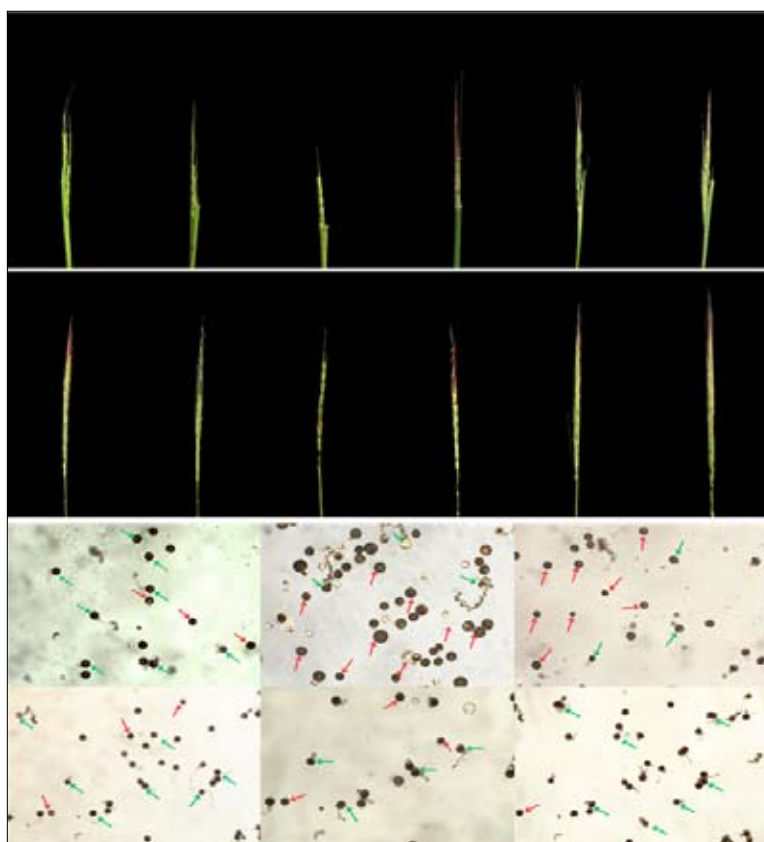


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Bottom: Representative pollen germination images. Green and red arrows indicate germinated and non-germinated pollen grains, respectively.

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EDITORIAL

Scientific literacy and public trust in science

Surveys attempting to obtain measurements of civic scientific literacy and public trust in science have been carried out in several countries, but not as yet in Sri Lanka. Although there is a general understanding that these two features of a society are multidimensional and that any numerical measure obtained needs to be interpreted with caution, there is no general agreement on the extent and manner in which scientific literacy impacts on the nature of public trust in science.

Public trust in science can exist without a high level of scientific literacy, due to the appreciation of the positive applications of science-based technologies which the public comes in contact with as individuals, in almost all aspects of their daily lives. Thus, the thematic content for the programme initiated by the National Academy of Sciences Sri Lanka (NASSL) on rebuilding trust in science is ‘How science/scientists generate wealth for the nation’.

However, public opinion based only on trust without scientific literacy can be manipulated to obtain support for irrational and polarized positions on controversial issues by appealing to the inherent biases of individuals. In contrast, public opinion based on trust grounded in an understanding of the processes involved in the creation of scientific knowledge, as well as an acquaintance with basic scientific facts and theories will be more robust and less susceptible to manipulation.

The National Science Foundation (NSF) and the Sri Lanka Association for the Advancement of Science (SLAAS) are two of the leading organizations in Sri Lanka that have been involved over the years, in activities which would serve to enhance civic science literacy. It is evident that the NASSL has recognized the importance of scientific literacy in the development of public trust in science, by supporting and collaborating with the ‘Popularization of Science’ programme of the SLAAS, as a component of its effort at rebuilding trust in science.

Ajit Abeysekera

REVIEW ARTICLE

Food Microbiology

Antibiotic resistance of *Listeria monocytogenes* present in raw milk and milk products: mechanisms, prevalence and implications for food safety.

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Submitted: 18 February 2025; Revised: 24 April 2026; Accepted: 29 April 2026

Abstract: Antibiotic resistance has emerged as a major global concern in both clinical and food safety contexts leading to a reduction in the effectiveness of treatments against bacterial infections and contaminations. *Listeria monocytogenes*, which causes severe food borne infections is particularly important due to its increasing tendency to develop resistance to commonly used antibiotics, posing significant challenges to public health. To gain a deeper insight into this issue, a comprehensive literature search was conducted. Based on this extensive review of the available literature, this article describes the mechanisms underlying antibiotic resistance in *L. monocytogenes*, with particular emphasis on its genetic basis and the role of horizontal gene transfer. The review also discusses the prevalence of antibiotic-resistant strains, with a special emphasis on their occurrence in raw milk and dairy products. In addition, key factors contributing to the development and spread of resistance including antibiotic exposure, food processing conditions, and bacterial stress responses were evaluated. Overall, this review highlights the growing public health significance of antibiotic-resistant *L. monocytogenes* and emphasizes the need for continuous surveillance, improved control strategies, and interdisciplinary collaboration to mitigate its emergence and ensure food safety.

Key words: *Listeria monocytogenes*, antibiotic resistance, raw milk, food safety, public health, genetic mechanisms

INTRODUCTION

Listeria monocytogenes, the causative agent of listeriosis, is a pathogenic Gram-positive coccobacillus widely

distributed in environmental sources such as food, water, soil, animal feed, and sewage (Gartley et al., 2022). This bacterium is capable of contaminating a wide range of food products and is responsible for listeriosis, a serious foodborne infection that poses significant health risks to humans. The disease primarily affects vulnerable populations, including pregnant women, neonates, the elderly, and immunocompromised individuals. In humans, listeriosis can result in severe clinical outcomes such as meningitis, meningoencephalitis, septicemia, abortion, and prenatal infections. The infection is associated with high mortality rates, reaching approximately 20–30% among high-risk groups (Disson & Lecuit, 2012; Choi et al., 2018; Hilliard et al., 2018).

L. monocytogenes can be shed in the milk of cows and buffaloes following miscarriage or udder infection, contaminating apparently normal milk with a significant bacterial load (Osman et al., 2016). The potential transmission of listeriosis through raw milk highlights the importance of monitoring and managing dairy herds to prevent outbreaks. Contaminated dairy products, introduced during processing or post-production handling, remain a major vehicle for human infection (Borucki et al., 2005; Wijendra et al., 2014; Ulusoy & Chirkena, 2019).

In recent years, increasing attention has been directed toward antimicrobial resistance in *L. monocytogenes*,

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particularly among isolates associated with food and dairy environments. Although the organism has been reported to be susceptible to first-line antibiotics such as ampicillin and penicillin (Morvan et al., 2010; Haase et al., 2014; Wilson et al., 2018), emerging resistance to commonly used antimicrobials, including tetracyclines, macrolides, and sulfonamides, has been reported (Tayeb et al., 2023; Díaz-Martínez et al., 2024; Ramatla et al., 2026). The presence of multidrug-resistant strains raises concerns regarding treatment efficacy and public health risk (Kayode et al., 2020). These factors collectively highlight the importance of integrating antimicrobial resistance surveillance into studies of listeriosis outbreaks, particularly those associated with milk and milk products.

Listeriosis outbreaks

Listeriosis is a severe foodborne infection caused by *L. monocytogenes*, a bacterium capable of surviving in diverse environments, including refrigerated foods (Choi et al., 2018; Gartley et al., 2022). Infection occurs mainly through contaminated food such as ready-to-eat products and unpasteurized milk (Disson & Lecuit, 2012; Osman et al., 2016). Clinical outcomes range from mild febrile illness to septicemia, meningitis, and miscarriage, with case fatality rates reaching 20–30% in high-risk groups (Hilliard et al., 2018; Choi et al., 2018).

L. monocytogenes outbreaks linked to milk and milk products (Table 1) reveal several key trends in product type, outbreak severity, and contamination pathways. Soft and fresh cheeses, including Brie, Queso fresco, Mexican-type cheese, and raw milk cheeses, were the most frequently implicated products. Outbreaks involving these products often resulted in the highest number of cases and deaths, reflecting their susceptibility to contamination and widespread consumption. For example, the 1985 outbreak in California associated with Mexican-type cheese caused 142 cases and 48 deaths (Linnan et al., 1988), while soft cheese outbreaks in Switzerland (1983–1987) and Sweden (2001) accounted for 122 and 120 cases, respectively (Bula et al., 1995; Carrique-Mas et al., 2003).

Pasteurized milk and pasteurized dairy products were also implicated in outbreaks, though typically with lower case numbers. Notably, the 1983 Maryland outbreak of pasteurized milk resulted in 49 cases and 14 deaths (Fleming et al., 1985), highlighting that post-pasteurization contamination remains a significant risk. Products such as butter, chocolate milk, ice cream, and fermented dairy were less frequently associated with outbreaks and generally resulted in fewer cases and

fatalities (e.g., 1987 California butter: 11 cases, Not Reported; 2009 Lebanon Kashk: 6 cases, Not Reported) (FDA, 2003; Harakeh et al., 2009).

Geographically, outbreaks were reported across North America, Europe, and the Middle East, with the USA, France, Canada, and Switzerland experiencing multiple large-scale events. Multi-state outbreaks in the USA (2003–2007, 2020–2024) frequently involved Hispanic-style soft cheeses and Brie/Camembert-type cheeses, reflecting distribution across wider regions (Smith, 2008; CDC, 2021).

Mortality trends indicate that raw milk and soft cheese outbreaks caused the greatest fatalities. Early high-mortality events included the 1985 Mexican-type cheese outbreak in California (48 deaths) and the 1983–1987 soft cheese outbreak in Switzerland (33 deaths) (Linnan et al., 1988; Bula et al., 1995). In contrast, outbreaks involving butter, pasteurized milk, or ice cream generally resulted in fewer deaths.

Contamination of dairy products with *L. monocytogenes* has been attributed to several factors reported in the literature. Raw or unpasteurized products are particularly susceptible due to higher bacterial loads and the absence of heat treatment, which allows the bacteria to survive and proliferate (Allerberger & Guggenbichler, 1989; Osman et al., 2016). Soft cheeses, characterized by high moisture content and nearly neutral pH, provide an environment favorable for bacterial growth (Linnan et al., 1988; Bula et al., 1995). Even pasteurized products are at risk through post-processing contamination, which can occur during handling, storage, or from equipment (Fleming et al., 1985; Schwartz et al., 1989).

Additionally, fresh or minimally aged cheeses often have limited acidity and short shelf lives, which fail to inhibit bacterial proliferation effectively (Harakeh et al., 2009; Osaili et al., 2012). Together, these factors highlight the multiple points at which *L. monocytogenes* contamination can occur in dairy production.

Contamination pathways included direct shedding from infected animals (such as mastitis or miscarriage) (Osman et al., 2016), cross-contamination during processing, and improper storage allowing bacterial proliferation. Multi-country outbreaks in recent years (e.g., in 2023, in Europe) underscore the transboundary risks associated with modern distribution chains. Both industrial and artisanal dairy products are vulnerable, and the absence of mortality reporting in some recent outbreaks may underestimate the public health burden.

Table 1: *Listeria* outbreaks caused by contaminated raw milk and milk products

Year	Country / Region	Type of Milk Product	No. of listeriosis cases and (deaths)	Reference
1979	Maryland, USA	Raw milk cheese	20 (3)	Ho et al., (1986)
1981	England	Dairy products	11 (5)	FDA (2003)
1983	Maryland, USA	Pasteurized milk	49 (14)	Fleming et al., (1985)
1983–1987	Switzerland	Soft cheese	122 (33)	Bula et al., (1995)
1985	California, USA	Mexican-type cheese	142 (48)	Linnan et al., (1988)
1986	Austria	Unpasteurized milk	28 (5)	Allerberger & Guggenbichler (1989)
1986–1987	Pennsylvania, USA	Ice cream, Brie	36 (4)	Schwartz et al., (1989)
1987	California, USA	Butter	11 (NR)	FDA (2003)
1989–1990	Denmark	Blue-mold / hard cheese	26 (6)	Jensen et al., (1994)
1989	Italy	Soft cheese	2 (NR)	Massa et al., (1990)
1991	England & Wales	Soft ripened cheese	63 (NR)	Greenwood et al., (1991)
1994	USA	Chocolate milk	45 (NR)	Dalton et al., (1997)
1995	France	Soft cheese	37 (11)	Goulet et al., (1995)
1997	France	Soft cheese	14 (NR)	Jacquet et al., (1998)
1998–1999	Finland	Butter	25 (6)	Lyytikäinen et al., (2000)
1999	England	Cheese	2 (NR)	Torner (2007)
2000	North Carolina, USA	Queso fresco	12 (5)	CDC (2001); MacDonald et al., (2005)
2001	Sweden	Soft cheese	120 (NR)	Carrique-Mas et al., (2003)
2001	Belgium	Frozen ice cream	2 (NR)	Yde & Genicot (2004)
2001	Japan	Washed-type cheese	38 (NR)	Makino et al., (2005)
2002	British Columbia, Canada	Cheese	47 (NR)	Pagotto et al., (2006)
2002	British Columbia, Canada	Pasteurized cheese	86 (NR)	Pagotto et al., (2006)
2002	Quebec, Canada	Soft & semi-hard cheese	17 (NR)	Gaulin et al., (2003)
2003	Texas, USA	Mexican fresh cheese	13 (2)	Swaminathan & Gerner-Smidt (2007)
2003–2007	USA (multi-state)	Queso fresco	74 (10)	Smith (2008)
2005	Brazil	Minas frescal cheese	6 (NR)	Brito et al., (2008)
2007	Massachusetts, USA	Pasteurized milk	5 (3)	CDC (2008)
2007	Ireland	Farmhouse cheeses	21 (NR)	O'Brien et al., (2009)
2008	Quebec, Canada	Cheese	38 (2)	Gilmour et al., (2010)
2008	Chile	Brie cheese	119 (5)	Jordan, 2016
2009	Lebanon	Kashk (fermented dairy)	6 (NR)	Harakeh et al., (2009)
2011	Jordan	Cheese	39 (NR)	Osaili et al., (2012)
2020	USA (multi-state)	Hispanic-style soft cheeses	13 (1)	CDC (2021)
2021	France	Raw milk soft cheeses	14 (NR)	Robinson et al., 2020)
2022	USA (Florida)	Ice cream	23 (1)	CDC (2022)
2022	USA (multi-state)	Brie & Camembert-style cheese	6 (1)	CDC (2022)
2023	Europe (multi-country)	Raw milk soft cheeses	16 (NR)	EFSA & ECDC (2023)
2024	USA (multi-state)	Cottage cheese & dairy desserts	8 (NR)	CDC (2024)

NR-Not Recorded

Treatment of listeriosis

Antibiotics are the primary therapeutic option for listeriosis, as no vaccines are currently available for its prevention. Early diagnosis of *L. monocytogenes* infection is critical due to the potentially severe outcomes of the disease, including septicemia, meningitis, and complications in pregnant women. One factor that complicates timely diagnosis and outbreak investigations is the pathogen's unusually long incubation period, which can last up to 70 days. Symptoms may appear weeks after exposure, and thus patients often fail to accurately recall, the relevant details making it difficult for public health authorities to trace the source of infection and implement control measures (Dortet et al., 2009; Rhoades et al., 2009). First-line treatments include penicillin, ampicillin, and gentamicin, while trimethoprim-sulfamethoxazole serves as an alternative for penicillin-allergic individuals. Erythromycin is often recommended for infected pregnant women, and vancomycin is used for cases of bacteremia. Other antibiotics, including rifampicin, doxycycline, chloramphenicol, and fluoroquinolones, may also be effective depending on the clinical context (Ramaswamy et al., 2007; Conter et al., 2009; Allerberger & Wagner, 2010; Alonso-Hernando et al., 2012).

However, the emergence of antibiotic-resistant strains of *L. monocytogenes* in raw milk and dairy products has complicated the control of this pathogen and may compromise the effectiveness of antimicrobial therapy for listeriosis, thereby posing significant public health concerns (Basu et al., 2017; Akrami-Mohajeri et al., 2018; Badawy et al., 2022).

Antibiotic resistance in *Listeria monocytogenes*

Antibiotic resistance in bacteria appears to be driven, in part, by the extensive and sometimes inappropriate use of antibiotics in both animal husbandry and human medicine, where they have been used not only for therapeutic purposes but also, in some regions, as growth promoters in livestock or applied without strict regulatory oversight (Marshall and Levy, 2011; Van Boeckel et al., 2015; O'Neill Review on Antimicrobial Resistance, 2016; Food and Agriculture Organization, 2016; World Health Organization, 2017). Exposure to sub-therapeutic antibiotic levels in animal production can select for resistant *L. monocytogenes* strains, and horizontal gene transfer via mobile genetic elements (plasmids, transposons), which further facilitates acquisition of resistance genes. In addition, stressful conditions in food-processing environments and the formation of

biofilms can enhance survival of resistant bacteria. As a result, multidrug-resistant *L. monocytogenes* isolates have been increasingly recovered from raw milk, pasteurized products, and other dairy foods, reducing the therapeutic options for listeriosis and underscoring the need for prudent antibiotic stewardship and ongoing surveillance across the farm-to-fork continuum.

Prevalence of antibiotic-resistant *Listeria monocytogenes* in raw milk and dairy products

Numerous studies over the past four decades have documented the prevalence of antibiotic-resistant *L. monocytogenes* in raw milk and dairy products (Akrami-Mohajeri et al., 2018; Aksem et al., 2018; Badawy et al., 2022; Kayode & Okoh, 2022; Harshani et al., 2022). More recent studies have reported multidrug-resistant (MDR) strains in raw milk and dairy products across multiple regions, with resistance commonly observed against penicillin, ampicillin, tetracycline, oxacillin, and aminoglycosides (Harshani et al., 2022, 2024; Suryawanshi et al., 2023; He et al., 2025). Many isolates also harbor virulence genes, underscoring the potential for both antimicrobial resistance and pathogenicity to co-exist in dairy-associated *L. monocytogenes* (Parussolo et al., 2021; Mohamed et al., 2022).

Mechanisms of antibiotic resistance in *Listeria monocytogenes*

Antibiotic resistance in *L. monocytogenes* is mediated by a combination of intrinsic and acquired mechanisms, involving horizontal gene transfer, mobile genetic elements, active efflux systems, and genetic modifications affecting antibiotic targets.

(i) Plasmid- and conjugation-mediated resistance

The acquisition of resistance genes via plasmids and conjugative transposons represents a major mechanism contributing to antimicrobial resistance in *L. monocytogenes*. Early studies demonstrated that resistance plasmids can be transferred between bacteria through conjugation (Evans & Macrina, 1983; Flamm et al., 1984). In addition, conjugative transposons carrying tetracycline resistance genes have been shown to transfer between *Listeria* species (Vicente et al., 1988). Certain studies indicate that such mobile elements continue to play a significant role in the dissemination of resistance traits, including fluoroquinolone resistance and multidrug resistance (Allen et al., 2016; Barker et al., 2025).

(ii) Mobile genetic elements in the gastrointestinal tract

Horizontal gene transfer within the gastrointestinal tract is another important pathway for the acquisition of resistance. In this environment, *L. monocytogenes* interacts with a diverse microbial community, facilitating the exchange of genetic material. Mobile genetic elements such as transposons and integrative conjugative elements have been reported to carry resistance genes, including those conferring resistance to tetracycline and ampicillin (Charpentier & Courvalin, 1999; Aminov et al., 2001; Walsh et al., 2001; Périchon & Courvalin, 2009). This contributes to the persistence and spread of resistance determinants across bacterial populations.

(iii) Efflux pumps and regulatory mutations

Efflux-mediated resistance is another key mechanism in *L. monocytogenes*. Efflux pumps, particularly MdrL (also referred to as Mdr1), actively expel antibiotics from the bacterial cell, thereby reducing intracellular drug concentrations. Resistance to fluoroquinolones such as ciprofloxacin has been associated with mutations in regulatory genes, including *fepR*, which control the expression of efflux systems (Romanova et al., 2006; Guérin et al., 2014; Wilson et al., 2018). These mutations can lead to overexpression of efflux pumps and enhanced resistance.

(iv) Modification of antibiotic targets and genetic determinants

Modification of antibiotic target sites is a major mechanism underlying resistance in *L. monocytogenes*. Genetic alterations can affect structures such as ribosomal subunits and penicillin-binding proteins, reducing the binding affinity of antibiotics, particularly macrolides and β -lactams.

At the molecular level, a wide range of antimicrobial resistance genes have been identified in *L. monocytogenes* isolates from diverse sources. Tetracycline resistance is among the most frequently reported and is associated with genes such as *tetA*, *tetC*, *tetD*, *tetG*, *tetK*, and *tetM* (Srinivasan et al., 2005; Kayode et al., 2022; Parra-Flores et al., 2022). Resistance to β -lactam antibiotics has been linked to genes such as *ampC* and *blaZ*, which may contribute to reduced susceptibility through target modification or enzymatic activity (Mohamed et al., 2022; He et al., 2025).

Aminoglycoside resistance is mediated by genes including *aph(3')-IIa* and *aad6*, which encode enzymes capable of modifying and inactivating these antibiotics (Kayode et al., 2022; Mohamed et al., 2022). Similarly, macrolide resistance involves genes such as *lnuG*, *mphB*, *msr(A/D)*, and *mef(A)*, which act through mechanisms including target modification, enzymatic inactivation, and active efflux (Barker et al., 2025; He et al., 2025).

In addition, other resistance determinants such as *fosX*, *lin*, *norB*, and *mprF* have been reported, reflecting a diversity of mechanisms including antibiotic inactivation, efflux activity, and cell membrane modification (Srinivasan et al., 2005; Parra-Flores et al., 2022).

(v) Multidrug resistance and environmental adaptation

Several studies have reported the occurrence of multidrug-resistant (MDR) isolates in dairy products, often associated with high antibiotic resistance indices and the ability to persist under environmental stress, including exposure to disinfectants (Frieri et al., 2017; Suryawanshi et al., 2023; Harshani et al., 2024). These findings highlight that both genetic determinants and environmental pressures play a critical role in the evolution and maintenance of antimicrobial resistance. Environmental conditions encountered during food processing and storage significantly influence the development of antibiotic resistance in *L. monocytogenes* and other foodborne pathogens. A range of biological, physical, and chemical stresses can trigger adaptive responses that enhance bacterial survival and tolerance to antimicrobial agents (Wesche et al., 2009). A key regulator in this process is the sigma factor B (SigB), which controls stress response pathways in Gram-positive bacteria. In *L. monocytogenes*, activation of SigB has been linked to increased expression of genes associated with cell wall integrity and resistance to antibiotics such as vancomycin (Palmer et al., 2009; Shin et al., 2010).

Exposure to environmental stressors including fluctuations in pH, temperature, and osmotic pressure has been shown to increase bacterial resistance to antibiotics. For instance, *L. monocytogenes* subjected to low pH, high salt concentrations, or refrigeration temperatures demonstrates enhanced tolerance to multiple antimicrobial agents (Al-Nabulsi et al., 2015; Olaimat et al., 2018). Similarly, prior exposure to certain antibiotics, such as ampicillin, tetracycline (achromycin), gentamicin, rifampicin, and trimethoprim-sulfamethoxazole, may

confer cross-protection against non-lethal stresses, including oxidative stress and sublethal heat (Faezi Ghasemi and Kazemi, 2015).

In addition, environmental stresses commonly applied in food preservation such as low water activity, acidic conditions, and high osmotic pressure can facilitate the horizontal transfer of conjugative plasmids carrying resistance genes (Beuls et al., 2012). Starvation stress further enhances bacterial resistance to preservation methods like heat and irradiation (Mendonca et al., 2004). Moreover, both horizontal and vertical gene transfer between *Listeria* spp. and other Gram-positive bacteria, including *Staphylococcus* and *Enterococcus*, contribute to the dissemination of antimicrobial resistance determinants (Charpentier and Courvalin, 1999; Lyon et al., 2008).

The use of approved food antimicrobials, such as sodium diacetate, potassium lactate, and nisin, may also influence resistance by inducing efflux pump activity (Stasiewicz et al., 2011). Additionally, natural plant-derived antimicrobials such as clove oil, cinnamaldehyde, and vanillin are increasingly used in food preservation; however, their potential to induce cross-protection against clinically relevant antibiotics in *L. monocytogenes* remains limited (Oussalah et al., 2005; Zanini et al., 2014).

Biofilm formation further complicates control strategies, as *L. monocytogenes* embedded within biofilms exhibit intrinsic resistance to disinfectants and sanitation procedures. Mechanisms such as exopolysaccharide production, persister cell formation, quorum sensing, and efflux pump activity enhance bacterial persistence in food processing environments, posing ongoing challenges for food safety and public health (Jahid and Ha, 2012; De La Fuente Nunez et al., 2013; Buchanan et al., 2017; Li et al., 2023).

Implications of the emergence of antibiotic-resistant strains of *Listeria monocytogenes* in raw milk and milk products

The emergence of antibiotic-resistant strains of *L. monocytogenes* in raw milk and milk products presents a complex public health challenge with significant implications for food safety (Harakeh et al., 2009; Wilson et al., 2018). Consumption of products containing these strains exposes diverse populations to antibiotic-resistant pathogens, potentially resulting in severe and difficult-to-treat infections, particularly among immunocompromised individuals, (Ramaswamy et al., 2007; Choi et al., 2018).

The well-documented link between raw milk and milk products and listeriosis outbreaks further amplifies the risk, highlighting the need for robust public health interventions (Dalton et al., 1997; Osman et al., 2016). Moreover, the potential for horizontal gene transfer of antibiotic resistance genes among microbial communities raises broader concerns regarding the dissemination of resistance determinants (Charpentier & Courvalin, 1999; Aminov et al., 2001).

This scenario underscores the necessity for responsible antibiotic use in both clinical and agricultural settings, alongside effective monitoring, surveillance, and intervention strategies to limit the spread of antibiotic-resistant *L. monocytogenes* within the food supply chain (Frieri et al., 2017; Guérin et al., 2014). The safety of milk and dairy products is therefore a critical concern, as the presence of resistant *Listeria* strains threatens both consumer health and the integrity of the dairy industry (Noll et al., 2017; Harshani et al., 2022). Continuous surveillance, strong collaboration between food regulatory authorities and the dairy industry, and the adoption of innovative strategies are crucial to effectively mitigate this threat. Alternative antimicrobials such as bacteriophages, plant extracts, and essential oils have shown potential in controlling resistant strains, while advanced detection methods, including biosensor technologies, enable rapid and accurate identification of contamination (Oussalah et al., 2005; Stasiewicz et al., 2011; Zanini et al., 2014). Predictive analytics further support proactive interventions, helping to prevent outbreaks and safeguard public health (Jahid & Ha, 2012).

The integration of alternative antimicrobials, advanced detection techniques, and predictive tools represents a paradigm shift in combating antibiotic-resistant *L. monocytogenes*, addressing immediate risks while promoting long-term food safety. Collectively, these strategies highlight the importance of One Health approaches, ongoing surveillance, stricter food safety regulations, and consumer education to ensure the safety of milk and milk products and the protection of public health (Buchanan et al., 2017; Li et al., 2023).

Study gaps in antibiotic resistance of *Listeria monocytogenes* in raw milk and milk products

Despite reported antibiotic resistance, critical gaps exist in understanding genetic mechanisms driving resistance development in *L. monocytogenes* in raw milk and milk products. Comprehensive studies exploring these pathways are inadequate for understanding how

L. monocytogenes acquires resistance genes, how these genes are transferred between strains or species, and how resistant strains persist and spread in dairy environments and the food chain.

Limited research on environmental conditions within dairy production facilities has also limited the understanding of the factors that influence the survival and persistence of antibiotic-resistant *L. monocytogenes*. Variables such as temperature fluctuations, sanitation practices, biofilm formation on equipment, and exposure to disinfectants or sub-lethal stressors can affect the ability of resistant strains to survive and spread, but these factors remain poorly characterized. Understanding these factors is essential for insights into resistance development within raw milk environments. Further investigations are necessary in the realm of DNA repair following exposure to such environmental conditions. Enhanced comprehension of this pathogen would advance our understanding of how its adaptation to anaerobiosis contributes to its pathogenesis (Roberts et al., 2020).

There is still a significant lack of knowledge regarding the functions of specific proteins and their contributions to the pathogenesis and virulence of *L. monocytogenes*. Additionally, understanding how this information relates to strains obtained from various environments remains incomplete (Wiktorczyk-Kapischke et al., 2023).

Additional research is essential to quantify the release of antibiotics into agricultural ecosystems and to assess the extent of human exposure not only to antibiotics but also to antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). While much of the focus has been on *L. monocytogenes*, similar concerns apply to other foodborne and environmental bacterial species, including *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus*, and *Enterococcus spp.*, which can acquire and disseminate resistance genes. Comprehensive studies are needed to evaluate the potential risks that antibiotics, ARB, and ARGs in the environment pose to human health and ecological systems, as well as to inform strategies for mitigating the spread of resistance across bacterial populations (Iwu et al., 2020).

In summary, targeted research efforts are needed to address these gaps in understanding antibiotic resistance in *L. monocytogenes* associated with raw milk, offering insights into mechanisms, drivers, and effective mitigation strategies.

CONCLUSIONS

In conclusion, antibiotic-resistant *L. monocytogenes* in raw milk poses significant challenges for public health, food safety, and the dairy industry. Resistance mechanisms, including mobile genetic elements and efflux pumps, lead to multidrug resistance. Environmental stressors like pH, temperature, and salt contribute to antibiotic resistance, highlighting the pathogen's adaptability. Implications include challenges in clinical management, increased healthcare burdens, and strains on public health resources. Horizontal gene transfer underscores the need for comprehensive strategies, including responsible antibiotic use, continuous surveillance, and interventions. Despite progress, gaps persist in understanding genetic mechanisms, environmental factors, and clinical implications of antibiotic resistance in raw milk strains. Continuous surveillance, innovative strategies, and interdisciplinary collaboration are crucial. Alternative antimicrobials, advanced testing, and predictive analytics offer promising avenues. The convergence of these strategies emphasizes prevention, consumer safety, and dairy industry sustainability. Targeted research is essential to fill knowledge gaps and develop effective mitigation strategies for antibiotic-resistant strains in raw milk.

Based on guidance from the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), addressing antibiotic-resistant *L. monocytogenes* in raw milk and milk products requires coordinated, risk-based interventions across the food chain. Joint FAO/WHO expert reports emphasize strengthening integrated surveillance systems to monitor *L. monocytogenes* contamination and antimicrobial resistance trends in dairy production, processing environments, and final products (FAO/WHO, 2020; FAO, 2022). The adoption of a One Health approach is strongly recommended, recognizing the interconnected roles of human health, animal husbandry, food safety, and the environment in the emergence and dissemination of antimicrobial resistance (WHO, 2017; FAO/WHO, 2021). Responsible and regulated use of antibiotics in livestock production, coupled with strict enforcement of good hygienic practices, good manufacturing practices, and Hazard Analysis and Critical Control Point (HACCP) based controls, is critical to reducing selective pressure that drives resistance (FAO/WHO, 2019). Furthermore, FAO/WHO reports advocate pasteurization as a key control measure and stress the importance of consumer risk

communication, particularly for vulnerable populations, regarding the hazards of raw milk consumption. Continued research into alternative antimicrobials, rapid detection tools, and updated quantitative microbial risk assessments is also encouraged to support evidence-based policy development and ensure long-term protection of public health and dairy food safety (FAO/WHO, 2022).

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RESEARCH ARTICLE

Forestry

Influence of climate change on the geographic distribution of forest fires and their potential scale in Sri Lanka

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Abstract: Forest fires pose a significant threat to the conservation of forest resources in Sri Lanka. Forest fire modeling is a novel technique to study and predict the behavior of forest fires. This study utilizes simulation techniques to investigate the relationship between forest fire occurrence and climate change particularly in the dry zone of Sri Lanka. The frequency and duration of phenomena such as sea current oscillation *El Niño* southern oscillation (ENSO) change due to climate change, can create extreme dry conditions that increase the risk of intensified fires, especially in the dry zone of Sri Lanka. To assess fire vulnerability under prevailing weather conditions and projected climate change scenarios, the FConstMTT simulation algorithm was used with 1,000 iterations per scenario. In this analysis forest types across Sri Lanka were classified into six classes based on elevation, rainfall, and fuel characteristics. Customized fuel models were created using BehavePlus and spatial data was processed through the ArcFuel extension of ArcGIS. The results indicate that under projected climate scenarios, fire hazards in the dry zone particularly in the districts of Mullaitivu, Monaragala, Anuradhapura, Polonnaruwa, and Badulla, are likely to worsen in the future. In the climate change scenarios, Monaragala district accounted for approximately 53% of total fire damage, with Badulla district showing a significant baseline vulnerability. Anuradhapura district also showed significant levels of fire vulnerability under the projected climate change conditions. Based on fire frequency and intensity, the 25 administrative districts in Sri Lanka were categorized into high, moderate, and low-risk areas. Forest types such as dry monsoon forests, shrublands, open and

sparse forests, and savannahs are particularly vulnerable under the predicted climate change scenarios.

Keywords: FConstMTT, fire simulation, dry monsoon forest, ArcFuel, climate change

INTRODUCTION

The world has been experiencing an increase in forest fires due to drier climates. Extreme drought conditions, which are one of the main effects of climate change (Groves et al., 2012) have affected the eruption and spread of forest fires.

In Sri Lanka, forest fire damages are very high in the months between July and October. This period falls within the southwest monsoon season where the dry and intermediate zones of Sri Lanka face dry winds that carry less moisture content favoring fire ignition. Further, less rain and prevailing high atmospheric temperatures in this period make the fuel load drier, creating an optimum condition for forest fires (Ariyadasa, 2002). It is reported that dry monsoon forests and monoculture homogenous forests such as forest plantations (pine) have high dry fuel loads ranging from 35 to 42 tons/ha (Ariyadasa, 2002), compared to other forest types in Sri Lanka.

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When considering forest fires in Sri Lanka, temperature, precipitation, relative humidity, and wind are the four main weather variables that control fires (Hapuarachchi et al., 2015). It has been reported that there are no natural causes such as lightning, heavy winds, or volcanic eruptions for forest fires in Sri Lanka. Anthropogenic factors such as negligence, slash and burn cultivation, and animal husbandry cause forest fires. In dry zone forests weather factors and human impacts are the main contributors to forest fire damages. (Bandara, 2019). According to the Forest Department, approximately 100 ha to 2000 ha of forest and forest plantations are destroyed due to forest fires annually in Sri Lanka showing an increasing trend in the occurrence and extent of fire damage over time. Figure 2 illustrates the extent of the area damaged by forest fires from 2002 to 2019 (source: Forest Department). Usually, forest fire damages are high in pine forest plantations (Pathirana, 1998). However, in recent years' forest fire damages in the dry monsoon forests have also increased significantly.

There are several forest simulation models developed by the United States Department of Agriculture (USDA) to predict risk areas for wildfire under prevailing conditions and potential climate change scenarios. These models are Fsim and FConstMTT, FSpro and FlamMap (Scott et al., 2013). In Sri Lanka, investigations on modeling the risk of forest fires have not been done before. As some forest types in Sri Lanka are comparable to the forest types in Brazil and the USA, studies done in those countries are applicable here. The study conducted by Guedes et al. (2019) in the Paraiba do Sul River Valley in Brazil to identify the vulnerability of small forest patches for forest fires, using the FConstMTT fire simulation algorithm can be taken as an example.

The objectives of our study were to identify the relationship between climate change and forest fire occurrence in Sri Lanka, to develop a fire model platform capable of simulating a library of plausible fires under prevailing and projected conditions, and to identify and map forest fire vulnerable areas in Sri Lanka.

MATERIALS AND METHODS

Study area

The total land area of Sri Lanka was selected as the study area for this study. The study covered all forest types managed under the Department of Forest Conservation (DFC) and the Department of Wildlife Conservation (DWC) (Figure 1).

Data collection

Weather data including rainfall, temperature, relative humidity, wind speed and wind direction were obtained from the Meteorological Department of Sri Lanka. Data pertaining to the extent of forest fire damage in different forest types, forest fuel loads, canopy height, location of fire ignition, perimeter of damage, value of fire damage, fire control efforts, and the distance to the nearest village or residential area from the location of fire were obtained from the DFC. All weather and fire data were collected over a 20-year period, from 2000 to 2020. Data on forest canopy cover, canopy height and canopy base height, were taken from the National Forest Inventory data collected by the DFC from 2017 to 2018.

As data on canopy bulk density and fuel moisture content were not available in Sri Lanka for the relevant forest types concerned, this information was sourced from foreign studies conducted in Brazil (White et al., 2012), Netherlands (Oswald et al., 2017), USA (Scott et al., 2005) and India (Mondal et al., 2016). Since field level data for fuel moisture content was unavailable for herbaceous and woody vegetation at 1 hr, 10 hrs and 100 hrs time lag periods, relevant data were taken from the above published studies.

Preparation of forest fire vulnerable area maps

Vulnerable areas for forest fire were mapped using ArcGIS geographic information system, and vulnerable forest areas were identified in a sequential manner.

Identification of climate change impact on forest fire occurrence and distribution

Since dry weather reduces fuel moisture content, wind speeds were used for fire simulations.

Preparation of landscape files (LCP)

A digital elevation model of Sri Lanka obtained from the DFC was used to derive raster layers of elevation, slope, and aspect, using ArcGIS Pro. An Excel spreadsheet was prepared for canopy cover, canopy bulk density, canopy base height, and fuel model, listing the magnitude for each parameter for each forest type. Data on canopy cover, canopy base height, and canopy height were taken from the National Forest Inventory data collected in 2017 and 2018. Custom fuel models were prepared to represent each forest type in Sri Lanka.

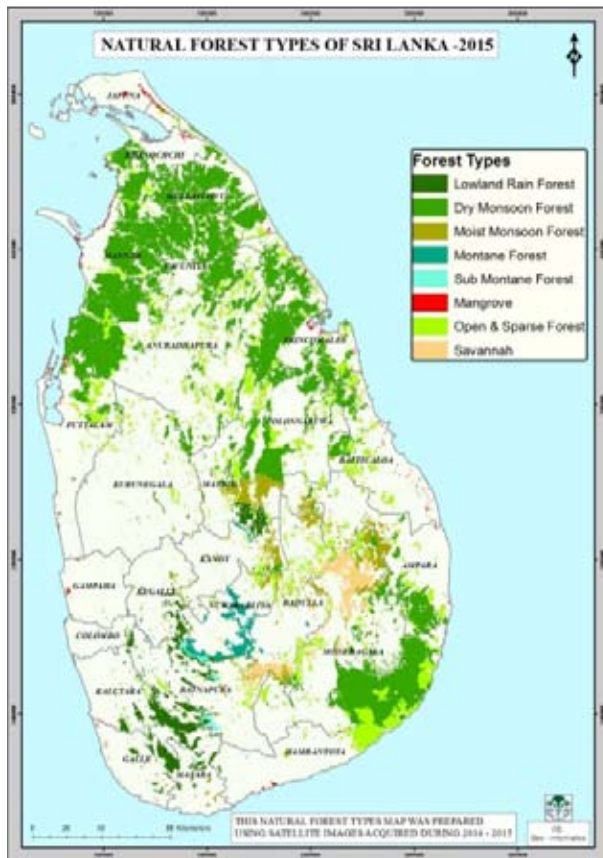


Figure 1: Forest cover map of Sri Lanka based on the 2015 forest inventory data (Source: Department of Forest Conservation, Sri Lanka).

Custom fuel models

Custom fuel models were derived based on Scott et al. (2005), White et al. (2012) and Oswald et al. (2017). Sri Lankan forest types were categorized based on elevation and rainfall pattern as (i) dry monsoon forest (ii) forest plantation (iii) high elevation wet forest (iv) low elevation wet forest (v) scrubland and (vi) open & sparse forests. The custom fuel models were numbered from 30 to 36 except number 33. The 'BehavePlus' computer application was used to develop these custom fuel models. It has been reported that the surface fuel load of a tropical forest ranges from an average of 12.5 t/ha to 40 t/ha (Ariyadasa, 2002) while forest plantations have an average of 10 t/ha to 15 t/ha. The surface fuel load in a low elevation forest ranged from 13 t/ha to 17 t/ha while in scrubland and grassland it averaged 9.18 t/ha, and 3.7 t/ha, respectively. The fuel bed depth in wet forests

and dry forests was measured at 0.6 - 0.9 m and 0.45 m, respectively, while in forest plantation it was 0.3 m.

Fire simulation

Fire simulation was conducted using FConstMTT fire simulation executive files, which uses the minimum travel time (MTT) algorithm (Finney, 2002). This algorithm is commonly used to model fire spread and analyze burn probability in the United States Forest Service. The FConstMTT wildfire simulation algorithm uses mandatory switches as inputs for the simulation process including Landscape Files in .lcp format, resolution of the simulations (cell size), grid distance unit (either in meters or feet), wind speed (miles per hour), wind direction, number of fire iterations, fuel moisture files in fms format, FConstMTT output file name and locations, and duration of the fire in hours. Since the dry and intermediate zones face dry wind conditions during the southwest (SW) monsoon, the wind direction was considered as of 225 with a wind speed of 4 miles per hour based on data from the Meteorological Department of Sri Lanka. Data for fuel moisture content was taken from the study done by White et al., (2012) in the Itabaiana National Park, Brazil. Based on the simulation results, districts were categorized by the extent of damage, number of ignitions, and the extent of damage per ignition (Figure 3).

Justification for using Brazilian studies for fire simulation

- Both Sri Lanka and Brazil have tropical and subtropical forest ecosystems located near the equator, sharing similar climate characteristics such as high temperatures, seasonally variable rainfall, and relatively consistent humidity patterns that influence vegetation moisture dynamics.
- Cross regional data application is an accepted interim practice in ecological modeling, especially in regions where empirical data collection is limited. Previous studies (e.g., Guedes et al., 2019) have demonstrated the validity of using data from ecologically analogous regions to model fire behavior in data-scarce environments. While recognizing the inherent limitations of using proxy data, this study selected Brazilian fuel moisture content values due to ecological comparability and the urgent need to assess fire risk in Sri Lanka under climate change. The results offer a preliminary yet valuable baseline for understanding potential fire behavior, pending future field-based calibration and validation efforts in local forest ecosystems.

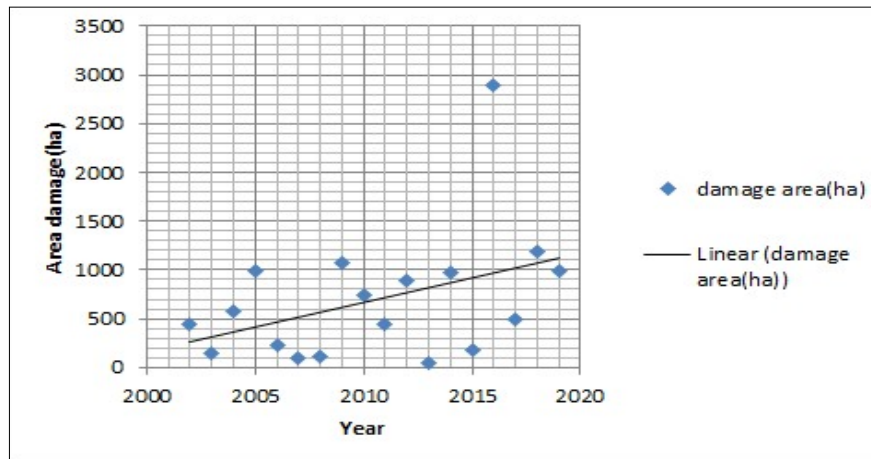


Figure 2: Extent of area damaged by forest fires from 2002 to 2019 (Source: Department of Forest Conservation, Sri Lanka).

Data for canopy bulk density were based on studies done in some forests in Brazil, the Netherlands, and the United States that are comparable to forests in Sri Lanka. These data were used for the analysis after some modifications together with the data obtained from the National Forest Inventory, which were done on Sri Lankan Forest types.

To identify the impact of climate change on forest fires, a simulation was conducted with 1000 iterations under dry weather conditions and low fuel moisture content (with 1hrs, 10 hrs and 100 hrs time lag periods, for both woody and herbaceous vegetation). These fire locations were determined based on an ignition grid (Figure 3) created using the kernel density tool in ArcGIS pro with historical fire data downloaded from the MODIS satellite platform and weather data.

The output from the fire simulation process for dry weather conditions was compared to scenarios to determine the impact of climate change on forest fire occurrence and spread in Sri Lanka. The simulated fire results were overlaid with the forest cover layer to identify the most affected forest types by forest fires under projected climate change scenarios. Since 1000 fire iterations would not be sufficient to saturate the forested landscape to obtain a precise value, the burn probability was calculated using equation 1 (Guedes et al., 2019) to calculate it numerically.

$$\text{Burn probability} = \frac{\text{Total Fire damage area(ha)}}{\text{Total forest land areas(ha)}} \quad \dots(01)$$

Based on equation 1, the burn probability for the entire forest area of Sri Lanka was calculated under the baseline and projected climate change scenarios for each district and forest type category. Results were visualized on a bar chart to get a clear understanding on the change that would occur under both situations.

Weather data for the past twenty years for temperature, wind speed and direction, and relative humidity in all districts were plotted using Excel to identify the weather variability in each district, particularly the districts falling within the dry zone and the intermediate zone. Statistical analysis was done to verify the significance of variations in temperature, rainfall and relative humidity.

The fire simulation results were compared with weather variables to identify areas prone to increased forest fire occurrence under impending impacts of climate change. Results were prepared using ArcGIS maps, graphs, and tables. Ground level fire data obtained from Badulla, Puttalam, Anuradhapura and Monaragala districts were used to validate the results. These data were converted to percentage values based on the total damaged area and compared with the values obtained for the two scenarios.

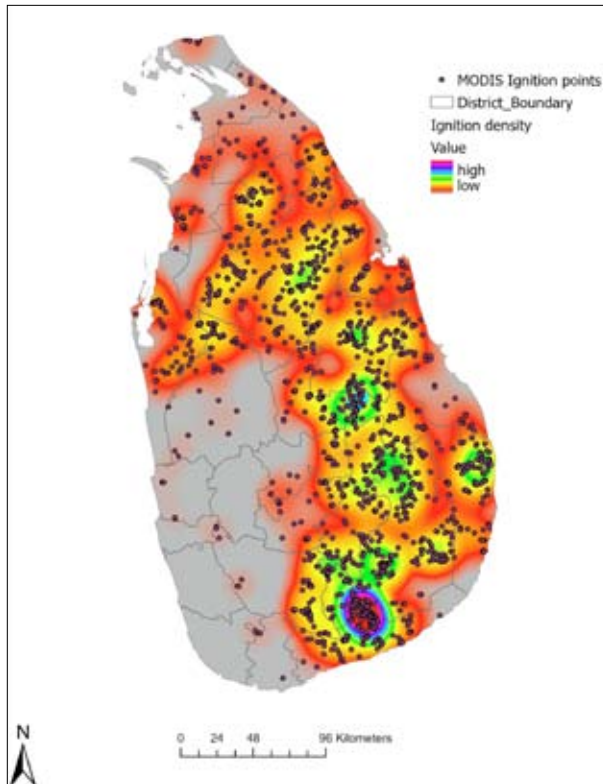


Figure 3: Ignition density map used to make an ascii grid file to be used as an input (as one of the switches in the simulation model) on FConstMTT fire simulation process.

RESULTS AND DISCUSSION

The landscape file representing Sri Lanka is shown in Figure 4. Historical fire data indicate that most forest fires fall under the surface fire category, with very rare occurrences of crown fires. In this context elevation, slope, aspect and fuel models play a more significant role in fire behavior than the canopy characteristics. Forests in the east, north, southeast and northeastern parts with high dry burnable fuel loads are categorized as the forest fire vulnerable areas in Sri Lanka. Figure 4 shows that the landscape file (LCP) derived from ArcFuel with eight raster layers including elevation, slope, aspect, fuel model, canopy cover, canopy height, canopy base height and canopy bulk density from the top left to the bottom right. The LCP reveals that most forest plantations, dry monsoon forests, and sub montane forests have high fuel loads (13-30 mt/ha) that become more inflammable under dry weather conditions. The fuel moisture content decreases over time periods of one hour, 10 h, and 100 h in woody and herbaceous vegetation.

Fire simulations were done under baseline and projected climate change scenarios. Figure 5 summarizes the simulation results showing that compared to the baseline condition, parameters such as fire intensity line and area damaged by fire increased in the predicted scenario for all districts. In Monaragala, Ampara and Anuradhapura districts, the projected climate change scenario significantly increased fire damage to forests (Figure 6). As shown in Figure 6 (left), the number of fires increased from 375 and 409 under the baseline condition and climate change scenario, respectively. For districts in Figure 6 (left), these numbers were as 316 and 324.

