

Characterizing microbiome ecology and developing targeted interventions for *Salmonella* control
in low-moisture wheat systems

by

Shivaprasad Doddabematti Prakash

B.Tech., Karnataka Veterinary, Animal and Fisheries Science University, 2015
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DOCTOR OF PHILOSOPHY

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Abstract

Low-moisture foods (LMFs), including wheat and wheat-based products, have been considered "microbiologically safe" due to their low water activity ($a_w < 0.85$). However, wheat grains and their products have emerged as a novel vehicle for the transmission of foodborne illnesses to humans. Current knowledge of microbiological hazards in this system relies heavily on culture-dependent, pathogen-specific methods that provide a very narrow view of the broader microbial ecology of the wheat milling system. Culture-independent approaches, such as 16S rRNA sequencing, can provide a more comprehensive characterization of microbial communities and can offer predictive knowledge on the wheat microbiome, which can aid in the development of mechanistically informed intervention strategies. However, the lack of systematic benchmarking in the LMF environment leaves significant uncertainty regarding the optimal bioinformatics workflow. To address these gaps, this research benchmarked a 16S rRNA sequencing protocol for microbiome analysis in wheat milling environment (Chapter 3); Characterize the wheat milling microbiome through taxonomic profiling, diversity analysis, and functional pathway prediction across different milling stages (Chapter 4); Determine the efficacy of sodium bisulfate (SBS) (Chapter 5); acidic tempering (Chapter 6) and non-thermal techniques (cold plasma, ultraviolet, and pulsed light) in reducing *Salmonella* in wheat during tempering and assess their impact on flour functionality, and baking quality (Chapter 7).

Chapter 3 systematically evaluated 32 bioinformatics workflows (eight 16S rRNA pipeline X four taxonomic database) using wheat milling samples ($n=160$). At the family level, diversity metrics were conserved across pipeline-database combinations (Chao1: 22.97 ± 2.20 - 24.92 ± 2.04 ; Shannon: 2.59 ± 0.19 - 2.74 ± 0.18 ; InvSimpson: 10.63 ± 1.25 - 11.27 ± 1.06 ; Bray-Curtis: 0.79 - 0.82 ; Jaccard: 0.79 - 0.80), whereas at the genus level both alpha and beta diversity exhibited wider ranges

and larger dispersion (Chao1: 45.27 ± 5.68 - 50.20 ± 5.64 ; Shannon: 2.54 ± 0.24 - 2.73 ± 0.22 ; InvSimpson: 10.37 ± 1.4 - 11.03 ± 1.05 ; Bray-Curtis: 0.79-0.82; Jaccard: 0.79-0.80). Furthermore, ASVs and OTUs were comparable across metrics; however, ASVs showed numerically higher values for some genus-level measures than OTUs due to the fact that they can resolve variation down to the single-nucleotide level, thereby retaining low-abundance features important for LMF safety. This work paves the way toward using bioinformatics and the 16S pipeline to characterize sparse, low-density, and uneven samples in low-moisture environments.

Building on this, Chapter 4 validated the DADA2_SILVA (ASV) workflow for profiling bacterial communities across different wheat milling stages: before milling (BM), after milling (AM), and the milled fraction (MF). 22 families and 132 genera were identified. Genus-level richness (alpha diversity) decreased by 41.5% as a result of the BM to AM transition. Similarly, beta-diversity analysis indicated significant differences in community composition across milling stages, with MF consistently forming a compositionally distinct cluster. Furthermore, PICRUSt2-based KEGG module predicted 20 KEGG pathways, enriched for stress- and biofilm-related pathways, indicating persistence. Overall, our findings reveal that wheat milling drives stage-dependent restructuring and selective redistribution of microbial communities with direct implications for food safety, contamination control, and risk management.

To address *Salmonella* contamination in wheat, Chapter 5 evaluated the effect of sodium bisulfate (SBS) tempering (17% moisture) on 4 different *Salmonella enterica* serovars. SBS tempering (1.5% SBS, w/v) reduced the *Salmonella* load by 4.3 log CFU/g after 24 h of tempering. Flour functionality and baking characteristics were comparable to those of the control ($p > 0.05$), indicating that SBS is a viable antibacterial tempering agent.

Chapter 6 employed a hurdle approach combining acidic water tempering with heat treatment (55 °C). Tempering alone with SBS, lactic acid (LA), and citric acid (CA) at 15% w/v reduced *Salmonella* load by 3.15, 3.23, and 2.91 log CFU/g, respectively ($p < 0.001$). Heating alone reduced *Salmonella* load by 4.1 lg CFU/g after 24 h. Combining both tempering and heat treatment, however, resulted in greater reduction as non-detectable levels (< 2 log CFU/g) of *Salmonella* in wheat were obtained after 12 h of tempering with LA (15%) + heat, while CA (15%) + heat and SBS (15%) + heat after 18 h of tempering. Flour functionality and baking characteristics remained comparable to the control across all treatments ($p > 0.05$), supporting commercial viability.

The final chapter 7 evaluated the antimicrobial potential of Pulsed light (PL) following a preliminary screening in which PL was the only non-thermal technology among cold plasma, ultraviolet, and PL to achieve ≥ 3 log CFU/g reduction under the evaluated conditions. PL at 0.329 J/cm², achieved 4.22 log CFU/g on xylose-lysine deoxycholate (XLD) selective agar medium alone and 3.91 log CFU/g on XLD with a tryptic soy agar overlay (XLD+T), indicating a 7.2% sublethal injury associated with permeabilization after 18 h of tempering ($p < 0.0001$). The Weibull model best described the non-first-order inactivation kinetics, predicting a 5-log reduction at 0.41-0.48 J/cm² (99-118 s), consistent with the experimental data. Bacterial lysis was induced by oxidative stress, which damaged membranes, led to leakage, and compromised DNA integrity. PL at 0.329 J/cm² did not affect particle size or dough rheology ($p > 0.05$); however, a significant difference in damaged starch content was observed ($p < 0.05$), affecting baking and C-cell characteristics.

Together, this work establishes a validated bioinformatic workflow for microbiome analysis in LMF environments, such as the wheat milling system, revealing a stage-dependent restructuring of the wheat microbiome. The study also demonstrated that acidic tempering, hurdle

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Approved by:

Major Professor
Dr. Kaliramesh Siliveru

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Dedication

I dedicate this dissertation to my parents, Umakka and Prakash, for their unconditional love and support.

Chapter 1 - Introduction

1.1 Problem Statement

Low-moisture foods (LMFs), including wheat and wheat-based products, have been considered "microbiologically safe" due to their low water activity (a_w ; <0.85). However, wheat grains and their products have emerged as a novel vehicle for the transmission of foodborne illnesses in humans. Since 2005, over 27 food recalls and more than 15 illness outbreaks linked to enteric pathogens like *Salmonella* and Shiga toxin-producing *E. coli* (STEC) have been traced back to wheat flour and wheat flour-based products (e.g., dough premixes, bake mixes, cookie dough). (Rivera et al., 2025; FDA, 2025). These pathogens can survive in wheat flour for up to 12 months despite their low water activity (Gill et al., 2020; Lauer et al., 2021), because there is no established pathogen reduction treatment applied to the wheat flour during the milling process, contamination from pre- and post-harvest, including processing stages, can distribute these contaminants into wheat flour fractions, further increasing the food safety risk for the product (Rivera et al., 2022). This risk is also worsened by unsafe consumer practices of consuming raw dough and batters, along with low consumer awareness of the food safety hazards associated with wheat-based foods (Feng & Archila-Godinez, 2021).

Even though the microbial risk associated with wheat- and flour-based products has been increasingly recognized, current knowledge largely relies on culture-based detection techniques that are pathogen-specific (Gurtler et al., 2019). Such techniques, although valuable, provide a very narrow view of the broader microbial ecology in the wheat milling setting. Conversely, culture-independent techniques such as 16S rRNA gene amplicon sequencing can provide a more comprehensive overview of microbiome structure, including taxa that are difficult to recover with conventional techniques (Marizzoni et al., 2020; Srinivasan et al., 2015). This is particularly

important in wheat milling settings, as milling can cause a stage-dependent restructuring of the microbial community across samples and can directly influence food safety risk and management.

Despite the growing interest in using the microbiome tool in the food system, systematic benchmarking data specific to the LMF wheat milling environment remains absent, leaving uncertainty about which analytical approach, either amplicon sequencing variable (ASV) or operational taxonomic unit (OTU), is best suited to characterize the microbiome in the wheat milling environment. This illustrates a major gap, as the choice of the bioinformatic pipeline and database choices can significantly influence results for community composition and diversity metrics (Daly et al., 2024; Marizzoni et al., 2020; Sierra et al., 2020). Additionally, 16S rRNA sequencing can provide critical insight into the predicted functional potential of the wheat milling microbiome, providing essential information on pathways enriched for stress- and survival-related functions, such as biofilm formation, osmoprotectant, and multidrug efflux (Douglas et al., 2020). Without these ecological and predictive functional understandings, intervention development remains largely empirical rather than microbiome-informed. Therefore, there is a clear demand to understand the taxonomic composition and its influence on milling by establishing a suitable bioinformatic workflow and using this knowledge to develop science-based intervention techniques to improve the microbial safety of wheat flour and its products.

1.2 Research hypothesis

This research hypothesized that systematic benchmarking and validation of the 16S rRNA bioinformatic workflow would help establish a reliable framework for characterizing microbial communities in low-moisture grain settings and further elucidate the stage-dependent restructuring and selective redistribution of the microbiome across different milling samples, with direct relevance to food safety. Further, the research hypothesized that antimicrobial tempering with

acids (organic and inorganic) and non-thermal treatments would help control *Salmonella* in wheat without significantly compromising wheat flour functionality or baking characteristics.

1.3 Research Objectives

This research focused on understanding the wheat milling microbiome, developing interventions to combat pathogens, and evaluating the effects of these interventions on wheat flour functionality and baking characteristics. The specific objectives of this research were to:

1. Benchmark a 16S rRNA sequencing protocol for microbiome analysis in a low-moisture grain environment.
2. Characterize the wheat milling microbiome through taxonomic profiling, diversity analysis, and functional pathway prediction across different milling stages.
3. Determine the efficacy of sodium bisulfate (SBS) in reducing *Salmonella* in wheat during tempering and assess its impact on flour milling, functionality, and baking quality.
4. Assess the efficacy of organic (citric and lactic acid) and inorganic acids (sodium bisulfate), alone and in combination with heat, as tempering treatments for reducing *Salmonella* in wheat during tempering, and evaluate their impact on flour milling, functionality, and baking quality.
5. Evaluate the potential of non-thermal techniques (cold plasma, ultraviolet, and pulsed light) in reducing *Salmonella* in wheat during tempering and assess their impact on flour milling, functionality, and baking quality.

1.4 Dissertation outline

The dissertation is divided into seven chapters, excluding the introduction chapter. Chapter 2 provides a comprehensive overview of potential sources of enteric pathogen contamination in wheat milled products. This chapter reviews available information on potential pre-harvest and

post-harvest sources of microbial contamination and on food-borne pathogens present in wheat-based systems.

Chapter 3 discusses the impact of methodological choices, specifically clustering and denoising algorithm (OTU versus ASV), and taxonomic richness, relative abundance, and diversity metrics derived from 16S rRNA amplicon sequencing of the wheat milling environment. This chapter will help provide key insight into using bioinformatics and the 16S pipeline to characterize sparse, low-density, and uneven samples in a low-moisture environment.

Chapter 4 validates the DADA2_SILVA bioinformatics workflow for characterizing the taxonomic composition of bacterial communities and diversity metrics across milling stages (before milling, after milling, and milled fractions). This study will also apply the PICRUSt2-based predictive tool to identify enriched metabolic and functional pathways across different samples.

Chapter 5 describes the significance of sodium bisulfate (SBS) tempering as an intervention to reduce the pathogenic *Salmonella* load in wheat during tempering, as well as its effect on wheat flour quality.

Chapter 6 evaluates the use of acidic water tempering and heat treatment as a hurdle approach to reduce wheat *Salmonella* load during tempering, and their effects on flour quality.

Chapter 7 compares the efficacy of different non-thermal technologies, such as cold plasma, ultraviolet, and pulsed light, as interventions for reducing *Salmonella* during wheat tempering. The selected pulsed light technique was further evaluated to determine its effect on time-kill kinetics and wheat-flour quality.

Chapter 8 elucidates the summary of this research work, and recommendations for future work based on the findings from this research are described.

Together, the findings presented across all chapters collectively advance the understanding of microbial safety in wheat milling and provide a scientific basis for developing effective, multi-faceted intervention strategies to reduce pathogen load throughout the grain-flour continuum.

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Chapter 2 - From Wheat Grain to Flour: A Review of Potential Sources of Enteric Pathogen Contamination in Wheat Milled Products.

Abstract

The number of food safety issues linked to wheat milled products have increased in the past decade. These incidents were mainly caused by the contamination of wheat-based products by enteric pathogens. This manuscript focused on reviewing the available information on the potential pre-harvest and post-harvest sources of microbial contamination, and potential foodborne pathogens present in wheat-based products. Potential pre-harvest sources of microbial contamination in wheat included animal activity, water, soil, and manure. Improper grain storage practices, pest activity, and improperly cleaned and sanitized equipment are potential sources of post-harvest microbial contamination for wheat-based foods. Raw wheat flour products and flour-based products are potentially contaminated with enteric pathogens such as Shiga toxin-producing *E. coli* (STECs) and *Salmonella* at low concentrations. Wheat grains and their derived products (i.e., flours) are potential vehicles for foodborne illness in humans due to the presence of enteric pathogens. A more holistic approach is needed for assuring the food safety of wheat-based products in the farm-to-table continuum. Future developments in the wheat supply chain should also be aimed at addressing this emerging food safety threat.

Journal paper:

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2.1. Introduction

Foodborne illness is a prevalent cause of morbidity and loss of productivity for the global population. Annually, foodborne illnesses affect ≥ 600 million people worldwide, with $\geq 420,000$ cases resulting in death (WHO, 2022). In the U.S., 48 million cases of foodborne illnesses are documented every year with most cases associated with 31 major foodborne pathogens (Centers for Disease Control and Prevention, 2024). The enormity of the food supply chain exacerbates the risk of food products being contaminated with pathogenic microorganism.

Wheat grains, and its products have emerged as a novel vehicle for foodborne illnesses in humans. Such products were previously deemed as “microbiologically safe” products due to their water activity being non-conducive to pathogenic microorganisms. The frequency of recalls and foodborne illnesses linked to these products have increased in recent years (Food and Drug Administration, 2024). A potential reason for this trend is the complexity of the wheat supply chain, which makes wheat grains vulnerable to pathogenic contamination. Even prior to harvest, wheat grains are exposed to multiple sources of microbial contamination (shown in **Figure 2.1**) from biotic and abiotic factors (Brar & Danyluk, 2018). In addition, weather conditions (i.e., precipitation, humidity, and temperature) also contribute the extent of microbial contamination present in wheat grains (Sabillón et al., 2016b). Post-harvest operations could also expose wheat grains to potential microbial contamination sources. Typical milling processes do not incorporate antimicrobial interventions. Instead, its physical nature (i.e., size-based separation) facilitates the

redistribution of the microbial contamination present in the grains into the wheat flour and other milling fractions (Rivera et al., 2022a and 2022b). Hence, the risk of pathogen contamination in wheat flours increases when contaminated wheat grains are inadvertently milled.

This manuscript aims to review available information on the potential pre-harvest and post-harvest sources of pathogen contamination in wheat grains, and milled wheat products. In addition, this manuscript also reviewed the results of survey studies on the level of pathogenic microorganisms (e.g., STECs and *Salmonella*) present in wheat grains and milled wheat products. The findings from this review can be used as a basis for the implementation of control measures to address the potential contamination of wheat-based products by pathogenic microorganisms.

2.2 Pre-harvest sources of microbial contamination in wheat grains

As a field crop, the cultivation of wheat grains in open environments increases the risk of microbial contamination on the wheat grains from different sources during the pre-harvest stage. These sources include activity from wild and domesticated animals, soil, manure, and water (Sabillón et al., 2016a). Environmental factors such as temperature, precipitation rates, and relative humidity, as well as extrinsic grain characteristics (physical damage, immature grain, infected grain etc.) can also contribute to the amount, type and survivability of microbial contaminants associated with wheat grains in the field (Alegbeleye et al., 2018; Sabillón et al., 2016a, 2016b; Weidenbörner et al., 2000).

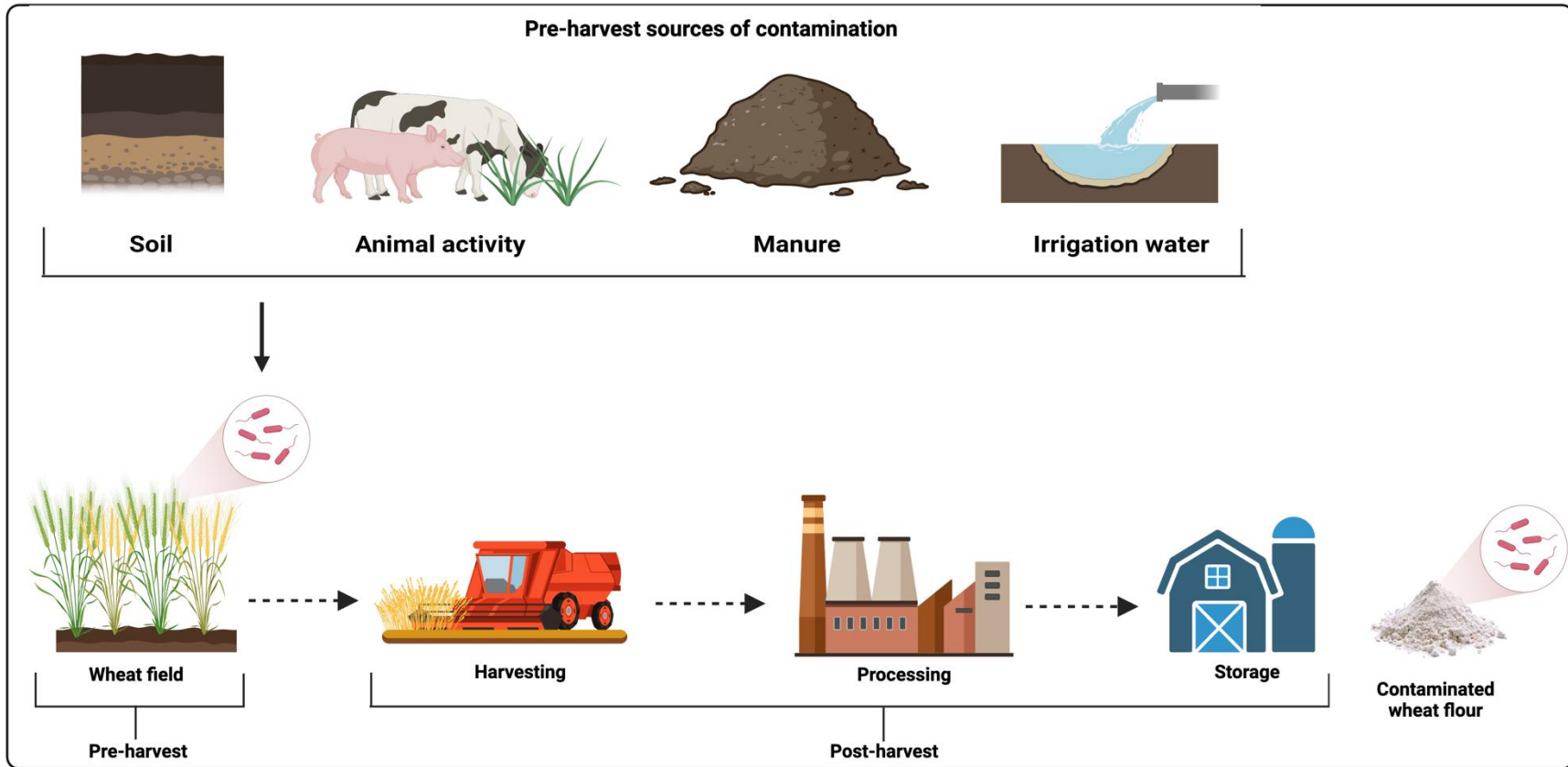


Figure 2.1: Wheat flour production chain; Potential source of microbial contamination in wheat grains from the pre-harvest and post-harvest steps.

2..2.1. Soil

Contamination of wheat grains by pathogenic microorganisms could occur prior to harvesting due to contaminated soil (Janssen et al., 2019). Pathogens like *E. coli* and *Salmonella* may be present naturally or introduced to the agricultural soil once it is treated with animal manure. Pathogenic microorganisms can be found in agricultural soils as they are continuously introduced by both direct and indirect sources including animal manure, irrigation water, water run-offs from adjacent animal operations, and free-ranging animals (Alegbeleye et al., 2018; Iwu & Okoh, 2019).

The prevalence of pathogenic microorganisms in the soil is also dependent on its inherent characteristics such as temperature, pH, porosity, particle size, and water retention capacity (Ackers et al., 1998; Atkinson et al., 2010; van Veen et al., 1997). For example, Lang & Smith (2007) reported that *E. coli* may survive in both sandy loam and silty clay soil for around 31 weeks; greater survivability for *E. coli* was observed in sandy loam soil compared to silty clay soil. In a more recent study, Underthun et al. (2018) reported that *Salmonella* Javiana and *E. coli* O144:H4 and O145 can persist in Candler sand and Orangeburg sandy loam for at least 168 and 224 days, respectively. The use of manure and its different types could also influence the occurrence of pathogens in the soil. For example, the use of poultry waste over horse waste favored the occurrence of *E. coli* O157:H7 at a higher rate in clay loam, slit loam, and sandy loam soils (Whyte et al., 2014). Depending on abiotic conditions and the interactions with soil indigenous microbiome, enteric pathogens may survive in manure-amended soils for long periods, which threatens the safety of agricultural commodities throughout several production cycles (Gagliardi & Karns, 2002; Holley et al., 2006; Semenov et al., 2008; You et al., 2006). Martinez et al. (2015) evaluated the survival and translocation of *E. coli* cells into wheat plant tissues by sowing sanitized wheat seeds in soil inoculated with *E. coli* O157:H7. The results from their study showed that 5

out of 100 wheat seedlings tested positive for *E. coli* O157:H7 after 9 days of sowing. Similarly, Kutter et al., (2006) reported that *Salmonella* and *Listeria* spp. can colonize the intracellular spaces in the root cortex of barley although only *Salmonella* was capable of further internalization into plant tissues. These research findings demonstrate that the soil used for cultivation is a viable vehicle for pathogen transmission and propagation in agricultural products during the pre-harvest stage. Most wheat varieties are grown best in well-drained loamy soil (Aloi, 2023). However, routine field-wide testing of soil for foodborne pathogens may not be practical or reliable for wheat production because pathogens are often present at low levels and may be heterogeneously distributed across large acreage. Rather than testing entire fields, a more realistic approach would be to use risk-based soil assessment in research or targeted surveillance studies. Such studies could focus on fields with known risk factors, including recent manure application, use of untreated biological soil amendments, flooding or run-off from nearby animal operations, wildlife intrusion, or irrigation water concerns. In such fields, composite and/or stratified sampling of topsoil from areas of high risk (for example, field edges, in run-off areas, along run-off channels, and on manure-amended sections of the field) could determine whether soil contamination correlates with pathogen presence on harvested wheat grains. Accordingly, microbiological testing of field soils could be better suited as a specific research and risk-assessment tool than as a practical field-scale monitoring program.

2.1.2. Manure

The application of animal manure for soil amendment and fertilization is a common agricultural practice (Szogi et al., 2015). For instance, a 2006 survey on wheat cultivation practices reported that manure treatment was used in 2.4% (winter wheat), 1.1% (spring wheat), and 1.2% (durum wheat) of the acreage used for wheat cultivation, respectively (MacDonald et al., 2010).

In these cases, most of the manure used for fertilizing fields was mainly sourced from livestock animals mainly from dairy cattle operations. Livestock animals, such as cattle, are regarded as asymptomatic carriers of enteric pathogens such as Shiga toxin-producing *E. coli* (STEC) and *Salmonella enterica* (Stein & Katz, 2017). These pathogens are considered commensal organisms living in the gastro-intestinal tract of livestock animals (Karmali, 2004). Hence, these pathogens are typically shed into the environment through the fecal matter produced by these animals (Sapountzis et al., 2020). In addition, healthy cattle could occasionally excrete *E. coli* O157:H7 in their stool due to intermittent shedding (Kulow et al., 2012; Sharma et al., 2012). Once shed, these pathogenic bacteria can survive in manure for extended durations depending on factors such as pH, moisture content, and manure treatment (Ingham et al., 2004). Semenov et al. (2011) demonstrated that anaerobic conditions facilitate the survival of *E. coli* in slurry (94.5% water content; 50% liquid manure & 50% urine) and farmyard manures. Hence, the use of untreated manure is a potential food safety risk for agricultural commodities (Brar & Danyluk, 2018). Due to this, various manure treatment regimens have been developed to reduce the pathogenic load of manure used for soil amendments. These treatments include lime (calcium oxide or calcium hydroxide) treatment (Gerba & Smith, 2005), hydrogen peroxide (Bilotta & Kunz, 2013), pasteurization (Marañón et al., 2006), air-drying (US Environmental Protection Agency, 2023), UV-irradiation (Oni et al., 2013), aerobic and anaerobic composting (Spiehs & Goyal, 2007) and aerobic and anaerobic digestion (Babaei & Shayegan, 2011; US Environmental Protection Agency, 2023).

Despite the use of manure treatments and adherence to the composting regulations, the risk of *E. coli* contamination in the soil is still present and has become more prevalent (Anderson, 2018). Jiang et al. (2002) demonstrated that *E. coli* persists in soil-treated manure for as long as

231 days, with its survival dependent on temperature. A study by Thiel et al. (2020) also reported that manure bacteria are more at risk of being aerosolized in fertilized soil; this is further worsened during dry seasons where airborne dust from cattle operations are easier to generate (Berry et al., 2015). Farms that use manure treatment for wheat cultivation operations produce their own manure, which indicates that the wheat fields and livestock areas are close to each other (MacDonald et al., 2010). Hence, wheat fields that are treated with manure or near cattle feedlots have a higher risk of contamination of pathogens from fecal matter. This contamination can occur either through direct deposition of feces in the soil or indirectly through the transport of soil, dust, and water that was previously contaminated with fecal matter (Sabillón, 2018). An additional consideration in some wheat-producing regions is dual-purpose wheat production, in which cattle graze winter wheat during the vegetative stage and are removed before the crop reaches grain harvest. The impacts of dual-purpose wheat have been reviewed by a variety of aspects related to agronomic measures and animal performances, such as forage production, weight gain of cattle, grain yield as well as grain quality (Netthisinghe et al., 2020). However, direct studies evaluating whether cattle grazing in dual-purpose wheat systems affects the prevalence or levels of foodborne pathogens in soil, wheat plants, or harvested wheat grain appear limited. This represents a relevant knowledge gap because cattle can shed enteric bacteria in feces, and wheat grazing has been shown to influence fecal bacterial populations, including antimicrobial-resistant *E. coli* and enterococci (Agga et al., 2022a; Agga et al., 2022b). Related integrated crop-livestock studies have also shown that grazing can introduce fecal indicators into soil, although pathogen recovery from harvested produce has often been low or absent when sufficient intervals occur between grazing and harvest (Cheong et al., 2024). Therefore, future studies should evaluate whether grazing termination timing, fecal deposition, environmental die-off, rainfall/runoff, and

the interval between cattle removal and wheat harvest influence the potential transfer of enteric pathogens from grazed wheat fields to mature wheat grain. The improper management of cattle manure in crop fields poses significant risks to the microbiological quality and safety of harvested agricultural products (Black et al., 2021). In the case of wheat grains, the outer layer of wheat grains (germ and bran) protects the interior from direct contamination with enteric pathogens (Hocking, 2003). Although this seems beneficial, the presence of pathogens in the exterior of wheat grains has negative implications on the food safety of milled wheat products.

Overall, the microbial quality of soil also influenced the microbial quality of the wheat grains being cultivated. Future studies relating the microbial quality of soil and wheat grains are still needed along with quantifying the pathogen load transferred into wheat grains from the soil. Such studies could help in the development of risk assessment models for pathogen contamination of wheat-based foods.

2.1.3. Water

According to a 2005 United States Geological Survey, agriculture uses 80% of the U.S. water supply, with irrigation alone accounting for 37% of the country's freshwater withdrawal (Kenny et al., 2005). Typical bodies of water used for irrigation include surface water, groundwater, rain, ponds, municipal water, and reclaimed water (Subedi, 2022). Furthermore, ground water is regarded as a safer source of irrigation water as its enclosed nature makes it less prone to microbial contamination. However, contamination of groundwater can still occur through seeping of toxic pollutants from cultivated lands, industries, landfills, and sewage (Iwu & Okoh, 2019). For example, a study in Canada observed that a higher frequency and concentration of *E. coli* in well water occurred after manure application in the fields (Vander Zaag et al., 2010).

Surface water comprises freshwater sources including rivers, ponds, streams, lakes, and creeks, which represent another potential route for enteric pathogen transmission to wheat grains and other agricultural products (Sabillón, 2018). Using effluents from farms and industries for irrigation can be dangerous if not treated correctly. According to a report from New Zealand by Manono (2016), farm effluents are sometimes sprayed onto wheat fields as a way of irrigation. Their report mentioned that this practice could potentially carry *Salmonella* that could contaminate wheat grains prior to harvest. As for generic *E. coli*, Martinez et al. (2015) showed that the bacteria can survive and grow in wheat crops for up to 15 days when sprayed with contaminated water. The result of their study is evidence that the use of contaminated water for irrigation is a potential route for pathogen contamination of wheat grains prior to harvest. Their results indicate that testing the microbial load and/or pathogenic load of irrigation water in wheat farms is needed. Furthermore, quantifying the extent of transfer and quantity of pathogens from irrigation to harvest is needed in future studies. Results from these tests could help further understand the potential relationship of the microbial quality of wheat grains and irrigation water quality and provide more data for risk assessment with regards to wheat grains.

2.3. Post-harvest sources of contamination for wheat grains

Wheat grains undergo several aggregation steps from the wheat supply chain through the series of transport and storage steps as they make their way to milling facilities. Unclean transport, handling, and processing equipment are the potential contributors for pathogen contamination during the grain handling process. In addition, inappropriate storage conditions could lead to pest infestation and cross-contamination (i.e., from equipment and other grains in the facility). Upon reception of wheat grains into milling facilities, they undergo a series of cleaning, tempering, and milling processes to eventually produce wheat flour. Laca et al. (2006) reported that most of the

microorganisms present on the wheat grain are on its surface layer. Hence, all these processes contribute to the microbial load of wheat grains and wheat flours due to the risk of cross-contamination and worsened with improper grain handling practices. This is important as the food safety issues of low-moisture foods (LMF) are typically caused by cross-contamination (Carrasco et al., 2012; Podolak et al., 2010). The role of grain handling, storage, cleaning, tempering, and milling as a source of post-harvest contamination will be discussed in this section.

2.3.1. Role of grain handling and storage practices

After harvest, the stored wheat grains from the farms are moved to local elevators, local mills, and port terminals (i.e., export purposes) through a network of transportation vehicles (e.g., trains, trucks, barges, etc.). The repeated use of these vehicles for transporting wheat grains leads to the accumulation of dust and dirt deposits, microbial contaminants, and insect and pest activity (Perez-Mendoza et al., 2004). Currently, no published studies have directly linked this factor to pathogen contamination in wheat grains. Published studies have shown that unclean transport vehicles could potentially serve as vectors for microbial contamination of wheat grains. Repeated handling of wheat grains produces dust emissions, which can serve as a route for microbial contamination. Gutarowska et al. (2018) assessed the microbiological hazards of dust from various sources and reported that dust coming from cultivated areas had the highest number of microbial contaminants including bacteria (3.8×10^2 to 1.6×10^8 CFU/ g) and fungi (1.5×10^2 to 6.5×10^6 CFU/ g). Various types of pathogenic microorganisms such as *Bacillus*, *Actinomyces*, *Corynebacterium*, *Prevotella*, *Rickettsia* and *Clostridium* species were also identified in the dust samples. Similar observations were also made in previous studies, wherein *Salmonella* (Morita et al., 2006) and non-pathogenic *E. coli* (Berry et al., 2015) were also detected in dust samples. In grain handling facilities, dust is mainly contained through pneumatic systems (Posner & Hibbs,

2005. Majchrzycka et al. (2018) reported that generic *E. coli* and other microorganisms could survive and grow in dust filtration systems. Butcher & Miles (2018) also noted that dust was a major cause of *Salmonella* contamination in feed mills. Hence, dust collection systems should regularly be maintained as it could serve as a reservoir for pathogenic microorganisms. Currently, there are no studies directly investigating the role of dust in pathogen contamination of wheat grains. Hence, investigating this matter in future studies could be beneficial in further understanding the role of dust emissions in transmitting pathogens into wheat grains, and eventually be used for optimizing dust control systems for improving food safety.

Grain storage is another crucial aspect of wheat handling in the supply chain; in between transport, wheat grains are typically stored in different storage facilities (e.g., bins, grain elevators, silos). Wheat grains could remain stored in these facilities for extended periods of time to compensate poor harvest years (Hagstrum et al., 1999). The presence of stored-product insects is a major concern in these storage facilities. For instance, several surveys have reported significant presence of insects in stored wheat grains (Babarinde et al., 2017; Flinn et al., 2010; Hamel et al., 2020; Reed et al., 2003); the extent of infestation is typically dependent on the moisture and temperature conditions in these storage facilities (Riaz et al., 2014). Insect infestation is a concern from both quality and safety viewpoints. From a quality issue, insect infestation results in poorer grain quality, and eventually poorer wheat flour quality (Keskin and Ozkaya, 2015; Stathers et al., 2020). Insect infestation causes the formation of moisture, and heat gradients inside the storage bin due to their metabolic activity (Hagstrum et al., 1999); these lead to the higher localized temperature and heat moisture levels, which could encourage the growth of pathogenic microorganisms such as STECs and *Salmonella*.

Another concern for insect infestation is their ability to transmit pathogenic microorganisms into clean stored wheat grain through their progeny (Hazeleger et al., 2008; Laroche et al., 2018; Simothy et al., 2018). For example, Husted et al. (1969) reported that rice weevils (*Sitophilus oryzae* L.) retained *Salmonella* Montevideo for at least 1 week after being in *Salmonella*-inoculated wheat for 7 days; longer retention times (> 5 weeks) observed when the rice weevils were reared in contaminated wheat for greater durations (14 to 21 days). Crumrine et al. (1971) exposed several common stored-product insects (rice weevil, saw-toothed grain beetle, and red flour beetle) to *Salmonella* Montevideo-inoculated wheat for 21 days and transferred them sequentially into three more batches of clean wheat. All the stored-product insects tested transmitted the pathogen into the first batch of clean wheat but not into the succeeding wheat batches. The studies that covered the role of insects in pathogen transmission are relatively old (published > 30 years ago); hence, conducting similar studies, which mimic the conditions available in modern grain storage practices and facilities, could be done to further understand the role of insects in pathogen contamination of wheat grains.

The relevance of grain handling and storage practices for wheat grains highlights the importance of management practices (e.g., cleaning, pest control, and product scheduling). The presence of spilled grains, and residual grain materials in the storage facilities and grain handling equipment present a risk of microbial cross-contamination due to the presence of stored-product insects. Dowdy & McGaughey (1998) reported that all areas of the grain elevators surveyed had detectable populations of stored product insects that could migrate into the stored grain. The consistent presence of insects in grain residue samples from the discharge spout of used and unused storage bins, and commercial elevators were also observed in previous studies (Reed et al., 2003; Arthur et al., 2006). Aside from potential insect activity, grain residues could also serve as food

for common pests such as rodents, birds, and cockroaches. Rodent and bird droppings could find their way to the grains and contaminate it with foodborne pathogens such as *Salmonella* and STECs (Kilonzo et al., 2013; Navarro-Gonzalez et al., 2019). In addition, Stejskal et al. (2014) reported the presence of rodent droppings in stored grains (6.90 feces/ m²) and conveyors (34.8 feces/ m²) used in silos for barley storage; their results also highlighted the similarities of the sizes of fecal droppings and grains, which makes physical separation difficult. Cockroaches are likely carriers of pathogenic microorganisms (Donkor, 2020): these could be shed into the stored grains and equipment surfaces as they come into contact. These studies highlight the potential role of pests in transmitting pathogenic microorganisms into stored wheat grains. Future studies could be done on relating the level of pest infestation in wheat grain storage facilities to the pathogen and overall microbial load of stored wheat grains.

2.3.2. Role of the wheat cleaning process

The first step in the milling process is cleaning, wherein foreign materials (e.g., metals, stones, soil, dead insects, and fecal droppings), and unsound wheat grains (e.g., damaged, shrunken, broken) are removed from the wheat batch (Posner & Hibbs, 2005). The wheat cleaning step was reported to effectively reduce mycotoxin contamination in wheat due to the removal of damaged kernels (Tibola et al., 2016). However, the effects of cleaning on the microbial load of wheat grains are reported to be minimal. Manthey et al. (2004) reported a reduction of ~1.0 log CFU/g in the overall wheat microbial load after cleaning. In another study, Corina et al. (2012) reported a < 1 log (i.e., 90%) reduction in the mold (76.9%) and aerobic bacteria (81%) load of wheat grains after undergoing an industrial-scale cleaning process. However, no studies have directly investigated the effects of cleaning steps on the pathogen load of contaminated wheat

(Manthey et al., 2004; Rose et al., 2012). Hence, future studies could be done on evaluating the effects of different cleaning steps on reducing the pathogenic load of wheat grains.

Published studies have also reported that wheat cleaning equipment are likely sources of microbial contamination due to their high microbial load from processing unclean wheat grains. For example, Dacarro et al., (2005) reported that the cleaning sector of the grain milling facility has the highest levels of microbial contamination (i.e., 11.41 times higher than the external environment). In a more recent study, Sabillón et al. (2021) observed substantial microbial contamination levels in wheat cleaning equipment from aerobic mesophilic bacteria, *Enterobacteriaceae*, and molds. Further, *E. coli* was detected at two instances on the cleaning equipment (0.4 log CFU/ 10 cm²). Overall, the published studies on the effects of the wheat cleaning process on microbial load indicate that the process, as it is designed, cannot be used as a reliable measure for mitigating pathogen contamination in wheat grains prior to milling. Studies investigating potential directions for changing steps in the wheat cleaning process (i.e., inclusion of non-thermal antimicrobial treatments) are needed to improve its effectiveness in reducing the pathogenic and overall microbial load of wheat grains.

2.3.3. Role of the wheat tempering process

Once grains are cleaned, they undergo a tempering process prior to milling. This step involves adding controlled amounts of water to the wheat grains (i.e., increasing their moisture content to a target range of ~14–17%, depending on wheat class), and resting the grains (4 to 24 h) to toughen the bran and soften the wheat endosperm (Posner & Hibbs, 2005). The tempering step is a critical step from a quality (i.e., improves milling yield, and lower flour ash content) and safety point of view. Improper tempering (i.e., too much moisture added, use of dirty water, and too long resting time) could result in higher water activity, and temperature, which can encourage

the proliferation of microorganisms in wheat grains and on equipment surfaces. In the tempering step of commercial milling operations, chlorinated water (400 to 700 ppm) is typically used for microbiological safety as it can inhibit yeast and mold growth on the wheat (Lin et al., 2022; Sabillón et al., 2020). However, the presence of organic matter in the wheat grains limits the effectiveness of chlorinated water as an anti-microbial agent (i.e., lowers free chlorine concentration for disinfection). This was evidenced in the findings of survey studies on microbial contamination of wheat grains and equipment from the tempering step. For instance, Berghofer et al. (2003) reported that higher ($> 10^4$ CFU/g) mesophilic aerobic counts were more frequent in tempered wheat grains. In addition, the number of non-pathogenic *E. coli*-positive wheat samples after tempering also increased (0 to 14%), with counts ranging from 10^0 to 10^2 CFU/g being more frequent. The observations were attributed to the cross-contamination of microorganisms from the encrusted grain residues inside the bins, conditioning augers, and elevators. Hence, pathogenic bacteria such as STECs and *Salmonella*, if present, could also cross-contaminate wheat grains from unclean tempering equipment. The tempering system may also be considered a potential microbial harborage point as it combines moisture addition, deposits of residual grain materials, dust buildup, and repeated contact with product. These effects may be conducive to localized microbial persistence on equipment surfaces. Nonetheless, published evidence of STEC or *Salmonella* biofilm development within commercial wheat tempering systems are sparse. This represents an important knowledge gap because moisture introduction into otherwise low-moisture processing environments can promote microbial persistence when equipment design, cleaning access, and drying are inadequate. The results of these published studies indicate that the conventional tempering process is inadequate in maintaining or lowering the microbial quality of wheat grains. In addition, their results show the importance of properly cleaning tempering

equipment and using grains with low microbial contamination levels. Published information on standardized cleaning and sanitation practices for commercial wheat tempering vessels is limited. Standard procedures are likely facility-specific, depending on equipment design, access schedules, standard operating procedures, and the ability to dry equipment thoroughly. Wheat milling is a low-moisture process environment, so practice tends to focus on minimizing water use, removing residual grain and dust by dry cleaning wherever possible, and using wet cleaning strategically after thorough draining, cleaning, and drying of equipment. Cleaning of tempering equipment can involve dislodging any residual grain and encrusted debris through the use of brushes, scrapers, vacuum equipment, disassembly, etc., and then targeted use of sanitizer by whatever means of application is compatible with equipment configuration and process constraints. Future industry surveys could provide insight into how tempering bins, dampeners, conditioning augers, and downstream conveyors are cleaned in wheat mills. Future studies could be done on developing anti-microbial methods to reduce the pathogenic and overall microbial load of wheat grain during the tempering step. These methods could then be used in milling operations to validate their effects on reducing the microbial contamination on tempering equipment surfaces, and microbial quality of wheat grains.

2.3.4. Role of the wheat milling process

Tempered grains are processed into wheat flour through the milling process. This includes a series of grinding, sieving, and purification steps to extract flour from other wheat grain components. Grinding steps are conducted using corrugated or smooth roller mills, while sieving is done using nylon/steel meshes to separate the ground materials based on size (Posner & Hibbs, 2005). Laca et al. (2006) established that grain microbial contamination is mainly concentrated on the bran layer of wheat grains. Hence, the comingling of the bran and endosperm parts of the grain

presents a cross-contamination risk for the microorganisms (including pathogens) initially present on the wheat grains. In a lab-scale milling simulation, Rivera et al. (2022a) observed a substantial *E. coli* load (1.9-3.6 log CFU/g) in the milling fractions (flour and non-flour) produced from non-pathogenic *E. coli*-inoculated wheat (at 3 and 6 log CFU/g). The results from this study indicate that cross-contamination occurred between the bran and the endosperm particles resulting in the *E. coli*-load being recovered in the flour fractions.

The accumulation of grain residues on the mill equipment surfaces is another potential source of contamination. The continuous action of the rollers during grinding generates heat, which results in moisture condensation on the cooler surfaces of the mill equipment. This promotes the accumulation of the grain residues on the interior walls of the equipment. These then become a viable reservoir for pathogens (if present) and contaminate the succeeding streams of mill fractions. This was demonstrated in the study by Rivera et al. (2022a), where the non-pathogenic *E. coli* load recovered from flour (1.0-1.7 log CFU/g) and non-flour (0.9-1.7 log CFU/g) milling fractions increased with greater quantities of inoculated wheat milled. The presence of accumulated grain residues also resulted in greater contamination levels on the surfaces of milling equipment where the accumulation was observed to take place (i.e., hoppers, rollers, sieving surfaces). In the study by Rivera et al. (2022b), non-pathogenic *E. coli* was recovered from the surfaces of the milling equipment used (0.5-3.6 log CFU/100 cm²) after milling inoculated wheat grains. The contaminated equipment surfaces could also become a source of pathogen contamination in the milling fractions produced. In the same study, surface contamination on the milling equipment dropped to non-detectable levels after using the contaminated milling equipment to mill non-inoculated wheat grains. In conjunction with this, Rivera et al. (2022a) recovered decreasing levels non-pathogenic *E. coli* (0.5-2.5 log CFU/g) in the milling fractions

produced from the non-inoculated wheat grains after milling. The results of this study indicate that grain residues and mill equipment are viable sources of microbial contamination during milling. These present a food safety risk as pathogen contamination of bulk cereal products with the “cleaning remains” from the mill equipment has been cited as a reason for product recalls (Rushdy et al., 1988). The loading and transportation for finished products are also potential routes for microbial contamination. These steps are often done in partially exposed or open areas, which presents a risk for microbial contamination. Overhead equipment installation and roof structures could harbor pests that could transfer microorganisms (including pathogens) into the grains or finished products. In addition, weather conditions could also increase the risk of pathogen contamination. Colder seasons could result in moisture condensation and eventual caking of the flour powder, which in turn could facilitate microbial proliferation, and serve as a source of contamination in succeeding batches of finished products.

Overall, published literature has established the role of milling in the contamination of wheat grains and flours with pathogenic microorganisms. However, there is still a lack of knowledge on investigating cross-contamination of pathogens during the milling phase. Recent studies that directly investigated pathogen contamination were done in a smaller capacity (lab-scale/ pilot scale) mill. Hence, it would be beneficial if such studies could be done on a larger scale to better approximate cross-contamination in commercial milling operations. In addition, evaluating the effect of different milling conditions (i.e., wheat type, mill flow sheet, tempering conditions, and contamination level) on cross-contamination are needed to fully determine if certain milling processes are more prone to cross-contamination. Such studies could then be used as a basis for developing food safety measures specific to wheat milling. Lastly, combining milling

processes that limit cross-contamination along with potential antimicrobial interventions could be a great help in limiting pathogen contamination of milled wheat products.

2.4. Pathogenic microorganisms in wheat grains and flours

Pre-harvest and post-harvest provide plenty of opportunities for enteric foodborne pathogens such as *Salmonella* and STECs to get into wheat grains and wheat flours. The presence of these pathogens compromises the food safety of wheat-based foods. In the food industry, coliforms and generic *E. coli* are typically used as indicators of hygiene and safety of raw materials and processing conditions (National Research Council, 1985). High levels of these indicator microorganisms in wheat grains or flours are potential indicators of unsanitary handling or fecal contamination of grains. From a food safety standpoint, the detection of *E. coli* could signify a higher likelihood of hazard compared to other coliform bacteria. This section will cover the results of survey studies pertaining to the presence of enteric pathogens and/or indicator organisms in wheat grains and wheat flour. A summary of this section is included in **Table 2.1**.

Table 2.1: Prevalence and contamination levels of enteric microorganisms in wheat grains and wheat flours.