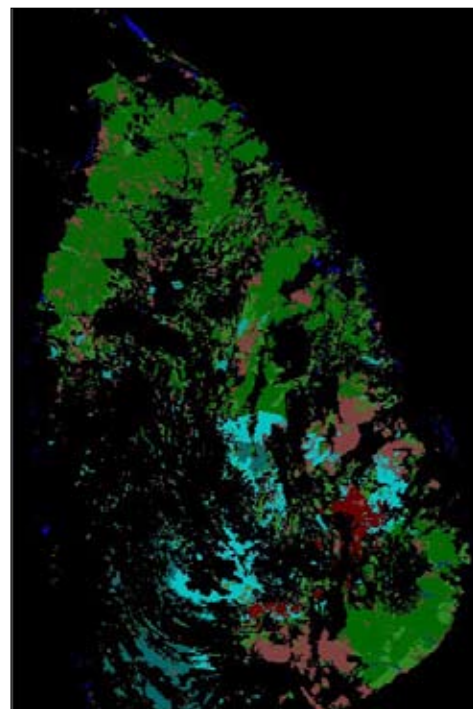


Figure 4: Landscape file map with the eight themes elevation, slope, aspect, fuel model, canopy cover, canopy height, canopy base height and canopy bulk density (raster layers).

Monaragala district had the highest simulated forest fire damage under baseline conditions as well as the projected climate change scenario, which were about 726 acres and 387 acres, respectively. For the northcentral and north eastern parts of the country, the simulated fire damage was 247 acres and 518 acres, respectively, under both conditions (Figure 6 (left)).

The fire intensity level (FIL) is the rate of heat energy released per unit length of the fire front (Byram, 1959;

Scott, 2012), which depends on the rate of spread (ROS) of the flame, forest fuel type, fuel load, and forest fuel moisture content (McEvoy et al., 2020). In this study, the fire intensity level increased, but not significantly, with the projected climate change scenarios compared to the baseline condition. This means that the intensity of fire damage due to fire occurrence has increased making the fire more hazardous for forest resources under the impact of climate change.

In the Anuradhapura district, the FIL increased slightly under the simulated climate change scenarios (Figure 7). However, despite this slight increase, the ROS and flame length increased significantly in the Anuradhapura district. In the Monaragala, Badulla and Ratnapura districts, FIL3 was slightly higher under climate change conditions when compared to the baseline level (Figure 8).

However, these figures are considerably higher in average fire intensity level (FIL) and FIL3 under climate change conditions than the baseline level (Figure, 8) but the rate of spread (ROS) of the fire, flame length (FL) and heat release per unit area, which determine the fire severity, smoke production and crown scorch height (Scott, 2012) increased significantly under the projected climate change scenarios when compared to the baseline condition. This means that the fire hazard has increased significantly under the climate change scenario indicating that forest resources are facing a serious threat. The vulnerability of forest resources in the country will be increased in the future under drier and hotter weather conditions.

From 2006 to 2009 there were no records reporting damages to the dry monsoon forest due to forest fires (Figure 9). During this period, forest plantations were the most vulnerable forest category. On the other hand, after 2009, a significant increase in forest fire damage was recorded in the dry monsoon forests. Burn probability was calculated from ratios between the total burned area and total forested area for a particular district. As shown in Figure 10, Badulla district has the highest burn probability under the baseline conditions. This means that the future risk for fire vulnerability under climate change scenarios in the Badulla district is significantly reduced. Due to the probability of rainfall occurrence increasing and changing wind characteristics in the Badulla district, the fuel moisture content will increase. Therefore, the burn probability in the Badulla district will be drastically reduced with the modeled climate change scenario.

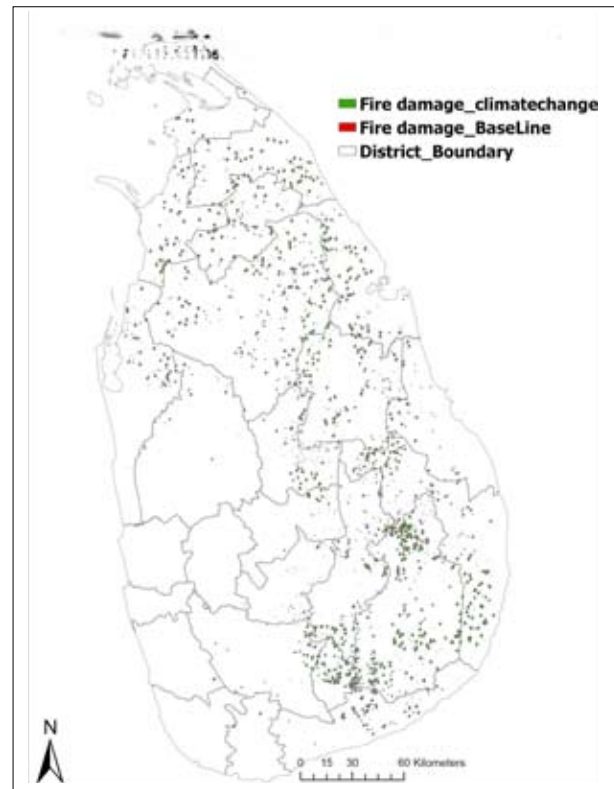


Figure 5: Fire simulation output result under the projected climate change and baseline scenario (Total simulated damages for the 1000 fire iterations are 293,758 acres and 56,617 acres on both projected climate change and baseline scenario.)

Forests in the Monaragala, Anuradhapura and Mullaitivu districts have become the most vulnerable forests for forest fires under impending climate change scenarios. In the Matale and Ampara districts, the burn probability is less severe under the climate change scenario. Currently the Matale district is considered a moderately forest fire vulnerable district. It is shown that Matale has become a less vulnerable district for forest fires under the future climate change scenario. When the burn probability of different forest types is compared (Figure 11), dry monsoon forests show the highest burn probability followed by open and sparse forests, and forest plantations under baseline conditions, while open and sparse forests show the highest level of burn probability under projected climate change scenarios. Thus, it can be concluded that dry monsoon forests, and open and sparse forests show the highest vulnerability for forest fires in impending climate change events (Figure 11). Based on the baseline scenario, the Monaragala district shows the highest fire damage.

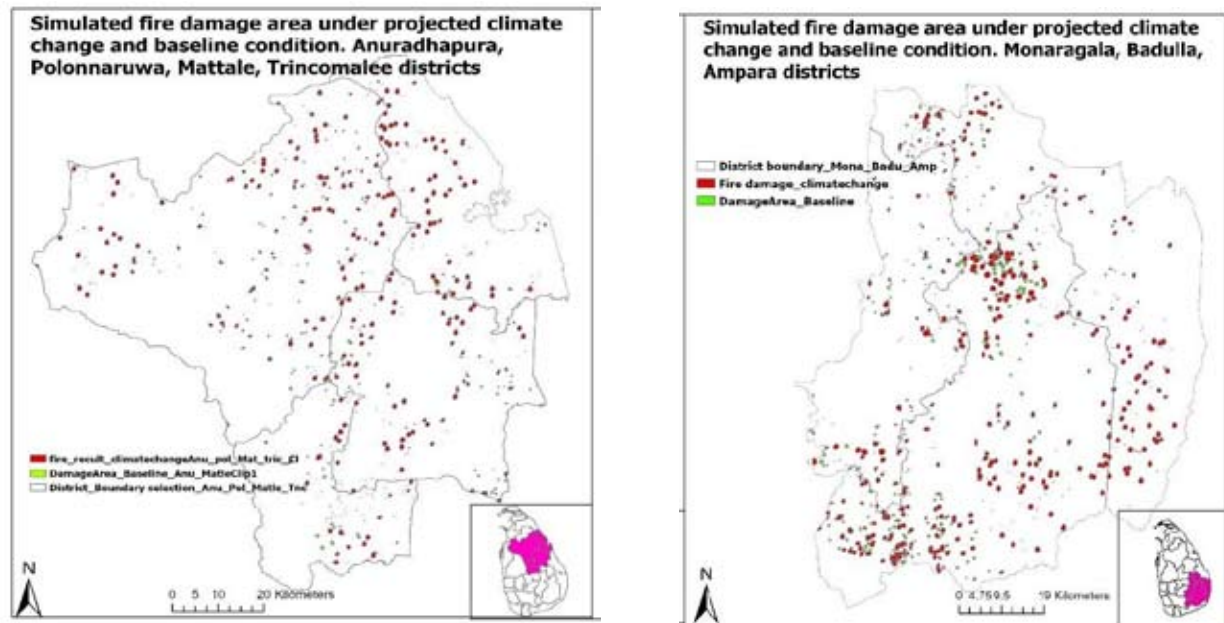


Figure 6: Forest area damaged by simulated fire under baseline conditions and the projected climate change scenario in (left) Anuradhapura, Polonnaruwa, Matale and Trincomalee districts and (right) Monaragala, Badulla and Ampara districts.

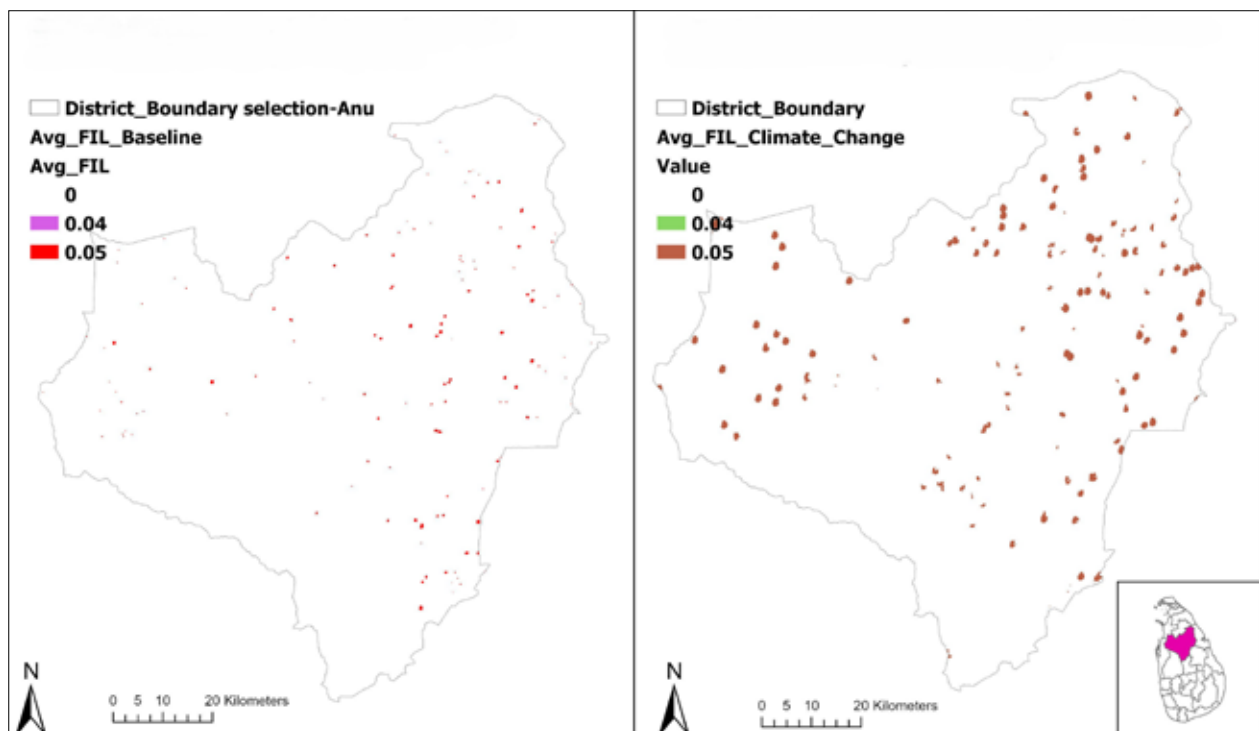


Figure 7: Average Fire intensity level (FIL) under (left) the baseline condition and (right) projected climate change scenario in the Anuradhapura district.

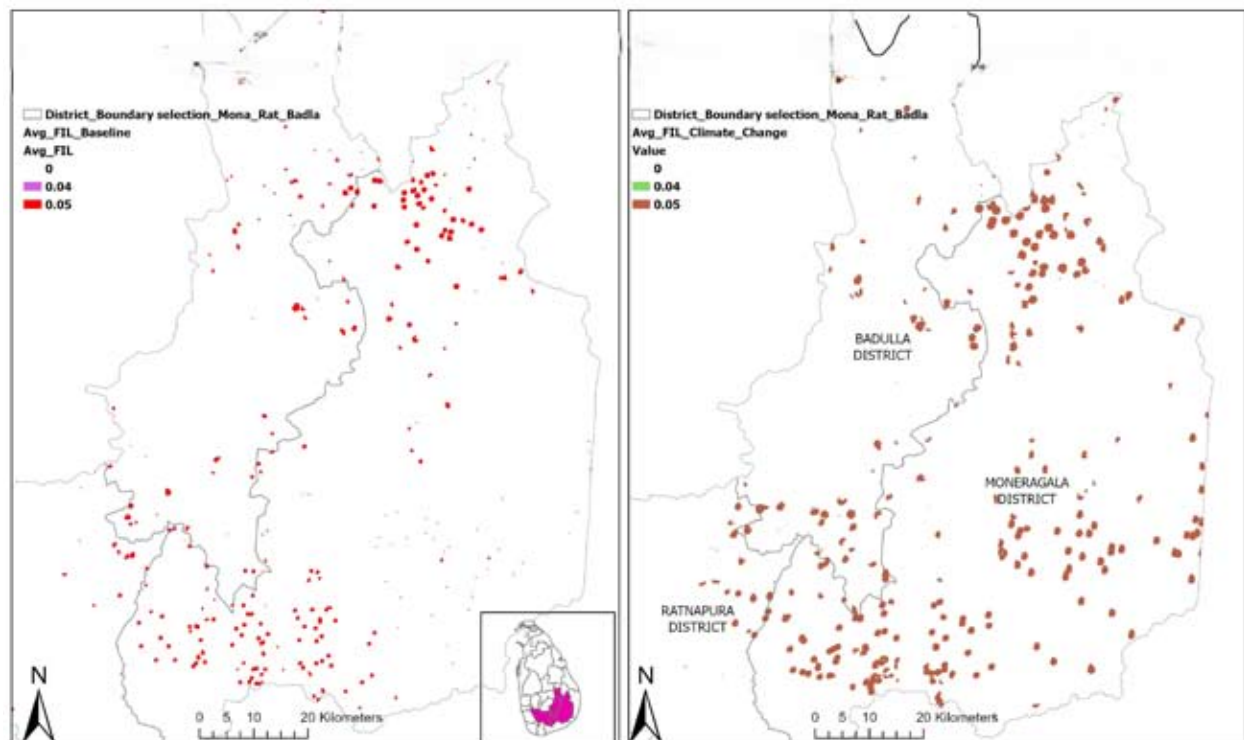


Figure 8: Fire intensity level 3 (FIL3) under (left) the baseline condition and (right) projected climate change scenario in Monaragala, Badulla and Ratnapura districts

Risk areas were categorized as low, moderate and high forest fire areas, based on the total forest area damaged by fire, number of fire ignitions, and the area damaged per one fire incidence (Figure 12). The threshold level was decided based on the total extent of the damaged area and the number of fire ignitions. It was found that under projected climate change scenarios, the severity of fire damage and fire hazard increased drastically in the Monaragala district.

When the projected climate change scenario is considered, Figure 12 (left) shows that Monaragala, Ampara and Anuradhapura districts are at high risk based on the total damaged area while Figure 12 (right) shows that Monaragala, Ampara, Anuradhapura, Polonnaruwa, Trincomalee, and Mullaitivu districts are at high risk based on the number of fire ignitions.

The total simulated fire damage area was approximately 293,758 acres and the Monaragala district accounted for 41% of the damage. Assessment of data in the past few years shows that the dry monsoon forest has the highest forest fire damage. Figure 13 (left) shows that dry monsoon and open and spare forests are

prone to the highest forest fire damage. Forest types in the districts in Figure 13 (right), including dry monsoon forests, scrubland and savannah, open and sparse forest and forest plantations reported the highest vulnerability for forest fire damages in descending order. In this study, the simulated baseline results were validated with actual field data obtained from the DFC.

Field data on the extent of forest fire damage for districts in this study obtained for the years 2016 and 2018 were used for validation. These two years were selected because they reported the highest forest fire damages in the country compared to other years. The fire results obtained in this study by simulation under the baseline conditions were clearly validated with the field level data. In Sri Lanka, one of the main causes of forest fire damage is human involvement as there are no natural causes for forest fires such as heavy winds and lightning. The forest fire season in Sri Lanka is from July to September. Some of the causes of forest fires are careless behavior by tourists in forested areas, intentional or accidental ignition of fires to clear land for chena cultivation to chase animals away, or to create new pasture land for cattle herding.

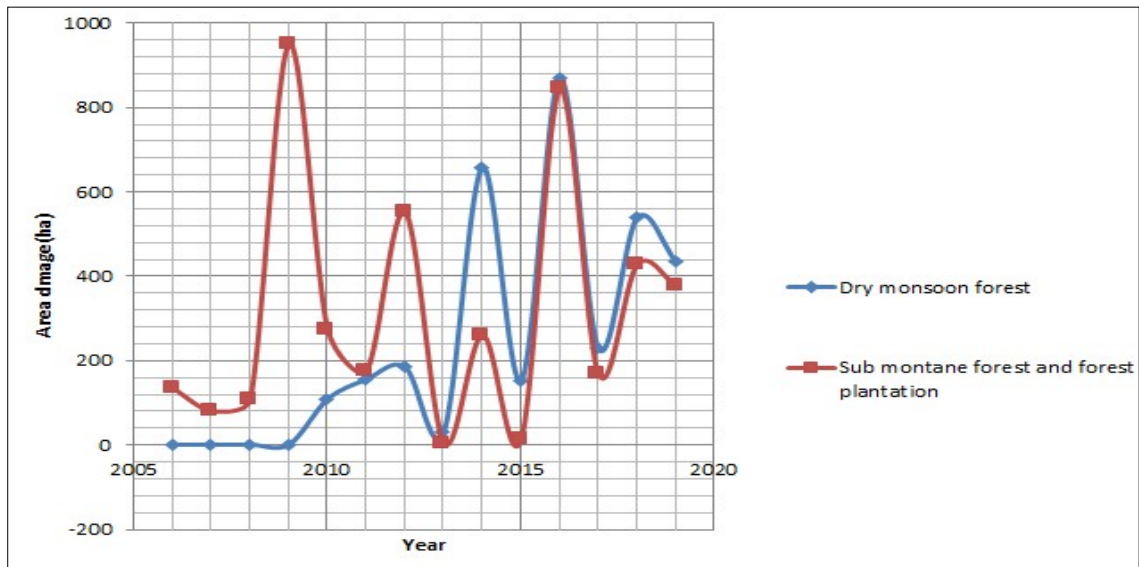


Figure 9: Forest fire damages from 2006 to 2019 in the dry monsoon forest and forest plantation and sub- montane forest (Source: Department of Forest Conservation)

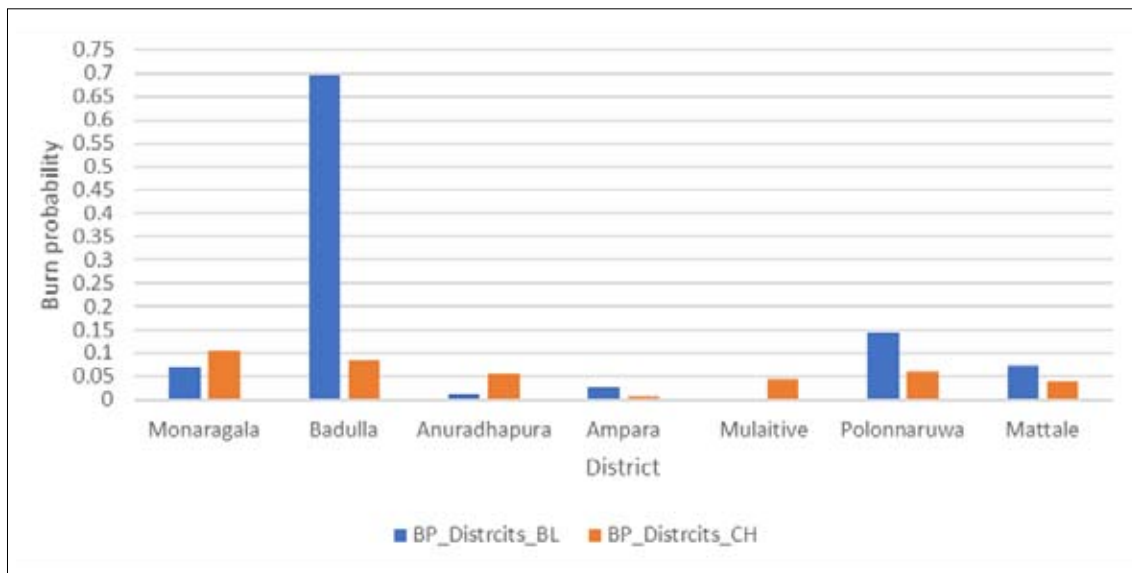


Figure 10: Burn probability under baseline scenario (BL) and projected climate change scenario (CH) in Monaragala, Badulla, Anuradhapura, Ampara, Mullaitivu, Polonnaruwa and Mattale districts

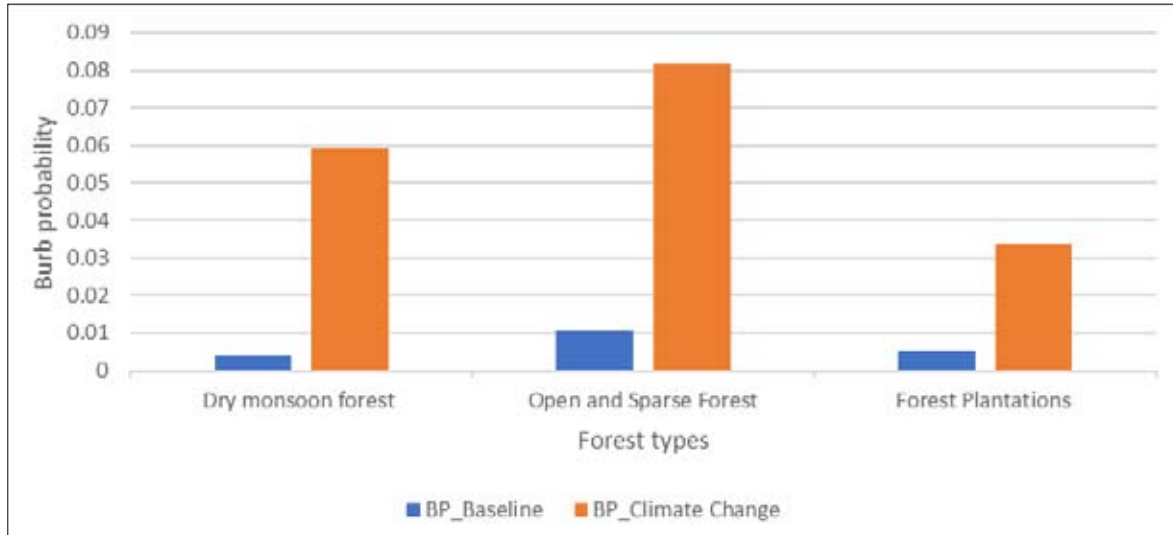


Figure 11: Burn Probability of simulated fire under baseline condition and projected climate change scenario for different forest types in the study.

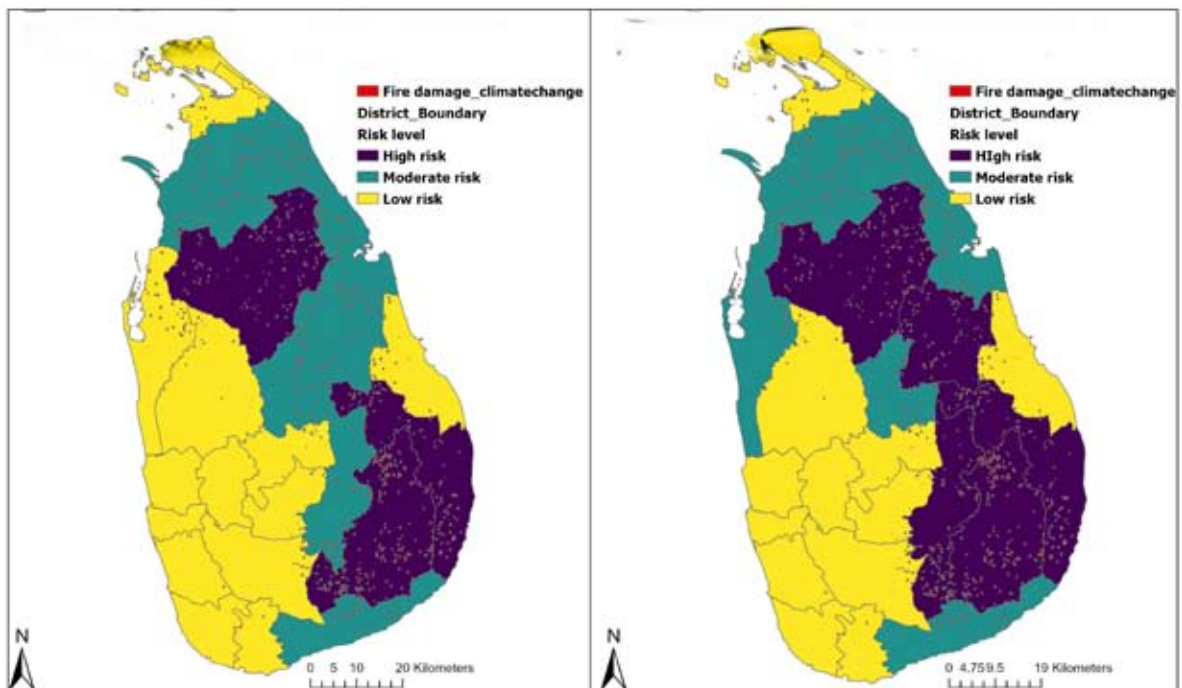


Figure 12: Risk area assessment in fire simulation for the country under the projected climate change scenario based on (left) the total area damaged by forest fires and (right) the number of fire ignitions.

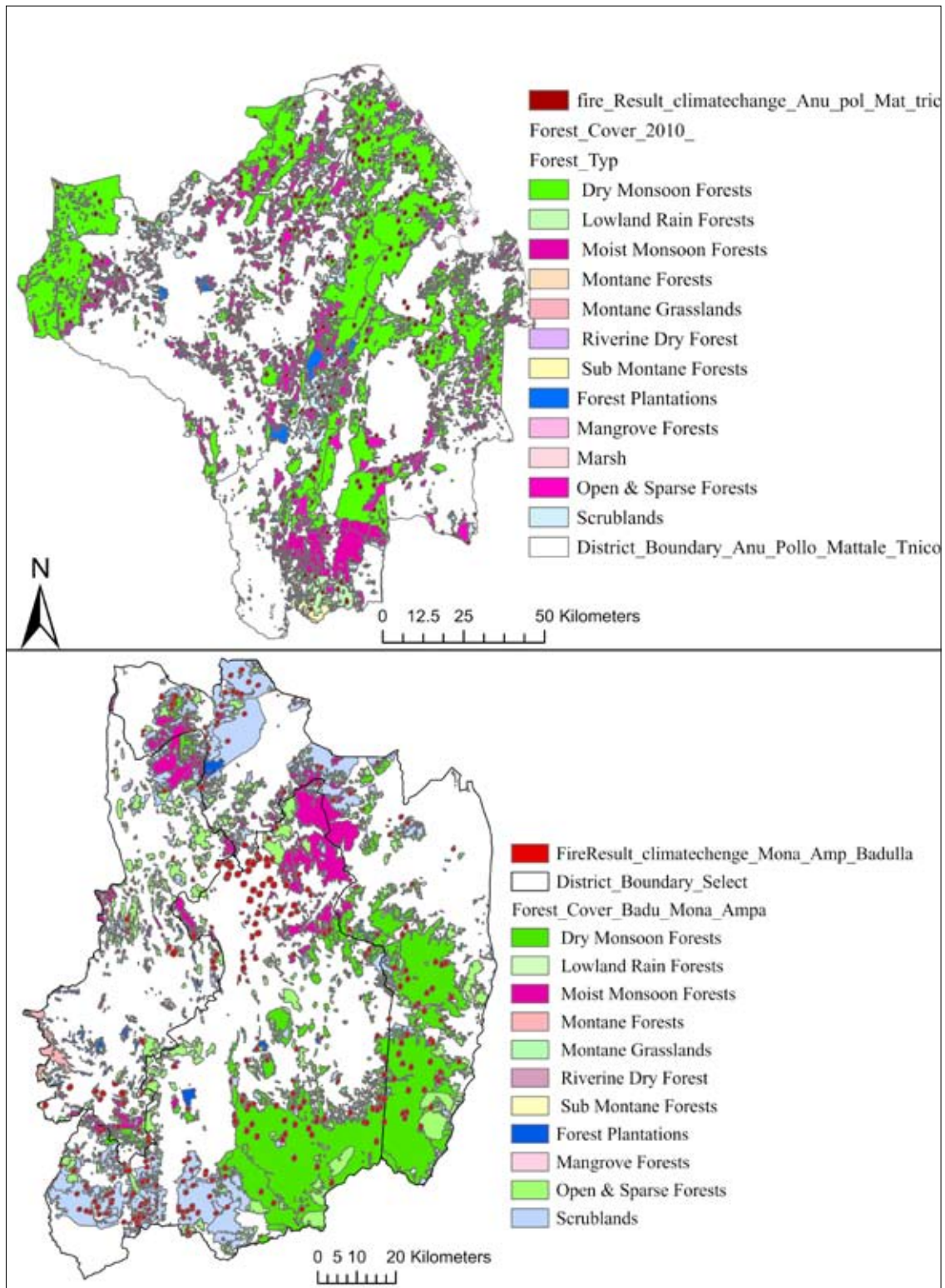


Figure 13: Risk forest types for forest fire damages under the projected climate change scenario (upper) Anuradhapura, Matale, Polonnaruwa and Trincomalee districts (lower) Monaragala, Badulla and Ampara districts.

It was noted that most forest fire ignitions start from open and sparse forests and spread to dry monsoon forest types, which are often located adjacent to them. This is the main reason for dry monsoon forests having the highest forest fire damages under both climate change and baseline scenarios. Analysis of historical fire occurrence data indicates that many ignition locations are associated with open and sparse forest areas. These vegetation types are often located adjacent to dry monsoon forests, facilitating the spread of surface fires into these forests and resulting in higher fire damage levels in dry monsoon forest ecosystems under both baseline and projected climate change scenarios. Previous studies have shown that forest fire ignition in Sri Lanka is strongly associated with human activities such as chena cultivation, tourism-related negligence and livestock grazing practices (Ariyadasa, 2002; Bandara et al., 2019). Similar patterns have been observed in this study where many ignition points occur in accessible forest landscapes. This suggests that human accessibility may contribute to fire ignition in these areas, although the present study primarily focuses on fire spread dynamics rather than ignition mechanisms.

Fire simulation can also be used to evaluate how fire is expected to spread across the landscape including the likelihood and intensity of expected fires (McWethy et al., 2018). The projected increase in the number of simulated fire occurrences under the climate change scenario does not represent an increase in human ignition sources. In the simulation framework used in this study, ignition locations were kept constant while climatic variables influencing fire behavior such as fuel moisture content, wind speed and burning duration were modified to represent projected climate conditions. Under drier and warmer conditions, fire spread characteristics such as rate of spread, flame length and burn probability increase significantly. Therefore, once ignition occurs, fires are more likely to propagate across larger areas, resulting in a higher number of simulated fire events and increased burned area under the projected climate change scenario. The study reported a 3.8-fold increase in fire damaged areas over the whole island under the projected climate change scenario. In this analysis, the burning period of the baseline condition was set to 180 minutes, which aligned with the 2016 fire information obtained from the DFC and the burn period of the projected climate change scenario was set to 210 minutes with a slight increase in wind speed. Burn probability in the Monaragala, Anuradhapura and Mullaitivu districts increased significantly under the projected climate change scenario.

In addition to the increase in forest fire hazards, the fire size also increased significantly in almost all the districts in the dry zone of Sri Lanka. The distribution of fire ignitions is influenced by several causes such as climate factors (rainfall and temperature) and vegetation type that determine the continuity of fire, significance, fuel characteristics, and population density and level of human activities (Holz et al., 2012). Hot, dry and windy conditions accelerate large fires in dry monsoon forests, scrublands, forest plantations, including savannah forests that can be found in Badulla and Monaragala districts. It was evident that warming and drying trends that are projected to continue in the future under the climate change scenario, would affect a major part of the dry zone to promote fires in its fuel-rich forests, such as dry monsoon forests and forest plantations.

CONCLUSIONS

Forest fire modeling conducted in this study aimed to explore the behavior of forest fires in the dry and intermediate zones of Sri Lanka under potential climate change scenarios. The results have shown that forests in these zones are at a significant risk of increased fire damage in the future due to climate change.

Dry monsoon forests are the most vulnerable to forest fires under projected climate change conditions, followed by open and sparse forests and scrublands. Monaragala and Anuradhapura districts show a higher vulnerability compared to other districts. Forests within and around places where there are areas with high human impact are more prone to fire, with ignition usually beginning near roads and spreading into forested areas. In such instances, fire spreads initially affect village-type forests such as open and sparse forests before spreading into nearby dry forests, scrublands and forest plantations.

This study reveals a significant increase in burn probability, damaged area and fire intensity under the projected climate change scenario. This suggests that the dry zone will be threatened by more fire hazards in the future necessitating more focused forest fire management strategies to address this situation.

This study provides valuable insights for identifying high-risk areas for forest fires in the country. Effective forest fire management strategies should include guarding high-risk ignition locations, maintaining existing fire belts, establishing new fire belts, managing fuel in vulnerable forests and plantations at risk, and establishing

fire protection units by the communities affected by forest fires. Findings generated by this research study can be used to anticipate, prevent, and control forest fires more efficiently and develop more effective forest fire management strategies aimed at mitigating forest fire damage.

Acknowledgement

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RESEARCH ARTICLE

Entomology

Insecticidal, repellent and histopathological effects of selected botanical extracts on the rice leaf folder (*Cnaphalocrocis medinalis*)

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Abstract: The rice leaf folder (*Cnaphalocrocis medinalis*) is a key pest in Asian rice fields that causes significant yield loss. This study evaluated the insecticidal potential of 10 methanolic botanical extracts from *Azadirachta indica*, *Calotropis* sp., *Ricinus communis*, *Justicia adhatoda*, *Aristolochia bracteolata*, *Cassia auriculata*, *Acorus calamus*, *Nicotiana tabacum*, *Pongamia pinnata*, and *Lawsonia inermis*. The extracts were tested at concentrations of 1%, 3%, 5%, 10%, 15% under laboratory conditions. Larval mortality was recorded at 24, 48, and 72 h and repellency was assessed using the filter paper disc method. Histopathological changes were examined in thoracic muscles, gastrointestinal tracts, and Malpighian tubules. The overall findings demonstrated that *A. indica* and *J. adhatoda* consistently caused the most larval mortality across all concentrations and time intervals, with one-way ANOVA indicating highly significant effects at 24, 48, and 72 h ($p < 0.001$). Probit analysis established lethal concentration values with an LC_{50} value of 28.5 ppm for *A. indica*, which is consistent with the previously reported efficacy of neem-based botanicals against rice leaf folder larvae. *A. indica* and *P. pinnata* showed 100% repellent activity while *C. auriculata* and *L. inermis* were the least effective (<30%). *A. indica* extracts caused intense histopathological effects resulting in significant tissue alterations with an H-score ranging from 255 to 285 while a lower H-score was recorded for the control. Overall, the selected botanical extracts caused significant larval mortality and pronounced histopathological alterations in *C. medinalis* larvae.

Keywords: Botanical extracts, *Cnaphalocrocis medinalis*, eco-friendly pest control, larval mortality

INTRODUCTION

Rice is a way of life and a cultural heritage that is essential in most Asian countries (Samarasekara et al., 2018). In Sri Lanka, paddy cultivation occupies ~29% of the total cultivated area and is the main source of food security, feeding most people and providing vital income for many farming families. According to statistics from 2022-2023, the annual average yield was 3.68 million tons (Department of Agriculture, Sri Lanka, 2025). Although Sri Lanka is able to cultivate rice intensively due to its favourable weather and climate, recurring insect pests are a source of biotic stress affecting both yield and quality of rice (Deutsch et al., 2018; Jena et al., 2018).

One such key pest is the rice leaf folder (*Cnaphalocrocis medinalis*), a common lepidopteran pest that has become widespread in Asia. The infective stage is the larva, which is able to roll the leaf and scrape off the green mesophyll tissues affecting photosynthesis, retarding plant growth and causing significant damage to yield (Zhuang et al., 2021; Bairwa et al., 2023).

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When leaf folders appear, farmers resort to synthetic insecticides as their main form of protection. However, the instantaneous results of chemical insecticides come with long-term consequences to the environment such as the presence of persistent chemicals in the soil and water, elimination of other helpful insects such as pollinators, and potential health concerns to people (Kampermann et al., 2023; Kashyap et al., 2024; Shareefdeen & Elkamel, 2024). Additionally, using synthetic chemicals in farming leads to the development of resistance in pests so that common pest treatments cease to function. This highlights the urgent need for environmentally safe, flexible and practical pest control methods (Nile et al., 2019; Fenibo et al., 2022).

The use of botanical insecticides shows promise in pest control. These extracts, derived from plants, have a broad range of effects, including insecticidal, ovicidal, repellent, growth regulatory and antifeedant actions. Studies have shown that plant extracts that include bioactive phytochemicals like alkaloids, flavonoids, terpenoids, tannins and saponins have an important role in controlling pests (Damalas & Koutroubas, 2020; Ahmed et al., 2022). More importantly they are biodegradable, safe for non-target organisms and usually slow down the development of resistance. In addition, using native or traditional plants makes their application more practical and economical for smallholder farmers (Monsreal-Ceballos et al., 2018; Ngegba et al., 2022).

However, the current literature lacks consistent and in-depth comparative analysis. Most studies consider a limited set of plant species or pests with little replication across geographical areas or environmental settings. Incompatible results on efficacy are not uncommon, often attributed to variations in extraction procedures, solvents, and bioassay conditions, which prevent the generalisation of the results (Nikolaou et al., 2021; Assadpour et al., 2023). Moreover, although larva mortality rates are widely used parameters, there are few studies addressing the physiological or histopathological alterations that could explain the described phenomenon. To address this gap, the present study tested the effectiveness of different botanical extracts to control *C. medinalis* larvae under laboratory conditions. The study also determines the bioactive phytochemicals present in the most effective extracts and examine how they affect larval tissue. By linking the activity of botanical insecticides with insect physiology through tissue studies, this research aims to contribute to eco-friendly pest control and a better understanding of their mode of action. The research results will help to promote the use of botanicals to control pests as part of eco-friendly rice farming in Sri Lanka.

Our hypothesis is that methanolic extracts of selected botanical species have significant insecticidal and repellent activities against *C. medinalis* larvae and induce distinct histopathological alterations in their larval tissues. These findings will provide scientific evidence supporting the potential application of these botanical extracts for environmentally sustainable rice pest management.

MATERIALS AND METHODS

Rearing of rice leaf folder larvae

Field collection

Rice leaf folder larvae and pupae were randomly collected from rice fields by hand-picking infested rice leaves. After collection, the insects were transferred onto clean potted rice plants placed inside net cages and labelled with the collection date.

Growing of host plant

Larvae and pupae were raised on rice seedlings in cages at a temperature of 27 ± 2 °C and a 12 h light cycle. Rice seeds of a susceptible variety (Bg352) were soaked in water for 3 – 4 days, placed on moss at 5 °C for 2 – 3 weeks and then planted in peat moss trays. The trays were kept at 25 °C under a 16 h light and 8 h dark period. The plants were watered well to keep them moist.

Introduction of adult rice leaf folder to the host plant

Male and female adult moths were confined in polyethylene cages containing cotton soaked with a 5% honey solution as a food source. The cages were placed over potted rice plants to allow controlled oviposition under laboratory conditions. Adults were not released freely as confinement was necessary to prevent flight and ensure uniform egg laying on rice leaves.

After 5-7 days, eggs were laid on the rice leaves, and larvae emerged naturally, resulting in uniform larval infestation of the rice plants. The emerged larvae were reared and maintained on rice plants within the cages and subsequently used for bioassay and histopathological studies.

Pupae were collected approximately on day 20 and transferred to separate containers lined with moistened cotton and supplied with a 5% honey solution until adult emergence. The rearing cycle was continuously

maintained through laboratory handling to obtain the required number of larvae throughout the study period. All rearing procedures and developmental timelines were followed according to standard protocols (Tsuda et al., 2005; Padmavathi et al., 2013).

Preparation of plant extracts

In this study, 10 plant species were chosen and identified based on their English common names and scientific names as follows: Neem (*Azadirachta indica*), Giant milkweed (*Calotropis* sp.), Castor (*Ricinus communis*), Malabar nut (*Justicia adhatoda*), Worm killer (*Aristolochia*

bracteolata), Tanner cassia (*Cassia auriculata*), Sweet flag (*Acorus calamus*), Tobacco (*Nicotiana tabacum*), and Pongam (*Pongamia pinnata*),

Specific plant parts (leaves, flowers, or roots) were collected based on the species of the plants (Table 1). The plant materials were thoroughly washed with tap water followed by distilled water and allowed to dry in the shade for 3-4 weeks at room temperature. The dried plant materials were ground to a fine powder using an electric grinder, sieved and stored in dark airtight containers before extraction.

Table 1: Selected plant species, and their parts used in the study

Scientific name	Family	English common name	Plant part used
<i>Azadirachta indica</i>	Meliaceae	Neem	Leaves
<i>Calotropis</i> sp.	Apocynaceae	Giant milkweed	Leaves
<i>Ricinus communis</i>	Euphorbiaceae	Castor	Leaves
<i>Justicia adhatoda</i>	Acanthaceae	Malabar nut	Leaves
<i>Aristolochia bracteolata</i>	Aristolochiaceae	Worm killer	Leaves
<i>Cassia auriculata</i>	Fabaceae	Tanner's cassia	Flowers
<i>Acorus calamus</i>	Acoraceae	Sweet flag	Rhizome
<i>Nicotiana tabacum</i>	Solanaceae	Tobacco	Leaves
<i>Pongamia pinnata</i>	Fabaceae	Pongam	Leaves
<i>Lawsonia inermis</i>	Lythraceae	Henna	Leaves

Stock solution preparation

A sample of 25 g of the powdered plant part was mixed with 250 ml of methanol and allowed to shake at 350 rpm on a mechanical shaker for two days. The concentrate was obtained after filtration with Whatman No.1 filter paper and a crude extract was prepared after evaporating the filtrate in a rotary evaporator at 40 °C. Then, 10 g of the crude extract was dissolved in 10 ml of methanol to prepare the 1 g/ml stock solution. The five bioassay concentrations of the extract (10 mg/ml or 1%, 30 mg/ml or 3%, 50 mg/ml or 5%, 100 mg/ml or 10%, 150 mg/ml or 15%) were prepared using the stock solution. These specific concentrations were determined based on previous range-finding bioassays and previously reported dose-response data for botanical extracts for lepidopteran pest control (Ghabbari et al., 2018; Ahmed et al., 2022; Vasantha-Srinivasan et al., 2023). The 150 mg/ml concentration is considered the most effective (non-phytotoxic) concentration that produces biological

activity under laboratory conditions, as suggested by standard procedures for botanical bioassays (Sarangi et al., 2025; Seni et al., 2025). In order to determine how different doses produce various physiological responses, the above concentrations were used for the evaluation of dose-dependent responses and provide evidence of both sub-lethal and lethal effects.

Bioassays

Larvicidal bioassay

Plant extracts were added to each beaker containing 100 ml of paddy water. Fourth instar larvae of leaf folders were then exposed to the plant extracts in the beaker. A control was maintained without adding any plant extract. For each trial, 10 ± 2 larvae were used per concentration. The larvae were fed with dry yeast powder (25 mg/100 ml) during the exposure period. The motility of larvae in the test and control beakers was observed at

24, 48 and 72 h. Five replicates were conducted for each concentration of all 10 plant extracts.

The percent mortality was calculated using the following formula [1] (Abbott, 1925).

$$\text{Corrected mortality} = \frac{(x-y)}{(100-y)} \times 100 \quad \dots(01)$$

Where,

X = Percent mortality in the treatment

Y = Percent mortality in the control

Repellent activity

The filter paper disc method was used to evaluate repellent activity. Petri dishes containing filter paper discs (9 cm in diameter) were prepared for each of the five different concentrations. Each filter paper disc was divided into two equal halves, one half with the plant extract and the other half treated with methanol as the control. Ten fourth-instar larvae were placed on the central line of each filter paper disc per replicate, and the experiment was conducted with five replicates for each concentration. After 2 h of exposure, the position of larvae on the treated and untreated halves was recorded, and repellency percentage was calculated using the standard formula (2) (Loko et al., 2017),

$$PR = \frac{(Nc - Nt)}{(Nc + Nt)} \times 100 \quad \dots(02)$$

Where,

PR = Percentage of repellence

Nc = Number of insects on untreated seedlings.

Nt = Number of insects on treated seedlings.

Histopathological analysis

Tissues from treated larvae were immersed in 10% neutral-buffered formalin for 7 days and then dehydrated, embedded, sectioned using a microtome and stained with haematoxylin and eosin. Parts of the gut thoracic muscles and Malpighian tubules were examined under a light microscope. The tissues were graded using an H-score (0–300), reflecting the level of severity of the plant extracts on the larval tissues (Christina and Walker, 2014; Pintong et al., 2020). The H-score was calculated by multiplying the percentage of affected cells by the severity score of tissue damage, where 0 = no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage.

RESULTS AND DISCUSSION

Concentration depended larval mortality analysis

The risk of larval mortality increased as the dose was increased (Figure 1). The control group (0 mg/L) showed the lowest mortality ($M = 1.31$, $SD = 0.788$), proving minimal natural mortality and showing the accuracy of the experiments, an increased number of larval deaths were observed as the concentration of extracts increased and significant mortality was observed at 150 mg/L

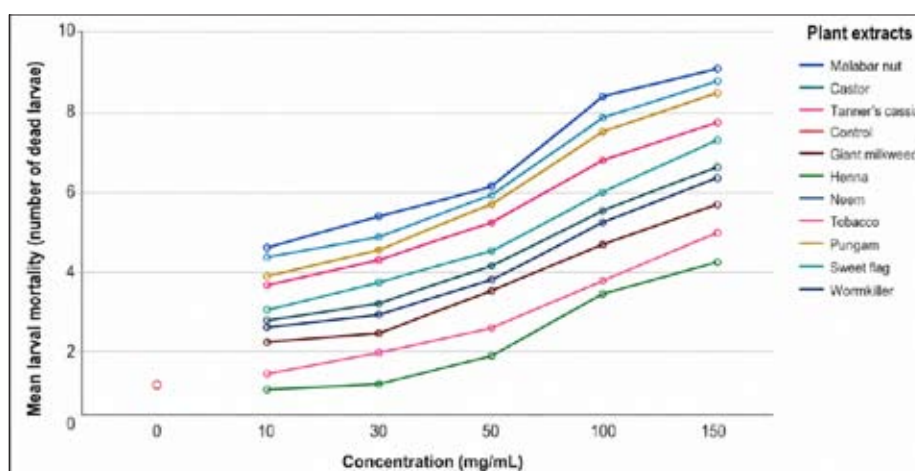


Figure 1: Larvicidal effect of various plant extracts at different concentrations (10 mg/ml (1%), 30 mg/ml (3%), 50 mg/ml (5%), 100 mg/ml (10%) and 150 mg/ml (15%))

($M = 6.97$, $SD = 1.778$). This shows a close positive relationship between the concentration of the extract and its larvicidal strength.

Out of all the plant extracts tested, Neem (*Azadirachta indica*) performed the best with a mean mortality of 6.75 ($SD = 2.014$) followed in efficiency by Malabar nut (*Justicia adhatoda*) with a mortality mean of 6.43 ($SD = 1.918$) and Pungam (*Pongamia pinnata*) with a mean of 6.12 ($SD = 1.945$). The potent bio-compounds such as azadirachtin, vasicine and karanjin in these plants contributed to larval mortality. In contrast, Henna (*Lawsonia inermis*) and Tanner's cassia (*Cassia auriculata*) showed the lowest average mortality with 2.35 and 2.97 respectively, revealing weaker action against rice leaf folder larvae.

The one-way ANOVA showed that larval mortality significantly varied among the different concentration groups, indicating that concentration is a major factor affecting mortality. Evidence of a strong impact of concentration on mortality was also found using the Welch test, which corrected for unequal variances (Welch's $F(5, 369.38) = 296.75$, $p < 0.001$). All groups in the concentration experiments differed significantly from the control group ($p < 0.001$). The risk of mortality was higher in larvae exposed to higher concentrations than to lower concentrations. At 150 mg/L, there was a marked increase in mortality compared to 100 mg/L (by an average of 0.95, $p < 0.001$) and all lower concentrations, demonstrating a strong dose-response relationship. These results indicated that smaller concentrations of extracts have reduced lethality.

Table 2: Summary of two-way ANOVA

Tests of Between-Subjects Effects					
Dependent variable: Mortality (number of dead larvae)					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3993.173 ^a	50	79.863	121.027	.000
Intercept	16411.117	1	16411.117	24869.873	.000
Extracts	1471.115	9	163.457	247.708	.000
Concentration	1656.941	4	414.235	627.744	.000
Extracts * Concentration	34.339	36	0.954	1.445	.046
Error	510.747	774	0.660		
Total	21062.000	825			
Corrected Total	4503.920	824			

a. R Squared = 0.887 (Adjusted R Squared = 0.879)

Two-way ANOVA analysis was used to examine how plant extract type and concentration independently and together affect larval mortality. As shown in Table 2, both main factors, i.e., plant extract type and concentration had significant effects on larval mortality (Extracts: $F(9, 774) = 247.708$, $p < 0.001$; Concentration: $F(4, 774) = 627.744$, $p < 0.001$). Additionally, there was a significant difference ($p = 0.046$) in the efficacy of extracts due to changes in the concentrations used in testing. For instance, Neem and Malabar nut caused high mortality at all tested levels, compared to Henna and Tanner's cassia, which required

higher doses to achieve similar mortality rates.

An excellent fit and reliability of results were demonstrated by the fact that the overall model accounted for 88.7% of the larval mortality variation ($R^2=0.887$, Adjusted $R^2=0.879$). The data indicate that some plant extracts are consistently effective, while higher concentrations of some extracts are needed to achieve similar outcomes. This shows that the botanical pesticides could be tailored with specific concentrations to control pests more effectively (Tiyagi et al., 2004)

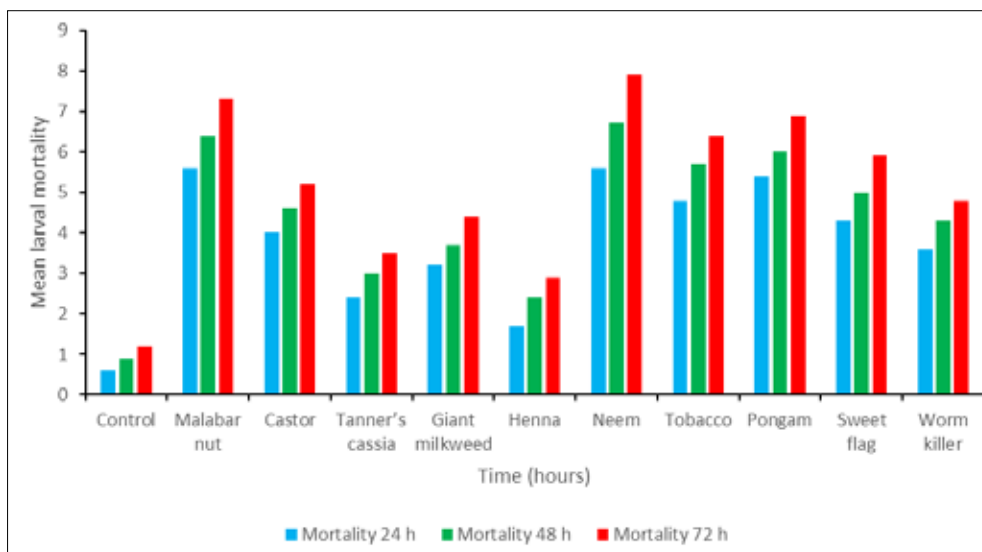


Figure 2: Mean larval mortality of *Cnaphalocrocis medinalis* after treatment with various plant extracts at 24, 48, and 72 hours.

Time depended larvicidal analysis

A repeated measures analysis of variance (ANOVA) was used to study the impact of different plant extracts on insect mortality at 24, 48 and 72 hrs. Significant differences were shown for the time and type of extract.

Most extracts showed a progressive increase in mortality. At 24 h, the highest average death count occurred among treated groups ($M=5.68$ for Malabar nut and Neem; and 5.40 for Pongam) while the control group had the lowest mortality ($M = 0.60$). After 72 h, the Neem extract treatment had the highest mortality. Meanwhile, both Malabar nut and Pongam had slightly lower mortality rates compared to Neem (about 70% and 69% of mortality) with insignificant mortality in the control group, showing the effectiveness of these plants over the treatment period.

Results from the multivariate tests showed that time played a highly significant role (Wilks' Lambda = 0.197, $F(2,288) = 588.41$, $p < .001$, $\eta^2 = .803$) in explaining differences in mortality among different time periods. An important relationship between time and extracts (Wilks' Lambda = 0.666, $F(20,576) = 6.49$, $p < .001$, $\eta^2 = .184$) indicated that insecticidal activity, as measured by time, was affected by the plant extracts. Subsequent analysis revealed that Neem, Malabar nut and Pongam extracts

significantly differed from the control and from several others, showing higher insecticidal activity. Therefore, it is evident from Figure 2 that prolonged exposure to botanical insecticides is more important for their effectiveness than short exposure times, and pest control efforts may be more effective if repeated applications are utilized (Scott et al., 2005)

Probit analysis

The results of the probit analysis for the larvicidal action of ten botanical extracts on *C. medinalis* larvae are summarized in Table 3, showing LC_{50} , LC_{90} , and LC_{99} values together with lower and upper confidence limits (LCL – UCL).

There is a significant variability in the larvicidal toxicity among the tested botanical extracts, indicating differences in the dose-response relationship. Neem had the highest larvicidal efficacy as shown by the lowest values for LC_{50} (25.8 ppm), LC_{90} (75.6 ppm), and LC_{99} (120.0 ppm). Lower concentrations of neem extract were required to produce larval mortality at 50%, 90%, and 99%, highlighting its toxic potential. On the other hand, Henna demonstrated the highest lethal concentrations ($LC_{50} = 70.1$ ppm; $LC_{90} = 150.3$ ppm; $LC_{99} = 250.0$ ppm) of the tested botanical extracts, which indicates lower larvicidal effectiveness under experimental conditions.

Table 3: Probit analysis of larvicidal activity of selected botanical extracts against *Cnaphalocrocis medinalis* larvae

Plant extract (English common name)	LC ₅₀ (ppm) (LCL-UCL)	LC ₉₀ (ppm) (LCL-UCL)	LC ₉₉ (ppm) (LCL-UCL)
Malabar nut	35.2 (30.1-40.5)	95.8 (90.0-100.8)	155 (150.0-160.5)
Worm killer	45.1 (40.0-50.5)	110.2 (105.0-115.8)	180.5 (175.0-185.8)
Castor	50.4 (45.0-55.8)	120.3 (115.0-125.8)	200 (195.0-205.8)
Tanner's cassia	60.3 (55.0-65.5)	135.4 (130.0-140.5)	220.5 (215.0-225.8)
Giant milkweed	55.6 (50.0-60.8)	128.5 (123.0-133.8)	210 (205.0-215.8)
Henna	70.1 (65.0-75.5)	150.3 (145.0-155.8)	250 (245.0-255.8)
Neem	25.8 (20.0-30.8)	75.6 (70.0-80.5)	120 (115.0-125.8)
Tobacco	40.7 (35.0-45.8)	105.7 (100.0-110.5)	175.5 (170.0-180.8)
Pongam	30.9 (25.0-35.8)	85.4 (80.0-90.5)	140 (135.0-145.8)
Sweet flag	38.5 (33.0-43.8)	98.7 (93.0-103.5)	160.5 (155.0-165.8)

The remaining botanical extracts demonstrated a moderate level of larvicidal activity, with lethal concentration values falling between those of Neem and Henna. For example, Pongam and Malabar nut had lower LC₅₀ values than Castor, Giant milkweed, and Tanner's cassia, which indicated higher toxicity at lower concentrations. The progressive increase in LC values among the different botanical extracts demonstrates a consistent dose-dependent response, which is an essential characteristic of a valid probit model.

In addition, the relatively narrow LCL-UCL confidence intervals for the LC₅₀, LC₉₀, and LC₉₉ values among all plant extracts demonstrated the precision and

reliability of the probit estimates. The lack of excessively wide confidence limits also suggested consistent larval mortality responses across replicates and concentrations. In general, the probit analysis confirms the significant differences in larvicidal potency among the tested botanical extracts, providing a quantitative basis for comparing the toxic effect on *C. medinalis* larvae.

Repellent activity of selected botanical extracts against rice leaf folder larvae

Repellency plays a vital role in suppressing larval settlement, feeding damage and subsequent crop injury by rice leaf folder larvae, even in the absence of direct

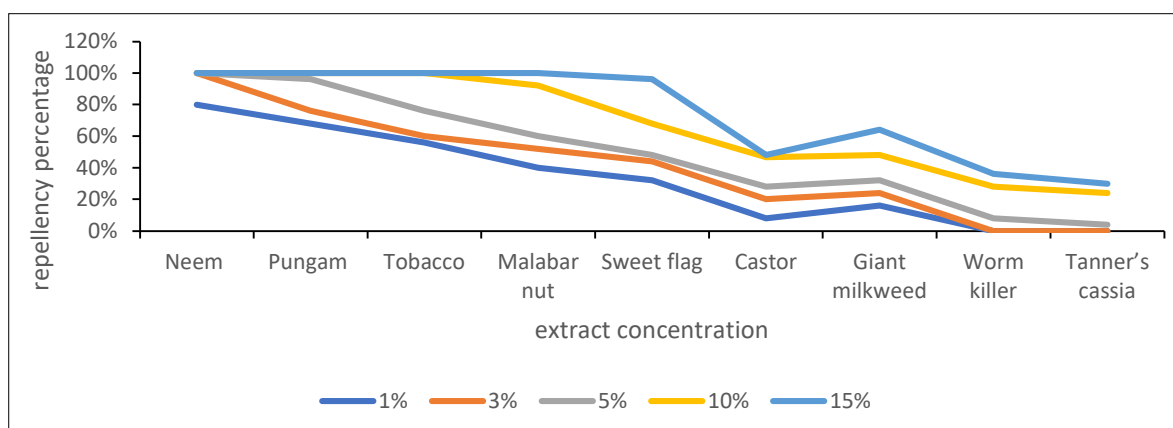


Figure 3: Repellency percentage of ten plant extracts against rice leaf folder (*Cnaphalocrocis medinalis*) larvae at five different concentrations (10 mg/ml (1%), 30 mg/ml (3%), 50 mg/ml (5%), 100 mg/ml (10%) and 150 mg/ml (15%))

lethality. Hence, repellency trials were incorporated as complementary assessments to mortality analysis offering insights into the behavioural disruption potential of botanical extracts in an integrated pest management (IPM) program.

Figure 3 shows the percentage of repellence of ten botanical extracts on rice leaf folder larvae at five concentrations (1, 3, 5, 10 and 15%). Repellency response depended on concentrations in all tested extracts with highly different levels of activity across plant species.

Out of the botanicals tested, Neem and Pongam showed good repellent activity with maximum repellency recorded at 3%. The repellency levels remained high at higher concentrations implying a strong repulsive action on larval movement and feeding behaviour. There was also significant repellent behaviour observed with tobacco, especially at 10% and 15%, implying that there was a concentration level beyond which strong behavioural avoidance was elicited.

Malabar nut and Sweet flag exhibited moderate repellency with high levels of activity at the highest concentration (15%). Castor, showed a gradual and steady increase in repellency with increasing concentration ranging with increasing values of about 32% at a 1% concentration to 96% at a showing a dose-dependent behavioural response.

Reduced repellence was observed in Giant milkweed, *Aristolochia bracteolata* (Worm killer) and *Cassia auriculata* (Tanner cassia). Even though repellence was concentration-dependent, the highest repellency values were comparatively low (48, 64 and 36%, respectively),

indicating that there was no significant deterrent capacity under the experimental circumstances. *Lawsonia inermis* (Henna) was the most inactive, with no more than about 30% repellency at the highest concentration. This implies that its use as a repellent against rice leaf folder larvae is limited.

The variation in repulsive behaviour between plant extracts could be due to the differences in the nature and concentration of bioactive phytochemicals, including alkaloids, terpenoids, and phenolic compounds which are known to influence insect sensory perception and behaviour. In general, the findings of the repellency experiments indicate that some botanical extracts can alter larval behaviour, which reduces feeding pressure and complements their larvicidal properties. These results show the potential of repellency as an additional mode of action to botanical-based management of rice leaf folder.

Histopathological effects of Neem extract on larval tissues

Histopathological analysis indicated significant tissue-based effects in the neem extract-treated larvae compared to the untreated control larvae (Figure 4). The degree of tissue injury was evaluated semi-quantitatively based on an H-score from 0 (no injury) to 300 (maximum tissue damage).

Histologically, in the control larvae, the structure of each of the three tissues was viz, gut, thoracic muscle and Malpighian tubule well preserved. In addition, the epithelial cells lining the midgut had normal organization,

Table 4: Histopathological damage scores (H-score) of gut, thoracic muscle, and Malpighian tubules of *Cnaphalocrocis medinalis* larvae treated with Neem extract.

Sample ID	Parts of larvae	% at 0 (No damage)	% at 1 (Mild)	% at 2 (Moderate)	% at 3 (Severe)	H-Score (% of damage cells*score)
Control	Gut	5	-	-	-	0
	Thoracic muscle	-	25	-	-	25
	Malpighian tubules	8	-	-	-	0
Neem Extracts	Gut	-	-	-	95	285
	Thoracic muscle	-	-	-	85	255
	Malpighian tubules	-	-	-	86.67	260

Note. H-score values ranged from 0–300, with higher scores indicating greater tissue damage.

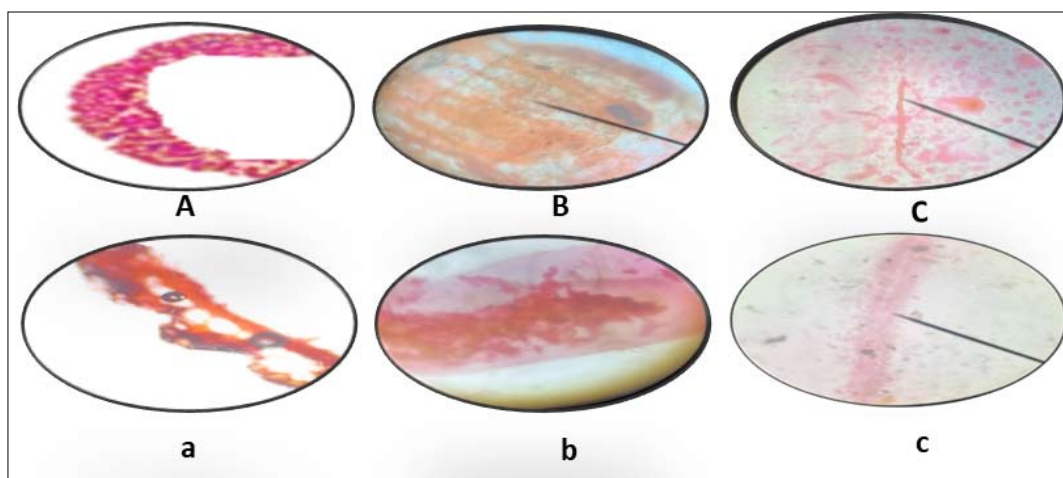


Figure 4: Histological changes in the larvae: Control groups. A: Gut, B: Thoracic muscle, C: Malpighian tubules. Neem extract treated larvae. a: Gut, b: Thoracic muscle, c: Malpighian tubule

with only slight alteration of less than 5% of the cells and the Malpighian tubules had only slight structural changes affecting < 10% of cells. As such, the H-scores for these two tissues were 0, indicating that the tissues were healthy. The thoracic muscles in the control larvae also had slight histological alterations with some breakage of the individual muscle fibres but this represented a low H-score of 25. These observations suggested that the tissues studied were not injured during the preparation for microscopy, and thus represent a valid reference point for comparing the effect of the neem treatment.

On the other hand, the larvae that were treated with neem extract had extensive histopathological injury to all the tissues that were studied. In particular, the midgut had a high level of cellular injury with >95% of the epithelial cells showing extensive damage including epithelial disintegration, cytoplasmic vacuolization, and loss of normal tissue structure and architecture, and consequently received an H-score of 285. It is reasonable to assume that the extensive damage to the midgut epithelial cells would cause an impairment of nutrient absorption and, subsequently, a reduction in digestive capacity and increased likelihood of larval death.

Similar to the midgut, the thoracic muscles also had extensive histological alterations due to the neem extract, including massive fragmentation and disintegration of the

individual muscle fibres, granulation of the cytoplasm, and necrotic areas, resulting in an H-score of 255. Injury to the thoracic muscles could reduce or eliminate the ability of larvae to move and feed, and therefore enhance the toxic effects of the extract. Finally, the Malpighian tubules had extensive structural injury to approximately 87% of the cells, including shrinkage and vacuolation of the cytoplasm, and degradation of the tubular architecture, and therefore received an H-score of 260. This suggested that the excretory and osmoregulatory functions of the Malpighian tubules were impaired.

In general, the microscopic studies demonstrate that epithelial rupture occurred in the midgut, degeneration and necrosis occurred in the thoracic muscle fibres, and extensive vacuolization and tissue degeneration occurred in the Malpighian tubules of the larvae treated with the neem extract. The histopathologic alterations to the tissues supported the previously described larvicidal activity of the neem extract. The histopathologic effects are in agreement with the mechanism of action of azadirachtin, a major active compound of neem, which damages cellular membranes, causes cell death through apoptosis and reduces membrane fluidity in insect tissues (Shao et al., 2016). Therefore, the results of the histopathological studies provide additional evidence supporting the toxic mechanism of action of neem extract at the cellular and tissue level against *C. medinalis* larvae.

Possible mechanisms of bioactive compounds which act as larvicidal compound

The larvicidal effect of botanical extracts is due to the synergistic effect of a variety of phytochemicals that are known to interfere with the biochemical and physiological functions of insects. These chemicals can disrupt neural control, hormonal balance, energy production and the integrity of the cell membranes, which can result in larval death.

Alkaloids are known to inhibit acetylcholinesterase (AChE), an important enzyme involved in the termination of nerve impulses at cholinergic synapses. AChE inhibitors prevent the breakdown of acetylcholine (ACh), leading to continual nerve stimulation, paralysis of the insect's nervous system, and death. Additionally, certain alkaloids can act on ion channels and neurotransmitter receptors, which also interfere with the normal neural function of the brain (Colović et al., 2013; Abou-Donia et al., 2016).

Flavonoids can exhibit toxicity to insects through the generation of oxidative stress in insect cells. High concentrations of reactive oxygen species (ROS) may cause damage to lipids, proteins, and DNA, leading to mitochondrial dysfunction, and triggering apoptosis and necrosis. Larval tissues are more vulnerable to oxidative damage, particularly in the Malpighian tubules and midgut, which could be the reason for histopathological changes observed in the present study (Mao et al., 2024).

Terpenoids and saponins may cause leakage of ions and enzymes from cellular and mitochondrial membranes, leading to the loss of electrochemical gradients and ultimately causing cell lysis. Tannins can bind digestive enzymes or even structural proteins in the insect gut, reducing the absorption of nutrients and damaging the gut epithelium (Konuk & Ergüden, 2020; Ergüden, 2021).

Together, these mechanisms can affect several organ systems such as digestive, nervous, muscular and excretory, which can lead to poor feeding, diminished mobility, physiological dysfunction and mortality of the larvae.

Comparison with existing studies

The final results of this study conclude a high insecticidal potential of neem, malabar nut and pongam at all concentrations. These findings align with the existing literature, which has demonstrated comparable larvicidal

activity of neem on lepidopteran pests (Charleston et al., 2005; Kamaraj et al., 2008; Dua et al., 2009; Nicoletti et al., 2012; Adhikari et al., 2020). The study included neem and also evaluated the larvicidal activity of various native botanical extracts, including malabar nut, pongam, tobacco and castor under laboratory conditions. These extracts showed different degrees of larvicidal activity on rice leaf folder larvae, and thus have potential applications in botanical pest control.

Histopathological observations revealed severe tissue changes in neem-treated larvae characterized by gut epithelium degeneration, as well as Malpighian tubules and muscle fibre disruption. These results indicate systemic cellular toxicity. The comparable pathological alterations were identified in experimental studies with neem-based extracts (Coventry & Allan, 2001, Agbaje et al., 2023, Nagini et al., 2023), which suggests to the hypothesis that the abnormal digestive and excretory processes are caused by active phytochemicals, including azadirachtin and flavonoids. Similar to the histological changes reported in previous studies, the present study also observed comparable alterations in the gut, which may be attributed to oxidative damage as well as inhibition of digestive enzymes; the degeneration of Malpighian tubules indicated a disruption of osmoregulation and excretion. Neuromuscular dysfunction may occur through interference with neurotransmission leading to muscle tissue damage. These processes can have a strong connection with larval behavioural pattern such as decreased feeding and locomotion throughout the bioassays.

CONCLUSIONS

The present study evaluated the larvicidal and repellent activity as well as the histopathological effects of ten botanical extracts against *Cnaphalocrocis medinalis* under laboratory conditions. neem and malabar nut showed the highest larvicidal activity resulting in significant mortality at all tested concentrations and exposure times ($p < 0.001$). These extracts additionally exhibited lower LC_{50} values compared to other botanical treatments, implying greater potency at lower concentrations, as confirmed through probit analysis.

In repellency assays, neem and pongam demonstrated strong repellent effects, suggesting their potential to reduce larval settlement and feeding damage. Histopathological examination of larvae treated with neem extract revealed severe damage to the midgut, thoracic muscles, and Malpighian tubules, providing mechanistic evidence for its toxic effects at the tissue level.

In general, the results support the efficacy of selected botanical extracts against *C. medinalis* and suggest their use in environmentally friendly rice pest management strategies. However, additional research is needed to test these extracts under field conditions, develop appropriate formulaions, isolate the active ingredients, and evaluate their non-target effects before practical field applications.

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RESEARCH ARTICLE

Agricultural and Veterinary Sciences

Prevalence of subclinical mastitis in a selected group of cows in Matara district of Sri Lanka: Risk factors, pathogens and antimicrobial resistance

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Abstract: This study aimed to determine the prevalence of subclinical mastitis (SCM), identify associated risk factors, characterize major bacterial pathogens, and evaluate their antimicrobial resistance in dairy cows in the Matara district, Sri Lanka. The study was conducted from October 2024 to March 2025, involving 220 lactating cows from 60 randomly selected groups. SCM was diagnosed using the California mastitis test (CMT), and positive milk samples were subjected to bacteriological identification. Antimicrobial susceptibility testing was performed following Clinical and Laboratory Standards Institute (CLSI) guidelines. Farm- and animal-level data were collected using a structured questionnaire and analyzed using descriptive statistics, chi-square tests, and binary logistic regression using IBM SPSS (version 25) Statistics. The overall cow-level prevalence of SCM was 30.0%, with a quarter-level prevalence of 11.5%. Logistic regression analysis identified older age (>5 years), higher parity, early lactation stage, and high milk yield as significant animal-level predictors of SCM ($p < 0.05$). Friesian crossbred cows showed comparatively higher susceptibility than other breeds. At the farm-level, larger herd size, once-daily milking, poor udder hygiene, poor housing cleanliness, and absence of supplementary feeding were significantly associated with increased SCM risk. *Staphylococcus aureus* (54.6%) was the predominant isolate, followed by *Escherichia coli* (19.7%) and *Streptococcus* spp. (13.6%). Antimicrobial susceptibility testing revealed high resistance of *S. aureus* to amoxicillin, while *E. coli* showed high resistance to enrofloxacin and amoxicillin. However, gentamicin and neomycin remained highly effective against *E. coli*, and enrofloxacin showed complete efficacy against

Streptococcus spp. In conclusion, SCM remains a significant constraint to dairy productivity in southern Sri Lanka. The findings highlight the need for improved milking hygiene, routine bacteriological screening, and prudent antimicrobial use to mitigate antimicrobial resistance and improve udder health within a One Health framework.

Keywords: Antimicrobial resistance, dairy cattle, risk factors, subclinical mastitis, *Staphylococcus aureus*.

INTRODUCTION

Mastitis, an inflammation of the mammary gland, remains one of the most economically impactful diseases in dairy production worldwide. It significantly reduces milk yield and quality, increases veterinary expenses, leads to premature culling, and contributes to substantial economic losses for dairy farmers (Seegers et al., 2003; Ruegg, 2017). Mastitis manifests in two primary forms. Clinical mastitis is characterized by visible signs such as udder swelling, heat and abnormal milk secretion. The subclinical mastitis (SCM) lacks visible symptoms but silently affects milk production and facilitates the transmission of pathogens within the herd (Halasa et al., 2007; Sharma et al., 2011).

SCM is considerably more prevalent than its clinical counterpart. It represents a major challenge to udder

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health particularly in low- and middle-income countries (LMICs) where resources for routine surveillance and control are limited (Mekonnen et al., 2017). A global meta-analysis estimated the average prevalence of SCM at approximately 42.0%. However, some regions had reported even higher rates, such as 86.2% in Uganda and 60.0% in Egypt (Abrahmsén et al., 2014; Bonatto et al., 2021). The absence of visible signs often delays detection, allowing infections to persist and spread unnoticed thereby undermining herd health interventions. Early detection and effective management of SCM are essential to sustain dairy productivity and ensuring milk safety, particularly in LMICs (Molina-Flores et al., 2020).

The most common pathogens responsible for mastitis include *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli*. The distribution of these pathogens varies with geography, hygiene, and farm management practices (Bradley, 2002; Kalmus et al., 2011). Among them, *S. aureus* is often linked to persistent subclinical infections due to its ability to evade host immune defenses through intracellular survival and biofilm formation (Zaatout et al., 2020).

Antibiotic therapy remains the cornerstone of mastitis treatment. However, the excessive and often unregulated use of antimicrobials in dairy production has led to the emergence of antimicrobial resistance (AMR). It is posing risks to both animal and public health (WHO, 2016). Resistance among mastitis pathogens, including *S. aureus*, *E. coli*, and *Streptococcus* spp., to widely used antibiotics such as tetracyclines, β -lactams and fluoroquinolones has been increasingly reported (Zhang et al., 2021; Velasco et al., 2025). Resistance genes such as blaZ, blaSHV, and blaTEM contribute to this trend. AMR surveillance in dairy systems is now recognized as an integral part of global One Health strategies to mitigate risks of transmission via the food chain (Van et al., 2015; WHO, 2025).

In Sri Lanka, the dairy sector is a vital component of rural livelihoods and national food security. Yet, the country meets only about 40.0% of its domestic milk requirement. The remainder is met through imports (Department of Animal Production and Health, 2019). SCM remains a major but underdiagnosed issue among local herds particularly under small- and medium-scale farming systems. Studies have shown that *S. aureus* is the predominant SCM pathogen, with increasing resistance to commonly used antibiotics such as cephalixin and

amoxicillin (Jayaweera et al., 2018; Priyantha et al., 2021). However, comprehensive regional data on SCM prevalence, bacterial aetiology, and AMR remain limited and fragmented. According to Bari et al. (2022), only two published studies in Sri Lanka have investigated SCM pathogens with considerable variation across regions, underscoring the need for localized research.

The Matara district, situated in the Southern province, features predominantly small to medium dairy herds managed under extensive, semi-intensive, or intensive systems. While dairy farming is expanding in this region, constraints such as poor milking hygiene, inadequate veterinary support and lack of awareness hinder effective mastitis control. Understanding the epidemiology and AMR patterns of SCM in this context is essential for developing targeted interventions and guiding rational antibiotic use. Therefore, this study was undertaken to: (i) determine the prevalence of SCM, (ii) identify the associated bacterial pathogens and their antimicrobial resistance profiles, and (iii) assess animal and farm-level risk factors (determinants) associated with SCM. The findings aim to inform evidence-based mastitis control strategies and promote sustainable dairy development at the regional level.

MATERIALS AND METHODS

Ethical clearance statement

This study involved no experimental intervention on animals. All procedures were conducted as part of routine veterinary investigation services. Informed verbal consent was obtained from all participating farmers. The study adhered to the guidelines set by the Department of Animal Production and Health, Sri Lanka, for animal welfare and field data collection.

Study area and design

This cross-sectional study was conducted from October 2024 to March 2025 in 12 veterinary surgeon (VS) divisions of the Matara district of Sri Lanka.

Sample size determination

A prevalence of 50.0% was assumed for the purpose of sample size calculation as this provides the maximum variability and yields the most conservative estimate. The desired margin of error was set at 7.0% (0.07), with a 95.0% confidence level.

Although dairy farmers are typically registered under VS divisions, updated and reliable records of lactating cow populations at the district level were not available at the time of the study. Therefore, the total cattle population ($n = 7,580$) reported by the Department of Census and Statistics (Department of Census and Statistics, 2024) was used to estimate the lactating population. Based on regional herd structure and management practices, it was assumed that approximately 40.0% of cattle were lactating, yielding an estimated population size of 3,032 cows.

The initial sample size for an infinite population was calculated using the standard formula for prevalence studies.

$$n = \frac{Z^2 \times P \times (1 - P)}{d^2}$$

Where:

$Z = 1.96$ (95% confidence)

$p = 0.5$ (assumed prevalence; 50.0%)

$d = 0.07$ (precision)

n = population size (3032)

This value was adjusted for the finite population of lactating cows using the finite population correction formula.

$$n_{adj} = \frac{n \times N}{N + n - 1}$$

Where,

n_{adj} = adjusted sample size for finite population

n = initial sample size calculated for an infinite population

N = total population size (estimated number of lactating cows in the study area)

Accordingly, the minimum required sample size was estimated at 184 cows. To enhance the statistical power and allow for potential exclusions, a total of 220 lactating cows with about 880 teat quarters were considered for the study. The 220 cows were distributed across 12 VS divisions in the Matara district, representing similar agro-climatic conditions. All study locations fall within the low-country wet zone, characterized by high rainfall and humidity, minimizing climatic variation across sampling sites. The number of cows sampled from each division varied depending on herd availability and farmer participation (Table 1).

Table 1: Distribution of sampled lactating dairy cows across Veterinary Surgeon (VS) Divisions in the Matara District, Sri Lanka ($n = 220$)

VS Division	Sampled cows (n)	Percentage (%)
Akuressa	09	4.1
Dikwella	04	1.8
Hakmana	10	4.5
Kamburupitiya	26	11.8
Kekanadura	26	11.8
Malimbada	20	9.1
Matara	28	12.7
Mulatiyana	11	5.0
Pasgoda	17	7.7
Pitabeddara	16	7.3
Weligama	26	11.8
Welipitiya	27	12.3
Total	220	100.0

Detection of subclinical mastitis (SCM)

All lactating cows were screened during the morning milking period. Cows exhibiting clinical signs of mastitis were excluded. SCM was diagnosed using California mastitis test (CMT), as described by Quinn et al. (1999). Equal volumes of milk and CMT reagent (Chimertech, Chennai, India) were mixed in a four-compartment paddle and gently swirled for 10-15 seconds. Reactions were visually evaluated. Each quarter was scored from 0 to 4 based on gel formation. A score of ≥ 2 in any quarter in the absence of visible abnormalities in milk or udder was considered positive for SCM. All CMTs were conducted by the same trained veterinarian to ensure consistency. All four quarters of each selected cow were tested and evaluated separately.

Composite foremilk samples (15 mL) from CMT positive quarters were aseptically collected into sterile glass vials after cleaning the teats with 70.0% ethyl alcohol and discarding the initial streams. Samples were kept on ice and transported to the Matara Veterinary Investigation Centre (VIC) for microbiological analysis.

Questionnaire survey

A questionnaire was designed to gather farm and animal-level data through face-to-face interviews with the farmer during the farm visit. It was pretested with 10 randomly selected farmers. Animal-level data included age, breed, parity, lactation stage, body condition score (BCS), and daily milk yield. Farm-level information captured herd size, farming system, milking frequency, udder hygiene practices, supplementary feeding, and house cleanliness. Interviews were conducted in the local language by a trained investigator. Clarifications and follow-up visits were conducted to ensure data accuracy.

Defining categorical variables

Categorical variables used in the analysis were defined based on field observations and standard management practices. Age was categorized as ≤ 3 years, 3–5 years and > 5 years. Parity was grouped as 1, 2 and ≥ 3 . Lactation stage was classified as early (≤ 3 months), mid (3–6 months) and late (> 6 months). Daily milk yield was categorized as low (1–4.9 L/day), average (5–6.9 L/day) and high (7–12 L/day). BCS was classified as poor (≤ 2.5), average (2.75–3.0) and good (≥ 3.5) following Edmonson et al. (1989). The breed of the animals was recorded as Jersey cross, Friesian cross, and Sahiwal cross.

Udder hygiene was categorized as ‘good’ when teats were washed and dried before milking, and ‘poor’ when no cleaning or inadequate cleaning was observed. House cleanliness was classified as good (clean, dry housing with regular manure removal), average (moderately clean with occasional manure accumulation) and poor (dirty, wet conditions with visible manure accumulation). Milking frequency was categorized as once or twice daily. Supplementary feeding was recorded as ‘yes’ when concentrate or additional feed was provided and ‘no’ otherwise. Herd size was categorized as small (≤ 10 cows) and medium (11–50 cows). Farming systems were classified based on animal housing and grazing practices. Management systems were defined as intensive where there is zero-grazing (animals housed and fed for 24 hours), semi-intensive where cows were tethered in the morning (around 08:00 h) and returned to sheds by 14:30 h (Bandara et al., 2011), and extensive where animals were predominantly managed under free grazing conditions with minimal housing.

Bacteriological examination

Milk samples were processed at VIC, Matara within six hours of collection. Samples were brought to room temperature and aseptically streaked onto blood agar

and MacConkey agar plates. The plates were incubated aerobically at 37 °C for 24–48 hours. Colonies were characterized based on morphology and haemolysis.

Gram staining was used to classify isolates. Gram-positive cocci were subjected to catalase testing to differentiate *Staphylococcus* spp. (catalase-positive) from *Streptococcus* spp. (catalase-negative). Coagulase testing was used to identify *Staphylococcus aureus*. Gram-negative isolates were sub-cultured on MacConkey agar and presumptively identified as *E. coli* based on lactose fermentation. Final confirmation was done using standard biochemical tests including triple sugar iron (TSI), urease, citrate, and indole tests.

Antimicrobial sensitivity testing (ABST)

Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion method (Bauer et al., 1966) following the Clinical and Laboratory Standards Institute (CLSI - 2023) guidelines. Five commonly used antibiotics were tested, including gentamicin (10 µg), oxytetracycline (30 µg), amoxicillin (10 µg), enrofloxacin (10 µg) and neomycin (30 µg). Antibiotic discs (Himedia, India) were placed on Mueller-Hinton agar inoculated with overnight cultures grown in Luria-Bertani broth. The plates were incubated at 37 °C for 18–24 hours. Zones of inhibition were measured by one technician to reduce observer bias. The zone of inhibition was measured in millimeters and interpreted based on CLSI breakpoint standards for each species (CLSI, 2023).

Statistical analysis

All data collected from field observations, laboratory analysis, and questionnaire surveys were coded and entered into IBM SPSS Statistics Version 25 for statistical analysis. Descriptive statistics were used to summarize the data. SCM prevalence was calculated at both the cow and quarter levels according to Gómez-Quispe et al. (2015).

$$\text{Cow level prevalence} = \frac{\text{No. of CMT positive cows}}{\text{Total cows examined}} \times 100$$

$$\text{Quarter level prevalence} = \frac{\text{No. of positive quarters}}{\text{Total quarters examined}} \times 100$$

The association between explanatory variables and SCM status (positive/negative) was initially assessed using the Chi-square (χ^2) test of independence. Variables with a significance level of $p < 0.05$ in the Chi-square analysis were considered for further modeling.

To identify independent risk factors associated with SCM, binary logistic regression analysis was performed using a backward stepwise approach. Separate models were developed for animal and farm-level variables to avoid multicollinearity and improve interpretability. The dependent variable was SCM status (1 = positive, 0 = negative). Independent variables were entered as categorical predictors based on predefined classifications. The general form of the binary logistic regression model is expressed as follows:

$$\text{logit}(p) = \ln\left(\frac{p}{(1-p)}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \dots$$

where,

p = Probability of SCM occurrence

β_0 = Intercept

β_i = Regression coefficient

X_i = Independent variables

The strength of association was expressed using Adjusted Odds Ratios (AORs) with corresponding 95%

confidence intervals (CI). Statistical significance was declared at $p < 0.05$.

Model adequacy was evaluated using standard diagnostic measures including the Hosmer–Lemeshow goodness-of-fit test, and multicollinearity among predictors was assessed using variance inflation factors (VIF), ensuring that all included variables met acceptable thresholds.

All statistical tests were two-tailed and results were presented in tables with corresponding test statistics, p -values, and confidence intervals.

RESULTS AND DISCUSSION

Prevalence of subclinical mastitis (SCM)

The overall prevalence of SCM in the present study was 30.0% (66/220 cows) as determined by the CMT. Of the 880 quarters examined, 101 quarters (11.5%) were found positive for SCM. These results indicate a considerable burden of subclinical infections in lactating cows in the Matara district. Compared to previous studies in

Table 2: Association between animal-level factors and subclinical mastitis (SCM) prevalence in lactating dairy cows ($n = 220$).

Variable	Category	n	%	SCM positive (n)	Prevalence (%)	X ²	P value
Age	≤3 years	97	44.1	18	18.6	14.68	0.001
	3-5 years	93	42.3	32	34.4		
	> 5 years	30	13.6	16	53.3		
Breed	Jersey cross	100	45.5	20	20.0	20.22	0.000
	Friesian cross	36	16.4	6	16.7		
	Sahiwal cross	84	38.2	40	47.6		
Parity	1 parity	71	32.3	17	23.9	8.90	0.012
	2 parity	65	29.5	14	21.5		
	≥ 3 parity	84	38.2	35	41.7		
Lactation stage	Early	109	49.5	46	42.2	15.31	0.000
	Mid	55	25.0	10	18.2		
	Late	56	25.5	10	17.9		
BCS	Poor (≤ 2.5)	89	40.5	28	31.5	2.67	0.263
	Average (2.75-3.0)	125	56.8	38	30.4		
	Good (≥ 3.6)	6	2.7	0	0.0		
Milk yield	Low (1-4.9 l/d)	84	38.2	15	17.9	13.89	0.001
	Average (5-6.9 l/d)	101	45.9	33	32.7		
	High (7-12 l/d)	35	15.9	18	51.4		

BCS: body condition score

Sri Lanka, the observed prevalence is relatively lower. For instance, Gunawardana et al. (2014) reported prevalence rates ranging from 43.0% to 71.0% while Rahularaj et al. (2019) recorded a prevalence of 49.0% based on CMT testing. This variation may be attributed to differences in study settings, herd sizes and management practices including hygiene and milking systems.

Internationally, reported SCM prevalence varies widely. In Bangladesh, studies have shown prevalence rates ranging from 20.0% to 75.0% depending on herd characteristics and sampling methodology (Sarker et al., 2013; Bari et al., 2022). A meta-analysis by Krishnamoorthy et al. (2021) documented a global SCM prevalence of 42.0% with higher prevalence in India (45.0%) and African countries like Uganda (86.2%, Abrahmsén et al., 2014).

Association between animal-level factors and subclinical mastitis (SCM)

Results of the Chi-square test revealed significant associations between SCM prevalence and age, breed, parity, lactation stage and milk yield. No statistically significant association was found with BCS (Table 2). These significant variables were subsequently included in a binary logistic regression model to identify independent predictors.

Animal-level determinants of subclinical mastitis (SCM)

The binary logistic regression model identified age, breed, parity, milk yield, and lactation stage as statistically significant animal level determinants of SCM ($p < 0.05$) (Table 3).

Table 3: Binary logistic regression analysis of animal-level determinants of subclinical mastitis (SCM) in lactating dairy cows ($n = 220$)

Variable	B	S.E.	P value	AOR (Exp(B))	95% CI
Age					
≤3 years	-2.415	0.587	0.000	0.089	0.028-0.282
3-5 years	-0.590	0.540	0.275	0.554	0.192-1.598
Breed			0.002		
Sahiwal cross	-1.420	0.413	0.001	0.242	0.107-0.543
Jersey cross	-1.106	0.573	0.054	0.331	0.108-1.017
Parity			0.004		
Parity 1	-1.586	0.512	0.002	0.205	0.075-0.559
Parity 2	-1.426	0.516	0.006	0.240	0.087-0.660
Milk yield			0.002		
Low (1-4.9 l/d)	-2.050	0.570	0.000	0.129	0.042-0.394
Average (5-6.9 l/d)	-1.249	0.489	0.011	0.287	0.110-0.747
Lactation stage			0.020		
Early lactation	1.408	0.507	0.005	4.088	1.514-11.036
Mid lactation	0.934	0.623	0.134	2.545	0.751-8.626

AOR: Adjusted Odds Ratio; CI: Confidence interval; Reference groups: Age: > 5 years; Breed: Friesian cross; Parity: ≥ 3 parity; Milk yield: High (7-12 l/d); Lactation stage: late lactation. The Hosmer–Lemeshow test indicated good model fit ($p > 0.05$), and no significant multicollinearity was detected among predictors ($VIF < 10$).

Logistic regression analysis revealed that older cows (>5 years) were significantly more prone to SCM, likely due to age-related teat and immune changes (Rainard &

Riollet, 2006; Radostits et al., 2007; Moges et al., 2011; Zeryehun et al., 2013). Younger cows had significantly lower odds of infection, supporting earlier findings.

Breed also significantly influenced SCM risk with Sahiwal and Jersey crosses being less susceptible than Friesian crosses. Breed-related differences reflect both genetic susceptibility and variations in management practices. Friesian crosses, being higher-yielding, showed greater risk of SCM, particularly under sub-optimal conditions like outdoor milking and temporary housing with poor hygiene (Curone et al., 2018; Ranasinghe et al., 2021). The present study observed that farmers used outdoor areas for milking and brought the cows outside the rearing pens and tethered them to a tree before milking. Those farmers were poor and had minimal facilities such as temporary housing. Further, it was observed that those farmers were not concerned about the hygienic aspects of milking. Although these farmers are encouraged to keep high-yielding exotic animals, this is not accompanied by the awareness of proper prevention and control of mastitis in the study area.

Cows with higher parity had greater SCM risk, which is consistent with prior findings (Zeryehun et al., 2013). Some studies reported that compared with primiparous cows, multiparous cows were 2.51 times more likely to have SCM (Ranasinghe et al., 2021). Age-related teat end changes, tissue degeneration and pendulous udders may predispose to infection (Neijenhuis et al., 2001; Bhutto et al., 2010; Zeryehun et al., 2013).

High yielding cows had increased SCM risk, likely due to elevated metabolic stress and udder pressure

(Taponen et al., 2006; Biswas & Sarker, 2017). This association is likely due to the elevated nutrient demands in high producing cows which can compromise the immune system and make the mammary glands more susceptible to infection (Schalm et al., 1971; Sharma et al., 2011). However, some studies have reported that the incidence rate of mastitis was higher in low (55.2%) and moderate (58.0%) yielders than high (41.0%) yielders (Jingar et al., 2014; Elmaghraby et al., 2017).

Early lactation was significantly associated with SCM (AOR = 4.09). Zeryehun et al. (2013) also reported that the early lactation stage had higher relative prevalence (87.2%) than late (73.1%) and mid (65.9%) lactation stages. The absence of a dry cow therapy regime could possibly be the major factor contributing to high prevalence at early lactation and early infection associated with delayed diapedesis of neutrophils into the mammary glands (Schalm et al., 1971). This may also due to physiological stress and immunosuppression of cows in the study region (Schalm et al., 1971; Sharma et al., 2011).

Poor BCS has been suggested as a risk factor in other studies. For instance, Fesseha et al. (2021) have reported that poor BCS had higher (60.4%) relative prevalence than that of moderate (52.5 %) and good (30.0%) BCS. However, it was not significant in the present analysis possibly due to limited sample representation.

Table 4: Association between farm-level factors and subclinical mastitis (SCM) prevalence in lactating dairy cows (n = 220).

Variable	Category	n	%	SCM positive (n)	Prevalence (%)	X ²	P value
Herd size	Small (≤ 10)	70	31.8	8	11.4	16.86	0.000
	Medium (11-50)	150	68.2	58	38.7		
	Intensive	33	15.0	17	51.5		
Farming system	Semi intensive	162	73.6	44	27.2	9.08	0.011
	Extensive	25	11.4	5	20.0		
Milking frequency	Once daily	126	57.3	52	41.3	17.83	0.000
	Twice daily	94	42.7	14	14.9		
Udder hygiene	Good	97	44.1	10	10.3	32.03	0.000
	Poor	123	55.9	56	45.5		
Supplementary feeding	Yes	137	62.3	26	19.0	21.00	0.000
	No	83	37.7	40	48.2		
House cleanliness	Good	36	16.4	6	16.7	49.03	0.000
	Average	117	53.2	18	15.4		
	Poor	67	30.5	42	62.7		

SCM: Subclinical mastitis

Association between farm-level factors and subclinical mastitis (SCM)

Chi-square analysis revealed significant associations between SCM and several farm-level variables, including

herd size, farming system, milking frequency, udder hygiene, supplementary feeding and house cleanliness (Table 4). These variables were subsequently included in the logistic regression model (Table 5).

Table 5: Binary logistic regression analysis of farm-level determinants of SCM in lactating dairy cows (n = 220)

Variable	B	S.E.	P value	AOR (Exp(B))	95% CI
Herd size (Small)	-1.892	0.516	0.000	0.151	0.055-0.415
Milking frequency (once daily)	1.375	0.469	0.003	3.954	1.576-9.920
Udder hygiene (Good)	-1.535	0.469	0.001	0.216	0.086-0.541
Supplementary feeding (No)	1.299	0.417	0.002	3.666	1.618-8.308
House cleanliness			0.001		
House cleanliness (Good)	-1.771	0.649	0.006	0.170	0.048-0.607
House cleanliness (Average)	-1.470	0.425	0.001	0.230	0.100-0.529
Farming system			0.033		
Farming system (Intensive)	1.444	0.872	0.098	4.239	0.767-23.433
Farming system (Semi Intensive)	0.084	0.772	0.913	1.088	0.240-4.938

AOR: Adjusted odds ratio; CI: Confidence interval; Reference groups: Herd size: Medium herd (11-50); Milking frequency: twice daily; Udder hygiene: poor; Supplementary feeding: Yes; House cleanliness: Poor; Farming system: Extensive. The Hosmer–Lemeshow test indicated good model fit ($p > 0.05$), and no significant multicollinearity was detected among predictors ($VIF < 10$).

Cows reared in small herds (≤ 10 cows) had significantly lower odds of SCM compared to those in medium-sized herds. This supports findings by Islam et al. (2012) and Ranasinghe et al. (2021), suggesting that larger herds may face increased pathogen exposure due to crowding, shared equipment, and less individualized attention to udder health and hygiene. Bari et al. (2022) also explained that the larger herds are usually managed under an intensive management system to be commercially viable. Therefore, they are likely to have increased pathogen exposure due to high stocking density and dirty condition of the sheds or equipment.

Poor udder hygiene, poor house cleanliness, and absence of supplementary feeding were all significantly associated with higher SCM risk. Cows milked once daily had a significantly higher likelihood of SCM compared to those milked twice daily. This may be due to increased intramammary pressure and bacterial proliferation caused by prolonged milk retention (Stelwagen & Lacy-Hulbert, 1996; Green et al., 2006). Schreiner & Ruegg (2003) demonstrated that dirty udders were associated with higher pathogen load in milk (1.5 times more likely to have major pathogens compared to clean udders).

Post-milking teat disinfection, use of gloves and keeping cows standing after milking are effective preventive measures (Dufour et al., 2011). In the present study, cows that did not receive supplementary feeding exhibited a significantly higher prevalence of SCM compared to those that received supplementary feeding. This suggests that improved nutritional support may enhance immune function and udder health, thereby reducing susceptibility to infection (Green et al., 2007; Sordillo et al., 2009). Farms with good and average hygiene practices had substantially lower odds of SCM. Dirty housing environments contribute to increased bacterial load on the udder and teat surface facilitating intramammary infections (Abrahmsén et al., 2014; Mureithi & Njuguna, 2016; Kagira et al., 2022).

Although there was a significant difference among farming systems, pairwise comparisons indicated no significant difference between intensive and extensive systems. An intensive system may contribute to more frequent infections as the cow stays longer within a small area that may be less clean and in addition, cows may be more stressed because of overcrowding (Barker et al., 2001).

Bacterial pathogens of milk

Bacteriological examination of milk samples from SCM positive cows revealed four major bacterial groups: *Staphylococcus aureus*, *Streptococcus* spp., *E. coli* and other coagulase negative *Staphylococcus* species (CNS) (Table 6). Based on Gram staining, colony morphology, and biochemical tests, *S. aureus* was confirmed by its Gram-positive cocci appearance in clusters, catalase and coagulase positivity, and golden-yellow colonies on nutrient agar. *Streptococcus* spp. appeared as Gram-positive cocci in chains, were catalase negative, and produced small translucent colonies. *E. coli* was identified as Gram-negative rods producing pink lactose fermenting colonies on MacConkey agar and confirmed by gas production on triple sugar iron (TSI) slants and negative urease and citrate tests.