Location	Sample size (n)	Pathogens Tested	Prevalence (%)	Contamination Level	Reference
Wheat grains					
Australia	58	Coliforms	ns	10 ⁰ to 10 ³ log CFU/g	Berghofer et al. (2003)
		E. coli	ns	10 ⁰ to 10 ² CFU/g	
Australia	50	E. coli	2%	≤ 0.6 log CFU/g	Eglezos (2010)
		Salmonella	2%	ns	
North America	3350	E. coli	12.8%	ns	Richter et al. (1994)
	3040	Salmonella	1.32%		
United States of America	3891	Salmonella	1.23%	0.110 MPN/g	Myoda et al. (2019)
		EHEC	0.44%	0.039 MPN/g	
Wheat flours					
Australia	412	Salmonella	0.49%	ns	Berghofer et al. (2003)
		E.coli	ns	10 ⁰ to 10 ¹ CFU/g	
Australia	300	E.coli	0.7%	0.80 – 0.96 log CFU/g	Eglezos (2010)
	150	Salmonella	< 0.7%	ns	
Turkey	142	E.coli	ns	0.33-1.37 MPN/g	Aydin et al. (2009)
		Salmonella	nd	nd	
	4210	Salmonella	1.05%	ns	Sperber (2007)
	2921	E.coli	ns	0.74 log CFU/g	
North America	3735	E. coli	ns	0.23 MPN/g	
	2921	E.coli	ns	2.99 log CFU/g	
	2467	Coliforms	ns	2.65 log CFU/g	
	3688	Coliforms	ns	1.63 MPN/g	

	2467	Coliforms	ns	3.64 log CFU/g	
Germany	62	STEC	39%	ns	Mäde et al. (2017)
Germany	ns	STEC	ns	ns	Projahn et al. (2021)
North America	46	STEC O121	ns	0.15 to 0.42 MPN/g	Gill et al. (2019)
		Coliforms	ns	2.25 to 2.96 log CFU/g	
		<i>Enterobacteriaceae</i>	ns	3.21 to 4.55 log CFU/g	
		<i>E. coli</i>	ns	< 0.3 to 0.38 log CFU/g	
Switzerland	70	STEC	12.9%	ns	Kindle et al. (2019)
Canada	347	Non-O157 STECs	1.7%	ns	Zhang et al. (2021)
Sweden	200	STEC	4%	ns	Soderlund et al. (2023)

ns - not specified; **MPN** - Most Probable Number; **STEC** – Shiga toxin-producing *Escherichia coli*; **EHEC** – Enterohemorrhagic *E. coli*

2.4.1. Prevalence in wheat grains

In an Australian wheat survey (n = 58 samples), Berghofer et al. (2003) reported coliform counts ranging from 10^0 to 10^3 log CFU/g in wheat grains. Eglezos (2010) surveyed wheat grains in flour mills in Australia (n = 50) and observed low prevalence for *E. coli* and *Salmonella* as only 2% (1 out of 50) of the samples tested positive for both enteric bacteria. As for North American wheat, an earlier survey by Richter et al. (1994) reported generic *E. coli* prevalence at 12.8% (n = 3350 samples tested), while *Salmonella* was detected in 1.32% (n = 3040 samples tested). In a more recent survey on U.S. wheat, Myoda et al. (2019) reported that 1.23% (48 samples; n = 3891) of wheat were positive for *Salmonella* at an average contamination level of 0.110 MPN/g., Enterohemorrhagic *E. coli* (EHEC) was also sporadically detected in 0.44% (17 samples; n = 3891) of wheat samples with an average contamination level of 0.039 MPN/g; *E. coli* O157 was not detected in the wheat samples tested. Furthermore, PFGE fingerprinting of the recovered isolates revealed high genetic diversity, with all EHEC patterns (100%; 22 out of 22) and the majority of *Salmonella* patterns (83%; 39 out of 47) being unique, indicating that the isolated pathogenic microorganisms came from diverse sources, which could be from pre-harvest or post-harvest (Myoda et al., 2019). This observation indicates that the isolated pathogenic microorganisms came from diverse sources, which could be from pre-harvest or post-harvest.

2.4.2. Prevalence in wheat flour

Like wheat grains, the incidence and contamination levels of enteric bacteria in wheat flour were also low. Berghofer et al. (2003) reported *Salmonella* was isolated from 2 out of 412 milling samples analyzed from Australian wheat milling facilities. In addition, *E. coli* was sporadically detected at low levels (10^0 to 10^1 CFU/g) in samples taken from the early stages of milling, and end-products derived from the outer grain layer (i.e., bran). In a more recent survey, Eglezos (2010)

detected a low prevalence of generic *E. coli* in flour milling fractions at only 0.7% (2 out 300 samples; 0.8 log CFU/g; max count: 0.96 log CFU/g). Aydin et al. (2009) surveyed wheat flour samples from Thrace, Turkey (7 locations) and reported low counts for generic *E. coli* (0.33-1.37 MPN/g; n = 142 samples) while *Salmonella* was not detected in the samples (25 g sample/ test). Sperber (2007) reported that from the period of 1984 to 1991, 1.05% (44 out of 4210 samples) of wheat flour samples tested from North America were positive for *Salmonella*. In addition, generic *E. coli* was detected in wheat flours from 2003 to 2005 at a geometric mean level of 0.74 log CFU/g (max level: 2.99 log CFU/g; n = 2921 samples), while coliforms were detected at a geometric mean level of 2.65 log CFU/g (n = 2467). From the same study, *E. coli* was present in whole wheat flours at a geometric mean level of 0.84 log CFU/g, while coliforms were present at a geometric mean level of 3.64 log CFU/g. The low levels of pathogenic bacteria in whole wheat flour were also observed by Cardoso et al. (2019), wherein *Salmonella* was not detected (25 g analytical units) in 7 commercial samples of whole wheat flour.

In the following studies, the presence of STECs in retail wheat flour samples was investigated. In a survey of wheat flours and flour-based products from Germany (2015-2019), Projahn et al. (2021) isolated 123 STEC strains. Their whole genome sequencing (WGS) revealed high diversity for the isolated serotypes including O157:H7, O145:H28, O146: H28, and O103:H2; all of which are frequently associated with foodborne illness outbreaks in humans. However, these isolates were reported broadly as STEC based on the presence of Shiga toxin genes and associated virulence markers and should not all be interpreted as confirmed enterohemorrhagic *E. coli* (EHEC) strains. Mäde et al. (2017) reported that for wheat flour samples from Germany, 39% (n = 62) of the samples tested were positive for STEC through molecular detection with 105 different isolates detected. This relatively high prevalence compared with other surveys may be partly

explained by the detection methodology used, as enrichment followed by molecular screening can detect STEC-associated genes even when culture-confirmed recovery is lower. Therefore, the reported prevalence should be interpreted as evidence of potential STEC contamination rather than direct evidence that all positive samples contained viable, clinically significant EHEC strains. The results from both studies indicate that STEC contamination in German wheat flour samples could occur. Gill et al. (2019) analyzed retail wheat flour samples implicated in an *E. coli* O121 outbreak from 2016 to 2017 in North America and reported STEC O121 levels of 0.15 to 0.42 MPN/g. Mean counts (log CFU/ g) for coliforms (2.25 to 2.96), enteric bacteria (3.21 to 4.55), and generic *E. coli* (< 0.3 to 0.38) were also observed for the retail flour samples tested. Kindle et al. (2019) reported that 9 (12.9%; n = 70 samples) tested positive for *stx* gene, while STEC was only recovered in eight (88.9%) of the *stx*-positive samples. The isolates recovered include STEC O11:H48 (n =1; *stx1c/stx1d*), O146:H28 (n = 2; *stx2b*), O103:H2 (n = 1; *stx1a* and *eae*). The O nontypeable STECs were also recovered including Ont: H12 (*stx2a*), Ont: H14 (*stx2a* and *stx2g*), and Ont: H31 (*stx1c* and *stx1d*). Among these isolates, STEC O103 was significant as it is one of the “Big Six” STEC serotypes responsible for majority of STEC-related foodborne illness outbreaks. Zhang et al. (2021) isolated and identified non-O157 STECs (1.7%; n = 347) in retail wheat flour samples from Canada. In their study, one of the isolates carried the *stx1a* and *eae* genes, while the rest carried the *stx2* gene. These virulence genes (*stx1* and *stx2* – toxin; *eae* – attachment) are important in the major STEC serotypes relevant to humans as they facilitate the development of foodborne illness (e.g., bloody diarrhea) (Boerlin et al., 1999). In another study, Söderlund et al. (2023) analyzed 200 bags of Swedish-produced wheat flour for the presence of prevalent STEC genes (*stx1*, *stx2*, and *eae*). Their analysis (real-time PCR after enrichment) revealed that 12% and 11% of the analyzed samples were positive for both *stx* genes and *eae* gene,

respectively. Further, 8 STEC isolates (4%) were recovered from the samples although none of them were linked to previous STEC outbreaks. Within the limitations of the sample size and the low number of culture-confirmed isolates, no clear association was observed between flour production type (organic, small-scale, or whole grain) and detection of the targeted STEC genes.

Overall, the results from the survey studies indicate that enteric pathogens such as *Salmonella* and STECs are present in wheat grains and wheat flours at low prevalence and quantity. In addition, most of the studies have focused on the presence of non-O157 STECs compared to O157 and *Salmonella* as they are more prevalent in milled wheat products. Although documented raw wheat flour-associated outbreaks have primarily involved STEC and *Salmonella*, future studies could also consider other dry-tolerant or cereal-associated foodborne hazards, such as the *Bacillus cereus* group and *Cronobacter* spp. These organisms should be discussed differently from STEC and *Salmonella*, because the outbreak evidence directly linked to raw wheat flour is stronger for STEC and *Salmonella*. The *B. cereus* group is relevant because it has been detected frequently in cereal flour and starch-rich foods and can persist in dry food systems, while *Cronobacter* spp. have been detected in cereal kernels, cereal flour, and flour-based products, although illness and outbreak concerns are more strongly associated with powdered infant formula. Therefore, future surveillance should distinguish between pathogens with confirmed evidence of raw flour-associated outbreaks and organisms that represent plausible hazards due to their occurrence and persistence in low-moisture cereal systems. This could help manufacturers get ahead in addressing potential food safety issues for wheat grains and wheat flours. Due to wheat grains/ wheat flour consisting of individual granules, pathogens could be non-homogeneously distributed throughout the bulk of these products. Hence, “false negative” results could occur when samples taken are not properly representative of the bulk quantity of grains or flours. This could be addressed in future

survey studies to be conducted by using larger sample sizes (number and analytical unit) of grains or wheat flour. This could help in improving the detection rates for these pathogens of concern as the heterogeneity of milled wheat flour/ grains could hinder the detection capability of detection methods used. For instance, both the studies by Projahn et al. (2021) and Made et al. (2017) demonstrated that increasing the number of subsamples tested resulted in higher detection rates. In addition, conducting more microbiological surveys focused on quantity of these pathogens (non-O157 STECs, O157 STECs, and *Salmonella*) in wheat grains and wheat flours could be done in the future. This could help establish a baseline level of contamination that could aid in designing more effective anti-microbial methods, sanitation controls, and “Good Manufacturing Practices” (GMPs) in wheat milling operations to minimize the risk of pathogen contamination.

2.5. Conclusions and Future Outlook

This literature review has discussed the potential contamination sources in the pre-harvest and post-harvest stages in the wheat supply chain. However, there are still knowledge gaps to be addressed including the identification of significant factors in these contamination sources that contribute to the extent of contamination in wheat grains and wheat flours. In addition, microbiological survey studies have also established the presence of pathogenic microorganisms in wheat grains, and wheat flours. Pathogens such as STECs (O157 and non-O157), and *Salmonella* were the most common pathogens detected in wheat grains and flours from these survey studies. The detection of these pathogens indicates that the conventional milling process is inadequate in reducing/ eliminating these pathogens in wheat flour products. Although it is established that they are present in low quantities and prevalence, there is still a need for establishing the baseline (minimum / maximum) levels for these pathogens through microbiological surveys of grains and wheat flours from different geographical locations. The

results from these surveys would help the development of potential milling-specific mitigation solutions such as developing antimicrobial treatments, improving sanitation controls, and tailoring GMPs to milling facilities. The presence of these pathogens in wheat grains and wheat flours shows that these products have emerged as a viable vehicle for foodborne illness in humans; efforts should also be made to increase consumer awareness regarding the food safety issues of wheat-based foods. Future developments in the wheat supply chain should also aim in addressing these emerging food safety threats in milled wheat products. Implementing food safety measures from the pre-harvest and post-harvest stages of the wheat supply chain can provide a holistic approach in mitigating pathogen contamination.

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Chapter 3 - Benchmarking a 16S rRNA Sequencing Protocol for Microbiome Analysis in Low-Moisture Grain Environments

Abstract:

Microbial amplicon sequencing studies are an important tool in food and biomedical research. However, accurate interpretation of the 16S rRNA gene survey requires specialized software and an algorithm to convert raw sequencing data into reliable taxonomic profiles. Given the existence of multiple bioinformatics pipelines varying in sequence aggregation strategies, reference databases, and filtering parameters, there is little to no consensus on best practices for LMF processing systems. In this study, we systematically assessed discrepancies in taxonomic composition, alpha diversity, and beta diversity across 32 combinations of bioinformatics workflows, based on eight widely used 16S rRNA pipelines and four taxonomic databases, applied to 16S rRNA gene sequences extracted from wheat milling environments (n=160). Weighted average scores were used to select the top 10-performing workflow combinations for downstream analysis. Taxonomic assignments were broadly similar across workflows at the family and genus levels; however, diversity metrics at the genus level showed greater sensitivity to workflow choice. At the family level, diversity metrics were conserved across pipeline-database combinations (Chao1: 22.97±2.20-24.92±2.04; Shannon: 2.59±0.19-2.74±0.18; InvSimpson: 10.63±1.25-11.27±1.06; Bray-Curtis: 0.528-0.556; Jaccard: 0.557-0.582), whereas at the genus level both alpha and beta diversity exhibited wider ranges and larger dispersion (Chao1: 45.27±5.68-50.20±5.64; Shannon: 2.54±0.24-2.73±0.22; InvSimpson: 10.37±1.4-11.03±1.05; Bray-Curtis: 0.79-0.82; Jaccard: 0.79-0.80). Furthermore, ASV vs. OTU workflows were comparable across the different evaluated metrics; however, the ASV showed numerically higher values for some genus-level measures compared to OTUs because they can resolve variation down to the single-

nucleotide level, thereby retaining low-abundance features important for LMF safety. This work paves the way toward using bioinformatics and 16S pipelines to characterize sparse, low-density, and uneven samples in low moisture environments.

3.1. Introduction

Low-moisture foods (LMF) have been considered microbiologically safe due to their low water activity (a_w) (Shivaprasad et al., 2024). This attribute has led to a false impression among the general public that they pose a low microbiological risk (Rivera et al., 2024). Wheat and wheat-flour-based products are prominent members of this category and have been increasingly recognized as vehicles for enteric pathogens, including *Salmonella* and Shiga toxin-producing *E. coli*, which have been linked to several illness outbreaks and recalls in the United States over the past decade (CDC, 2024; CDC, 2023; CDC, 2023a). This is mainly due to the complexity of the wheat supply chain, which can allow contamination at the pre-harvest stage and enable its persistence through post-harvest handling and processing, with pathogens redistributed into milled fractions. Given the absence of a defined lethality step during the milling of wheat grains into wheat flour, it is increasingly important to elucidate the composition and dynamics of milling-associated bacterial communities (Doddabematti Prakash et al., 2024). This understanding is essential for identifying potential contamination pathways and supporting the development of science-based microbial control strategies for low-moisture wheat products.

Traditional culture-based techniques are fundamentally inadequate for characterizing microbial communities in LMF environments, including wheat grains, because these organisms are often present at very low abundance and almost always require an enrichment step to increase their numbers to a detectable level (Gurtler et al., 2019). Without enrichment, these methods recover only a small fraction of the viable microbial population and fail to capture viable but non-

culturable and fastidious taxa, resulting in an incomplete and potentially biased representation of community structure (Rizal et al., 2020; Trinh & Lee, 2022). To address these limitations, culture-independent approaches based on high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene have emerged as an effective alternative for identifying phylotypes in samples (Marizzoni et al., 2020). The 16S rRNA gene is a short, conserved marker that can identify bacterial genera with up to 96% accuracy and, in some cases, species with up to 87.5% accuracy (Srinivasan et al., 2015). However, processing 16S rRNA sequences requires a complex bioinformatics workflow, such as merging forward and reverse sequence reads, denoising, quality filtering, trimming sequence reads (Lehr et al., 2025), and clustering into operational taxonomic units (OTUs) at defined similarity thresholds or resolving amplicon sequence variants (ASVs) representing exact biological sequences. Commonly used pipelines (e.g., DADA2, UNOISE3, Deblur, MED, MOTHUR, UCLUST, UPARSE, and RDADA2) employ distinct algorithms for these tasks, and taxonomic assignment relies on reference databases including SILVA, Greengenes, or BLAST. Several comparative studies have demonstrated that the choice of bioinformatics pipeline and analysis tools significantly influences results for community composition and diversity metrics, even when the datasets analyzed are identical (Daly et al., 2024; Marizzoni et al., 2020; Sierra et al., 2020). These pipeline-dependent effects are especially concerning for low-biomass communities in LMF processing environments, where identifying low-abundance taxa, such as potential foodborne pathogens, is essential for food safety assessment.

Despite this recognized variability (both in pipelines and databases), systematic benchmarking data specific to LMF wheat-milling environments remain absent, leaving uncertainty about which analytical approaches are best suited to characterizing the microbiota in low-moisture grain systems. This study aims to (i) assess the impact of methodological choices,

specifically clustering and denoising algorithms (OTU versus ASV) and taxonomic richness, relative abundance, and diversity metrics derived from 16S rRNA amplicon sequencing of wheat milling samples, and (ii) establish the most appropriate combination of bioinformatics analysis framework for future microbiota surveillance in wheat milling and other low-moisture food processing environments. This study closes a critical methodological gap and provides a validated foundation for standardized, reproducible surveillance of the microbiota in wheat milling and other LMF processing environments.

3.2. Materials and methodology

3.2.1. Study design

This study used a factorial benchmarking approach to evaluate the effects of the amplicon-processing pipeline and reference-formatted taxonomic database on the wheat mill microbiome. Two computational factors, pipelines and databases, were evaluated in an 8×4 matrix, yielding 32 discrete combinations. All 32 combinations were applied to each sample, yielding repeated measurements. All combinations were executed on the same set of raw demultiplexed FASTQ files to ensure that differences in performance metrics were attributable to pipeline-database differences rather than input variability. Since Greenegenes2, SILVA, and GTDB are all built on a pre-trained Naïve Bayes classifier, and BLAST is an alignment-based taxonomy assignment workflow, the aim is to compare the workflow combinations rather than database-only factors in a fully crossed way.

3.2.2. Sample collection and DNA extraction

The study focused on identifying potential sites that could harbor or support microbial growth. A total of 160 (n=160) samples were collected from a wheat milling facility in Kansas, which comprised (i) *surface samples* collected from Food Contact Surfaces (FCS) and Non-Food

Contact Surfaces (NFCS), and (ii) *millstream fraction samples* collected from product and process streams. The temperature in the facility was maintained at 20-24 °C during sample collection. Surface samples were collected using BLU-DRY PUR-Blue Swab samplers (World Bioproducts LLC, WA, USA) pre-moistened with PBS (pH 7.4) (BD Difco; Franklin Lakes, NJ, USA). For each sampling point, an area of approximately 100 cm² was swabbed. Millstream fraction samples were collected by aseptically scooping material into sterile tubes or sterile stomacher bags. All samples were maintained at 4 °C and processed for DNA extraction within 24 h of collection.

gDNA extractions were performed on the samples using DNeasy® Blood and Tissue Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol with minor modifications to accommodate extended cell lysis (1h). Absorbance (Abs) at 260, 230, and 280 nm, using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), was used to assess the DNA concentration and purity of the extracts. Furthermore, Qubit 4.0 Fluorometer (Thermo Fisher Scientific) was used to quantify DNA concentration, and the samples were normalized to 5 ng/μL.

3.2.3. Sequencing and library preparation

Bacterial community profiles were generated by amplifying the V4 hypervariable region of the 16S rRNA gene using degenerate primers (515F/806R) with Illumina overhangs (Caporaso et al., 2012). The V4 region is a ~254-bp region that spans a broad taxonomic breadth across Archaea and Bacteria and has been used in the Earth Microbiome Project (EMP) (Thompson et al., 2017). Additionally, the short amplicon length is compatible with short read paired-end sequencing and facilitates taxonomic assignments and community-level comparisons (Hu et al., 2025). The V4 region was PCR amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for

bacterial community profiling. Amplicon library preparation (including index addition and clean-up steps) followed the manufacturer's recommended workflow described in the Illumina 16S Metagenomic Sequencing Demonstrated Protocol (Illumina). A total of 35 PCR cycles (27 cycles for amplicon and 8 cycles for indexing) were used, with an annealing temperature of 55°C. Because the wheat milling dataset represents a low-biomass setting, contamination assessment was further evaluated using both DNA extraction blank and no-template controls (NTCs). Extraction blanks and NTC libraries were handled alongside biological samples, and the results were illustrated using post-filter-retained reads, assigned-read proportions, and the most common control-associated genera across different pipeline-database combinations. The taxa associated with control and recurring in blanks and NTCs were considered as contamination background candidates, and their corresponding signal was removed during the downstream pre-processing before ecological interpretation of the biological samples.

Resulting libraries were assessed for size distribution and quality using a DNA 7500 chip on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Libraries were then pooled in equimolar amounts. The final pooled library (including the negative control) was sequenced on the Illumina MiSeq i1000 Plus using a 25 million-cycle, 600-cycle kit (paired-end sequencing). Sample preparation was performed according to the 16s Metagenomic Sequencing Demonstrated Protocol recommended by Illumina.

3.2.4. Bioinformatic analyses

The same set of demultiplexed FASTQ files was used for all runs to ensure homogeneity across procedures. Unless otherwise indicated (**Table 3.1**), the default parameters were used to conduct each workflow. Primers were removed during pre-processing prior to denoising or clustering using standard primer trimming during demultiplexing and quality filtering (**Figure**

3.1). Eight amplicon-processing pipelines were evaluated: DADA2, Deblur, MED, mothur, rDADA2, UCLUST, UNOISE3, and UPARSE, since the choice of amplicon-processing pipeline influences feature inference and diversity estimation. These eight were selected because they represent widely adopted and well-documented approaches spanning both major paradigms in amplicon processing, such as single-nucleotide resolved denoising/sequence-variant inference (DADA2, Deblur, UNOISE3) and traditional OTU clustering workflows (UPARSE/UCLUST and mothur), which enabled comparison across methods known to differ in their error-handling assumptions, feature definitions (ASVs, ZOTUs vs OTUs), and downstream diversity outputs (Callahan et al., 2016; Nagai et al., 2024; Odom et al., 2023). The interpretability and comparability of inferred features across studies are also determined by subsequent taxonomic assignment; hence, four reference databases were evaluated: SILVA, Greengenes2, GTDB (version r220), and the BLAST reference database. These databases were selected because they are widely used but also differ substantially in underlying curation and source material. Evaluating taxonomic assignment across multiple databases is important because database-specific differences in naming conventions, taxonomy structure, update frequency, and reference coverage can change which label a sequence receives and at what rank, which in turn alters reported community composition and diversity (Almeida et al., 2018; McDonald et al., 2024; Parks et al., 2022).

3.2.4.1. Sequence aggregation method

Each pipeline was run independently to generate (i) a feature table and (ii) representative sequences for taxonomy assignment (**Figure 3.1**). Pipelines were grouped into two feature inference paradigms: (i) operational taxonomic units (OTUs) clustering methods, and (ii) variant-based methods, since they vary in the way features are defined. OTU-based pipelines reduce

complexity by clustering similar sequences using a similarity threshold, whereas variant-based processes aim to recover true biological sequence variants by distinguishing true differences from sequencing noise. Both paradigms were compared as they generate distinct feature tables from identical FASTQ files.

(i) OTU clustering methods: By grouping reads into OTUs based on a predetermined identity criterion (97% or 99%), feature complexity can be decreased. Reads were quality-filtered and trimmed; paired reads were merged where applicable and then clustered into OTUs at 97% identity (de novo) using the QIIME 2 vsearch plugin. The OTU processing pipelines UCLUST, UPARSE, and MOTHUR were assessed in this study.

(ii) Variant-based methods: These workflows help in determining denoised sequence characteristics that preserve genuine biological variation while suppressing variance caused by sequencing errors. The outputs were reported as ASVs or SVs (DADA2, Deblur, rDADA2), ZOTUs (UNOISE3), and MED nodes (MED). For consistency, all variant-based feature outputs are referred to collectively as ASVs in the remainder of the manuscript, regardless of whether the original pipeline reported them as ASVs, SVs, ZOTUs, or MED nodes.

Pre-processing stages within each pipeline (read filtering, trimming, paired-end merging, dereplication, and chimera detection) were executed using default settings unless otherwise indicated (**Table 3.1**). The parameters for feature inference and clustering (ASV, SV, ZOTU inference parameters, OTU identity thresholds) are detailed in **Table 3.2**. The pipeline retained its original features to preserve process functionality.

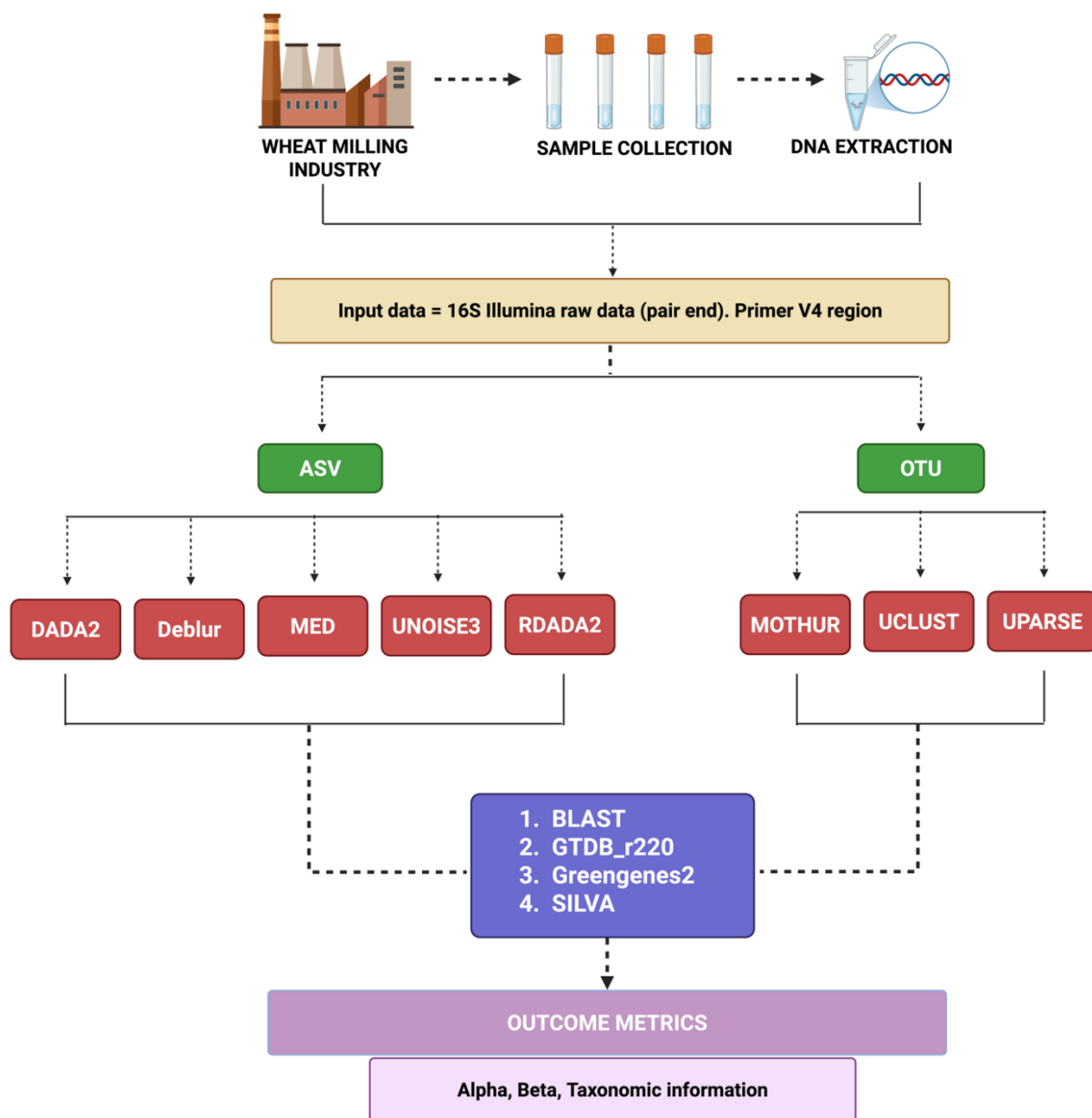


Figure 3.1: Overview of the bioinformatic benchmarking workflow used to evaluate 32 pipeline-database combinations for 16S rRNA gene analysis of wheat milling microbiota. Eight amplicon-processing pipelines were compared, including five variant-based methods and three OTU-based methods, and each was combined with four reference resources: SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, and a BLAST 16S reference database.

Table 3.1: Read pre-processing settings for pipelines

Pipeline	Software version	orPrimer removal	Trim/trunc (F/R or fixed)	Quality filter	Merge	Chimera method
DADA2	QIIME2 2024.2	Yes	240/200	maxEE default	default	consensus
rDADA2	DADA2 1.26	Yes	240/200	maxEE=2	default	consensus
Deblur	QIIME2 2024.2	Yes	150	default	n/a	built-in
UNOISE3	USEARCH proxy	Yes	fixed	default	yes	built-in
MED	MED proxy	Yes	fixed	default	yes	n/a
UCLUST	<i>vsearch</i>	Yes	fixed	default	yes	uchime
UPARSE	<i>vsearch</i>	Yes	fixed	default	yes	uchime
mothur	<i>vsearch proxy</i>	Yes	fixed	default	yes	uchime

DADA2: Divisive Amplicon Denoising Algorithm 2; UCLUST: Ultra-fast Clustering; UPARSE: Ultra-fast PARSE; MED: Minimum Entropy Decomposition; trim/trunc (F/R): trimming/truncation lengths for forward (F) and reverse (R) reads; maxEE: maximum expected errors allowed per read; QIIME: Quantitative Insights Into Microbial Ecology v2

Table 3.2: Feature inference or clustering controls across pipelines

Paradigm	Pipeline	Feature type	Identity threshold	Key inference parameters
Variant based	DADA2	ASVs	N/A	trunc-len-f=240; trunc-len-r=200; max-ee-f/r=default; pooling=Independent
Variant based	rDADA2	ASVs	N/A	truncLen=c(240,200); maxEE=c(2,2); pool=FALSE; chimera=consensus
Variant based	Deblur	SVs	N/A	trim length=150
Variant based	UNOISE3	ZOTUs	N/A	(α) alpha=default; minsize=8
Variant-based	MED	MED nodes	N/A	M=100
OTU based	UCLUST	OTUs	97	Identity=97%; de novo
OTU based	UPARSE	OTUs	97	Identity=97%; de novo
OTU based	mothur	OTUs	97	Average neighbor identity=97%; de novo (proxy),

N/A: Not applicable; OTU: Operational Taxonomic Unit; ASVs: Amplicon Sequence Variants; ZOTUs: Zero-radius OTU; SVs: *Sequence Variants*; DADA2: Divisive Amplicon Denoising Algorithm 2; UCLUST: Ultra-fast Clustering; UPARSE: Ultra-fast PARSE; MED: Minimum Entropy Decomposition; maxEE: maximum expected errors allowed per read.; trim/trunc (F/R): trimming/truncation lengths for forward (F) and reverse (R) reads.

3.2.4.2 Reference databases and taxonomic assignment

The SILVA database is a widely used, comprehensive repository of ribosomal RNA sequences. SILVA encompasses Bacteria, Archaea, and Eukaryota, enabling broad coverage. Greengenes2 (2022) is curated specifically for bacterial and archaeal 16S rRNA sequences and therefore requires minimal filtering. GTDB r220 is a database constructed from whole-genome data, filtered for Archaea and Bacteria. We used SILVA v138 SSU NR99 (16S) for the analysis and filtered the dataset to retain Bacteria and Archaea, excluding Eukaryotes. In addition, non-target organelle sequences, such as chloroplast and mitochondrial sequences, were removed. Post filtering, the final SILVA reference contained approximately 430,000 sequences derived from the filtered FASTQ used to train/classify, and taxonomic assignment was performed using a pretrained Naive Bayes classifier with the default confidence cut-off (0.7). Likewise, Greengenes2 (2022) and GTDB r220 were used with pretrained classifiers under a Bacteria+Archaea scope, yielding reference FASTA sequences of ~331,000 (Greengenes2) and 317,000 (GTDB) sequences post-filtering, respectively. The BLAST reference database, comprising bacterial 16S reference sequences, contained ~400,000 sequences and was formatted for nucleotide alignment using *makeblastdb*. Representative sequences produced by each pipeline were annotated against each reference resource, yielding 32 pipelines $\times$ database combinations (**Table 3.2**).

Taxonomy was assigned using a consensus BLAST approach with thresholds of $\geq 97\%$ identity, ≥ 100 bp minimum alignment length, and $E\text{-value} \leq 1e-20$. Taxonomic outputs were summarized at the phylum, family, and genus ranks. Assignments were propagated upwards via lineage inheritance such that features assigned at a lower rank (genus) were also considered assigned at all higher ranks (family, phylum). Reads that did not meet the specified criteria for a rank-resolved label were recorded as unclassified or unknown at that rank. Each combination

is referred to using a standard convention: Pipeline_Database: (e.g., *DADA2_GTDB_r220*; *UNOISE3_SILVA*).

3.2.5. Benchmarking metrics for pipeline filtering and down selection

We used a tiered quality and interpretability framework to select 10 workflows from 32 combinations, based on read-level quality retention and taxonomic interpretability metrics derived from each workflow's feature table, taxonomy assignments, and QC summaries. For each method, input reads were recorded as demultiplexed paired-end read counts per sample (MiSeq 2×300) and reads retained after quality filtering and denoising/clustering were recorded. Taxonomic assignment depth was summarized as reads assigned at phylum, family, and genus (mean ± SD) across, and the complementary Unclassified reads (%) at each rank. Community complexity was captured as the total number of distinct taxa observed across all samples at each rank (No. of phylum/family/genus). Chimera control was assessed using the workflow-reported Chimera rate (%). Finally, an integrated signal-to-noise ratio (S/N) was calculated to jointly penalize ambiguous classification and chimeric artifacts (**Eq. 1**), defined as:

$$\frac{S}{N} = \frac{\overline{\text{Genus assigned reads}}}{\text{unknown} + \text{chimera} + \epsilon} \quad (1)$$

where ϵ is a small constant to avoid division by zero; higher S/N indicates greater genus-level signal relative to noise. To shortlist analytical workflows, signal-to-noise ratio, unknown rate, and chimera rate were combined into a heuristic composite score (0-100) after min-max normalization. Signal to noise ratio was assigned the highest weight (0.50) because it corroborated with taxonomic discrimination power, whereas unknown assignment rate and chimera rate were each assigned a penalty weight of 0.25 as it can dispose the results to be less interpretable and inflate false diversity in the samples (**Eq. 2**). Additionally, to evaluate workflow performance without imposing a prior importance or bias on any single metric, an unweighted composite score was also calculated as the mean of signal to noise ratio, inverse

unknown rate and inverse chimera rate without weight (**Eq. 3**). This provided a complementary ranking in which all quality criteria contributed equally. Workflow-database combinations were ranked according to the weighted score and the top 10 workflows were retained for downstream analyses. Notably, both the weighted and unweighted scoring approaches yielded the same top 10 shortlisted workflows.

The weighted score approach and unweighted scoring was calculated as following.

$$\text{Weighted Score} = 100 \left[0.5 \left(\frac{S}{N} \right) + 0.25(1 - \text{Unknown rates}) + 0.25(1 - \text{Chimera rate}) \right] \quad (2)$$

$$\text{Unweighted Score} = 100 \left[\left(\frac{S}{N} \right) + (1 - \text{Unknown rates}) + (1 - \text{Chimera rate}) \right] \quad (3)$$

3.2.6. Diversity analyses

Feature tables were rarefied prior to the calculation of alpha diversity and presence/absence-based beta diversity metrics to control for unequal sequencing depth. For abundance-based analyses (Bray-Curtis dissimilarity, relative abundance summaries, and differential abundance testing), non-rarefied tables were normalized to relative abundances or analyzed using compositional methods.

3.2.6.1. Alpha diversity

Alpha diversity was quantified for each combination across three milling fractions using four complementary indices (Shannon diversity, Inverse Simpson diversity, Chao1 richness, and Fisher's alpha) calculated from feature count tables. Based on rarefaction curve inspection, 30,000 reads per sample were selected for primary rarefied analyses and were used for alpha diversity analysis. Shannon and Inverse Simpson were used to characterize diversity structure, incorporating richness and evenness, whereas Chao1 was included as a richness-

oriented estimator to facilitate direct comparison of the number of taxa recovered across workflows at the family and genus levels.

3.2.6.1.1. Shannon diversity

It quantifies the ecological diversity of the wheat mill by accounting for both taxonomic richness (the number of family/genus) and evenness (the relative abundance of family/genus).

$$H' = -\sum_{i=1}^S p_i \ln(p_i) \quad (4)$$

Where H' stands for Shannon diversity,

3.2.6.1.2. Inverse Simpson diversity

Inverse Simpson diversity emphasizes dominant features and is less sensitive to rare features.

$$\frac{1}{D} = \frac{1}{\sum_{i=1}^S p_i^2} \quad (5)$$

Where D stands for Simpson diversity; $D = \sum_{i=1}^S p_i^2$

3.2.6.1.3. Fisher's alpha

Fishers' alpha (α) is a diversity parameter derived from the log-series model of feature abundances and is estimated from the relationship between richness and sequencing depth.

$$S = \alpha \ln \left(1 + \frac{N}{\alpha} \right) \quad (6)$$

S denotes observed features (ASVs/OTUs) with counts n_i for the feature i , the total reads $N = \sum_{i=1}^S n_i$, α =Fisher's alpha parameter, and the relative abundance of feature i $p_i = n_i/N$.

3.2.6.1.4. Chao1 richness

The nonparametric Chao1 index estimates total species richness with an equation that generates a ratio of the number of singletons squared divided by the number of doubletons multiplied by two. This value is then added to the number of observed species to generate a species richness prediction (Deng et al., 2024)

$$S_{Chao1} = S_{obs} + \frac{f_1^2}{2f_2} \quad (7)$$

S_{obs} denotes the total number of species observed, f_1 is the number of singletons, and f_2 is the number of doubletons

3.2.6.2. Beta diversity

To reduce detection bias due to unequal sequencing depth, beta diversity was assessed using both presence- and absence-based, as well as abundance-based, dissimilarity metrics. Specifically, Jaccard distances were computed from rarefied tables (30,000 reads/sample) after converting to presence/absence using a $\geq 0.1\%$ relative abundance threshold. Additionally, a sensitivity test was also carried out with a threshold of 0.01% relative abundance as this threshold will account for the low- abundance taxa. Their corresponding effect on the family and genus level beta diversity as well as ASV-OTU differences were also evaluated. Bray–Curtis distances were computed from non-rarefied tables normalized to relative abundance. Pairwise Mantel tests were used to compare workflow-specific beta-diversity structures directly across pipeline×database combinations by calculating correlations between different workflow distance matrices. These analyses were conducted separately for the family and genus level profiles by using either (i) *Jaccard dissimilarity matrix* (presence or absence) and (ii) a *Bray-Curtis dissimilarity matrix* (abundance-weighted). The resulting Mantel correlation coefficients were visualized as clustered heatmaps, and the corresponding p-values are provided in **Supplementary Figure S3.2**. For compositional analyses based on centered log-ratio (CLR) transformed data, Euclidean distance was used, corresponding to Aitchison distance. CLR transformation was carried out using *phyloseq* package in R (RStudio v2022. 12. 0+353).

3.2.7 Statistical analyses

All statistical analyses were performed in R (RStudio). Alpha diversity (Chao1, Shannon, inverse Simpson, and Fisher’s alpha) was calculated from rarefied tables (30,000 reads per sample) and analyzed using linear mixed-effects models with pipeline and database as fixed

effects. Tukey-adjusted pairwise comparisons were used for post hoc testing. Beta diversity was assessed using Jaccard dissimilarity (rarefied presence–absence) and Bray–Curtis dissimilarity (relative abundance or centered log-ratio [CLR]-transformed data). Differences in community composition were tested using PERMANOVA (adonis2; 9,999 permutations), and homogeneity of dispersion was evaluated using PERMDISP (betadisper). Genus-level differences in taxonomic composition were evaluated using ANOVA on CLR-transformed abundances, with p-values adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR). Core wheat taxa (defined as the cumulative abundance of the top 10 genera per method) and signal-to-noise ratios were compared across workflows and correlated with chimera and unknown rates using Spearman correlation. Comparisons between ASV- and OTU-based approaches (including taxonomic resolution and diversity metrics at genus and family levels) were performed using Welch’s t-tests. Unless otherwise stated, multiple comparisons were controlled using the Benjamini–Hochberg FDR, and a significance threshold of $p < 0.05$ was applied.

3.3. Results and discussion

3.3.1 Weighted-score-based identification of top-performing bioinformatic workflows

LMF processing environments, such as wheat-milling facilities, remain under-characterized using culture-independent approaches; consequently, no consensus or validated 16S rRNA gene bioinformatics workflow has been established for LMF systems. The lack of methodological standardization has significant implications; previous benchmarking studies in clinical and environmental contexts have shown that the selection of sequence aggregation methods, taxonomic reference databases, and quality-filtering parameters can influence diversity estimates, differential abundance, and ecological inferences, potentially impacting biological conclusions (Abellan-schneyder et al., 2021; Caruso et al., 2019; Lloréns-Rico et al.,

2021; Nearing et al., 2022). However, it remains unclear whether such method-dependent differences translate to low-moisture grain-processing environments, which include microbial diversity with plant-associated endotypes (Makar et al., 2021), soil-derived taxa (Solanki et al., 2021), and environment-adapted bacteria (Magallanes López & Simsek, 2021; Rivera et al., 2024). To explore this, we tested 32 method-database pairs across eight widely used bioinformatic pipelines (DADA2, UCLUST, Deblur, UNOISE3, RDADA2, MED, MOTHUR, and UPARSE) combined with four reference databases (SILVA, BLAST, Greenegenes2, and GTDB r220) (**Table 3.3**). These were applied to 160 samples collected from FCS, NFCS and milling streams at a commercial mill in Kansas, United States. To systematically compare these pipelines and streamline downstream analysis to a select few effective workflows, we implemented a weighted scoring system to rank all 32 pipeline-database combinations and select the top ten workflows for detailed evaluation. This heuristic composite score, ranging from 0 to 100, integrates multiple quality and interpretability metrics, rewarding pipelines with greater taxonomic assignment depth and accurate representation of core wheat-associated genera, while penalizing elevated rates of unclassified taxa and chimeric sequences. Similar multi-metric composite indices have been employed in 16S rRNA benchmarking studies to evaluate bioinformatics pipelines across different datasets, balancing sensitivity, specificity, and error profiles within a unified ranking system (Caruso et al., 2019; Prodan et al., 2020).