Table 6: Distribution of bacterial pathogens isolated from SCM positive milk samples (n = 66)

Bacteria species	Number of isolates (n)	Prevalence (%)
<i>S. aureus</i>	36	54.6
<i>Staphylococcus</i> spp (CNS)	8	12.1
<i>Streptococcus</i> spp.	9	13.6
<i>E. coli</i>	13	19.7
Total	66	100.0

CNS: Coagulase-negative *Staphylococcus*

Several studies in Sri Lanka have reported the major causal agents for SCM to be *Staphylococcus* spp., *Klebsiella* spp., *E. coli*, *Streptococcus* spp., *Pasteurella* spp., *Corynebacterium* spp., and *Proteus* spp. (Samarakoon et al., 2014).

S. aureus was the most prevalent isolate among SCM cases followed by *E. coli*, *Streptococcus* spp. and other coagulase-negative *Staphylococcus* species in this study. This aligns with previous studies in Sri Lanka and South Asia, where *S. aureus* is consistently recognized as the primary pathogen associated with SCM (Bhattacharyya et al., 2016; Bari et al., 2022). The dominance of *S. aureus* is attributed to its strong udder colonization capacity, intracellular persistence and resistance to immune clearance enabling it to cause chronic subclinical infections and facilitate transmission during milking (Radostits et al., 2007).

E. coli and *Streptococcus* spp. were also important pathogens identified. These are often classified as environmental mastitis pathogens, commonly originating from contaminated housing, bedding, or milking equipment. The presence of *E. coli* highlights poor hygiene or water contamination in some farms. Similar bacterial profiles have been reported across tropical dairy systems in Bangladesh (Islam et al., 2014), India (Shome et al., 2012; Prabhu et al., 2013), and Pakistan (Khan et al., 2013), where *S. aureus*, NAS, and *Streptococcus* spp. predominate.

The identification of both contagious pathogens like *S. aureus* and environmental pathogens such as *E. coli* and *Streptococcus* spp. in the current study highlights the dual challenge of mastitis control. Effective prevention must therefore integrate milking hygiene, cow cleanliness, housing sanitation and targeted treatment strategies to mitigate both routes of infection.

Antimicrobial resistance patterns

Staphylococcus aureus

Among 36 *S. aureus* isolates, high susceptibility was observed to enrofloxacin, gentamicin and neomycin (Table 7), which is consistent with previous findings that aminoglycosides and fluoroquinolones retain significant efficacy against *S. aureus* infections (Pyorala & Taponen, 2009; Kalmus et al., 2011). However, marked resistance to amoxicillin was observed raising concern regarding the sustained effectiveness of β -lactam antibiotics in mastitis control. Moderate resistance levels to oxytetracycline reflect its widespread and often indiscriminate use raising concerns about its future efficacy in mastitis management (Barkema et al., 2006) (Table 7).

Table 7: Antimicrobial susceptibility profile of *S. aureus* isolates (n = 36)

Antimicrobial agent	Disc concentration (μ g)	Susceptible (%)
Enrofloxacin	10	94.9
Amoxicillin	10	35.9
Gentamicin	10	84.6
Neomycin	30	82.1
Oxytetracycline	30	59.0

Interpretation of antimicrobial susceptibility was performed according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Intermediate results were not observed.

Escherichia coli

All 13 *E. coli* isolates demonstrated complete susceptibility to gentamicin and neomycin (Table 8) reinforcing the continued value of these antimicrobials for treating Gram-negative infections. Similar susceptibility patterns have been reported in calf isolates (Orden et al., 2000; Diwakar, 2014). However, resistance to enrofloxacin was notably high, a troubling observation given the drug's broad-spectrum profile. The emerging resistance to fluoroquinolones may be attributed to their frequent and indiscriminate use in veterinary practices within the study area. Furthermore, field observations revealed that antibiotics were often self-administered by farmers without adequate veterinary supervision. High resistance was also observed against amoxicillin, which is consistent with studies in France and South Asia (Islam et al., 2014; Boireau et al., 2018). Moderate resistance to oxytetracycline underscores the growing concern about tetracycline-class antibiotic efficacy, as reflected in resistance trends reported by Johns & Adams (2015) (Table 8).

Table 8: Antimicrobial susceptibility profile of *Escherichia coli* isolates (n = 13)

Antimicrobial agent	Disc concentration (μg)	Susceptible (%)
Enrofloxacin	10	9.1
Amoxicillin	10	27.3
Gentamicin	10	100.0
Neomycin	30	100.0
Oxytetracycline	30	72.7

Interpretation of antimicrobial susceptibility was performed according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Intermediate results were not observed.

Streptococcus spp.

All 9 *Streptococcus* spp. isolates were fully susceptible to enrofloxacin indicating robust activity of this fluoroquinolone against streptococcal pathogens (Table 9). This finding is in consistent with earlier findings of Kaczorek et al. (2017) and Yurin et al. (2020). However, amoxicillin and oxytetracycline exhibited equal rates of resistance and susceptibility (50%) indicating variable therapeutic outcomes depending on strain and context. Such inconsistencies have also been reported by Ceniti et al. (2017) and Diri et al. (2022). Gentamicin and

neomycin were not tested due to the absence of CLSI-approved interpretive breakpoints for *Streptococcus* spp. (CLSI, 2023) to be in accordance with best practices to avoid misleading interpretations.

Table 9: Antimicrobial susceptibility profile of *Streptococcus* spp. isolates (n = 9)

Antimicrobial agent	Disc concentration (μg)	Susceptible (%)
Enrofloxacin	10	100
Amoxicillin	10	50.0
Oxytetracycline	30	50.0

Interpretation of antimicrobial susceptibility was performed according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Intermediate results were not observed.

Overall, the findings suggest that enrofloxacin demonstrated high *in vitro* efficacy against major mastitis pathogens of *S. aureus* and *Streptococcus* spp., while gentamicin or neomycin could be considered for *E. coli* infections. In situations where mixed bacterial infections are suspected, a combination approach may offer broader efficacy.

Implications for mastitis management and public health

The observed antimicrobial resistance patterns in the present study may be attributed to multiple factors, including frequent empirical treatment without culture-based diagnosis, incomplete dosage regimens and the widespread availability of antibiotics without veterinary prescription. The high resistance observed against amoxicillin and enrofloxacin suggests prolonged and possibly indiscriminate use of β-lactams and fluoroquinolones in the study area, leading to increased selective pressure and the emergence of resistant strains. In smallholder dairy systems, such practices are often exacerbated by limited access to veterinary guidance.

Environmental factors may also contribute to resistance development, as improper disposal of drug residues, manure contamination, and horizontal gene transfer among bacterial populations facilitate the persistence and spread of resistant organisms. These findings highlight the urgent need for antimicrobial stewardship programs, farmer education and stricter regulatory control of veterinary drug use.

It is important to note that *in vitro* antimicrobial susceptibility does not necessarily translate into clinical efficacy due to host factors, drug pharmacokinetics, and infection dynamics. From a One Health perspective, the detection of antimicrobial resistance in milk pathogens poses potential risks to public health through the transmission of resistant bacteria or resistance genes via the food chain. This aligns with global concerns regarding the role of livestock in the dissemination of antimicrobial resistance (WHO, 2025). Therefore, integrating mastitis control strategies with prudent antibiotic use, farmer training, and routine surveillance is essential for safeguarding both animal and human health.

CONCLUSIONS

The present study revealed that the overall and quarter-level prevalences of SCM in the Matara district of Sri Lanka are 30.0% and 11.5% respectively. Key animal-level factors including age, breed, parity, lactation stage, and milk yield, as well as farm-level practices such as herd size, milking frequency, udder hygiene, supplementary feeding, and housing cleanliness were significantly associated with SCM prevalence. *S. aureus*, *E. coli*, and *Streptococcus* spp. were identified as the predominant pathogens of SCM. High resistance rates to commonly used β -lactam antibiotics and moderate resistance to oxytetracycline raise concerns about the continued efficacy of empirical treatment protocols and emphasize the need for antibiotic stewardship in dairy farming. These findings highlight the importance of targeted management interventions, routine bacteriological testing and integrated AMR surveillance as part of a One Health approach.

This study relied solely on the CMT for the detection of SCM. It is inherently subjective and may fail to detect infections with low somatic cell count elevations or pathogens. Furthermore, the cross-sectional nature of the study limits the ability to capture seasonal patterns in SCM prevalence and AMR over time. Additionally, the study did not include molecular characterization of the bacterial isolates. Furthermore, the study did not assess farmer knowledge, attitudes, or practices regarding mastitis control and antibiotic usage which could significantly influence treatment outcomes and resistance development.

Future research should employ longitudinal designs to monitor seasonal variations in SCM prevalence and AMR trends. Incorporating molecular tools for pathogen identification and resistance gene profiling

is recommended. In addition, integrating socio-behavioral assessments such as farmer knowledge and antibiotic usage practices would provide a more holistic understanding. It would support the design of effective, locally adapted mastitis control interventions.

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RESEARCH ARTICLE

Agronomy

Long-term effects of rice husk biochar on the milling traits of rice (*Oryza sativa* L.) grown in the low country dry zone of Sri Lanka: evidence from a four-season field experiment with urea application

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Abstract: A four-season consecutive field experiment was conducted from the 2023 *Yala* season to the 2024/25 *Maha* season under low country dry zone conditions in Sri Lanka. The objective was to evaluate the combined effect of rice husk biochar (RHB) and urea fertilization on the rice milling traits hardness, chalkiness, bran weight (BW), total milled rice (TMR), hull percentage, degree of milling (DM), brown rice (BR), and head grain (HG) percentages. The rice variety At 362 was used as the test cultivar and the experiment followed a split-plot design with three replicates. The main plot treatments consisted of four RHB levels: 0, 1, 2, and 3 t ha⁻¹ (designated as BC0, BC1, BC2 and BC3) with partially burnt paddy husk (PBPH) applied at the Department of Agriculture recommended rate of 625 kg ha⁻¹. Sub-plot treatments included four levels of urea fertiliser: 0, 30, 70, 100 % of the recommended dose (U0, U30, U70, U100, where U100 = 225 kg ha⁻¹). Agricultural-grade urea commonly used by local farmers was applied following standard local practice. Results indicated that a one-time application of RHB combined with seasonal urea significantly enhanced total grain yield peaking at 6.50 t ha⁻¹ in season 3 and improved rice milling traits. Higher biochar and urea application rates led to consistent improvements in grain yield and all positive milling attributes, while significantly reducing hull percentage and grain chalkiness across all seasons, demonstrating a residual biochar effect. In comparison, PBPH resulted in significantly lower grain yield and quality improvements compared to RHB treatments. Correlation analysis revealed a strong positive relationship between grain hardness, DM % and TMR %, and

a strong negative relationship between hull percentage and BW %. Forecasting models suggest that rice milling traits can be further optimized with RHB application rates exceeding 3 t ha⁻¹. Overall, the integration of RHB with urea provides greater and more sustainable benefits for rice milling traits while increasing productivity under low-country dry zone conditions in Sri Lanka.

Keywords: Rice biochar, grain milling traits, partially burnt paddy husk, urea, rice husk

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for over half of the world's population. Ninety percent of rice production and consumption occurs in Asia (Ali et al., 2022). Rice plays a key role in Sri Lankans' daily diets and rural livelihoods. With the increase in the local population, the demand for food increases. Therefore, improving rice productivity and quality has become an urgent requirement in Sri Lanka. Due to the heavy use of synthetic fertilizers and intensive cultivation practices, existing paddy production methods are increasingly unsustainable. Fertilizer leaching and pollution are other issues related to the heavy use of synthetic nitrogen (N) fertilizers (Lai et al., 2023; Gowthami et al., 2022a;

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Adebajo et al., 2022). Industrial urea, the major nitrogen fertiliser used in paddy cultivation, is synthesized from atmospheric nitrogen via ammonia and generally contains negligible concentrations of heavy metals. Therefore, urea itself is not considered a major direct source of heavy metals, especially compared to phosphate fertilizers, which are the primary contributors of cadmium (Cd) and lead (Pb) in agricultural soils. However, in tropical rice-growing systems, heavy metal accumulation may still occur through long-term fertiliser use, irrigation with contaminated water, soil acidification, and the use of other agrochemicals, particularly phosphate fertilizers containing trace Cd and Pb. However, due to the naturally low fertility of many paddy soils, the application of synthetic fertilizers is essential for higher production. Repeated cultivation often worsens this issue, resulting in high acidity and toxic levels of aluminum (Al), iron (Fe), and manganese (Mn) (Butnan & Vityakon, 2023). These challenges highlight the need for sustainable soil management practices to restore soil health, maintain productivity, and protect environmental quality.

One promising solution is biochar, a carbon-rich byproduct produced by pyrolysis, where biomass is burned at high temperatures, usually between 300 and 1000 °C, with limited or no oxygen (Lehmann & Joseph, 2009; Verheijen et al., 2010; Karam et al., 2022). Although biochar research has become popular among scientists in recent years, it has been used since ancient times as a soil amendment. The Terra Preta highly fertile man-made soils found in the Amazon Basin, are a type of biochar used in pre-Columbian civilizations. One of the key characteristics of the carbon contained in biochar is its long-term stability, which helps to improve soil fertility and mitigate climate change (Koyama et al., 2016; Abukari et al., 2018). The high surface area and porous structure of biochar is also useful as a sustainable soil amendment.

Rice husks, the main byproduct of the rice milling process, can be converted into biochar, which reduces agricultural waste by providing an eco-friendly recycling method. Research on the application of rice husk biochar (RHB) has shown significant improvements in various physical, chemical, and biological attributes of paddy soils (Hammer et al., 2015; Rawat et al., 2019). As an example, RHB can improve soil structure, porosity, water retention, and aggregate stability, which are essential attributes for relieving the effects of water stress (Lehmann & Joseph, 2009; Jin et al., 2020) in dry zone rice cultivation. Additionally, applications of RHB can increase pH, cation exchange capacity, and carbon content, which are essential in ameliorating acidic soils. Moreover, RHB can increase soil nutrient retention and availability (Abrishamkesh

et al., 2015; Ding et al., 2022; He et al., 2017; Karam et al., 2022). In addition to improving nutrient retention, RHB may reduce heavy metal bioavailability in paddy soils through adsorption within its porous structure (Asadi et al., 2021), surface complexation by oxygen-containing functional groups (Asadi et al., 2021; Chen et al., 2023), ion exchange, mineral precipitation, and pH-induced immobilization (Asadi et al., 2021). The high silicon (Si) content of RHB may further help to reduce heavy metal uptake in rice by strengthening root tissues and reducing the mobility of toxic metals such as Cd (Karam et al., 2022; Tan et al., 2024), Pb (Asadi et al., 2021), and Cr (Karam et al., 2022; Knoblauch et al., 2021). Further, biochar has shown significant enhancement in soil microbial biomass and activity following its application (Steinbeiss et al., 2009; Ali et al., 2021). Because of these positive effects, many field and pot experiments applying RHB have demonstrated improved growth and yield parameters in rice (Asai et al., 2009; Lai et al., 2023; Jeffery et al., 2017).

When considering consumer preferences and market prices, grain milling properties such as hardness, chalkiness, head grain recovery, and milled rice percentage are important parameters. Ali et al. (2022) reported that rice husk biochar (RHB) applications can enhance brown rice recovery, milled rice percentage, and head rice recovery. They also indicated a positive correlation between these parameters and grain composition and starch deposition during grain filling. For example, Ali et al. (2022) observed reductions in chalkiness and higher head rice recovery, while Ali et al. (2020a) and Gong et al. (2020) reported improvements in starch quality parameters such as gel consistency and viscosity with the application of RHB. Other studies on the biochar effect on grain quality concluded that grain quality improved because of better nitrogen availability and enzyme activity in soils treated with biochar (Wang et al., 2014; Ali et al., 2020b). Improved nitrogen availability may also enhance grain protein content (Ali et al., 2020a; 2020b; Li et al., 2023), thereby increasing the nutritional value of rice, which is particularly important for populations relying on rice as a staple food. Although higher grain nitrogen may improve protein content, excessive nitrogen fertilization can compromise food safety and human health indirectly through nitrate contamination of groundwater and enhanced uptake of toxic heavy metals into rice grains (Ali et al., 2020a), soil acidification, and humification (Ali et al., 2020b). Soil acidification increases the solubility and bioavailability of toxic heavy metals (Asadi et al., 2021; Tan et al., 2024). Therefore, improving nitrogen use efficiency while maintaining grain quality remains a critical objective in sustainable rice production. The effects of biochar on soil properties and crop

performance can be seen after several cropping cycles due to its resistance to breakdown by microbes (Lehmann & Joseph, 2009; Bi et al., 2021). Also, the benefits of biochar are not always immediate and can vary based on soil conditions, biochar types, and management practices. Therefore, long-term field studies are needed to understand the cumulative and residual effects of biochar amendments. Although many research findings are available on the application of biochar in tropical countries, there are limited long-term studies over multiple growing seasons especially on the milling properties of rice. Milling properties significantly influence farmers' economic returns and the quality of rice available. The low-country dry zone of Sri Lanka, known for its water shortage and fragile soils, requires more research on sustainable solutions for soil improvement. Therefore, in this study, we conducted four seasonal field experiments to understand the lasting benefits of a single RHB

application in season 1 (S1), followed by another three consecutive seasons on rice milling properties under low country dry zone conditions in Sri Lanka.

MATERIALS AND METHODS

The field experiment was conducted at the Rice Research Station in Ambalantota, Sri Lanka to determine the interactive impact of biochar and urea on rice crops cultivated during four consecutive seasons: 2023 *Yala* (S1, from June to September), 2023/24 *Maha* (S2, from November to March), 2024 *Yala* (S3), and 2024/25 *Maha* (S4). The climate is classified as tropical monsoon and the mean maximum and minimum temperatures, mean relative humidity, and mean precipitation values throughout the experimental period are shown in Figure 1.

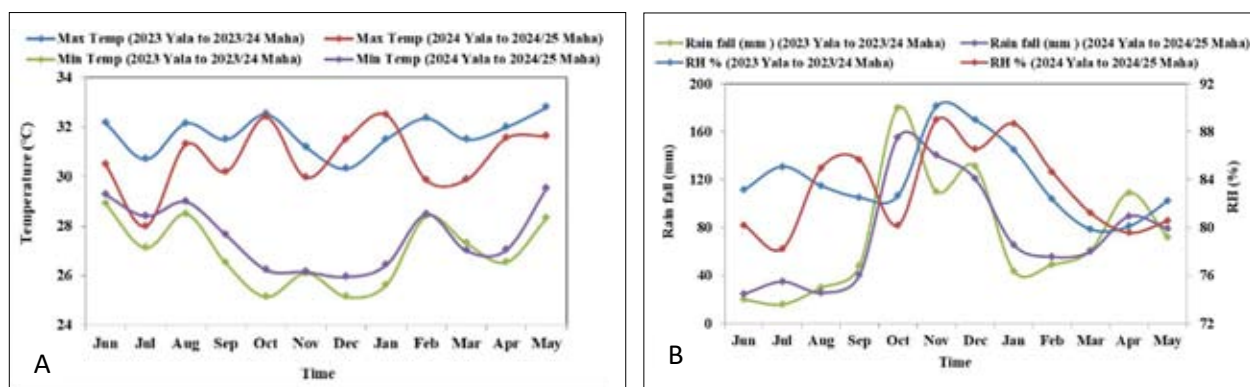


Figure 1: The variation of mean maximum and minimum temperatures (A), relative humidity, and total rainfall (B) during the experimental period from 2023 *Yala* to 2024/25 *Maha* seasons.

The soil is characterized as Grumusol (Mapa et al., 2010) and is of near natural acidity (pH 6.54), electrical conductivity $210 \mu\text{S cm}^{-1}$, soil organic matter 1.03%, soil organic carbon 0.74 % with a bulk density of 1.41 g cm^{-3} . The rice variety At 362 was used for the experiment. Four different levels of RHB at 0, 1, 2, 3 t ha^{-1} (denoted as BC0, BC1, BC2 and BC3 respectively) and the Department of Agriculture recommended level of partially burnt paddy husk (PBPH) (625 kg ha^{-1}) with four levels of urea at 0, 30, 70, 100 % (U0: without urea fertiliser, U30: 67.5 kg ha^{-1} , U70: 157.5 kg ha^{-1} , U100: 225 kg ha^{-1}) were used as treatments in this experiment. The Department of Agriculture recommended urea fertiliser amount for paddy was considered 100% (DOA, 2013). A commercially available agricultural-grade urea commonly used by local farmers was used in the experiment. Except for

urea, all other nutrients were added to each experimental unit according to the recommended rates. The treatment combinations were arranged in a split-plot design with three replicates, with biochar levels as the main-plot factor and urea levels as the subplot factor. The biochar was derived from rice husk. The rice husk was burned in a pyrolyser (thermally insulated chamber) for 8 hours in the absence of oxygen at approximately 500°C . PBPH was prepared by open environmental burning without temperature control. RHB was added as a one-time application in the first season (S1) and urea was applied in every season. Weather parameters were collected through equipment located in the research field. Milling qualities were determined through standard calculations described by Sultana et al. (2022).

At maturity, the rice plants were harvested from the entire plot and rice grain yields were weighed. The dry weight of the rice grain was determined at 14% moisture content and expressed as kg ha⁻¹. A clean paddy sample of 150 g was de-husked in a milling machine (SATAKE - THU 358, Japan) and the brown rice and hulls were separated. The brown rice (BR) percentage and hull percentage were calculated as described in Equations 1 and 2. The bran was removed from 100 g of BR and polished for 75 seconds in a grain polishing machine (SATAKE TM050C, Japan). The percentage of total milled rice (TMR) was determined from the weight of the polished rice (Equation 3). The polished rice was separated into whole and three-quarter(³/₄) kernel grains to calculate the percentage of head grains (HG) (Equation 4). The degree of milling (DM) was determined using the amount of TMR and BR using Equation 5. A sample of 100 g of the 75-second milled rice grains was counted and placed in the grain scope (TX - 200) under blue light to calculate chalkiness. Grains showing translucency with a white belly, white back, and white center were counted, and chalkiness was calculated using Equation 6. Harvested grain samples were air-dried for 36 hours until the moisture level reached 13%, after which a grain hardness tester (KIYA 174886, Japan) was used to measure the hardness values. Twenty readings from each parameter were recorded for each replicate.

$$\text{BR (\%)} = \frac{\text{Brown rice weight}}{\text{Paddy weight}} \times 100 \quad \dots(01)$$

$$\text{Hull (\%)} = \frac{\text{Weight of hull}}{\text{Weight of paddy}} \times 100 \quad \dots(02)$$

$$\text{TMR (\%)} = \frac{\text{TMR weight}}{\text{Paddy weight}} \times 100 \quad \dots(03)$$

$$\text{HG (\%)} = \frac{\text{Head grains weight}}{\text{Paddy weight}} \times 100 \quad \dots(04)$$

$$\text{DM (\%)} = \frac{\text{Weight of total milled rice}}{\text{Weight of brown rice}} \times 100 \quad \dots(05)$$

$$\text{Chalkiness (\%)} = \frac{\text{Number of translucent grains}}{\text{Total number of sample grains}} \times 100 \quad \dots(06)$$

Data from the experiments were statistically analyzed, including analysis of variance (ANOVA) and the Least

Significant Difference (LSD) test to determine the significance of the effects between biochar treatments. To compare the effects of RHB and PBPH, Dunnett's test (with PBPH as the control) was used. Analysis of variance and graphical presentations were performed using Minitab 21 software for the Windows operating system.

RESULTS AND DISCUSSION

Grain yield was significantly affected by biochar (BC), urea level (U), and season (S), with significant BC × U and BC × U × S interactions (Figure 2). Yield increased across all four seasons with increasing urea level, and this response was further enhanced by higher biochar rates. The highest grain yield was consistently recorded in the BC3U100 treatment, increasing from 5.39 t ha⁻¹ in S1 to 6.05 t ha⁻¹ in S4, indicating a strong residual effect of the one-time RHB application. In contrast, the lowest yields were observed in the zero-urea treatments across all biochar levels. Notably, in the later seasons, BC3U70 produced yields close to those of BC3U100, suggesting that residual biochar improved nitrogen use efficiency and sustained productivity even at reduced urea rates.

Figure 3 shows the biochar effects on rice grain hardness across four consecutive seasons. Application of biochar had a consistent positive effect on grain hardness. Biochar and season significantly influenced the hardness, but there was no significant effect of urea. Grain hardness increased with the application of higher rates of biochar (3 t ha⁻¹) especially when combined with urea with the highest hardness value of 4.34 N in the BC3U100 treatment in S1. Hardness values showed a gradual reduction with the season. However, the gradual reduction was less with the application of biochar.

The variation of chalkiness during four consecutive seasons with the effect of biochar and urea is shown in Figure 4. Data indicate that the grain chalkiness decreased with increasing biochar levels, particularly under BC2 and BC3 with urea. The significantly highest chalkiness (18 %) was recorded in the BC0U100 treatment in S1, while the significantly lowest chalkiness (8.67 %) was recorded in the BC2U100 treatment in S2, which is not significantly different from the BC3U100 treatment in S2. Only biochar and season, significantly affected the chalkiness; urea alone did not have a significant effect (Figure 4).

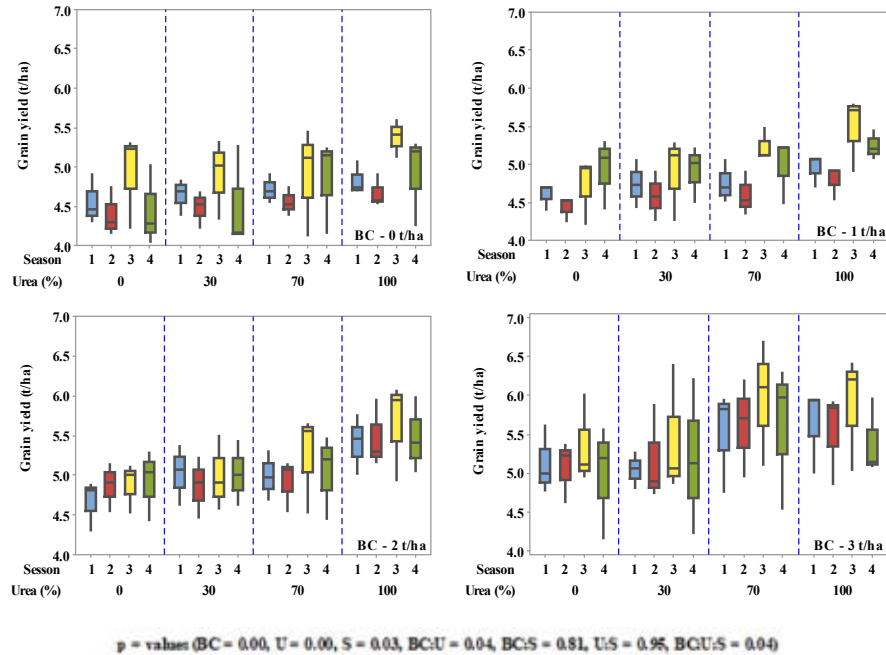


Figure 2: Effect of rice husk biochar and urea levels on grain yield in four consecutive seasons from 2023 Yala to 2024/25 Maha. The four graphs represent four biochar levels. Numbers from 1 to 4 and 0 to 100 on the x-axis represent the four consecutive cropping seasons and urea levels added, respectively. BC: rice husk biochar; U: urea; S: season.

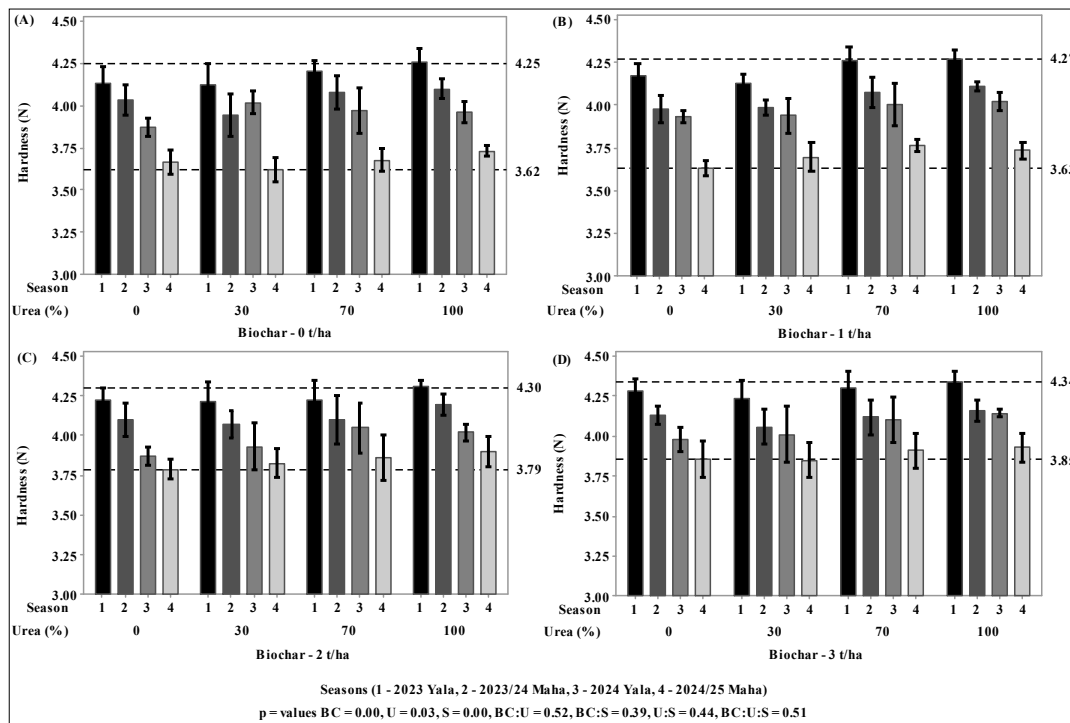


Figure 3: Effect of rice husk biochar and urea levels on grain hardness in four consecutive cropping seasons from 2023 Yala to 2024/25 Maha. The four graphs represent four biochar levels. Vertical bars represent the standard error of the mean. Numbers from 1-4 and 0-100 on the x-axis represent the four consecutive seasons and urea levels added, respectively. BC: rice husk biochar; U: urea; S: season.

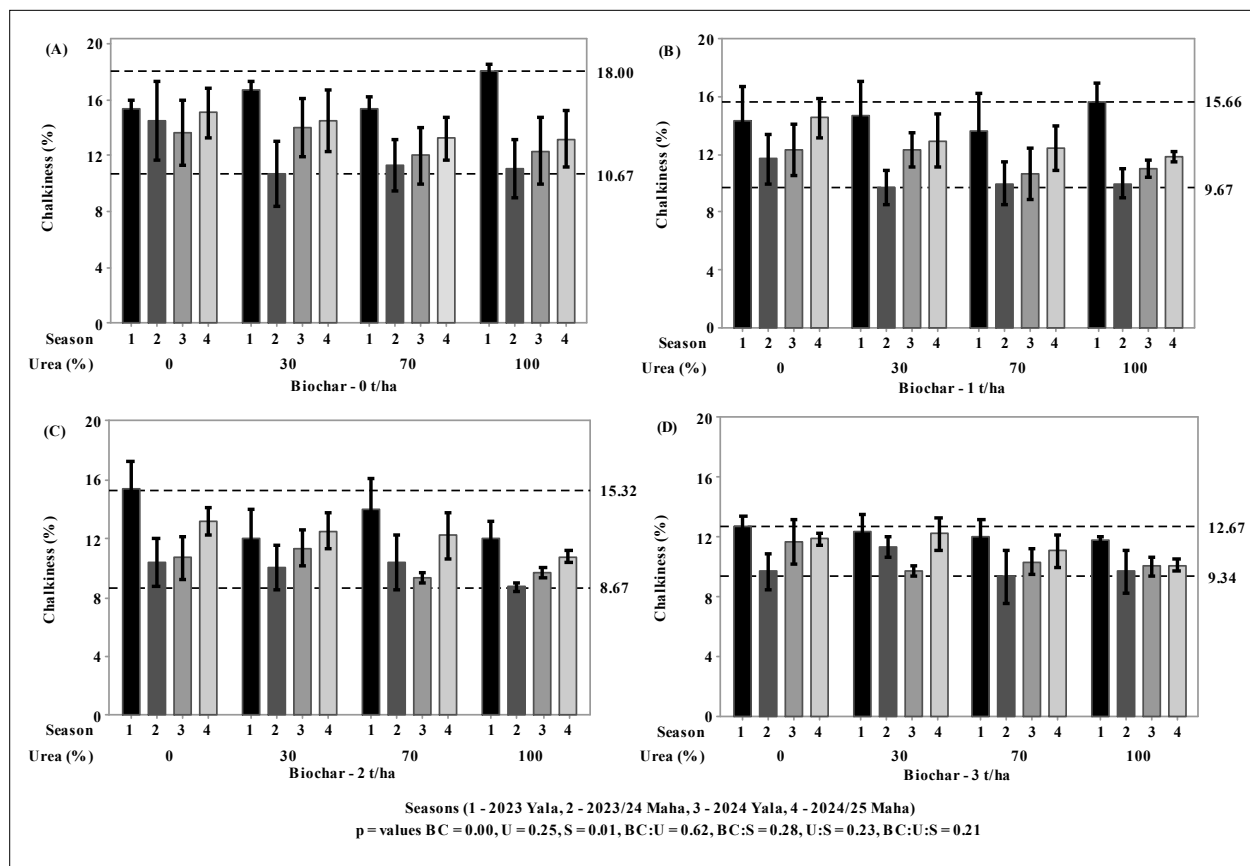


Figure 4: Effect of rice husk biochar and urea levels on grain chalkiness in four consecutive cropping seasons. The four graphs represent four biochar levels. Vertical bars represent the standard error of the mean. Numbers from 1 - 4 and 0 - 100 on the x-axis represent the four consecutive seasons and urea levels added, respectively. BC: rice husk biochar; U: urea; S: season.

Table 1 shows the variation of BR, TMR and HG percentages during four consecutive cropping seasons after the application of different levels of biochar in the first season and different levels of urea in each subsequent season. All three parameters were significantly affected by the rate of biochar, rate of urea, and the seasonal changes. The interaction between biochar and season (BC:S) significantly affected BR %, while the interaction between urea and season (U:S) significantly affected HG %.

The BR% increased gradually with higher biochar rates, reaching the highest levels in BC3U100 (78.53 %, 80.07 %, 80.65 %, and 77.84 % in S1 – S4, respectively). In contrast, the lowest BR % came from the BC0 treatments suggesting that biochar application improves husk removal efficiency, especially when paired with higher urea levels. TMR represents the overall recovery

of edible rice after milling and polishing. In all seasons, TMR % increased steadily with more biochar application, with the highest values recorded in BC3U100 (71.20 %, 72.18 %, 73.07 % and 70.49 % in S1 - S4, respectively). HG % represents the proportion of whole grains within milled rice reflecting resistance to breakage during milling. Higher HG % also increases the market price. The highest significant HG % was consistently recorded in the BC3U100 treatment across all seasons.

Table 2 shows the variation of BW, DM, and hull percentages with the application of biochar and urea during four consecutive seasons. Significantly highest BW % and DM % and lowest hull % were observed in the BC3 treatment with higher urea levels in every season. BC:S interaction significantly affected BW %, whereas U:S interaction significantly affected DM %.

Table 1: Brown rice, total milled rice and head grains percentages influenced by different rice husk biochar and urea fertilization levels from 2023 Yala to 2024/25 Maha

Treat.	BR (%) 2023 Yala	BR (%) 2023/24 Maha	BR (%) 2024 Yala	BR (%) 2024/25 Maha	TMR (%) 2023 Yala	TMR (%) 2023/24 Maha	TMR (%) 2024 Yala	TMR (%) 2024/25 Maha	HG (%) 2023 Yala	HG (%) 2023/24 Maha	HG (%) 2024 Yala	HG (%) 2024/25 Maha
BC0U0	76.63 ^d	78.84 ⁱ	78.58 ⁱ	74.64 ^e	67.73 ^{gh}	69.71 ^g	69.22 ^k	68.08 ^g	59.33 ^h	60.98 ^k	60.91 ⁱ	59.08 ^h
BC0U30	77.41 ^{bcd}	78.98 ^h	79.36 ^j	76.39 ^{bcd}	67.34 ^h	69.99 ^f	70.00 ^j	68.23 ^{fg}	59.91 ^{gh}	61.49 ^k	61.54 ^h	59.93 ^h
BC0U70	77.40 ^{bcd}	79.61 ^d	79.53 ^{ghij}	76.21 ^{bcd}	69.37 ^{de}	70.69 ^d	70.37 ⁱ	68.98 ^{cd}	61.35 ^{ef}	63.60 ^{gh}	62.10 ^{fg}	62.04 ^{ef}
BC0U100	77.36 ^{bcd}	79.74 ^c	79.72 ^{efg}	76.21 ^{bcd}	69.87 ^{bcd}	70.85 ^d	70.89 ^{gh}	68.82 ^{de}	63.07 ^{bce}	64.32 ^{def}	62.31 ^f	62.09 ^{ef}
BC1U0	76.87 ^{cd}	79.08 ^h	78.70 ⁱ	75.85 ^d	67.78 ^{gh}	70.42 ^e	69.36 ^k	68.37 ^{efg}	60.57 ^{gh}	62.16 ⁱ	61.91 ^g	60.41 ^{gh}
BC1U30	77.38 ^{bcd}	79.26 ^g	79.10 ^k	75.70 ^d	69.91 ^{bcd}	70.89 ^d	70.33 ⁱ	69.20 ^{bcd}	61.22 ^{ef}	63.14 ^h	62.43 ^{ef}	61.58 ^{fg}
BC1U70	77.51 ^{bc}	79.56 ^{de}	79.50 ^{hij}	76.26 ^{bcd}	69.72 ^{cde}	71.36 ^c	71.20 ^{ef}	69.47 ^{bc}	61.81 ^{de}	64.06 ^{efg}	62.79 ^{de}	62.33 ^{ef}
BC1U100	77.49 ^{bc}	79.95 ^b	79.68 ^{efgh}	77.41 ^{bc}	70.14 ^{bcd}	71.37 ^c	71.02 ^{fg}	69.68 ^b	63.03 ^{bce}	64.68 ^d	63.38 ^c	63.01 ^{de}
BC2U0	77.50 ^{bc}	79.04 ^h	79.43 ^{ji}	76.08 ^{cd}	68.37 ^{fg}	70.68 ^d	70.74 ^h	69.09 ^{cd}	60.38 ^{gh}	61.97 ^{ij}	62.45 ^{ef}	65.61 ^c
BC2U30	77.51 ^{bc}	79.39 ^f	79.80 ^{lef}	75.82 ^d	69.33 ^{lef}	70.64 ^{de}	71.51 ^d	68.78 ^{def}	60.89 ^{efg}	63.14 ^h	63.32 ^c	63.37 ^{de}
BC2U70	77.52 ^{bc}	79.55 ^{de}	79.87 ^{ale}	77.39 ^{bc}	70.16 ^{bcd}	71.47 ^c	71.98 ^c	69.78 ^b	62.64 ^{bcd}	63.89 ^g	63.57 ^e	64.38 ^{cd}
BC2U100	78.50 ^{bc}	79.96 ^b	80.02 ^{bc}	77.40 ^{bc}	69.76 ^{cde}	71.87 ^b	72.36 ^b	69.81 ^b	63.00 ^{bce}	64.57 ^{de}	64.10 ^b	64.15 ^d
BC3U0	77.34 ^{bcd}	79.21 ^g	79.60 ^{ghij}	76.28 ^{bcd}	69.09 ^{ef}	70.40 ^e	71.12 ^f	68.38 ^{efg}	61.88 ^{cde}	69.80 ^e	62.91 ^d	63.91 ^d
BC3U30	77.53 ^{bc}	79.47 ^{ef}	79.94 ^{bcd}	76.39 ^{bc}	69.52 ^{cde}	70.82 ^d	71.39 ^{de}	69.47 ^{bc}	62.41 ^{bcd}	71.23 ^b	63.30 ^c	67.67 ^b
BC3U70	77.74 ^{ab}	79.91 ^b	80.12 ^b	77.05 ^{ab}	70.78 ^{ab}	71.76 ^b	72.32 ^b	69.74 ^b	63.06 ^{bce}	71.54 ^b	64.03 ^b	68.65 ^{ab}
BC3U100	78.53 ^a	80.07 ^a	80.65 ^a	77.84 ^a	71.20 ^a	72.18 ^a	73.07 ^a	70.49 ^a	64.49 ^a	72.20 ^a	65.10 ^a	69.98 ^a
BC	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*
U	S**	S**	S**	S**	S**	S**	S**	S**	S**	S**	S**	S**
S	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*
BC:U	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
U:S	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
BC:S	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*
BC:U:S	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Note: BR: brown rice; TMR: total milled rice; HG: head grains; BC: rice husk biochar; U: urea; S: season; BC0, BC1, BC2 and BC3, represent 0, 1, 2 and 3 t ha⁻¹ biochar levels, respectively. U0, U30, U70 and U100 represent 0, 30, 70 and 100% urea levels based on recommended rates, respectively. Values followed by the same letters, within columns are not significantly different at $p \leq 0.05$. * Indicates significant difference at $p \leq 0.05$ and ** indicates $p \leq 0.01$.

Rice bran is a milling byproduct, and the BW % provides an estimate of the proportion of the outer layer (bran) removed during the polishing process. DM % reflects the extent of bran removal during milling; therefore, a higher

DM % corresponds to a higher BW %. DM% improved with increasing RHB, and the effect was more pronounced under higher urea content. However, the rate of milling gradually reduced across the seasons indicating the

Table 2: Bran weight, hull and degree of milling percentages as influenced by different rice husk biochar and urea fertilization levels from 2023 Yala to 2024/25 Maha season.

Treat.	BW (%) 2023 Yala	BW (%) 2023/24 Maha	BW (%) 2024/25 Maha	Hull (%) 2023 Yala	Hull (%) 2023/24 Maha	Hull (%) 2024 Yala	Hull (%) 2024/25 Maha	DM (%) 2023 Yala	DM (%) 2023/24 Maha	DM (%) 2024 Yala	DM (%) 2024/25 Maha
BC0U0	9.01 ⁱ	8.27 ^k	9.08 ^e	24.96 ^a	27.71 ^a	21.81 ^a	25.35 ^a	75.09 ^{hi}	74.17 ^{ef}	74.09 ⁱ	71.92 ^{def}
BC0U30	9.15 ^h	8.43 ^j	9.37 ^{cd}	23.15 ^{cd}	27.25 ^{bc}	21.39 ^b	23.94 ^{bc}	75.90 ^{defg}	74.28 ^e	74.73 ^{de}	72.02 ^{cdef}
BC0U70	9.22 ^{gh}	8.61 ^{hi}	9.43 ^c	23.18 ^{cd}	27.24 ^{bc}	21.20 ^c	23.05 ^{cde}	75.94 ^{defg}	74.35 ^e	74.16 ^{hi}	72.09 ^{cdef}
BC0U100	9.28 ^g	8.54 ⁱ	9.53 ^b	23.56 ^{bcd}	27.25 ^{bc}	20.74 ^d	22.48 ^e	75.97 ^{def}	74.68 ^d	74.37 ^{gh}	72.75 ^{bcd}
BC1U0	9.52 ^{ef}	8.60 ^{hi}	9.10 ^e	23.71 ^{bcd}	26.99 ^d	21.29 ^{bc}	24.14 ^b	76.18 ^{cde}	74.35 ^e	74.47 ^{fg}	71.35 ^f
BC1U30	9.53 ^{def}	8.65 ^{gh}	9.39 ^{cd}	23.8 ^{bcd}	26.83 ^{de}	20.89 ^d	24.30 ^b	76.18 ^{cde}	74.14 ^{ef}	74.81 ^{de}	71.81 ^{ef}
BC1U70	9.60 ^{bc}	8.71 ^{fg}	9.41 ^c	23.90 ^{bc}	26.89 ^d	20.49 ^{ef}	23.73 ^{bcd}	75.41 ^{ghi}	74.36 ^e	74.96 ^{bcd}	72.03 ^{cdef}
BC1U100	9.60 ^{bc}	8.78 ^{ef}	9.42 ^c	23.12 ^{cd}	27.31 ^b	20.31 ^{gh}	22.41 ^c	75.91 ^{defg}	74.69 ^d	74.89 ^{cde}	72.52 ^{bc}
BC2U0	9.54 ^{def}	8.71 ^{fg}	9.38 ^{cd}	24.09 ^b	27.01 ^{cd}	20.57 ^e	23.92 ^{bc}	75.32 ^{ghi}	74.06 ^f	74.67 ^{ef}	72.38 ^{bde}
BC2U30	9.57 ^{cdef}	8.81 ^e	9.43 ^c	24.07 ^b	27.33 ^b	20.20 ^{hi}	24.17 ^b	75.86 ^{defg}	74.27 ^{ef}	74.86 ^{cde}	72.41 ^{bde}
BC2U70	9.60 ^{bc}	8.95 ^{bc}	9.53 ^b	23.58 ^{bcd}	26.76 ^{de}	20.12 ^{ij}	23.02 ^{cde}	76.41 ^{bcd}	75.14 ^c	74.96 ^{bcd}	72.52 ^{bc}
BC2U100	9.60 ^{bc}	8.99 ^b	9.54 ^b	23.17 ^{cd}	26.60 ^e	19.97 ^{kl}	22.30 ^e	76.40 ^{bcd}	75.29 ^{bc}	74.96 ^{bcd}	72.52 ^{bc}
BC3U0	9.46 ^f	8.85 ^{de}	9.40 ^c	23.18 ^{cd}	27.26 ^{bc}	20.39 ^{fg}	23.68 ^{bcd}	75.72 ^{dgh}	74.79 ^d	74.74 ^{de}	71.87 ^{ef}
BC3U30	9.54 ^{def}	8.89 ^{cd}	9.53 ^b	23.65 ^{bcd}	27.30 ^b	20.06 ^{ji}	23.08 ^{cde}	76.34 ^{bcd}	75.39 ^b	74.86 ^{cde}	72.52 ^{bc}
BC3U70	9.70 ^{ab}	8.98 ^b	9.59 ^{ab}	23.51 ^{bcd}	26.58 ^e	19.88 ^k	22.94 ^{de}	76.73 ^{abc}	75.47 ^{ab}	75.12 ^{ab}	72.98 ^{ab}
BC3U100	9.74 ^a	9.09 ^a	9.63 ^a	23.05 ^d	26.55 ^e	19.35 ^l	22.15 ^e	77.13 ^a	75.54 ^a	75.34 ^a	73.62 ^a
BC			s**			s*				s**	
U			s*			s*				s*	
S			s**			s*				s*	
BC:U			ns			ns				ns	
U:S			ns			ns				s**	
BC:S			s*			ns				ns	
BC:U:S			ns			ns				ns	

Note: BW: Bran weight; DM: Degree of milling; BC: Rice husk biochar; U: Urea; S: Season; BC0, BC1, BC2 and BC3, represent 0, 1, 2 and 3 t/ha biochar levels, respectively; while U0, U30, U70 and U100 represent 0, 30, 70 and 100% urea levels based on the recommended rates, respectively. Values followed by the same letters, within the columns, are not significantly different at $p \leq 0.05$. * Indicates a significant difference at $p \leq 0.05$ and ** indicates $p \leq 0.01$.

reduction of residual effect with the season. Significantly lower DM % was recorded in BC0U0 treatments in every season. Reduced hull % indicates lower generation of agricultural waste during rice milling. Hull % has no

significant influence on final consumer preference. The significantly lowest hull% was observed in the BC3U100 treatments.

There was no significant 3-way interaction (BC:U:S) for the grain milling traits measured in this study, indicating that the treatment responses were consistent across seasons. In all seasons, the best milling quality was achieved from the BC3U100 treatment.

The correlation analysis of the heat map among rice grain milling properties is shown in Figure 5. The highest positive correlations were observed between grain hardness and DM % (0.74) and between TMR % and BR % (0.78). The highest negative correlation was observed between BW % and hull % (-0.73). Grain hardness showed a positive correlation with all milling parameters except chalkiness, whereas chalkiness showed positive correlation with all parameters except BW %.

In Sri Lanka, the recommended application rate of PBPH is 625 kg ha⁻¹, primarily to increase the pH of acidic soils. In the present study, the effects of the recommended PBPH rate were compared with different levels of RHB treatments. The comparative results of the RHB and PBPH treatments are presented in Table 3. Among the treatments, BC3 had significantly superior milling quality traits compared with the PBPH treatment, except for BW%, hull percentage, and chalkiness. For TMR%, all biochar treatments showed significantly higher values than the PBPH treatment, whereas grain hardness was significantly greater in BC2 and BC3 than in the PBPH treatment. The zero-biochar treatment showed a significantly lower BW% and higher chalkiness compared with PBPH application.

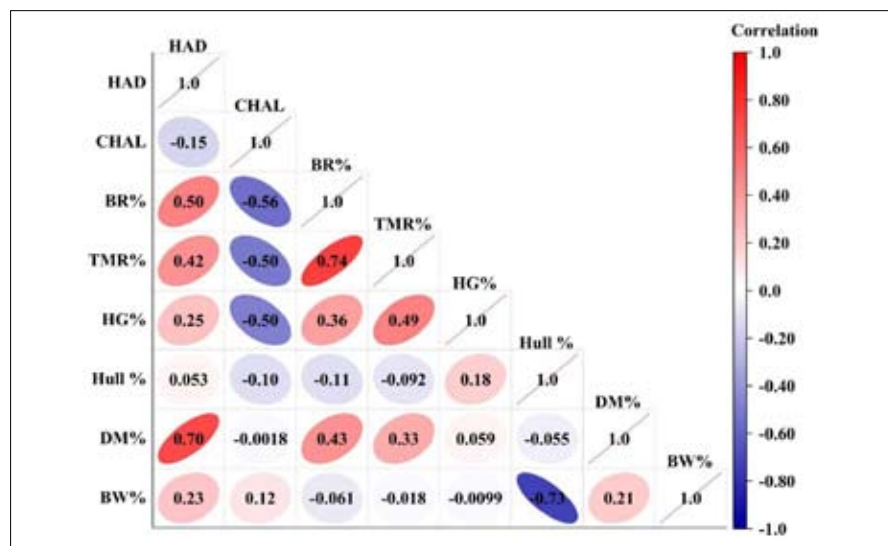


Figure 5: Heatmap correlation matrix representing Pearson correlation coefficients among grain milling traits of rice. CHAL: grain chalkiness; BR: brown rice; TMR: total milled rice; HG: head grain; Hull: rice husk; DM: degree of milling; BW: bran weight; HAD: grain hardness.

Table 3: Dunnet's comparison of milling properties of rice

Treatment	BW(%)	DM(%)	Hull(%)	HG(%)	TMR(%)	BR(%)	CHAL(%)	HAD(N)	GY (t/ha)
PBPH	9.26	74.07	24.18	63.48	68.96	77.89	11.64	3.89	4.36
BC0	9.03*	74.14	24.05	61.51	69.39	77.44	13.58*	3.90	4.68
BC1	9.21	74.24	23.79	62.43	70.03*	78.13	12.31	3.98	4.75
BC2	9.38	74.60	23.55	62.26	70.45*	78.40	11.25	4.04*	5.10*
BC3	9.40	74.90*	23.39	66.44*	70.74*	78.64*	10.58	4.09*	5.09*

Note: Means labeled with * are significantly different from the control treatment mean at $\alpha = 0.05$. BW: Bran weight; DM: Degree of milling; Hull: Hull amount; HG: Head grains; BR: Brown rice; CHAL: Chalkiness; HAD (N): Hardness in Newton; GY: Grain yield in t ha⁻¹; BC: Rice husk biochar; PBPH: Partially burnt paddy husk (the control treatment for Dunnett's test analysis).

The forecasting models of the effects of further increases in RHB on grain milling traits are presented in Figure 6. Increasing the biochar level from 3 to 4 t ha⁻¹ resulted in increases of 1.61% in grain hardness, 1.43% in HG,

and 0.41% in TMR, along with a 6.57% reduction in chalkiness (Figure 6). These findings further highlight the benefits of applying higher levels of RHB as a soil amendment for rice cultivation.

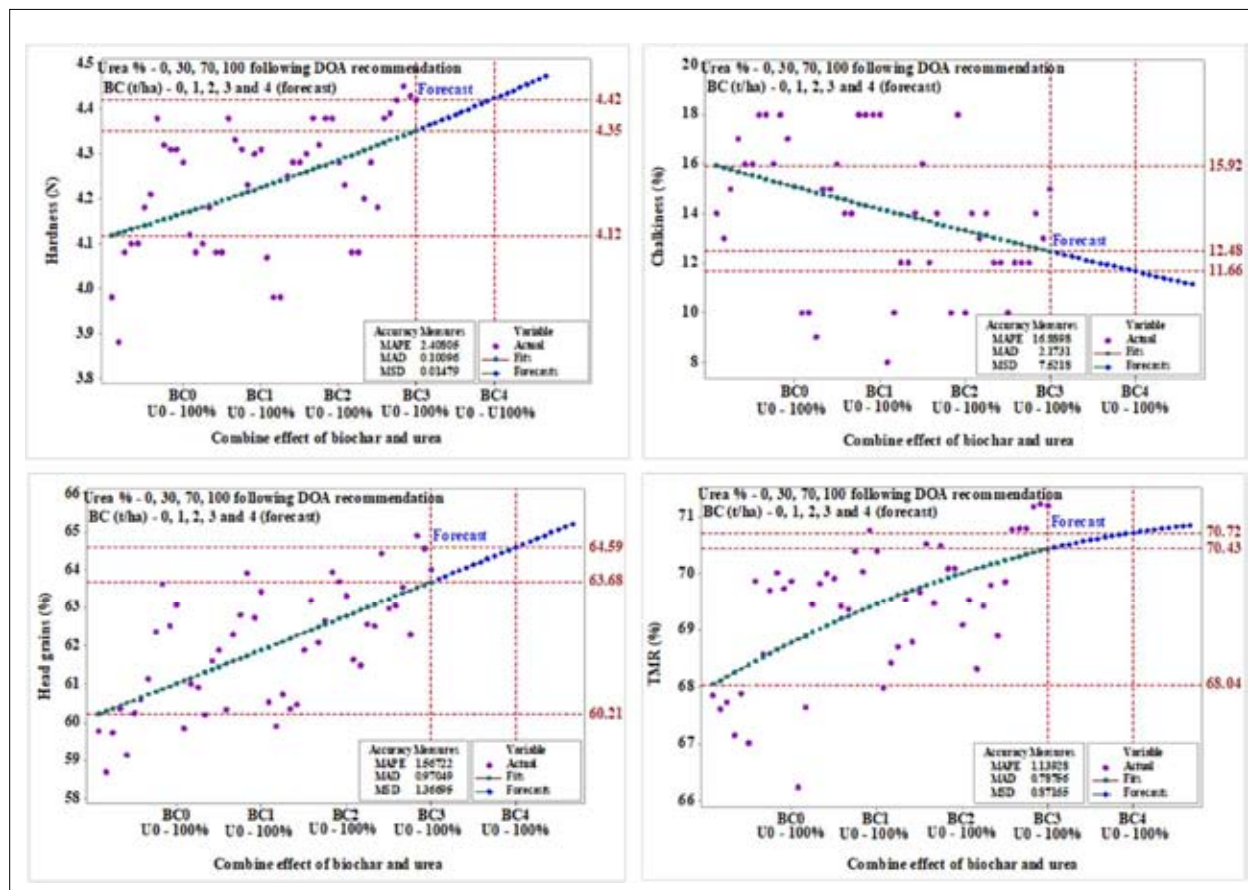


Figure 6: Forecasting the effect of further increments of rice husk biochar on grain hardness, chalkiness, head grain and total milled rice percentages (TMR%).

The data presented in this study indicated consistent positive effects on rice grain yield and milling traits with the application of higher levels of RHB. The residual effect of RHB across seasons shows that even a single application can sustainably enhance grain yield and milling traits over multiple cropping cycles. Similar to this study, many other researchers have reported that the application of biochar, combined with N fertiliser, often exerts positive effects on grain yield and quality traits (Ali et al., 2020a; Chen et al., 2023; Du et al., 2024; He et al., 2024). However, the specific impact of nitrogen on grain quality may depend heavily on the source and rate of application. Earlier studies have reported that

synthetic nitrogen fertilization alone can deteriorate rice cooking quality by reducing apparent amylose content and gel consistency (Zhang et al., 2008; Bryant et al., 2012; Iqbal et al., 2022), while excessive nitrogen may also interfere with starch structural order (Zhang et al., 2023). In contrast, under integrated biochar–urea systems, the increased grain nitrogen and protein contents are often associated with improved milling recovery, reduced chalkiness, and better grain appearance (Iqbal et al., 2022; Zhang et al., 2023). Therefore, the positive effects observed in the present study may be linked with balanced nitrogen availability under biochar-amended conditions rather than the effect of urea alone. This

improved nutrient balance also may have contributed to enhanced overall productivity (Figure 2), supporting sustained yield trends across multiple seasons. These results agree with previous findings that moderate nitrogen management can sustain grain yield at levels comparable to higher nitrogen rates while simultaneously improving grain texture and starch quality, indicating opportunities for more sustainable fertiliser strategies without yield penalties (Li et al., 2023). While urea contains a small fraction of carbon, most of which is rapidly lost as CO₂ upon application, contributes minimally to soil organic carbon dynamics.

Grain hardness is a critical determinant of rice milling quality and market value. It has significant influences on both HG % and TMR %. Physically harder grains are more resistant to breakage during the intensive hulling and polishing processes, resulting in a higher recovery of whole kernels which give superior selling prices. The results of the present study indicate that a single application of RHB can significantly influence grain hardness. The forecasting models (Figure 6) also suggest that further increments in biochar application beyond 3 t ha⁻¹ will continue to enhance grain hardness. The impact of biochar on grain structural integrity is possibly mediated through changes in starch and protein deposition. Gong et al., (2020) reported a direct impact of RHB on grain hardness and reduced chalkiness in several rice cultivars. They noted that grain hardness is closely associated with the structure of starch, especially the proportions of amylose and long-branched amylopectin. Furthermore, Li et al. (2023) reported that nitrogen-induced changes in starch structure may also contribute significantly to this response; higher N availability is associated with smaller, more uneven starch granules, an increase in short-chain amylopectin, and greater relative crystallinity. These structural changes promote a more compact starch organization, which physically harden the grain (Li et al., 2023).

High Si content of RHB also plays a vital role in enhancing grain quality (Karam et al., 2022). Si-rich amendments like RHB have been shown to reduced grain chalkiness, a trait that makes grains more fragile and prone to breakage during milling (Gong et al., 2020; He et al., 2024). The “nutrient sponge” effect of biochar further ensures better grain filling by improving soil nutrient and moisture retention (Karam et al., 2022; Selvarajh et al., 2024).

In this study, the seasonal application of urea may complement this effect by providing the nitrogen necessary for protein synthesis (Gowthami et al., 2022b).

Research by Gowthami et al., (2022c) confirms that higher nitrogen levels increase the density of protein bodies, which function as physical binders between starch granules, thereby increasing the mechanical resistance of the grain to milling breakage. However, it is essential to distinguish between the benefits of nitrogen for milling and its potential drawbacks for eating quality. While increased nitrogen contributes positively to raw grain hardness and milling recovery, excessive application can deteriorate cooking quality by reducing apparent amylose content and gel consistency, leading to a harder, less palatable cooked texture (Iqbal et al., 2022; Li et al., 2023). Therefore, the improved hardness and milling efficiency observed in the present study are best attributed to the synergistic effect of biochar-mediated nutrient stabilization and balanced nitrogen supply, rather than the effects of urea alone. Adherence to recommended urea rates in this study ensured optimised grain structural integrity while minimizing potential adverse impacts on soil health or biological activity.

Chalky grains are characterized by a loose and porous arrangement of starch granules in the endosperm, making them highly fragile and prone to breakage due to physical stresses of the milling process (Du et al., 2024). This fragility directly decreases both the HG % and sometimes TMR %. Statistical analysis in this study confirmed this relationship with correlation coefficients between chalkiness and HG % and TMR % recorded at -0.48 and -0.49, respectively (Figure 5). The forecast analysis (Figure 6) suggests that a further increment in biochar applications beyond 3 t ha⁻¹ will continue to reduce chalkiness while progressively increasing HG % and TMR %. Consequently, reducing grain chalkiness is a primary strategy for enhancing grain appearance, cooking quality and market value (Ali et al., 2022; He et al., 2024). Therefore, grains with low chalkiness can significantly increase the market price and consumer preference. The results of the present study demonstrate that higher levels of RHB and urea application effectively reduce chalkiness. These findings are consistent with recent reports showing that integrated biochar and nitrogen management can increase grain protein content while simultaneously reducing both the chalky grain rate and the degree of chalkiness (Khan et al., 2023; Du et al., 2024; Jin et al., 2024). In such systems, the higher grain nitrogen content should be interpreted as a marker of improved grain filling and grain quality rather than as a direct cause of quality decline (Ali et al., 2020a).

Beyond soil conditions, the environmental factors such as temperature and rainfall also significantly impact chalkiness formation. Chen et al. (2023) reported that

the application of biochar and nitrogen fertiliser can compensate for the environmental effects to some extent. Fahad et al. (2016) also showed that high night-time temperature can increase chalkiness, but the application of RHB and phosphorus fertiliser can reduce chalkiness possibly because of higher water availability. Similarly, Du et al. (2024) and Chakraborty et al. (2021) found that biochar can compensate for the increased chalkiness under no-tillage conditions and drought stress at the grain filling stage respectively. Zhang et al. (2023) also reported a decreasing trend in chalkiness following the application of crop straw biochar in fragrant rice. These inter-seasonal fluctuations are closely linked to the stability of the grain-filling process (Chen et al., 2023). Disruptions during this stage, such as those reported under combined salinity and heavy metal stress, lead to impaired structural integrity and significant reductions in head rice yield (Rifasa et al., 2025). Biochar's role in improving soil structure and nutrient use efficiency promotes more uniform grain filling, thereby preventing the formation of chalky regions (He et al., 2024).

Although heavy metal contamination was not explicitly assessed in the present study, previous research on the At 362 rice variety indicates that stressors such as Cd and As can severely disrupt energy metabolism during grain filling, further increasing grain fragility and chalkiness (Rifasa et al., 2025). Therefore, the use of RHB serves as a robust buffer against both soil and environmental stressors to maintain superior rice quality.

Grain hardness is a fundamental trait influencing milling quality, as demonstrated by its positive correlation with TMR % and DM % (Rifasa et al., 2025). Physically harder grains are more resistant to mechanical breakage during processing, resulting in a higher percentage of whole kernels that command superior market prices (Rifasa et al., 2025). The correlation coefficients of 0.45 and 0.78 in this study quantify the positive relationships of grain hardness of TMR % and DM %, respectively. Enhanced nitrogen availability is likely to contribute to this grain structural integrity by increasing the density of protein bodies, which function as binders between starch granules to harden the physical structure of the raw grain (Gowthami et al., 2022c; Li et al., 2023). The forecasting analysis (Figure 6) suggests that further increases in the application of RHB and urea will continue to improve these parameters. Higher TMR % and DM % scores indicate superior milling efficiency, resulting in a higher yield of polished rice with minimal breakage even if total production remains stable (Rifasa et al., 2025). Conversely, extreme environmental conditions, such as

salinity, Cd, and As stress, have been shown to drastically reduce head rice yield and milled grain weight while increasing the degree of milling (Rifasa et al., 2025).

The positive impact of integrated amendments is supported by Alli et al. (2020b) who reported a 5 % increase in TMR % when evaluating the combined effect of cassava straw biochar and nitrogen levels in a two-season study. Similarly, Ali et al. (2022) observed improvements in grain milling attributes following the co-application of biochar and N fertiliser. However, results may vary depending on the variety and management practices; for instance, Gowthami et al. (2022c) reported that while high nitrogen levels significantly increased TMR % in direct-seeded rice, the effect of RHB on the degree of milling was not statistically significant in their specific trial.

Biochar also serves as a critical buffer against environmental stressors that degrade quality. Fahad et al. (2016) observed that while temperature stress can negatively affect milling traits, the application of RHB combined with phosphorus fertiliser can compensate for these effects by improving water availability during grain filling (Ali et al., 2020b). Furthermore, Du et al. (2024) reported an enhanced fine rice rate as a key measure of milling recovery and increased protein content with the application of biochar across both the main season and ratoon seasons. These collective findings underscore that the synergistic effect of biochar and balanced nitrogen fertilization is essential for maintaining the structural maturity and milling efficiency of rice (Li et al., 2023).

Results of this study indicate that treatments receiving higher biochar and urea consistently had significantly lower hull % across all seasons. Although hull % may have less impact on the market price, compared to head rice yield, reducing the hull proportion is a critical indicator of processing efficiency. A significant increase in BR %, the grain remaining after husk removal, was observed with higher rates of RHB and urea treatments suggesting that the integrated application of these amendments promoted endosperm development, thereby lowering the proportion of hull. This improvement in BR % is economically significant, since a higher proportion of harvested paddy can be converted into a marketable product, thus indirectly contributing to the total usable yield (Zhang et al., 2023). These findings highlight that the synergistic effect of biochar and urea is a key driver of long-term productivity, grain filling and endosperm maturation (Ning et al., 2022; Du et al., 2024; Selvarajh et al., 2024).

The observed increase in BR % is corroborated by several recent studies: a meta-analysis by He et al. (2024) found that biochar application significantly elevated the brown rice rate by an average of 0.5 %, with the most favorable effects occurring in soils with low organic matter and high-temperature biochar derived from husks. Du et al. (2024) reported increases of 1.0 % and 1.5 % in BR % under rotary tillage and no-tillage conditions, respectively, following rice straw biochar application. Similarly, Zhang et al. (2023) observed that biochar significantly increased BR % and TMR % for the Meixiangzhan2 (MXZ2) cultivar, although responses can vary by genotype. Chakraborty et al. (2021) found that biochar application significantly increased BR % and HG % even under drought stress indicating that biochar stabilizes grain development during critical growth stages. While Chen et al. (2023) noted that the impact on TMR % can sometimes be inconsistent across seasons, they found that lower biochar dosages generally maintain higher quality of appearance. Furthermore, Gowthami et al. (2022c) noted that while rice husk biochar alone may not always show a statistically significant impact on the degree of milling, the application of urea significantly increased TMR % by enhancing grain boldness.

Results of the present study showed significant improvements in all evaluated milling traits. These findings imply that while urea application alone had a minimal impact, the integrated application of RHB and urea significantly enhances grain filling and minimizes kernel breakage during the milling process. Furthermore, the consistent improvement of milling traits across seasons in treatments with higher biochar and urea levels suggests that a single RHB application has a significant long-term cumulative effect on grain quality in the low country dry zone. These enduring enhancements in grain quality are likely complement the sustained yield increases observed across cropping cycles, highlighting the dual benefits of biochar for agricultural productivity and grain quality.

A comparison with available literature reveals mixed responses regarding the impact of integrated biochar-urea systems on rice milling traits (He et al., 2024). These inconsistencies may arise not only from variations in biochar feedstock, application rates, environmental conditions, and cultivars, but also from differences in fertiliser quality and potential trace-level impurities, which may vary among fertiliser sources. Although urea is generally not considered as significant source

of heavy metal contamination, variations in fertiliser composition and soil chemical conditions may indirectly influence enzymatic processes involved in grain development (Li et al., 2023; Rong et al., 2024; Rifasa et al., 2025). Since enzyme activity is highly sensitive to pH, temperature, and ionic strength, such factors may partially explain the discrepancies in grain hardness and other milling traits reported among different studies (Li et al., 2023; Rifasa et al., 2025). While the present study did not assess trace metal concentrations in the applied fertiliser, such effects are likely minimal in urea and remain a speculative but plausible factor for future investigation. Additionally, previous research suggests that varietal differences in resilience to environmental and heavy metal stressors strongly influence milling outcomes, with specific cultivars maintaining greater stability in head rice yield and grain physical traits even under severe stress conditions (Rifasa et al., 2025).

The Department of Agriculture, Sri Lanka through the Yaya programme recommends the application of PBPH at a rate of 625 kg ha⁻¹. Since PBPH is produced from uncontrolled incomplete burning with an ample amount of oxygen, the carbon conversion efficiency is as low as 55% with a high amount of ash (Asadi et al., 2021). Therefore, PBPH paddy husk is technically described as a mixture of ash and high-carbon char (Asadi et al., 2021). Results of the present study indicate that RHB particularly at the 3 t ha⁻¹ rate, often outperforms PBPH in enhancing rice milling traits. This superior performance is likely due to the structural stability and high porosity of RHB, which are optimised under controlled pyrolysis conditions (typically 300 – 600 °C without oxygen) to improve soil quality and nutrient retention (Adebajo et al., 2022; Ali et al., 2022b). In contrast, the agricultural benefits of PBPH are less predictable and can vary significantly due to its lack of uniform quality. This inconsistency arises from a higher proportion of unburnt organic matter and volatile compounds, which may negatively affect soil nitrogen transformations and plant growth (Asadi et al., 2021). Furthermore, because of its high ash content and inconsistent chemical nature, PBPH may even cause detrimental effects such as nutrient imbalances or the introduction of phytotoxic compounds, compared to the stable aromatic skeleton of well-produced biochar (Asadi et al., 2021; Butnan and Vityakon 2023; Jin et al., 2024). Ultimately, while stabilized biochar supports consistent improvements in yield and grain quality across multiple seasons, the variable nature of PBPH may constrain its contribution to long-term sustainable productivity.

CONCLUSION

The findings of this study showed significant and sustainable improvements in total grain yield and grain milling traits over four consecutive seasons following the application of RHB in the low country dry zone of Sri Lanka. While urea application alone had a limited impact, its integration with RHB particularly at the rate of 3 t ha⁻¹ produced a synergistic effect that significantly enhanced grain yield and milling traits. All measured milling traits showed significant improvements, alongside significant declines in grain chalkiness and hull % in all seasons. These results indicate the residual effect of one-time application of RHB, which maintains grain structural integrity and processing efficiency over multiple cropping cycles. Furthermore, the results confirm that carbon-rich RHB is more beneficial than the PBPH application. In conclusion, the combined application of RHB with the recommended level of urea fertilizer provides a sustainable solution to optimise not only total grain production but also rice milling recovery, quality of appearance, and ultimately the market value of the rice.

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RESEARCH ARTICLE

Agriculture

Phenotypic characterization of flag leaf and pollen germination behavior in relation to grain yield in selected *Oryza nivara* populations in Sri Lanka

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Abstract: The flag leaf, the most important functional leaf in the rice plant, positively influences the photosynthetic process and yield components. Meanwhile, pollen germination is a critical indicator of species compatibility for successful seed setting. This study analyzed the correlation between flag leaf traits and panicle traits and pollen germination characteristics of six wild rice (*Oryza nivara*) populations. Plants from six populations (N1 to N6) were collected from different geographical locations and established in a common garden. Morphological traits of the flag leaf were evaluated shortly after heading and yield traits were measured at the maturity stage. Agro-morphological, reproductive, and physiological traits related to grain yield were recorded. Data were analyzed using one-way ANOVA, followed by Duncan's multiple range test ($p < 0.05$), least significant difference (LSD) ($p < 0.05$), and Pearson's correlation test ($p \geq 0.05$ to $p < 0.001$). The Kilinochchi (N6) population recorded the highest significant values for flag leaf length (26.58 ± 0.49), flag leaf area (23.04 ± 1.22), number of seeds per panicle (71.40 ± 3.52), panicle length (24.55 ± 0.38), 100-seed weight (1.76 ± 0.027), and pollen germination (65.74 ± 3.51) compared to other populations. The Anuradhapura (N1) population had the highest ratio of fertile seeds per panicle (0.75 ± 0.023). The Kurunegala (N3) population showed the lowest seed fertility (0.11 ± 0.012) after removal of the flag leaf, showing a 71.8% reduction in seed fertility compared to the intact flag leaf. According to Pearson's correlation analysis, the highest positive correlation was observed between flag leaf length ($r = 0.79$, $p < 0.001$) and panicle length, followed by chlorophyll content ($r = 0.26$, $p < 0.05$) and pollen germination

($r = 0.36$, $p < 0.01$) with the ratio of fertile seeds per panicle. Results suggested that flag leaf length, chlorophyll content, and pollen germination could be the best yield-attributing indicators for selecting *O. nivara* wild rice populations to adopt in rice pre-breeding studies.

Keywords: Chlorophyll content, flag leaf excision, *Oryza nivara*, pollen germination, seed fertility, wild rice

INTRODUCTION

The leaf is the primary organ that intercepts light energy and converts it into organic compounds through photosynthesis. In particular, the flag leaf characteristics of the Poaceae species act as a key determinant of the grain yield and biomass (Liu et al., 2015). As a cereal crop, the grain yield of rice directly relies on photosynthetic efficiency, with particular emphasis on the morphological characteristics of the flag leaf (Yue et al., 2006). The flag leaf is the uppermost leaf, subtending the panicle, directly associated with panicle development and grain filling. Reports have underscored the significance of the flag leaf in rice productivity, attributing up to 45% to grain filling (Abou Khalifa et al., 2008; Mahesh et al., 2022) due to its position and assimilation-related characteristics. Traits such as length, width, angle, and chlorophyll content of the flag leaf during the reproductive period have been

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positively correlated with rice yield (Rahman et al., 2014; Zhang et al., 2015). Further, the angle of the flag leaf directly affects light interception and significantly contributes to determining rice grain yield (Mahesh et al., 2022). Leaf chlorophyll content also has positive impacts on leaf photosynthesis (Takai et al., 2010; Rahman et al., 2014). Studies have shown that leaf photosynthesis and chlorophyll content are higher in the flag leaf of rice at the heading stage (Takai et al., 2006; Ohsumi et al., 2007; Takai et al., 2010), suggesting a strong correlation with yield characteristics. However, the flag leaf has been identified as one of the most variable phenotypic traits among populations of *Oryza nivara* (Sandamal et al., 2022).

Pollen viability is essential for successful fertilization, which is directly linked with seed fertility of rice. Thus, pollen viability is a critical indicator for evaluating reproductive success, as it directly influences grain yield (Dafni & Firmage, 2000; Rahman et al., 2014).

Wild relatives of rice serve as natural germplasm resources that play a crucial role in rice breeding by providing an alternative source of resistance or tolerance to abiotic and biotic stresses (Vaughan, 1994; Jena, 2010) with genomes AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ serving as an important reservoir of useful genes (Brar and Khush, 1997). Sri Lanka hosts five wild relatives of rice species, namely, *Oryza nivara*, *Oryza granulata*, *Oryza eichingeri*, *Oryza rufipogon*, and *Oryza rhyzomatis* (Vaughan, 1994). Among these, *O. nivara*, the main wild ancestor is closely related to cultivated rice (*O. sativa*) with the AA genome (Wang et al., 2017; Sandamal et al., 2018). This species has been extensively utilized in rice breeding programs (Bhasin et al., 2012; Yamagata et al., 2019; Zheng et al., 2024), strong potential for use in breeding programs. *O. nivara* populations from diverse geographic locations in Sri Lanka show significantly higher phenotypic diversity among populations than within populations (Sandamal et al., 2022; Sandamal et al., 2024). Additionally, *O. nivara* is known to possess fertility restoration (Rf) genes, and new and useful alleles that can improve grain yield, grain quality, and yield-contributing traits (Kaladhar et al., 2008; Gaikwad et al., 2014; Haritha et al., 2018).

There is a significant research gap particularly in Sri Lanka, concerning the yield potential associated with flag leaf characteristics in wild rice germplasm. This study aims to evaluate the flag leaf characteristics and yield traits of *O. nivara* populations across different geographical locations in Sri Lanka. Additionally, we

assessed correlations of chlorophyll content, pollen viability, and analyzed spikelet fertility after flag leaf removal in selected superior *O. nivara* populations to assess their yield potentials (Zheng et al., 2024). This study highlights how flag leaf characteristics, chlorophyll content, and pollen viability can influence yield traits in *O. nivara* populations.

MATERIALS AND METHODS

Sample collection and field establishment

Mature seeds were collected from six natural habitats of *O. nivara* across different geographical locations in Sri Lanka, including both the intermediate and dry zones, showcasing a wide diversity of typical environments (Sandamal et al., 2022). Each population consisted of 20 individuals, ensuring a minimum distance of 5 m between individuals to avoid the multiple sampling of the same genet. The six *O. nivara* populations (N1-N6) were established in a common garden at the Faculty of Agriculture, University of Ruhuna (latitude: 06.060337°N, longitude: 80.5681455°E) in Sri Lanka, using one plant per cement pot (16"x16"x18") arranged as a single experiment unit in a completely randomized design (Table 1). The garden conditions included an annual average air temperature of 29.16 ± 0.44 °C and a relative humidity level of 83.08 ± 1.21 % in an open field (Data source: Faculty meteorology unit, Faculty of Agriculture, University of Ruhuna). The established plants were allowed to grow for one year, from 2024 to 2025. Flag leaves of ten randomly selected plants were excised at the panicle insertion stage, and the remaining ten were kept with the flag leaf as a set of control treatment for each population. Morphological descriptors (both qualitative and quantitative) such as flag leaf traits, pollen viability, and yield were assessed in all populations, as described by the guidelines of the International Rice Research Institute (IRRI) (Brewbaker & Kwack, 1963; Rahman et al., 2014).

Phenotypic characterization of the flag leaf and panicle

Phenotypic characterization of the flag leaf and panicle for wild rice was performed following the guidelines set by the IRRI descriptors. The measurements included flag leaf length, flag leaf width, flag leaf area, flag leaf angle, number of spikelets per panicle, ratio of fertile spikelets per panicle, panicle length, and 100-seed weight detailed in Table 2.

Table 1: Geographical and ecological information of six *Oryza nivara* populations collected from different locations and subsequently established in a common garden for flag leaf characterization.

Label	Location	GPS coordinates		Agro-ecological zone*	Annual mean temperature* of the district	Natural habitat description
		Latitude	Longitude			
N1	Anuradhapura	8.135274	80.553759	DL _{1b}	26.5°C	Shallow water lake
N2	Kataragama	6.443126	81.309686	DL ₅	26.8°C	Roadside, waterlogged, marshy area
N3	Kurunegala	7.704122	80.163938	IL ₃	26.4°C	Roadside, waterlogged, marshy area
N4	Batticaloa	7.623656	81.716753	DL _{2b}	27.5°C	Shallow water lake
N5	Mannar	9.016355	80.066568	DL ₄	29.3°C	Shallow water lake
N6	Kilinochchi	9.341323	80.409326	DL ₃	29.0°C	Shallow water lake

*Source: Department of Meteorology in Sri Lanka

Table 2: Methods for measuring traits and corresponding code details

Traits	Code	Method of trait measurement
Flag leaf length (cm)	FLL	Measured from tip to leaf base
Flag leaf width (cm)	FLW	Measured at the widest part of the leaf
Flag leaf area (cm ²)	FLA	$= k \times FLL \times FLW \text{ (k; 0.75)}$... (01) (Yoshida et al., 1976) (FLL - Flag leaf length, FLW - Flag leaf width)
Flag leaf angle (°)	FLAN	Measured at the collar as the angle of attachment between the flag leaf blade and the main panicle axis using the Angulus (version 5.1.0) mobile application (Porkodi et al., 2023).
Number of spikelets per panicle	NSP	Count of the individual seeds in the panicle
Ratio of fertile spikelets per panicle	RFSP	Count of the fertile spikelets by pressing the spikelets with fingers and noting those that do not have grains at the mature stage. $= \frac{\text{Number of fertile seeds per panicle}}{\text{Total number of seeds per panicle}}$... (02)
Panicle length (cm)	PL	Measured panicle base to tip
100 seed weight (g)*	STW	Weight of 100 well-developed whole grains, dried to 13% moisture content, and weighed on a precision balance.
Chlorophyll content (SPAD value)	CC	Measured using the CCM 200 plus SPAD meter at the heading stage of the flag leaf
Pollen germination (%)	PG	Recorded using the in vitro pollen germination method $\text{Pollen germination (\%)} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$... (03)

*100-seed weight was used instead of 1000-seed weight due to biological constraints (low seed fertility, and high seed shattering) in wild rice populations.

Excision of the flag leaf

At the heading stage (panicle insertion stage), the flag leaves were carefully removed and the number of spikelets per panicle was counted at the fully mature stage (after 21 days) to determine the ratio of fertile spikelets to the total number of spikelets per panicle (Rahman et al., 2014; Das et al., 2018).

Total chlorophyll content

Total chlorophyll content was measured using the CCM 200 plus SPAD meter at the heading stage of the flag leaf, as a higher photosynthesis rate can be observed during this time, which is related to yield (Takai et al., 2006; Ohsumi et al., 2007; Takai et al., 2010).

Pollen viability

Pollen viability was recorded using the *in vitro* pollen germination method described by Brewbaker and Kwack (Brewbaker & Kwack, 1963). Briefly, the protocol included the following steps: Brewbaker and Kwack medium was prepared using 10% sucrose, 100 mg/L boric acid, 300 mg/L calcium nitrate, 200 mg/L magnesium sulfate, and 100 mg/L potassium nitrate. Pollen grains were placed on a slide covered with one to two drops of Brewbaker and Kwack medium using a

fine brush. The slide was then placed on a wet filter paper in a watch glass to maintain high humidity and kept at room temperature for 40 minutes. Finally, the number of germinated pollen grains was observed using a light microscope (Jayaprakash, 2018).

Data analysis

The data were analyzed using SPSS version 25 and R Studio version 4.4.2. An initial ANOVA was conducted to analyze all variables, including flag leaf and yield traits. Additionally, the least significant difference (LSD) test was used to identify statistically significant differences between means at a 5% level among populations. Pearson's correlation was performed for correlation analysis ($p < 0.05$) using the R programming language. The correlation matrices for flag leaf traits and yield components were visualized using a heatmap created in R Studio (Turney, 2024; Howlader et al., 2025). Pearson's coefficient of correlation (r) was calculated using the formula given below (Turney, 2024).

$$r = \frac{n \sum xy - (\sum x)(\sum y)}{\sqrt{[n \sum x^2 - (\sum x)^2][n \sum y^2 - (\sum y)^2]}} \quad \dots(04)$$

Here, r = Pearson coefficient of correlation, n = sample size, x = each x-variable value, and y = each y-variable value.

Table 3: Variation of flag leaf morphological traits, yield attributed traits, and pollen germination characteristics of six *Oryza nivara* populations.

Variable	Anuradhapura	Kataragama	Kurunegala	Batticaloa	Mannar	Kilinochchi	LSD*
FLL (cm)	21.76 ± 0.52 ^b	10.63 ± 0.27 ^a	21.27 ± 0.38 ^b	10.84 ± 0.39 ^a	22.05 ± 0.58 ^b	26.58 ± 0.49 ^c	1.27
FLW (cm)	0.85 ± 0.022 ^c	0.72 ± 0.025 ^b	0.85 ± 0.022 ^c	0.61 ± 0.028 ^a	1.15 ± 0.043 ^c	1.05 ± 0.034 ^d	0.085
FLA (cm ²)	13.92 ± 0.62 ^b	5.78 ± 0.34 ^a	13.61 ± 0.56 ^b	5.03 ± 0.39 ^a	17.41 ± 0.84 ^c	23.04 ± 1.22 ^d	2.053
FLAN (°)	37.28 ± 4.58 ^a	27.38 ± 3.51 ^a	50.16 ± 5.62 ^b	69.69 ± 3.93 ^c	34.38 ± 3.88 ^a	39.82 ± 1.63 ^{ab}	11.46
NSP	48.70 ± 2.74 ^d	23.40 ± 1.09 ^b	35.00 ± 1.89 ^c	16.60 ± 1.14 ^a	42.90 ± 2.62 ^d	71.40 ± 3.52 ^e	6.63
RFSP – IFL	0.75 ± 0.023 ^d	0.48 ± 0.029 ^b	0.39 ± 0.028 ^a	0.64 ± 0.027 ^c	0.50 ± 0.035 ^b	0.51 ± 0.019 ^b	0.080
RFSP – FLE	0.17 ± 0.026 ^b	0.21 ± 0.009 ^b	0.11 ± 0.012 ^a	0.30 ± 0.031 ^c	0.23 ± 0.024 ^b	0.44 ± 0.027 ^d	0.063
PL (cm)	21.44 ± 0.30 ^d	16.80 ± 0.27 ^b	16.66 ± 0.20 ^b	12.33 ± 0.25 ^a	19.13 ± 0.39 ^c	24.55 ± 0.38 ^e	0.87
100-STW (g)	1.70 ± 0.031 ^b	1.46 ± 0.038 ^a	1.70 ± 0.042 ^b	1.48 ± 0.044 ^a	1.75 ± 0.028 ^b	1.76 ± 0.027 ^b	0.102
CC (SPAD value)	11.00 ± 0.35 ^b	7.32 ± 0.25 ^a	8.51 ± 0.32 ^a	12.89 ± 0.44 ^c	14.79 ± 0.86 ^d	13.42 ± 0.40 ^c	1.36
PG (%)	42.50 ± 3.38 ^b	20.62 ± 2.02 ^a	16.31 ± 1.40 ^a	37.55 ± 3.98 ^b	24.72 ± 1.71 ^a	65.74 ± 3.51 ^c	8.06

Note: FLL: flag leaf length, FLW: flag leaf width, FLAN: flag leaf angle, NSP: number of seeds per panicle, RFSP – IFL: ratio of fertile seeds per panicle with an intact flag leaf, RFSP-FLE: ratio of fertile seeds per panicle, flag leaf excised, PL: panicle length, 100-STW: 100 seed weight, CC: chlorophyll content, and PG: pollen germination. Within a row, means followed by the same letters are not significantly different by Duncan's multiple range test (DMRT) at $p > 0.05$.

RESULTS AND DISCUSSION

Variation of flag leaf and yield traits of *Oryza nivara* populations

Results indicated significant differences among the populations for the assessed quantitative traits of flag leaf and yield (Table 3).

Morphological traits of the flag leaf such as flag leaf length (FLL), flag leaf width (FLW), flag leaf area (FLA), flag leaf angle (FLAN), and chlorophyll content (CC) were significantly different among the populations. In terms of yield traits, the number of seeds per panicle (NSP), ratio of fertile seeds per panicle with an intact flag leaf (RFSP - IFL), ratio of fertile seeds per panicle to flag leaf excised (RFSP-FLE), panicle length (PL), 100-seed weight (STW), and pollen germination percentage (PG) were distinct parameters in differentiating populations.

Within the populations studied, the Kilinochchi population had the longest flag leaf (FLL - 26.58 ± 0.49), highest flag leaf area (FLA - 23.04 ± 1.22) and longest panicle length (PL - 24.55 ± 0.38), showing significant

differences compared to the other populations ($p < 0.05$). On the other hand, the Kataragama (FLL - 10.63 ± 0.27 ; FLA - 5.78 ± 0.34) and Batticaloa (FLL - 10.84 ± 0.39 ; FLA - 5.03 ± 0.39) populations had the shortest flag leaves among the populations studied.

The Mannar population showed an intermediate flag leaf length of 22.05 ± 0.58 ; however, it had the highest flag leaf width at 1.15 ± 0.043 , which was significantly different from other populations. Conversely, the Batticaloa population had the lowest flag leaf width at 0.61 ± 0.028 , which was also significantly different from other populations.

The characteristics of the flag leaf are modified by genetic and environmental factors. It has been reported that the size of the flag leaf is controlled by several genes (Zhang et al., 2015). According to Zhao et al. (2011), the observed SNPs at 31 Mb on chromosome 4 are significantly associated with flag leaf width in 413 diverse accessions. Additionally, the NARROW LEAF1 (*NALI*) gene is linked to flag leaf size (Taguchi-Shiobara et al., 2015). Among environmental factors low light intensity has been found to enhance flag leaf area and width (Evans & Poorter, 2001; Fabre et al., 2016).

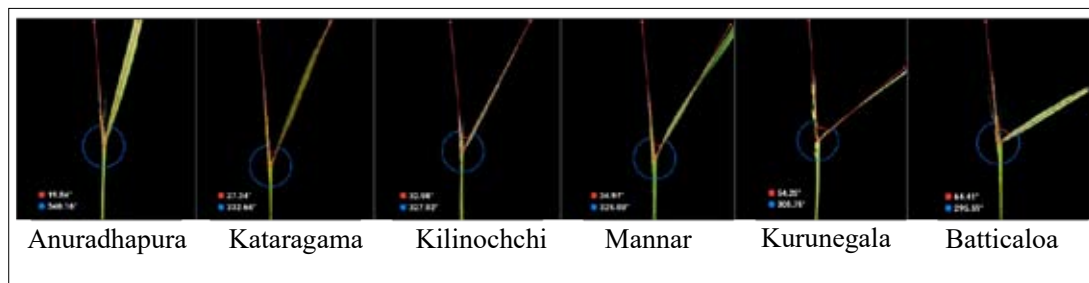


Figure 1: Variation in flag leaf angle of six *Oryza nivara* populations studied.

Significant variation in flag leaf angle was observed among the six *O. nivara* populations, as shown in Figure 1. The Flag leaf angle has been classified into four groups based on the IRRI standard descriptors (Chang & Bardenas, 1965; Mahesh et al., 2022): erect ($0^\circ - 30^\circ$), intermediate ($31^\circ - 60^\circ$), horizontal ($61^\circ - 90^\circ$), and descending ($\geq 91^\circ$). The Batticaloa population exhibited a horizontal flag leaf angle (69.69 ± 3.93), while the Kataragama population showed the lowest flag leaf angle (27.38 ± 3.51), indicating an erect flag leaf angle. The Anuradhapura (37.28 ± 4.58) and Mannar populations (34.38 ± 3.88) showed an intermediate flag

leaf angle (Figure 1). The genetic basis of flag leaf angle largely relies on *qFLANF.1A*, which is a major QTL associated with flag leaf angle and consistently shows a strong signal on chromosome 1A that explain 9 - 13 % of the variation across different environments (Mantilla-Perez & Salas Fernandez., 2017; Kumar et al., 2024). Moreover, similar variations in the flag leaf angle have been observed in different wheat accessions (Kumar et al., 2024). These studies have identified three stable genomic regions (MTAs) on chromosomes 1A, 5A, and 5B that regulate flag leaf angle, and remain stable across various environments in wheat plants (Kumar et al., 2024).

The Kilinochchi population had significantly higher values for the number of seeds per panicle and panicle length, with averages of 71.40 ± 3.52 and 24.55 ± 0.38 , respectively. In contrast, the Batticaloa population had the lowest values, with an average of 16.60 ± 1.14 for the number of seeds per panicle and 12.33 ± 0.25 for panicle length. *qTSN4* is involved in both flag leaf and panicle traits, including flag leaf size and the number of spikelets per panicle (Fujita et al., 2013; Takai et al., 2013). According to Panda et al. (2018), the *Gn1a* gene is associated with an increase in the number of spikelets, while a loss-of-function mutation in the *LOG* gene results in several floral defects, such as a reduction in panicle size and the number of spikelets. The influence of environmental factors on flag leaf and panicle traits is well studied (Evans & Poorter, 2001; Fabre et al., 2016). For instance, plant grain production can be affected by environmental factors, particularly light levels, which affect panicle size (Fabre et al., 2016). These findings clearly demonstrate that the morphological traits of flag leaves and panicle characteristics vary according to environmental and genetic factors. In this study, *O. nivara* collected from six different geographical locations in Sri Lanka clearly showed significant differences in flag leaf traits and panicle morphology among populations, indicating adaptive phenotypic plasticity in various geographic locations.

Excision of the flag leaf affects the ratio of fertile seeds per panicle

The effect of flag leaf excision on panicle development varied significantly among the six *O. nivara* populations, as shown in Figure 2.

The impact of flag leaf removal on seed fertility differed significantly across populations, ranging from pronounced decreases to negligible effects (Figure 3). The Anuradhapura population exhibited the most severe reduction in seed fertility (77.3 %), indicating that the flag leaf (FL) contributes substantially to final yield in this population. In contrast, the Kilinochchi population displayed the highest seed fertility (0.44 ± 0.027) in the flag leaf excised treatment, with only a 13.73 % reduction compared to the treatment with an intact flag leaf, suggesting that the FL made the least contribution to grain yield. Additionally, the Kurunegala population

exhibited the lowest seed fertility (0.11 ± 0.012) in the flag leaf excised treatment, with a 71.8 % reduction in seed fertility compared to the seed fertility of the treatment with an intact flag leaf treatment. According to Rahman et al. (2014), yield was reduced in rice cultivars after flag leaf excision, with varying effects among different cultivars. Furthermore, removing the flag leaf at the booting stage can decrease the yield capacity of wheat plants by more than 25%. The relationship between flag leaf and panicle characteristics depends on the variety of the plant and the environmental conditions (Racz et al., 2022).

The chlorophyll content index (CCI value) reflected by SPAD values showed significant variation among different populations. The Mannar population had the highest chlorophyll content (14.79 ± 0.86), followed by Kilinochchi (13.42 ± 0.40) and Batticaloa populations (12.89 ± 0.44). In contrast, the Kataragama population had the lowest chlorophyll content (7.32 ± 0.25), followed by the Kurunegala population (8.51 ± 0.32), with no significant difference between them. Over 900 quantitative trait loci (QTL) for chlorophyll content have been identified in rice, which include at least eight stay-green-related genes such as *DYE*, *SGR*, and *EF8* (Feng et al., 2014; Yamatani et al., 2018). Additionally, QTL linkage mapping has been used to explore the genetic architecture of natural variation in chlorophyll content in rice (Wang et al., 2015). Moreover, different levels of phosphorus can affect chlorophyll content, with phosphorus deficiencies potentially reducing chlorophyll levels in the flag leaf (Yang et al., 2022). Populations with higher chlorophyll contents (Mannar, Kilinochchi, Batticaloa, and Anuradhapura populations) recorded a greater number of fertile seeds compared to populations with lower chlorophyll contents (Kataragama, Kurunegala), indicating a strong link between chlorophyll levels in the flag leaf and the number of fertile seeds.

The Kilinochchi population had the highest 100-seed weight (1.76 ± 0.027) among all populations. In contrast, there were no significant differences in 100-seed weights among the Mannar (1.75 ± 0.028), Anuradhapura (1.70 ± 0.031), and Kurunegala populations (1.70 ± 0.042). The lowest 100-seed weights were found in the Batticaloa population (1.48 ± 0.044) and the Kataragama population (1.46 ± 0.038).

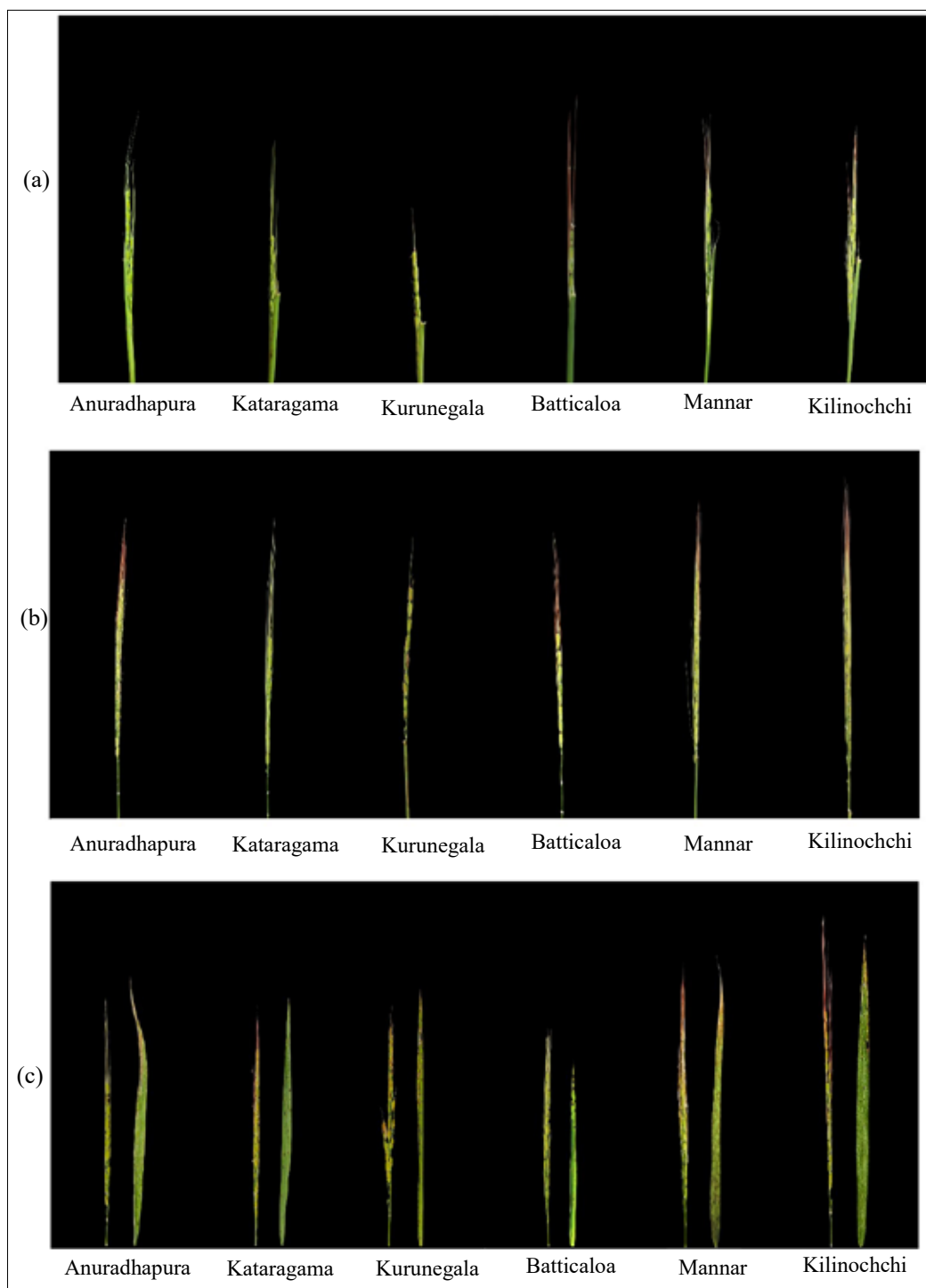


Figure 2: Effect of flag leaf excision on panicle development of six *Oryza nivara* populations. (a). Excised flag leaf at the booting stage (panicle insertion stage), (b). Panicle development after flag leaf excision. (c). Variations in flag leaf length and panicle length of six *O. nivara* populations in Sri Lanka.