Table 3.3: Weighted-score metrics used to identify top-performing bioinformatic workflows

Method	Pipeline	Database	S/ N	Unkno wn rates	Input read	Reads after filtering/denoi sing	Reads assigned at genus (mean±SD)	Unclassifi ed reads at genus % (mean±S D)	Chime ra rate %	Weight ed scores	Unweight ed scores
DADA2_BLAST	DADA2	BLAST	12. 1	0.04	79173±11 380	74583±11012	74162±11 037	9.6±1.6	2.09	78.9	82.2
DADA2_GTDB_r22 0	DADA2	GTDB_r2 20	6.9	0.10	79074±12 205	70831±11134	70374±11 168	10.2±2.2	2.66	46.3	53.2
DADA2_Greengene s2	DADA2	Greengen es2	7.3	0.08	78149±13 338	69512±11871	69063±11 894	3.5±2.9	3.38	46.2	51.9
DADA2_SILVA	DADA2	SILVA	15. 8	0.01	80649±12 684	74827±12061	74418±12 103	9.4±2.5	3.11	92	89.3
Deblur_BLAST	Deblur	BLAST	8.0	0.08	79142±99 88	70542±9076	70081±90 98	4.0±1.6	2.32	55.5	62.3
Deblur_GTDB_r22 0	Deblur	GTDB_r2 20	5.4	0.14	78587±11 996	67611±10402	67133±10 438	6.1±2.8	3.12	32.3	38.7
Deblur_Greengenes 2	Deblur	Greengen es2	5.6	0.12	78612±10 909	68402±9733	67926±97 58	8.5±2.6	3.13	35.6	42.5
Deblur_SILVA	Deblur	SILVA	8.5	0.07	79662±12 647	73491±11703	73022±11 746	6.6±1.1	2.41	58.2	64.6

MED_BLAST	MED	BLAST	5.6	0.11	80116±10991	69112±9224	68602±9258	7.7±0.7	4.39	29.3	34.1
MED_GTDB_r220	MED	GTDB_r220	4.2	0.17	79642±12395	67281±10821	66764±10867	10.0±2.1	5.94	6.7	7.8
MED_Greengenes2	MED	Greengenes2	4.7	0.15	81367±11286	69701±9831	69182±9875	9.4±1.4	3.48	26	32.2
MED_SILVA	MED	SILVA	5.7	0.10	79815±12624	70782±10994	70261±11042	11.3±2.7	5.07	26.9	30.6
MOTHUR_BLAST	MOTHUR	BLAST	6.0	0.10	81329±12046	72521±10701	71998±10748	9.6±1.0	4.33	32.6	37.3
MOTHUR_GTDB_r220	MOTHUR	GTDB_r220	4.4	0.16	79702±12142	65841±10437	65312±10488	6.9±0.6	5.19	13.2	16
MOTHUR_Greengenes2	MOTHUR	Greengenes2	4.8	0.14	80129±12077	69503±10741	68972±10789	4.1±2.2	4.57	21.1	25.4
MOTHUR_SILVA	MOTHUR	SILVA	6.1	0.09	80111±12649	71233±11142	70698±11191	9.3±2.9	5.16	29.3	32.6
RDADA2_BLAST	RDADA2	BLAST	4.6	0.15	80263±13207	64912±10621	64382±10673	5.6±1.4	5.71	12.2	14.1
RDADA2_GTDB_r220	RDADA2	GTDB_r220	3.8	0.21	81129±11574	63911±9043	63381±9092	6.8±1.0	5.54	2.4	3.2
RDADA2_Greengenes2	RDADA2	Greengenes2	4.0	0.19	79758±13247	63284±10564	62741±10608	3.9±1.7	4.43	12.4	16

RDADA2_SILVA	RDAD A2	SILVA	4.9	0.14	80290±12 584	67602±10936	67071±10 988	4.7±2.2	4.90	19.6	23
UCLUST_BLAST	UCLUS T	BLAST	9.5	0.05	79716±12 938	72174±11894	71736±11 941	6.5±2.6	3.23	60	64.2
UCLUST_GTDB_r 220	UCLUS T	GTDB_r2 20	6.3	0.11	81311±11 387	73211±10803	72771±10 852	8.9±1.3	3.29	38.8	44.8
UCLUST_Greenge nes2	UCLUS T	Greengen es2	6.9	0.09	82009±11 537	74186±10664	73742±10 712	10.0±2.5	3.28	43.9	49.9
UCLUST_SILVA	UCLUS T	SILVA	12. 4	0.04	79975±13 575	74368±12974	73911±13 023	6.1±1.2	1.77	82.1	85.6
UNOISE3_BLAST	UNOIS E3	BLAST	7.0	0.09	80888±11 075	70721±9716	70283±97 62	8.7±0.8	3.13	21.0	51.4
UNOISE3_GTDB_r 220	UNOIS E3	GTDB_r2 20	5.2	0.15	79020±12 551	67602±10752	67161±10 803	4.5±0.5	2.78	15.2	39.1
UNOISE3_Greenge nes2	UNOIS E3	Greengen es2	5.2	0.13	80228±12 392	69152±10661	68692±10 708	9.7±2.2	4.28	11.9	30.5
UNOISE3_SILVA	UNOIS E3	SILVA	7.5	0.08	79620±11 401	72038±10163	71579±10 211	8.9±2.5	3.26	23.5	53.4
UPARSE_BLAST	UPARS E	BLAST	5.3	0.13	78620±12 245	65502±10311	64971±10 358	6.8±1.9	3.76	13.5	34.9
UPARSE_GTDB_r 220	UPARS E	GTDB_r2 20	3.9	0.19	79423±11 733	65941±9804	65412±98 48	4.0±0.8	4.90	5.3	11.9

UPARSE_Greengenes2	UPARSE	Greengenes2	4.3	0.17	82354±11779	69482±9935	68941±9982	8.7±1.7	4.39	8.0	20.4
UPARSE_SILVA	UPARSE	SILVA	5.9	0.12	78811±11360	68621±9888	68082±9934	7.8±2.4	3.03	17.2	44.1

S/N (signal to noise): (mean genus assignment)/(unknown+chimera+ small constant); **Unknown rates:** Approximate fraction of higher-rank taxonomic label remaining “Unassigned”; **Input reads:** Raw reads per sample, typical for MiSeq 2×250 sequencing after demultiplexing; **Reads after filtering/denoising:** Retained reads per sample following quality filtering and denoising, values depend on the bioinformatic pipeline and database tier; **Reads assigned at genus (mean ± SD):** Retained reads multiplied by the percentage of abundance assigned (i.e., not Unassigned) at the respective rank; **Unclassified reads at genus % (mean ± SD):** Calculated as 100–assigned percentage at the respective rank; **Chimera rate (%):** Proportion of reads identified as chimeric; expected to vary by denoising or clustering method; and **Weighted scores:** Heuristic composite score (range 0–100) that rewards higher assignment rates and dominance of core taxa, and penalizes higher unknown and chimera rates; **Unweighted score:** Heuristic composite score (range 0–100) that rewards all metrics equally.

A worked example for DADA_BLAST for the ranking approach

Let us take DADA_BLAST; S/N= 12.1; Unknown rate= 0.04; Chimera= 2.09;

$$(1) \text{ Normalization} = \frac{12.1-3.8}{15.8-3.8} = \mathbf{0.69}; \text{ Unknown rate} = \frac{0.04-0.01}{0.21-0.01} = \mathbf{0.15}; \text{ Chimera rate} = \frac{2.09-1.77}{5.94-1.77} = \mathbf{0.077}$$

$$(2) \text{ Weighted Score} = 100 [0.5(0.69) + 0.25(1 - 0.15) + 0.25(1 - 0.077)] = \mathbf{78.825}$$

$$(3) \text{ Unweighted Score} = 100 [(0.69) + (1 - 0.15) + (1 - 0.077)] = \mathbf{82.2}$$

Weighted scores ranged from 2.4 to 92 across 32 combinations (**Table 3.3**), indicating approximately a 24-fold difference in the effectiveness of standard workflows for extracting taxonomic signals from identical sequence data. The top-performing group included DADA2-SILVA (92), UCLUST-SILVA (82.1), DADA2_BLAST (78.9), UCLUST_BLAST (60), Deblur-SILVA (58.2), Deblur_BLAST (55.5), UNOISE_SILVA (47.7), and DADA2_GTDB_r220 (46.3), with DADA2_Greengenes2 and S1UNOISE_BLAST scoring 46.2 and 45.2, respectively (**Table 3.3**). Across workflows, high-performing combinations consistently exhibited three characteristics critical for reliable 16S rRNA gene profiling: (i) effective retention of quality-filtered reads (up to 92.6% of input sequences assigned to bacterial taxa), (ii) low levels of genus-level unclassified reads (3.8-10.7%), and (iii) low chimera rates consistent with benchmark values for well-optimized DADA2, UNOISE3, and UPARSE workflows. In contrast, several MED, MOTHUR, and RDADA2-based methods scored lower due to higher unknown rates (up to 21%) and chimeric fractions exceeding 5%, despite similar input read counts. These observations are consistent with prior benchmarking studies that demonstrate that database selection and error-model configuration substantially influence taxonomic assignment accuracy, clustering behavior, and downstream ecological inference (Edgar, 2016; Molano et al., 2024; Tikhonov et al., 2014). Importantly, although absolute scores varied across workflows, the relative ranking of high-performing pipelines was stable across milling stages, supporting the robustness of the weighted scoring framework.

3.3.1.1 Negative control profiling and contamination handling

Extraction blanks and NTCs saw non-zero read counts after filtering across all workflow combinations, reflecting a measurable low-biomass background signal. Control profiles across methods, however, were consistently composed of a limited number of recurrent genera commonly identified with reagent or kit background in low-biomass studies, such as *Ralstonia*, *Sphingomonas*, *Cutibacterium*, *Methylobacterium*, *Pseudomonas*, *Acinetobacter*,

and *Stenotrophomonas*. While the principal control genus and the total control burden shifted slightly among the workflow combinations, these taxa were consistently more abundant in the blank and NTC libraries than in the biological samples. Thus, the control-associated signal was considered during data preprocessing to make the final biological interpretation. The measurement of background contamination was expected for a low-biomass dataset, and it is appropriate that the main diversity and taxonomic comparisons presented here came from the contamination-controlled dataset rather than from the raw control-associated signal. Detailed workflow-level summaries of retained blank and NTC reads, dominant control genera, and overall contamination burden are provided in **Supplementary Figure S3.5**

3.3.2 Impact of bioinformatic pipeline choice on family- and genus-level taxonomic identification

All 10 top-performing workflows showed strong concordance in family-level community composition and consistently identified 22 bacterial families in wheat-milling samples (family- and genus-level taxonomic identification across 32 pipelines; **Supplementary Figure S3.6**). This taxonomic agreement, largely independent of pipeline or reference database, reflects the evolutionary conservation and balanced variability in the 16S rRNA V4 region used for taxonomy. This amplicon provides sufficient phylogenetic signal for reliable family-level classification across diverse bacterial communities. Consistent with this observation, Liu et al. (2020) reported that the V4 region produced the most accurate and consistent taxonomic profiles among the other amplicon strategies tested, including V3-V4 for a defined sample. The five most common families across all samples were almost always *Aerococcaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, and *Leuconostocaceae* (**Figure 3.2a**). Similarly, the total number of detected genera ranged from 131 to 132 depending on the database used, reflecting minor annotation differences rather than biological discrepancies. The top five genera identified across workflows were *Aerococcus*, *Arthrobacter*,

Clostridium, *Corynebacterium*, and *Lactococcus* (**Figure 3.2b**). Together, these families and genera represent the core bacterial microbiota associated with wheat, although their relative abundances differ. The percentage of unassigned sequences at the family level ranged from 27.02% (DADA2_Silva, lowest) to 31.72% (UNOISE_Silva, highest). At the genus level, this ranged from 24.42% (UNOISE_BLAST, lowest) to 27.78% (Deblur_SILVA, highest) (**Table 3.2**). No significant differences ($p > 0.01$) in the proportion of unassigned sequences among the ten methods were observed for either family or genus classification. The narrow variation in abundance values for families and genera across methods is mainly due to the chosen taxonomic classification method, which can significantly influence the results (Marizzoni et al., 2020). As noted by Shah et al. (2019), the intrinsic resolution limits of the 16S rRNA gene and incompleteness or bias in reference databases can restrict confident genus-level assignment, resulting in a substantial fraction of reads remaining unclassified across most pipelines. Taken together, our results indicate that while family-level taxonomic structure is highly robust to choices in the bioinformatic workflow, genus-level resolution remains constrained by limitations in marker and reference databases rather than by pipeline choice alone. Overall, **Figure 3.2** provides a qualitative overview of the 10 most dominant taxa recovered across workflows; however, inferential statistical comparisons of method-dependent differences are provided in the subsequent alpha- and beta-diversity analyses (Section 3.3.3).

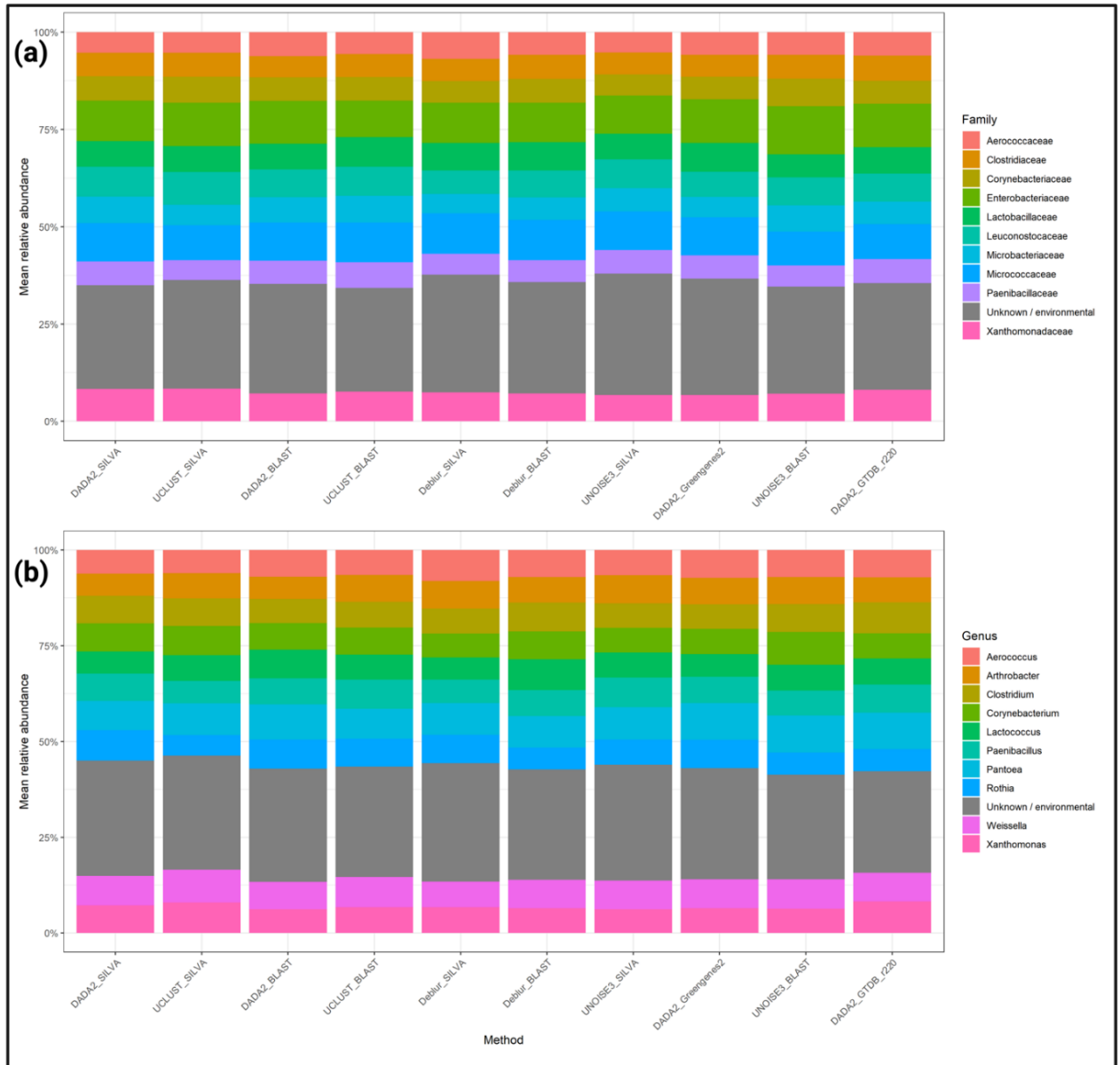


Figure 3.2: Descriptive overview of the impact of 16S rRNA analysis methods on the taxonomic characterization of low-moisture grain processing microbiota. Stacked bar plots represent the mean relative abundance of the 10 most abundant bacterial (a) families and (b) genera identified across various bioinformatics pipelines (x-axis). Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow. This figure is intended for qualitative comparison of averaged profiles; formal statistical testing of workflow effects is presented in Figures 3.3 and 3.4.

3.3.3 Bacterial Alpha and Beta Diversity Patterns Across Bioinformatic Workflows

3.3.3.1 Alpha diversity

Alpha diversity was useful for assessing the richness and evenness of the microbiota in pre- and post-milling environments and in millstream fractions. Alpha diversity values reported here represent pooled analyses across all milling stages to evaluate workflow-dependent variability independent of biological grouping. Alpha-diversity metrics, including Chao1, Shannon diversity, Simpson's inverse, and Fisher's alpha, were used to evaluate the effect of the bioinformatics workflow on diversity (**Diversity metrics across 32 workflows is provided in the supplementary figure S3.4**). At the family level, a stable community diversity signal was observed across workflows (**Figure 3.3**). Chao1 richness ranged from 22.97 ± 2.20 in UCLUST_BLAST to 24.92 ± 2.04 in DADA2_SILVA. Shannon diversity ranged from 2.59 ± 0.19 (UCLUST_BLAST) to 2.74 ± 0.18 (DADA2_SILVA), while the majority of the estimates for workflow combinations grouped around 2.6-2.7 (**Figure 3.3a**). The narrow range across workflows indicates that richness and evenness estimate at the family level were largely insensitive to processing strategy. Parallely, trends observed in complementary indices supported the same conclusion. InvSimpson values were constrained, ranging from 10.63 ± 1.25 (UCLUST_BLAST) to 11.27 ± 1.06 (UNOISE3_SILVA) (**Figure 3.3b**). Since InvSimpson is more sensitive to dominant taxa, the limited variation across workflows suggested that community evenness and dominance structure were preserved regardless of the processing strategy. Additionally, Fisher's alpha exhibited the narrowest range (2.21 ± 0.04 to 2.37 ± 0.31), which further corroborated that richness-associated inference at the family level was robust across all workflows (**Figure 3.3c**). However, UCLUST-based workflows, in particular UCLUST_BLAST and UCLUST_SILVA, yielded lower richness and evenness estimates

across most indices than the Deblur, DADA2, and UNOISE3 combinations. DADA2_SILVA consistently produced the highest estimates for Chao1, Shannon, and Fisher's alpha, while UNOISE3_SILVA gave the highest value for InvSimpson, indicating greater recovery of dominant taxon evenness. The relatively narrow workflow ranges observed for the family are consistent with the completeness of taxonomic assignments at higher taxonomic ranks. The ecological conclusions based on within-sample diversity are unlikely to be altered by workflow selection at the family level. The shortlisted workflows recovered a consistent diversity signal, with modest shifts in absolute magnitude across methods. The ecological conclusions based on within-sample diversity are unlikely to be altered by workflow selection at the family level. The shortlisted workflows recovered a consistent diversity signal, with modest shifts in absolute magnitude across methods. These observations were consistent with beta-diversity analyses, where PERMANOVA indicated no significant differences in community composition across workflows at the family level (PERMANOVA, $R^2 = 0.64$, $p = 0.001$), and dispersion among groups was not significantly different (PERMDISP, $p = 0.007$). While family-level alpha diversity provided an insight into within-sample diversity across workflows, the genus-level output offered better ecological resolution but was more sensitive to workflow-database differences (Campos et al., 2022; von Meijenfeldt et al., 2019).

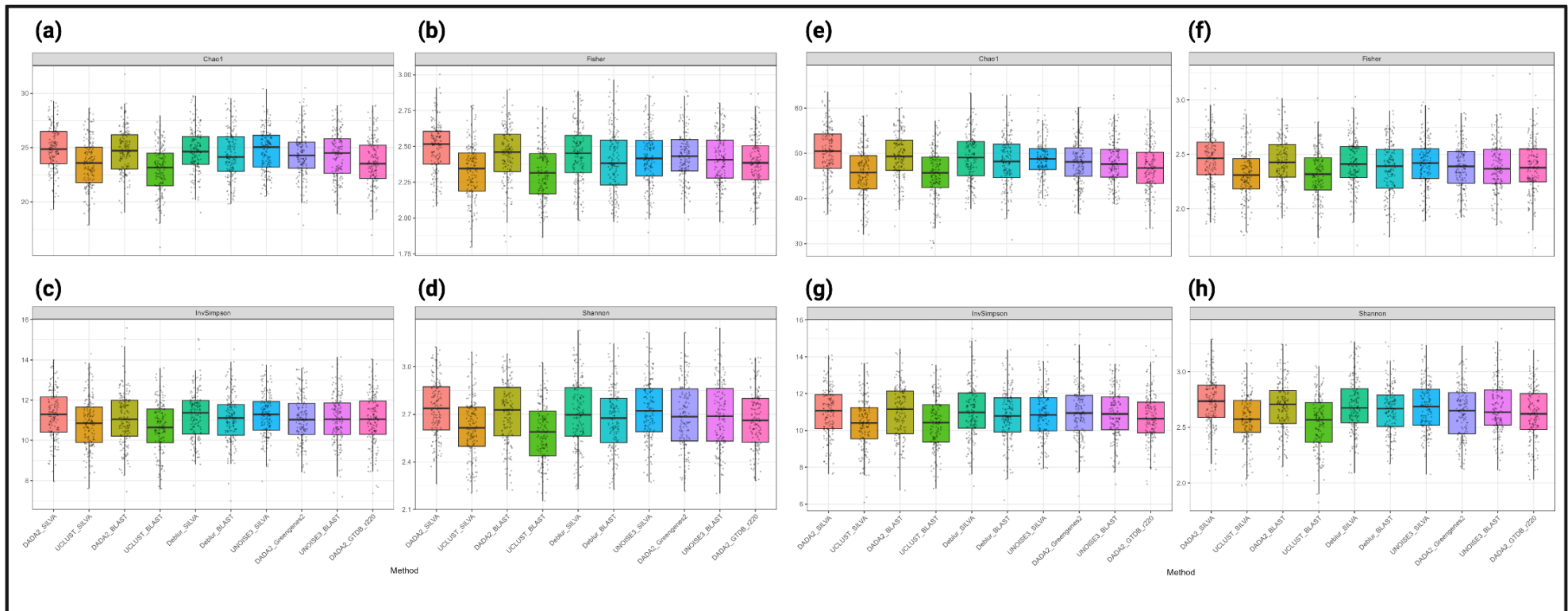


Figure 3.3: Alpha-diversity across bioinformatic workflow-database combinations at the family and genus levels. Metrics shown include Chao1 richness, Fisher's alpha, Inverse Simpson diversity, and Shannon diversity for the family level (a–d) and genus level (e–h). Values are presented as mean $\pm$ SD. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB was assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow

In contrast, at the genus level, alpha diversity was more sensitive to workflow and exhibited greater variation in both absolute magnitude and dispersion (**Figure 3.3e-h**). This is attributed to improved classification success at higher taxonomical ranks (Serrana et al., 2022; Smith et al., 2022). Chao1 richness ranged from 45.27 ± 5.68 in UCLUST_BLAST to 50.20 ± 5.64 in DADA2_SILVA, indicating modest but consistent differences in richness estimation across workflows. A similar pattern was observed in Shannon diversity, which ranged from 2.54 ± 0.24 (UCLUST_SILVA) to 2.73 ± 0.22 (DADA2_SILVA). This dispersion was more pronounced for InvSimpson, which ranged from 10.37 ± 1.4 (UCLUST_SILVA) to 11.03 ± 1.5 (Deblur_SILVA and DADA2_SILVA), indicating that the choice of workflow could influence evenness and dominance inference at lower ranks. Additionally, Fisher's Alpha also exhibited a similar trend with estimates ranging from 2.31 ± 0.22 (UCLUST_SILVA) to 2.44 ± 0.24 (DADA2_SILVA). It is to be noted that UCLUST_SILVA consistently yielded the lowest richness/evenness estimates across all the measured indices of alpha diversity, while DADA2_SILVA workflows tended to produce comparatively higher genus-level diversity. Among these, DADA2_SILVA yielded the highest Chao1, Shannon, and Fisher's alpha values, suggesting greater recovery of low-abundance and evenly distributed genera, while Deblur_SILVA produced the highest InvSimpson estimate, indicating slightly greater evenness among dominant taxa. Database effects were also more apparent at the genus level than at the family level. For example, within DADA2, Chao1 decreased from 50.20 ± 5.64 with SILVA to 46.90 ± 5.14 with GTDB r220, and Shannon decreased from 2.73 ± 0.22 to 2.62 ± 0.23 , indicating that reference database choice can influence fine-scale taxonomic resolution more strongly than at the family level. These differences were supported by beta-diversity analyses, where PERMANOVA detected significant variation in community composition across workflows and databases at the genus level (PERMANOVA, $R^2 = 0.69$, $p = 0.0001$),

while PERMDISP indicated that group dispersions were [not significantly/significantly] different ($p = 0.01$).

3.3.3.2 Beta Diversity

The distinction among community profiles of milling samples was assessed using Bray-Curtis dissimilarity (abundance weight) and Jaccard distance (presence and absence) at both the family and genus levels (**Figure 3.4a-d**) (**Pairwise Mantel comparisons across all 32 pipeline × reference-resource combinations is provided in supplementary figure S3.1 and S3.2**). Consistent with the alpha-diversity observation, the effect of workflow choice depended on taxonomic rank. Family-level distances for beta diversity were more conserved than genus-level distances, which were higher and more dispersed. Mean Bray-Curtis dissimilarity increased from 0.528-0.556 at the family level to 0.799-0.820 at the genus level, while mean Jaccard dissimilarity increased from 0.557-0.582 to 0.792-0.807 indicating that finer taxonomic resolution at genus level captured substantially greater compositional difference. This systematic increase indicates that more compositional heterogeneity in the dataset was expressed at finer taxonomic resolution and became partially obscured when sequence data were collapsed to the family rank. At the family level, mean Bray-Curtis values ranged from 0.5276 in Deblur_BLAST to 0.5560 in DADA2_SILVA while family-level Jaccard ranged from 0.5574 in UNOISE3_BLAST to 0.5822 in DADA2_GTDB_r220. (**Figure 3.4b**). The minimal mean distances and overlapping of variability across all workflows indicated that the family rank is not sensitive to pipeline-database choices. Despite differences in processing and annotation, the workflows yielded similar beta-diversity estimates at the family level, suggesting that beta-diversity patterns at this rank are primarily driven by the underlying biological heterogeneity of the pooled samples rather than by the choice of bioinformatics pipeline (Shah et al., 2019b).

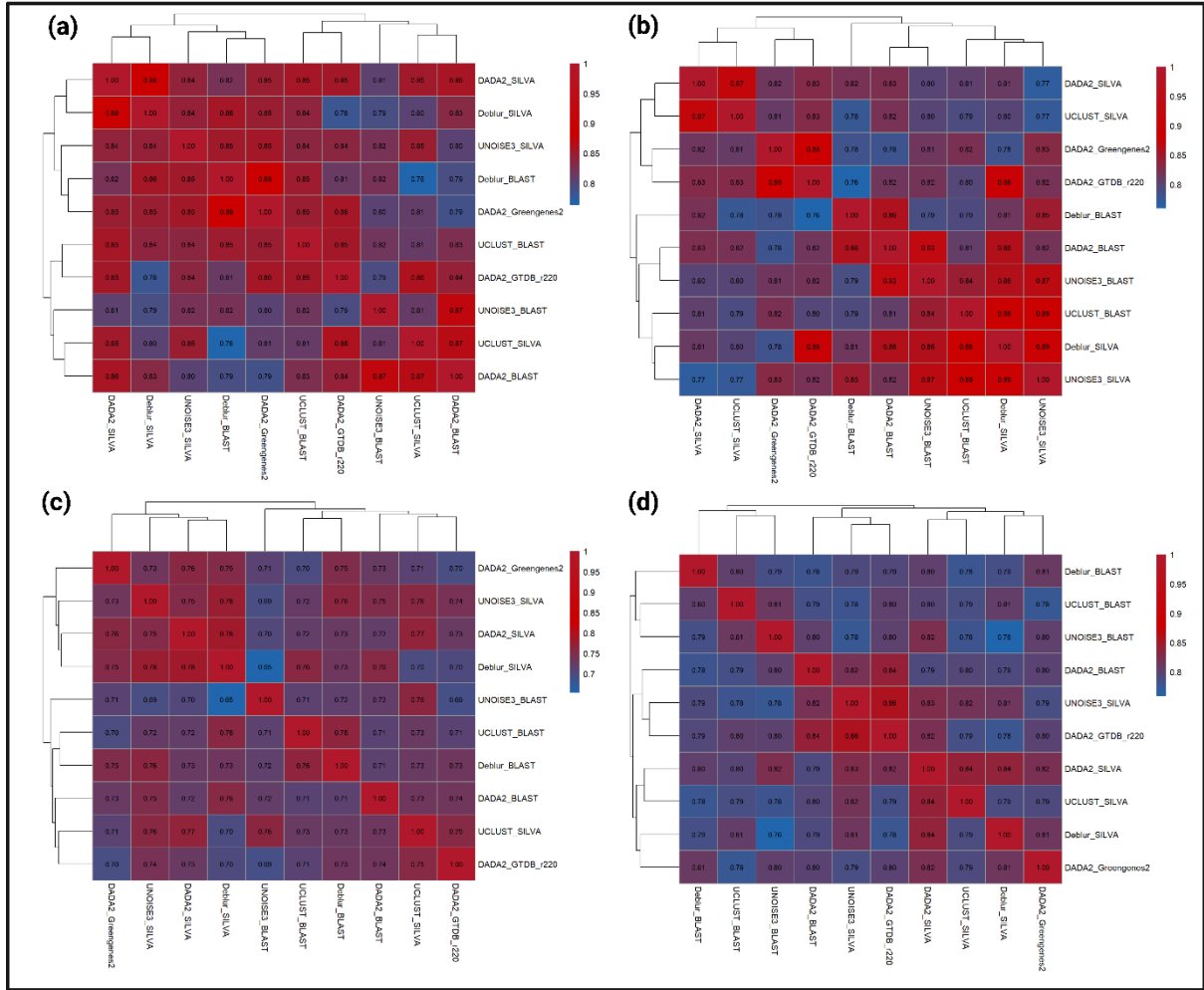


Figure 3.4: Beta-diversity concordance across the top 10 bioinformatic workflow combinations, assessed by Mantel correlation I between workflow-specific distance matrices. Heat maps show Bray–Curtis and Jaccard comparisons at the family level (a–b) and genus level (c–d). Higher Mantel r values indicate greater similarity in the sample-to-sample ecological structure recovered by two workflow combinations. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB was assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow

At the genus level, the strongest dissimilarity was generally observed for Deblur_SILVA for both Bray-Curtis (0.820) and Jaccard (0.807) with DADA2_GTDB_r220 and UNOISE3_SILVA also yielding higher values, whereas DADA2_BLAST and UNOISE3_BLAST were consistently conservative. At the genus level, both distance metrics (Bray-Curtis and Jaccard) increased and exhibited a wider spread across workflow combinations (**Figure 3.4c-d**). For Bray-Curtis, the strongest genus-level separation was produced by Deblur_SILVA (0.8196), followed by UNOISE3_SILVA (0.8131) and DADA2_GTDB_r220 (0.8117). The lowest abundance-based genus dissimilarities were obtained with DADA2_BLAST (0.7995) and UNOISE3_BLAST (0.8012). A similar trend appeared for Jaccard, where Deblur_SILVA (0.8066), DADA2_GTDB_r220 (0.8058) and UNOISE3_SILVA (0.8055) were among the highest, while UNOISE3_BLAST (0.7918) and DADA2_BLAST (0.7926) were the lowest (**Figure 3.4d**). Deblur_SILVA consistently produced the strongest genus-level separation for both abundance-weighted and presence-absence metrics. Comparison across taxonomic ranks suggested that database effects were modest at the family level but amplified at the genus level. For example, the difference between Deblur_BLAST and Deblur_SILVA was relatively small at the family level for Bray-Curtis (0.5276 vs 0.5331) but became substantial at the genus level (0.8072 vs 0.8196). Additionally, at the family rank, the Jaccard dissimilarities exceeded Bray-Curtis dissimilarities with mean values of 0.568 and 0.536, respectively, which indicated that incidence-based community differentiation was more pronounced than abundance-weighted differentiation. This divergence was especially evident for UNOISE3_SILVA (0.575 vs 0.530) and Deblur_SILVA (0.575 vs 0.533), which suggested that family-level differences can be partly attributed to low-abundance or inconsistently detected taxa. By contrast, at the genus level, the two metrics were much more closely aligned, with overall means of 0.808 for Bray-

Curtis and 0.799 for Jaccard. Metric-specific discrepancies were likewise minimal, ranging from 0.006 in DADA2_GTDB_r220 (0.812 vs 0.806) to 0.013 in Deblur_SILVA (0.820 vs 0.807).

Furthermore, a sensitivity check at a threshold of 0.01% relative abundance was performed. From a descriptive perspective, reducing the threshold did not substantially change the family- or genus-level composition pattern across workflow combinations, indicating that the main taxa were largely unaltered even when the rare taxa were retained (**Supplementary figure S3.3**).

3.3.4 Comparison of ASV versus OTU aggregation methods and their impact on characterization of low-abundance microbiota

When comparing 8 ASV (DADA2, UNOISE, & Deblur) and 2 OTU pipelines (UCLUST), overall performance differences were minimal across all 10 combinations. OTU-based methods exhibited slightly higher average weighted scores and signal-to-noise ratios than ASV-based methods, although these differences were not statistically significant ($p > 0.01$; **Table 3.4**). Importantly, the unknown taxon rates for ASV v/s OTU ($p=0.0753$), chimera rate ($p=0.7561$), and family & genus level unclassified reads proportions ($p=0.081$; and $p=0.204$, respectively) were relatively similar, indicating that appropriate implementations of both models recover similar taxonomical signal from wheat milling samples. The lower S/N ratio for ASVs was accompanied by a large coefficient of variation (ASV/OTU: 35.9/19.3). This variability is not attributable to noise alone but may also reflect rare taxa or low-abundance variants, which is of particular concern when screening for LMF safety. Several outbreaks involving LMF foods, such as chocolate, peanut butter, flour, and infant milk powder, have been reported to have very low concentrations of implicated etiological agents, which are often present at low abundance (FAO/WHO, 2022). The ability to identify foodborne pathogens in low-moisture environments is often complicated by background communities, such as *Bacillus*

and *Pseudomonas*, that dominate the signal and may obscure the signals of *Salmonella* and STECs, which are present at low abundance. This is mainly because OTUs rely on clustering (97% in our study) and often discard singletons, which can lead to low-abundance pathogens (*Salmonella* and STECs) being wrongly considered as noise or merged with common sequences based on OTU clustering (Aladhadh, 2023; Elbehiry et al., 2023; Karstens et al., 2019). In the context of food safety hazard screening, loss of such important taxa could pose a risk, as these pathogens are clinically relevant and can cause outbreaks or trigger recalls. In contrast, ASV methods can retain single-nucleotide variants while accounting for sequence errors. However, sequencing depth, filtering thresholds, and contamination control are primary considerations for pathogen detection, and pipelines can only improve the data's resolution potential (Callahan et al., 2017a; Nikodemova et al., 2023a).

OTUs or ASVs did not influence alpha- or beta-diversity at the family level or genus level ($p > 0.01$). However, ASV detected numerically higher genus-level estimates for Shannon and Chao1 than OTU aggregation, indicating a greater genus-level diversity and richness under the ASV approach, which could be attributed to the finer resolution and less clustering (Chiarello et al., 2022). Shannon diversity is sensitive to low-abundance taxa and uniformity in the distribution of counts. In contrast, dominance-weighted metrics, such as the inverse Simpson index, remained consistent, indicating that both ASV and OTU captured abundant communities (Chiarucci et al., 2011; García-López et al., 2021). However, regarding beta diversity (Bray-Curtis and Jaccard), no community dissimilarities were observed ($p < 0.01$). Moreover, both ASV- and OTU-based methods were consistent in classifying the most abundant genera ($>1\%$ relative abundance) across samples (**Figure 3.2**). This suggested that the ecological diversity of the samples across milling stages was preserved irrespective of the processing paradigm.

Table 3.4: Alpha diversity and taxonomic metrics comparing ASV and OTU aggregation methods.

	Parameters	Mean, ASV/OTU	CV (%) ASV/OTU	p value, mean contrast (ASV/OTU)
Taxonomical parameters	Weighted scores	52.69/71.1	47.2/22.08	0.300
	Signal/Noise	9.1/10.9	35.9/19.3	0.4108
	Unknown rates	0.06/0.04	42.8/15.7	0.0753
	Chimera	2.7/2.5	17.4/41.2	0.7561
	Unknown family	29.5/28.4	5.2/0.9	0.081
	Unknown genus	7.6/6.3	34.2/4.4	0.204
	No of unique species	280.8/286.5	1.1/1.2	0.2190
Alpha diversity (Family level)	Shannon	2.6/2.6	7.3/7.1	0.592
	Chao1	24.4/23.27	8.5/9.4	0.579
	InvSimpson	11.1/10.7	10.8/11.6	0.737
	Fishers alpha	2.4/2.3	7.8/8.1	0.559
Alpha diversity (Genus level)	Shannon	2.6/2.5	8.5/9.1	0.624
	Chao1	48.5/45.5	10.6/12.4	0.580
	InvSimpson	10.9/13.4	12.8/13.4	0.695
	Fishers alpha	2.4/2.3	9.6/9.8	0.685
Beta diversity (Family level)	Bary Curtis	0.5/0.5	20.3/20.1	0.952
	Jaccard	0.5/0.5	19.1/19.2	0.993
Beta diversity (Genus level)	Bary Curtis	0.8/0.8	10.5/10.5	>0.999
	Jaccard	0.7/0.8	8.4/8.3	0.956

Abbreviation: CV, coefficient of variation

Additionally, the OTU and ASV methods consistently classified the most abundant genera (>1% relative abundance). Some non-overlapping genera were detected between the OTU and ASV methods, which are common in low-density samples (Daly et al., 2024). Notably, *Enterobacteriaceae* consistently accounted for >3% relative abundance across both ASV- and OTU-based workflows; however, high family-level abundance did not necessarily translate into confident genus-level resolution. This is partly attributable to commonly applied abundance thresholds (e.g., $\geq 0.1\%$ relative abundance) used to define taxon presence (Nikodemova et al., 2023b), which effectively may exclude the genus like *Salmonella* (if present in low abundance), an emerging pathogen of concern in low-moisture foods such as wheat grains and their product (Balyatanda et al., 2026; Doddabematti Prakash et al., 2024). Therefore, care must be taken when sampling food products with low microbial abundance, as

differences among sequence aggregation methods must be considered. The chosen method will directly influence alpha and beta diversity. To address this concern, a sensitivity analysis at 0.01% relative abundance was conducted to account for low-abundance taxa; however, the ASV- and OTU-based methods did not alter the representation of rare taxa. The relative merits of ASV- and OTU-based inference remain debated, with advantages and limitations depending on ecological context, sequencing depth, and study objective. (Daly et al., 2024; Fasolo et al., 2024; Prodan et al., 2020). Instead of clustering sequences based on an identity threshold as the OTU method does, the ASV method infers exact amplicon sequence variants and can resolve sequence differences as small as a single nucleotide across the sequenced region (Callahan et al., 2017b; Porter & Hajibabaei, 2020). However, the ASV method may inflate the number of taxonomic units derived from 16S rRNA sequences because multiple 16S rRNA gene copies within a single genome can be treated as distinct features (Schloss, 2021). But samples may contain more than one copy of the 16S rRNA sequence (up to 15) (Klappenbach et al., 2001), and ASV typically considers each of these copies as a separate taxonomic unit, while the OTU methods consolidate all these copies into one taxonomic unit (Daly et al., 2024). This study yielded more taxonomic units for ASVs, higher alpha- and beta-diversity metrics, and fewer unknown family- and genus-level assignments than OTU methods (**Table 3.4**). However, it is important to note that, in general, ASV methods did not improve taxonomic classification or diversity metrics, as OTU methods produced virtually the same or slightly lower percentages of unassigned family and genus assignments, as well as alpha and beta diversities ($p>0.01$). After accounting for 16S rRNA gene copy-number effects, alpha and beta diversity estimates converged between ASV- and OTU-based workflows, indicating that the observed differences were methodological rather than biological. Daly et al. (2024) observed inflated alpha and diversity values as they did not adjust for the copy corrections. The study also noted that to improve the specificity of the OTU method, it is important to reduce

microbiota size, thereby increasing the accuracy of microorganism identification and enabling more effective monitoring of spoilage and potentially pathogenic bacteria in the food processing environment.

Additionally, there are several other challenges when using 16S rRNA amplicon sequencing to study the microbiota in low-density food or environmental samples: (i) the 16S rRNA techniques do not provide enough information to identify the microbes at the species level (Rizal et al., 2020), (ii) and are unable to reliably distinguish DNA from viable versus non-viable cells. These constraints partially explain the limited adoption of amplicon-based approaches in routine food industry monitoring. Nevertheless, 16S rRNA sequencing and complementary next-generation sequencing (NGS) approaches provide valuable insights into newly identified taxonomic and ecological features of the microbiota in food-processing environments (Zwirzitz et al., 2020). By improving understanding of how processing environments influence microbiota characteristics, these NGS methods hold promise for more accurately identifying the sources and mechanisms of cross-contamination (Augusto et al., 2022; Mcmurdie & Holmes, 2014).

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3.4. Conclusion

This study offers a detailed benchmarking of amplicon-based bioinformatic techniques to investigate bacterial populations in LMF processing environments using a commercial wheat milling plant as a case study. By methodically analyzing 8 popular pipeline and 4 databases, we show that overarching ecological patterns are unchanged irrespective of the analytical method used; nevertheless, the choice of the workflow has a strong influence on taxonomic assignment depth, the proportion of unclassified reads, and alpha and beta diversity estimates, especially at the genus level, where database coverage, clustering strategy, and the abundance thresholds directly impacted ecological interpretation. Additionally, our study showed that ASV-based methods slightly outperformed OTU-based methods, largely since ASVs can distinguish variations down to the single-nucleotide level and thus can keep low-abundance features, which may be of importance to LMF safety, especially after removing the effect of 16S rRNA gene copy number, which lowered the inflation of richness and aligned alpha and beta diversity measures more closely with OTU workflows. The findings thus suggest that in sparsely populated LMF processing environments where hazard detection might rely on rare taxa and subtle sequence variants, ASV-based aggregation could be the right choice for taxonomic and ecological characterization, provided sequencing depth, contamination control, and filtering thresholds are carefully optimized. Collectively, this benchmarking framework provides a practical and reproducible foundation for selecting and validating bioinformatics

strategies in low-moisture food microbiome studies and contributes to methodological standardization in food-processing environments.

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Chapter 4 - Selective Redistribution of Bacterial Communities

Across Wheat Milling Stages and Fractions

Abstract

Microbial contamination in wheat and wheat products has increased over the past decade, yet microbial community dynamics across commercial wheat milling stages remain poorly characterized. This study aimed to characterize the bacterial communities associated with three key stages of wheat processing: before milling (BM), after milling (AM), and milled fractions (MF) in a commercial milling facility in Kansas, USA. Using 16S rRNA sequencing, we identified 22 unique families and 132 different genera across all milling stages. The core microbiota identified across all milling stages included *Enterobacteriaceae*, *Xanthomonadaceae*, *Lactobacillaceae*, *Microbacteriaceae*, and *Leuconostocaceae*, accounting for 31.4% of the total relative abundance. Alpha diversity varied across milling stages, with genus-level richness declining by 41.5% from BM (19.22 ± 3.06) to AM (11.24 ± 2.24) ($p < 0.001$). Beta diversity analyses further demonstrated significant compositional shifts across stages, with MF forming the most distinct microbial communities ($p < 0.001$). Functional predictions using PICRUSt2 identified 20 enriched KEGG modules associated with stress tolerance and survival, including pathways related to biofilm formation, osmoprotection, sporulation, and multidrug efflux, particularly in AM and MF samples. This study provides critical information that can serve as a foundation for developing standard operating procedures tailored to wheat milling facilities, where physical cleaning remains the primary sanitation method. Overall, our findings reveal that wheat milling drives stage-dependent restructuring and selective redistribution of microbial communities with direct implications for food safety, contamination control, and risk management. These results also lay the groundwork for developing targeted sanitation strategies in milling environments and effective interventions for wheat-based products.

4.1 Introduction

Wheat (*Triticum* spp.) plays a critical role in global food security, providing sustenance for approximately 80 million people (Javid Iqbal et al., 2022; Sharma & Kumar Sharma, 2025), serving as a staple food for a substantial proportion of the global population. It is also the primary raw material processed in commercial milling systems to produce wheat flour and other processed foods. Wheat and wheat-based products are categorized as low-moisture foods (LMFs) and have historically been considered microbiologically low-risk (Shivaprasad et al., 2024); however, this assumption has been challenged in the last two decades. Wheat-based foods have emerged as an important source of foodborne illness transmission in humans. With over 27 recalls and 15 outbreaks since 2005, wheat and wheat-milled products are currently under extensive review despite their low water activity ($a_w < 0.85$) (Balyatanda et al., 2026; Doddabematti Prakash et al., 2025; Rivera et al., 2025). In most cases, *Salmonella* and Shiga toxin-producing *E. coli* (STEC) were isolated from these samples (Myoda et al., 2019). A critical vulnerability underlying these events is that, unlike high-moisture foods subjected to thermal lethality, wheat flour lacks a validated kill step (Sabillón Galeas, 2014). Therefore, enteric pathogens such as *Salmonella* and STEC can enter the wheat system via pre-harvest and post-harvest contamination. Recent evidence indicates that these pathogens can survive in wheat grains and flour for over 12 months and are primarily concentrated during milling (Gill et al., 2020; Lauer et al., 2021). Therefore, there is a critical need to characterize the composition, diversity, and functional potential of the wheat-associated microbiome across milling stages, an important prerequisite for developing an evidence-based intervention strategy.

Commercial milling is a multi-stage process that transforms wheat grains into distinct milling fractions such as bran, germ, and endosperm through successive cleaning, tempering, and grinding (breaking, sizing, and reduction) (Doddabematti Prakash et al., 2023). Given the

absence of a pathogen-reduction step during milling, pathogens present on the wheat kernels are redistributed across milling fractions as a consequence of the physical fractionation process (Magallanes López & Simsek, 2021; Rivera, Deliephan, et al., 2021; Rivera, Phebus, et al., 2021). Culture-based investigations have shown that the milling process preferentially partitions microbial contaminants into bran and germ fractions while delivering a relatively lower load to the endosperm-derived fraction, including wheat flour (Sabillón Galeas, 2014). A recent investigation by Solanki et al. (2021) noted that most common wheat-associated microbiomes belong to *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Planctomycetes*; however, how these communities shift during milling remains poorly understood. This gap limits our understanding of changes in microbial dynamics during milling, particularly regarding the taxa that harbor stress-adaptive functions and persistence.

Culture-independent techniques, such as 16S ribosomal RNA (rRNA) gene sequencing, enable high-resolution characterization of microbial communities (Beaudry et al., 2021). However, the reliability of ecological inferences from these techniques depends on several factors, including the choice of bioinformatic pipeline (ASVs vs. OTUs) and the type of databases used (Lehr et al., 2025; Marizzoni et al., 2020). Several comparative studies have shown that the choice of bioinformatics pipeline significantly influences results in terms of community composition and diversity (Daly et al., 2024; Marizzoni et al., 2020; Sierra et al., 2020). Our previous chapter 3 on the 16S benchmarking study systematically evaluated the performance of the competitive pipeline X database combination (32 combinations in total) for 16S rRNA amplicon data from environmental and product samples from a commercial wheat milling setup. The study found that DADA2_SILVA was an optimal choice for the LMF setting, as it preserved family- and genus-level taxonomic assignments. This chapter 4 is an extension of our previous work, where we validated the analytical framework using the

DADA2_SILVA workflow to characterize the ecological niche across different milling samples.

16S rRNA sequencing data provides taxonomic resolution primarily at the family and genus levels, and occasionally at the species level (Johnson et al., 2019); however, it does not directly capture metabolic and functional potential represented within that community (e.g., stress tolerance, multi-drug resistance, biofilm formation). Predictive metagenomic tools like PICRUST2 (Douglas et al., 2020), Tax4Fun2 (Wemheue et al., 2020), and MicFunPred (Mongad et al., 2021) can translate 16S amplicon phylogeny into functional predictions by assigning Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs to infer pathway-level predictions. For food-related metagenomics, PICRUST2 is widely preferred because of its accuracy and flexibility (Choi et al., 2020; Shingling & Tamang, 2021). Additionally, it also uses more than 20,000 reference genomes and gene families (Douglas et al., 2020). To our knowledge, no study has integrated culture-independent (16S rRNA) data with PICRUST2-based predictive functional KEGG pathway for the wheat milling setting. This study aims to address this gap. Specifically, we (i) characterize the taxonomic composition of bacterial communities across BM, AM, and MF samples at both family and genus levels; (ii) assess alpha and beta diversity to evaluate changes in richness and community composition across milling stages; and (iii) apply PICRUST2 to predict the enriched metabolic and functional pathways across samples.