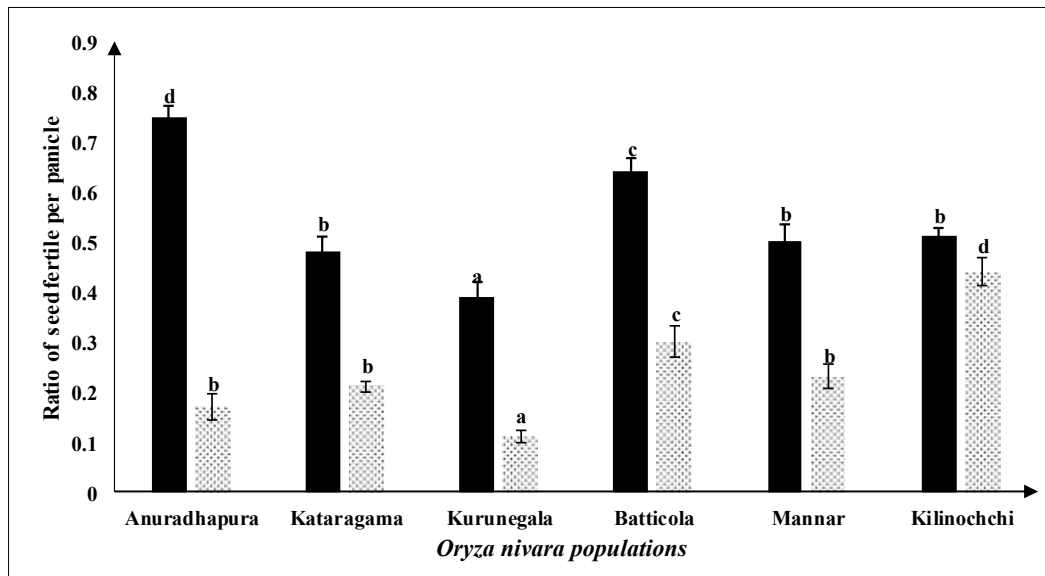


Figure 3: Comparison of the ratio of fertile seeds per panicle with an intact flag leaf; RFSP-IFL (black filled) and with the flag leaf excised; RFSP-FLE (dotted).

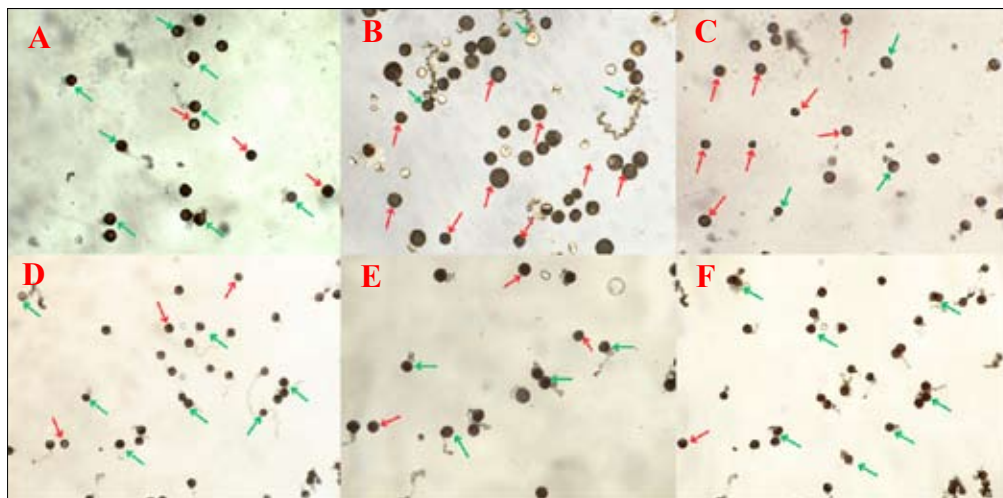


Figure 4: Pollen germination of six *Oryza nivara* populations in Sri Lanka. (A) Anuradhapura population. (B) Kataragama population. (C) Kurunegala population. (D) Batticaloa population. (E) Mannar population. (F) Kilinochchi population. The green and red arrows indicate germinated and non-germinated of pollen grains, respectively.

Variation in Pollen Germination among Six *O. nivara* Populations

Pollen germination percentages were observed across the six populations of *O. nivara*, as illustrated in Figure

4. The highest pollen germination was observed in the Kilinochchi population (65.74 ± 3.51), followed by the Anuradhapura population (42.50 ± 3.38) and the Batticaloa population (37.55 ± 3.98), both of which were not significantly different from each other. The lowest

pollen germination was observed in the Kurunegala population (16.31 ± 1.40), which was not significantly different from the Kataragama (20.62 ± 2.02) and Mannar (24.72 ± 1.71) populations. Environmental conditions significantly influence pollen development and germination, with temperature being a key factor (Coast et al., 2016). High temperatures during anthesis can reduce both pollen viability and germination (Tran Loc Thuy et al., 2020). However, some studies suggest that certain rice cultivars exhibit strong pollen germination even under high temperatures (Zakaria et al., 2022). Additionally, research has shown that

both environmental and genetic factors affect pollen germination (Hu et al., 2025). Genetic factors such as the *OsSTA*, *OsAP65*, *Cdi*, and *MGP2* genes may play a role in pollen germination (Ling et al., 2015; Huang et al., 2013; Li et al., 2012). In this study, all populations were established in a common field providing equal conditions. All collected populations were from the dry zone and intermediate zone of Sri Lanka where high temperatures prevail for most of the year. Therefore, considering both environmental and genetic factors, it is likely that genetic factors have influenced pollen germination across the six populations.

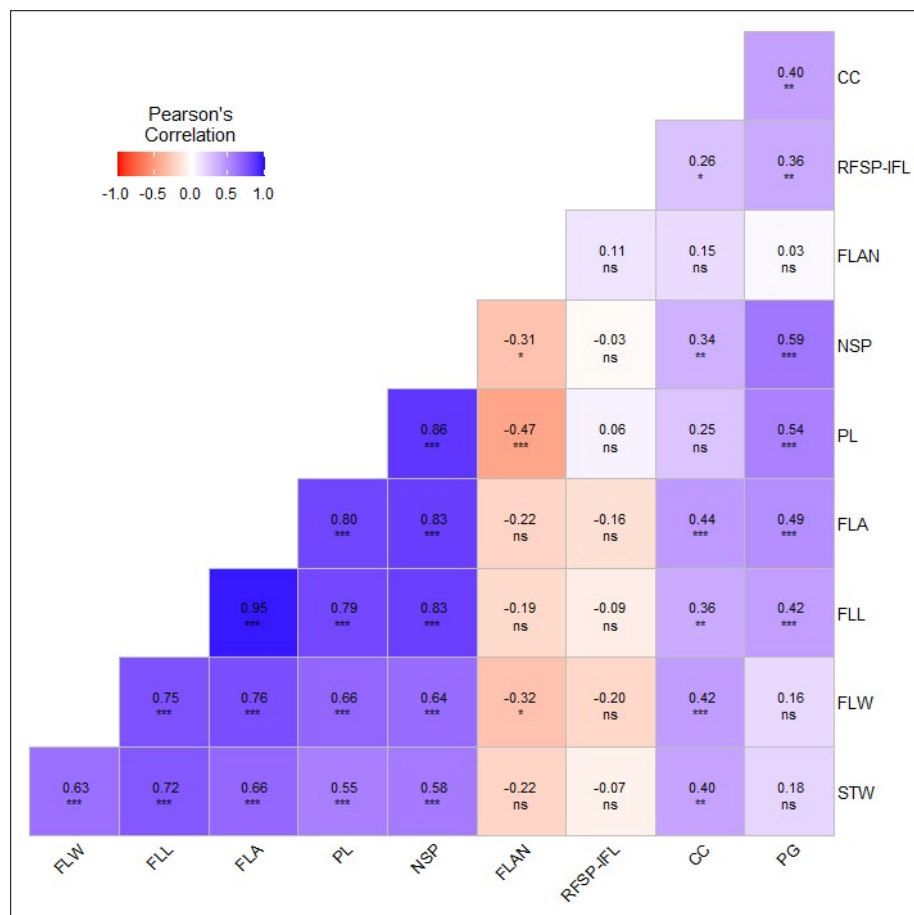


Figure 5: Correlations between flag leaf related traits and yield related traits. The number in the middle of the cell is the correlation coefficient; 'ns', '*', '**', and '***' indicate levels of significance ($p \geq 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.001$). FLW, flag leaf width; FLL, flag leaf length; FLA, flag leaf area; PL, panicle length; NSP, number of seeds per panicle; FLAN, flag leaf angle; RFSP-IFL, ratio of fertile seeds per panicle with an intact flag leaf; CC, chlorophyll content; PG, pollen germination; STW, seed test weight.

Correlations among the flag leaf traits with yield

The correlation between flag leaf related and yield related traits are given in Figure 5. The FLL significantly and highly positively correlated with FLA ($r = 0.95$, $p < 0.001$). Additionally, FLL is also significantly and highly positively correlated with panicle length ($r = 0.79$, $p < 0.001$). This positive correlation between FLL and panicle elongation is consistent with findings in rice (Rahman et al., 2014). Furthermore, FLA ($r = 0.83$, $p < 0.001$), FLL ($r = 0.83$, $p < 0.001$), and FLW ($r = 0.64$, $p < 0.001$) are significantly and positively correlated with the number of seeds per panicle. According to Wang et al. (2022), FLL, FLW, and FLA showed highly significant positive correlations with spikelet number per spike in wheat. When FLL, FLW and FLA increase, the number of seeds per panicle also increases. Additionally, FLW is positively correlated with FLL ($r = 0.75$, $p < 0.001$), FLA ($r = 0.76$, $p < 0.001$), PL ($r = 0.66$, $p < 0.001$), and chlorophyll content ($r = 0.42$, $p < 0.001$). In wheat, FLL showed a strong positive correlation with FLW, FLA, and FLAN, indicating comparable patterns in other *Poaceae* species. In our study, we found that FLL was strongly positively correlated with FLW ($r = 0.75$, $p < 0.001$) and FLA ($r = 0.95$, $p < 0.001$), but it exhibited a negative correlation with FLAN (Liu et al., 2018; Wang et al., 2022). The ratio of fertile seeds per panicle is significantly positively correlated with pollen germination ($r = 0.36$, $p < 0.01$) and chlorophyll content ($r = 0.26$, $p < 0.05$), although the r values are relatively low. Furthermore, the number of seeds per panicle is positively correlated with chlorophyll content ($r = 0.34$, $p < 0.01$). Moreover, several studies showed a strong correlation between leaf chlorophyll content and grain yield (Reynolds et al., 2000; Rosyara et al., 2010; Lopes et al., 2012). The 100-seed weight was positively correlated with FLW ($r = 0.63$, $p < 0.001$), FLL ($r = 0.72$, $p < 0.001$), FLA ($r = 0.68$, $p < 0.001$), PL ($r = 0.55$, $p < 0.001$), and the number of seeds per panicle ($r = 0.58$, $p < 0.001$).

Furthermore, PL was negatively correlated with FLAN ($r = -0.47$, $p < 0.001$). Meanwhile, FLW ($r = -0.20$, $p \geq 0.05$), FLL ($r = -0.09$, $p \geq 0.05$), and FLA ($r = -0.16$, $p \geq 0.05$) were negatively correlated with the ratio of fertile seeds per panicle. The number of seeds per panicle ($r = -0.03$, $p \geq 0.05$) was also negatively correlated with the ratio of fertile seeds per panicle. This implies that as the number of seeds per panicle increases, the ratio of fertile seeds per panicle tends to decrease. This may occur due to a low pollen germination percentage in the wild relatives of rice (Hu et al., 2025). Additionally, the ratio of fertile seeds per panicle with an intact flag leaf (RFSP-IFL) negatively correlated with 100-seed weight

(STW) ($r = -0.07$, $p \geq 0.05$). Furthermore, according to Khaliq et al. (2008), the association between fertile grains per panicle and 1000-grain weight is negatively correlated and highly significant. In particular, flag leaf traits are often positively correlated with yield traits in many cereal crops, including rice. Additionally, chlorophyll content varies among populations, varieties, and cultivars; however, it shows a positive correlation with yield traits (Rahman et al., 2014).

CONCLUSION

This work presents the first investigation into how yield-related flag leaf traits and pollen germination correlate with seed setting in wild rice. The flag leaf traits and yield characteristics significantly varied among six populations of *O. nivara*, showing phenotypic adaptation to diverse environments. Key traits, including flag leaf length, flag leaf area, flag leaf width, the number of seeds per panicle, the ratio of fertile seeds per panicle, and panicle length, were highly different among the populations and could be utilized as key indicators in selecting wild geographic locations in future studies. Conversely, traits such as flag leaf angle, 100-seed weight, chlorophyll content, and pollen germination showed minimal variation across populations, making them unsuitable for selection criteria. Variation in seed fertility across populations points to either differential dependency on flag leaf photosynthesis or the activation of population-specific compensatory mechanisms, governed by underlying genetic and physiological plasticity. Consequently, further research is required to elucidate the underlying physiological and genetic mechanisms. These findings provide phenotypically promising *O. nivara* populations serving as candidate populations for future pre-breeding studies.

Habitat-specific phenotyping of wild rice is recommended to capture the significant trait diversity of *O. nivara* populations across Sri Lanka. This approach is essential for developing targeted conservation strategies and promoting the sustainable management. Further research should focus on linking morphological traits to adaptive significance, rather than relying solely on descriptive trait variation.

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RESEARCH ARTICLE

Electrical and Electronic Engineering

Advanced cell balancing strategies for scalable lithium-ion battery management in electric vehicles

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Abstract: The growing demand for efficient and reliable energy storage in electric vehicles has led to significant advancements in battery management systems (BMS), particularly in cell balancing. While existing research has primarily focused on balancing techniques using a small number of cells (typically 2 to 4), or lead-acid batteries, and employed algorithms like Kalman filters and state of charge (SoC)-based estimations for passive resistor-based or simple inductor/capacitor-based balancing, these approaches often face limitations in energy efficiency and scalability. In contrast, this study presents a comprehensive analysis of 13 lithium-ion cells using both passive and active balancing, as well as an advanced active staggered cell balancing strategy. The proposed system re-allocates the charge more efficiently among cells, minimizing thermal loss and improving overall energy utilization. Using MATLAB®/Simulink® simulations, we evaluate and compare the simultaneous start and staggered start methods under various load conditions. Results show that active balancing methods can reduce energy loss from 15–25 W (typical in passive systems) to 3–7 W and decrease balancing time from 120–180 minutes to 45–80 minutes. Notably, the staggered start method delivers superior thermal management and extended battery life, confirming its suitability for high-performance and scalable BMS in electric vehicles.

Keywords: Fast charging battery management systems, hybrid cell balancing techniques, electric vehicles, lithium-ion batteries, next-generation electric vehicles, energy efficiency.

INTRODUCTION

Efficient battery management is significant for keeping electric vehicle (EV) batteries safe, reliable, and long-

lasting. Batteries are the core of EVs, storing energy to run the vehicle's motor and other systems (Chavan et al., 2024). The lithium-ion cells are connected to form the battery pack, but how well the pack performs affects key factors such as how far the car can go, how long it takes to charge, and how long the vehicle will last (Fichtner, 2022). These cells can have voltage, capacity, and resistance differences because of how they are made, how old they are, and how they are used. A big challenge is to ensure each cell in a battery pack has the same charge level (Zhang et al., 2023). If the cells are not balanced, some might get overcharged or undercharged, reduce the battery's life, or even make it unsafe. Overcharging speeds up aging and can lead to overheating, while undercharging reduces how much energy the battery can store and use (Khan et al., 2024). To address these challenges, cell balancing methods are employed to equalize the voltages or state-of-charge (SOC) of cells within a pack, ensuring optimal performance and safety (Sharma et al., 2024).

Traditional passive cell balancing methods dissipate excess energy from high-voltage cells as heat using resistors. Although passive balancing is simple and worthwhile, they are naturally inefficient due to power dissipation and slow response times (Ali et al., 2024). On the contrary, active cell balancing methods dynamically transfer charge between cells. This process distributes energy from the high-voltage cell to the low-voltage cell. This saves energy and achieves balance more quickly (Sugumaran & Prabha, 2022). Active balancing is

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especially useful for applications that require high energy efficiency and fast response for EVs (Thiruvonasundari & Deepa, 2023). Lithium-ion batteries are needed for EVs, due to the density of stored energy, long service life, and low loss rates. However, the chemicals of lithium-ion cells make them too sensitive to charging and depth (Rahman & Alharbi, 2024). Therefore, a strong battery management system (BMS) must check and control its work. They can balance the longevity of consumer concerns during the construction of infrastructure. The cost of charging the battery is important in the development of the latest technological progress, such as a DC-DC converter in two directions, the prediction and learning of the machine have given new opportunities for balanced system innovation (Wongdet et al., 2023). But the cells are enabled, inspecting, and controlling the dynamic conditions to be appropriate and confident that the best performance utilization is achieved from the software, MATLAB®/Simulink® (Farooq et al., 2024).

This study is positioned within ongoing efforts to improve scalable and energy-efficient battery management systems for EVs. While recent literature has explored passive dissipation-based techniques, converter-based active balancing, and algorithmic estimation strategies, many investigations remain limited to small cell configurations or focus primarily on hardware topology optimization. The present work extends these studies by evaluating balancing performance in a 13-cell lithium-ion configuration representative of practical EV battery modules. Furthermore, it introduces a staggered-start active balancing strategy that complements existing simultaneous transfer approaches by improving thermal behaviour, reducing switching stress, and enhancing energy utilization. By providing a unified comparative simulation framework and quantitative benchmarking, this work contributes system-level insights that support scalable BMS design.

A comparative analysis of active and passive cell balancing techniques was conducted by Rout & Samal (2024) using a two-cell lithium-ion battery pack for maintaining the battery performance and prolonging battery life in electric vehicles. A novel approach for active cell balancing was proposed by Sharma et al. (2024), utilizing the Kalman filter algorithm to estimate the state of each battery cell and determine the optimal charging and discharging currents, thereby improving the efficiency of BMS, and extending the battery lifespan. The bidirectional active balancing solution was developed by Qi (2024) for the transit of power bidirectionally between the vehicle's 12V bus and the high-voltage bus for the addition and removal of charges to any cell in the

energy storage system. This also acts as a charger for the 12V lead acid battery. A single inductor-based active cell balancing topology was explored by Kaushik et al. (2024) for a series of connected four-cell batteries to address the state of charge imbalance among the cells. It employed a min-max state of charge (SoC) control algorithm to balance the cells effectively during static, charging, and discharging modes. The novel two-layer multi-inductor and single-layer multi-inductor active cell balancing was developed by Venkatasatish & Dhanamjayulu (2024) with a SoC controller for 52V battery systems, and tested with the OPAL-RT 5700 simulator for effectiveness. This paper studied the implementation and simulation of an active balancing technique in a 13-cell lithium-ion battery pack, a configuration commonly used in EVs, by comparing three common methods: passive balancing, staggered active balancing (upon initiation), and active balancing (simultaneous start). Studying the principles, efficiency, and practical meaning provides a comprehensive understanding. The goal is to identify an efficient and scalable balancing solution. This is in line with the increasing demand for sustainable and high-performance EV technology. This study developed a simulation model to assess the effectiveness of different balancing techniques, providing valuable in-depth information for researchers and engineers in EVs.

From the reviewed literature, it is evident that prior studies predominantly investigate topology-level innovations or estimation-based control applied to limited cell counts. However, fewer works examine scalability-oriented comparative strategy evaluation under unified simulation environments representative of EV battery modules. Addressing this gap, the present study complements existing research by integrating passive and two active balancing strategies within a single modeling platform and extending analysis to a larger cell configuration. This enables direct benchmarking of efficiency, thermal impact, and balancing duration, thereby advancing understanding of practical deployment trade-offs in scalable BMS architectures.

MATERIALS AND METHODS

The proposed method analyzes the simulated operations of a 13-cell lithium-ion battery pack using passive, active simultaneous start, and active staggered start configurations. The study explores different methods for achieving balanced voltage distribution and deals with real-life situations in battery cells regarding response time, ease of use, and operational efficiency. Table 1 indicates the notations used in the proposed system.



Figure 1: Flowchart of the proposed system

Table 1: Notations of the proposed system

Symbol	Description	Unit
P_{loss}	Power loss due to passive balancing	W
$V_{Cell, max}$	Maximum Cell Voltage	V
$V_{Cell, min}$	Minimum Cell Voltage	V
R_{bal}	Balancing Resistor	Ω
$E_{transferred}$	Energy transferred during balancing	J
C_{bal}	Capacitance	F
I_{ind}	Inductor Current	A
L	Inductance for energy transfer	H
t	Time step for charge transfer	s

The flowchart in Figure 1 shows the battery cell balancing strategy selection between passive balancing and active simultaneous balancing, followed by active staggered balancing.

- The method starts by creating an initial phase, includes measuring cell voltage levels and setting voltage balance limits.
- The balancing method selection occurs by considering voltage discrepancies together with available power outputs.
- The procedure of passive balancing allows energy dissipation into resistors, yet it creates power wastage.

- Active simultaneous balancing requires a large amount of power for its operation because it distributes energy to all cells at the same time.
- Active staggered balancing controls the delay timing of energy flow operations to enhance the distribution of energy while minimizing power spikes.
- All cells require equalization through the balancing process before carrying out performance evaluations.
- Step four examines and declares the best balancing method by measuring efficiency results, temperature regulation, and time requirements.

The structured method enables researchers to select the battery balancing method with maximum efficiency, thermal performance, and energy utilization benefits.

Simulation setup

The simulation setup using MATLAB®/Simulink® software for the cell balancing method of 13 lithium-ion cells in Figure 2 shows the Simulink model of the proposed balancing system, in that all three balancing methods have been incorporated using balancing logic. Table 2 provides the simulation parameters used in the proposed system.

Once the simulation has been started with switches, the balancing happens in three levels and the balancing methods are explained as follows. The balancing techniques consist of some equations and their notations are given in Table 3.

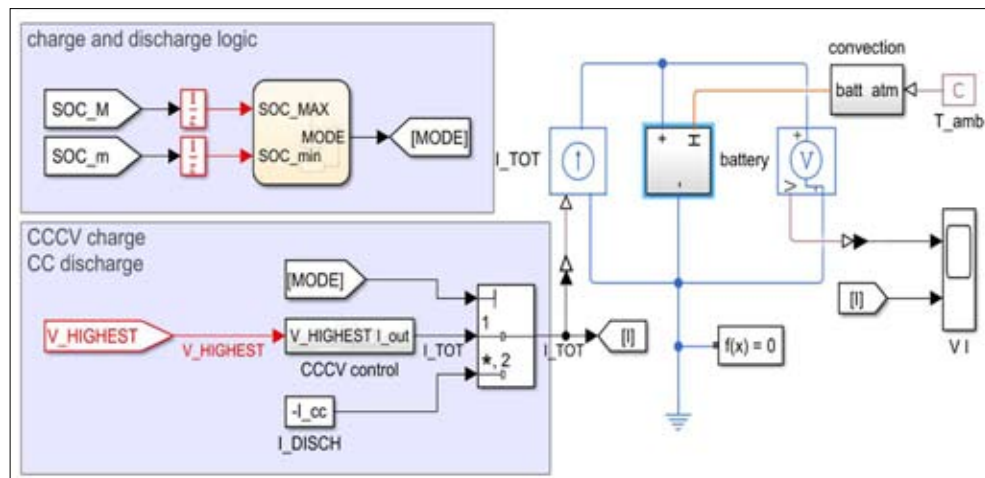
**Figure 2:** Simulation model of the Lithium-ion battery cell

Table 2: Simulation components and description

Serial No.	Components	Description
1.	Battery	Represents the lithium-ion battery with a nominal voltage of 3.7V per cell.
2.	Load Resistor	Simulates the battery discharge process by providing a resistive load.
3.	Passive Balancing Resistors	Used for passive balancing, ensuring equal charge distribution among cells.
4.	DC-DC Converter	Implements active balancing by transferring energy between cells to maintain voltage balance.
5.	Voltage Measurement Sensor	Measures the voltage across different battery cells for monitoring and control.
6.	Current Sensor	Measures the current flow in the system to analyze energy distribution.
7.	Scope	Displays real-time voltage and current waveforms for system performance analysis.
8.	Simulink-PS Converter	Converts Simulink signals to physical signals for interaction with Simscape blocks.
9.	PS-Simulink Converter	Converts physical signals from Simscape back into Simulink signals for data analysis.
10.	Display	To display the values during the simulation.

Table 3: Notations of the proposed system

Serial No.	Notation	Description
1.	I_{bal}	Balancing Current
2.	$V_{cell,max}$	Highest voltage cell
3.	$V_{cell,min}$	Lowest voltage cell
4.	R_{bal}	Balancing Resistor Value
5.	P	Power dissipated in the resistor
6.	I	Current Value
7.	R	Resistance value
8.	P_{loss}	Power loss during balancing
9.	E_{loss}	Energy loss during balancing
10.	Δt	Time interval
11.	Q_{bal}	Total charge transferred during balancing
12.	t_{bal}	Balancing time
13.	C_{cell}	Cells Capacitance
14.	E	Energy stored in the cell
15.	V	Cell voltage
16.	E_{max}	Total energy stored in the highest voltage cell before balancing
17.	E_{min}	Total energy stored in the lowest voltage cell before balancing

Passive balancing

Passive balancing as in Figure 3 is performed by resistors through the process of energy surplus dissipation. The passive cell balancing circuit discharges energy from higher-voltage cells to resistors for distributing charge evenly across the battery pack. A control system utilizes voltage sensors to collect continuous data about every cellular level. The control system identifies high-voltage cells after which the control method activates specific switches that enable the cells to connect to resistors so excess energy can turn into heat. This basic approach succeeds through simplicity and low cost, yet produces inefficient operation because energy conversion into heat uses power, resulting in poor performance in high-power situations. Unfortunately, passive balancing has its constraints, but it functions to stop battery overcharging and increases the life span by keeping all cells at uniform voltage levels.

To calculate the power loss during passive balancing, the fundamental things related to power loss are current flow, energy dissipation, and power transfer in the resistors. When a switch is closed for passive balancing, the excess charge flows through the balancing resistor R_{bal} . The following equations are used to derive the power loss. Ohm's law is used for balancing circuits and is represented in equation (1) because if a switch is closed for passive balancing, the excess charge flows through the balancing resistor R_{bal} .

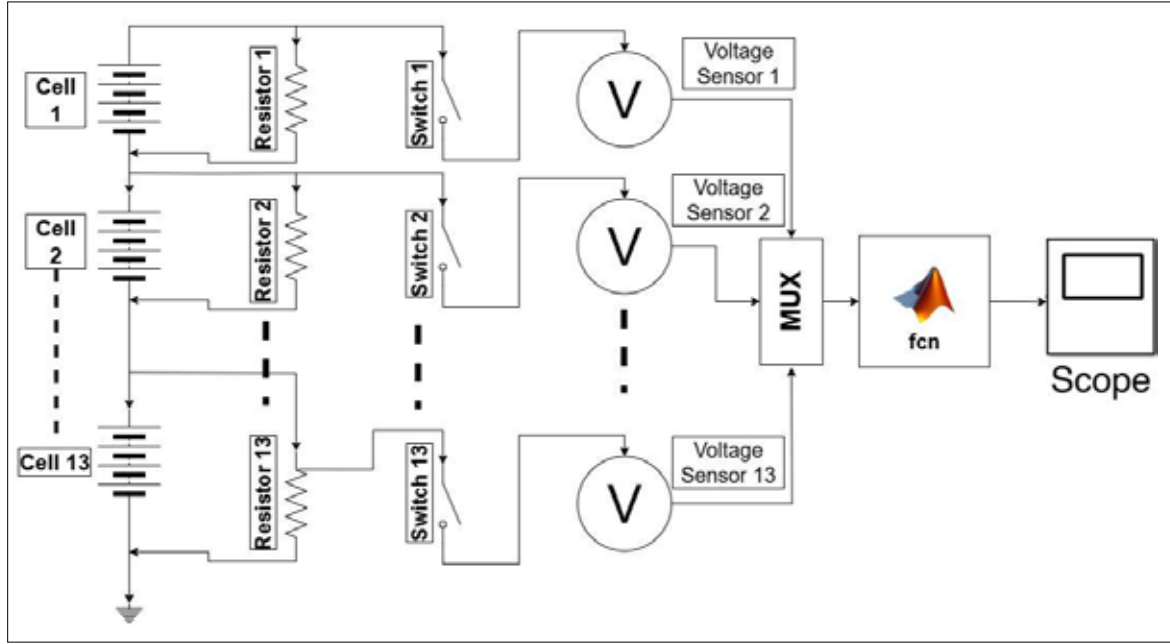


Figure 3: Proposed simulation circuit for passive balancing

$$I_{bal} = \frac{V_{cell,max} - V_{cell,min}}{R_{bal}} \quad \dots(01)$$

The next step is to derive the power dissipation in the resistor through equation (2)

$$P = I^2 R \quad \dots(02)$$

By substituting I_{bal} from equation (1), we get equation (3)

$$P_{loss} = \frac{(V_{cell,max} - V_{cell,min})^2}{R_{bal}} \quad \dots(03)$$

The energy dissipation over time due to balancing is given by equation (4)

$$E_{loss} = \int P_{loss} dt \quad \dots(04)$$

By assuming a constant power loss over a small-time interval Δt in equation (5), and it is shown that the energy wasted as heat is directly proportional to time

$$E_{loss} = \frac{(V_{cell,max} - V_{cell,min})^2}{R_{bal}} \cdot \Delta t \quad \dots(05)$$

The steps to find out the total charge transferred during balancing are derived by following equation (6) and (7). The charge Q_{bal} removed from a higher voltage cell during balancing is expressed as:

$$Q_{bal} = I_{bal} \cdot \Delta t \quad \dots(06)$$

By substituting I_{bal} :

$$Q_{bal} = \frac{V_{cell,max} - V_{cell,min}}{R_{bal}} \cdot \Delta t \quad \dots(07)$$

This indicates that the charge transfer depends on the voltage difference, resistor value, and balancing time.

Since balancing follows an exponential discharge behaviour, the time required to equalize the voltages can be approximated using the equation (8)

$$t_{bal} \approx R_{bal} C_{cell} \ln \left(\frac{V_{cell,max}}{V_{cell,min}} \right) \quad \dots(08)$$

The technique of passive balancing releases higher-voltage cell energy as heat through resistors. It is a simple implementation at a reasonable cost, but major energy loss and slow balancing rates are important disadvantages. The time it takes for balancing depends on both the chosen

resistor value and the cell capacitance, which determines overall efficiency. The technique functions for minor cell imbalances, although it proves unfit for commercial applications because of its excessive power usage and thermal defects. The basic hardware limitations restrict this method because the dissipated heat energy goes to waste instead of supplying cells with redistributed power. This technique requires better equipment to operate effectively for battery energy management, therefore it fails to meet the requirements of fast rebalancing and long battery life in specific applications.

In the MATLAB®/Simulink® implementation, these analytical expressions are directly used to evaluate power loss during simulation. Cell voltages and balancing currents are measured through voltage and current sensor blocks, and the instantaneous power dissipation is computed using embedded mathematical function blocks based on the derived relations. The cumulative energy loss is then obtained by numerical integration over time. This approach ensures consistency between theoretical formulation and simulation-based performance evaluation, enabling direct comparison of balancing strategies under identical operating conditions.

Active balancing (simulation start)

DC-DC bidirectional converters and MOSFETs are the power electronics components of active balancing

systems that can perform multiple simultaneous operations by relocating electronic charges between cells. At the very first step, each cell separately starts the balancing process with the rest of the cells at the very same moment. By targeting individual cells, the charge within the neighboring cells dynamically alters voltages. They aim to attain an equal charge in all the cells with minimal energy loss. The proposed simulation circuit for the active balancing using simultaneous start is shown in Figure 4.

The active cell balancing circuit performs an important function through its mechanism, transferring extra power from cells with higher voltage to those with lower voltage thereby maintaining a balanced distribution of energy throughout the battery pack. The controlled switching mechanism along with a DC-DC converter enables this method to efficiently transfer energy instead of heat-based dissipation found in passive balancing techniques. The battery pack maintains continuous voltage sensor input which leads the control algorithm to determine ideal balancing operations. The active balancing uses adaptable charging management capabilities to cut down power loss, decrease heat effects and extend battery life. Active cell balancing demonstrates remarkable advantages within power systems that need high operational efficiency and extended battery usage, like EVs, alongside energy storage systems.

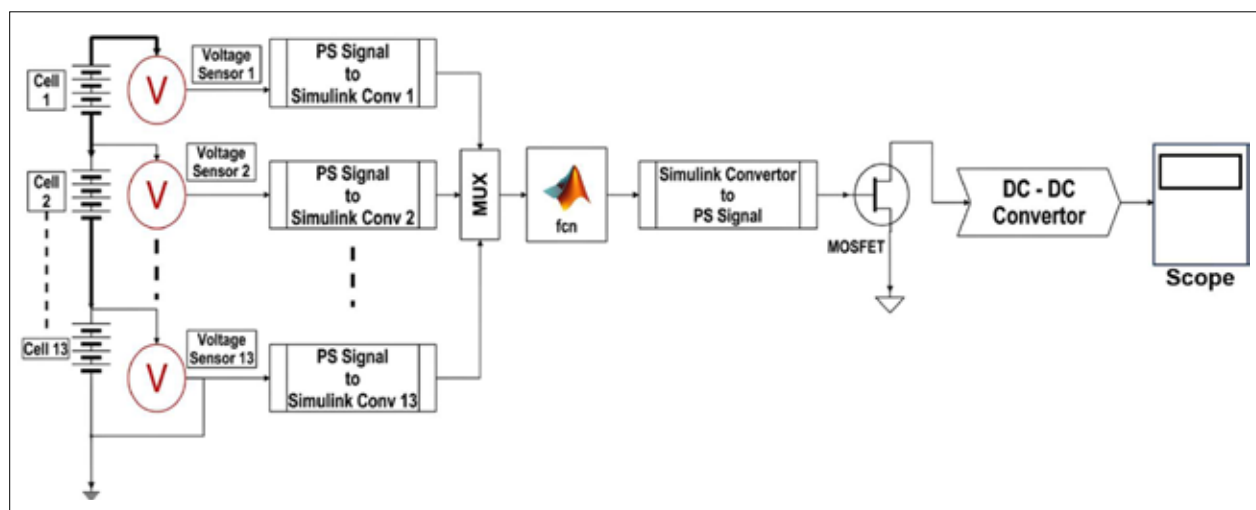


Figure 4: Proposed simulation circuit for active balancing

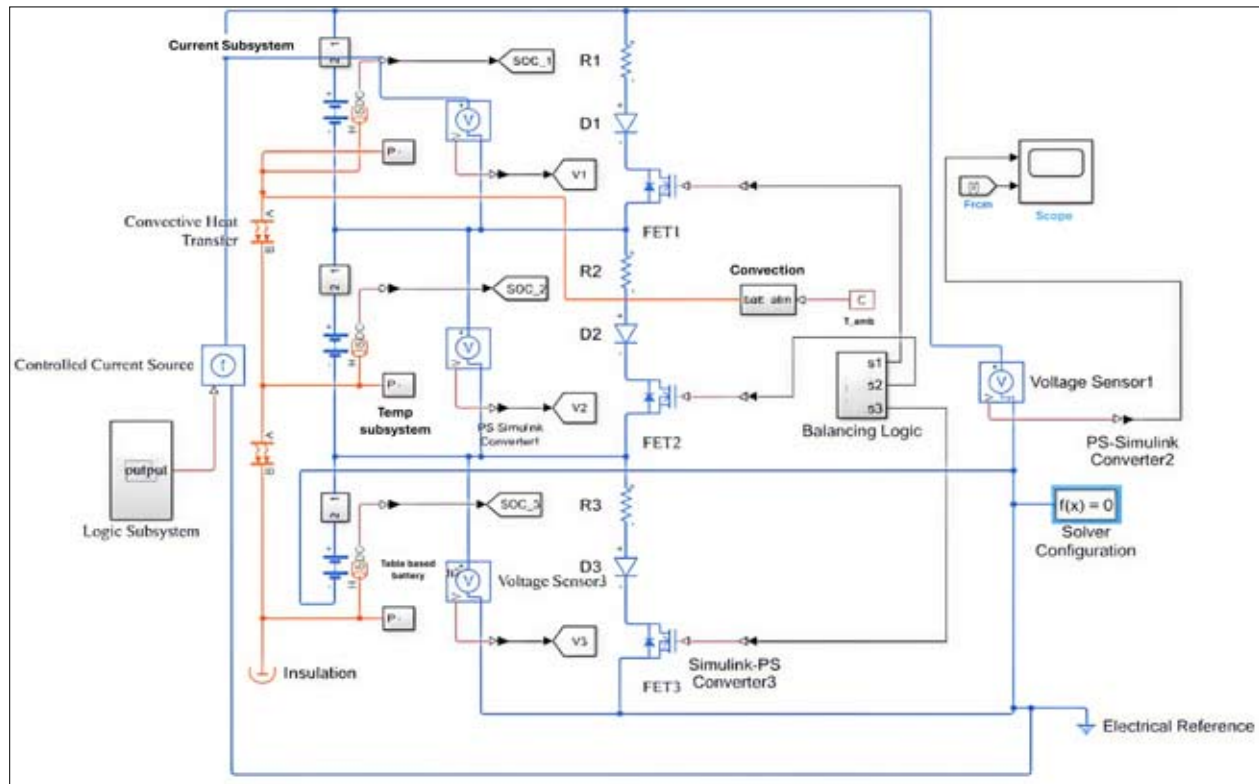


Figure 5: ACB circuit with MOSFET transistor

Figure 5 illustrates the MOSFET-based ACB sub-circuit implemented in MATLAB®/Simulink®. Each lithium-ion cell is modeled with associated sensing and switching elements enabling controlled energy redistribution. The balancing structure consists of:

- **MOSFET switches (FET1–FET3):**
These act as controllable bidirectional current paths driven by the balancing logic. When activated, they enable charge transfer from higher-voltage cells toward lower-voltage cells through the balancing network.
- **Balancing resistors (R1–R3):**
These components regulate current magnitude and limit transient surges during switching events, ensuring numerical stability and realistic dissipation modeling.
- **Protection diodes (D1–D3):**
These are included to prevent reverse current flow and protect switching devices from voltage transients during charge redistribution.

- **Voltage sensing blocks:**
Cell voltages are continuously monitored and forwarded to the balancing control logic, enabling adaptive switching decisions based on imbalance thresholds.
- **Logic control subsystem:**
This implements the decision algorithm that determines MOSFET gating signals using SOC and voltage deviation metrics. This ensures selective activation of simultaneous or staggered balancing modes.
- **Isolation and interfacing elements:**
Converter blocks and electrical references ensure compatibility between physical-domain signals and Simulink control signals, maintaining simulation integrity.

Overall, the circuit models realistic switching-based energy equalization where MOSFET activation dynamically redistributes charge across cells, enabling performance evaluation of balancing strategies under controlled simulation conditions.

In this method, we define each lithium-ion battery cell as a capacitor because the charge stored in a cell is proportional to its voltage. Therefore, the energy stored in a capacitor is given by equation (9):

$$E = \frac{1}{2} C_{bal} V^2 \quad \dots(09)$$

If the battery pack is imbalanced, some cells have high voltage, other cells remain with a lower voltage. To transfer the excess energy from the higher voltage cell to the lower voltage cell is the important objective. The total energy stored in the highest voltage before balancing is given in equation (10)

$$E_{max} = \frac{1}{2} C_{bal} V_{cell,max}^2 \quad \dots(10)$$

Likewise, the total energy stored in the lowest voltage cell before balancing is given in equation (11):

$$E_{min} = \frac{1}{2} C_{bal} V_{cell,min}^2 \quad \dots(11)$$

By substituting the energy expressions, we obtain equation (12):

$$E_{transferred} = \frac{1}{2} C_{bal} (V_{cell,max}^2 - V_{cell,min}^2) \quad \dots(12)$$

The calculated formula demonstrates that capacitance along with squared voltage differences controls the energy movement between voltage extremities. Power electronics utilized for active balancing allow efficient energy charge redistribution thus outperforming the regressive heat dissipation method of passive balancing. The continuous dissipation method in passive balancing produces large energy wastage yet active balancing accomplishes fast convergence along with maximal energy efficiency. This approach restores equal cell charging levels through its method and enhances both battery efficiency and operational life extensions. The active balancing method stands as the best solution for high-performance electric vehicles since it offers automatic speed-efficient cell rebalancing, optimizes the usable energy and ensures long-term battery system reliability.

For active balancing configurations, power loss is calculated from converter current flow and switching behaviour rather than resistive dissipation alone. Sensor outputs capturing current transfer and voltage

differences are processed using equivalent energy-transfer formulations implemented within the Simulink model. These measurements allow estimation of energy redistribution efficiency and residual losses associated with switching and component resistance. Consequently, the reported loss values represent simulation-derived metrics grounded in the same analytical framework used for passive balancing.

Active balancing (staggered start)

The active balancing method employs an additional practicality with the staggered start technique consisting of the same circuit diagram as in Figure 3 but only the MATLAB® code is modified according to the staggered start. Active balancing using staggered start improves efficiency together with redistributing battery cell charges at increased speed. The method enables a step-by-step charge movement instead of simultaneous transfers thus minimizing the circuit peak load while improving thermal control capacities. A controlled switching sequence dictates the balancing process which moves energy from the most elevated cell voltage through an inductor until reaching the smallest voltage cell. The developed equation shows how cells create current flux through the inductor as a function of their differential tension and the balance integration speed. Staggered activation within this model reduces power switching losses, distributes energy evenly, and grows battery lifetime. Such a control system brings exceptional benefits to platforms combining electric vehicles with large-scale energy storage because it provides exact management and efficiency.

RESULTS AND DISCUSSION

For each technique, the voltages of all 13 cells are recorded at each time step over a simulated duration of 2 hours. The results are visualized as separate plots in Figure 6 for each technique, showing how cell voltages evolve and eventually converge to the target voltage. The x-axis represents time, while the y-axis displays cell voltages, providing a clear comparison of the dynamics and efficiency of each method.

Although the results are generated through MATLAB®/Simulink® simulation, the performance trends reflect realistic operational conditions due to hardware-representative component modeling and practical parameter selection. Metrics such as voltage convergence rate, power loss, and balancing duration correspond to observable characteristics in experimental battery pack testing. Consequently, the simulation outcomes serve as a reliable indicator of

comparative strategy effectiveness. However, absolute numerical values may vary in physical deployment due to temperature effects, manufacturing variability, and

electrochemical aging, which are beyond the scope of the present system-level study.

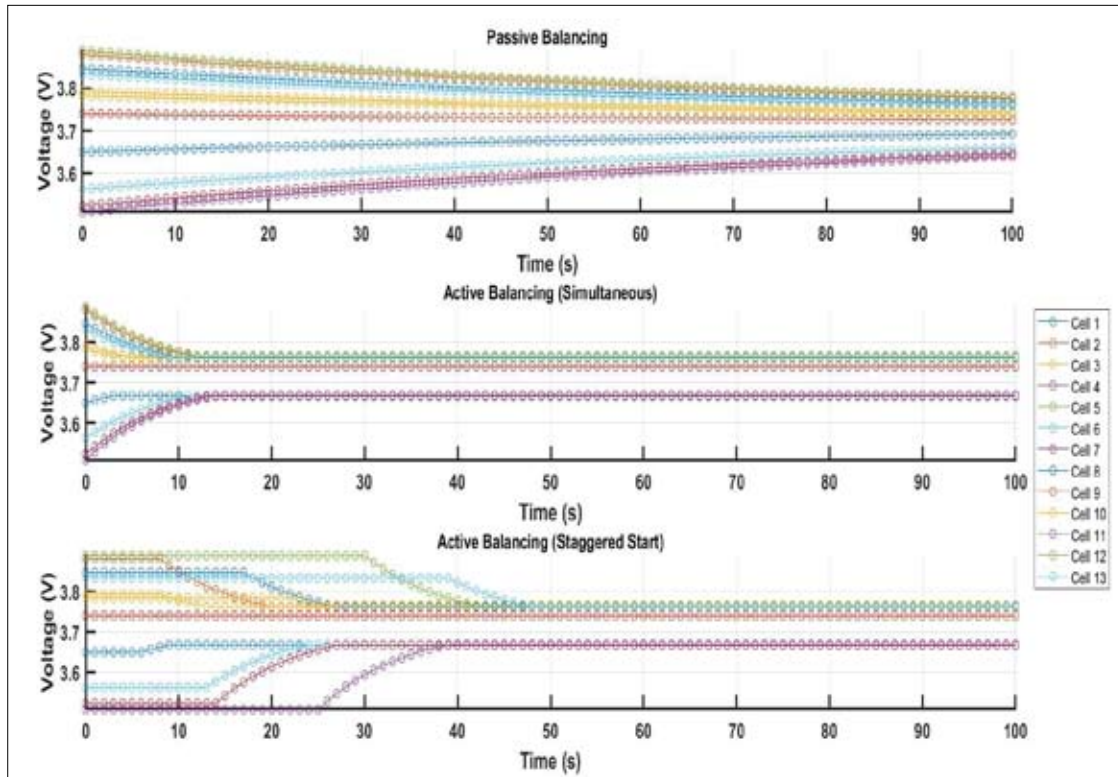


Figure 6: Simulation output of the proposed balancing techniques

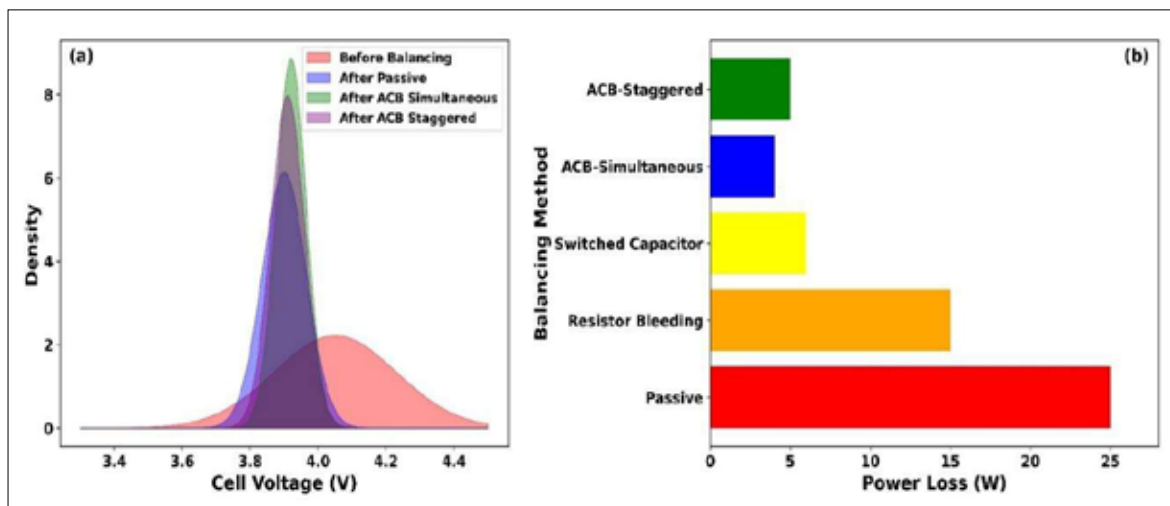


Figure 7: (a) Voltage distribution before and after balancing; (b) Power loss comparison

The analysis presents three cell balancing approaches: passive balancing and active balancing via the simultaneous method along with the staggered start method. The process of passive balancing (top graph) produces heat through resistors to dissipate excess energy from higher voltage cells, thus causing cells to become equalized at a slow and inefficient rate over time. Through a capacitor system, simultaneous active balancing (middle graph) enables direct voltage exchanges from high-voltage to low-voltage cells which results in a rapid equilibrium that stabilizes quickly. The charge transfer within staggered start active balancing (bottom graph) is distributed sequentially between cells at different intervals during the balancing operation. This approach enables better control of the balancing process to minimize energy waste, while preventing sudden current surges, resulting in enhanced voltage equalization between cells.

The red distribution in Figure 7 (a) reveals how the voltage ranges from high to low before balancing occurs because different cells possess various voltage levels. The difference in cell voltage levels destabilizes battery performance while shortening its overall operational time. Different balancing methods cause voltages to move toward proximity with each other. Passive balancing (green) works under a system where voltage differences are reduced through time yet it maintains heat dissipation as a side effect.

The active balancing method (blue) transfers energy between cells instantaneously which produces rapid and even voltage spread throughout the system. Staggered active balancing through the purple sequence provides a methodical balance process that controls the performance while maintaining consistent current flow. All balancing approaches together drive the voltages of cells toward equilibrium yet active methods provide more efficient and effective results than passive methods.

Different battery balancing techniques appear in Figure 7 (b) showing their power loss measurements. Red in the graph indicates passive balancing which results in the maximum power consumption totaling almost 25W. The production of heat through resistors results in energy waste in passive balancing since it extracts energy from higher voltage cells to balance of their voltage. The power loss for resistor bleeding (orange) stands out since this method performs energy dissipation like its counterpart. The yellow method of switched capacitor balancing performs better than passive balancing in terms of power loss because it can redirect cell charge between cells yet it does lose some energy through operation. The implementation of ACB Simultaneous (blue) decreases power waste and uses active components to move energy between cells without requiring heat dissipation (under dynamic conditions, it may slightly increase and exceed up to 6 W). The ACB Staggered approach (green) proves to be the most power-efficient method among all the

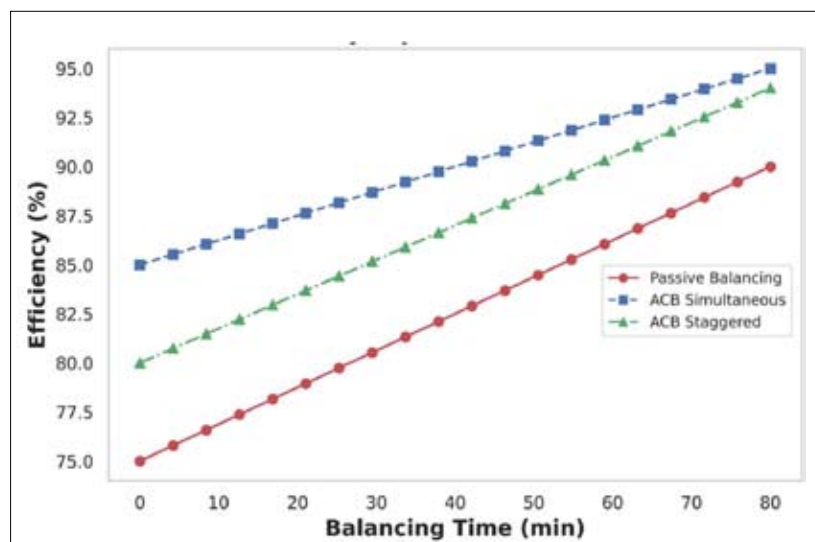


Figure 8: Efficiency improvement over time

solutions. Series connection of MOSFETs occurs in a timed sequence to stop momentary high currents and reduce the production of heat. The technique increases total energy efficiency but maintains balanced cell operations.

The power-loss values illustrated in Figure 7(b) are derived from simulation measurements processed through the analytical formulations presented in the methodology sections. Real-time voltage and current data obtained during balancing are applied to the loss equations to compute instantaneous dissipation and aggregate energy loss over the balancing period. This integration of theoretical equations with sensor-based simulation outputs ensures that reported performance metrics are not solely graphical observations but quantitatively grounded calculations.

Figure 8 shows how efficiency improves over time for different balancing methods. Initially, efficiency is lower (around 75-85%), but as balancing progresses, it gradually increases to nearly 95%. ACB Staggered achieves the highest efficiency by optimizing energy flow and minimizing unnecessary energy loss.

ACB Simultaneous balances the cells quickly but loses more energy in the process. Passive Balancing, on the other hand, improves efficiency at a much slower rate due to continuous minor energy losses. Overall, ACB Staggered provides the best balance between speed and energy efficiency, making it the most effective method over time.

Figure 9 compares the switching patterns of MOSFETs for different balancing methods. Passive balancing enables frequent switching of its switches (every 5 minutes) which results in substantial energy losses. ACB Simultaneous operates all MOSFETs as a complete group at 10-minute intervals while using increased energy.

ACB Staggered presents the most efficient configuration which applies power to MOSFETs in sequential order at 20-minute intervals to minimize power waste and extend battery life. ACB staggered delivers the optimal solution for long-lasting battery performance as well as reliability.

Here is Table 4 summarizing the design and implementation of the three balancing techniques:

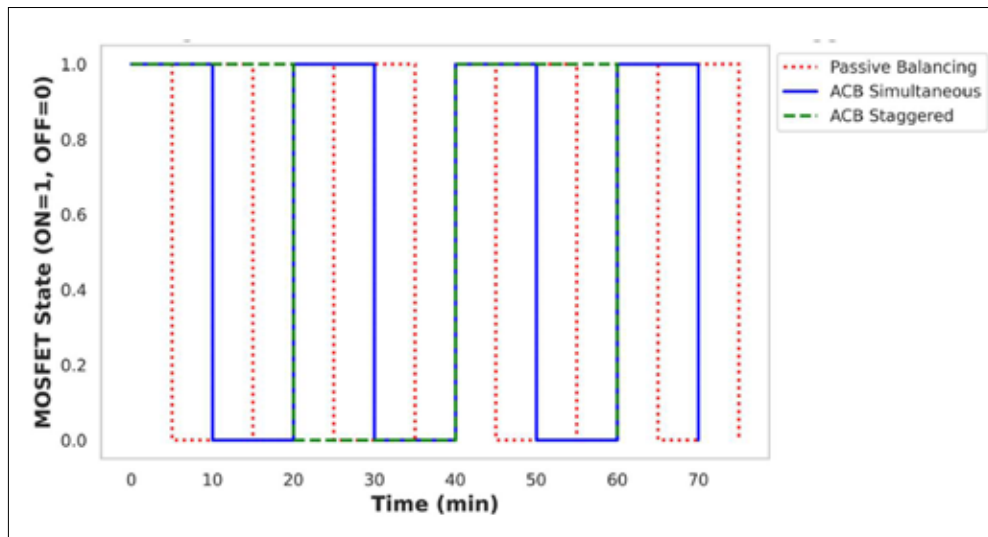


Figure 9: MOSFET switching pattern- passive vs simultaneous vs staggered

Table 4: Features of balancing techniques

Feature	Passive balancing	Active balancing (simultaneous start)	Active balancing (staggered start)
Balancing principle	Excess energy is dissipated as heat through resistors.	Charge is redistributed among cells using DC-DC converters.	Charge redistribution is like simultaneous balancing but starts at different times for each cell.
Energy efficiency	Low (energy is wasted as heat).	High (minimal energy loss).	High (like simultaneous balancing).
Balancing speed	Slow, due to energy dissipation rate.	Fast, as all cells start balancing at the same time.	Gradual, as each cell starts balancing at staggered intervals.
Control logic	Simple: proportional dissipation based on voltage.	Uses a control block to balance voltage differences actively.	Adds staggered start times to the active balancing logic.
Simulation components	- Battery blocks - Resistor blocks - Scope blocks	- Battery blocks - DC-DC converters - MATLAB® Function for logic - Scope blocks	- Battery blocks - DC-DC converters - MATLAB® Function for staggered start logic - Scope blocks
Key parameters	Resistance factor for energy dissipation.	Voltage threshold and balancing step size.	Voltage threshold, balancing step size, and staggered start times.
Expected output	Gradual convergence of voltages, with energy loss as heat.	The rapid convergence of all voltages with minimal energy loss.	Gradual convergence, with delayed balancing for specific cells.
Visualization	Voltage profiles of all cells over time.	Voltage profiles show fast convergence of all cells.	Voltage profiles show staggered convergence of cells.

Table 5: Comparison with existing systems

Balancing type	Power level (W)	Energy loss (W)	Balancing time (min)	Efficiency (%)	Complicity	Source
Switched capacitor	5	6-10	70-90	85-90	Medium	(Sun <i>et al.</i> , 2021)
Inductor capacitor	5	2-4	50-70	92-97	High	(Guo <i>et al.</i> , 2023)
Resistor bleeding	5	20-30	150-200	50-60	Very low	(Duraisamy & Deepa, 2021)
Passive balancing	5	15-25	120-180	60-70	Low	Proposed
Active balancing (Simultaneous start)	5	3-5	45-60	90-95	High	Proposed
Active balancing (Staggered start)	5	4-7	60-80	88-92	Medium	Proposed

Comparison with the existing systems

Table 5 compares different battery balancing techniques based on energy loss, balancing time, efficiency, and complexity. Passive methods, such as resistor bleeding, have high energy loss and longer balancing times but are simpler. In contrast, active balancing methods, especially inductor-capacitor and simultaneous start approaches, offer higher efficiency with lower energy loss, though

they require more complex circuitry. Now, let us analyze these results in detail.

The comparative evaluation of balancing techniques using a normalized reference power level was used to ensure fair benchmarking against previously reported studies. The proposed strategies were simulated under identical electrical conditions corresponding to a representative 5 W cell-level balancing transfer

capability. The resulting loss values reflect system-level dissipation that includes converter switching losses, sensing overhead, and control interactions rather than idealized transfer-only losses. Consequently, absolute magnitudes exceed theoretical converter efficiency estimates but remain consistent in relative ranking across methods.

Passive techniques exhibit substantially higher dissipation due to resistive energy dumping, whereas active simultaneous balancing achieves the lowest loss and highest efficiency. Staggered balancing shows moderate overhead attributable to sequential control coordination. This normalized evaluation framework ensures that performance improvements observed for the proposed methods arise from architectural advantages rather than differing operating scales, thereby strengthening the validity of cross-study comparison.

Observations

Figure 10 shows the comparison with the existing systems, and the key findings are:

- Efficiency is marginalized with passive balancing owing to energy dissipation.
- The performance obtained by active balancing (simultaneous start) can significantly reduce the

balancing time and the internal drive voltage deviation.

- Staggered start active balancing provides a balance with moderate performance improvements.

The complexity scale illustrated in Figure 8 is intended to provide a qualitative comparison of implementation efforts across different balancing strategies. The classification is established based on three technical considerations: (i) circuit topology requirements, including the number of passive and active components, magnetic elements, and switching devices; (ii) control and sensing requirements, representing the level of monitoring, synchronization, and algorithmic coordination involved; and (iii) implementation overhead, encompassing driver circuitry, design effort, and integration challenges within a battery management system environment. Using these criteria, methods employing minimal circuitry and straightforward control logic are categorized as low complexity, whereas approaches requiring additional switching coordination or energy-transfer components are designated as moderate complexity. Techniques incorporating magnetic elements or tightly coordinated switching control are classified as high complexity. This normalized qualitative framework facilitates comparison of design trade-offs alongside efficiency and loss metrics without implying a hardware-specific cost or precise numerical measure.

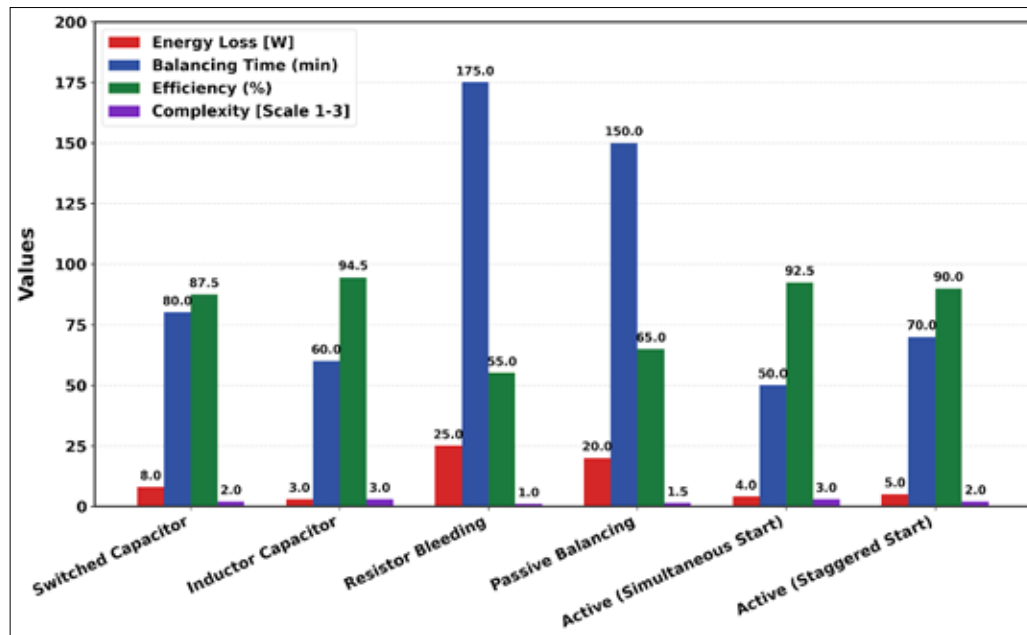


Figure 10: Comparison of the proposed system with existing methods

A three-level ordinal scale (1–3) is adopted to represent these categories, as implementation complexity is not directly quantifiable through physical measurement. Employing a limited number of levels avoids artificial numerical precision while preserving interpretability in graphical representation. The selected structure provides adequate differentiation among balancing architectures and aligns with qualitative benchmarking practices commonly used in BMS research.

From a practical EV deployment perspective, the most critical performance metric highlighted in Figure 8 is energy efficiency, represented by reduced power loss during balancing. In real-world EV battery packs, minimizing dissipative losses directly preserves usable stored energy, thereby extending driving range and reducing charging frequency. Additionally, lower energy dissipation mitigates thermal buildup, which is strongly linked to accelerated battery aging, safety risks, and cooling system burden. Although balancing time influences responsiveness and circuit complexity affects implementation cost, efficiency remains the dominant system-level priority because it simultaneously impacts operational safety, lifetime reliability, and energy utilization. The superior loss characteristics observed in the staggered active balancing strategy therefore indicate strong practical suitability for scalable EV BMS.

The possible ways to further develop the current work and improve the performance of battery management systems based on innovative methods and new technologies are:

Integration with advanced BMS: Active cell balancing has the potential to be integrated into next-generation BMS for EVs, electric bikes, and energy storage systems (ESS). With improvements in BMS algorithms, active balancing can be modified to work under real-time load and charging conditions of the system to enhance performance.

Machine learning and AI for predictive balancing: Introducing machine learning algorithms can facilitate predictive cell balancing by flagging potential imbalances before they happen. AI can use historical data to enhance the efficiency of balancing and minimize energy waste.

Compatibility with solid-state batteries: As solid-state batteries become more mainstream, methods of active cell balancing can be altered for smooth control of solid electrolytes, higher energy density, and a broader operating voltage range.

Scalability for large battery packs: Future work can be chosen to increase the scope of active balancing techniques for heterogeneous battery systems, like grid-level energy storage, where thousands of cells must be optimized.

CONCLUSIONS

The research investigates passive and active balancing technologies for EV battery packs, evaluating their advantages and disadvantages. The findings reveal that active balancing techniques, specifically the simultaneous start and staggered start methods, excel in performance by reducing energy waste, shortening balancing periods, and enhancing operational efficiency. Active battery balancing reduces energy loss to between 3 and 7 watts, with balancing durations typically ranging from 45 to 80 minutes. In comparison, traditional passive balancing results in higher losses of 15 to 25W and extended balancing periods of 120 to 180 minutes. The staggered start method facilitates automated energy distribution, minimizing cell temperature variations and extending battery life expectancy. This study establishes that advanced active balancing techniques significantly improve lithium-ion battery packs in EVs compared to traditional methods such as switched capacitors and resistor bleeding techniques. Moreover, it highlights that intelligent battery management strategies enhance performance in sustainable battery control systems. Future efforts will concentrate on hardware-in-the-loop testing or experimental validation to verify the simulation results in real-world operating conditions. Future work incorporating machine learning is aimed at creating predictive balancing techniques, which will improve energy efficiency, extend battery life, and increase system reliability in EVs.

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RESEARCH ARTICLE

Statistics

Spatiotemporal techniques for imputing sequential missing values in rainfall data collected from Ratnapura area: A comparative study

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Abstract: Modeling and forecasting rainfall is vital for issuing early flood warnings, but missing data, particularly sequential gaps, make this task challenging. Even though there are several techniques proposed in the literature aiming at the estimation of sequential missing values, selecting the most suitable technique is challenging. It is worth considering both temporal patterns and spatial patterns in such imputations. This study evaluates four widely used spatiotemporal techniques, K-nearest neighbors (KNN), MissForest, denoising autoencoder, and spatiotemporal kriging, to identify the best approach for imputing sequential missing values of rainfall at Ratnapura. Analysis was carried out using daily rainfall data of Rathnapura and six neighboring gauging stations, collected from 2015 to 2019. The accuracy of each estimated series was evaluated against the actual data using root mean square error (RMSE) and mean absolute error (MAE). The results show that the KNN is the most suitable technique under both wet and dry seasons, showing lower RMSE and MAE values. MissForest also performed well, surpassing denoising autoencoder and spatiotemporal kriging, though it generally fell slightly short of KNN. Among the methods compared, spatiotemporal kriging demonstrated the lowest accuracy in both wet and dry seasons due to a lack of reference stations and other meteorological variables. However, none of the techniques were successful in accurately imputing sudden extreme rainfall events, highlighting an important limitation. Forecasted rainfall at the Ratnapura station using both the imputed data and the actual data revealed that the forecasts obtained using the imputed data were not significantly different from those obtained using the actual data.

Keywords: Spatiotemporal data, sequential missing value imputation, rainfall data, machine learning, deep learning

INTRODUCTION

Modeling and forecasting rainfall in a particular area is essential for issuing early warnings of floods in that area. However, in a developing country like Sri Lanka, modeling rainfall is very complicated as these series consist of missing values, especially when data are missing continuously in time for considerably long periods as a result of natural events or technical failures. If data of a series are missing continuously in time for a period, such missing values can be identified as sequential missing values.

Even though a few techniques proposed in literature aiming the estimation of sequential missing values (Feng et al., 2014; Mital et al., 2020; Saubhagya et al., 2024), selecting the most suitable technique is challenging, as the performance of the techniques will depend on the nature of the missing data series as well as the characteristics of the aiding data, which are used to estimate the missing values. In estimating sequential missing values of rainfall data, it is worth considering not only their temporal patterns but also their spatial patterns as well (Li et al., 2020; Mital et al., 2020; Saubhagya

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et al., 2024). However, studies which evaluate advanced spatiotemporal techniques for imputing missing values, especially a sequence of missing values in rainfall data, are extremely rare.

In the Sri Lankan context, one study (Silva et al., 2007) focuses on three conventional methods, namely the arithmetic mean method, the normal ratio method, and the inverse distance method, and they introduced a new method called the aerial precipitation ratio method. However, according to the RMSE and mean absolute percentage error (MAPE) accuracy of these methods was very low. In another study, Saubhagya et al. (2024) developed a new hybrid method which combines spatial kriging and artificial neural networks. However, it does not examine how the seasonal variation of data affects missing value imputation and also does not compare the novel technique developed with widely used spatiotemporal missing value imputation techniques. In the global context, (Mital et al., 2020) developed a novel sequential imputation algorithm with the aim of imputing missing values in spatiotemporal daily rainfall measures. They used a sequential imputation algorithm, which was incorporated within a spatial interpolation method based on random forests. However, they did not compare this algorithm with other available techniques.

The main objective of this study is to compare widely used spatiotemporal missing value imputation techniques with the intention of identifying the most suitable missing value imputation technique to estimate the sequential missing values of the rainfall at Rathnapura gauging station. Rathnapura was selected as it is the most vulnerable area for floods in Sri Lanka (Thevakaran et al., 2019; Weerakoon & Dharmapriya, 2021). Moreover, it is important to mention that there is a research gap in identifying the most suitable spatiotemporal missing value technique to estimate sequential missing values of rainfall data.

Apart from finding a more accurate spatiotemporal missing data imputation technique, this study examines how different imputation techniques perform during different seasons and investigates the sensitivity of the selected spatiotemporal imputation techniques to different levels of missingness. Furthermore, this study will recommend the imputation technique that better estimates sequential missing data in rainfall records in the Rathnapura area. Finally, it investigates the pertinence of the imputed missing values in developing the forecasting models.

The rest of the paper is organized as follows: the next section describes the materials and methods used in achieving the objectives of this study. The theories behind the selected widely used spatiotemporal techniques for imputing sequential missing values are also briefly presented in this section. The third section presents and interprets the results obtained. The final section presents the conclusion derived and proposes directions for future research.

MATERIALS AND METHODS

Four widely used sequential missing value imputation techniques, namely, K-nearest neighbors (KNN) (Zhu and Cheng, 2016), Missforest (Zhang et al., 2021), denoising autoencoder (Wardana, Gardner and Fahmy, 2022) and spatiotemporal kriging (Bae et al., 2018; Yang et al., 2018) were identified from a comprehensive literature review. Sequential missing value imputation for Rathnapura gauging station was carried out using daily rainfall data of Rathnapura and six neighbouring gauging stations (Moralioya, Detanagala, Keragala, Elston, Balangoda, and Ilubbuluwa) from 2015 to 2019. This period was selected as complete rainfall data records (i.e. without missing values) were available for all selected stations. Initially, a preliminary analysis was carried out using the selected dataset as spatial analysis and temporal analysis. As the Spearman correlation coefficient has the ability to measure linear as well as non-linear correlation between variables, it was applied to identify relationships between rainfall records in Rathnapura and other neighbouring stations (i.e. spatial dependencies).

Rainfall records are deleted during selected periods of different lengths to artificially create sequential missing values. Then, sequential missing values were estimated using the four selected imputation techniques. The accuracy of each estimated series of missing values was evaluated against the actual data using RMSE and MAE. Sequential missing values were imputed with a focus on both wet and dry seasons. For each season, missing periods of different durations (one week, two weeks, and one month) were examined. The selection of missing periods for wet and dry seasons was carried out according to Priyakumara and Ranasinghe (2023), considering the rainfall values (whether it is high (wet season) or low (dry season)) in the dataset. They mentioned that the primary source of rainfall for the Rathnapura area is the southwest monsoon, typically occurring from May to September. According to this statement, September was selected to represent the wet season, and January was

selected to represent the dry season. Therefore, missing values were generated for both September (representing the wet season) and January (representing the dry season) in the years 2015, 2017, and 2019. This was done for varying durations, including one week, two weeks, and one month, to evaluate how the imputation techniques performed under different lengths of missing data. To implement KNN, Missforest, and spatiotemporal kriging, daily data from 2015 to 2019 were used without considering the train-test split since these techniques only need datasets with missing values. However, the denoising autoencoder was trained using data from 1994 to December 2014 since it needs a large number of data points to avoid overfitting, while the test sample included data from January 2015 to December 2019. The model accepts input as a collection of 7×7 matrices, where each matrix consists of 7 columns representing rainfall data from different stations, including Ratnapura. Each matrix also contains 7 rows, where the first 6 rows correspond to the previous 6 days of observed data, and the final row represents the current day. This setup allows the autoencoder to impute sequential missing values during both the wet and dry seasons in the test set.

The following sub-sections briefly explain the theory behind four selected techniques relevant to estimating sequential missing value imputation of rainfall at Rathnapura guaging station.

K nearest neighbors

The KNN algorithm used in this study applies a weighted mean from a sample of data at the target location. This requires the presence of data at the multi-site station network considered. Let us define a dataset where the rows represent days (r_i ; $i = 1, \dots, i_0, \dots, I$) and the columns represent stations (c_s ; $s = 1, \dots, s_0, \dots, S$). Each entry in the dataset corresponds to the value of a variable $X_{i,s}$, observed for a specific station (s) on a particular day (i). Consider a row vector (r_{i_0}) containing a missing value in the target station (s_0). This row vector is compared with other row vectors (r_i) by computing a distance measure ($d_i|r_{i_0}$) which measures dissimilarity between the day with a missing value (r_{i_0}) and a particular day (r_i). For this purpose, the commonly used Euclidean norm is adopted with a weight (defined by equation (2)) (Juna et al., 2022).

$$d_i|r_{i_0} = \sqrt{\text{weigh} \times \text{sqaured distance from persent coordinates}} \quad \dots(01)$$

$$\text{sqaured distance from present coordinates} = \sum_{s=1}^S (x_{i,s} - x_{i_0,s})^2 \quad \dots(02)$$

$$\text{weight} = \frac{\text{total number of stations without the target station}}{\text{present coordinates}} \quad \dots(03)$$

Then, data at the target station (c_{s_0}) are ranked according to the ascending order of $d_i(c_{s_0}|d_i)$.

$$c_{s_0}|d_i \rightarrow x_{s_0,k} = \{x_{s_0,1}, \dots, x_{s_0,k}, \dots, x_{s_0,I}; d_k \leq d_{k+1}\}$$

Then, the target datum is estimated as the weighted mean:

$$\hat{x}_{i_0,s_0} = \sum_{k=1}^K d_k^{-1} x_{s_0,k} \quad \dots(04)$$

where K is the parameter indicating the number of nearest neighbours considered for the estimation.

Missforest

The Missforest imputation (S. Zhang et al., 2021) procedure starts by initializing an estimate for the missing values in the dataset. This initial estimate is commonly obtained through a simple imputation method such as mean imputation. Additionally, the variables in the dataset are sorted based on their respective counts of missing values, arranging them from lowest to highest. Each variable undergoes an imputation process in which missing values are imputed by employing a random forest. This involves training the random forest model with observed responses and predictors for the given variable. Subsequently, the trained random forest is applied to predict the missing values by utilizing the corresponding set of predictors for the missing entries. This procedure is repeated until the difference (normalized mean square error (NMSE)) (defined by equation (5)) between the imputed data (X_{new}^{imp}), and the previous iteration output data (X_{old}^{imp}) do not increase.

$$NMSE = \frac{\sum_{j \in N} (X_{new}^{imp} - X_{old}^{imp})^2}{\sum_{j \in N} (X_{new}^{imp})^2} \quad \dots(05)$$

where X = Matrix of data points, N = Number of data points.

Spatiotemporal kriging

In this study, a spatiotemporal process $\{Z(s, t): (s, t) \in D \subseteq \mathbb{R}^d \times \mathbb{R}\}$ was assumed, where $s \in \mathbb{R}^d$, $d=2$ represented the two dimensions in space (latitude and longitude), and $t \in \mathbb{R}$ represented only one dimension, namely time. For this spatiotemporal process, spatiotemporal kriging can be performed mainly in two ways.

- Directly fit the variogram to the original data and perform kriging to impute missing values (Yang et al., 2018)
- Initially, fit a multiple linear regression model to the data and fit the variogram to the residuals of the multiple linear regression model (de Medeiros et al., 2019)

In the first approach, using original rainfall data ($Z(s, t)$), the sample variogram $\gamma_{ss}(h_s, h_t)$ was calculated. Here, h_s refers to a predefined spatial lag, representing a fixed distance interval between pairs of rainfall stations, while h_t refers to a predefined temporal lag, representing a fixed time interval between observations. Then the theoretical model was selected that was best suited for the sample variogram based on the smallest mean square error (MSE) between the sample variogram and fitted theoretical variogram (this is achieved by selecting a variogram model (such as spherical, exponential, or Gaussian) and adjusting its parameters (range, sill, and nugget) to closely match the empirical or sample variogram, which is calculated from the observed data). After that, use the least-squares approach or maximum likelihood estimation (MLE) to fit the theoretical variogram model to the sample variogram. Then the fitted variogram can be used to impute the missing rainfall values.

In the second approach, the spatiotemporal variation $Z(s, t)$ was established by the decomposition of the deterministic $m(s, t)$ and stochastic residual $\varepsilon(s, t)$ as follows:

$$Z(s, t) = m(s, t) + \varepsilon(s, t) \quad \dots(06)$$

Where $m(s, t)$ is a deterministic part of the $Z(s, t)$ which can be estimated as follows (See equation (7)). Here $\varepsilon(s, t)$ is the residual term.

$$\hat{m}(s, t) = \sum_{k=0}^K \hat{\beta}_k X_k(s, t);$$

$$\hat{\beta}_k = \text{Estimated Coefficients of a multiple linear regression model} \quad \dots(07)$$

Where $X_k(s, t)$ represents the observed predictors at the location s and time t in the dataset.

Usually, $m(s, t)$ component is determined by an ordinary least square regression model. After that for the residual part, the same procedure was applied as in approach one.

Denoising autoencoder

Denoising autoencoders commonly use convolutional layers to build the neural network. It consists of two main parts: an encoder and a decoder (as shown in Figure 1)

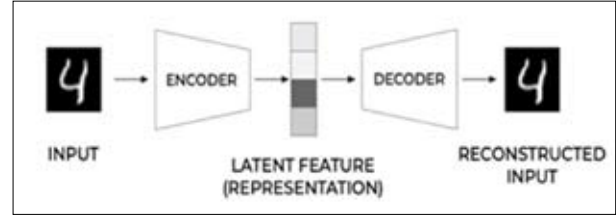


Figure 1: General structure of an autoencoder (Source: Michelucci, 2022, (<http://arxiv.org/abs/2201.03898>))

In general, the encoder can be written as a function g that will depend on some parameters as in equation (8):

$$h_i = g(x_i) \quad \dots(08)$$

Where $h_i \in \mathbb{R}^q$ (latent feature representation) is the output of the encoder (i.e. $g: \mathbb{R}^n \rightarrow \mathbb{R}^q; q < n$, where n is the original dimension of the data). The decoder (and the output of the network can be indicated as $\tilde{x}_i$) can be written then as a second generic function f of the latent features as in equation (9):

$$\tilde{x}_i = f(h_i) = f(g(x_i)) \quad \dots(09)$$

here $\tilde{x}_i \in \mathbb{R}^n$. Training and autoencoder simply means finding the functions $g(\cdot)$ and $f(\cdot)$ that satisfy,

$$\arg \min_{f, g} \langle \Delta(x_i, f(g(x_i))) \rangle \quad \dots(10)$$

where Δ indicates a measure of how the input and the output of the Autoencoder differ (basically the loss function will penalize the difference between input and output) and $\langle \cdot \rangle$ indicates the average over all observations (Baldi, 2012).

Further verification of imputed values

Finally, to clarify whether the imputed dataset gives accurate forecasting results, LSTM (long-short-term memory) models were fitted using actual data and imputed data under different scenarios, and the forecasted results obtained from two types of models were compared by

means of MAE. LSTM was selected as the past studies (Zhang et al., 2020) confirm its suitability in forecasting time series data for short as well as long horizons.

Three LSTM models were trained using three overlapping time periods for the training and test sets with the aim of obtaining generalized results. The training and test sets for each model were as follows:

- **Model 1:** The training set covered from January 2015 to December 2016, and the test set included a three-month period from January 2017 to March 2017.
- **Model 2:** The training set spanned from January 2016 to December 2017, with the test set covering January 2018 to March 2018.
- **Model 3:** The training set ranged from January 2017 to December 2018, while the test set included January 2019 to March 2019.

Forecasting accuracy was evaluated for models trained with actual data and imputed data, considering different random seeds and various lengths of missing data periods (one month, two weeks, and one week). These missing periods were selected because, in the Ratnapura area, sequential missing values in rainfall typically occur for a maximum duration of about one month. Therefore, the

chosen scenarios reflect realistic conditions, covering up to one month of continuous missing data.

- a) For ten random seeds, generate five one-month missing periods.
- b) For ten random seeds, generate five two-week missing periods.
- c) For ten random seeds, generate ten weekly missing periods.

RESULTS AND DISCUSSION

Results of the preliminary analysis

As the relationships between daily rainfall at the target station, Ratnapura, and its neighboring stations are not linear (for example, see Figure 2), Spearman's correlation coefficient was used to measure the correlations. Figure 3 shows the calculated correlation measures of these stations. It shows positive correlations between the target station, Ratnapura, and all reference stations, especially strong correlations (>0.6) with Keragala, Moraliya, and Elston. Therefore, it is worth incorporating the rainfall readings of these reference stations in imputing missing rainfall values at Ratnapura station.

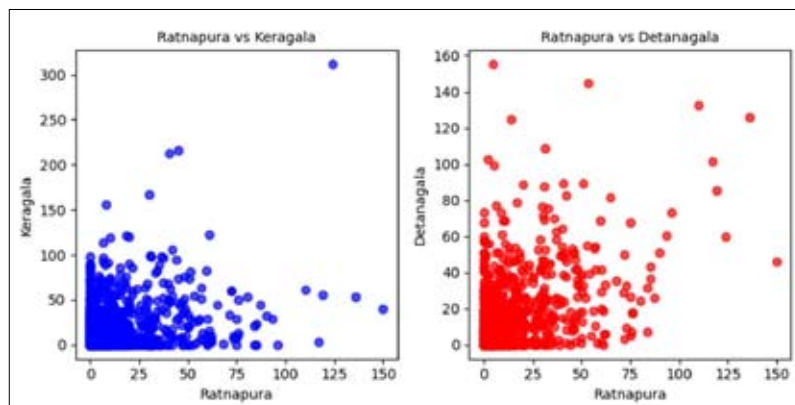


Figure 2: Scatter plots of daily rainfall at Ratnapura versus Keragala and Detanagala (2015–2019)

Figure 4 shows the distribution of daily rainfall at Rathnapura station by month. The plot shows high occurrences of rainfall from May to October. Additionally, an extreme rainfall event, which was very high compared to the other values, occurred in May. Conversely, during January to April and November to December, rainfall is lower compared to the other months. The details observed in the graph align with findings from previous

studies (for example, Priyakumara & Ranasinghe, 2023). These studies have pointed out that the primary source of rainfall for the Ratnapura area is the southwest monsoon, typically occurring from May to September. Figure 4 confirms this matter, as it clearly illustrates high rainfall events during these months, consistent with the influence of the southwest monsoon.

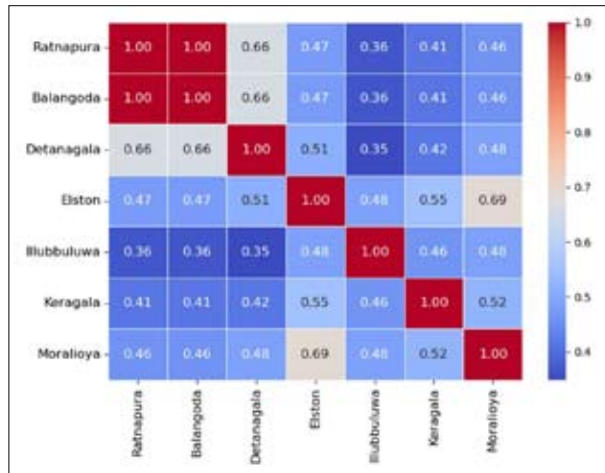


Figure 3: The heatmap of the correlation between rainfall gauging stations

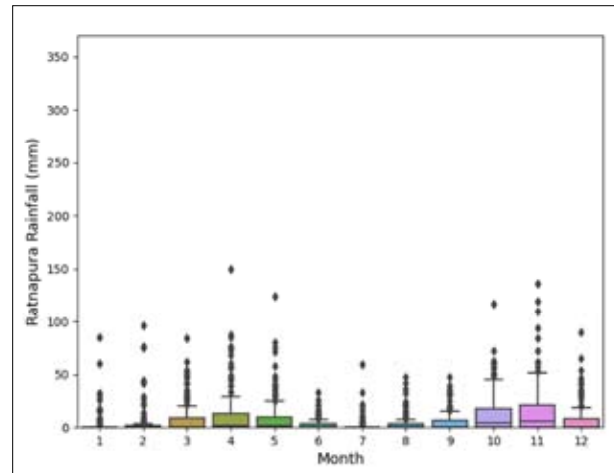


Figure 4: Distribution of daily rainfall at Rathnapura station by months (from 2015 to 2019)

Figures 5 and 6 present the distributions of daily rainfall at reference stations by month. It indicates that other stations exhibit similar rainfall patterns as Rathnapura station, with minor deviations in some months. Since this section mainly focuses on identifying the wet and dry seasons in the Rathnapura area, the variation of rainfall

in other stations is considered less significant. In here, the wet season, characterized by high rainfall events, occurs from May to September, while the dry season, with lower rainfall, takes place from January to April and from October to December.

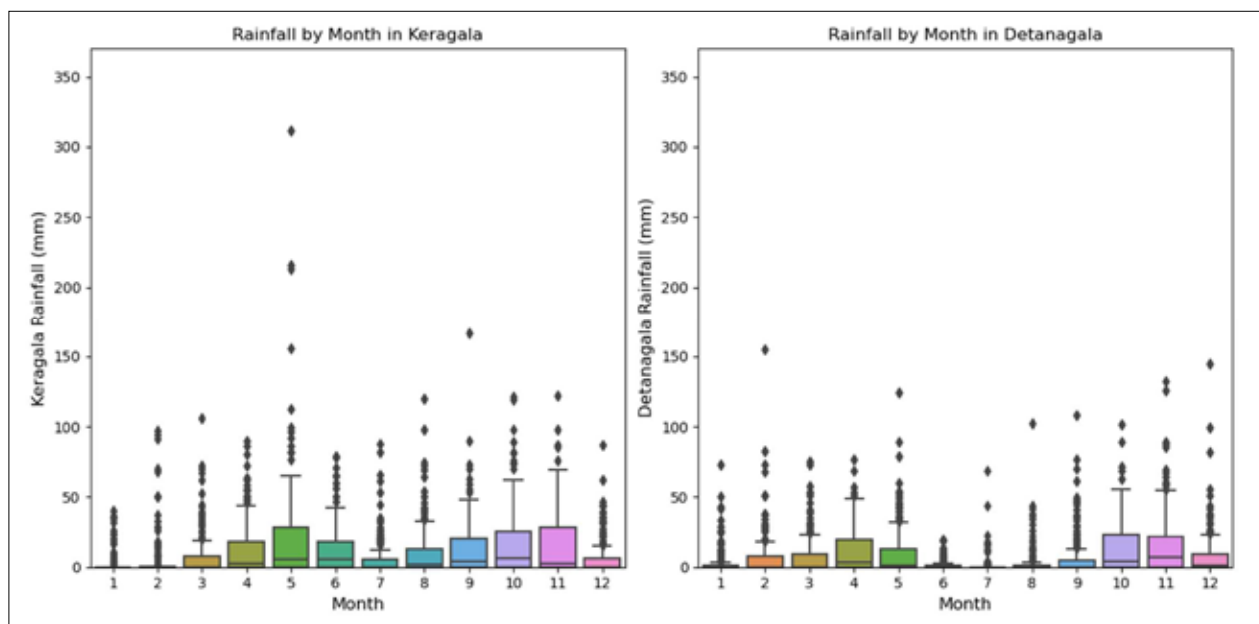


Figure 5: Distribution of daily rainfall of Keragala and Detanagala stations by months (from 2015 to 2019)

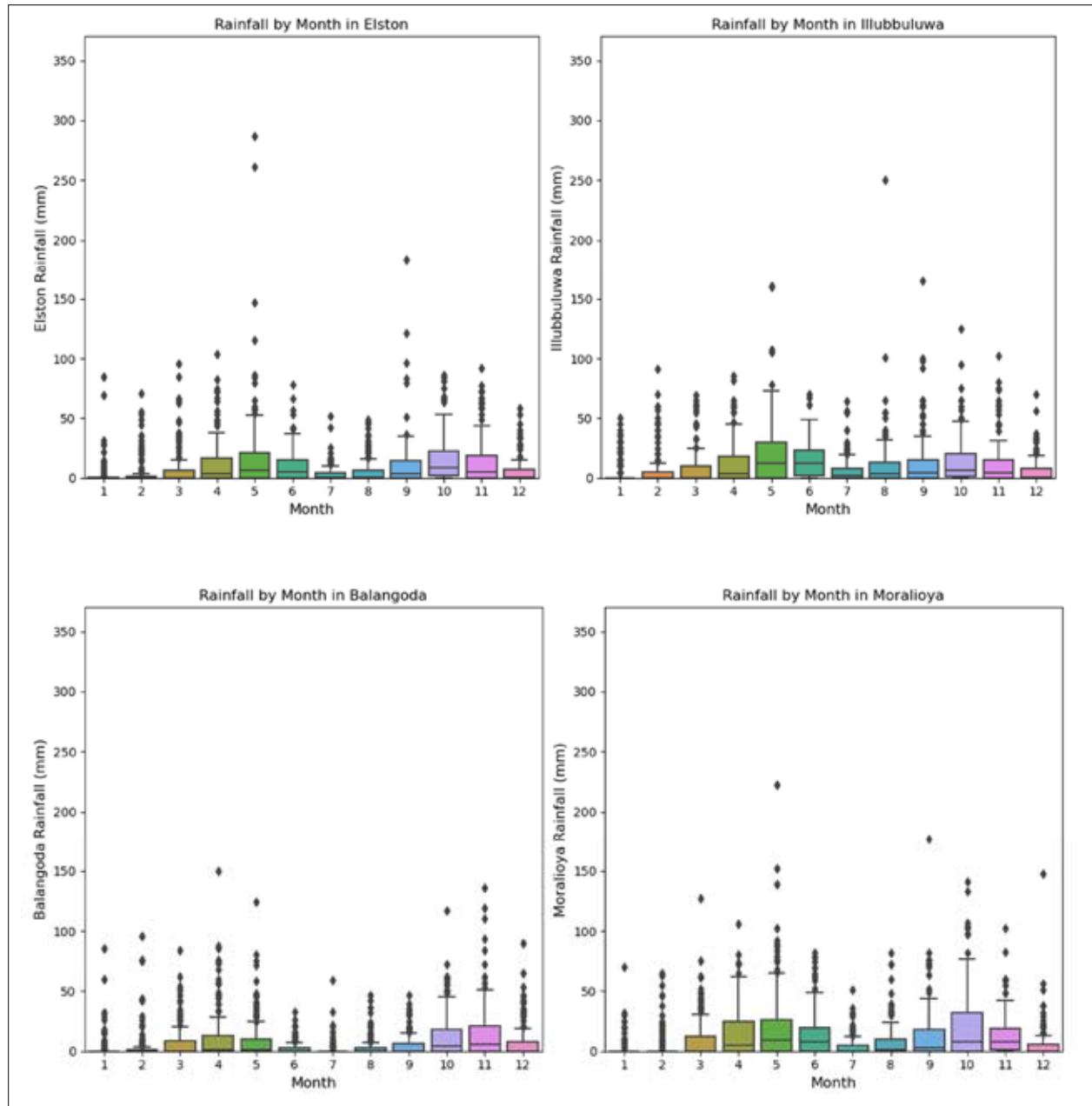


Figure 6: Distribution of daily rainfall of Elston, Illubbuluwa, Balangoda, and Moraliya stations by months (from 2015 to 2019)

Evaluation of underlying assumptions for imputation methods

For spatiotemporal kriging, the validity of underlying assumptions, including second-order stationarity and spatial isotropy (Gräler et al., 2016) was assessed prior to application. To assess spatial isotropy, directional spatial semi-variograms were computed (see Figure 7). The

semi-variograms differed across directions, indicating the presence of anisotropy (Oliveira et al., 2022), which was subsequently incorporated into the fitted theoretical variogram models. In addition, the temporal stationarity of each station's time series was evaluated using the augmented Dickey-Fuller (ADF) test. All series returned p-values close to 0, indicating that all the time series were stationary.

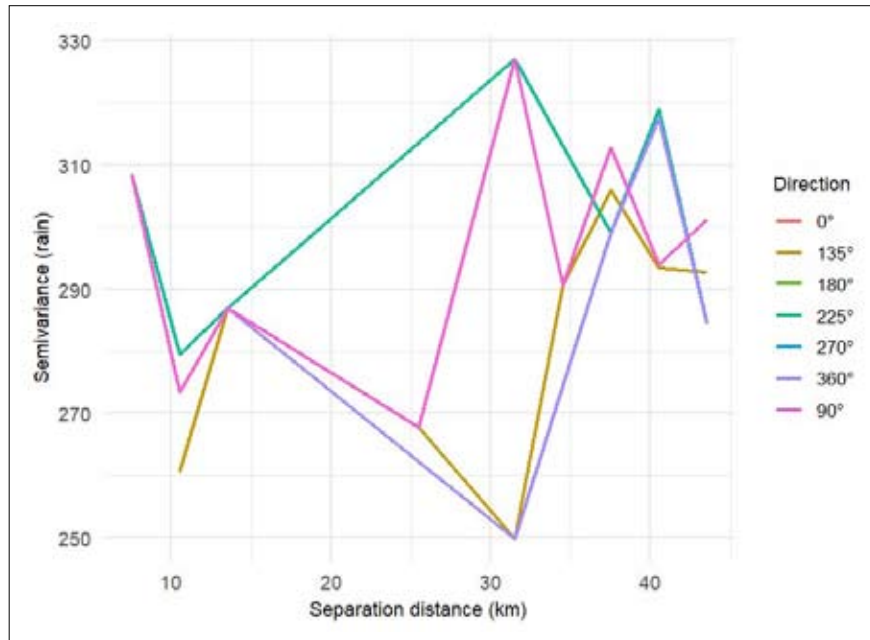


Figure 7: Directional spatial semi-variograms

In contrast, MissForest (Stekhoven & Bühlmann, 2012), KNN, and denoising autoencoder methods do not require any prior assumptions regarding the data distribution or correlation structure. Consequently, these nonparametric

and machine learning-based approaches were applied directly, and the evaluation of imputation accuracy was performed without preliminary assumption checking.

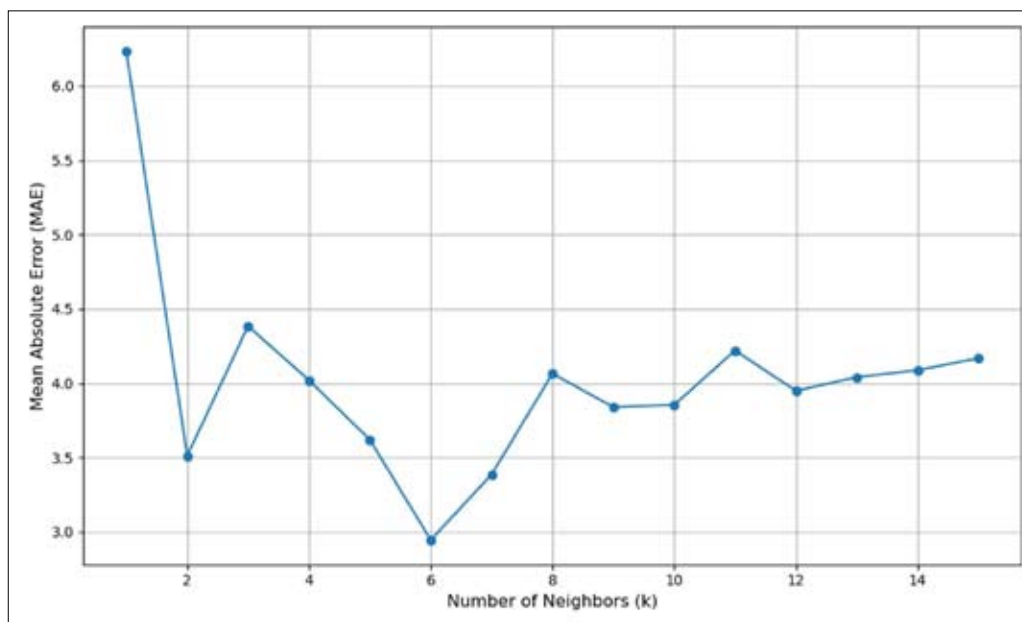


Figure 8: Plot of K values vs MAE values in KNN imputation

Comparison of performance of the four missing value imputation techniques

Table 1 presents the MAE and RMSE values obtained to compare the accuracy of imputed missing values for the wet and dry seasons in 2019. For KNN and MissForest, no train-test split was used, as these lazy learning (instance-based) algorithms automatically identify and iteratively impute missing values directly on the dataset without producing a reusable model. Therefore, these techniques were implemented using data from 2015 to 2019, the period in which sequential missing values are included. Furthermore, when performing KNN, the optimal K value was selected using the MAE vs K value plot (see Figure 8). In this study, the range of K values considered spans from 1 to 15. The criterion for selecting the optimal K value is based on identifying the K value that yields the minimum MAE for the imputation.

For spatiotemporal kriging, to fit the variogram, rainfall values from 2015 to 2019 of reference stations were used, as it needs to have complete rainfall records of other stations to impute Ratnapura station's (unknown location according to the kriging theory) rainfall missing values in the period of 2015 to 2019. However, for the denoising autoencoder, training was conducted using data from 1994 to 2014, with the test set used for imputation

spanning 2015 to 2019, as discussed in Section 2. The reason for using a full dataset to implement this method is that denoising autoencoders consist of convolutional layers with more parameters (weights) to tune during the model-training procedure. Therefore, this model needs to have sufficient data to overcome the overfitting problem when training.

Table 1 shows that the MAE and RMSE values of the KNN algorithm (in the wet season) are the lowest. Therefore, it is evident that for sequential missing value imputation for the wet season, KNN outperformed the other three techniques. For the dry season, KNN outperformed all the other techniques when the missing period is one week and one month. However, there is a conflict between KNN and Missforest imputation results for the two-week missing value period. In this case, Missforest performed better than KNN according to both the RMSE and MAE values. This is because both methods imputed a low rainfall event (2019-Jan-11) as a high rainfall event and an extreme rainfall event (2019-Jan-14) as a low rainfall event (See Figure 9). Referring to Table 1, it can be mentioned that both KNN and Misforest produce better imputation results in dry periods than wet periods. This may be due to the lower variability of rainfall in dry seasons.