4.2. Materials and Methods

4.2.1 Sample collection and DNA extraction

A total of 160 (n=160) samples were collected from a wheat milling facility in Kansas, which comprised (i) *surface samples* collected from Food Contact Surfaces (FCS), Non-Food Contact Surfaces (NFCS), and (ii) *millstream fraction samples* collected from product and process streams (AM, BM, and MF). Ambient facility temperature during sampling was

maintained at 20-24 °C. Surface samples were collected using BLU-DRY PUR-Blue Swab samplers (World Bioproducts LLC, WA, USA) pre-moistened with PBS (pH 7.4) (BD Difco; Franklin Lakes, NJ, USA). For each sampling point, an area of approximately 100 cm² was swabbed. Millstream fraction samples were aseptically collected by scooping material into sterile tubes or sterile stomacher bags. All samples were maintained at 4 °C and processed for DNA extraction within 24 h of collection.

Genomic DNA (gDNA) extractions were performed on the samples using DNeasy® Blood and Tissue Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol with a modified lysis step (1 h incubation) to improve cell disruption. Absorbance at 260, 230, and 280 nm was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to assess the DNA concentration and purity of the extracts. In addition, DNA concentration was quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific), and the samples were normalized to 5 ng/μL for downstream processing.

4.2.2 16S rRNA gene sequencing and library preparation

Bacterial community profiles were generated by amplifying the V4 hypervariable region of the 16S rRNA gene using degenerate primers (515F/806R) with Illumina overhang adapters (Caporaso et al., 2012) targeting the V4 region (~254 bp) as established in the Earth Microbiome Project (Thompson et al., 2017). Additionally, the short amplicon length is compatible with short read paired-end sequencing and facilitates taxonomic assignments and community-level comparisons (Hu et al., 2025). The V4 region was PCR amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for bacterial community profiling. Amplicon library preparation (including index addition and clean-up steps) followed the manufacturer's recommended workflow described in the Illumina 16S Metagenomic Sequencing Demonstrated Protocol (Illumina). PCR amplification consisted of 27 cycles for amplicon

generation and eight cycles for indexing, with an annealing temperature of 55°C. Given the low-biomass nature of wheat milling samples, contamination assessment was further evaluated using both DNA extraction blank and no-template controls (NTCs). Extraction blanks and NTC libraries were handled alongside biological samples, and the contamination was assessed based on retained read counts, assigned-read proportions, and recurring taxa in controls. Taxa consistently detected in extraction blanks, and NTCs were considered background contaminants and were removed during downstream preprocessing prior to ecological analyses.

Resulting libraries were assessed for size distribution and quality using a DNA 7500 chip on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Libraries were then pooled in equimolar amounts. The final pooled library (including the negative control) was sequenced on the Illumina MiSeq i1000 Plus using a 25 million-cycle, 600-cycle kit (paired-end sequencing). Sample preparation was performed according to the 16S Metagenomic Sequencing Demonstrated Protocol recommended by Illumina.

4.2.3 Taxonomic assignment

Raw sequencing data were processed with QIIME2 (v2024.10.1) (Licata et al., 2025). Sequences were denoised using DADA2 to generate amplicon sequence variants (ASVs) (Callahan et al., 2016). ASVs representing >0.005% of total reads and present in $\geq 1\%$ of samples were retained for downstream analysis. The taxonomic annotations of ASVs were performed using QIIME2 using the SILVA database (v.138).

Alpha and beta diversity analyses were performed in QIIME2 using the *q2-diversity* plugin. Samples were rarefied to an even sequencing depth of 5000 reads per sample prior to diversity analysis. Alpha diversity was assessed using Shannon diversity, observed richness, and inverse Simpson indices, and beta diversity was evaluated using Bray–Curtis dissimilarity, Aitchison distance, and Jaccard distance. Group differences in alpha diversity were tested using

the QIIME2 alpha-group-significance method, and beta diversity differences were tested using permutation-based methods such as PERMANOVA.

4.2.4 Functional prediction of wheat microbiome based on 16S rRNA gene sequencing data

PICRUSt2 (v2.5.3) was applied to predict the functional potential of the wheat microbiome based on 16S rRNA gene sequencing data (Douglas et al., 2020). ASV abundance tables were input into PICRUSt2. Functional profiles were inferred from Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog predictions. Predicted genes were mapped to KEGG pathways for downstream analysis (Kanehisa et al., 2025).

4.2.5 Statistical analysis

All statistical analyses were conducted in R (v4.5.3) and QIIME2 (v2024.10.1). Alpha diversity metrics, including Shannon diversity, observed richness, and Inverse Simpson index, were calculated using the *q2-diversity* plugin in QIIME2. Differences in alpha diversity across milling stages (BM, AM, and MF) were assessed using the Kruskal–Wallis test, followed by pairwise Wilcoxon rank-sum tests with Benjamini–Hochberg false discovery rate (FDR) correction for multiple comparisons. Beta diversity was evaluated using Bray–Curtis dissimilarity, Jaccard distance, and Aitchison distance (centered log-ratio transformed data). Permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function (vegan package) with 999 permutations to test for differences in community composition across milling stages. Homogeneity of group dispersion was assessed using PERMDISP (*betadisper* function) to confirm that observed differences were not driven by differences in within-group variance. Relative abundance differences at the family and genus levels were assessed using non-parametric statistical tests. Differential abundance analysis was performed using the Kruskal–Wallis test with FDR correction, and taxa with adjusted p-values < 0.05 were considered significantly different across groups. Functional potential of the

bacterial communities was inferred using PICRUSt2 (v2.5.3). ASV sequences and their corresponding abundance table were used as input to generate predicted gene family abundances based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs). These predictions were subsequently collapsed into higher-level KEGG pathways (Level 2 and Level 3) for downstream analysis.

Prior to statistical analysis, predicted functional profiles were normalized to relative abundances to account for differences in sequencing depth across samples. To evaluate differences in functional profiles across milling stages (BM, AM, and MF), nonparametric statistical approaches were used because the microbiome data were non-normally distributed. Differences in individual KEGG pathway abundances between groups were assessed using the Kruskal–Wallis test. Where significant differences were detected ($p < 0.05$), pairwise comparisons between BM, AM, and MF were performed using Wilcoxon rank-sum tests with Benjamini–Hochberg false discovery rate (FDR) correction to control for multiple testing. Pathways with adjusted p-values (q-values) < 0.05 were considered significantly different across milling stages. To identify pathways enriched at specific milling stages, median relative abundances were compared across groups, and enrichment was defined as a significantly higher abundance at one stage relative to the others following post hoc testing. All statistical tests were two-sided, and significance was defined at $\alpha = 0.05$ unless otherwise stated. Data visualization was performed using *ggplot2* and *phyloseq* packages in R.

4.3. Results and Discussion

4.3.1 Taxonomic composition of AM, BM, and MF at the family and genus level

The bacterial composition of the wheat-milling samples was evaluated using the DADA2–SILVA workflow. In brief, a total of 3793 ASVs were obtained from all samples prior to quality filtering. After quality control and downstream preprocessing, 3744 ASVs were retained for further analysis. Most ASVs showed low prevalence across samples, with 3738

ASVs detected in fewer than 50% of samples, consistent with the high-resolution characteristics of ASV-based inference; however, only a small fraction of ASVs (0.02%) were detected in the majority of samples. Following taxonomic assignment, a total of 22 families and 132 genera were identified. At the family level, six families (27.2%) were detected in more than 90% of samples, while eight families (36.3%) were detected in fewer than 50% of samples. Likewise, at the genus level, three genera (2.3%) were found in >90% of samples, while 94 genera (71.2%) were detected in <50% of samples.

At the family level, the most abundant taxa across different samples included *Enterobacteriaceae*, *Lactobacillaceae*, *Xanthomonadaceae*, *Leuconostocaceae*, and *Microbacteriaceae*, which collectively accounted for 31.4% of the total relative abundance. At the genus level, the dominant taxa included *Lactobacillus*, *Weissella*, *Paenibacillus*, *Xanthomonas*, and *Corynebacterium*, collectively accounting for 16.6% of the total relative abundance. In addition to the overall taxonomic composition, the stacked bar profiles also revealed distinct disparities among BM, AM, and MF. At the family and genus levels, BM and AM exhibited similar community structures, whereas MF displayed a distinct relative abundance profile with a lower cumulative contribution of dominant taxa (**Figure 4.1**). These results imply that milling drives taxonomic restructuring with MF representing a compositionally distinct fraction rather than a direct extension of BM and AM communities. These changes likely reflect the physical redistribution of microorganisms among the grain-associated niches during milling, as well as the differential retention of taxa within flour-associated fractions.

It's also worth noting that many of the dominant taxa identified in the current study are consistent with earlier findings, showing that wheat and other cereal-related microbiomes are typically dominated by plant- and grain-associated *Proteobacteria*, *Actinobacteria*, *Bacillota*, and lactic acid bacteria. For instance, *Pantoea* and *Paenibacillus* were identified as the most

prominent members of the wheat seed microbiota in *Triticum aestivum* L. and *Triticum spelta* L. (Kuźniar et al., 2020). Similarly, Díaz Herrera et al. (2016) reported *Paenibacillus*, *Bacillus*, and *Pantoea* as the dominant microbiome in the spring wheat endosperm microbiome. In addition, *Lactobacillus*, *Weissella*, and *Lactobacillaceae* (lactic acid bacteria-related taxa) are commonly associated with cereal grains and cereal-derived products (Milanovic et al., 2020; Tsafrakidou et al., 2020).

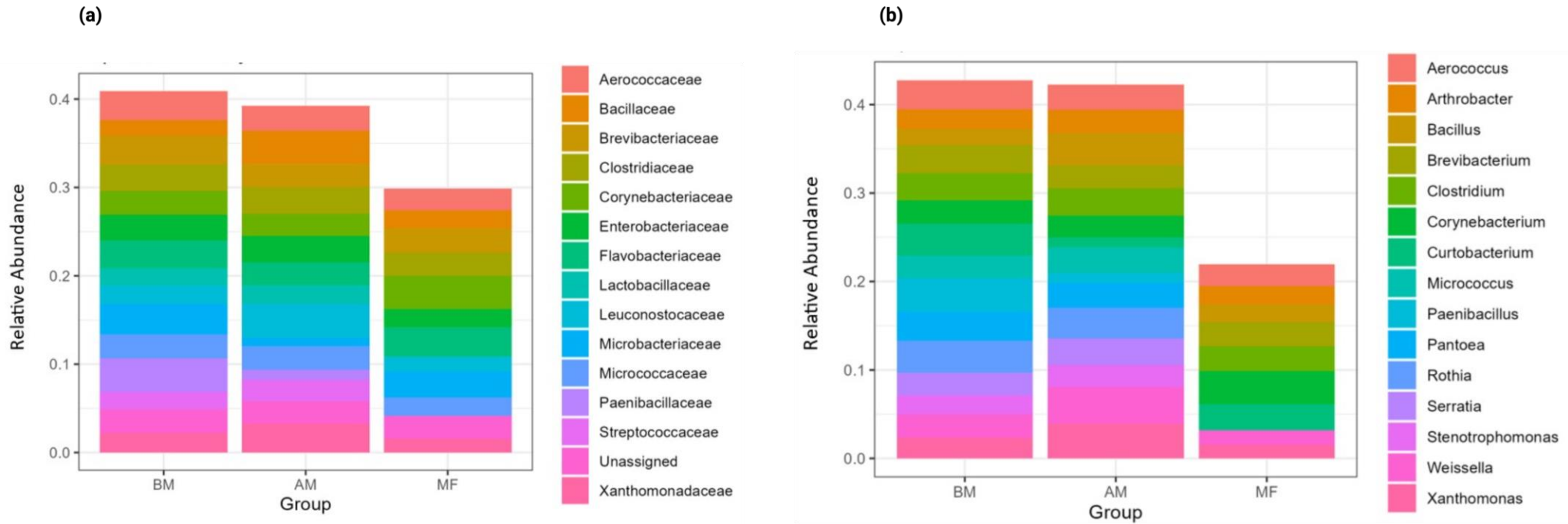


Figure 4.1: Relative abundance of the most dominant bacterial taxa at the family and genus levels across BM, AM, and MF sample groups. Stacked bar plots show the mean relative abundance of the top taxa identified in each group, with (a) representing family-level composition and panel (b) representing genus-level composition. Each colored segment corresponds to a taxon listed in the legend, and bar heights represent the cumulative relative abundance of the displayed taxa within each sample group.

4.3.2 Alpha and Beta diversity

4.3.2.1 Alpha Diversity

Alpha diversity metrics provided critical insight into microbial community structure across milling stages (BM, AM, and MF) (**Figure 4.2**). Shannon diversity indices at the family level (**Figure 4.2a**) followed the pattern $AM > BM > MF$, with AM exhibiting the highest diversity and MF the lowest ($BM=1.93 \pm 0.2$; $AM=2.01 \pm 0.2$; $MF=1.35 \pm 0.32$). A similar trend was observed for family-level richness (**Figure 4.2b**), with AM ranking highest, BM intermediate, and MF lowest ($AM=11.82 \pm 2.1$; $BM=10.18 \pm 1.9$; 6.32 ± 1.8). Collectively, these findings indicate MF harbored a narrower taxonomic spectrum than BM and AM samples. However, Inverse Simpson values (**Figure 4.2c**) were substantially higher for MF (1.56 ± 0.31) compared to BM and AM (1.21 ± 0.06 and 1.22 ± 0.09 , respectively). This higher inverse Simpson value reflects lower richness, indicating the presence of fewer families, while dominant taxa were more evenly distributed. This pattern is ecologically consistent and likely reflects the physical redistribution of microbial populations during milling (Sabillón, 2018; Sabillón & Bianchini, 2016). Therefore, each MF acquires grain-surface microflora and becomes dominated by a few stress-resistant taxa.

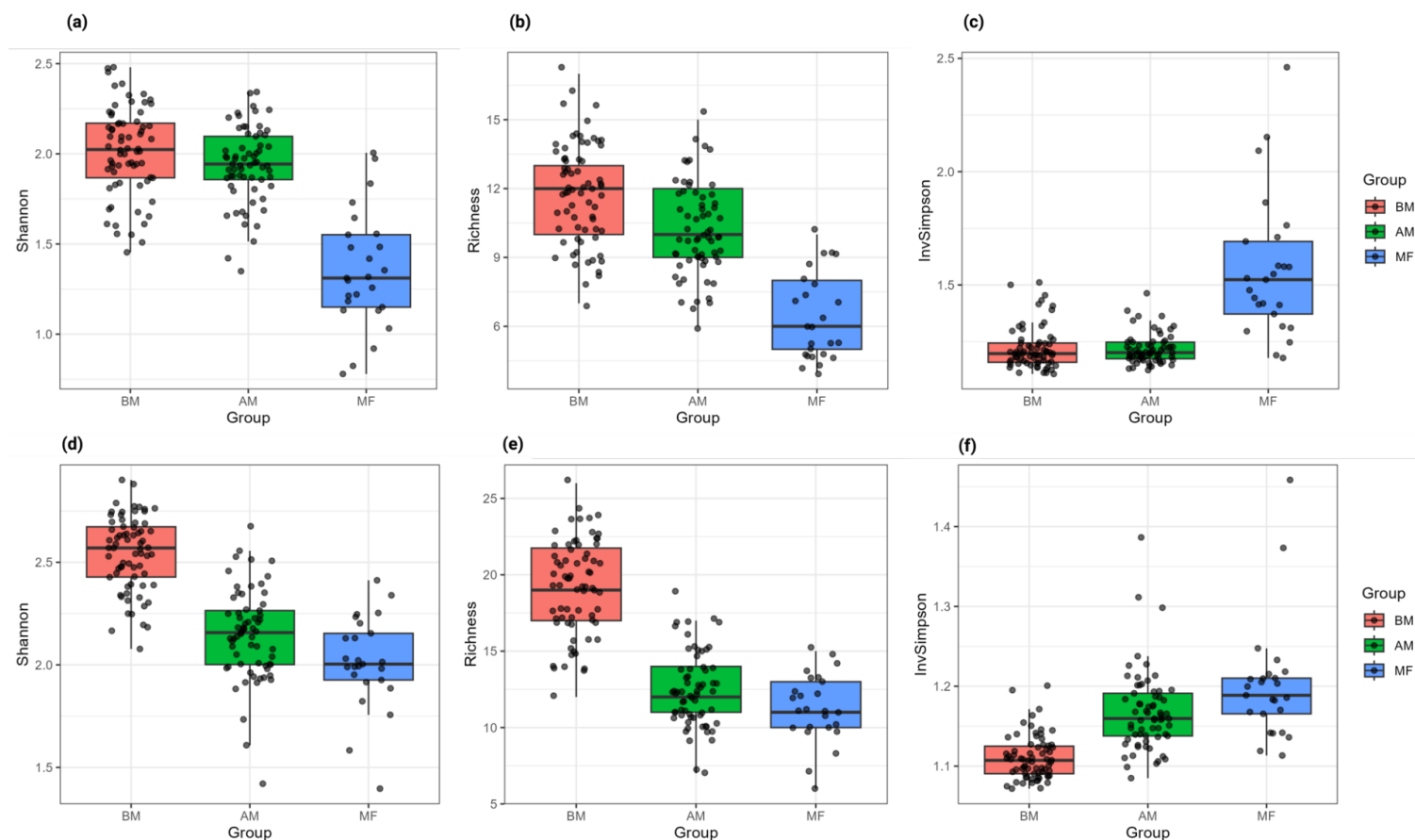


Figure 4.2: Alpha diversity of bacterial communities across BM, AM, and MF sample groups at the family and genus levels. Boxplots show Shannon diversity, richness, and inverse Simpson diversity for the family level in panels (a–c) and the genus level in panels (d–f). Values are presented as means $\pm$ SD.

At the genus level (**Figure 4.2d–f**), diversity differences were most pronounced in BM samples, which exhibited significantly higher diversity across all indices (Shannon, richness, and inverse simpson; $p < 0.001$), whereas no significant differences were observed between AM and MF in any metric ($p > 0.05$). Specifically, BM samples showed higher Shannon (19.22 ± 3.06), richness (10.58 ± 2.11), and inverse simpson (2.54 ± 0.18), indicating approximately a 41.5% loss in genus-level richness post-milling (MF = 11.24 ± 2.24). However, the absence of significant differences between AM and MF suggests that the major loss of diversity occurs during the transition from BM to AM, rather than progressively across downstream fractions. This finding is consistent with previous studies (Rivera et al., 2022b, 2022a), which reported that the *E. coli* recovered in the milled fractions (flour) differed significantly from those in the non-flour fractions (bran and shorts). This indicates that bran is the main reservoir of wheat grain contamination, and removal of the bran during the milling process significantly removes a larger contamination source. Overall, these findings suggest that environmental and product sampling in wheat processing environments are highly variable across sample types and milling stages. Moreover, these communities are characterized by a small number of dominant genera but alongside several low-abundance taxa reflecting high unevenness.

4.3.2.2 Beta diversity

Beta diversity was assessed using Bray-Curtis (abundance-weighted), Jaccard (presence-absence), and Aitchison distances (based on centered log-ratio transformed data) (Martino et al., 2022). Principal Coordinates Analysis (PCoA) plots of beta diversity metrics are presented in **Figure 4.3**. These ordinations revealed three distinct clusters corresponding to BM, AM, and MF, with minimal overlapping among group centroids, and clear compositional shifts in bacterial communities across milling stages.

At the family level, the PCoA ordination showed clear separation among BM, AM, and MF (**Figure 4.3a-c**). BM and AM were closer to each other relative to MF. In the Bray-Curtis, BM (red) occupied a central position, with AM (green) clustering to one side, and MF (blue) forming a distinct cluster on the opposite side, indicating an abundance-driven compositional divergence among all three milling stages. In the Jaccard dissimilarity, substantial overlap between AM and BM ellipses was observed, suggesting that these milling share similar bacterial family composition, whereas a clear displacement was observed in MF, as seen in the alpha diversity analysis. This was further supported by the Aitchison-based ordination, which showed a broad yet clearly distinct clustering of MF despite greater dispersion. A similar but more pronounced separation was observed at the genus level in beta-diversity ordinations (**Figure 4.3d-f**), with MF consistently forming a compositionally distinct cluster, whereas BM and AM remained closely clustered. These differences were statistically supported with PERMANOVA and betaspread ANOVA, both indicating significant differences for Bray-curtis ($p=0.001$; $p=7.16E-07$), Aitchison ($p=0.001$; $p=3.48E-48$), and Jaccard ($p=0.001$; $p=5.28E-12$), respectively.

Overall, these data indicate that milling significantly alters microbial community composition at both the family and genus levels, with MF emerging as the most compositionally distinct group. These findings support our conclusion that removing the outer layer of the wheat kernel significantly alters microbiome diversity, and that subsequent processing leads to redistribution and selective partitioning of bacterial taxa across milled fractions.

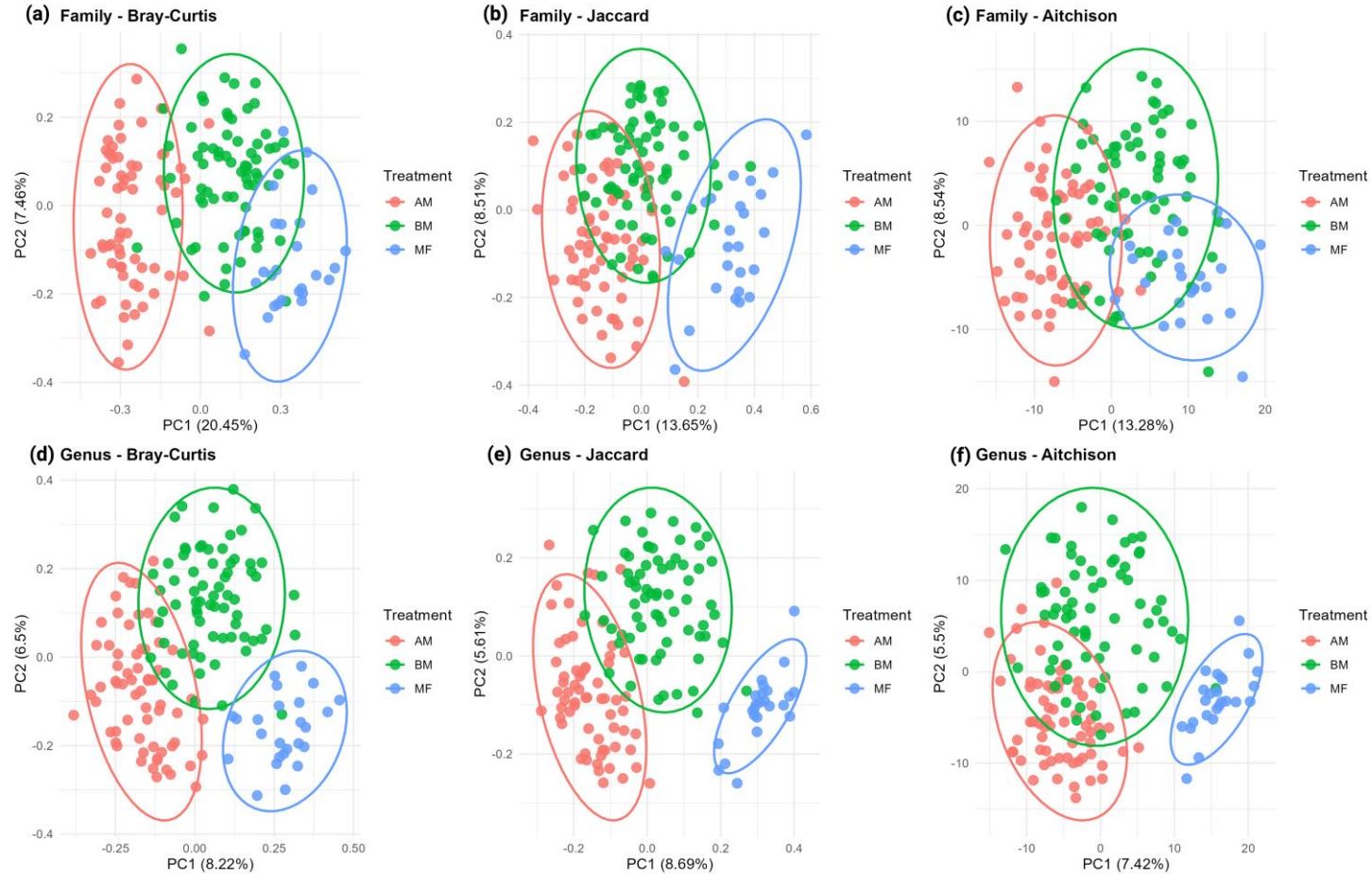


Figure 4.3: Beta diversity of bacterial communities across BM, AM, and MF sample groups at the family and genus levels based on Bray–Curtis, Jaccard, and Aitchison distance metrics. Principal coordinates analysis (PCoA) plots for the family level are shown in panels (a–c), and those for the genus level are shown in panels (d–f). Values are presented as means $\pm$ SD.

4.3.3 Predicted functional capacity of the wheat milling microbiome and implications for intervention development

To extend the taxonomic and diversity patterns identified above, the functional potential of the wheat milling microbiome was inferred from 16S rRNA gene data using PICRUSt2-based KEGG module prediction. Because amplicon sequencing does not directly measure gene content or expression, these results are interpreted as predicted functional capacities rather than direct measurements of metabolic activity. Despite this limitation, the stage-wise distribution of predicted modules remained informative and suggested that restructuring of the microbiome across BM, AM, and MF was accompanied by corresponding shifts in ecological function. PICRUSt2 is a widely used tool that predicts functional potential from 16S rRNA gene sequences based on reference genomes (Douglas et al., 2020). However, it lacks the resolution to represent strain-level genomic variation or transcriptional dynamics. Therefore, inferences derived from these predictions remain provisional and should be considered testable hypotheses rather than definitive conclusions.

Further, PICRUSt2 predicted top 20 KEGG functional modules across different samples (BM, AM, and MF) (**Figure 4.4**). The results indicated that the core metabolic (housekeeping) pathways, such as Bacterial riboflavin biosynthesis (M00125), Citrate cycle (TCA)(M00009), Ribosome (M00185), threonine biosynthesis (M00018), pentose phosphate pathway (M00036), type VI secretion system (M00334), two-component system (M00579), fatty acid biosynthesis (M00083), and UDP-N-acetylglucosamine biosynthesis (M00052), were consistent across the milling stages, indicating that the fundamental energy and biosynthetic capacity of wheat microbiome are largely conserved across processing stages.

In contrast, a clear shift in stress tolerance and persistence-associated pathways was observed across milling stages. In particular, a pronounced enrichment of pathways related to biofilm formation, sporulation, oxidative stress response, trehalose biosynthesis, heat shock protein chaperones, LPS biosynthesis, quorum sensing, peptidoglycan biosynthesis, glycine betaine biosynthesis, and multidrug efflux was observed in AM and MF samples relative to BM. These patterns suggest an increased functional potential for microbial taxa to detoxify ROS, accumulate protective compatible solutes, and refold damaged proteins in AM and MF samples (Eriksson De Rezende et al., 2001; Ulfig & Jakob, 2024). Overall, this pattern indicates that the functional restructuring of the bacterial community in wheat across different milling stages was not random but reflects selective enrichment of stress-associated traits that may facilitate survival under low-moisture and processing-related stress conditions.

The enrichment of glycine betaine/proline transport and trehalose biosynthesis in AM and MF is particularly relevant in LMF systems. Previous studies have identified osmoprotection as a key mechanism enabling *Salmonella* survival under low-moisture conditions by regulating cellular water balance through the accumulation of solutes such as proline, glycine betaine, and ectoine (Csonka & Hanson, 1991; Finn, Händler, et al., 2013). Similarly, Finn et al. (2013) reported that trehalose contributes to the stabilization of *Salmonella* cells under conditions of desiccation by stopping the migration of water/solutes and balancing the internal and external osmolality. Supporting this, Li et al. (2012) reported global transcriptome changes in *Salmonella* under desiccation (air-drying at 11% relative humidity), with upregulation of the trehalose biosynthesis gene (*otsB*). Together, these findings are consistent with our results, which show enrichment of osmoadaptive traits in AM and MF compared to BM.

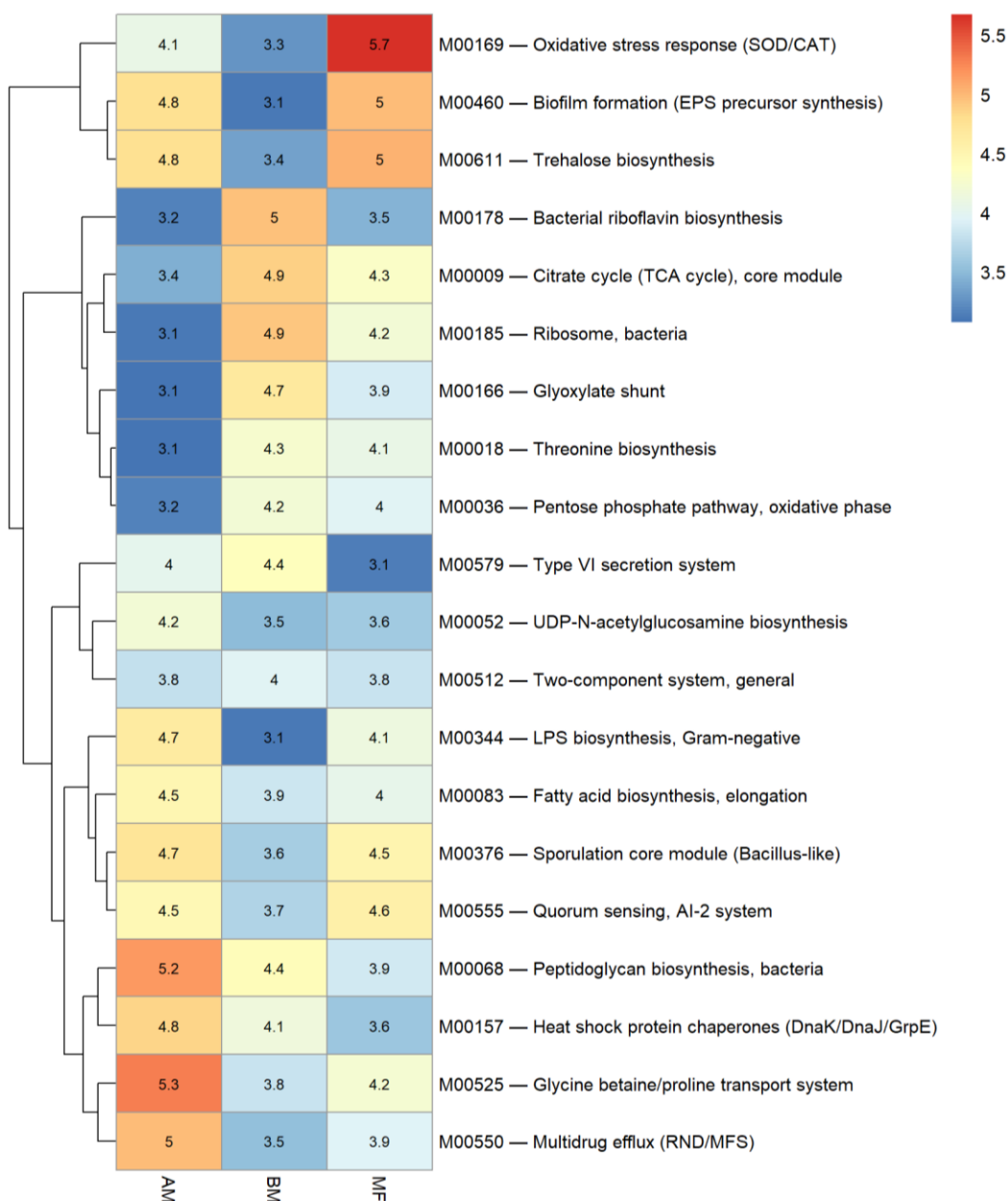


Figure 4.4: Heatmap of the top 20 KEGG functional modules with the highest absolute mean abundance (%) across the three sample groups, AM, BM, and MF. Rows represent KEGG modules, and columns represent sample groups. Cell colors show row-scaled relative abundance patterns, indicating enrichment or depletion of each module across groups, while the numbers within each cell display the absolute mean percentage for that module in each group. Hierarchical clustering of modules is shown on the left to illustrate similarities in functional distribution patterns across sample types.

Similarly, MF and AM samples showed higher enrichment of pathways associated with persistence and sanitation resistance. Dry surface biofilms (DSBs) are an emerging food safety threat in LMF and are typically formed when cells are subjected to osmotic stress (Rahman et al., 2022). In wheat milling systems, where physical sanitation methods are often the primary control strategy (Alonso et al., 2023), these persistent DSBs can pose a significant food safety risk, leading to contamination of wheat flour and its products. Evidence also indicates that DSB can survive for more than 12 months without any hydration (Hu et al., 2015). Thus, enrichment of M00460, M00555, M00169, and M0050 suggests an increased potential for biofilm formation during milling. However, to identify the taxa more closely associated with these persistent functions, taxon-pathway correlation analysis was performed, as shown in **Figure 4.5**. Genera such as *Bacillus* and *Paenibacillus*, commonly associated with wheat microbiota (Mulk et al., 2022), showed a positive correlation with trehalose biosynthesis and other stress-related pathways. Among Bacilli, *Bacillus cereus* is commonly associated with wheat (Eglezos, 2010). Although typically present at low levels, *B. cereus* can pose a food safety risk at higher concentrations; at higher levels, it may cause food poisoning (Eglezos, 2010). A 2003 Australian wheat grains and flour survey reported 2 positive samples for *B. cereus* in wheat grains; however, it was absent in all the flour samples (Berghofer et al., 2003). The positive samples were reported to contain fewer than 1 spore/g. Though lower, the spore count is particularly relevant to fermented bakery products, where spores can germinate and proliferate under favorable conditions (Berghofer et al., 2003).

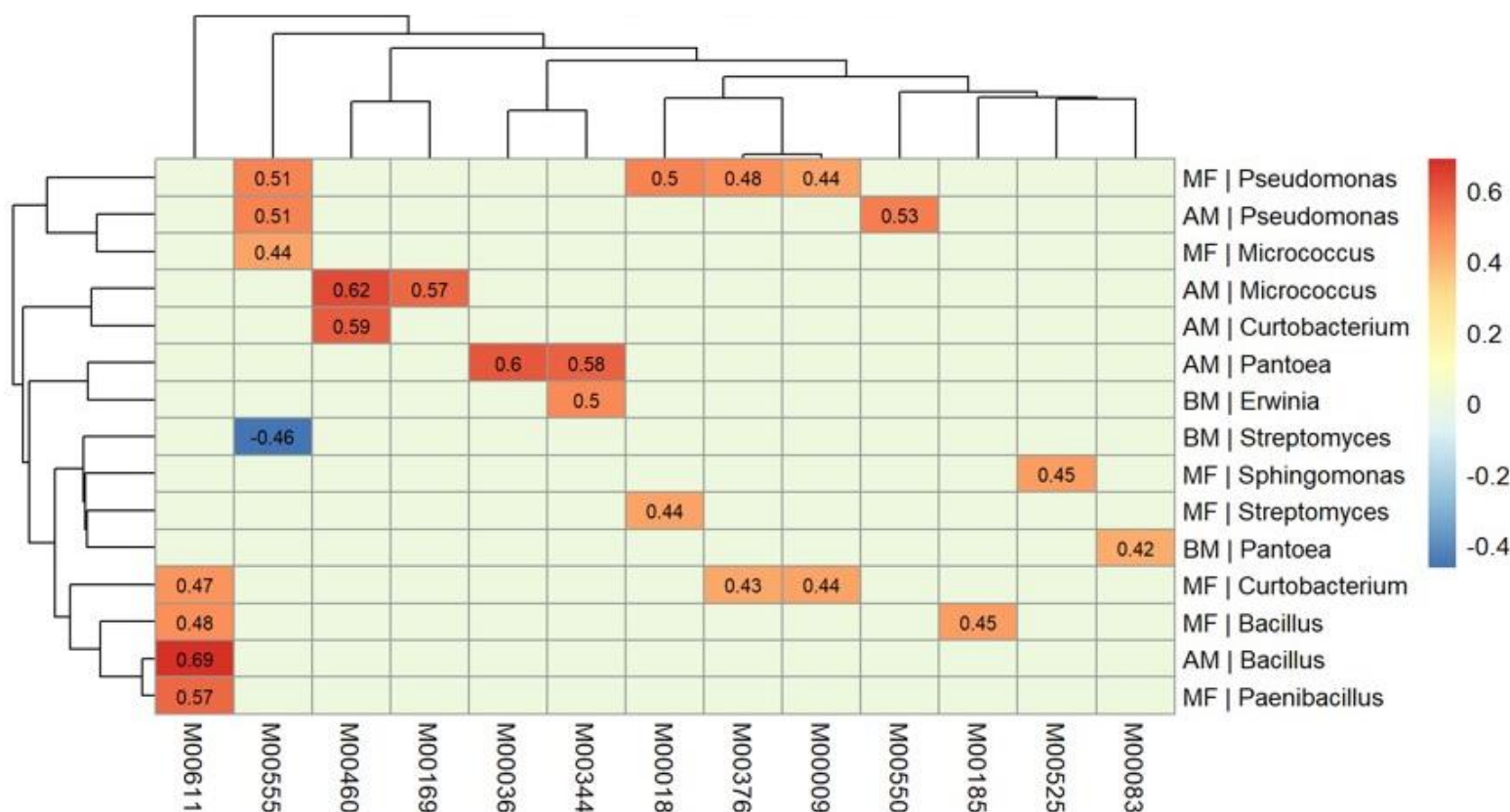


Figure 4.5:Heatmap illustrating the strongest correlations between dominant bacterial taxa identified by 16S rRNA gene sequencing and predicted KEGG functional modules across the AM, BM, and MF sample groups. Rows indicate sample-specific taxon combinations, and columns show KEGG modules. Only the top taxon pathway associations are depicted. Cell colors represent the direction and strength of correlations: red indicates positive correlations, and blue indicates negative correlations, with the numerical values within cells showing correlation coefficients. Hierarchical clustering was used on both rows and columns to group taxa and pathways with similar correlation patterns.

Similarly, *Pseudomonas* (MF and AF; **Figure 4.5**) was also positively associated with multidrug efflux (0.23) and quorum sensing (0.43), consistent with its known resilience and persistence in food-processing settings. *Pseudomonas* spp. are also excellent biofilm formers in both dry and wet conditions (Aparecida et al., 2025; Nkemngong et al., 2020), making them tolerant to cleaning interventions and contributing to their persistence in food-processing environments through adaptive stress responses and surface colonization (Møretro & Langsrud, 2017; Sterniša et al., 2023). Other species, such as *Micrococcus*, *Pantoea*, and *Curtobacterium*, were strongly correlated with biofilm and oxidative stress (**Figure 4.5**). These dominant taxa are not only taxonomically abundant but also functionally associated with persistence. Rather than showing isolated pathway enrichment, these patterns suggest that the wheat milling microbiome exhibits coordinated enrichment of survival-associated functions.

Overall, these findings suggest that microorganisms persisting in wheat-milling systems show enrichment in stress- and survival-related pathways. Our results invite the need for targeted, mechanistically informed intervention strategies to reduce persistence within the milling environment. The observed functional profiles suggest that tempering with acids and non-thermal treatments may represent effective control points for several reasons. First, tempering is a common unit operation in the wheat milling industry; therefore, integrating antimicrobial tempering represents a practical opportunity for industrial intervention (Qin et al., 2022). Secondly, acidic tempering leverages rehydration to alter intracellular pH, osmotic balance, and membrane integrity, thereby interfering with cells that depend on osmoadaptation and stress responses for survival (Guan, 2020). In parallel, emerging non-thermal processing techniques such as UV-C, pulsed light, and cold plasma can help inactivate pathogens through oxidative stress, membrane disruption, and DNA damage (White et al., 2025). Combined, these observations suggest

that antimicrobial tempering is not just a processing step but rather a competitive advantage used to disrupt the survival mechanisms of the microbes that enable them to persist in the wheat milling environment.

4.4. Conclusions

This study provides a comprehensive 16S rRNA gene-based characterization of the wheat microbiome across different milling stages (BM, AM, and MF) using the DADA2-SILVA-based analytical workflow. A total of 22 unique families and 132 distinct genera were identified, with a core microbiota comprising *Enterobacteriaceae*, *Xanthomonadaceae*, *Lactobacillaceae*, *Microbacteriaceae*, and *Leuconostocaceae*, collectively representing 31.4% of the total relative abundance. A significant reduction in alpha diversity was observed, with genus-level richness decreasing by 41.5% during the transition from BM to AM. Similarly, beta diversity using PCoA and PERMANOVA (Bray-Curtis, Jaccard, and Aitchison distances; $p=0.001$) demonstrated significant differences in community composition across milling stages, with MF consistently forming a compositionally distinct cluster. These findings suggest that milling induces stage-dependent restructuring of microbial communities with direct implications for food safety and risk management in wheat processing systems. Functional potential was further inferred using PICRUSt2-based KEGG module predictions to assess the wheat milling microbiome. With 20 predicted KEGG modules, enrichment of stress-related and biofilm-related pathways was observed. However, as PICRUSt2 provides predictive rather than direct functional measurements, it cannot confirm active gene expression or metabolic activity. Therefore, further validation through metagenomics or RNA sequencing is warranted. Nonetheless, these predicted functional profiles provide a foundation for developing targeted, mechanistically informed intervention strategies in wheat milling systems.

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Chapter 5 - Control of *Salmonella* in wheat grains with sodium bisulfate (SBS) tempering and its impact on flour quality

Abstract:

In the last decade, the incidence of foodborne illness outbreaks and recalls connected with wheat flour and flour-based goods infected with enteric bacteria such as *Salmonella* has grown. This study investigated the activity of sodium bisulfate (SBS) as an antibacterial agent against four different serovars of *Salmonella enterica*. The dose-dependent, anti-*Salmonella* concentration of SBS revealed a minimum inhibitory activity (MIC) of 0.32%. At this concentration, SBS induced the production of reactive oxygen species (ROS), demonstrating that lysis of bacterial cells has occurred. SBS tempering (1.5% SBS, w/v) reduced wheat *Salmonella* load by 4.3 log CFU/g after 24h of tempering. Additionally, tempering at 0% (control) and 1.5% SBS (17% moisture, 24 h) showed similar flour functionality (rheology and composition) and baking characteristics (volume, texture, and crumb structure) ($p > 0.05$). These results suggest that SBS is a viable antibacterial tempering agent to reduce *Salmonella* contamination in wheat before milling. Acidic water tempering, as opposed to the traditional water tempering method, may result in milled products with improved microbiological quality.

Keywords: Sodium bisulfate (SBS), antibacterial, tempering, wheat, *Salmonella*

Journal article

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5.1 Introduction

Foodborne illnesses and diseases have long been a topic of concern for both developed and developing countries. Food-related illness is responsible for 128,000 hospitalizations and 3000 fatalities in the United States alone each year (CDC, 2011). Food-borne disease is predicted to cost the United States (US) \$7 billion in economic losses (Sharma et al., 2019). Low moisture foods (LMF) such as wheat flour on the other hand are regarded as low-risk foods because of their low water activity (a_w) which suggests that they would not support the growth of pathogens. However, in recent years outbreaks linked to LMF have significantly increased and the preponderance of outbreaks are linked to *Salmonella* contamination (Finn et al., 2013). This pathogen is known to be present in raw ingredients including wheat grain and wheat flour and can survive for periods in harsh, dry conditions (Podolak et al., 2010; Van Doren et al., 2013). Wheat flours have been increasingly implicated in severe foodborne illness outbreaks, and food recalls (Forghani et al., 2020). In the past years, at least eight foodborne illness outbreaks linked to the consumption of contaminated wheat flour, or its products have been identified in the U.S. and Canada alone; these events have also led to several recalls wherein the presence of pathogens such as Shiga toxin-producing *E. coli* (STEC) and *Salmonella* have been cited as the cause of the recall (Jung & Harris, 2022). The recently reported outbreak was in 2018 in a popular cereal brand involving *Salmonella* Mbandaka, which lead to 135 cases of infection with 34 people being hospitalized (CDC, 2018).

Wheat, being a raw agricultural commodity, is prone to microbial contamination not just in the field, but throughout the supply chain. As a result, enteric pathogens such as *Salmonella* can contaminate the grains in both pre and/or post-harvest stages presenting a public health threat (Sabillón et al., 2020). Most of the wheat microorganisms, according to Laca et al., (2006), are found on its surface. However, mechanical injury to the kernel allows entry of microorganisms

into the grain endosperm. In this sense, wheat debranning prior to milling could significantly reduce the potential microbial contamination in the wheat flour. However, debranning would need further modifications in the milling process which affects its feasibility as an intervention. Pathogens that may be present in grains are more easily redistributed into milling fractions, including wheat flour, upon milling. This was demonstrated in the studies conducted by Rivera et al., (2022) & Sabillón et al., (2020), who reported that milling leads to redistribution of pathogenic microflora into the milling fractions.

Considering the increasing food safety issues linked to wheat flour, numerous investigations have been conducted to possibly lower the pathogen load of the wheat before milling. Several antimicrobial interventions have been introduced such as use of the salt, and chlorine, including non-thermal treatments such as ozone, atmospheric plasma, organic acid, and pulsed electric field (Aron Maftai et al., 2014; Dhillon et al., 2007; Ibanoglu, 2001 and Los et al., 2018); However, these interventions significantly increased the moisture content, requiring the addition of a drying step as an additional unit operation, which increases the milling industry's financial burden. Additionally, these interventions were said to have had a considerable impact on the physical, chemical, and functional characteristics of the wheat flour (such as altered sensory properties, decreased pH of the wheat flour, and change in the gluten strength), which in turn affect how well the flour performed in baking. In addition, use of the high acid concentrations might negatively impact the milling equipment leading to the corrosion of metal surfaces (Rivera et al., 2021).

Sodium bisulfate (SBS, NaHSO_4), referred to as bisulfate of soda, sodium acid sulfate, or sodium hydrogen sulfate, emerged as an acidulant in 1988 (Pszczola, 2007). It has since gained recognition as a Generally Recognized as Safe (GRAS) food preservative by FDA (FDA, 2020).

Its GRAS designation indicates that no pre-market review is needed prior to its use and that it is safe to be used in accordance with Good Manufacturing Practices (GMP). The Environmental Protection Agency of the United States has also recently designated SBS as a "Safer Choice" antimicrobial and processing aid or additive (EPA, 2020). SBS possesses a pKa of 1.99 (Weeraratne et al., 2021) and exhibits acidity like phosphoric acid (Pszczola, 2007). In its role as a potential antimicrobial wash solution, research has showcased its efficacy in reducing *E. coli* O157:H7, *L. monocytogenes* and *S. Enteritidis* on whole chicken drumsticks (Weeraratne et al., 2021; Bodie et al., 2019; Dittoe et al., 2019). With this background, the present study aimed to determine the efficacy of SBS in reducing *Salmonella* in wheat during tempering and assess its impact on flour milling, functionality, and baking quality. The results from this study can be used to address *Salmonella* contamination in wheat milling thereby improving the food safety of wheat flour and its products.

5.2. Material and Methods

Organic hard red spring (HRS) wheat was used for the present study (purchased from Great River Organic Milling Company, Arcadia, Wisconsin, USA) as use of conventionally grown wheat might introduce extraneous compounds (fertilizers, pesticides, and herbicides) that could confound the study's findings. SBS used for the present study was provided by Jones-Hamilton Co. (Walbridge, OH, USA). All the media and other chemicals were purchased from Fisher Scientifics, Illinois, USA unless mentioned otherwise.

5.2.1 Bacterial strains

Four frozen (-80°C) isolates of *Salmonella enterica* of different serotypes (Newport - ATCC 6962, Typhimurium - ATCC 14802, Kentucky - ATCC 9263, and Enterica - ATCC 8326) were procured from the Feed Toxicology and Microbiology Laboratory, Kansas State University

(Manhattan, KS) for the current study. The bacterial stock cultures were kept at -80° C in tryptic soy broth supplemented with 30% glycerol until further use. Selected strains were previously linked to foodborne illness outbreaks and/or recalls. The cultures were prepared and used as individual strains or as cocktails in the experiments conducted in the current study.

5.2.2 Inoculum preparation

The inoculum for the present study was prepared in accordance with the procedure outlined by Rivera et al., (2021). In short, the frozen bacterial stock cultures maintained at -80 °C were revived by streaking on tryptic soy agar (TSA) plates followed by propagation onto xylose lysine deoxycholate (XLD) plates and incubation (24 h at 37 °C). A single well-isolated black colony of individual strains was inoculated into individual tryptic soy broth tubes (TSB;10 ml) followed by incubation (24 h at 37 °C). Centrifugation (2500 x g, 10 mins; Sorvall X1R, Waltham, MA, USA) was performed to collect the cell pellet that was further inoculated into a sterile TSB medium (600 ml). Following the incubation (24 h at 37 °C), the cells were pelleted down again by centrifugation; harvested cells were washed (2x) in PBS and resuspended in sterile phosphate-buffered saline (PBS). An equal amount of cell suspension from each serovar prepared was combined to make the working culture/inoculum for the experiment ($\approx 7 \log$ CFU/ml). The prepared inoculum was later used for inoculating the wheat kernels.

5.2.3 Minimum Inhibitory Concentration (MIC) of SBS

A standard micro broth dilution method as per Clinical and Laboratory Standard Institute (CLSI) was performed to evaluate the MIC of SBS against 4 different strains of *Salmonella*. A two-fold serial dilution of the SBS stock was prepared in fresh TSB and freshly prepared inoculum with a cell density of $\approx 7 \log$ CFU/ml was added into a 96-well microtiter plate. For the control wells, only culture and media were added. The plates were incubated at 37° C for 24 h under static

conditions followed by serial dilution and plating on TSA plates. The percentage survival was determined by dividing the number of CFU recovered after SBS treatment by the number of CFU recovered from untreated controls. The MIC was expressed as the lowest SBS concentration that impedes the growth of *Salmonella* after incubation.

5.2.4 Reactive Oxygen Species (ROS) measurement

A 2', 7' -dichlorofluorescein diacetate (DCFDA) assay was used to measure the ROS generated as described earlier by Shivaprasad et al. (2021). Bacterial cells treated with or without SBS were incubated at 37 °C/4 h. The cells were harvested by centrifugation at 2795× g at 4 °C/10 min. Each supernatant was later incubated for 1h at 37 °C with 10 mM DCFDA dye. The amount of ROS produced was measured at $E_x=485$ nm and $E_m=520$ nm using an LS50B Luminescence Spectrometer (PerkinElmer, Waltham, Massachusetts, USA).

5.2.5 Preparation and application of inoculum and tempering solution

The wheat kernels were autoclaved (121°C for 15 minutes) to minimize the natural microflora before the tempering experiment. The wheat kernels were wrapped in aluminum foil and placed in a tin container before autoclaving. The moisture increase was very minimal thus avoiding the chances of gelatinization of wheat kernels. The cooled wheat kernels (room temperature) were later used for the experiment. The moisture content of the sterile wheat was determined according to the American Society of Agriculture Engineers (ASAE) 352.2 method.

Sterile wheat grains (200 g) were tempered to 17% moisture using a tempering solution (13.66 ml), which was calculated based on the initial moisture content of the wheat (11.33%). SBS was applied as a tempering solution to yield a concentration of 0.32, 0.625, 1.0, 1.25, and 1.5% SBS. The SBS solution concentration required to achieve the SBS tempering concentration was calculated using the equation below (Rivera et. al., 2021). The final tempering conditions (final

moisture 17% and resting time 24 h) were selected to mimic the standard industrial practice (Alfin, 2020; Dubat & Bock, 2019; Değirmencilik, 2014).

$$\%SBS \left(\frac{w}{v}\right) = \frac{(\%SBS_{wheat\ basis}) \times (Weight_{tempered\ wheat})}{Volume_{tempering\ solution}}$$

The sterile wheat (200 g) was later spray inoculated with the prepared *Salmonella* cocktail (section 2.2) (6.83 ml) using a sterile bottle sprayer (cleaned by washing 2x with 70% ethanol and sterile water) thus yielding a concentration of ≈ 7 log CFU/g. A resting period of 30 min was provided to ensure cell attachment to the wheat kernels. Following this SBS solution (6.83 ml) was spray inoculated using a sterile sprayer giving a desired final concentration (0.32, 0.625, 1, 1.25, and 1.5%) of SBS. The metal container was then closed, sealed, and shaken vigorously for 3 min to ensure proper distribution of the tempering solution. Wetted kernels were then held at 25 °C for 24 h for conditioning.

5.2.6 Enumerating *Salmonella* load of wheat during tempering

During tempering, 25 g of tempered wheat samples under each treatment were aseptically drawn at various time intervals (0, 0.5, 2, 6, 12, 18, and 24 h). Samples were homogenized for 120 s in a stomacher bag (VWR, Radnor, PA, USA), using 225 ml of sterile buffered peptone water. The homogenate was serially diluted with 0.1% peptone water and appropriate dilutions were spread-plated (100 µl, duplicates) on XLD plates with a TSA overlay for enumeration before incubation (37 °C for 24 h). Enumerated *Salmonella* colonies were then expressed as log CFU/g and reductions were estimated as the difference between the *Salmonella* load at each of the chosen time points and at time zero (before tempering).

5.2.7 Experimental milling

5.2.7.1 Tempering

Based on the reductions observed in the inoculated wheat tempering experiment, effective SBS concentrations were selected, and assessed for their impact on flour quality. In this part of the study, non-inoculated wheat was used. The amount of tempering solution was calculated based on the initial moisture content of the wheat (11.33%) and the target wheat moisture for tempering (17%). Tempering solutions were prepared similarly to that of the inoculated wheat tempering experiment (section 2.5). The tempered wheat kernels (1500 g) were mixed in a rotary mixer for 30 min after dispensing the prepared tempering solutions. Tempered kernels were transferred into re-sealable bags and held at ambient conditions for 24 h prior to milling. Wheat kernels tempered with distilled water were maintained as the control treatment. The wheat tempering experiments were conducted in triplicates.

5.2.7.2 Milling

A lab-scale roller mill (Chopin LabMill, Chopin, France) was used for milling the tempered wheat. The equipment was cleaned (vacuum cleaning) and sanitized (wiping with 70% ethanol) before milling each sample. The wheat milling process was done with 6 roller milling steps (2 breaks, 1 sizing, and 3 reduction steps). A feed rate of 5 g/s was used for the first break step while a feed rate of 2.5 g/s was maintained for the subsequent milling steps. The obtained flour fractions were mixed (break, sizing & reduction flour) in re-sealable bags to produce the straight-grade flour for quality evaluation. The milling yield was calculated using the below formula (Shivaprasad et al., 2022). Wheat flours produced were refrigerated (≈ 4 to 6°C) until further analysis.

$$\text{Milling Yield (\%)} = \frac{\text{Weight of milling fraction}}{\text{Weight of wheat}} \times 100$$

5.2.8 Physico-chemical analysis of wheat flour

5.2.8.1 Particle size analysis

The particle size distribution of the wheat-milled samples was assessed using a vacuum sieve, Alpine jet sieve (Hosokawa Micron, E200 LS). Roughly, 100 g of the wheat flour was sieved through sieves (32, 53, 75, 90, 125, 150, 180, and 212 μm) in ascending order.

5.2.8.2 Compositional analysis

To determine any variations in the composition of wheat flours milled from SBS - treated and untreated wheat, a proximate analysis of wheat flour was carried out through an external laboratory. Moisture analysis of the wheat flour was performed in accordance with standard ACC 44-15.02 (AACC, 2010). As for the other proximate composition, analysis was done according to AOAC procedures - AOAC 922.06 (crude fat), AOAC 962.09 (crude fiber), AOAC 923.03 (ash), and AOAC 984.13 (crude protein). Carbohydrate content was determined by calculation ($\text{Carbohydrate}\% = 100\% - (\text{moisture}\% + \text{protein}\% + \text{fat}\% + \text{ash}\%)$).

5.2.8.3 Gluten quality, and solvent retention capacity (SRC)

Wet, dry, and gluten index of the wheat flours were evaluated using a Glutomatic device (Perten Instruments, USA) in line with AACC method 38-12.04. On the other hand, the AACC technique 56-11.02 was used to analyze the SRC profiles of the wheat flours using four solvents (50% sucrose, 5% Na_2CO_3 , 5% lactic acid, and deionized water).

5.2.8.4 pH measurement

The pH of the tempering solution, wheat samples at the beginning (30 min) and at the end of tempering (24 h) including the wheat flour was measured (Rivera et al., 2021).

5.2.8.5 Rheological, and pasting properties of wheat flour

The rheological properties of flour samples were evaluated using a MixoLab (Chopin Technologies, France) as per AACC method 54-60.01 under the “Chopin +” protocol. The pasting

properties of the wheat flours were evaluated using an RVA instrument model 4500 (Perten Instrument, Australia) as per AACC method 76-21.02.

5.2.8.6 Baking characteristics

The bake test was performed as bread loaf according to the AACC method 10-10.03 method. Briefly, 100% flour was blended with a mixture of sugar (6%), salt (1.5%), yeast (5.3%), shortening (3.0%), and water (% based on absorption from Mixolab) for 10 min. The dough was fermented (90 min at 37 °C & 85% RH) before sheeting and molding into a baking pan. This is followed by proofing (33 min at 37 °C & 85% RH) and baking at 420 °F for 20 min. The breads produced were cooled and analyzed for bread volume, c-cell characteristics, and texture analysis as described in the methods by Rivera et al. (2021) and Shivaprasad et al. (2022).

5.2.9 Data analysis

In all cases, unless otherwise specified, three experimental replications were conducted. The measured responses were expressed as mean $\pm$ standard deviation (SD). Significant differences among treatment means were determined using paired *t*-test at a significance level of $p < 0.05$ using SAS Statistical Software 9.3 (SAS Institute, Cary, NC).

5.3. Results and Discussion

5.3.1 Antimicrobial activity of SBS on *Salmonella*

Minimum inhibitory concentration (MIC) is defined as the lowest antimicrobial concentration that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). This study examined the antimicrobial activity of SBS against 4 different *Salmonella enterica* serovars (**Figure 5.1**). Notably, we found a MIC of SBS at 0.15625% concentration that inhibited *Salmonella enterica* serovars. Additionally, lower concentrations of SBS exhibited a concentration-dependent effect on the growth of *Salmonella* bacterium, with a

99% reduction (MIC₉₉) observed at 0.078% concentration. The findings by Micciche et al., (2019) who demonstrated a MIC of 0.375% SBS against *S. Typhimurium*, are supported by the observed data in this study. In a similar study by Laranja et al. (2021), MIC of 1.5% SBS was reported for the *S. enterica* serovars alone and in combination (*S. Minnesota* 7,301,007, *S. Typhimurium* L12031, *S. Saintpaul*, *S. Heidelberg* 22,295, and *S. Enteritidis* SE86) which is high compared to the current study. The observed difference can be attributed to the variation in the culture type, experimental conditions, cell denseness, and test concentrations.

Table 5.1: Representative pH values determined before and after tempering

Treatment (%SBS)	pH (mean ± Standard Deviation)			
	pH ¹	pH ²	pH ³	pH ⁴
0%	6.95±0.02 ^A	6.88±0.01 ^A	6.95±0.02 ^A	6.60±0.02 ^A
1.50%	0.85±0.00 ^B	3.13±0.02 ^B	3.12±0.00 ^B	5.32±0.01 ^B
P value (p<0.05)	<0.0001	<0.0001	0.0413	0.0002

Note: Values were expressed as means ± standard deviation from 3 replicates; means with a different letter in the column are significantly different ($p < 0.05$). pH¹ corresponds to pH of the tempering solution; pH² corresponds to pH of the wheat at the beginning of tempering (30 min); pH³ corresponds to pH of the wheat at the end of tempering (24 h); pH⁴ corresponds to pH of the wheat flour upon milling.

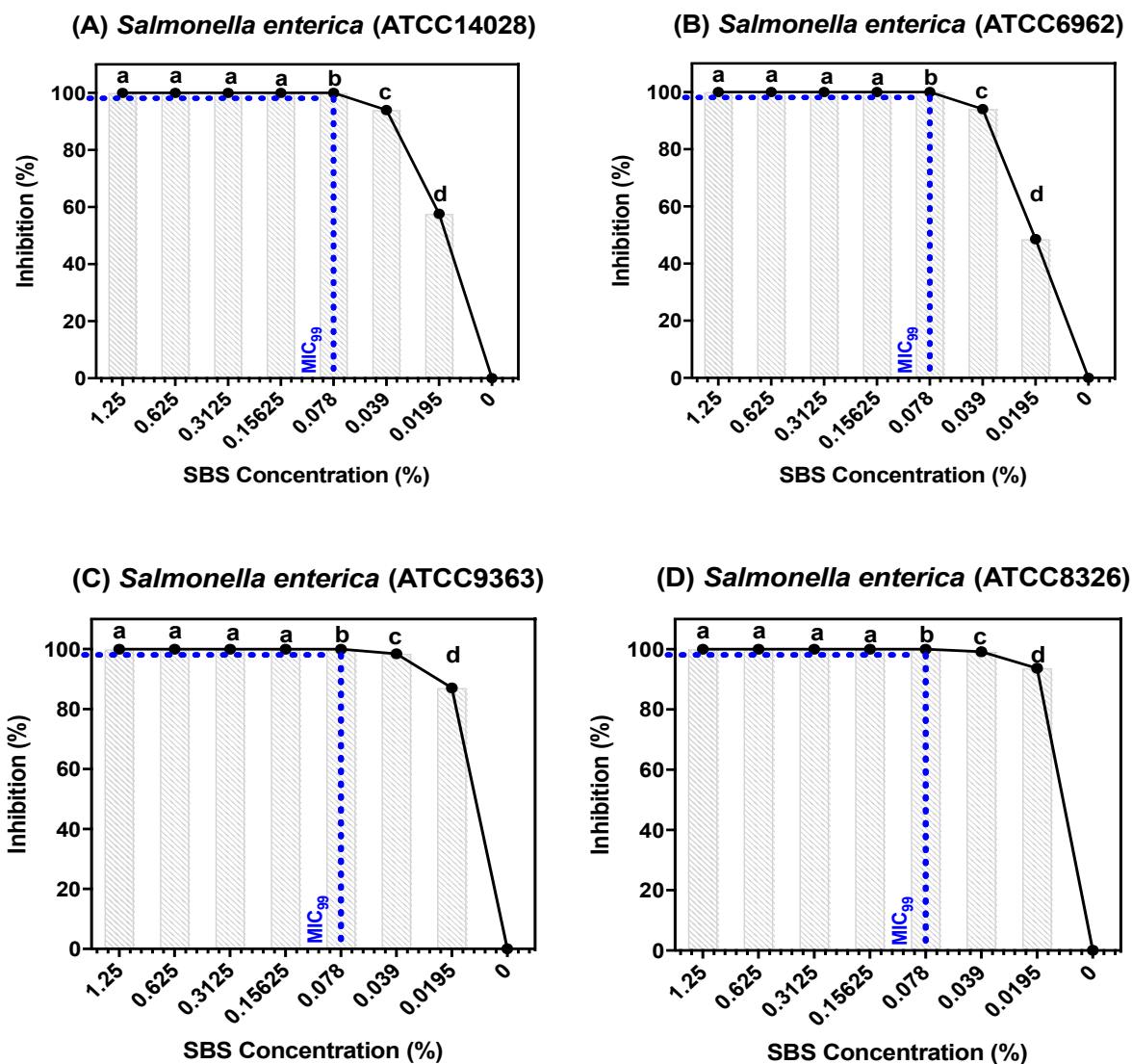


Figure 5.1: Antimicrobial potency of sodium bisulfate on *Salmonella enterica* serovars. (A) *Salmonella typhimurium* (ATCC 14028); (B) *Salmonella enterica* (*Salmonella enterica* subsp. Enterica ser. Newport (ATCC 6962); (C) *Salmonella enterica* serovar Kentucky (ATCC 9263); (D) *Salmonella enterica* subsp. Enterica (ATCC 8326). Means $\pm$ Standard deviation from 2 replicates are plotted. Means with a different letter are significantly different ($p < 0.05$).

5.3.2 Bactericidal mechanism of SBS

Efficient maintenance of the pH homeostasis in a bacterial cell and its environment is critical for its survival (Francois & Kappock, 2007). Therefore, a decrease in the pH of the bacterial environment can have lethal effects on the cell as acidic pH is known to generate ROS which damages the cell's genetic material thus leading to its lysis (Bruno-Bárcena et al., 2010; Kim et al., 2006; Lund et al., 2020; Schellhorn & Stones, 1992). The present study hypothesized that the decrease in pH (**Table 5.1**) and subsequent generation of ROS was responsible for the antibacterial activity of SBS. To prove this, intracellular accumulation of ROS in the bacterial cell was evaluated by measuring the fluorescence intensity using the DCFDA assay. The results showed that the SBS treated *Salmonella* cocktail resulted in a 4.5-fold increase in fluorescence in contrast to the control (**Figure 5.3a**). Several studies have reported that oxidative stress caused by the production of reactive oxygen species and free radicals causes disintegration of the cellular membrane, disruption of biosynthesis pathways, disruption of iron inflow, DNA damage, and iron-sulfur cluster eventually killing the microbes (Ghosh et al., 2019; Shivaprasad et al., 2021). Additionally, Rivera et al. (2021) in their recent finding observed that as the concentration of SBS increased, there was a corresponding enhancement in the reduction of STEC load. According to their findings, this reduction can be attributed to the increased acidity and osmolarity resulting from the increased concentration of SBS within the tempering solution. Furthermore, the hygroscopic properties of SBS played a significant role in enhancing the reduction process by facilitating the drying of the kernel surface. Further studies on evaluating the effect of SBS on the ROS-neutralization pathways or components in food borne pathogens could be worthwhile to fully understand the lethality of acid treatment against food borne pathogens. A detailed mechanism depicting oxidative stress induced by ROS accumulation in bacterial cell is illustrated in **Figure**

5.3b. It should be noted that the antimicrobial response observed in the present study, being laboratory-based, may not reflect contamination events occurring in the field, where the *Salmonella* cells would likely be desiccation-adapted, attached to wheat kernels for a longer duration, and protected within surface irregularities and organic residues. Desiccation adaptation has provided cross-tolerance of *Salmonella* cells to numerous environmental stresses and limitations of chemical treatment (Wang et al., 2021; Gruzdev et al. 2011). Cells of desiccation adaptation up-regulated oxidative stress resistance genes (e.g., *cysM*, *pspE*), and formed dry surface biofilms with an extracellular polymeric matrix, which could physically hinder the penetration of acidified SBS solution (Wang et al., 2021; Lin et al., 2024). Additionally, differences in inoculum preparation and drying time prior to wheat tempering have been reported to influence pathogen reduction, with dried, low-moisture-adapted cells being more representative of the contamination conditions encountered on wheat grain (Lin et al., 2025). While increased intracellular ROS presents a clearly observable response in this laboratory study, future validation should be conducted using aged, desiccated wheat-associated inocula in pilot or commercial wheat-tempering conditions.

5.3.3 Effect of SBS on *Salmonella* load of wheat during tempering

SBS is a GRAS food additive commonly used as a pH control agent in beverages and confections, leavening agents in cake mixes (Pszczola, 2007), and anti-browning agent (Calder et al., 2012; Xie et al., 2017). The current study assessed the reduction in the *Salmonella* load of the wheat when SBS was added to the tempering water used (**Figure 5.2b**).

We believe that this is the first investigation into the effectiveness of SBS against *Salmonella* in wheat grains. By tempering wheat with lethal doses of SBS (0.3, 0.6, 1, 1.25, and 1.5%) at predetermined intervals, the time-kill kinetics were ascertained. The schematic

representation of the experimental setup is shown in **Figure 5.2a**. The study revealed that the bactericidal activity of the SBS began early on with a 1.13 log CFU/g reduction at the lowest concentration (0.3%) after 0.5 h of tempering. The reductions observed increased with higher SBS concentration used with a 2.29 log CFU/g reduction observed from the highest concentration (1.5% SBS) used. Following the tempering process (24 h), the use of 1.5% SBS concentration yielded an average reduction of 4.3 log CFU/g in the wheat *Salmonella* load. The reduction in the *Salmonella* load can be attributed to the ROS production, by virtue of which an inhibition in the growth was observed. Further, the produced ROS cause oxidative damage thus resulting in the lysis of the bacteria as mentioned in the **Figure 5.3**.

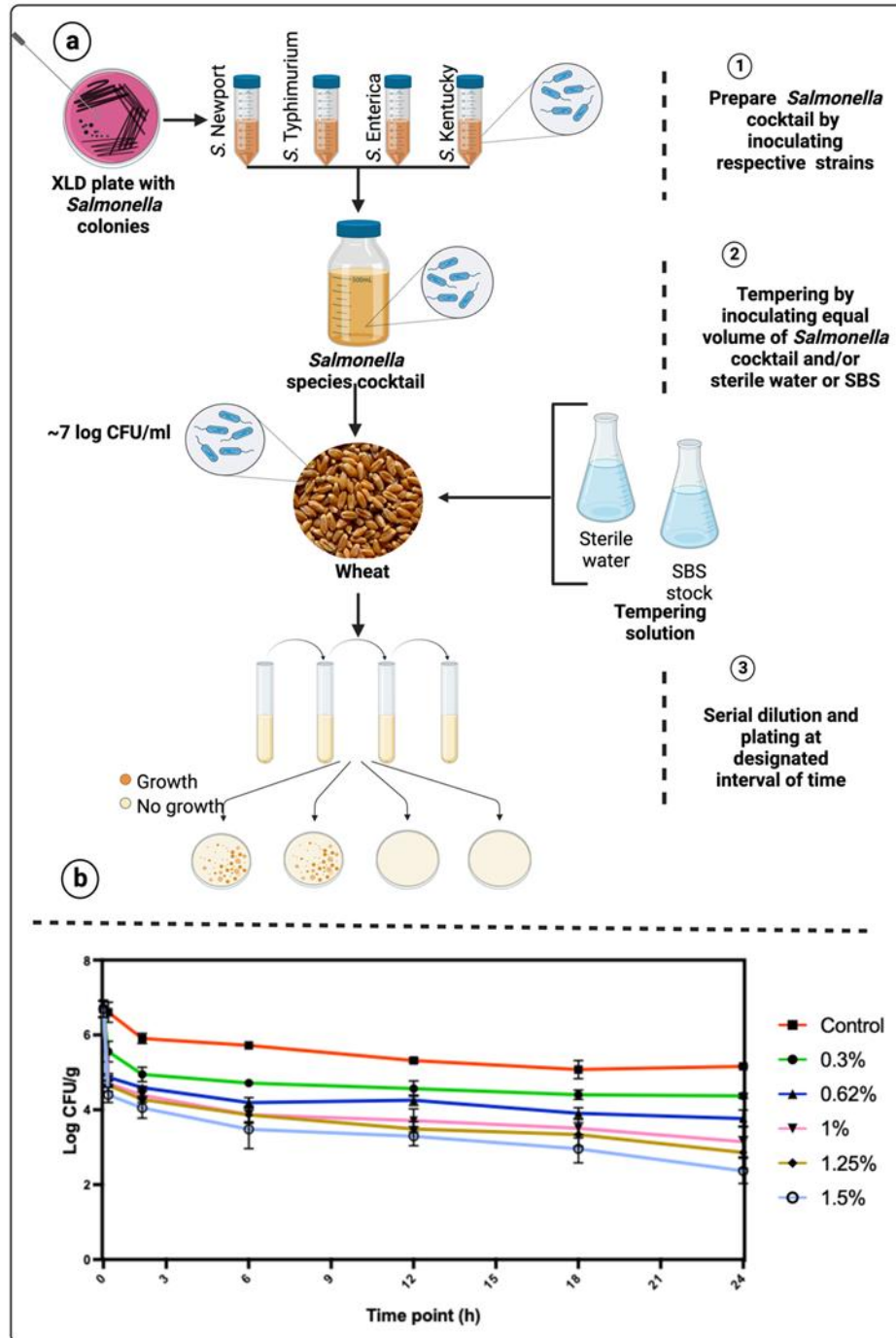


Figure 5.2: Survival of *Salmonella* serovars in wheat. (a) Experimental design ensued to test the efficacy of sodium bisulfate against *Salmonella* serovars in wheat when applied as a tempering solution. (b) Time-kill kinetics of sodium bisulfate against *Salmonella* serovars. Means $\pm$ Standard deviation from 2 replicates are plotted. Means with a different letter are significantly different ($p < 0.05$).

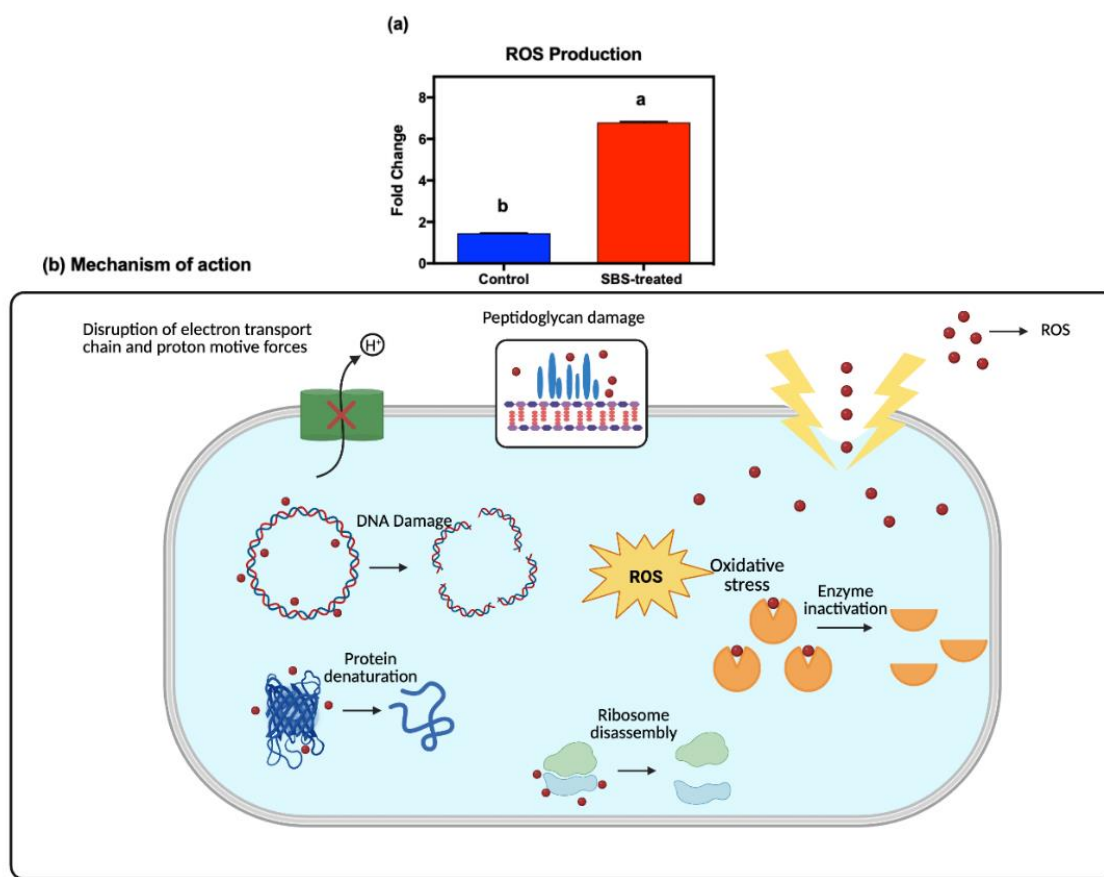


Figure 5.3: Bactericidal mechanism of sodium bisulfate. (a) ROS measured using DCFDA assay. (b) Schematic representation of bactericidal mechanism of SBS as a result of oxidative stress induced by acidic pH. Means $\pm$ Standard deviation from 3 replicates are plotted. Means with a different letter are significantly different ($p < 0.05$).

The observed reduction is consistent with the results published by Rivera et al. in (2021), who observed a 3.5 log decrease in the *E. coli* O121 and O26 population when 1.25% of SBS was used. The observed differences can be attributed to the differences among the bacterial culture used, the density of the bacterial population, the type of wheat used, and the methodologies adopted. An additional study by Pineda et al., (2021) revealed a significant decrease in the *Salmonella* Heidelberg biofilm by 2.16 and 1.04 logs when exposed to SBS at a pH of 3.5 for

8 and 24 h respectively. In a recent study by Chen et al. (2022), SBS solution at a concentration of 1.5% w/v was used as an alternative sanitizer for washing cherry tomatoes. Their findings revealed that soaking cherry tomatoes in SBS solution (1.5% w/v) for 1 min can lead to complete decontamination of the *E. coli* O157:H7 thus enhancing produce safety.

5.3.4 Flour quality evaluation

Antimicrobial interventions that yield at least a 3-log CFU/g reduction in pathogen loads after treatment are preferred in the milling industry (Rivera et al., 2021). The impact of SBS tempering (1.5%) on wheat flour functionality was assessed as it yielded the maximum reduction in the wheat *Salmonella* load among all the tested concentrations.

The milling yields (% as-is) for bran, shorts, and straight-grade flour are presented in **Table 5.2**. The moisture content of the wheat flour treated with 0% (control) and 1.5% of the SBS concentration was within the legal limit as stipulated by FDA (21CFR137.200). Bran yields in SBS treated (1.5%) wheat decreased ($p < 0.05$) compared to untreated (0%). This was contrary to the yield of the shorts wherein SBS-treated wheat had higher yields relative to the untreated sample. A similar trend was observed by Rivera et al., (2021) who explained that SBS tempered wheat induces bran friability which leads to an increase in the shorts fraction. In terms of individual flour yields, the SBS treatment yielded higher sizing ($p < 0.05$) flour yield compared to the control sample, although the straight-grade flour yield for both treatments was similar ($p > 0.05$).

The particle sizes distribution (**Figure 5.4**) of both SBS – treated and untreated milled fractions were between 50 and 180 μm which satisfies the regulatory requirements with most particles size (>98%) to be < 212 μm (21CFR137.105). Additionally, a bimodal distribution of particle size was observed in both cases (treated and untreated wheat flour) which corroborates the results reported by Rivera et al., (2021) and Nakamura, (2015).

Table 5.2: Milling yield of tempered wheat

Milling fractions	Treatments (%SBS)		P value (p<0.05)
	0%	1.50%	
Bran	11.00 ± 0.68 ^A	8.83± 0.22 ^B	0.0408
Shorts	9.34± 0.54 ^B	10.42±0.07 ^A	0.0788
Break flour ¹	21.86 ± 0.07 ^A	21.34± 0.43 ^A	0.2956
Sizing flour ²	9.064 ± 0.28 ^B	10.25± 10.25 ^A	0.0227
Reduction flour ³	33.46 ± 0.47 ^A	33.06± 0.22 ^A	0.6765
Straight grade flour ⁽¹⁺²⁺³⁾	64.39 ± 0.76 ^A	64.65 ± 0.69 ^A	0.0511
Nutritional characteristics			
Moisture (% wet basis)	13.41± 0.19 ^A	13.2 ± 0.19 ^A	0.2204
Crude protein (%)	14.77±0.05 ^A	14.92±0.07 ^A	0.0164
Crude fat (%)	nd	0.11±0.16	0.0422
Crude fiber (%)	0.17±0.04 ^B	0.32±0.04 ^A	0.1235
Ash (%)	0.58±0.00 ^B	0.77±0.00 ^A	0.0028
Carbohydrate (%)	71.23±0.24 ^A	70.99±0.16 ^A	0.3707
Total starch (%)	51.64±3.35 ^A	49.88±1.32 ^A	0.6409
Damaged starch (UCD)	15.83±1.75 ^A	16.83±1.26 ^A	0.5458
Falling number (s)	362±9.41 ^A	318±2.05 ^B	0.1066

Note: Carbohydrate = 100%- (moisture%+protein% +fat% + ash%). Values were expressed as means ± standard deviation from 3 replicates; means with a different letter in the row are significantly different ($p < 0.05$);

Abbreviation: nd= not detected

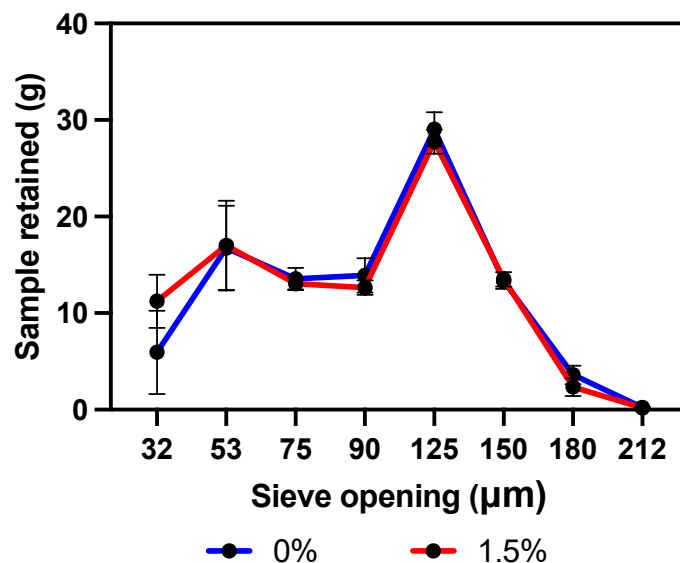


Figure 5.4: Particle size distribution of wheat flour treated with sodium bisulfate (%). Means $\pm$ Standard deviation from 3 replicates are plotted.

The proximate composition of both treated and untreated wheat flour is presented in **Table 5.2**. The proximate compositions of the flours were also similar except for crude fat and ash content ($p < 0.05$) wherein the wheat flour from SBS tempered wheat had higher fat and ash content. Both treated and untreated wheat flour were found to have similar moisture contents, measuring $13.41 \pm 0.19\%$ and $13.2 \pm 0.19\%$, respectively ($p > 0.05$), which is within the legal limit stipulated by FDA (21CFR137.200). Even though there was a considerable difference in the crude fat and ash contents, the differences observed are practically similar in industrial settings. Ash is an important attribute of flour as it denotes the extent of bran contamination in the flour which can bring negative effects to its functionality (e.g., inhibit gluten formation) (Best et al., 2023). Changes in the flour milling yields could be attributed to the bran contamination as there is a limit on how much endosperm could be extracted from the grains. As a result, any increase in yield can be linked to the bran particles transferring into the flour, which results in higher ash content. As the flour

yields were similar ($p > 0.05$), the higher ash content from the SBS treatment can be attributed to the increased salt concentration brought by using SBS. These findings and those by Rivera et al. (2021) were similar. As for the starch composition, lower total starch, and higher damaged starch ($p < 0.05$) were observed for the wheat flours from the treated wheat although the differences were not significant. Damaged starch is an important parameter for wheat flour as it influences the flour baking performance. Barak et al., (2014) reported that high damaged starch content will have a substantial effect on the quality of baked cookies as the damaged starch promotes moisture absorption resulting in dough stiffness affecting cookie spread and diameter. In terms of falling number (FN) value, both treated and untreated flour samples had FN values above 350 s which indicates minimal amylase activity. However, tempering with SBS resulted in a lower ($p < 0.05$) FN value compared to the control. This can be attributed to the lower pH of the flour (**Table 5.1**) as lower flour pH can reduce viscosity (Rivera et al., 2021; Suo et al., 2022).

Flour functionality was assessed, and the results are indicated in **Table 5.3**. Wheat flour gluten index (GI) is a criterion defining gluten quality as weak ($GI < 30\%$), normal ($GI = 30\text{--}80\%$), or strong ($GI > 80\%$) (Oikonomou et al., 2015). It is an important quality test as gluten is one of the major flour components responsible for wheat bread-making properties as it forms the bread structure. Similar ($p > 0.05$) wet gluten (36.43 to 37.7%), dry gluten (13.6 to 14.53%), and gluten index (94.12 to 94.78%) values were observed for the wheat flours indicating that SBS tempering did not impact the gluten quality. The high gluten index values measured for both flours ($> 90\%$) demonstrate a strong gluten formation. The wet and dry gluten values were satisfactory as recommended minimum values for wheat flours are 24 and 8% respectively (Singh, and Singh, 2006).

Table 5.3: Physicochemical characteristics of wheat flour

Treatment variables	Treatment (%SBS)		P value (p<0.05)
	0%	1.50%	
Rapid Visco analyzer (RVA) characteristics			
Peak viscosity (RVU)	1992.5±88.50 ^A	2148±30.00 ^A	0.1474
Trough (RVU)	1509±70.00 ^A	1587.5±73.50 ^A	0.6813
Breakdown (RVU)	483.5±18.50 ^A	560.5±43.50 ^A	0.1999
Final viscosity (RVU)	2385.5±102.50 ^A	2430±78.00 ^A	0.3204
Setback (RVU)	876.5±32.50 ^A	842.5±4.50 ^A	0.4386
Peak time (min)	6.4±0.00 ^A	6.5±0.03 ^A	0.1855
Pasting temperature (°C)	89.15±0.45 ^A	89.17±0.47 ^A	0.9828
Solvent retention capacity (SRC)			
Water	79.71±3.80 ^A	79.55±3.57 ^A	0.9775
50% sucrose	110.24±4.21 ^A	113.52±1.51 ^A	0.2266
5% Lactic Acid	156.67±2.15 ^A	93.79±5.44 ^B	0.0063
5% sodium carbonate	87.88±3.76 ^A	85.55±3.63 ^A	0.2846
Gluten Properties (%)			
Wet gluten	37.7±0.14 ^A	36.43±2.43 ^A	0.5589
Dry gluten	13.6±0.26 ^A	14.53±0.55 ^A	0.2662
Gluten Index	94.78±0.68 ^A	94.12±3.76 ^A	0.3966
Mixolab characteristics			
Water absorption (%)	55.3 ± 0.00 ^B	57.26±0.04 ^A	0.0280
Development time (min)	8.51±0.17 ^A	8.85±0.97 ^A	0.0002
Stability (min)	11.16±0.12 ^A	9±0.08 ^B	0.0002
Amplitude (Nm)	0.07±0.02 ^A	0.07±0.01 ^A	0.8928
C1 (Nm)	1.132±0.01 ^A	1.125±0.01 ^A	0.7542
CS (Nm)	1.12±0.01 ^A	1.11±0.01 ^A	0.8348
C2 (Nm)	0.53±0.00 ^A	0.52±0.00 ^A	0.3397
C3 (Nm)	1.03±0.16 ^A	1.16±0.03 ^A	0.4317
C4 (Nm)	1.71±0.02 ^B	1.79±0.01 ^A	0.0120
C5 (Nm)	2.59±0.09 ^A	2.7±0.03 ^A	0.3265

Note: Values were expressed as means ± standard deviation from 3 replicates; means with a different letter in the row are significantly different ($p < 0.05$).

In addition to the gluten quality, the solvent retention capacity (SRC) profiles of the wheat flours were established to evaluate the effects of SBS on different flour components. Similar ($p > 0.05$) SRC profiles for water, 50% sucrose, and 5% sodium carbonate was observed although the 5% lactic acid SRC (LA SRC) of the SBS wheat flour was lower ($p < 0.05$) than the control. The decrease in the 5% LA SRC value can be attributed to the added SBS, as the addition of acid can disaggregate the gluten matrix and reduces the degree of cross-linking in the protein network. This result corroborates the findings from Gomez-Martinez et al., 2009 and Chabrat et al., 2012. This might be concerning because a low glutenin content can adversely affect the baking quality of bread. Ideally, a 5% LA SRC value of $\geq 100\text{g}/100\text{ g}$ is desirable for bread making (Gaines 2000; Duyvejonck et al., 2012). Each solvent used in the SRC test reflects the quality of flour polymers including gliadin and pentosans (sucrose), damaged starch (sodium carbonate), and gluten (lactic acid) (Labuschagne et al., 2021). Water SRC reflects the flour water holding capacity which is the function of all flour polymers. Based on the SRC profiles, SBS tempering did not affect the water-holding capacity, gliadin, arabinoxylans, and damaged starch quality; however, SBS tempering resulted in lowered gluten strength based on lactic acid SRC.

The RVA profiles of the wheat flours are presented in **Table 5.3**. Flour RVA profiles reflect the flour pasting behavior (in slurry form) under heating/ cooling, and mixing stresses. Similar ($p > 0.05$) RVA values were found for both wheat flours tested. The SBS - treated flour had higher peak viscosity (2148 RVU) than the control indicating higher starch swelling capacity compared to untreated (1992.5 RVU). The observed difference can be attributed to the difference in the pH levels between the SBS-treated and control samples, as lower pH levels are known to correlate with higher peak viscosity. Specifically, the SBS-treated flour exhibits a lower pH (5.32) in comparison to the control (6.60); however, the viscosity observed contradicts the data obtained

from the Mixolab analysis. This inconsistency could be attributed to the different conditions under which the RVA and Mixolab tests were conducted. Mixolab test, the examined matrix is a dough with limited available water for starch gelatinization, while the RVA experiments use a suspension with sufficient water for starch gelatinization, as highlighted in previous studies (Bucsella et al., 2016; Chen et al., 2020). A similar change was observed in a study by Zhou et al., (2012) who reported an increased peak viscosity upon addition of phosphate salts to the wheat flour suspension. Breakdown viscosity measures how much the gelatinized starch granules collapse when heated and stirred continuously, resulting in a reduced viscosity. The SBS - treated wheat flour had higher breakdown viscosity in comparison to the untreated wheat flour. Setback viscosity represents starch retrogradation (the difference between trough and final viscosity) and final viscosity refers to the ability of the flour to form a viscous slurry after cooling. The results revealed that treated flour had a comparable setback and final viscosity to the control. The obtained RVA profiles revealed that SBS tempering did not significantly alter the pasting properties of wheat flour. The mixolab characteristics of both wheat flours are shown in **Table 5.3** and these reflect the dough consistency under constant mixing and heating conditions. In terms of mixing properties, significant ($p < 0.05$) but small differences in water absorption, development time, and stability was observed for both wheat flours. The increase ($p < 0.05$) in the water absorption of the SBS-treated wheat flour can be attributed to the structural weakening of the starch granules brought in by the acid-tempering (Chen et al., 2020). No significant difference was observed with C2 values for the flour indicating no changes in protein quality. The C3 and C4 values were slightly higher for the treated sample compared to untreated flour which shows higher hot gel viscosity, and stability respectively. Lastly, the C5 value of the treated flour was higher than the control indicating increased starch retrogradation during the cooling phase compared to control. The trends

observed in the Mixolab profiles agree with the RVA results as the treated sample had higher peak viscosity, trough, and final viscosity values which correspond to the C3, C4, and C5 values respectively.

The bread-baking characteristics of treated and untreated wheat flour samples are shown in **Table 5.4**. Similar ($p > 0.05$) bread volumes were observed for the bread produced from both flours. The values observed from this study were comparable to the results reported by Rivera et al., (2021). As for texture characteristics, similar ($p > 0.05$) values for hardness, cohesiveness, resilience, chewiness, and springiness were found, demonstrating that SBS tempering did not alter bread texture attributes. Hardness, chewiness, and cohesion relate to the bread properties when masticated while springiness, and resilience relate to the strength of the bread structure specifically on its ability to retain its shape once compression is removed and resistance to compression.

The crumb structure was analyzed, and the C-cell images of treated and untreated bread slices are shown in **Figure 5.5**. The cells (holes) in the bread slices are formed because of the evaporation of both water and alcohol during baking. Following this process, the gluten structure stretches and sets as the moisture is removed during baking creating the breadcrumb structure. Based on **Table 5.4**, the crumb structure of breads made from SBS-tempered wheat was comparable to the control. This is evidenced by the similar ($p > 0.05$) cell number, volume, diameter, and wall thickness values. The extent to which cells form in wheat bread is determined by the gluten-starch interactions that provide the viscoelastic properties of the dough (Lagrain et al., 2012; Trappey et al., 2015). Furthermore, Trappey et al. (2015) established the relationship between cell wall thickness (mm) and the number of cells per slice of bread wherein bread slices with greater wall thickness will have a higher number of cells per slice which was observed in the presented study.

Table 5.4: Baking performance of flours from SBS treated (%), and control wheat samples

Test variable	Treatment (%SBS)		P value (p<0.05)
	0%	1.50%	
Texture properties			
Hardness (N)	5.27±0.67 ^A	5.91±1.78 ^A	0.7218
Resilience (%)	40.29±1.7 ^A	36.37±3.59 ^A	0.3521
Cohesion	0.78±0.01 ^A	0.76±0.03 ^A	0.4546
Springiness (%)	119.42±13.82 ^A	117.35±32.59 ^A	0.9484
Chewiness (N)	4.91±0.47 ^A	4.95±0.5 ^A	0.9490
C cell characteristics			
Number of cells	3389.16±138.98 ^A	3627.83±178.48 ^A	0.0941
Cell diameter (mm)	1.67±0.08 ^A	1.71±0.14 ^A	0.6487
Wall thickness (mm)	0.39±0.00 ^A	0.39±0.01 ^A	0.3054
Cell volume (cc)	5.57±0.31 ^A	5.92±0.63 ^A	0.3715
Bread Characteristics			
Bread Volume (cc)	535±17.79 ^A	516.66±8.49 ^A	0.1107
Specific Volume (cc/g)	3.69±0.11 ^A	3.52±0.05 ^A	0.0684

Note: Values were expressed as means ± standard deviation from 3 replicates; means with a different letter in the row are significantly different ($p < 0.05$).