Table 1: Sequential missing values imputation results for four methods in 2019

Missing Rates	KNN		Missforest		Spatiotemporal Kriging		Denoising Autoencoder	
	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)
Wet: Week	1.85	2.99	4.26	5.72	2.90	4.94	1.99	2.51
Dry: Week	0.22	0.31	0.35	0.38	5.86	6.60	1.08	1.09
Wet: Two Weeks	4.60	7.88	6.78	10.88	7.87	12.89	6.87	12.12
Dry: Two Weeks	3.21	6.48	2.54	5.37	6.25	7.32	3.71	5.55
Wet: Month	6.21	9.13	7.82	10.66	11.47	19.39	9.13	13.86
Dry: Month	1.87	4.52	2.24	4.50	7.91	8.81	2.69	4.13

MAE and RMSE represent mean absolute error and Root mean square error, respectively

To verify the performance of the four techniques, the experiments were repeated for another two years. RMSE and MAE were used to evaluate performance. For this

purpose, 2015 and 2017 were selected. Tables 2 and 3 compare MAE and RMSE values obtained from the four techniques for 2015 and 2017, respectively.

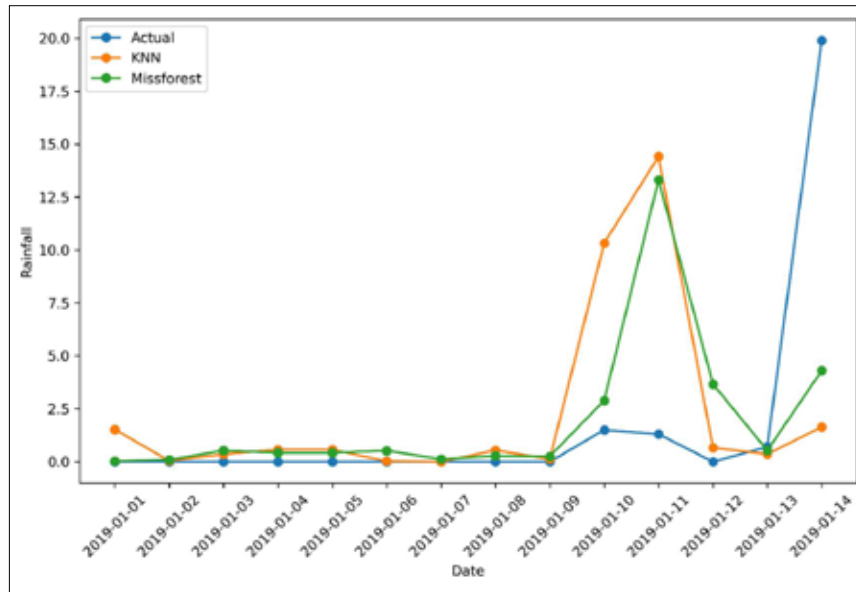


Figure 9: Actual vs imputed rainfall values with KNN and Missforest (1st to 14th January 2019)

According to Tables 2 and 3, in most of the missing scenarios, KNN outperformed the other three techniques in the aforementioned two years, as well as in 2019. Another noticeable point is that the MAE and RMSE values of all the techniques showed higher values in the wet season of 2017. The reason for this is that there is an extreme rainfall (120mm) compared to other observations in that period.

Although spatiotemporal kriging was expected to outperform MissForest and KNN, as reported in previous research (Yang et al., 2018), it exhibited the lowest performance in this study, with higher MAE and RMSE

values across all missing data scenarios (Tables 1–3) in both wet and dry seasons. As per (de Medeiros et al., 2019), this can be due to the lack of capturing spatiotemporal rainfall variability of the fitted variogram, and also due to the small number of locations. As a solution for the lack of capturing spatiotemporal rainfall variability, fitting a deterministic trend component to the rainfall data to capture some of the spatiotemporal variability that cannot be captured by the spatiotemporal variogram was suggested by several researchers (de Medeiros et al., 2019; Hu et al., 2017; van Zoest et al., 2020). However, fitting a deterministic component $m(s, t)$, as defined in sub-section 2.3 and Equation (6), was not appropriate,

Table 2: Sequential missing values imputation results for four methods in 2015

Missing rates	KNN		Missforest		Spatiotemporal kriging		Denoising autoencoder	
	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)
Wet: Week	6.12	10.65	5.27	7.05	8.52	13.66	8.54	11.56
Dry: Week	2.07	5.08	2.75	4.89	5.39	6.08	2.25	3.45
Wet: Two weeks	5.11	7.40	5.10	6.37	7.80	11.30	8.33	10.68
Dry: Two weeks	1.04	3.59	1.49	3.52	6.55	7.02	1.55	2.30
Wet: Month	7.27	10.26	7.65	10.24	10.48	15.93	8.54	16.60
Dry: Month	2.40	4.62	3.09	5.67	10.05	12.24	2.97	6.20

as most of the coefficients in the deterministic regression model were not significant, the model explained little

of the variability ($R^2 = 0.2$), and the residuals violated assumptions such as normality.

Table 3: Sequential missing values imputation results for four methods in 2017

Missing rates	KNN		Missforest		Spatiotemporal kriging		Denoising autoencoder	
	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)	MAE (mm)	RMSE (mm)
Wet: Week	30.84	48.17	34.73	51.31	41.89	67.18	35.23	56.14
Dry: Week	0.71	0.36	0.93	1.28	5.98	6.66	0.821	0.91
Wet: Two weeks	18.32	35.49	22.87	38.37	27.80	49.35	24.83	42.82
Dry: Two weeks	0.23	0.80	0.99	1.06	8.25	9.37	0.974	1.06
Wet: Month	12.84	25.32	13.24	26.04	22.15	36.96	15.86	30.98
Dry: Month	1.76	3.89	3.71	6.65	10.08	11.81	1.36	2.24

In general, KNN performs better than the other three imputation techniques considered in this study.

Forecasting results of the LSTM model

A similar procedure to the study done by Ahn et al. (2021), which was conducted to compare missing data imputation methods in time series forecasting, was followed. Here, forecasted values from LSTM models trained on actual and KNN-imputed datasets were compared to examine

the appropriateness of using imputed values for rainfall forecasting. The three LSTM models were fitted, and forecasting was carried out according to the procedure described in sub-section 2.5. For all the missing data scenarios tested in this study, a single set of parameters was found to be optimal from the search space considered. Table 4 summarizes the optimal configuration, which was applied consistently across all scenarios and the search space of each parameter where these optimal values were obtained.

Table 4: Optimal parameters for LSTM models and search space

Parameter	Optimal value	Search space
Look-back period	4 days	2, 3, 4, 5, 6
Number of LSTM layers	1	1, 2, 3
LSTM units per layer	32	16, 32, 64, 128
Batch size	32	16, 32, 64, 128
Learning rate	0.001	0.0001, 0.001, 0.005, 0.01
Dropout rate	0.2	0.1, 0.2, 0.3, 0.4
Epochs	100	50, 100, 150, 200

Table 5 compares the MAE values calculated from forecasted values obtained from the two types of LSTM models (fitted with actual and imputed data). Only the test data sets are used for calculating MAE. Results show that MAE values obtained from the LSTM models trained with imputed data do not deviate much from those

obtained from the counterpart models. This suggests that the KNN-imputed data are closely aligned with the actual dataset, consistent with findings from previous studies (Ahn et al., 2021), which reported KNN as one of the most accurate imputation techniques for time series forecasting.

Table 5: MAE values (for test data) of the LSTM Models trained with imputed and actual data

Train/Test period	Missing period length	Without missing values	Seed									
			1234	2222	2789	3456	4821	5093	6178	7342	8569	9027
2015-2016/ 2017 Jan-March	One week	6.67	6.59	6.50	6.66	6.55	6.72	6.76	6.66	6.62	6.66	6.60
	Two weeks		6.60	6.72	6.60	6.49	6.53	6.56	6.63	6.51	6.64	6.59
	One month		6.62	6.55	6.61	6.55	6.28	6.61	6.34	6.40	6.57	6.28
2016-2017/ 2018 Jan-March	One week	6.84	6.83	6.88	6.50	6.54	6.61	6.61	6.54	6.61	6.61	6.62
	Two weeks		6.90	6.74	6.49	6.55	6.60	6.63	6.51	6.59	6.61	6.56
	One month		6.82	6.52	6.42	6.49	6.53	6.65	6.45	6.58	6.63	6.54
2017-2018/ 2019 Jan-March	One week	11.00	11.04	10.76	10.99	10.88	10.87	10.99	10.79	10.98	10.99	11.01
	Two weeks		11.17	10.95	11.01	10.96	10.95	10.97	11.01	11.02	11.01	10.99
	One month		10.91	11.01	11.10	10.98	11.13	11.10	10.99	10.97	10.95	10.93

CONCLUSIONS

This study investigated the performance of different spatiotemporal imputation techniques for sequential missing values in rainfall data collected from the Ratnapura gauging station, with particular attention to how imputation accuracy varies across wet and dry seasons and to the sensitivity of methods to varying durations. According to the imputation results, it can be concluded that KNN outperformed the other three techniques in both wet and dry seasons in imputing sequential missing values of rainfall series collected from Ratnapura gauging station. The Missforest algorithm showed better performance compared to denoising autoencoder and spatiotemporal kriging during both wet and dry seasons, closely matching the results of the KNN algorithm, but worse than those of KNN in general. However, spatiotemporal kriging showed weaker performance in both wet and dry seasons compared to KNN, MissForest, and denoising autoencoder. Due to its limitation in capturing rainfall variability through the deterministic component, future studies are recommended to include additional meteorological variables when modeling spatiotemporal variability to improve imputation accuracy. Even though these techniques showed reasonable accuracy, they were not successful in imputing sudden extreme rainfall events accurately. Therefore, it is vital to further improve the methods applied to this study or develop novel techniques to impute such sudden extreme rainfall events accurately. Another important observation is that as the duration of missing values increases, the accuracy of the imputation methods decreases consistently across all techniques.

The LSTM forecasting results for both the imputed and actual datasets show very similar MAE values. This indicates that the imputation process, particularly using the KNN technique, has a minimal impact on the forecasting accuracy. Therefore, KNN can be recommended as a reliable method to impute sequential missing values of rainfall data at the Rathnapura gauging station, and such imputed values can be used in building flood forecasting models for Rathnapura.

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RESEARCH ARTICLE

Data Science and Analytics

Network analysis of tuberculosis spread with forecasting and technique for order preference by similarity to ideal solution

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Abstract: Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major health challenge, particularly in low income and middle-income countries. This study focuses on the Western province of Sri Lanka, which has high population density, trade, and high human mobility, all contributing to TB transmission. This research aims to strengthen TB control strategies through several key objectives. The TB contact network model was developed and analyzed using graph theoretical measurements such as degree centrality, closeness centrality and betweenness centrality, to identify influential nodes, and structural metrics such as modularity and clustering coefficient to understand transmission patterns. The TOPSIS (technique for order preference by similarity to ideal solution) method was applied to rank nodes based on their potential to spread TB, improving targeted intervention strategies. The novelty of this research is to apply graph theoretical measurements together with the TOPSIS method to identify the highly influential nodes and control the spread of disease by isolating those nodes from the community. Epidemic data extracted from the National Programme for Tuberculosis Control and Chest Diseases (NPTCCD) annual reports (2012–2023) revealed higher projected TB incidences among males and individuals belong to the age group 45–54. The synthetic dataset constructed using percentage-based forecasting data underwent 5-fold cross-validation, and the random forest model achieved an accuracy of 81.5%, indicating strong reliability of the forecasting model. Network analysis indicated the sparse local connections (clustering coefficient: 0.002) and strong community structures (modularity: 0.856). Comparative analysis before and after applying the TOPSIS method demonstrated the improved identification of influential spreaders. Sensitivity analysis showed that the model remained stable under small variations in data and weights, confirming the robustness of the

findings. The outcome of this research provides guidance to health authorities for allocating resources, prioritizing high risk groups, initiating strengthening programmes, and designing more effective community-based interventions to strengthening TB control strategies in the Western province.

Keywords: Centrality measures, cluster analysis, Western province, TOPSIS method, tuberculosis, contact network.

INTRODUCTION

Tuberculosis, abbreviated TB, is an infectious disease usually caused by *Mycobacterium tuberculosis* (MTB) bacteria. Tuberculosis (TB) is primarily spread through the air when a person with TB coughs, sneezes, or talks, expelling tiny droplets containing the bacteria into the air. If another person inhales these droplets, they can become infected with TB. Tuberculosis may infect any part of the body but most commonly affects the lungs; this is known as pulmonary TB. Extrapulmonary TB occurs when tuberculosis develops outside of the lungs, in organs such as lymph nodes, kidneys, bones, and brains (Kant et al., 2021). The World Health Organization (WHO) estimates 10.7 million persons acquired TB in 2024 worldwide and global death among patients with TB exceeds 1.23 million.

Understanding TB transmission dynamics is essential for developing targeted interventions. Over the past decades, researchers have employed various analytical

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methods to study the spread of TB. As a South Asian country, Sri Lanka is also facing substantial challenges in the fight against TB. Sri Lanka has made considerable progress in reducing the burden of TB over the past few decades. In the year 2018, Sri Lanka's WHO estimated new and relapsed TB cases were 64 per 100,000 population. By the end of 2018, the estimated number of TB deaths was 3.8 per 100,000 population (excluding TB/HIV deaths) (Ministry of Health, Sri Lanka, 2022). According to the weekly epidemiological reports of Ministry of Health of Sri Lanka, the incidence of TB in 2023 was 41.6 per 100,000 population (Epidemiology Unit of Ministry of Health, 2023), indicating a significant decline in disease transmission.

However, when analyzing TB cases in the Western province, it is required to pay attention to its geographical location, trade, and commerce due to the dense population and significant human mobility.

Several studies were conducted globally to reduce the transmission of TB using statistical approaches as well as social network analysis.

Molecular epidemiology techniques, such as whole-genome sequencing (WGS), help to identify transmission clusters and track strain movements across regions. Studies in Spain (Abascal et al., 2019) and Ghana (Asare et al., 2020) have demonstrated WGS's effectiveness in detecting outbreaks and guiding surveillance efforts. Contact tracing remains a fundamental approach in TB control, identifying individuals exposed to infected patients. (Baluku et al., 2022) have assessed its effectiveness by using logistic regression and Begun et al. (2013) used modeling techniques to optimize detection strategies.

Epidemiological modeling applies mathematical models, including SIR (Side, 2015), SEIR (Side et al., 2017) and agent-based models (Tuite et al., 2017), to simulate TB transmission dynamics and predict disease spread. These models provide insights into infection rates and control strategies. Spatial analysis using GIS (Munch et al., 2003) helps to map TB cases, revealing high-transmission areas. Research in South Africa demonstrated the utility of spatial epidemiology in identifying risk factors and transmission hotspots. Statistical analysis methods, including regression and time series analysis, help assess TB trends and risk factors. Various studies have applied survival models (Burusie et al., 2023) hidden Markov models (Rafei et al., 2012), and Box-Jenkins forecasting (Zheng et al., 2020) to improve TB surveillance. Network analysis examines social interactions that contribute to TB transmission.

Studies using social network analysis (SNA) (Pando et al., 2023) highlighted the role of high-risk communities and provided insights for targeted interventions. When considering the previous studies done in Sri Lanka, most TB studies rely on statistical analysis, with limited adoption of machine learning techniques. Some research has used SPSS for epidemiological assessments, while others have explored deep learning for TB detection via chest X-rays.

The current study is limited to the Western province of Sri Lanka, but can be extended for the whole country as a future work. By leveraging network-based approaches, the study aims to provide a deeper understanding of TB transmission patterns and influential spreaders in this region.

Additionally, the study incorporates the technique for order of preference by similarity to ideal solution (TOPSIS) method with a novel formula proposed to control COVID-19 transmission by isolation of influential nodes (Chaharborj et al., 2022). Rather than treating all contacts equally, decision makers can monitor and prioritize preventive treatment for individuals with the highest transmission potential.

According to the existing literature on TB analysis, social network analysis has been used to explore contact structures and identify high-risk communities, while statistical approaches have been widely used to analyze risk factors, temporal trends, and survival outcomes. Although community detection techniques have been applied in some studies, node-based analysis within detected communities, especially the identification and isolation of influential spreaders, has not been adequately investigated in TB transmission studies. To address this gap, and motivated by recent research that successfully employed TOPSIS-based formulations to control COVID-19 transmission through the identification of influential nodes, the present study integrates TB contact network analysis with the TOPSIS decision making criterion method. This combined framework provides a novel approach for identifying influential TB spreaders within contact networks, offering new insights into TB transmission dynamics in Sri Lanka.

MATERIALS AND METHODS

Data collection involved gathering annual TB reports from the National Programme for Tuberculosis Control and Chest Diseases (NPCCD) website, Ministry of Health, Sri Lanka (Ministry of Health, Sri Lanka, 2022), spanning 2012 to 2023. Based on the annual number of patients

reported during 2012–2023, separate autoregressive integrated moving average (ARIMA) forecasting models were developed using R software to predict the expected TB cases for the year 2025 by gender, age groups, and TB type. The ARIMA forecasting approach assumes that the time series becomes stationary after appropriate differencing, and that the model residuals are independent, normally distributed, and constant variance, and each subgroup model was validated independently using residual diagnostics and forecast accuracy metrics to ensure reliable predictions.

A synthetic dataset of 200 patients was generated based on the forecasted TB cases for 2025, including patient ID, age, gender, contact ID, TB type, and relationship type. The dataset was validated using a Random Forest classifier with 5-fold cross-validation to ensure its predictive stability.

Graph theoretical principles, including centrality measures (betweenness, closeness, degree) and TOPSIS-based node rankings, were applied to analyze TB transmission dynamics. Sensitivity analysis was conducted by perturbing criterion weights and input data to evaluate the robustness of the rankings and disease spread predictions. In this study, graph variants called centrality measures are used to determine the most influential nodes in a given network. Identifying such nodes is important to design resilient networks and identify targets for disease control in epidemic networks. The following gives a brief explanation about the graph theoretical concepts used in this study.

Graph theoretical concepts

Definition 1: Graph

The TB contact network is modelled as a directed graph, where nodes represent patients and directed edges indicate contact or transmission pathways.

Definition 2: Shortest path

The shortest path between two nodes is the minimum number of edges connecting them, representing the most efficient potential route of TB transmission.

Definition 3: Degree

The degree of a node is the number of edges connected to it; in directed graphs, this includes in-degree and out-degree.

Definition 4: Distance

The distance between two nodes is defined as the length of the shortest path separating them in the network.

Definition 5: Betweenness centrality (Wasserman, 1994)

The betweenness centrality (BC) measures the importance of a node in a graph based on its ability to act as a bridge along the shortest paths between pairs of other nodes.

The formula for the betweenness centrality of a node u in a graph is given by,

$g(u) = \sum_{s \neq u \neq t} \frac{\sigma_{st}(u)}{\sigma_{st}}$, where σ_{st} is the total number of shortest paths from node s to t , and $\sigma_{st}(u)$ is the number of those paths passing through the node u . Individuals with high betweenness centrality may not have highest number of contacts. However, they can significantly influence disease diffusion due to the prolonged infectious period of the disease.

Definition 6: Closeness centrality (CC) (Rochat, 2009)

The closeness centrality (CC) measures how close a node is to all other nodes in the network on average. Nodes with high closeness centrality can reach other nodes quickly.

The formula for the closeness centrality of a node v in a graph is given by,

$C_{closeness}(v) = \frac{1}{\sum_{u \neq v} dist(u, v)}$, where $dist(u, v)$ is the shortest path distance between nodes u and v .

Definition 7: The degree centrality (DC) (Chaharborj et al., 2022)

The degree centrality (DC) measure is used in network analysis to quantify the number of connections or ties a node has in a network. Nodes with high degree centrality have more close or prolonged interactions, increasing both their risk of acquiring TB and their potential to transmit it to others.

The degree centrality (DC) of node i , denoted as $C_d(i)$, is defined as $C_d(i) = \frac{1}{N} \sum_{j=1}^N x_{ij}$, where, N is the total number of nodes, i is the focus node, j represents all other nodes, x_{ij} represents the connection between node i and node j , and $x_{ij} = \begin{cases} 1, & i \text{ is connected to } j \\ 0, & \text{otherwise} \end{cases}$.

Definition 8: The Clustering coefficient (Watts & Strogatz, 1998)

The Clustering coefficient is a measure of the tendency of nodes to cluster together, while the average clustering

coefficient is the average of these measures across. This explains how disease spreads fast inside the small group but slowly in between groups.

The formula for the clustering coefficient $C(v)$ of a node v in a graph is given by

$C(v) = \frac{2T(v)}{\deg(v)(\deg(v)-1)}$, where $T(v)$ is the number of triangles centered at node v , and $\deg(v)$ is the degree of node v .

Definition 9: The modularity (Newman, 2006)

The modularity is a measure that quantifies how well a network can be divided into distinct modules (also called groups, clusters, or communities). High modularity shows a clear strong community.

For a weighted, directed network, the modularity Q is calculated as follows:

$$Q = \frac{1}{W} \sum_{ij} \left(A_{ij} - \frac{k_i^{\text{in}} k_j^{\text{out}}}{W} \right) \delta(c_i, c_j).$$

Where: A_{ij} is the weight of the edge from node i to node j , k_i^{in} is the in-degree of node i , k_j^{out} is the out-degree of the node j , W is the total sum of edge weights in the network, $\delta(c_i, c_j)$ is the Kronecker delta function, where c_i and c_j are the communities to which nodes i and j belong, respectively.

Definition 10: K-fold cross-validation

K-fold cross-validation is a resampling method used to evaluate the predictive performance of a model. Suppose the dataset D contains N observations, and k is the number of folds. The dataset is partitioned into k approximately equal subsets $\{D_1, D_2, \dots, D_k\}$. For each fold $i = 1, \dots, k$, the model is trained on $D \setminus D_i$ and tested on D_i . Let Err_i denote the prediction error on the i -th fold. Then the overall cross-validated error is computed as:

$$CV_k = \frac{1}{k} \sum_{i=1}^k \text{Err}_i$$

This process ensures that each observation is used exactly once for testing, providing a reliable estimate of model performance (Hastie et al., 2009).

Definition 11: The TOPSIS method

This method is used for ranking in social or complex networks by evaluating each alternative based on its distance from two ideal solutions (Chaharborj et al., 2022):

the positive ideal solution (PIS) and the negative ideal solution (NIS). The positive ideal solution represents the best possible values for each criterion, while the negative ideal solution represents the worst.

Let S_i^+ be the distance from the positive ideal solution and S_i^- be the distance from the negative ideal solution. Then closeness coefficient C_i is $\frac{S_i^-}{S_i^+ + S_i^-}$.

The closeness coefficient calculates how close an alternative is to the ideal solution. This value serves as the basis for ranking the alternatives, with higher C_i indicating a more influential alternative.

The criteria used in this study (degree centrality, betweenness centrality, closeness centrality, clustering coefficient, and modularity) were selected based on their relevance to TB transmission dynamics within the contact network. These criteria quantify the influence of a patient (node) on disease spread, reflecting public health priorities by identifying individuals most likely to contribute to TB transmission. Sensitivity analysis was performed by perturbing weights and input values to ensure the robustness of the TOPSIS rankings under varying assumptions.

Definition 12: Disease spread probability in a complex network

The disease spread probability formula proposed by Chaharborj et al. (2022) is for modeling the transmission dynamics in complex networks. Suppose that G is the proposed graph, N is the total number of nodes, and E is the number of influential nodes with top ranking. Then the pandemic spread probability $P(G_i)$ for the graph G_i can be defined as follows:

$$P(G_i) = \frac{N - i + 1}{N} \times \frac{\sum_{k=1}^N \text{Degree}(G_i)}{\sum_{k=1}^N \text{Degree}(G_0)}, i = 1, \dots, E; G_0 = G$$

Definition 13: Sensitivity analysis (weight- and data-based perturbations)

Sensitivity analysis is a method used to evaluate how variations in model inputs affect the resulting outcomes or rankings. Let a decision matrix $R = [r_{ij}]$ $i = 1, \dots, N, j = 1, \dots, m$, represent N alternatives evaluated under m criteria, and let

$$w = \{w_1, w_2, \dots, w_m\}$$

denote the original criterion weights used in the TOPSIS method.

- **Weight-based perturbation:**

To assess sensitivity with respect to criteria weights, each weight is perturbed as

$$w'_j = w_j + \delta_j, \quad j = 1, \dots, m,$$

subject to the normalization condition

$$\sum_{j=1}^m w'_j = 1$$

where δ_j is a small random perturbation. Using the perturbed weight vector

$$w' = \{w'_1, w'_2, \dots, w'_m\}$$

the TOPSIS procedure is repeated to compute the perturbed closeness coefficient

$$C_i^w = \frac{S_i^-(w')}{S_i^+(w') + S_i^-(w')}, i = 1, \dots, N$$

where $S_i^+(w')$ and $S_i^-(w')$ denote the distances of alternative i from the positive and negative ideal solutions, respectively, calculated using the perturbed weights.

The rankings obtained from C_i^w are then compared with the original TOPSIS rankings to evaluate the stability and robustness of the decision outcomes.

- **Data-based perturbation:**

$$r'_{ij} = r_{ij}(1 + \epsilon_{ij}), \quad \epsilon_{ij} \sim U(-\alpha, \alpha)$$

where ϵ_{ij} is a small random noise added to each criterion value and α is the maximum perturbation magnitude. The perturbed decision matrix $R' = [r'_{ij}]$ is then used to recompute the TOPSIS ranking through the data-perturbed closeness coefficient C_i^d , where C_i^d represents the closeness coefficient obtained after data (decision matrix) perturbation

This process allows assessment of the robustness of TOPSIS rankings and disease spread predictions under small variations in criteria weights and input data, ensuring the stability of the model outcomes (Saltelli et al., 2008; Behzadian et al., 2012; Demir & Arslan, 2022).

The contact network in Figure 1 is a visualization in Gephi that consists of 200 nodes representing patients and 199 edges representing contacts between them. Node

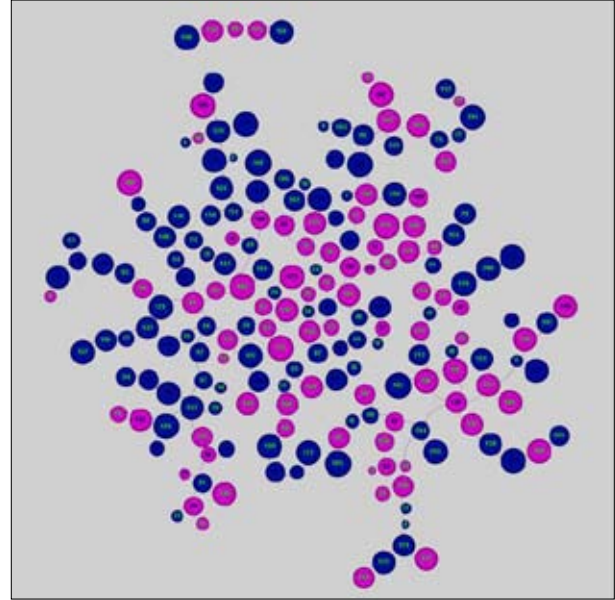
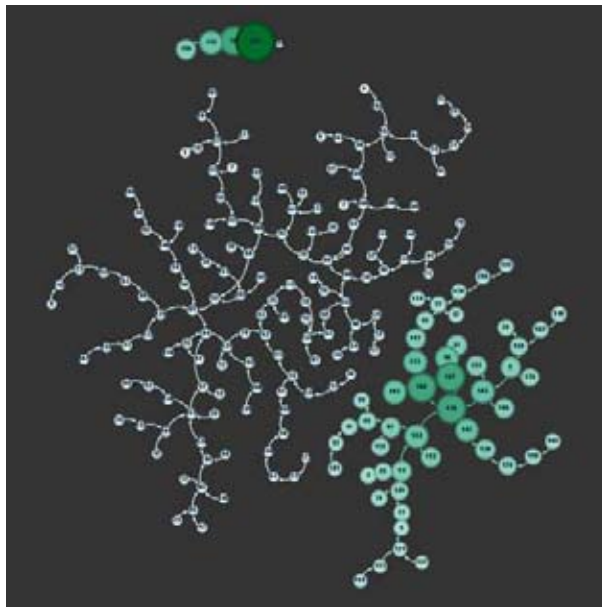


Figure 1: Contact network showing relationship between patients and contacts

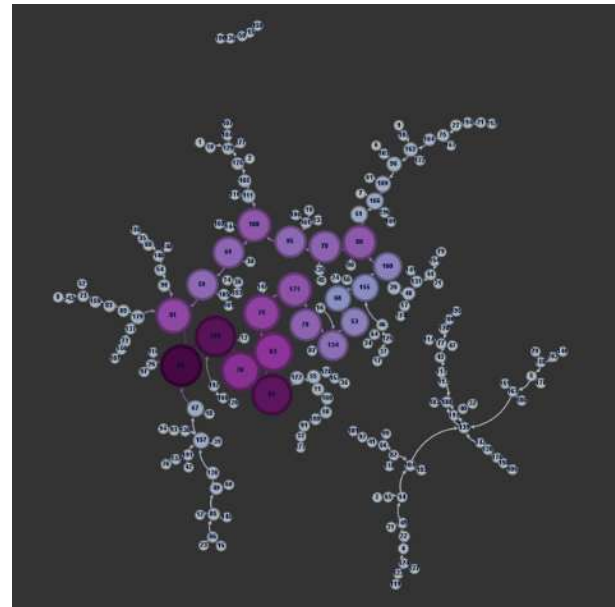
size is used to visualize patient age, with larger nodes indicating older patients and smaller nodes indicating 19 younger patients, providing a clear representation of the age distribution within the network. Gender is visualized using node color, with blue nodes representing male patients and pink nodes representing female patients, allowing for easy identification of the gender distribution in the network. TB type is represented by node label color, with green labels indicating PTB patients and black labels indicating extrapulmonary tuberculosis (EPTB) patients, facilitating the differentiation between different types of TB patients within the network. The visualization reveals interesting patterns, such as higher connectivity among older patients and a balanced distribution of male and female patients in the network

Centrality measures, including closeness centrality and betweenness centrality, were utilized to identify the most influential patients (nodes) in the network. Network visualization of centrality measures is shown in Figure 2.

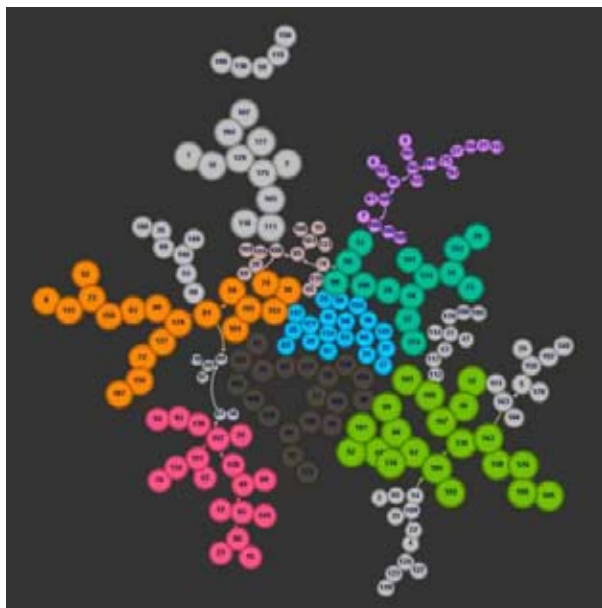
Cluster analysis was carried out to detect densely connected subgroups within the network as shown in Figure 3, and the calculation of average cluster coefficient and modularity provided insights into the network's structural characteristics.



(a) closeness centrality values



(b) betweenness centrality values

Figure 2: Network visualization of highlighting centrality measures**Figure 3:** Network visualization illustrating clustering within the network

The TOPSIS (technique for order of preference by similarity to ideal solution) method was utilized to rank and prioritize nodes based on their likelihood of spreading TB, incorporating multiple criteria for a comprehensive evaluation. The network with fifteen node ranks are highlighted in red colour and represented in Figure 4.

RESULTS AND DISCUSSION

According to the statistical forecasting results, out of a total of 3666 patients, 2439 (67%) were male, and 1227 (33%) were female. Given the lack of real-time data resources, ARIMA models were used to predict the expected number of male and female TB cases for 2025. The forecasted values indicate that male cases are expected to remain higher than female cases, suggesting a persistent gender disparity in TB distribution. Model validation metrics showed RMSE (root mean square error) = 209 for male cases and 127 for female cases, MAE (mean absolute error) = 155 and 94, and MAPE (mean absolute percentage error) = 7.4% and 7.8%, respectively, indicating that the forecasts closely matched historical data.

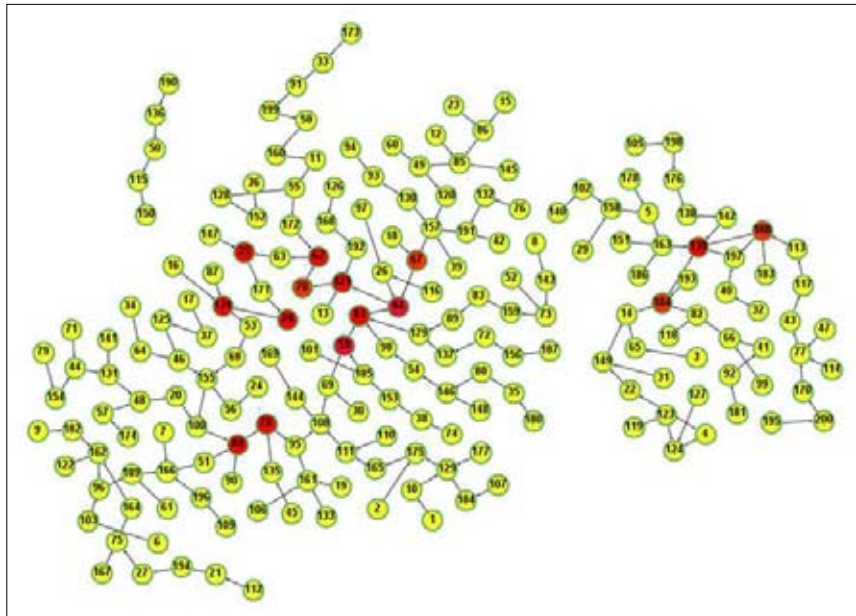


Figure 4: The network visualization of contact network with node ranking

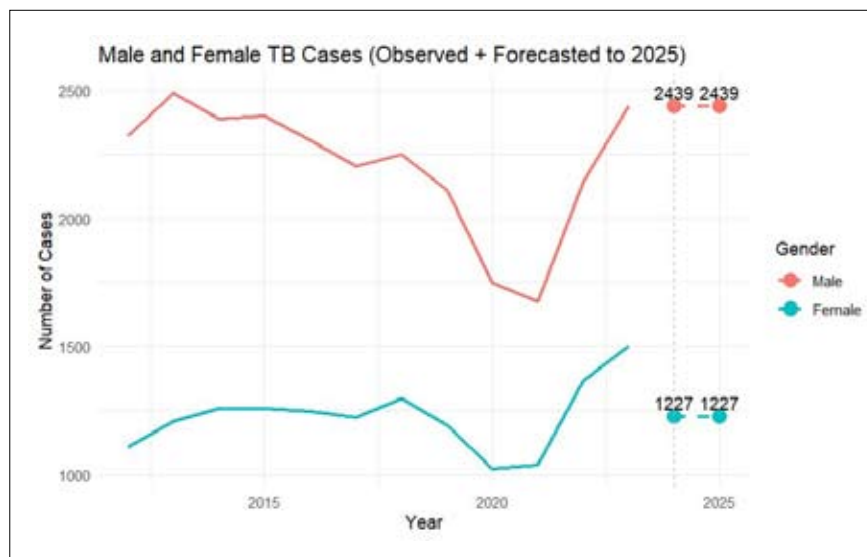


Figure 5: Trend plot of male and female TB cases (2012 – 2023) with forecasts for 2024 - 2025

The age distribution of patients was as follows: 0–14 years: 81 (2%), 15–24 years: 352 (10%), 25–34 years: 387 (11%), 35–44 years: 548 (15%), 45–54 years: 748 (20%), 55–64 years: 708 (19%), 65–74 years: 666 (18%) and 75+ years: 166 (5%). Figure 6 illustrates that the majority of forecasted TB cases in 2025 are expected

to be in the 45–54 age group, accounting for 21% of the overall forecasted cases. Validation of each age-group ARIMA model showed satisfactory predictive performance, with RMSE between 17 and 37 and MAPE between 5.8% and 18.1%, indicating that the forecasts closely matched historical data and are reliable.

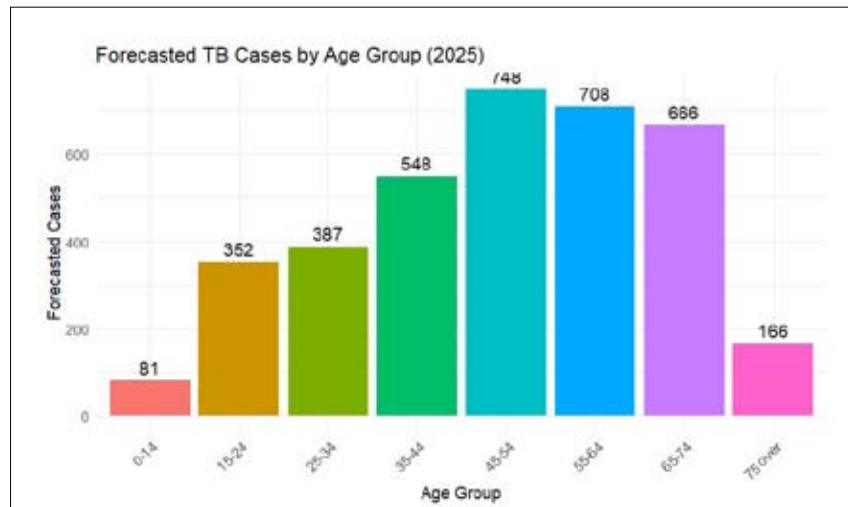


Figure 6: Forecasted TB cases by age group for 2025.

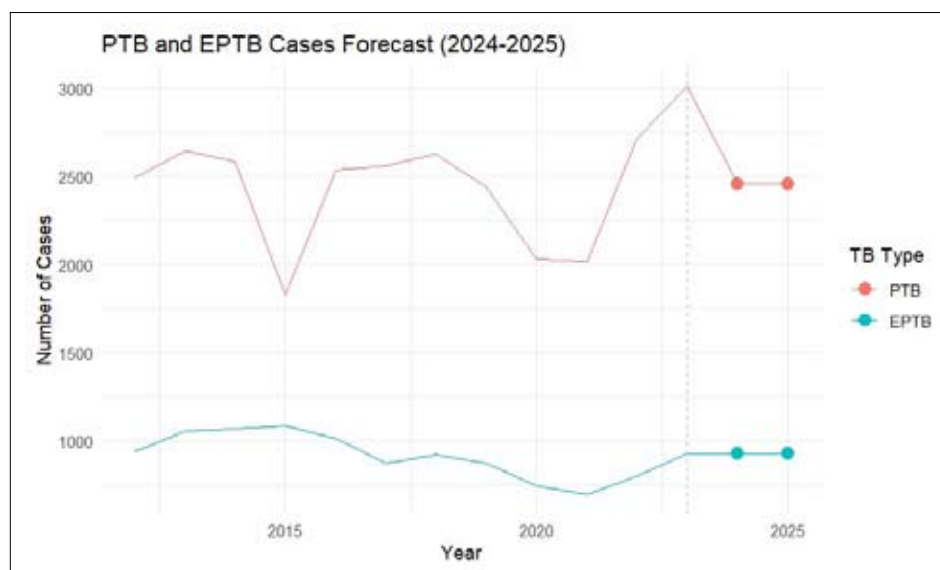


Figure 7: Trend plot of pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) cases with forecast for 2025.

Regarding TB types, 2457 (73%) had pulmonary TB (PTB) and 926 (27%) had extrapulmonary TB (EPTB). Forecasted values for trends of PTB and EPTB are shown in Figure 7. Both PTB and EPTB cases fluctuated over the years, with PTB cases consistently higher than EPTB. Forecast validation showed RMSE values of 322 for PTB and 86 for EPTB, with MAPE values of 11.1% and 8.3%, indicating that the forecasts closely matched

historical data. PTB cases are expected to remain the majority of TB cases in 2025.

Based on the forecasting data, a comprehensive data table was constructed using R software with a 200-patient sample representing the forecasting data population. A Random Forest model was applied to classify TB type (PTB vs EPTB) using three predictors. The model was

tuned with 5-fold cross-validation, and the optimal mtry = 2 (number of predictors randomly selected at each split) achieved an accuracy of 81.5%, indicating reliable predictive performance for distinguishing between PTB and EPTB cases.

We visualize high centrality values using larger nodes and darker colours. Nodes with high closeness values are in central positions within the network, allowing them to reach other nodes quickly. Nodes with high betweenness centrality, marked with special visual cues, act as important links in the network, connecting different groups of nodes.

The average clustering coefficient of 0.002 in the TB network for the Western Province indicates a low level of local clustering among TB cases, suggesting relatively few clusters of interconnected cases. In contrast, the modularity value of 0.856 signifies a high level of community structure within the network, indicating that TB cases are more densely connected within specific communities or groups. These findings reveal that TB cases are not highly connected locally but tend to form distinct, well-defined groups or communities.

Table 1: Node ranking based on relative closeness to the ideal solution

Nodes	S_i^+	S_i^-	C_i	Ranking
1	0.0252	0.0269	0.5158	14
11	0.0246	0.0283	0.5357	2
14	0.0247	0.0276	0.5276	7
15	0.0247	0.0274	0.5261	9
17	0.0248	0.0276	0.526	10
31	0.0249	0.0266	0.5164	13
42	0.0245	0.0283	0.5353	4
49	0.0246	0.0279	0.531	6
53	0.0249	0.0274	0.5247	11
56	0.0248	0.0272	0.5232	12
61	0.0246	0.0284	0.5354	3
82	0.0254	0.0267	0.5127	15
103	0.0245	0.0284	0.5365	1
170	0.0248	0.0281	0.5312	5
180	0.0247	0.0276	0.5272	8

Here, S_i^+ = The distance from the positive ideal solution, S_i^- = The distance from the positive ideal solution and C_i = Closeness coefficient.

Table 2: Disease spread probability in the network with varying numbers of nodes removed.

Number of removed nodes	Disease spread probability
1	0.9900
2	0.9751
3	0.9554
4	0.9407
5	0.9163
6	0.8970
7	0.8827
8	0.8733
9	0.8688
10	0.8500
11	0.8408
12	0.8221
13	0.8037
14	0.7948
15	0.7859

The results of node ranking based on relative closeness to the ideal solution are presented in Table 1, identifying top-ranking individuals crucial for understanding TB transmission pathways within the network. The highest C_i value 0.5365, is ranked first among the 200 nodes, indicating its significant influence on the network. The disease spread probability decreases as more top-ranking nodes are removed, dropping from 0.99 with one node removed to 0.7859 with fifteen nodes removed, as shown in Table 2.

The results reveal key trends in TB transmission in the Western province of Sri Lanka, indicating a shift that requires gender-specific interventions. Most TB cases forecasted for 2025 are expected in the 45-54 age group, emphasizing the need to target this age group for effective TB control. Pulmonary TB (PTB) remains more prevalent than extrapulmonary TB (EPTB), but both types have decreased since 2020, suggesting improvements in TB control measures.

Figure 8 shows how the chance of the disease spreading changes under three situations: original, data perturbed, and weight perturbed, as more nodes are removed. It shows that the model is strong and reliable even if the patient data or the criteria weights are slightly changed. In all three cases, the chance of disease spreading goes down as more important nodes are removed, meaning that isolating key patients or reducing their contacts can

help control the spread. The three lines are very similar, which means the results are consistent. Small differences appear: the data perturbed line is a little lower, and the

weight perturbed line is sometimes a little higher, but these differences are very small and do not change the main trend.

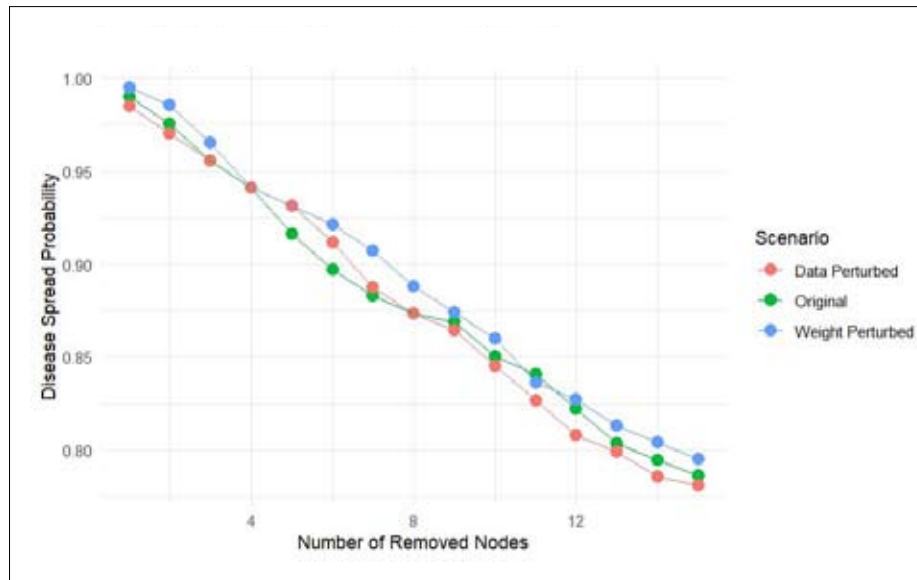


Figure 8: Sensitivity analysis of disease spread probability in the contact network

The data table and contact network visualizations highlight the demographics and relationships among patients, with older patients showing high connectivity and a balanced gender distribution. Close friends and household connections play a significant role in TB transmission, suggesting that interventions should target these relationships to mitigate spread. Network centrality measures provide further insights. Higher centrality values indicate key nodes that are more connected and influential; closeness centrality reflects how quickly a node can reach others, while betweenness centrality identifies key mediators of information flow. The low clustering coefficient and high modularity value suggest sparse local connections but strong community structures.

Identifying top-ranking nodes through the TOPSIS approach provides insights into potential TB transmission pathways. Removing these influential nodes effectively reduces TB spread, underscoring the importance of targeting key individuals and communities to improve TB control and treatment outcomes in the Western province of Sri Lanka. Overall, the sensitivity analysis confirms that the model is both responsive to interventions and

robust to small changes in inputs, supporting its reliability for guiding decision-making in intervention strategies.

This study focused only on the Western Province, which has high population density. Human mobility from outside the province could affect TB transmission but was not included in the analysis. Patient data collection was challenging because real healthcare data is not easily accessible, and public open-source data from websites or other sources are limited. Some cases may be underreported or reported late, which could affect data accuracy. Differences in contact patterns between individuals and unmeasured socio-economic or environmental factors could also influence TB spread.

Despite these limitations, the study provides guidance for health authorities. By discovering strong community structures, health authorities can prioritize influential spreaders for targeted screening and isolation, allocate limited resources more efficiently, and conduct community-wise awareness programs rather than applying uniform interventions. Overall, more effective age and gender specific interventions, as well as workplace or trade-based screening programs, can be planned.

CONCLUSION

This study integrated statistical forecasting, network analysis using graph theory, and the TOPSIS method to investigate TB transmission dynamics in Western province, Sri Lanka. Our study focused on analyzing TB spread in this region, utilizing data-driven methods to identify key trends and influential spreaders. Our analysis reveals that males have a higher percentage of TB cases in 2025. The 45-54 age group emerges as a significant demographic for TB incidence, with pulmonary TB (PTB) forecasted to constitute 73% of cases in 2025. Network analysis provided insights into disease transmission pathways, highlighting community structures and identifying influential nodes crucial for targeted interventions. The low average clustering coefficient suggests sparse local connections among TB cases, while the high modularity value indicates strong community structures where TB cases are more densely connected within specific groups than across the entire network. By focusing on key nodes and community structures, interventions can effectively mitigate TB spread. Given the lack of comprehensive data beyond 2025, our forecasting focuses on this year, ensuring that predictions are based on the most recent and reliable information available. Despite focusing on 2025, the methodological framework and insights from the study offer valuable guidance for future research and public health strategies. Additionally, the sensitivity analysis confirmed that the model's results remain stable under small data and weight perturbations, further strengthening the reliability of the findings. The findings of this research are helpful to health authorities to take quick decisions regards resource allocations, identify high risk group, and isolate influential patients, in order to control the spread of a disease.

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Capacity: kL, L, mL, µL

Volume: km³, m³, cm³

Mass: t, kg, g, mg, µg

Time: year(s), month(s), week(s),

day(s), hour(s), minute(s), second(s)

Concentration: M, mM, N, %, g/L, mg/L, ppm

Temperature: °C, K

Gravity: x g

Molecular weight: mol wt

Others: Radio-isotopes: 32P

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