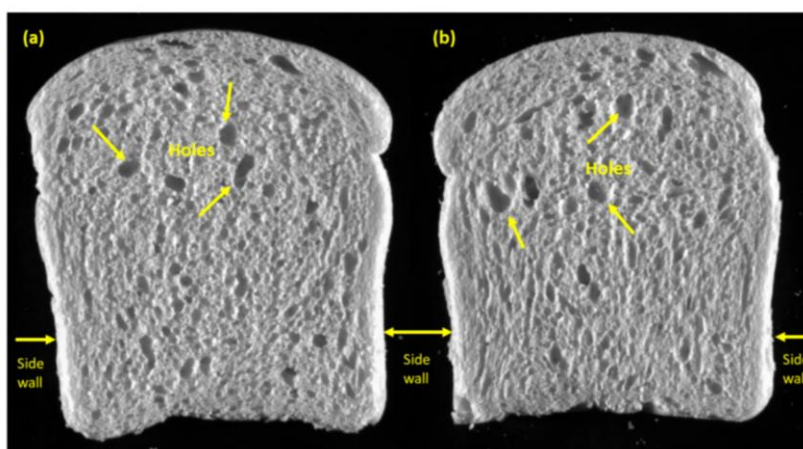


Figure 5.5: Representative c-cell images of bread slices treated with sodium bisulfate (%). (a) 0%; (b) 1.5%.

5.4. Conclusion

Effective interventions that demonstrate a broad antimicrobial spectrum are needed to address pathogen contamination in wheat flours. This study assessed the effectiveness of SBS, a GRAS additive, as a tempering intervention against *Salmonella* in wheat. The study revealed that by inducing oxidative stress by lowering pH, SBS produced ROS species resulting in the loss of cell viability. This was demonstrated in the tempering experiment as SBS tempering (1.5% SBS) reduced wheat *Salmonella* load by 3.0 log units after tempering. In addition, tempering wheat with SBS did not impact flour milling, and baking performance as the flour quality parameters were comparable to that of flours produced through conventional tempering. Further studies aiming to validate the commercial viability of this intervention on an industrial scale setting and assessing its effect on other wheat varieties and applications are warranted. Additionally, use of high concentration of acid can negatively impact the wheat flour quality and the milling equipment leading to corrosion of metal surfaces. More research in this area is needed to address these issues successfully. It is also critical to acknowledge that the use of SBS as a tempering agent could require its declaration as an additive on the product label to comply with labelling regulations. Overall, this study demonstrated the mode of action of SBS against *Salmonella*, and that SBS tempering is an effective antimicrobial intervention for mitigating *Salmonella* contamination in wheat, as it yielded significant reductions while having minimal effects on flour quality.

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Chapter 6 - Acidic water tempering and heat treatment, a hurdle approach to reduce wheat *Salmonella* load during tempering and its effects on flour quality.

Abstract

The cultivation and processing of wheat render it susceptible to microbial contamination from varied sources. Hence, pathogens such as *Salmonella* can contaminate wheat grains, which poses a food safety risk in wheat-based products. This risk is displayed by the incidence of foodborne illness outbreaks linked to *Salmonella*-contaminated wheat flour and flour-based products. The purpose of this study was to assess the effectiveness of combining acidic water and heat treatment in reducing the *Salmonella* load of hard red spring (HRS) wheat grains during tempering. Effective treatments were then evaluated for their effects on wheat flour quality. Tempering with sodium bisulfate (SBS), lactic acid (LA), and citric acid (CA) at 15% w/v alone reduced ($P < 0.001$) wheat *Salmonella* load by 3.15, 3.23, and 2.91 log CFU/g, respectively. Heat treatment (55°C) reduced ($P < 0.001$) wheat *Salmonella* load by 4.1 log CFU/g after 24 h of tempering. Combining both tempering and heat treatments resulted in a greater reduction in *Salmonella* load, as non-detectable levels (< 2 log CFU/g) of *Salmonella* in the wheat grains were observed after 12 h of tempering with LA (15%) + heat. Similar results were achieved for both SBS (15%) + heat and CA (15%) + heat treatments after 18 h of tempering. Applying the combined treatments in HRS wheat grains resulted in comparable wheat flour baking (volume, texture, and crumb structure) and physicochemical properties (rheology and composition) relative to the control (tempering with water alone). The results from this study have the potential to be utilized for developing more effective methods for improving the food safety of wheat flour against *Salmonella* contamination.

Journal article

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6.1 Introduction

Wheat grains have been regarded as “microbiologically-safe” foods due to their reduced water activity (a_w). This attribute has led to the general confidence that wheat flour and other flour-based products are low-risk foods from a microbiological safety standpoint (Jung & Harris, 2022). Research has shown that bacteria, including *Salmonella*, can persist for a considerable amount of time in reemerge once they are placed in more favorable environmental conditions for growth (Feng & Archila-Godinez, 2021; Finn et al., 2013). Over the past decade, several recalls and illness outbreaks reported in the United States have been linked to contaminated wheat flour or flour-based products (CDC 2019, 2022, 2023). In these events, *Salmonella* has been commonly isolated from the contaminated wheat flour flour-based products involved. For example, an ongoing Salmonellosis outbreak linked to contaminated wheat flour has been detected in the US in March 2023. This outbreak has so far caused 12 illnesses (from 11 US states) with 3 being hospitalized; affected consumers have also reported eating raw doughs or batters with flour being the only common ingredient in the doughs or batters eaten (CDC, 2023).

The cultivation practices render the wheat grains susceptible to microbial contamination during pre-harvest operations. Post-harvest operations also serve as viable sources of microbial contamination for wheat grains as a result of inadequately sanitized equipment, sub-optimal

storage practices, and pest activity. Hence, wheat kernels typically contain significant levels of microbial contamination (Sabillón & Bianchini, 2016). Pathogens including *E.coli*, *Salmonella*, and *Listeria* spp. have been isolated from raw wheat grains, with low prevalence and quantity observed (Myoda et al., 2019). Microbial contamination in wheat grains was reported to be condensed in the outer layer of the grains (Laca et al., 2006). This results in the wheat germ and bran having higher quantities of microbiological contamination compared to the wheat flour. Debranning (at 1.08% of the grain weight) was found to reduce the microbial contamination of wheat grains by 80.1% (Qi et al., 2022). However, debranning is only used in durum wheat milling as it minimizes bran contamination in semolina (Sarkar and Fu, 2022). The conventional wheat flour milling process uses successive grinding, sieving, and purification steps to separate the bran from the flour based on particle size. In these steps, co-mingling of the ground wheat fractions would result in the cross-contamination of microorganisms into the wheat flour. Moreover, it is also important to note that process of debranning is not omitted when producing whole wheat flour. Hence, milling facilitates the redistribution of the microbes from the wheat grain into the milled fractions produced (Rivera et al., 2021; Rivera et al., 2022a).

Tempering is a pre-milling step, wherein the grains are hydrated followed by a conditioning step that typically lasts up to 24 h depending on wheat hardness. This operation helps toughen the bran and soften the endosperm, which boosts flour extraction rates and quality. Commercial milling operations mostly use water for the tempering step. Therefore, the inclusion of antimicrobial agents at this stage could be a potential way to combat pathogen contamination in wheat grains and prevent their cross-contamination into milling products. Diverse tempering strategies such as using an ethanol-water mixture (Ari Akin et al., 2023), acidic organic acids (i.e., lactic acid, citric acid, peracetic acid) (Sabillon et al., 2016; Jung & Harris, 2022), inorganic acids

(sodium bisulfate) (Rivera et al., 2020), and oxidation agents (ozonated and chlorinated water) (Dhillon et al., 2007; Ibanoglu, 2001) have been employed to reduce the microbial contamination in wheat during the tempering process.

Recent evidence shows that bacteria might be capable of adapting to temperatures and pH levels that are not ideal, which could ultimately lengthen the time it takes to kill them (C. Kim et al., 2000). To overcome this problem simultaneous use of chemical treatments and physical processing can be an effective substitute strategy in food processing for controlling pathogens and enhancing microbiological safety (de Oliveira et al., 2019). This alternate method is founded on the synergistic antibacterial activity shown when certain physical processes, like heat and certain chemical treatments, are applied together. For instance, several studies have demonstrated that when used together, mild-temperature and essential oils can considerably increase microbial load inactivation in fruit juices as compared to either mild-temperature thermal processing alone or essential oil treatment alone. Similarly, the recent study by Rivera et. al., (2021) revealed that the use of acidic water tempering combined with heat treatment reduced the enterohemorrhagic *Escherichia coli* (EHEC) load to below the detection limit with 6 h of treatment. Overall, these new technologies have the advantage of improving microbial deactivation in food while utilizing mild levels of physical processing intensity, which reduces the overall negative effects of processing on food quality.

The present study investigated the efficacy of organic (citric acid and lactic acid) and inorganic acids (sodium bisulfate) alone and in combination with heat (55 °C) as a tempering treatment for reducing *Salmonella enterica* contamination in wheat. The selection of mild temperature and low acid concentration is ideal for evaluating the potential synergism between these treatments and minimizing the impact of processing on the quality of wheat flour.

Additionally, the study also evaluated the mechanism of action of acidic tempering agents on the bacterial cell by measuring the reactive oxygen species concentration (ROS). Furthermore, the study evaluated the impact of these treatments on the flour functionality and baking characteristics. Overall, this study assesses the synergistic use of mild physical and chemical processing technologies to improve the microbiological safety of wheat flour and the products there off.

6.2 Materials and Methods

6.2.1 Materials

Organic hard red spring (HRS) wheat (moisture 11.03% on wet basis and protein 15.9%) was used for the present study. The wheat was purchased from Great River Organic Milling Company, Arcadia, Wisconsin. Jones-Hamilton Co. (Walbridge, OH, USA) provided the SBS powder, while lactic acid and citric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the media and other chemicals were purchased from Fisher Scientific (Illinois, USA) unless otherwise mentioned.

6.2.2 Bacterial strain and inoculation preparation

Four frozen (-80 °C) isolates of *Salmonella enterica* of different serotypes (Newport - ATCC 6962, Typhimurium - ATCC 14802, Kentucky - ATCC 9263, and Enterica - ATCC 8326) were procured from the Feed Toxicology and Microbiology Laboratory, Kansas State University (Manhattan, KS) for the current study. The bacterial stock cultures were kept at -80 °C in tryptic soy broth supplemented with 30% glycerol until further use.

Frozen cultures were defrosted and streaked for activation on tryptic soy agar (TSA) plates for the inoculum preparation. Streak plates were incubated for 24 h at 37 °C. A single, well-isolated black colony of each *Salmonella enterica* serovars was inoculated into individual TSB (10 ml) media tubes and incubated for 24 h at 37 °C. Centrifugation (2500 x g, 10 min) was used to harvest

the cells, which were then inoculated into freshly prepared TSB (600 ml) and incubated at 37 °C for another 24 h. Cells were collected again by centrifugation at 4 °C (2500 x g, 10 min); pellets were washed and mixed in sterile 0.1% peptone water to obtain a final viable cell concentration of $\approx 10 \log$ CFU/ml. The cell suspension from each serovar prepared was combined in equal proportions to make the working inoculum for the experiment.

6.2.3 Inoculation and tempering of wheat grains

The four serovar cocktail of *Salmonella enterica* was used to inoculate the pre-dried wheat kernels (60 °C / 24 h) before tempering experiments. The amount of inoculum needed (13.73 mL) was calculated using the AACC method 26-95.01 (AACC, 2010). Briefly, a sterile bottle sprayer was used to spray the prepared inoculum into the 200 g of wheat kernels inside re-sealable bags (9x12", 4 MIL, ULINE). The kernels were hand-mixed and shaken for five minutes to evenly disseminate the inoculum after inoculation. A wheat inoculation level of 7.4 log CFU/g was achieved when the inoculated wheat kernels rested for 24 h (24 to 25 °C) while being periodically shaken.

Tempering of the prepared wheat kernels was carried out to a moisture content of 17% using the prepared acidic tempering solutions (**Table 6.1**). The amount of tempering solution needed was calculated based on the initial moisture content of original wheat kernels (11.3%) according to AACC method 26-95.01 (AACC, 2010). Using a clean sterile bottle sprayer, the tempering solutions were sprayed into the wheat kernels, which were then manually combined and agitated for five minutes. The tempered kernels were transferred to a metal container (12 cm dia and 4.3 cm deep) to mimic commercial tempering setups. For treatments that required heating, the tempered wheat kernels were placed in a forced air oven (Across International, Livingston, NJ, USA) kept at 55 °C. Treatments that did not include heating were rested in an incubator set at

ambient temperature (24 to 25 °C). Inoculated wheat kernels were tempered for 24 h. A schematic overview of the tempering experiment is shown in **Figure 6.1**.

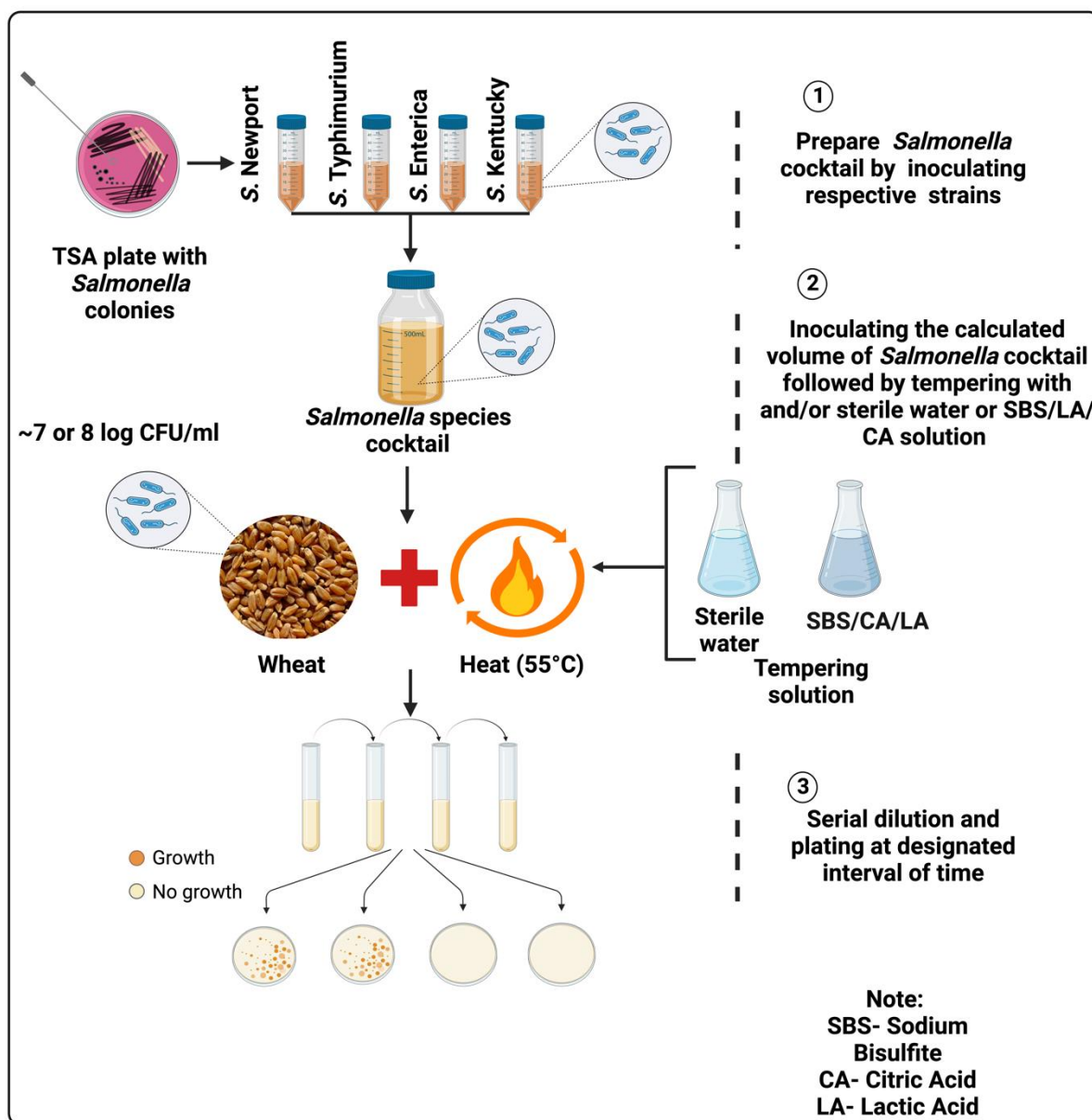


Figure 6.1: Experimental design used to test the efficacy of the antimicrobial tempering treatments used against *Salmonella* serovars in wheat.

Table 6.1: pH values of wheat grains during tempering and wheat flours after milling

Test variables	Control	15% SBS	15% CA	15% LA
pH1	6.95±0.02 ^A	1.27±0.00 ^C	2.05±0.02 ^B	1.62±0.40 ^{CB}
pH2	7.19±0.02 ^A	6.68±0.12 ^B	6.67±0.14 ^B	6.75±0.00 ^B
pH3	7.24±0.00 ^A	6.90±0.00 ^B	6.55±0.03 ^C	6.93±0.06 ^B
pH4	6.60±0.02 ^A	5.61±0.04 ^C	6.09±0.08 ^B	6.04±0.11 ^B

Note: Values were expressed as means ± standard deviation (SD) from three replicates; values with a different letter superscript in each row (test variable) are significantly different ($P < 0.05$); pH1- pH of tempering solution, pH2 - pH of wheat at the beginning of tempering (30 min), pH3 - pH of the wheat at the end of tempering (24 h), pH4 - pH of the wheat flour upon milling.

6.2.4 *Salmonella* load enumeration

Samples from each tempering treatment (25 g) were taken aseptically at 0.5, 2, 6, 12, 18, and 24 h intervals during tempering. Samples were placed in a stomacher bag (VWR, Radnor, PA, USA) and homogenized for 120 s with 225 ml of sterile buffered peptone water (BPW) using a stomacher (Seward, UK). Serial dilutions of the homogenized sample were done with 0.1% peptone water, and appropriate dilutions were spread-plated (100 µL duplicate) on XLD plates with a TSA overlay. Plates were then incubated at 37 °C for 24 h. Typical colonies for *Salmonella* (black colonies) were counted; counts were transformed into log CFU/g. Reductions were computed as the difference between the *Salmonella* load at the indicated time intervals and at time zero.

6.2.5 Reactive Oxygen Species (ROS) estimation

The ROS produced were measured using the 2', 7' -dichlorofluorescein diacetate (DCFDA) assay, as previously published by Shivaprasad et al. (2021). Bacterial cells were cultured at 37 °C for 4 h with or without acidic tempering agents (15% SBS, CA, and LA). The cells were extracted by centrifugation at 2795x g for 10 min at 4 °C. Each supernatant was then treated with 10 mM DCFDA dye for 1 h at 37 °C. The amount of ROS produced was determined using an LS50B Luminescence Spectrometer (PerkinElmer, Waltham, Massachusetts, USA) at Ex=485 nm and Em=520 nm.

6.2.6 Experimental milling and flour quality evaluation

6.2.6.1 Tempering and milling

The tempering treatments consisting of SBS 15% + heat, LA 15% + heat, CA 15% + heat, and heat alone were selected for this phase of the study as they resulted in > 3 log CFU/g reductions during the tempering experiment. The control was wheat that was only tempered with water at room temperature. Stock solutions of sodium bisulfate- SBS (50% w/v), lactic acid – LA (85% v/v), and citric acid – CA (50% w/v) were diluted with sterile water to prepare the desired concentration of the tempering solutions. The prepared solutions were applied to the wheat kernels (1500 g) and were mixed in a rotary mixer for 20 min before transferring them to a re-sealable bag. The tempered wheat kernels were held at a designated temperature of 24 -25 °C and 55 °C for 24 h (kernels held at 55 °C were allowed to cool to 24–25 °C before milling) before subjecting them to milling. A Chopin LabMill (Chopin, France) was used to mill the wheat flour with 6 grinding steps (2 breaks, 1 sizing, and 3 reductions). The initial break phase was done at a feed rate of 5 g/s, and the subsequent milling processes were done at a feed rate of 2.5 g/s. The straight-grade flour for evaluation was prepared by mixing the flour fractions (break, size, and reduction

flour) produced. Flour samples were kept at 4 °C until further evaluation. The milling yield of wheat-milled fractions was calculated using the formula (Eq. 1) below (Shivaprasad et al., 2022).

$$\text{Milling yield (\%)} = \frac{\text{weight of milling fractions}}{\text{weight of wheat}} \times 100 \quad \text{Eq. 1}$$

6.2.6.2 Flour quality evaluation

Proximate analysis of wheat flour was performed through an external laboratory. Moisture analysis was performed according to the standard AACC method 44-15.02 (AACC, 2010). The other components were estimated using AOAC protocols including AOAC 922.06 for crude fat, AOAC 962.09 for crude fiber, AOAC 923.03 for ash, and AOAC 984.13 for total composition (crude protein). Damaged starch and dough rheology analysis of the flours were carried out according to AACC method 76-33.01 and 54-60.01, respectively (AACC, 2010). The pasting quality of wheat flours was determined using a Rapid Visco Analyzer (Perten Instruments, Australia) according to AACC method 76-21.02 (AACC, 2010). Gluten quality (wet gluten, dry gluten, and gluten index) was determined according to AACC method 38-12.04 (AACC, 2010) using a Glutomatic 2000 system (Perten Instruments, USA).

6.2.6.3 Baking and bread quality evaluation

The baking quality of the wheat flour samples was evaluated according to the methods performed by Rivera et al. (2021). Bread volume, specific volume, C-cell (slice area, cell number, cell diameter, wall thickness), and textural characteristics (hardness, chewiness, cohesiveness, and springiness) were determined as described by Rivera, et al. (2022b).

6.2.7 Statistical analysis

All experiments for this study were conducted with three independent replications. The measured responses were represented as mean $\pm$ standard deviation (SD). For the tempering experiment, the mean *Salmonella* load of wheat was determined using the GLIMMIX method, and

Tukey's HSD ($P < 0.05$) test was used to compare the treatment means. Further, the significance of heat X acid X time was evaluated for all the tested concentrations. For all the other experiments significant differences among the mean values from each treatment were determined using the GLIMMIX method, and Tukey's HSD ($P < 0.05$) test was used to compare the treatment means. Further, for pattern recognition and dimensionality reduction, the data sets were subjected to principal component analysis (PCA). The experimental data consisted of 37 variables (responses) and 5 observations (samples). The number of essential principal components was decided based on the eigenvalues and their relative magnitude (i.e., values compared to the largest eigenvalue). SAS Statistical Software 9.3 (SAS Institute, Cary, NC, USA) was used to analyze all the data sets.

6.3.0 Results and Discussion

6.3.1 Time-kill kinetics study during tempering

The use of acids as an intervention for *Salmonella* decontamination emerged in the 1960's with the goal of decontaminating animal carcasses (Van Immerseel et al., 2006). Since then, organic acids have been used as antimicrobial agents for diverse purposes. The present study determined the antibacterial activity of the organic acid (LA, CA, and SBS) alone and in combination with heating (55 °C). **Figure 6.2** shows the time-kill kinetics of *Salmonella* in wheat grains during the tempering step. The bactericidal activity of the organic acid alone and in combination with heat decreased the *Salmonella* load of wheat during the tempering process at all the time points. The initial (0 h) post-inoculation population of *Salmonella* (control) on wheat grains was 7.47 ± 0.47 log CFU/g. Based on the results, an increase in wheat *Salmonella* load in the first 30 min of tempering as counts of 7.96 ± 0.18 log CFU/g were observed ($P < 0.001$). This could be because of the fact that tempering provides an adequate temperature, moisture, and proper humidity that are beneficial to microbial development at the initial stages. Past this, the *Salmonella*

counts for the tempered wheat decreased at all time points, with maximum reductions for all tempering treatments observed at 24 h (5.67 ± 0.27 log CFU/g). Jung & Harris, (2022) observed a 0.75 log CFU/g decrease in the *Salmonella* load of wheat grains after 48 h post-inoculation. Similarly, Sabillón et al. (2020) reported a 0.79 – 0.92 log CFU/g decrease in the *Salmonella* load of wheat after 7 days post-inoculation. This decrease could be explained by the dry nature of tempered wheat, which restricted the proliferation of *Salmonella* and resulted in cell death from dehydration (Rivera et al., 2021).

Acidic water tempering (SBS, LA, and CA) led to a greater reduction in wheat *Salmonella* load relative to the control. Tempering (at 15 %w/v) with SBS, LA, and CA resulted in reductions (log CFU/g) of 3.15, 3.23, and 2.91, respectively after 24 h of incubation ($P < 0.001$). These reductions agree with the results divulged by Rivera et al., (2022a), wherein tempering with SBS and LA (both at 10%) resulted in reductions of 2.6 and 2.0 log CFU/g in the STEC load, respectively after 24 h of tempering. A similar study by Sabillón et al., (2020) reported a 1.8 log CFU/g reduction of *Salmonella* load of wheat when treated with LA at 5% concentration. Further, the concentration of acid ($P < 0.001$), time ($P < 0.001$), heat ($P < 0.001$), and their interaction ($P=0.0029$) had a significant effect on the wheat *Salmonella* load. Using heat treatment (55 °C) along with acidic water (at 15%) during tempering resulted in the reduction of wheat *Salmonella* load to lower than detection limits (< 2 log CFU/ g) ($P < 0.001$). This was observed with the use of heating and LA after 12 h of tempering, while both SBS and CA required 18 h to achieve the same effect with heating. These reductions were higher compared to using heat treatment alone on tempered wheats as reductions of only ~ 3 to 4 log CFU/g after 24 h of tempering were achieved. The improved reductions of the combined treatments over individual treatments during tempering could be explained by the enhanced stress on *Salmonella* cells caused by heating. It is established

that elevated temperatures increase the pKa values of acids; lower values indicate that acids dissociate more when dissolved in water. In addition, the elevated temperatures could also damage the cell membrane resulting in increased permeability (Ebrahimi et al., 2018). Higher temperatures also lead to increased water penetration in grain kernels (Sfayhi-Terras et al., 2021). By these concepts, the improved reductions achieved by heating could be explained by the increased permeation of the acids into bacterial cells. This would in turn hasten the disruption of cell functions caused by acid permeation in the cell. Thermal treatment is historically known to be effective in reducing the pathogen load of food products or process equipment (Rivera et al., 2022a). Thus, combining this approach with other antimicrobial treatments would result in greater efficiency. Sabillón et al. (2020) studied the effects of seasonal temperatures on the efficacy of acid water tempering against *Salmonella* in wheat. It was observed that the increased temperature had a greater effect in reducing the bacterial load of wheat. Hyun et al., (2018) also reported that increased storage temperatures increased the reduction observed in the *E. coli* population in dried food products. Liu et al. (2020) revealed that the combined effect of heat with electrolyzed acidic water is effective in killing *E.coli* O157:H7 by reducing its cellular metabolic activity. Rivera et al. (2021) revealed that the use of an acidic tempering technique in combination with heat treatment reduced the STEC load of wheat to nondetectable levels ($<1.6 \log \text{ CFU/g}$).

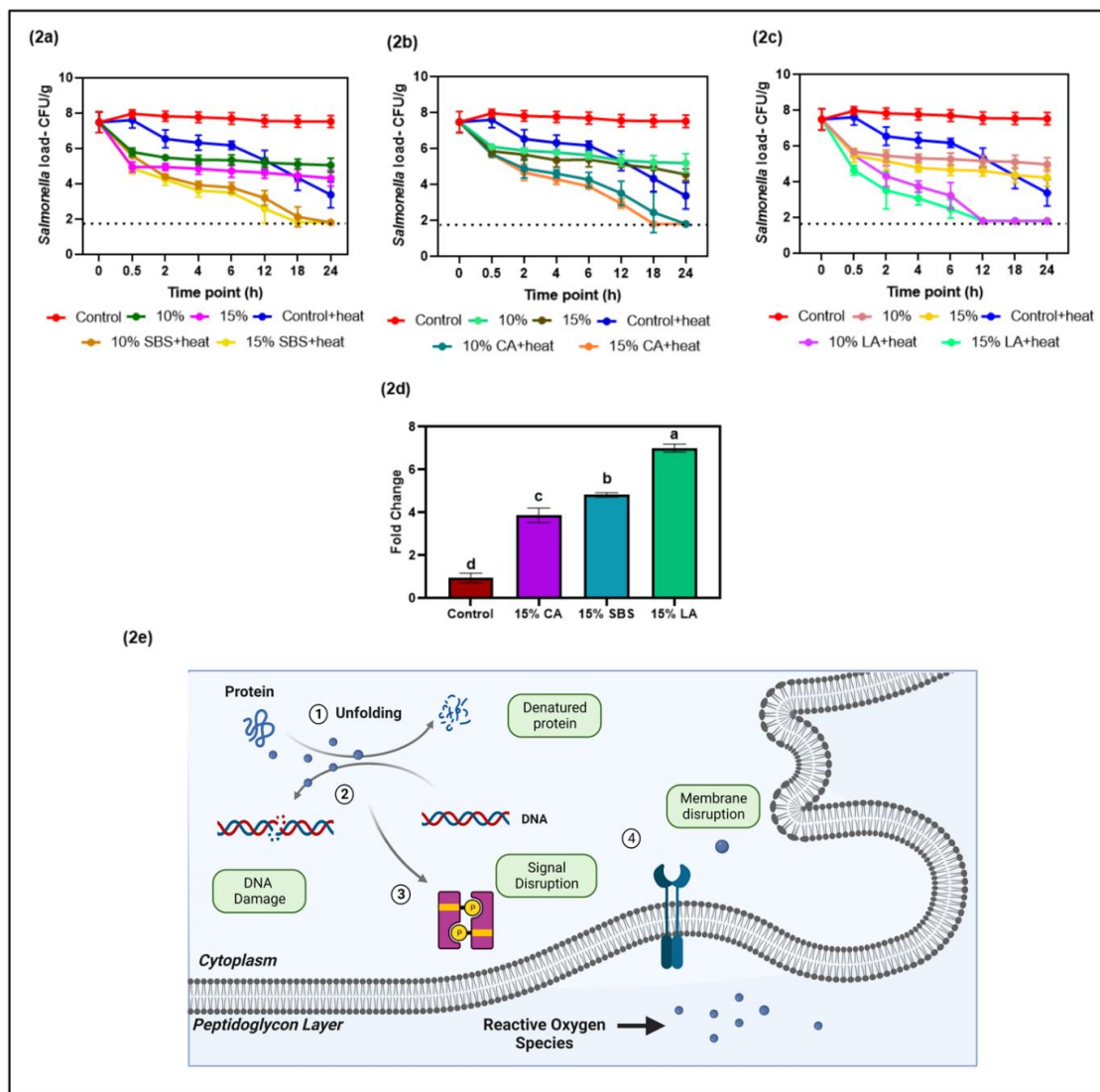


Figure 6.2: Population (log CFU/g) of *Salmonella* load of wheat during tempering using different organic and in-organic acids: (A) Sodium bisulfite (SBS), (B) Citric acid (CA), (C) Lactic acid (LA); means $\pm$ standard deviation from 3 replicates is plotted. Bactericidal mechanism of acidic tempering agents: (D) Reactive oxidative species (ROS) measured after tempering treatments using DCFDA assay. (E) Schematic representation of bactericidal mechanisms of acidic tempering agents as a result of oxidative stress from acidic pH levels

The antimicrobial properties of acids (organic or inorganic) stem from their ability to lower the pH of the bacteria environment. This is shown in **Table 6.1**, wherein wheat grains tempered with acidic water had lower ($P < 0.05$) pH values compared to the control (sterile water). Acids can also permeate cell membranes as their dissociated forms are lipophilic, which causes changes in the ion (protons and cations) of the cell cytoplasm (Gómez-García et al., 2019). This creates an imbalance in cellular homeostasis as the internal cell pH is lowered by the entry of H^+ ions (Francois & Kappock, 2007); this leads to the disruption of normal cell functions, enzymes, and cell membrane damage. **Figure 6.2D** shows the production of ROS species (measured by DCFDA assay) in the bacteria under different acidic water tempering treatments. Treatment with LA (7.00) resulted in the highest fold change in ROS production followed by SBS (4.82) and CA (3.87); all acidic water treatments resulted in higher ($P < 0.001$) fold change in ROS species compared to the control (0.95). The increased ROS production of *Salmonella* under acid treatments is an indicator of bacterial response to acid stress (brought by acidic water tempering). Bacteria produce ROS, through aerobic or non-aerobic metabolic pathways, to combat lethal environmental stress such as temperatures, reactive chemicals, pH changes, and nutrient depletion (Rhen, 2019; Zhao & Drlica, 2014). The ROS production of bacteria can also be detrimental as these compounds cause oxidative damage to critical cellular components such as the cell membrane, proteins, DNA, and lipids (**Figure 6.2E**). Hence, bacteria also need to neutralize these components to survive (Imlay, 2014). It can be assumed that the acids used in this study disrupted the protective proteins in *Salmonella* against ROS, which led to the accumulation of ROS under acidic water treatments. Hence, the reduction in the *Salmonella* load of wheat can be attributed to the accumulation of ROS compounds, leading to cell damage and oxidative stress (**Figure 6.2D**). In fact, ROS oxidative damage can also lead to puncturing of the cell membrane causing leakage of important cell

components; bacteria also use ROS components to trigger self-destruction in the presence of severe stress conditions (Zhao & Drlica, 2014). Further studies on evaluating the effects of acids on the ROS-neutralization pathways or components in foodborne pathogens could be worthwhile to fully understand the lethality of acid treatments against foodborne pathogens.

The results of the tempering experiments show that acidic water tempering and heating alone were more effective treatments in reducing wheat *Salmonella* load compared to tempering with water. Combining both treatments resulted in greater reductions in *Salmonella* load. These observations indicate that a hurdle approach would be an effective intervention for wheat grains prior to milling to eliminate *Salmonella* contamination.

6.3.2 Flour quality evaluation

The effects of heat treatment alone and in combination with 15% acidic water (SBS, CA, and LA) during tempering on the quality of wheat flour were assessed. These treatments were selected as they provided ample reductions (> 3.0 log CFU/g) compared to the other treatments used at a shorter tempering time. Currently, no mandated performance guidelines for pathogen reduction in wheat milling are present; flour producers prefer at least a 3.0 log CFU/g decrease for interventions used on wheat or wheat flour (Rivera et al., 2022a). **Table 6.2** displays the milling yields (% as-is) for bran, shorts, and straight-grade flour after milling the tempered HRS wheat. Lower ($P < 0.05$) bran yield was observed for the SBS-Heat treated wheat grains contrast to the other treatments; a similar trend was noticed by Rivera et al. (2021) with SBS-tempered wheat. This observation could be due to the increased bran friability caused by SBS, which could cause some bran particles to be transferred to the other milling fractions (Rivera et al., 2021). This also relates to the higher shorts yield for SBS-heat treated samples. Sodium bisulfate (SBS) has strong hygroscopic properties that enable it to absorb water from the bran later causing increased dryness

and friability. Significant differences among the individual flour yields (break and sizing), although these differences were minimal. Nevertheless, similar ($P > 0.05$) straight-grade flour yields were observed for all tempering treatments. This suggests that the combination of heating and acidic water tempering does not affect flour milling yields. These results were consistent with the findings by Kweon et al. (2009) and Rivera et al. (2021) who reported that temperature has no significant effect on wheat flour yield.

Table 6.2: Milling yield and physicochemical characteristics of the wheat flours produced

Test variables	Control	Heat alone	15% SBS+heat	15% CA+heat	15% LA+heat
<i>Milling yield (% as-is)</i>					
Bran	6.71 ± 0.29 ^A	6.41±0.11 ^A	5.09±0.13 ^B	7.23±0.55 ^A	7.34±0.39 ^A
Shorts	7.83± 0.16 ^{BC}	6.93± 0.47 ^{BC}	9.37±0.86 ^A	8.06±0.19 ^{BA}	6.50±1.55 ^C
¹ Break flour	23.08±0.39 ^{BA}	23.53±0.69 ^{BA}	21.47±0.16 ^C	22.33±0.19 ^{BC}	23.76±0.30 ^A
² Sizing flour	12.55±0.21 ^{BA}	12.27±0.43 ^B	12.27±0.43 ^A	13.99±0.62 ^{BA}	12.45±0.46 ^B
³ Reduction flour	35.91±0.26 ^A	37.23±0.17 ^A	34.65±1.36 ^A	37.71±4.1 ^A	35.28±0.15 ^A
Straight grade flour (1+2+3)	71.54±0.47 ^A	73.04±0.41 ^A	70.12±1.18 ^A	72.91±4.22 ^A	71.54±0.29 ^A
<i>Physico-chemical characteristics (as-is basis)</i>					
Moisture (%)	12.75±0.78 ^A	11.99±0.36 ^A	11.70±0.40 ^A	11.97±0.40 ^A	11.96±0.72 ^A
Crude protein (%)	14.86±0.01 ^A	14.88±0.04 ^A	14.77±0.00 ^{BA}	14.63±0.15 ^{BC}	14.58±0.02 ^C
Crude fat (%)	ND	ND	ND	ND	ND
Crude fibre (%)	0.29±0.02 ^A	0.30±0.05 ^A	0.37±0.02 ^A	0.30±0.02 ^A	0.32±0.02 ^A
Ash (%)	0.65±0.04 ^B	0.59±0.00 ^{CB}	0.79±0.01 ^A	0.57±0.01 ^C	0.61±0.01 ^{CB}
Carbohydrate (%)	72.50±0.05 ^B	72.54±0.05 ^B	72.74±0.02 ^A	72.83±0.09 ^A	72.85±0.03 ^A
Damaged starch (UCD)	16.8±0.37 ^A	17.13±0.16 ^A	15.16±0.62 ^A	17.16±0.77 ^A	17.53±1.91 ^A
Falling number (s)	433.5±25.48 ^A	407.33±51.38 ^A	397.33±36.73 ^A	371.33±41.24 ^{BA}	275±19.94 ^B

Note: Carbohydrate = 100%- (% moisture + %protein + %fat + %ash); values were expressed as mean ± standard deviation (SD) from three replicates; values with a different letter superscript for each test variable are significantly different ($P < 0.05$); Abbreviation: ND – not detected

The particle size distribution (PSD) of wheat flours produced from treated (acid + heat, heat alone) and untreated (sterile water) wheat grains is shown in **Figure 6.3**. Based on the PSD, all flour samples exhibited a bimodal distribution, which is typical for wheat flour (Moiraghi et al., 2019). The wheat flour particle sizes for the HRS wheat flours were between 50 and 180 μm ; this size range complies with the CFR requirement (21 CFR137.105) for wheat flours, wherein >98% of particles should pass through a 212 μm (FDA, 2023a). The findings on the flour PSD agree with the results reported by Nakamura, (2015) and Rivera et al., (2021). Interestingly, wheat flours produced using the combination of acidic water tempering and heating treatments (all acids) exhibited an increased percentage of finer particles compared to the control and heat treatment. This observation could be attributed to the use of heat treatment during tempering. Kim et al., (2004) reported that volume percentages in the first and second modes of the wheat (wild type) flours increased with elevated temperatures (30 and 50° C).

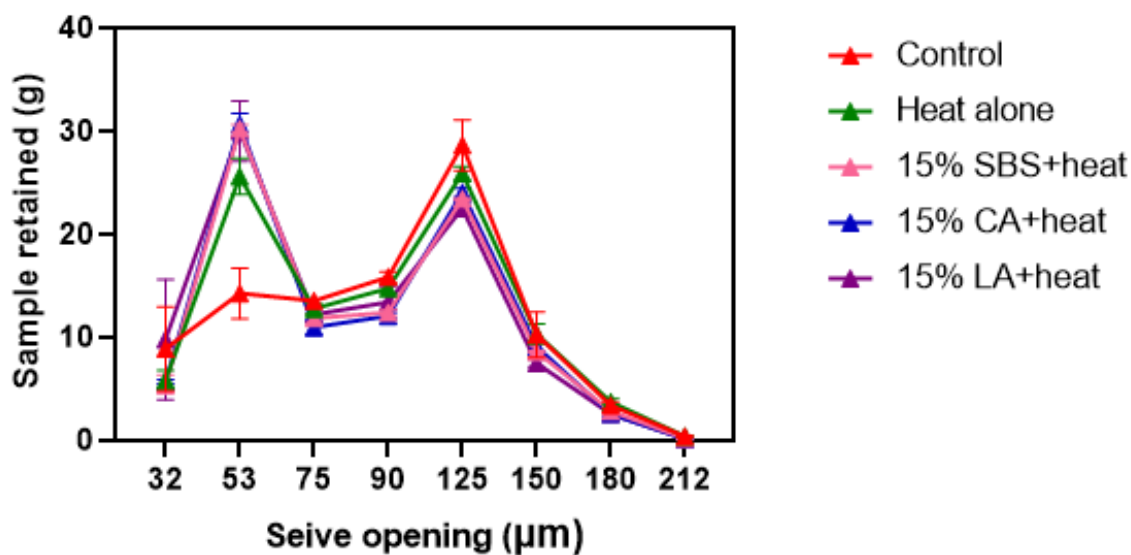


Figure 6.3: Particle size distribution of wheat flour. Means $\pm$ Standard deviation from 3 replicates are plotted

Table 6.2 shows the physicochemical properties of the wheat flour samples produced. All wheat flours produced had moisture contents that are below the permitted limit (14%) stated in the FDA CFR requirements (21 CFR137.200) (FDA, 2023b). Lower ($P < 0.05$) protein content was present in the LA- Heat (14.58%) compared to the other treatments (14.63 to 14.86%); these differences can be considered practically similar as they are all within the common protein content range (13 to 16%) for HRS wheat (US Wheat Associates, 2023). The ash content of the wheat flours was also different ($P < 0.05$) as the SBS-heat treated samples had the highest ash content among treatments. Ash content is typically used as an indicator of bran contamination of flours as the majority of the wheat grain minerals are in the bran layer (Stevenson et al., 2012). Hence, the higher ash content in the SBS-heat is due to excess bran contamination; this is also due to the increased bran friability due to the use of SBS which most likely increased the dryness of the bran layer. A similar trend was observed by Rivera et al., (2021), wherein higher ash content was present in the SBS-treated wheat flour. No Significant difference were observed for crude fiber and crude fat; however, significant different ($P < 0.05$) were observed for the carbohydrate content for all flour samples. Differences ($P < 0.05$) in the falling number (FN) values were also observed, with the LA-Heat and CA-Heat treatments producing the lowest FN values among treatments. These differences in the falling number values can be attributed to the greater acidity (lower pH) of the wheat flour (shown in **Table 6.1**); a more acidic pH is known to significantly decrease the falling number of wheat flour (Noll, 1985). Rivera et al. (2022a) also reported a decrease in the FN of the acid-tempered wheat flour. Their findings also revealed that upon acidic tempering, the acid present on the bran can dissociate into the endosperm of the wheat kernel resulting in lower flour pH. Nevertheless, the FN values for the flours produced using the tempering treatments could be considered practically similar. The FN values denote the extent of amylase activity in the wheat

grains due to pre-harvest sprouting; higher values (second) indicate lower enzyme activity, while lower FN values indicate high amylase activity. In general, wheat with FN values > 250, 300, or 350 seconds are considered high-quality wheat depending on the standards set in different countries (Mares & Mrva, 2008). An FN value of > 250 seconds for wheat flour is generally preferred for baking applications; FN values > 350 seconds indicate that enzyme supplementation is needed for wheat flour (Cauvain, 2017). All the flour samples produced had falling number (FN) values above 250 seconds which correspond to acceptable amylase activity. Hence, the FN values of the flours produced using the acidic water-heat treatments could be considered practically similar to the control. Similar ($P > 0.05$) damaged starch content (UCD) was observed in all the flour samples produced, which indicates that the hurdle approach used did not influence the damaged starch content. Damaged starch (DS) corresponds to the small starch particles that break away from the main starch granules during milling (Ma et al., 2016). It is important in bread making as it increases hydration and susceptibility to enzyme hydrolysis of the flour and serves as a food source for the yeast during leavening (Liu et al., 2014). Particle size also has an inverse relationship with DS as smaller particle sizes in wheat flours result in higher damaged starch content (Pang et al., 2021). The similar DS values show that the increase in the percentage of fine particle sizes of heat-treated flours (heat alone or with acid) did not significantly influence the flour DS values. Although the differences are not significant, an observed increase in DS was observed for heat-treated, CA-Heat, and LA-Heat tempering treatments. This can be due to the heat treatment used as it has a direct relationship with damaged starch content (Hassan & Musa, 2020; Kweon et al., 2009; Rivera et al., 2022a).

The pasting properties of the wheat flour produced are shown in **Table 6.3**. Similar ($P < 0.05$) peak viscosity, trough, and pasting temperature values were observed for all tempering

treatments used. Comparable peak times were obtained for all flour samples produced. Lower ($P < 0.05$) breakdown values were observed for wheat flours produced using the acidic water-heat tempering treatments compared to using water or heating alone. This trend indicates that the wheat flour from the hurdle treatments had better stability (holding strength) against shearing and heating (Balet et al., 2019). Lower ($P < 0.05$) final viscosity and setback values were also observed for wheat flour produced using the acid water–heat tempering treatment compared to the control. Both parameters relate to the increase in viscosity of the wheat flour paste after cooling, which indicates the ability of the starch components (amylose and amylopectin) to retrograde or re-associate when cooled (Kumar & Khatkar, 2017; Van Hung & Morita, 2005). These parameters also relate to the ability of the flour to form a viscous paste or gel after cooling, which can be important in various food applications. Flour-pasting properties are important as they correlate with bread-making quality. For example, Leon et al. (2010) reported that peak viscosity correlates with bread-firming behavior during storage. In another instance, Liu et al. (2022) reported that viscosity parameters correlate positively with biscuit diameter, spread ratio, and sensory scores and negatively correlate with hardness and thickness.

Table 6.3: Pasting, gluten quality, and dough rheology characteristics of wheat flours produced

Test variables	Control	Heat alone	15% SBS+heat	15% CA+heat	15% LA+heat
<i>Pasting characteristics (RVA)</i>					
Peak viscosity (cP)	2475.66±128.80 ^A	2495±14.16 ^A	2352.66±67.2 ^A	2384±12.02 ^A	2479±37.95 ^A
Trough (cP)	1854.66±155.30 ^A	1835.66±50.3 ^A	1783.66±78.0 ^A	1859±41.02 ^A	1903.33±88.78 ^A
Breakdown (cP)	621±33.74 ^{BA}	659.33±36.80 ^A	569±25.15 ^{BA}	525±36.35 ^B	575.66±59.65 ^{BA}
Final viscosity (cP)	2952.33±121.38 ^A	2953.33±8.17 ^A	2710±62.56 ^B	2754±9.93 ^{BA}	2848.33±29.84 ^A
Setback (cP)	1097±40.43 ^{BA}	1117.66±45.8 ^A	926.33±29.22 ^C	895.47±47.2 ^C	945±71.44 ^{BC}
Peak time (min)	6.513±0.06 ^{BA}	6.42±0.08 ^B	6.55±0.06 ^{BA}	6.66±0.05 ^A	6.55±0.06 ^{BA}
Pasting temperature (°C)	69.98±0.37 ^A	69.56±0.37 ^A	69.56±0.41 ^A	69.93±0.37 ^A	69.65±0.04 ^A
<i>Gluten properties (Gluten Washing)</i>					
Wet gluten (%)	41.06±0.80 ^A	37.4±2.27 ^A	38.43±1.56 ^A	39.36±0.75 ^A	40.16±1.24 ^A
Dry gluten (%)	14.16±0.20 ^A	13.5±0.86 ^A	14.96±0.80 ^A	14.83±0.37 ^A	15.27±0.69 ^A
Gluten Index (%)	94.08±0.58 ^A	95.34±2.52 ^A	97.42±1.00 ^A	95.76±0.51 ^A	98.36±0.98 ^A
<i>Dough rheology (Mixolab)</i>					
Water absorption (%)	55.3 ± 0.00 ^A	61.8 ± 0.00 ^A	58.76±1.12 ^B	61.7±0.00 ^A	61.7±0.00 ^A
Stability (min)	11.16±0.12 ^B	11.03±0.61 ^A	9.8±0.08 ^C	9.63±0.28 ^C	6.96±1.04 ^B
Amplitude (Nm)	0.07±0.02 ^A	0.07±0.00 ^A	0.07±0.00 ^A	0.07±0.01 ^A	0.06±0.01 ^A
C1 (Nm)	1.132±0.01 ^{BA}	1.02±0.08 ^A	1.15±0.00 ^A	1.07±0.016 ^B	1.08±0.01 ^A
CS (Nm)	1.12±0.01 ^A	1.01±0.08 ^A	1.11±0.01 ^A	1.05±0.00 ^A	1.07±0.00 ^A
C2 (Nm)	0.53±0.00 ^A	0.43±0.03 ^A	0.51±0.00 ^A	0.48±0.00 ^A	0.45±0.01 ^A
C3 (Nm)	1.03±0.16 ^{BA}	1.24±0.01 ^A	1.31±0.05 ^B	1.40±0.11 ^{BA}	1.04±0.39 ^A
C4 (Nm)	1.61±0.03 ^A	1.71±0.02 ^B	1.86±0.00 ^A	1.76±0.01 ^{BA}	1.73±0.10 ^{BA}
C5 (Nm)	2.42±0.03 ^A	2.59±0.09 ^B	2.77±2.77 ^A	2.69±0.00 ^A	2.55±0.22 ^A

Note: Values were expressed as mean ± standard deviation (SD) from three replicates; values with a different letter for each test variable are significantly different due to tempering treatment (P < 0.05).

The gluten properties of the wheat flour produced are also shown in **Table 6.3**. Similar ($P > 0.05$) wet gluten, dry gluten, and gluten index values were observed for all wheat flour samples. These trends indicate that the hurdle approach did not weaken the gluten of wheat flour. The flours produced from these tempering treatments had a similar quality to the control. Gluten-washing tests are used to determine these values, which involve washing away the starch and water-soluble components of wheat flour. After washing, the wet gluten is left, wherein its amount is an indicator of gluten quality as high-quality gluten tends to bind more water than poor quality ones (Finnie & Atwell, 2016). The wet gluten is then dried to obtain dry gluten; its amount corresponds to the amount of gluten present in the flour. Dry gluten corresponds to the quantity of gluten present in the flour, with higher values indicating greater flour affinity to bread making. The gluten content of HRS wheat flour tends to range from 12 to 15% (US Wheat Associates, 2023). Hence, the flour samples in this study have gluten contents typical for HRS wheat flour. Gluten index (%) is an indicator of gluten strength and quality. In the testing for this, wet gluten is placed in a sieve and centrifuged, and the gluten index is calculated as the percentage of gluten that remained in the sieve after centrifugation. Weak, normal, and strong gluten has GI (%) values of $< 30\%$, 30 to 80%, and $> 80\%$, respectively (Oikonomou et al., 2015). All the flour samples tested had GI values $> 80\%$ indicating that the hurdle approach did not contribute to weakening the gluten of the wheat flours. Although the differences were not significant, an increase in the GI index of flours produced with acid treatment is observable. This is due to the addition of acids, which are known to improve the stability of gluten (Jia et al., 2020).

Table 6.3 also shows the dough rheology of the wheat flour samples produced. These values were taken using a Mixolab, which tests dough rheology under simultaneous mixing and heating conditions. Higher ($P < 0.05$) water absorption and lower stability ($P < 0.05$) values were

observed for the heat-treated samples (alone or with acid) compared to the control. Water absorption refers to the amount of water needed by the flour to reach optimum dough consistency, while stability refers to the elapsed time during dough mixing before dough consistency falls below the optimum level. Both of these parameters are related to protein content, quality, damaged starch, and wheat polysaccharides (Alizadeh et al., 2022). Minimal differences in the protein content, gluten quality, and damaged starch values of the flour samples were present; hence, these observed differences could be due to differences in the particle size of the flour. Similar results were observed by (Sakhare & Inamdar, 2014), wherein finer particle-sized flours exhibited higher water absorption and lower stability during mixing. Similar amplitude, CS, and C2 values were observed for the wheat flours. These values represent the dough elasticity, dough consistency after mixing, and protein weakening the quality of the flour. The similar C2 value is important as it is known to correlate with bread-making quality (Hrušková et al., 2013). These values (amplitude, CS, and C2) are mostly determined by the protein quality of the flour. Hence, these observations can be linked to the similar protein quality and content of the wheat flours. Heat-treated flours (alone or in combination) had higher C3, C4, and C5 values compared to the control. C3 and C4 values indicate the hot gel viscosity and stability of the wheat flour during simultaneous mixing and heating conditions. C5 indicates the viscosity of the dough during cooling, which also reflects the ability of the flour components to re-associate. These values (C3, C4, and C5) correlate well with flour pasting properties obtained from RVA (Singh et al., 2019). Hence, the differences observed could also be explained similarly to that of the RVA results.

Table 6.4 shows the baking, texture, and crumb characteristics of the wheat flours produced using the selected tempering conditions. Differences ($P < 0.05$) in the specific volume values of the bread were observed. The CA-Heat ($3.47 \pm 0.16 \text{ cm}^3/\text{g}$) treatment had a similar

specific volume to the control ($3.46 \pm 0.06 \text{ cm}^3/\text{g}$), while the other treatments resulted in a lower specific volume (2.99 to $3.09 \text{ cm}^3/\text{g}$). The higher value for CA-heat is most likely due to the addition of citric acid, which is known to increase the protein stability of gluten (Jia et al., 2020). These improvements most likely resulted in increased gas retention and dough stability, which led to higher specific volumes. Acids are known to improve bread quality by lowering the intensity and density of the gluten network, which improves gas retention (Gupta et al., 2012; Su et al., 2019). The lower specific volume for the LA-heat, SBS-heat, and heat alone were most likely due to the heat treatment used as similar values for these treatments were observed. This indicates that the addition of the acid did not influence specific volume values. The heat treatment applied could have weakened the gluten structure causing lower specific volumes.

Table 6.4: Bread making quality of the wheat flours produced using the selected tempering

Test variables	Control	Heat alone	15% SBS+heat	15% CA+heat	15% LA+heat
<i>Textural properties</i>					
Specific volume (cc/g)	3.46 ± 0.06^A	3.09 ± 0.11^{BA}	3.03 ± 0.12^B	3.47 ± 0.16^A	2.99 ± 0.13^B
Hardness (N)	3.94 ± 0.32^B	6.77 ± 1.24^{BA}	10.10 ± 1.54^A	5.70 ± 0.68^B	7.56 ± 1.62^{BA}
Adhesiveness (N)	0.001 ± 0.00^A	0.001 ± 0.00^A	0.001 ± 0.00^A	0.002 ± 0.00^A	0.001 ± 0.00^A
Resilience (%)	40.68 ± 0.59^A	42.59 ± 0.92^A	37.08 ± 2.63^A	37.10 ± 2.05^A	39.87 ± 2.32^A
Cohesion	0.78 ± 0.00^A	0.80 ± 0.00^A	0.75 ± 0.02^A	0.74 ± 0.02^A	0.76 ± 0.025^A
Springiness (%)	106.46 ± 4.30^A	106.59 ± 10.72^A	106.49 ± 11.91^A	102.46 ± 5.75^A	106.47 ± 12.06^A
Gumminess	3.35 ± 0.10^B	5.42 ± 0.95^{BA}	7.53 ± 0.90^A	4.21 ± 0.46^B	5.74 ± 1.067^{BA}
Chewiness	3.56 ± 0.09^C	5.73 ± 0.99^B	7.92 ± 0.30^A	4.26 ± 0.32^{CB}	5.96 ± 0.54^B
Volume (cc)	513.33 ± 8.49^A	456.66 ± 16.99^{BA}	443.33 ± 16.99^C	510 ± 24.53^{BA}	450 ± 20.41^{BC}
<i>C-cell characteristics</i>					
Number of cells	2914.33 ± 190.4^A	2555 ± 40.04^A	2895.16 ± 180.81^A	2848.83 ± 57.8^A	2663.33 ± 50.4^A
Cell diameter (mm)	1.86 ± 0.11^A	$1.77 \pm 0.04^B^A$	1.537 ± 0.17^B	1.766 ± 0.04^{BA}	1.64 ± 0.04^{BA}
Wall thickness (mm)	0.42 ± 0.00^A	0.42 ± 0.00^A	0.40 ± 0.01^A	0.42 ± 0.00^A	0.42 ± 0.00^A
Cell volume (cc)	5.95 ± 0.348^A	5.62 ± 0.31^A	4.74 ± 0.69^A	5.49 ± 0.16^A	5.12 ± 0.11^A

Note: Values were expressed as means $\pm$ standard deviation (SD) from three replicates; values with different superscript letters are significantly different ($P < 0.05$) due to tempering treatment.

Textural qualities that are crucial to describing baked goods include hardness, springiness, cohesiveness, gumminess, chewiness, and resilience (Gao et al., 2022). Higher ($P < 0.05$) hardness and chewiness values were observed for the flours produced using heating (alone or with acid) compared to the control. The addition of organic acids in the dough results in higher viscosity, which leads to higher hardness and elasticity (Alkay et al., 2023; Moroni et al., 2012). However, no significant difference was observed in the springiness, cohesiveness, resilience, and adhesiveness values of the bread made. The treated samples also had higher ($P < 0.05$) gumminess (hardness*cohesiveness) compared to the control. This indicates that the hurdle approach during tempering resulted in more energy requirement for masticating the breadcrumbs. Similar observations were reported by Tien et al. (2018).

Crumb structures for the bread made were also included in **Table 6.4**; the c-cell images of the bread slices are also shown in **Figure 6.4**. The bread crumb structure forms during baking as both water and alcohol evaporate, which results in the formation of cells (holes) in the bread. During this procedure, the structure of the gluten expands and hardens as the moisture is drawn out during baking to form the bread structure. Similar ($P > 0.05$) cell number, wall thickness, and cell volume values were observed for the bread slices. The same observations were made by Rivera et al. (2022a), wherein no significant differences in the cell volume, number, and wall thickness were observed as a result of the hurdle tempering treatment. Lower ($P < 0.05$) cell diameter values were observed for the flours produced using the hurdle approach relative to the control. This indicates that the bread made from the tempering treatments has a finer crumb structure compared to the control (shown in **Figure 6.4**). This observation was most likely due to the addition of acids, which improved the stability of the gluten network. Variations in the results of this study and

published studies can be attributed to the differences in the culture, different concentrations of the acids, and the type of wheat used.

Overall, the use of a hurdle approach (acidic water + heating) as an intervention for wheat milling did not greatly influence the quality of the flour produced. Significant differences in flour quality parameters were observed; however, these differences did not cause adverse effects on the bread quality. Hence, the interventions described in this study are potential interventions for wheat milling to lessen *Salmonella* contamination in wheat kernels without compromising flour quality.

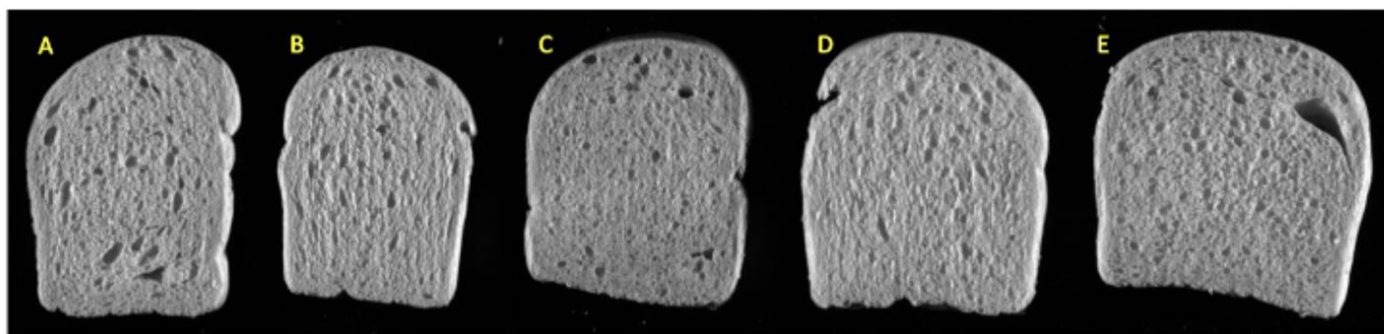


Figure 6.4: Representative C-cell images of bread slices. (A) Control; (B) Heat alone (55 °C); (C) 15% SBS+heat; (D) 15% CA+heat; (E) 15% LA+heat.

6.3. Principal component analysis (PCA)

PCA was used to better comprehend the variation in the flour quality and their interrelationships brought in by the different tempering conditions. During this procedure, the experimental dataset comprising of 37 variables and was compressed to 4 principal components (PCs) based on the eigenvalues obtained (Table 5). This allows for the identification of differences and similarities based on variable correlation and pattern recognition. The 4 PCs captured 99.99% of variabilities in the data. The first two PCs captured 66.16% of variance in the data, as shown in the **figure 6.5** (other combination of PCs and their variance can be found in the **supplementary**

figure S6.1). In PCA plots, positively correlated variables are positioned closely to each other, while negatively correlated variables are situated in a manner indicating an opposite correlation. From the figure it can be observed that cell diameter and volume ($e=0.24$ and $e=0.24$ respectively) correspond positively while the chewiness, gumminess, and hardness correspond negatively ($e= -0.20$, $e=-0.20$, and $e= -0.22$, respectively). Similarly, the trend in the A4 (15%LA+heat) and A1 (control+heat) are in the same direction as in the case of A2 (15%SBS+heat) and A3 (15%CA+heat). However, a negative trend is clearly seen between A1 (control+heat) and A2 (15%SBS+heat). Ultimately, the PCA biplot shows that each of the five samples resides in a distinct quadrant. This visual representation highlights the discernible variations in the quality attributes of these individual samples.

Table 6.5: Eigenvalues of the Correlation Matrix: Total = 37 Average = 1

	Eigenvalue (e)	Difference	Proportion	Cumulative
1	15.5947905	6.7121936	0.4215	0.4215
2	8.8825969	1.0138808	0.2401	0.6616
3	7.8687161	3.2148197	0.2127	0.8742
4	4.6538964	4.6538964	0.1258	1.0000

tempering. In terms of flour quality, the tempering treatments (heat alone, 15%SBS+heat, 15%CA+heat, and 15%LA+heat) produced wheat flours with comparable quality to the control (sterile water). Hence, combining acidic water tempering with heat treatment is a good intervention for reducing *Salmonella* contamination in wheat grains prior to milling. Finally, PCA was able to distinguish between the samples. Future studies can be conducted on validating this approach under different tempering conditions as milling facilities tend to have their own tempering guidelines to follow. In addition, future studies can also be done on improving the efficiency of the heat treatment process to further improve the economic viability of the tempering treatment.

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Chapter 7 - Evaluation of Pulsed Light as a Nonthermal Intervention for *Salmonella* Reduction During Wheat Tempering

Abstract

This study evaluated the antimicrobial potential of Pulsed Light (PL) exposure during wheat tempering to reduce *Salmonella* contamination. The preliminary screening of three non-thermal treatments (cold plasma, ultraviolet C, and PL) showed that only PL achieved a ≥ 3 log CFU/g reduction under the evaluated conditions. Hard red spring wheat was inoculated with a *Salmonella enterica* cocktail, tempered (17% moisture), and exposed to PL (0.164-0.329 J/cm²), and plating was performed on xylose-lysine deoxycholate (XLD) selective agar medium alone and XLD with a tryptic soy agar overlay (XLD+T) to account for the sub-lethally injured cells. The reduction of 4.22 log CFU/g (XLD) and 3.91 log CFU/g (XLD+T) at 0.329 J/cm² was observed after 18 h of tempering ($p < 0.0001$), indicating 7.2% sublethal injury-associated permeabilization. The Weibull inactivation model was the best fit for describing PL-induced *Salmonella* inactivation, indicated by a higher R^2 and a lower RMSE. The fitted shape parameter suggested a non-first-order inactivation with moderate shoulder/tailing. The model predicted a 5-log reduction in fluence would require approximately 0.41-0.48 J/cm² (99-118 s), consistent with experimental data. Bacterial lysis was caused by oxidative stress that damaged membranes, caused leakage, and compromised DNA integrity. The PL treatment at 0.329 J/cm² (80 s) had a significant effect on wheat flour functionality. Particle size distribution and dough rheology remained nearly identical ($p > 0.05$). However, a significant difference ($p < 0.05$) was observed in the damaged starch content, which further affected the bread's baking and C-cell characteristics. These findings pave the way for using PL as a potential intervention to reduce *Salmonella* in wheat during tempering.

7.1 Introduction

Global cereal consumption is expected to reach 2,943 million tons in 2025/26, with wheat grain dominating the worldwide food supply, totalling 805.3 million tons in 2025 and forecasted to increase to 810 million tons in 2026 (Balyatanda et al., 2026; FAO, 2026). Wheat-based food products have historically been regarded as microbiologically safe because of their low water activity (a_w ; <0.85), but recent events challenge this assumption. Since 2005, more than 27 food recalls and more than 15 illness outbreaks linked to enteric pathogens such as STEC and *Salmonella* have been traced to wheat flour and flour-based products, including dough premixes, cookie dough, pizza dough, and baking mixes (Doddabematti Prakash et al., 2025; Rivera et al., 2025). These pathogens can survive in wheat flour for up to 12 months despite their low water activity (a_w) (Du et al., 2020). Typically, there is no established pathogen reduction treatment applied to the wheat flour during the milling process (Rivera et al., 2022). The physical nature of the wheat milling process causes a redistribution of these pathogens across different milling fractions, which causes a food safety risk for the product (Eglezos, 2010). This risk is also worsened by the unsafe consumer of consuming raw dough and batters along with low consumer awareness on the food safety hazards associated with wheat-based foods (Feng & Archila-Godinez, 2021). *Salmonella* along with other pathogens, when present in low-moisture foods, exhibit increased thermal resistance (Archer et al., 1998; Bari et al., 2010). Therefore it is critical to explore innovative non-thermal mitigation strategies to enhance the safety of wheat-milled products.

Non-thermal preservation methods such as cold Plasma (CP), ultraviolet (UV), and pulsed light (PL) are potential alternative to the thermal technologies due to their efficacy against broad-spectrum inactivation of microbes with minimal to no effect on the food quality (Doddabematti

Prakash, Siliveru, et al., 2023; Levy et al., 2012; Magallanes López & Simsek, 2021). The PL treatment relies on intense, short-duration pulses of broad-spectrum light, UV (200-400 nm), visible (400-700 nm), near-infrared (700-1100 nm) using inert flash lamps or pulsed LEDs, with its energy extending up to 50 J/cm² (Elmnasser et al., 2007; Nicorescu et al., 2013). PL has been approved by the FDA for use in food production, processing, and handling since 1996, with a total dose limit of 12 J/cm² (Magallanes López & Simsek, 2021).

Previous studies have shown the potential of using acidic tempering (Doddabematti Prakash, Rivera, et al., 2023; Rivera, Deliephan, et al., 2021; Shivaprasad et al., 2024), thermal processes (Snelling et al., 2020), and non-thermal processes for decontamination of cereal grains (Lee et al., 2022; Subedi et al., 2020). However, thermal and acidic tempering interventions may adversely affect the product quality attributes at higher temperature and acid concentration. Alternatively, PL has emerged as a promising non-thermal decontamination technique in wheat grains and flour. For example Aron Maftei et al. (2014) reported a 4-log reduction in the naturally occurring molds in wheat when PL was used (51.2 J/g). Li et al. (2022) also reported a significant reduction in total plate count (TPC) and yeast and mold count (Y & M) in wheat grains. Although these studies demonstrate antimicrobial potency of PL, there is still limited evidence on its suitability as a pathogen reduction step in wheat milling steps such as tempering with regards to decontaminating *Salmonella*.

The present study was designed to address knowledge gaps in PL usage in wheat milling via (i) assessing and comparing efficacy of CP, UV-C and PL on *Salmonella* decontamination on inoculated wheat grains during tempering process; (ii) providing mechanistic evidence on *Salmonella* cells by measuring the cellular leakage and assessing the permeability and DNA integrity, and (iii) determining the extent of PL-induced changes on the physico-chemical

characteristics of wheat flour. The results from this study could provide basis on using PL-based processes for addressing *Salmonella* contamination on wheat grains during milling which can help improve the food safety of wheat-based products.

7.2. Materials and methods

7.2.1 Materials

Organic hard red spring (HRS) wheat grains (10.4% moisture, wet basis) were obtained from a domestic commercial source (Great River Organic Milling Company, Arcadia, Wisconsin). The raw wheat grains were pre-dried overnight at 60 °C for 24 h to offset the excess moisture added during the inoculation and tempering steps. The moisture content of both pre-dried ($6.92 \pm 0.1\%$) and original ($10.4 \pm 0.1\%$) wheat grains was measured using the standard forced-air oven method according to the ASAE S352.2 method (American Society of Agricultural and Biological Engineers) (ASAE, 1988). The pre-dried wheat grains were stored in resealable polyethylene bags at 5 °C until further use.

7.2.2 Preparation of working *Salmonella enterica* inoculum.

Four frozen cultures (-80 °C) of *Salmonella enterica* of different serotypes (Newport - ATCC 6962, Typhimurium - ATCC 14802, Kentucky - ATCC 9263, and Enterica - ATCC 8326) were obtained from the Feed Toxicology and Microbiology Laboratory at Kansas State University (Manhattan, KS). The *S. enterica* stock cultures were stored at -80 °C in tryptic soy broth (TSB) with 30% glycerol for later use. All microbial media used in this study were purchased from Difco (Beckton, Dickinson, and Company; Sparks, MD), unless otherwise specified.

Frozen cultures were thawed and then activated by streaking on tryptic soy agar (TSA) plates. The streak plates were incubated for 24 h at 37 °C. The culture preparation was performed as described by Doddabematti Prakash et al. (2024). Briefly, well-isolated colonies of *Salmonella*

enterica serovars were propagated into separate 10 mL TSB media and incubated for 24 h at 37 °C. Cell harvesting was done by centrifugation (4 °C, 2500 x g, 10 min), followed by inoculation into freshly prepared 600 mL TSB and further incubation at 37 °C for 24 h. Cells were collected again by centrifugation (4 °C, 2500 g, 10 min); pellets were washed twice with 0.1% peptone and then resuspended in 0.1% peptone to achieve a final viable cell concentration of approximately 10 log CFU/mL (plated in TSA). Equal amounts of cell suspension from each serovar were combined to create the working inoculum used for the experiments.

7.2.3 Inoculation and wheat tempering

The pre-dried wheat grains (250 g) were transferred into a sterile sample bag (9x12", 4 MIL, ULINÉ) and were spray-inoculated (Spray nozzle, Alpenko, CA) with *Salmonella enterica* cocktail (~10 mL). The wetted grains were hand-massaged and shaken for 3 min, then rested for 24 h at ambient temperature (24–25 °C) prior to tempering. 4 representative samples (10 g each) were drawn from the inoculated samples to enumerate for *Salmonella* load and to ensure homogeneity (defined as standard deviation of ± 0.3 log CFU/g among sub samples)(Snelling et al., 2020). This technique resulted in initial loads of 7.76 and 7.78 log CFU/g when plated on XLD selective agar medium and XLD with a TSA overlay, respectively. Tempering of the wheat kernels was performed in sterile distilled water to a moisture content of 17%.

7.2.4 Non-thermal treatment of tempered wheat

Three non-thermal treatment technologies were screened for their efficacy in reducing *Salmonella* population on tempered wheat. The treatment achieving ≥ 3 log CFU/g was considered efficacious and selected for the time-kill kinetics study. A schematic overview of non-thermal tempering experiment is shown in **Figure 7.1**.

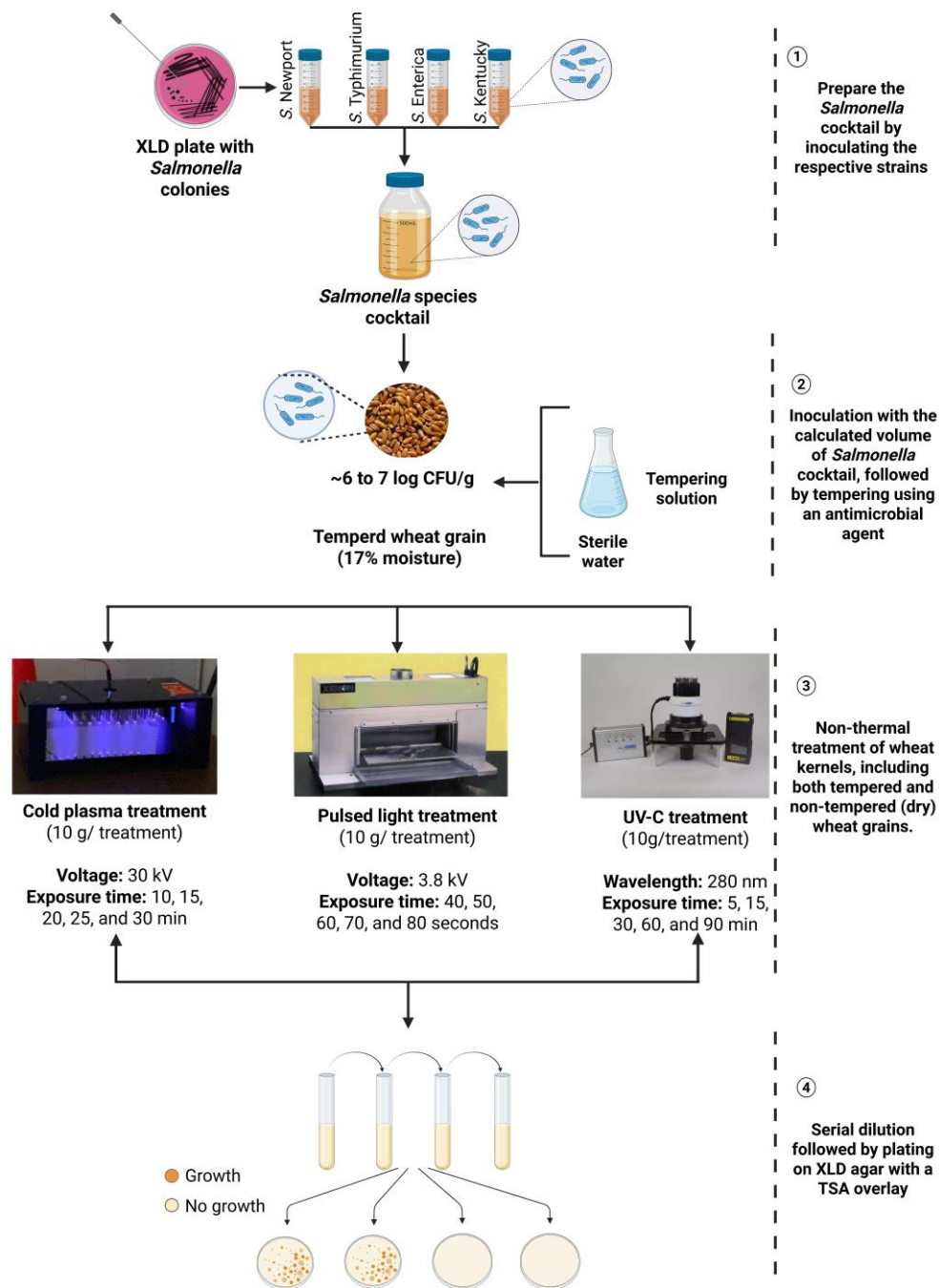


Figure 7.1: Experimental overview used to test the efficacy of different non-thermal tempering treatments against *Salmonella* in wheat

7.2.4.1 Cold plasma

A high-voltage atmospheric air multipin-plane CP reactor (40 cm x 25 cm x 20 cm; Ingenium Technologies, Odisha, India) was used in this study. Plasma was generated between two rectangular aluminium plate electrodes (top electrode = 88 pins and ground electrode) using two thick acrylic dielectric layers. The applied voltage to the reactor was provided by a high-voltage setup transformer and an electronic control panel. 10 g of tempered wheat kernels in an open glass Petri dish (100 mm x 15 mm) were exposed to CP generated in air (4.7 cm gap between the ground plate and the bottom edge of the pin) at 30 kV for 10, 15, 20, 25, and 30 min. The treated tempered wheat samples were stored for 18 h before enumerating for *Salmonella* survival.

7.2.4.2 UV-C

The inoculated wheat grains were exposed to UV-C using the PearlLab BeamTM UV Dose Response Tool (Aquisense; Charlotte, NC). The apparatus comprises a UV lamp (≈ 9 cm above the treated grains) and a collimating tube placed on an XY-Table (set on position zero). The radiation intensity (W/cm^2) was measured using a sensor connected to an optometer (ILT 2400; International Light Technologies, Peabody, MA), which was placed just under the XY-table surface. All the treatments were done at a wavelength (λ) of 280 nm was selected based on preliminary screening. Tempered wheat kernels (10 g) were placed in an open glass petri-dish (100 mm x 15 mm) and exposed to UV-C for 10, 15, 20, 25, and 30 mins. The total fluence (J/cm^2) for the treatment was measured as the product of the stabilized irradiance reading (W/cm^2) taken from the attached optical light meter and total exposure time (s). The total fluence for the UV-C increased over time; detailed fluence data are not shown, as this treatment did not advance beyond preliminary screening.

7.2.4.3 Pulsed light

PL treatment of tempered wheat grains was carried out using a lab-scale benchtop system (XENON Model, RC-847, Xenon Corporation, Wilmington, MA, USA) comprised of a controller, treatment chamber, and air-cooling module. A xenon flash lamp enclosed in a quartz window produced polychromatic light in the wavelength range of 240-1100 nm, with 40% of the radiation in the UV region (Lamp model, A type, mercury free). The frequency of the PL system was set to 3 Hz, with a pulse width of 360 μ s. From the manufacturer's description, a fluence of 12.7 J/cm² is produced per pulse at 1.905 cm distance when operating at 3800 V. The tempered wheat kernels (10 g) were placed in an open glass petri-dish (100 mm x 15 mm) and exposed to PL. Exposure was done at room temperature with samples placed at the shelf centre and 13 cm distance from the lamp. The number of pulses per second (3), distance (13 cm from lamp housing), fluence rate (3.81 W/cm²), and voltage (3800 V) were kept constant, while the exposure times of 40, 50, 60, 70, and 80 s were used, equivalent to 0.164592, 0.20574, 0.246888, 0.288036, 0.329184 J/cm², respectively.

The fluence rate (Equation 1) and effective fluence for each exposure was calculated using the following formula (Equation 2) (Shaik et al., 2024).

$$\text{Fluence rate (W/cm}^2\text{)} = \left[\text{Average fluence per pulse} \left(\frac{\text{J}}{\text{cm}^2} \right) \times \text{number of pulses} \right] \div \text{treatment time (s)} \quad (1)$$

$$\begin{aligned} \text{Effective fluence (F}_e\text{, J/cm}^2\text{)} \\ = [\text{Fluence rate (W/cm}^2\text{)}] \times \text{pulse width (s)} \times \text{number of pulses} \end{aligned} \quad (2)$$

The dosimetry for the PL treatments used were calculated and shown in **Table 7.1**. Overall, all treatment conditions used satisfy the 21 CFR 179.41 FDA (FDA, 2026c) regulation that limits

cumulative doses applied from PL treatments to 12 J/ cm². As the PL treatment was the most effective (consistent ≥ 3 -log reduction) among the non-thermal treatments used, the inactivation kinetics experiment was only done using PL treatment. In this experiment, the tempered wheat kernels were exposed to similar conditions and *Salmonella* enumeration was done at 0, 0.5, 6, and 18 h post-treatment.

Table 7.1: Dosimetry of PL on tempered wheat kernels

Time (s)	No. of pulses	Average fluence per pulse (J/cm ²)	Pulse width (μs)	Effective fluence (F _e , J/cm ²)
40	120	3.81	360	0.164592
50	150	3.81	360	0.20574
60	180	3.81	360	0.246888
70	210	3.81	360	0.288036
80	240	3.81	360	0.329184

7.2.5 *Salmonella* load enumeration

Samples (10 g) from each tempering treatment were collected aseptically (for preliminary screening, 18h; time kill kinetics = 0, 0.5 h, 6, and 18 h) during tempering. Samples were transferred to a stomacher bag (VWR Radnor, PA, USA) and homogenized for 120 s with 90 ml of sterile buffered peptone water (BPW) using a stomacher (Seward, UK). Serial dilutions of the homogenized sample were prepared in 0.1% peptone water, and suitable dilutions were spread-plated (100 μL, duplicate) onto XLD plates with TSA overlay for preliminary screening. For the time-kill kinetics study for PL, enumeration was performed on both XLD selective medium and XLD with a TSA overlay (hereafter referred to as XLD+T) to account for injured cells. The plates were then incubated at 37 °C for 24 h. Typical *Salmonella* colonies (black colonies) were counted

and expressed as log CFU/g. Reductions were calculated as the difference between the *Salmonella* level at the specified time intervals and at time zero. Sublethal injured cells (%) were calculated using **Equation 3** (Bevilacqua et al., 2021).

$$\% \text{ Sublethal Injury} = \left(\frac{N_{X+T} - N_{XLD}}{N_{X+T}} \right) 100 \quad (3)$$

7.2.6 Kinetic modelling of *Salmonella* inactivation on PL treatment

Survival data of *Salmonella* cocktail on wheat kernels after exposure to PL at five different treatment times (40, 50, 60, 70, & 80 s) and tempering intervals (0, 0.5, 6, & 18 h) were evaluated. Since the PL system was operated under fixed condition (distance and voltage), the cumulative frequency increased with exposure time; therefore, treatment time were converted to fluence value for kinetic modelling. The log-linear (first order) and Weibull mathematical models were fitted to log₁₀ survival ratios to characterize *Salmonella* inactivation via PL treatment. Although these models are commonly expressed as a function of treatment time, modified forms based on fluence are often used in PL studies. Accordingly, the log-linear model applied in this study was expressed as shown in **Equation 4**:

$$\ln \frac{N}{N_0} = -kF \quad (4)$$

where N is the surviving microbial population after PL treatment, N_0 is the initial microbial population, F is the fluence (J/cm²), and k (cm²/J) is the inactivation rate constant.

The Weibull model accounts for heterogeneity in microbial resistance within the population, as it can represent both concave and convex survival curves (**Equation 5**).

$$\ln \frac{N}{N_0} = - \left(\frac{-F}{\delta} \right)^\beta$$

(5)

where δ is the scale parameter, β is the shape parameter, and F represents effective fluence (J/cm^2). The parameter β indicates the form of the survival curve, where $\beta = 1$ describes a log-linear relationship, $\beta < 1$ indicates upward concavity or tailing, and $\beta > 1$ indicates downward concavity or shouldering (Van Boekel, 2002). Furthermore, the fluence (J/cm^2) required to achieve a 5-log (F_{5D}) reduction based on the Weibull model was calculated as shown in **Equation 6**.

$$F_{5D,t} = \delta_t 5^{1/\beta} \quad (6)$$

where $F_{5D,t}$ denotes the estimated fluence for 5-log microbial inactivation under condition t , δ_t is the corresponding Weibull scale parameter, and β is the Weibull shape parameter.

7.2.7 Matrix effect:

The matrix effect of wheat was quantified by comparing the fluence required to achieve an equivalent level of inactivation in wheat and aqueous systems. Aqueous data were best described by a log-linear model, and the inactivation of wheat by a Weibull model. Hence, model parameters were converted to a common lethality metric, F_{5D} , and the matrix effect was calculated as shown in **Equation 7** (Balyatanda et al., 2026). Comparisons were performed separately for XLD and XLD+T recovery media to avoid confounding matrix effects, because the media recovered different numbers of sub-lethally injured cells.

$$\text{Matrix effect (ME)} = \frac{F_{5D\text{wheat}}}{F_{5D\text{aqueous}}} \quad (7)$$

7.2.8 Mechanistic overview of PL-induced cellular damage

0.1 OD₆₀₀ (~1x10⁶ CFU/mL) of an overnight-grown culture of *Salmonella* was used for all the mechanistic studies described below. The cells were centrifuged (4 °C at 2500 rpm, 10 min) to obtain cell pellets, and they were resuspended in sterile PBS to obtain the desired OD.

7.2.8.1 Cell membrane integrity

The membrane integrity was measured using the crystal violet stain (CVS) assay, as previously described by Ren et al., (2025). Bacterial cells were prepared with or without PL treatment. The cells were harvested by centrifugation (4 °C, 6000 x g for 10 min). The precipitates were washed with PBS (3x) before resuspending in crystal violet solution (0.05%). The mixture was incubated at room temperature for 20 min before being washed (sterile distilled water, 2x) and resuspended in 95% ethanol. The membrane permeability was determined by measuring absorbance of supernatant (4 °C, 6000 x g for 10 min) with a UV Spectrophotometer (GENESYS Vis/UV–Vis Spectrophotometers, Thermo Fisher Scientific, Waltham, MA, USA) at 595 nm.

7.2.8.2 Cytoplasmic leakage analysis

Intracellular nucleic acid and protein leakage were measured using a previously described method by He et al. (2021). Bacterial cells were prepared with or without PL treatment. The cells were harvested by centrifugation (4 °C, 6000 g for 10 min). The supernatants were collected and measured for protein and nucleic acid at Abs = 280 nm and Abs = 260 nm using a UV Spectrometer (GENESYS Vis/UV–Vis Spectrophotometers, Thermo Fisher Scientific, Waltham, MA, USA). The results were represented as the leakage index (LI) (**Equation 8**) (Balyatanda et al., 2026)

$$LI = \frac{(OD_{260} + OD_{280})_{treated}}{(OD_{260} + OD_{280})_{control}} \quad (8)$$

7.2.8.3 DNA integrity

Bacterial cells for genomic extraction were prepared with or without PL treatment and precipitated by centrifugation (4 °C, 10000 g for 10 min). The pellets were then resuspended in sterile PBS before extraction using the DNeasy® Blood and Tissue Kit (Qiagen, Germantown, MD, USA), following the manufacturer's protocol. To assess the direct photochemical effect of PL on DNA, cell-free DNA from untreated cells was exposed to PL under similar conditions. Following this, gel electrophoresis (1% agarose gel; TAE 1x; 80V) confirmed DNA integrity (samples were loaded onto the gel with orange G loading dye and a 100 bp DNA ladder). DNA bands were visualised under UV illumination (Thamburaj et al., 2020).

7.2.9 Evaluation of milling and flour quality parameters

7.2.9.1 Milling

For milling study wheat kernels (1000 g) were tempered with sterilized water, mixed in a rotary mixer (20 min), treated with PL (80 s) before transferring them into a re-sealable bag. The tempered wheat kernels were kept at room temperature (24-25 °C) for 24 h before milling. The Chopin LabMill (Chopin, France) was used to mill the wheat flour, comprising 6 grinding steps (2 breaks, 1 sizing, and 3 reductions). Initially, the break phase was conducted at a feed rate of 5 g/s, and the subsequent milling steps were performed at 2.5 g/s. Flour for direct use was obtained by combining the generated flour fractions (break, size, and reduction flour). Flour samples were held at refrigerated condition (4 °C) until use. The milling yield of the wheat-milled fractions was determined as per **Equation 9** (Doddabematti Prakash et al., 2024).

$$\text{Milling yield (\%)} = \frac{\text{weight of milling fraction}}{\text{weight of wheat}} \times 100 \quad (9)$$

7.2.9.2 Physico-chemical analysis of PL treated wheat flour

The moisture content of the whole wheat flour was measured according to ASAE S352.2 method (ASAE, 1988). The particle size distribution (PSD) of straight grade wheat flour was evaluated through a vacuum sieve, Alpine Jet Sieve (Hosokawa Micron, E200 LS) (AACC method 55-60.01). The damaged starch content (UCDc) was measured using an SDMatic™ (Chopin Technologies; Villeneuve-la-Garenne, France) according to AACC method 76-33.01 (International, 2011). Rheological properties of the dough as a function of mixing and heating were measured using a Mixolab (Chopin technologies, France) as per AACC method 54-60.01 (AACC, 2011) using a Chopin + protocol. Solvent retention capacity (SRC) characteristics of wheat flour in water, 50% sucrose, 5% sodium carbonate, and 5% lactic acid solutions were measured according to AACC method 56-11.01 (AACC, 2011). Bake tests were performed in accordance with AACC method 10-10.03 (AACC, 2011). The quality evaluation of the baked breads were performed for (i) volume using rapeseed displacement method (AACC method 10-10.05) (AACC, 2011); (ii) crumb analysis of the sliced bread samples were carried out using C-cell image analyzer (CCFRA Technology Ltd.) and corresponding characteristics like diameter, wall thickness, volume and cell numbers were determined; and (iii) textural properties of the bread samples were measured using a TA.XT. Texture Analyser (Stable Micro System) with a 25 mm diameter cylindrical probe.

7.2.10 Statistical analysis

All experiments were conducted in triplicate, and results were expressed as mean $\pm$ SD. The initial screening of three non-thermal technologies, CP, UV-C, and PL, each at five exposure times, was analysed using one-way ANOVA ($\alpha = 0.05$) with Tukey's HSD post-hoc test. For the time-kill kinetics experiment a full factorial design was employed to assess the effect of PL on Salmonella in wheat with PL exposure time (40, 50, 60, 70, and 80 s) and tempering duration (0,

0.5, 6, and 18 h) as independent variables, following a 5×4 full factorial design evaluated across two recovery media (XLD and XLD+T overlay); data were analysed using two-way ANOVA with Tukey's HSD ($\alpha = 0.05$). The inactivation data were fitted in RStudio v4.4.0 using nonlinear least-squares regression with the nlsLM function from the minpack.lm package. Goodness of fit was evaluated using the residual sum of squares (RSS), coefficient of determination (R^2), adjusted R^2 , root mean square error (RMSE), and Akaike information criterion (AIC). Physical and functional properties of flour from PL-treated and control wheat were compared using unpaired t-tests with multiple testing controlled by the False Discovery Rate (FDR) two-stage step-up procedure ($Q = 0.05$). All statistical analyses were performed using GraphPad Prism 10 (Version 10.5.0, GraphPad Software, San Diego, CA, USA).

7.3. Results and Discussion

7.3.1 Comparative efficacy of non-thermal treatments for *Salmonella* inactivation on grains

Non-thermal decontamination methods have gained significant interest as innovative strategies for LMFs because of their ability to inactivate foodborne pathogens while preserving the nutritional and functional qualities of food products (Chacha et al., 2021). However, the effectiveness of these techniques is influenced by several factors such as treatment type, product characteristics, target pathogen, treatment mechanism, lethal dose, and exposure time. Therefore, a comparative evaluation of non-thermal methods, namely UV, CP, and PL, was performed to identify the most effective approach for decontaminating wheat from *Salmonella* (**Figure 7.2**). The CP treatment was the least effective under the tested conditions, reducing *Salmonella* by ≈ 0.8 log CFU/g ($p > 0.05$) after 30 min of treatment. The UV treatment showed a slightly higher reduction in *Salmonella* levels, with a maximum of 1.5 log CFU/g ($p = 0.02773$) after 90 min of

treatment, compared to the control (6.9 log CFU/g). PL, on the other hand, demonstrated superior efficacy among all the tested techniques, achieving up to 3.9 log CFU/g reduction after 80 s of exposure ($p=0.0277$) compared to the control (6.9 log CFU/g). Overall, the PL treatment achieved the greatest and most consistent reduction in *Salmonella* counts in the shortest treatment time relative to the CP and UV treatments.

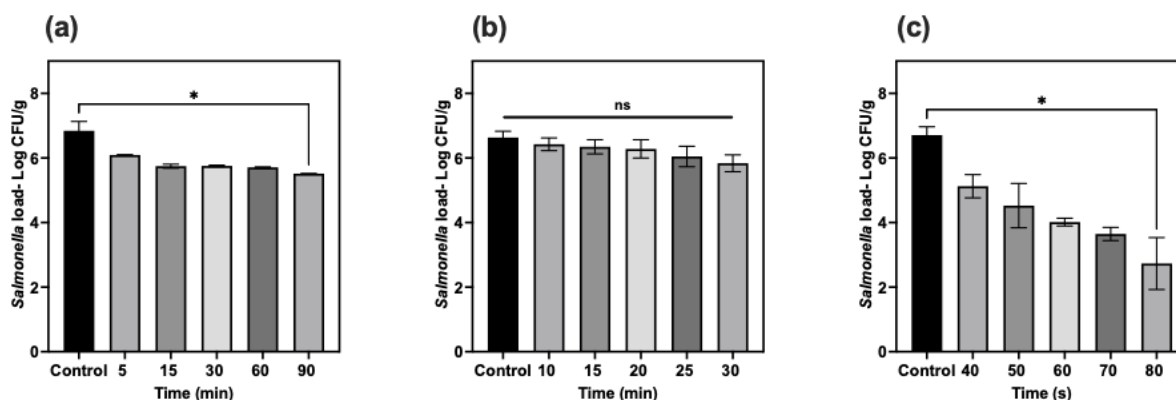


Figure 7.2: Non-thermal decontamination of tempered wheat grains (a) UV-C; (b) Cold plasma; (c) Pulsed light. The bar plots show mean *Salmonella* counts (Log CFU/g) $\pm$ standard deviation from 3 replicates plotted for all panels; An asterisk (*) indicates a treatment time whose mean *Salmonella* count differs significantly from the untreated control ($p < 0.05$), whereas “ns” denotes no significant difference ($p \geq 0.05$).

The differences in the antimicrobial effectiveness of these techniques lies in how they deliver their anti-microbial effects to the target microorganism. In bacterial cells, UV radiation induces pyrimidine dimers in their DNA, impairing replication and transcription and potentially leading to mutation or cell death (Cheon et al., 2015; Chevremont et al., 2012), while PL uses high-intensity, broad-spectrum pulses (200–1200 nm) that can cause photochemical, photothermal, and structural damage to microbial cells simultaneously (Bhujle et al., 2026). Therefore, the limited inactivation seen with UV can be due to its line-of-sight nature and poor penetration, especially on irregular surfaces. Evidence shows that rough or highly creviced foods

(peppercorn) can significantly reduce treatment effectiveness by shielding microorganisms from direct photon exposure (Bhavya & Umesh Hebbar, 2017; Bhujle et al., 2026). The CP treatment relies on the produced reactive species to diffuse and interact with target microorganisms. Similarly, the lower effectiveness of CP can be linked to the restricted and uneven interaction of reactive species with microorganisms on dry, granular surfaces (García Casado et al., 2024; Laroque et al., 2022). In contrast, PL shows increased inactivation because it concentrates light energy into high-intensity pulses, which, combined with the photo-thermal effect, produce a stronger antimicrobial action (Bhujle et al., 2026; Ye et al., 2025). Since PL demonstrated the most consistent and significant reduction in *Salmonella* count within the shortest treatment time, the rest of the treatments were excluded in the subsequent experiments conducted.

7.3.2 Time kill kinetics and sub-lethal injury of *Salmonella* during tempering

The time-kill kinetics of PL-treated *Salmonella* during tempering were evaluated by using both a selective (XLD) and overlay agar (XLD + T) mediums (**Figure 7.3**). All PL treatments caused a dose-dependent reduction in *Salmonella* populations in wheat grains compared to the untreated control (XLD=7.76 log CFU/g; XLD+T=7.78 log CFU/g). The inactivation efficacy increased significantly with longer exposure times (40 to 80 s) and longer tempering intervals (0.5 to 18 h) ($p < 0.0001$). This is mainly because the total effective fluence increases with treatment time at a constant voltage and distance (Harguindeguy & Gómez-Camacho, 2021; John & Ramaswamy, 2020). Exposing wheat grains to PL for 40 s reduced the *Salmonella* population by 1.07 log CFU/g on XLD and 0.71 log CFU/g on X+T, compared to control (0 h). This reduction improved with increased tempering time, with *Salmonella* cells recovered on XLD reporting 1.31 log CFU/g, while on X+T it was 1.29 log CFU/g. This explains the effect of tempering duration on *Salmonella* survival upon PL treatment. A similar trend was observed across all tested

conditions (dose and tempering time). Aron Maftai et al. (2014) reported that an increase in the effective fluence significantly improves the microbial inactivation. Similarly, Gyawali et al. (2024) reported a significant increase in *Salmonella* reduction with increased exposure time on pecans, from 1.58 log CFU/g (10s) to 4.55 log CFU/pecan (40 s).

Similarly, the percentage of sub-lethally injured cells varied non-monotonically with exposure time and tempering duration, ranging from 0.1% to 76.3% on XLD+T vs. XLD. The cells under low exposure time (40 s) showed an initial increase in injury (5.3% at 6 h); however, with increased tempering time (18 h), the cells recovered, with % injury dropping to 0.61%, reflecting a progressive repair induced by *Salmonella*. In contrast, at a higher dose (80 s), % injury increased from 4.25% to 8.52%, demonstrating a steady delay in membrane degradation following a progressive oxidative damage of the bacterial cell (Schottroff et al., 2018; Shao et al., 2023). Chen et al. (2023) reported that *Salmonella enteritidis* undergoes repair mechanism in three characteristics pathway: (i) repairing the damaged outer membrane through reassembly of porins, upregulating of fimbrial expression, and activating thioredoxin reductase (*TrxR*) to counteract oxidative damage caused by ROS; (ii) repairing the damaged DNA by halting DNA replication and initiating the SOS response system, while providing more ATP for DNA damage repair; (iii) reallocating metabolic resources to fuel cellular repair by upregulating aminoacidic biosynthesis and saccharide transport pathways to supply the ATP and nitrogen for membrane and DNA renewal. Therefore, cells that undergo sufficient recovery could survive XLD's selective pressure induced by sodium deoxycholate and bile salts, thereby further impeding membrane repair in already damaged cells. However, the TSA overlay provides a non-selective repair window during the early phase of the incubation period that allows the cell to restore membrane integrity before

encountering the components linked to the selective function of XLD agar (Kang & Fung, 2000). Therefore, recovery on XLD was limited compared with XLD+T.

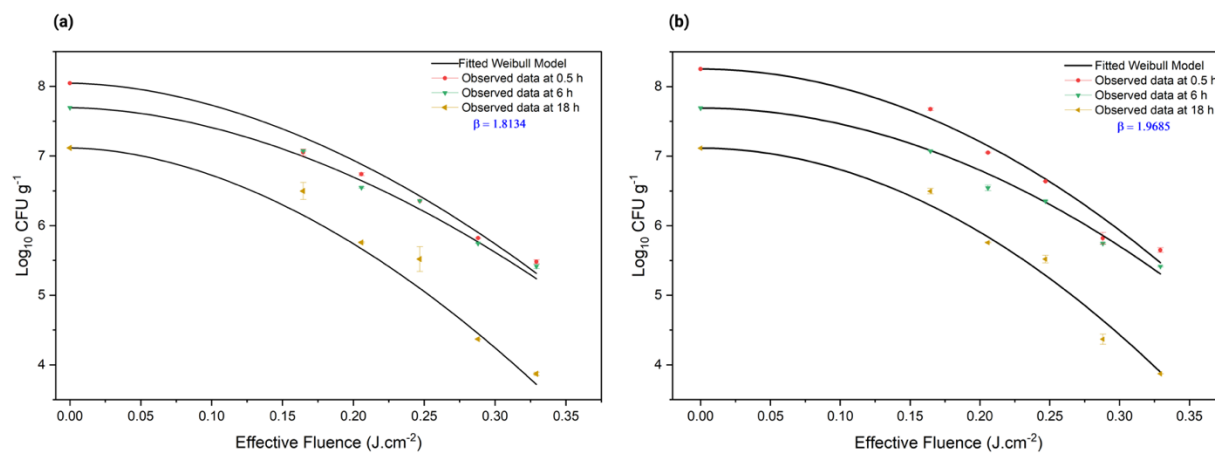


Figure 7.3: Predicted Weibull inactivation curves and experimental survivors of *Salmonella* on tempered wheat grains as a function of PL effective fluence, recovered on (a) XLD selective medium and (b) XLD supplemented with TSA overlay. Solid lines represent the fitted inactivation curve for tempered wheat kernels (0.5, 6, and 18 h). Survival is expressed as log CFU/g (mean of three independent replicates), plotted against effective fluence (J/cm^2); the coefficient of determination (R^2) for each model is shown in the corresponding panel.

7.3.3 Inactivation Kinetics of *Salmonella* During PL Tempering

Microbial inactivation kinetics are typically modelled as first-order (log-linear) kinetics as it is the least complicated model. However, deviations from linearity (e.g., shouldering / tailing, curvature) could occur once survival data are plotted on semi-logarithmic plots. These deviations are largely due to protective microenvironments, a heterogeneous resistance profile within a microbial population, and sub-lethal injuries (Fang et al., 2021; Peleg, 2000, 2021; Peleg et al., 2003). In our study, the survival of *Salmonella* on tempered wheat treated with PL was modelled using log-linear and Weibull models (**Table 7.2**) to account for this potential variability. In

addition to the selective recovery medium (XLD), a non-selective overlay recovery medium (XLD+T) was also employed. The Weibull model, irrespective of tempering time or recovery media, provided a superior fit to its counterpart (log-linear) as evidenced by higher R^2 , lower RMSE, and lower AIC values. Samples recovered on XLD+T; the log-linear model showed a moderate fit, with R^2 values ranging from 0.8361 to 0.9195. Conversely, the Weibull model considerably improved the fit ($R^2 = 0.9782$ - 0.9916 ; RSME 0.07-0.1378) while also achieving lower AIC at each time point. For example, at a tempering time of 6 h, the Weibull model remained superior ($R^2 = 0.9916$; RSME = 0.07; AIC = -10.8773) while the log-linear fit poorly ($R^2 = 0.5269$; RSME = 0.2174; AIC = 0.5269). A similar trend was also observed with the samples isolated on XLD medium alone. A log-linear model gave lower R^2 values (0.8347-0.9686) and higher RSME values (0.1487-0.4956), which suggested a poorer fit than the Weibull model that gave higher R^2 values (0.9929-0.9962) and lower RSME values (0.052-0.1027). Furthermore, the Weibull model yielded the lowest AIC values across all tempering times, further supporting its superiority over the log-linear model. Although the Weibull model offered a superior fit, its predictions have some limitations. Model fitting was based on only 4 experimental data points per condition. Additionally, the F_{5D} values were extrapolated as experimental data did not yield a 5-log reduction. Therefore, these results should be seen as approximate estimates rather than precise predictive thresholds.

The Weibull-derived fluence required for a 5-log reduction ranged from 0.4116 to 0.4795 J/cm² in XLD+T and from 0.4074 to 0.4873 J/cm² in XLD. For tempering, the 18 h wheat setting had the lowest F_{5D} and shortest treatment time in both cases, while the 6 h tempering needed the most energy. It is important to note that even though the spread across values was small, these results suggest that the efficacy of PL was modestly influenced by tempering duration, possibly

through changes in grain surface moisture distribution, microbial attachment, or optical exposure conditions during treatment.

The shape parameter (β) was greater than 1 for each recovery medium (XTD+T = 1.9685; XLD = 1.8134), indicating that the survivor curve had an upward concavity and a shoulder effect. This means that the *Salmonella* cells initially showed some resistance to the PL treatment, but the rate of cell death increased rapidly as the light dose increased. The scale parameter (δ), for XLD+T ranged from 0.1817-0.2117, and for XLD from 0.1677-0.2006, indicating that the time needed for inactivation varies depending on the tempering time. Accordingly, the Weibull model was selected as the best fit for describing *Salmonella* inactivation by pulsed light during wheat tempering.

Table 7.2: Inactivation model fit parameters for *Salmonella* on wheat at various tempering time

Recovery Media	Tempering time (h)	Model	Main parameters (s)	R ²	RMSE	AIC	$\Delta AIC_{\min}$	Weibull parameters ($\pm 95\%$ CI)	
								F_{5D} (J/cm ²)	Exposure time for 5D (s)
XLD+T	0.5	Log-linear	k = 7.1136	0.88	0.3237	5.6314	8.38	0.4430	108
		Weibull	$\beta = 1.9685$; $\delta = 0.1956$	0.9782	0.1378	-2.7542	0		
	6	Log-linear	k = 6.1232	0.9195	0.2174	0.5269	11.40	0.4795	117
		Weibull	$\beta = 1.9685$; $\delta = 0.2117$	0.9916	0.07	-10.8773	0		
	18	Log-linear	k = 8.1497	0.8361	0.4557	9.9799	11.9516	0.4116	100
		Weibull	$\beta = 1.9685$; $\delta = 0.1817$	0.9829	0.1471	-1.9717	0		
XLD	0.5	Log-linear	k = 7.2177	0.9686	0.1487	-1.8399	12.6081	0.4596	112
		Weibull	$\beta = 1.8134$; $\delta = 0.1892$	0.9962	0.052	-14.448	0		
	6	Log-linear	k = 6.4675	0.9491	0.175	0.3245	13.012	0.4873	118
		Weibull	$\beta = 1.8134$; $\delta = 0.2006$	0.994	0.0602	-12.6875	0		
	18	Log-linear	k = 8.7693	0.8347	0.4956	11.015	17.2953	0.4074	99
		Weibull	$\beta = 1.8134$; $\delta = 0.1677$	0.9929	0.1027	-6.2803	0		

Note: Values are parameter estimates for log-linear and Weibull models fitted to survival curves of *Salmonella* following PL treatment; k is the log-linear inactivation rate constant; β and δ are Weibull scale and shape parameters, R² is the coefficient of determination, RMSE is the root mean square error, AIC is Akaike's information criterion, and ΔAIC is the difference from the minimum AIC within each data set. Effective fluence (J/cm²) and exposure time (s) for 5 log reduction are calculated from the best-fitting model (Weibull model)

Comparing the PL lethality in wheat and aqueous medium using a common F_{5D} endpoint revealed significant matrix effects (**Table 7.3**). In demand-free aqueous medium, the inactivation rate constant was $k_{\text{aqueous}}=116.23$ on XLD agar (Adj $R^2=0.99$) and $k_{\text{aqueous}}=100.85$ (Adj $R^2=0.90$) in XLD+T medium, respectively. The aqueous system gave F_{5D} values of 0.0430 J/cm^2 for XLD+T and 0.0496 J/cm^2 for XLD, whereas the respective F_{5D} values in wheat were between 0.4116 to 0.4795 J/cm^2 and 0.4074 to 0.4873 J/cm^2 . Therefore, the matrix effects for XLD+T and XLD were 9.57 - 11.15 and 8.22 - 9.83 respectively, which means that wheat requires about 9-10 times higher fluence than water to get the same level (5-log) of inactivation. This significant matrix effect may well be due to the limited penetration of PL and the protective effect of the dark, uneven wheat surface, which limits microbes' exposure to light compared with transparent aqueous systems (Elmnasser et al., 2007; Kang & Fung, 2000). The fact that the matrix effect was slightly higher with XLD+T indicates that some cells that were injured but not killed could recover and were detected on XLD+T but not on XLD alone.

Table 7.3: Matric effect (ME) during PL tempering, calculated from F_{5D} values in water and wheat system

Medium	$F_{5D} \text{ (J/cm}^2\text{) Aqueous}$	$F_{5D} \text{ (J/cm}^2\text{) Wheat}$	ME
XLD+T	0.049580617	0.4430	8.93
		0.4795	9.67
		0.4116	8.30
XLD	0.043018091	0.4596	10.68
		0.4873	11.33
		0.4074	9.47

Note: $F_{5D} \text{ (J/cm}^2\text{) Wheat}$ = pulsed-light fluence required to achieve a 5-log reduction on tempered wheat kernels at tempering time; $F_{5D} \text{ (J/cm}^2\text{) Aqueous}$ = pulsed-light fluence required to achieve a 5-log reduction in demand free aqueous medium

7.3.4 Mechanistic insight

Figure 7.4 shows that the relative membrane damage and cellular leakage indices of PL-treated *Salmonella* cells increased with increasing effective fluence, indicating a dose-dependent disruption of bacterial cell integrity. Relative membrane damage increased from 2.33 to 2.8-fold, while the cellular leakage index increased from 1.3 to 2.05-fold. Therefore, an increase in the leakage index measured at Abs=260 nm (nucleic acid leakage) and Abs=280 nm (protein leakage) suggests a steady efflux of cellular components from the bacterial cell into the surrounding medium (Ji et al., 2023; Niu et al., 2025). Similarly, a higher uptake of CV can be attributed to a steady disruption of membrane permeability and cell envelope, which is an indicator of bacterial membrane damage (Halder et al., 2015; Wang et al., 2022). The observed results are also likely due to the synergistic photochemical, photothermal, and photophysical effect of PL. In general, the UV spectrum of PL typically generates ROS that causes lipid peroxidation and protein oxidation, while the visible and infrared spectrums induce localized heating and mechanical stress that further impairs membrane integrity (Bhavya & Umesh Hebbar, 2017; Elmnasser et al., 2007).

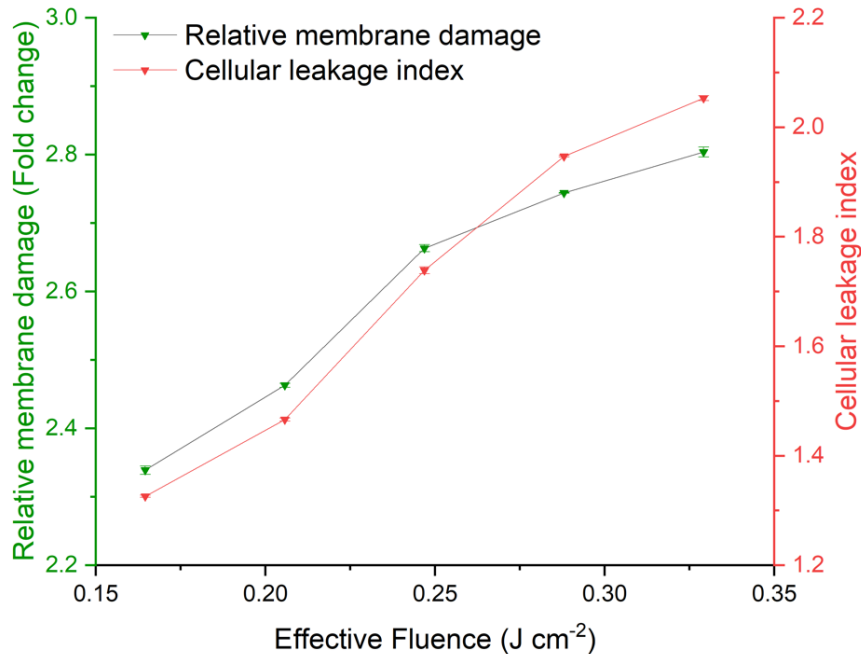


Figure 7.4: Assessment of *Salmonella* survival and membrane stability following PL treatment using crystal violet assay (CVS assay)

The genotoxic effects of PL were assessed on both on genomic DNA (TC) and naked DNA (DT) using agarose gel electrophoresis (Figure 7.5). The genomic DNA (control) appeared as a sharp, high-molecular-weight band with no smearing, which is indicative of an intact and unfragmented chromosomal DNA. The TC Lane (TC 40 s -TC 80 s) showed a progressive, dose-dependent increase in DNA degradation, with progressive smearing effect and loss of band intensity. This corroborates the reports that demonstrated formation of cyclobutane pyrimidine dimers (CPDs), double-stranded breaks, and (6-4) photoproducts resulting from UV-C component of PL (Chen et al., 2023; Kemp & Sancar, 2012). In the 70 and 80 s, the TC lanes displayed almost complete disappearance of the intact DNA band, signifying large-scale genomic fragmentation. The DT Lane, however, exhibited only a small amount of DNA degradation under similar exposure

conditions. The TC samples that exhibited complete fragmentation at 70 s and 80 s showed very minimal degradation in the relative DT lanes. This difference suggests that in-situ cellular photoinactivation caused by PL may be a result of not only direct photochemical DNA damage but also indirect oxidative damage as a result of oxidative stress induced by ROS accumulation produced as a result of the interaction between the PL and the cell membrane and cytoplasmic components, which together leads to further genomic degradation (Chen et al., 2023; Schottroff et al., 2018; Thamburaj et al., 2020).

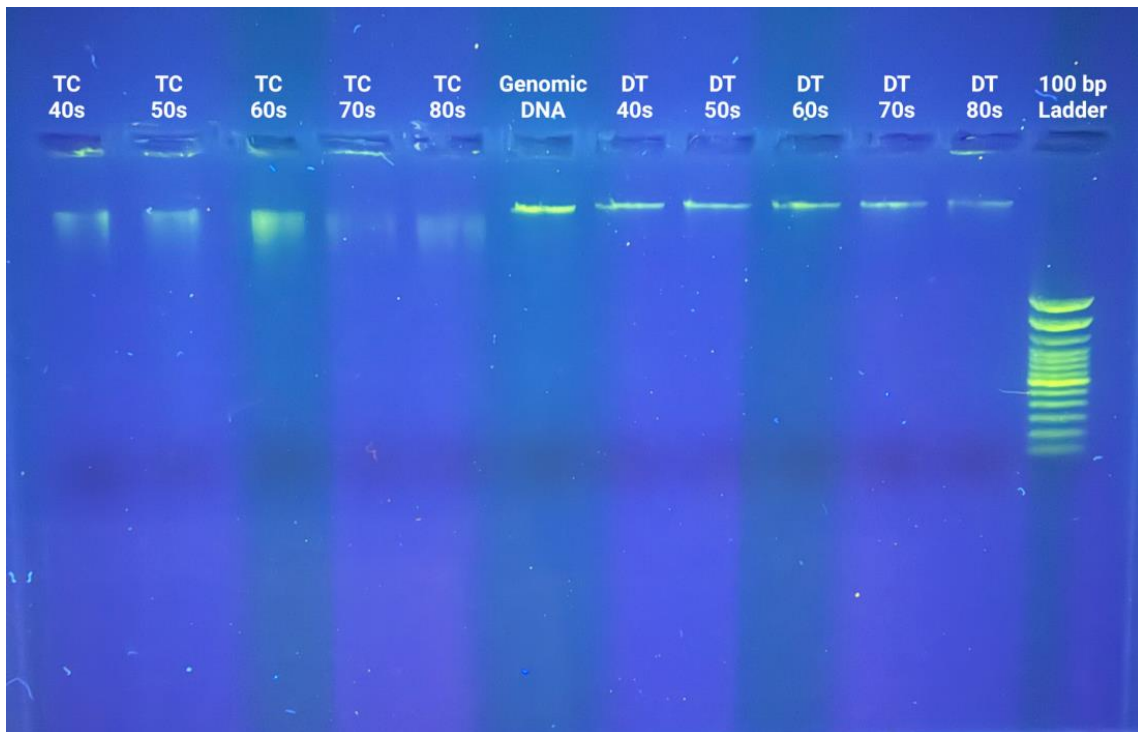


Figure 7.5: Oxidative damage of *Salmonella* genomic DNA following PL treatment. Genomic DNA was recovered either (TC lanes) from cells exposed to PL prior to lysis or (DT lanes) from naked DNA directly treated with PL under identical conditions. Lanes (left to right) correspond to genomic DNA extracted from treated cells for 40, 50, 60, 70, and 80 s (TC 40–80 s), untreated genomic DNA control, and naked DNA directly exposed for 40, 50, 60, 70, and 80 s (DT 40–80 s), followed by a 100-bp ladder. DNA fragments were resolved on a 1% agarose gel and visualized with SYBR-safe stain.

7.3.4 Flour quality evaluation

The particle size distribution (PSD) of straight grade flour from treated and untreated wheat grains is shown in Figure 7.6. Based on the PSD, all flour samples exhibited a bimodal distribution, which is typical for wheat flour as it typically contains finer protein fractions and coarser starch fractions after milling (Doddabematti Prakash et al., 2024; Moiraghi et al., 2019). The wheat flour particle sizes ranged from 50 to 180 μm , which complies with the CFR requirement (21 CFR137.105) (FDA, 2026a) for wheat flours, where more than 98% of particles should pass through a 212 μm sieve (FDA, 2026a). The similar PSD curves for treated and untreated samples suggest that PL treatment does not change endosperm fracture patterns. As a result, effects on dough hydration or rheology are unlikely to be caused by PL's influence on PSD. These results align with those reported by Rivera, Phebus, et al. (2021).

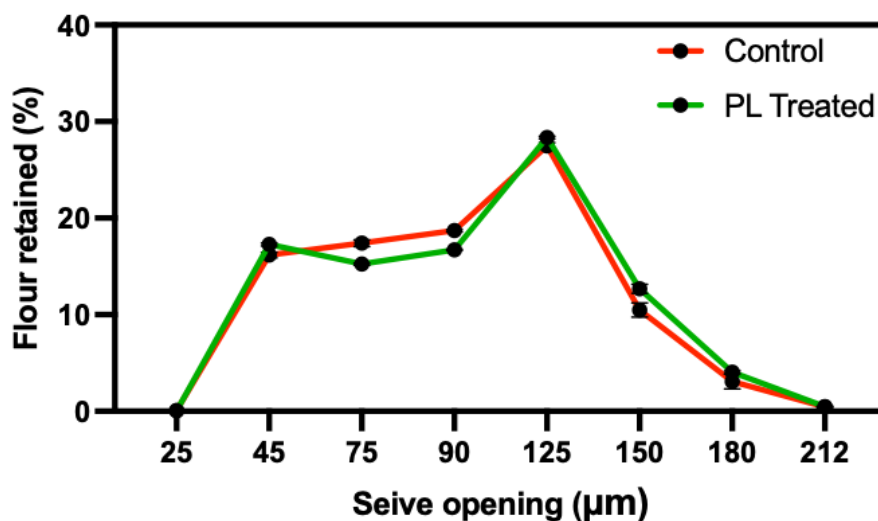


Figure 7.6: Particle size distribution (PSD) of wheat flour

The PL-assisted tempering effect on milling yields (% as-is) for bran, shorts, BK flour, SZ flour, reduction flour, and straight-grade flour is shown in Table 7.4. No significant difference in the milling yield fractions of the wheat samples was observed except for BK flour ($p=0.025833$). This overall preservation of the milling fractions after PL treatment suggests that surface-targeted light exposure does not degrade the wheat kernel's structural integrity or alter the milling process. This, in turn, restricts the penetration of the high-intensity pulse into the bran and endosperm layers. However, the PL-treated BK flour sample showed a significant reduction in BK. This change reflects localized surface alterations at the bran-endosperm level because of high-intensity broad-spectrum light pulses. Since no other fractions were significantly affected, this change is unlikely to have a major practical impact on the overall wheat flour composition profile. The moisture content of the wheat flour samples was within the legal limit set by the FDA (21 CFR 137.200) (FDA, 2026b). However, there was a significant difference in the damaged starch content in PL treated flour ($p=0.008$). The extent of damaged starch content can influence dough mixing, bread volume, water absorption, and crumb characteristics (Ghodke et al., 2009). Moderately damaged starch promotes fermentation by yeast, leading to gas production (CO_2), hydrates effectively, and is readily susceptible to enzymatic hydrolysis during dough preparation (Ma et al., 2016). Higher amounts of damaged starch can increase the reducing sugar content of the resulting flour (El-Porai et al., 2013), while excessive levels of damaged starch lead to much higher water absorption and increase enzymatic reaction rates causing a stickier dough and poorer crust color (Ghodke et al., 2009).

Table 7.4: Effect of PL treatment on wheat flour functionality

Test variables	Control	PL treated	p value
<i>Milling yield (% as-is)</i>			
Bran	17.57 ± 1.54	19.22±0.96	0.269655
Shorts	7.16±0.63	8.26±1.30	0.347097
BK flour¹	26.33 ± 0.70	24.56± 0.14	0.025833*
SZ flour²	6.70 ± 0.98	6.88± 0.23	0.815246
Reduction flour³	35.67 ± 2.46	33.67± 0.96	0.345662
Straight grade flour⁽¹⁺²⁺³⁾	68.71±3.17	65.12±0.81	0.196023
Moisture	13.55± 0.98	12.34± 0.70	0.160270
Damage Starch	13.1±2.38	25.23±3.57	0.008112*
<i>Dough Rheology</i>			
Water Absorption (%)	57.80 ±1.01	57.30±0.60	0.504788
Stability	9.167 ± 0.66	9.433±0.15	0.536039
Amplitude	0.08233± 0.01	0.07133±0.01	0.381834
C1	1.104± 0.04	1.094±0.01	0.714768
C2	0.4590± 0.04	0.4567±0.01	0.933780
C3	0.928± 0.35	0.4960±0.03	0.102154
C4	1.493± 0.44	0.8593±0.08	0.072644
C5	1.912± 0.54	1.204±0.16	0.098904
<i>Solvent Retention Capacity (SRC)</i>			
Water	52.37±3.44	51.89±2.72	0.857623
5% Lactic acid	122.13±4.69	119.88±4.24	0.571073
50% Sucrose	71.05±0.97	71.07±0.57	0.986295
5% Sodium Carbonate	76.23±1.61	84.08±1.66	0.004344*

Note: Values represent means ± standard deviation from 3 replicates. An asterisk (*) indicates a significant difference from the untreated control ($p < 0.05$).

Table 7.4 also shows the rheological properties of wheat flour samples measured with a Mixolab, which evaluates dough rheology during simultaneous mixing and heating. The results indicated no significant differences between the control and treated samples across all rheological parameters ($p > 0.05$). Water absorption was not significantly affected by PL treatment in either treated or untreated samples. A similar C2 value was observed, which is important because it

relates to bread-making quality (Hrušková et al., 2013). Additionally, amplitude, CS, and C2 are mainly influenced by the flour's protein quality. Therefore, these results suggest similar protein quality and content in the wheat flours. Although not statistically significant, the lower C3, C4, and C5 values in treated flour might point to a slight trend toward reduced gelatinization, hot-paste stability, and retrogradation. This trend may be associated with the observed increase in the damaged starch content in the PL treated wheat flour. A similar observation was noted by Singh et al. (2019).

Similarly, the SRC profile (**Table 7.4**) for water, 50% sucrose, and 5% lactic acid did not change significantly ($p>0.05$), indicating that PL-assisted tempering did not affect water-holding capacity, gluten properties, or gliadin and pentosans. However, the 5% sodium carbonate values were higher for the PL-treated wheat flour than in the control ($p=0.0043$). This increase in value can be attributed to the increased damaged starch (Kweon et al., 2011). Singh et al. (2022) reported that PL can alter the surface architecture and crystalline structure of starch granules, thereby increasing the swelling characteristics.

The baking, textural, and crumb characteristics of the treated and untreated wheat flour samples are shown in **Table 7.5**. The PL treatment significantly reduced bread loaf volume ($p<0.001$), a major quality change observed in this study. This is mechanistically linked to the changes seen in the SRC and milling yield. A notably higher 5% sodium carbonate value in PL-treated flour (84.08) indicates increased damaged starch. Barak et al. (2014) reported that excessive damage to starch can accelerate enzymatic reactions, leading to stickier dough, stronger proofing, and lower bread volume. This supports the C-cell data, which showed significantly higher cell numbers, wall thickness, cell diameter, and cell volume in PL-treated flour. A well-developed crumb typically features fewer, larger, evenly distributed gas cells with moderately

thick walls, indicating efficient gas retention during proofing and oven spring (Rathnayake et al., 2018; Shehzad et al., 2010). Conversely, the finer, more fragmented cell network observed in the PL-treated bread suggests reduced gas-holding capacity. Despite notable differences in volume and crumb characteristics, most textural parameters remained unchanged, indicating the internal integrity of the crumb matrix. Nonetheless, the adverse impact of PL-treated flour on the crumb structure might be alleviated by optimizing the PL processing conditions, especially effective fluence, exposure time, and grain moisture content during tempering. Additionally, process modification by supplementing with dough strengthener and enzymes can significantly improve the starch functionality and gas retention. Thus, in addition to being an effective antimicrobial treatment, PL treatment needs further optimization to reduce its negative impact on end-use quality.

Table 7.5: Effect of PL treatment on baking characteristics

Test Variables	Control	PL treated	p value
<i>Textural characteristics</i>			
Bread Volume (cc)	543.33±11.54	443.33±15.27	0.000828*
Hardness (N)	6.22±0.54	7.54±0.34	0.023919
Adhesiveness (N)	0.002±0.00	0.001±0.00	>0.999999
Resilience (%)	38.54±0.43	39.03±1.29	0.568491
Cohesion	0.74±0.00	0.74±0.00	0.351367
Springiness (%)	108.99±11.15	115.43±24.69	0.701687
Gumminess	4.60±0.40	5.62±0.18	0.016345*
Chewiness (N)	5.00±0.164	6.38±1.29	0.141768
<i>C-cell Characteristics</i>			
Number of Cells	3837.66±177.40	4775.5±374.17	0.006773*
Wall Thickness / mm	0.41±0.00	0.37±0.00	0.000261*
Cell Diameter / mm	1.94±0.11	1.45±0.08	0.000168*
Cell Volume /cc	7.40±0.51	5.12±0.36	0.000240*

Note: Values represent means $\pm$ standard deviation from 3 replicates. An asterisk (*) indicates a significant difference from the untreated control ($p < 0.05$).

7.6 Conclusion

This study aimed to evaluate the significance of PL tempering in reducing *Salmonella* contamination in wheat grains during tempering. The results indicate that PL treatment at 80 s for 18 h tempering resulted in the maximum reduction of *Salmonella* load by 4.22 log CFU/g (XLD+T) and 3.91 log CFU/g (XLD). Weibull kinetic modelling provided the best fit to describe survival curves of *Salmonella* inactivation under PL treatment across all recovery media (XLD and XLD+T) and tempering times, as indicated by higher R^2 and lower RMSE. The Weibull fit produced a consistent 5 log reduction fluence of approximately 0.41-0.48 J/cm² (99-118 s), suggesting a robust, non-first order inactivation with moderate shoulder/tailing. Mechanistic studies revealed that oxidative stress induced by the accumulation of reactive oxygen species is the lethal mechanism underlying PL-induced inactivation of *Salmonella*, as evidenced by membrane disruption and genomic DNA oxidation. PL treatment (80 s) did not adversely affect wheat flour functionality, as measured by milling yield and particle size distribution (PSD). However, a significant difference was observed in the damaged starch content, which further affected the bread's baking and C-cell characteristics. Future studies should focus on the hurdle approach, combining PL with acidic tempering techniques or supplementing with the dough strengtheners and enzymes to improve the observed changes in baking characteristics and upscaling the treatment process to handle large grain volumes efficiently. Overall, the study demonstrates the effectiveness of PL as an intervention to combat *Salmonella* in wheat grain during tempering.

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Chapter 8 - Overall Conclusion and Future Perspective

8.1 Conclusions

Microbial safety of low-moisture wheat and wheat-based products has become a growing concern, with increasing outbreaks and recalls linked to *Salmonella* contamination. This work was carried out to characterize the microbial community of the wheat milling environment using culture-independent techniques and develop target-specific intervention measures to reduce *Salmonella* during wheat tempering. Overall, the research has advanced both the foundational knowledge of wheat milling microbiology and the practical toolkit available for pathogen control, while demonstrating that efficient interventions can be implemented without affecting flour quality.

Chapter 2 presents a detailed benchmarking of 32 bioinformatics workflow combinations (eight 16S rRNA pipelines X four taxonomic databases), conducted on wheat milling samples ($n = 160$). The study found that diversity estimates depended on rank and workflow, with the genus level showing greater variability than the family level. Comparing ASVs and OTUs, it was found that ASVs are the best method for low-moisture food environments because they identify single-nucleotide variations and retain low-abundance taxa important for food safety characterization. These results provide a validated analytical framework that underpins the rest of the work.

Chapter 3 applied this framework to describe bacterial community dynamics in three stages of commercial wheat milling: before milling (BM), after milling (AM), and milled fractions (MF). 22 families and 132 genera were identified. The genus-level richness decreased by 41.5% from BM to AM ($p < 0.001$). Functional predictions were made using enriched KEGG modules associated with stress tolerance, such as biofilm formation, osmoprotection, sporulation, and multidrug efflux, especially in the post-milling samples (AM and MF). These ecological data

showed that milling changes the redistribution of stress-tolerant microbial communities, providing the scientific basis for subsequent intervention studies.

Chapter 5 presented Sodium Bisulfate (SBS) as an antimicrobial tempering agent, with a minimum inhibitory concentration of 0.32% against four *Salmonella enterica* serovars, and cell lysis was confirmed by reactive oxygen species production. Tempering with 1.5% SBS reduced wheat *Salmonella* load by 4.3 log CFU/g after 24 h of tempering. Additionally, flour functionality and baking quality were comparable to those of the controls ($p>0.05$). Building on this, *Chapter 5* applied a hurdle approach combining acidic water tempering (SBS, lactic acid, and citric acid at 15% w/v) with mild heat (55°C). The combined treatments resulted in non-detectable *Salmonella* levels (< 2 log CFU/g) within 12-18 h, a significantly better result than the individual treatments, while preserving the flour's physicochemical and baking properties. These two chapters together have shown that acidic and hurdle-based tempering interventions are not only effective but also versatile.

Chapter 7 focused on evaluating pulsed light (PL) as a non-thermal method selected from a preliminary screening of cold plasma, ultraviolet, and PL, as the only technology capable of achieving ≥ 3 log CFU/g reduction. PL dose of 0.329 J/cm² caused a 4.22 log CFU/g reduction in wheat *Salmonella* load during tempering by disrupting the cell membrane and damaging DNA through oxidative stress. Weibull model predicted a 5-log reduction at 0.41-0.48 J/cm² (99-118 s). While dough rheology and particle size were unaffected, an increase in damaged starch altered bread baking and crumb properties significantly, identifying an important quality threshold for process optimization.

Together, this dissertation work demonstrates that microbiome characterization and intervention development are complementary pillars of a comprehensive food safety approach for low-moisture wheat. The validated bioinformatics pipeline, identified ecological niche, and multiple intervention strategies discussed provide a scientific foundation that can be tailored for commercial wheat milling activities to improve the microbiological safety of wheat flour and wheat-based products.

8.2 Future Perspective

To advance the studies presented in this work, the following recommendations can be considered for future research:

8.2.1 Advanced microbiome and functional characterization

The PICRUSt2 functional prediction from Chapter 4 does not directly measure gene content or expression and therefore needs to be complemented with more direct experimental approaches. Shotgun metagenomics would enable strain-level resolution of antimicrobial resistance genes, antibiotic resistance, virulence, and mobile genetic elements, as well as 16S rRNA gene sequences that cannot be captured by 16S rRNA gene sequencing. In addition, whole genome sequencing (WGS) key identified taxa across different milling stages (BM, AM, and MF) is also necessary to characterize the evolutionary changes, such as the onset of stress resistance genes, horizontal gene transfer, and genomic signatures of selection, that enable food-borne pathogens to persist and survive in dry milling environments. Furthermore, metatranscriptomic analysis would reveal which genes and pathways are activated during milling and tempering, enabling us to identify metabolically active populations rather than dormant ones. This transcriptional perspective is important for understanding real-time stress responses, such as production of osmoprotectants, protection against oxidative stress, and biofilm formation, under the specific intervention conditions studied in this research. Altogether, these multi-omic methods would help us not only to describe microbial communities and predict their functions, but also to understand the mechanisms underlying their behavior in the wheat milling environment.

8.2.2 Pilot and commercial scale validation:

All the intervention strategies demonstrated at the laboratory scale must be evaluated in pilot- and commercial-scale settings, not only to confirm their efficacy but also to establish cost-effectiveness for industrial adoption.

8.2.3 Optimization of pulsed light parameters

The increase in damaged starch at higher PL levels warrants optimizing PL parameters, such as fluence rate, pulse frequency, and exposure geometry, to minimize the impact on the product quality. Combining PL with acidic tempering agents as a hurdle approach might offer a viable path.

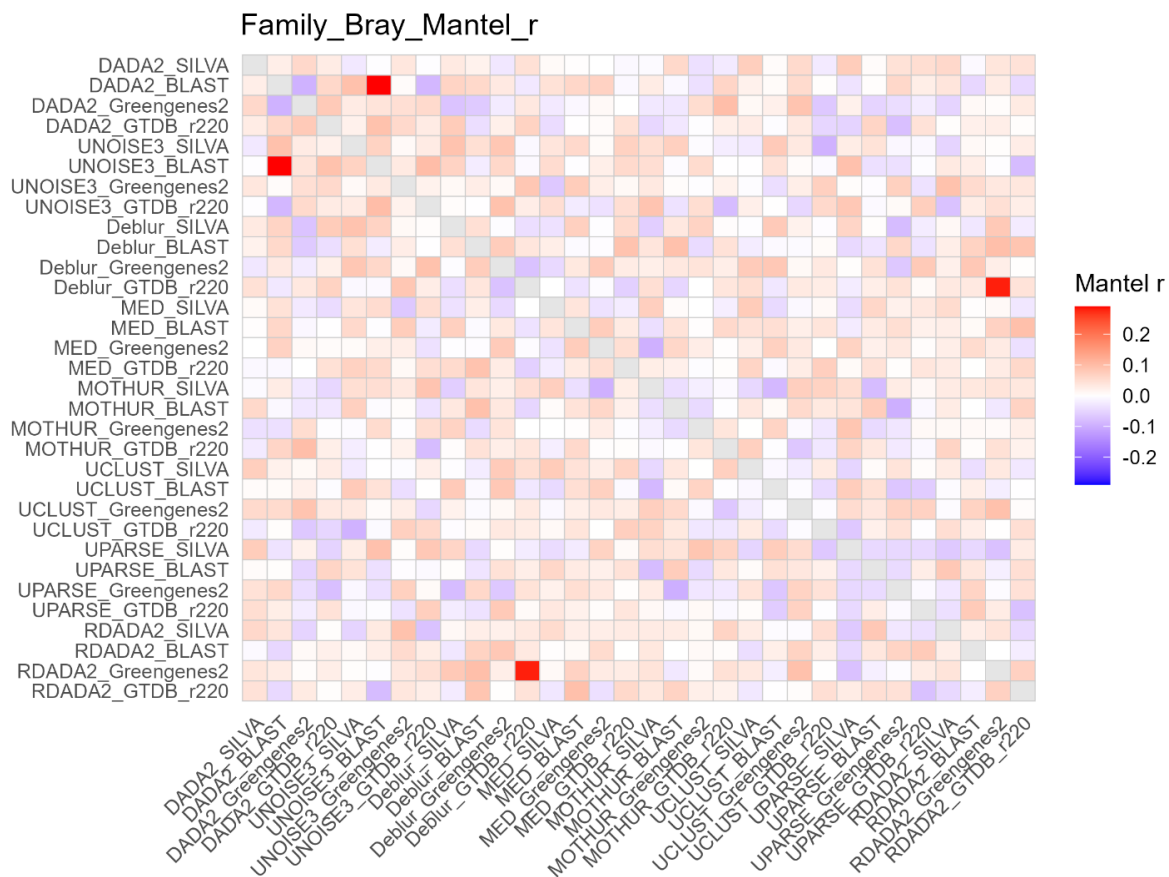
8.2.4 Isolation and characterization of stress-tolerant taxa

Chapter 4 identified specific taxa that were enriched for biofilm-forming and desiccation-tolerant capabilities in post-milling samples (AM and MF). This invites targeted culture-based isolation to study their phenotypic characteristics under conditions representative of milling environments. Isolates of interest, particularly those from families such as *Enterobacteriaceae* and *Bacillaceae*, should be evaluated for biofilm formation on milling-relevant surfaces (food contact and non-food contact), resistance to desiccation, and their potential to create harborage niches that shelter pathogens from intervention treatments. Co-culture studies examining whether these biofilm-forming residents facilitate *Salmonella* survival and persistence within milling equipment would be especially valuable, as resident biofilms may act as protective reservoirs for incoming pathogens.

Appendix - Supplementary Documents

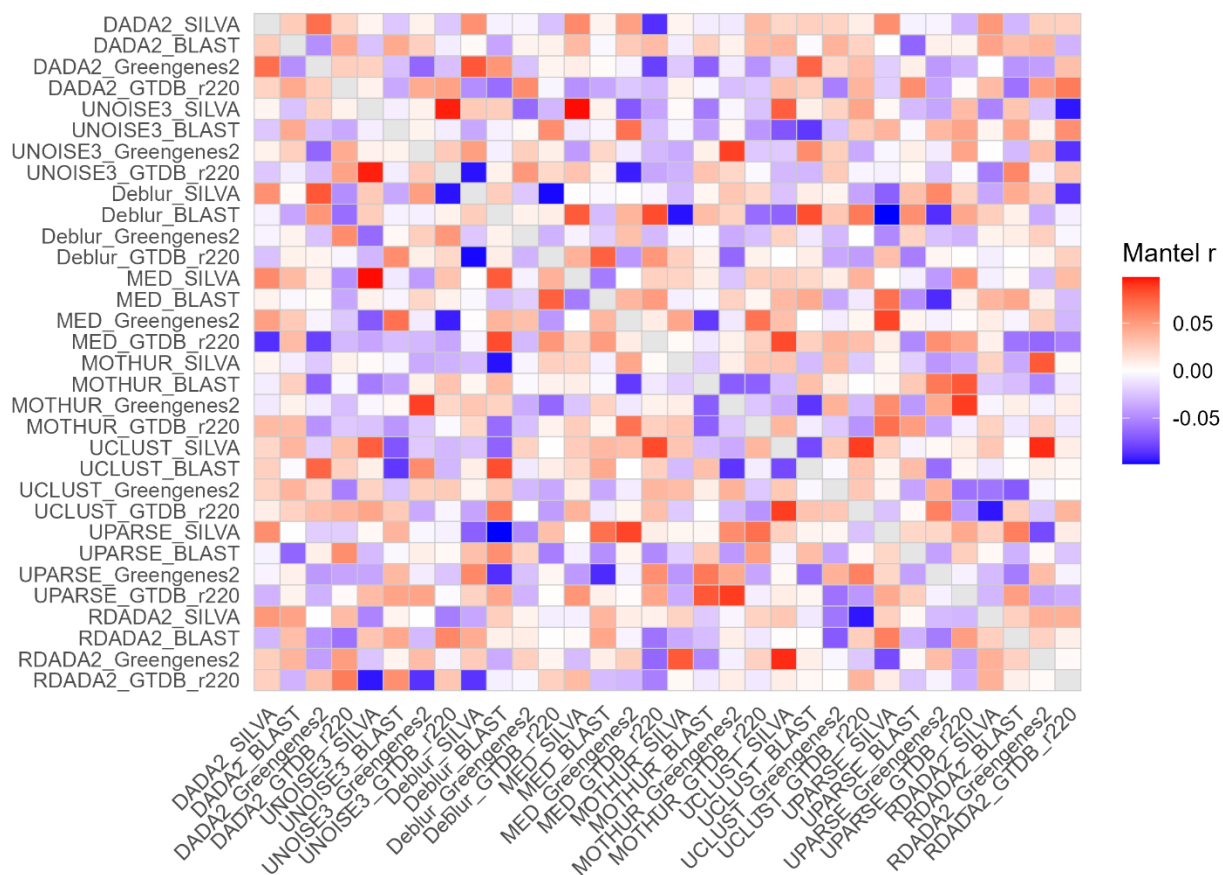
Chapter 1 Supplementary Figure S3. 1

Pairwise concordance of beta-diversity structure across all 32 pipeline $\times$ reference-resource combinations, assessed by Mantel correlation (r) between workflow-specific distance matrices. Heatmaps are shown for family-level Bray–Curtis (A), family-level Jaccard (B), genus-level Bray–Curtis (C), and genus-level Jaccard (D) comparisons. Higher Mantel r values indicate greater similarity in the sample-to-sample ecological structure recovered by two workflow combinations. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.

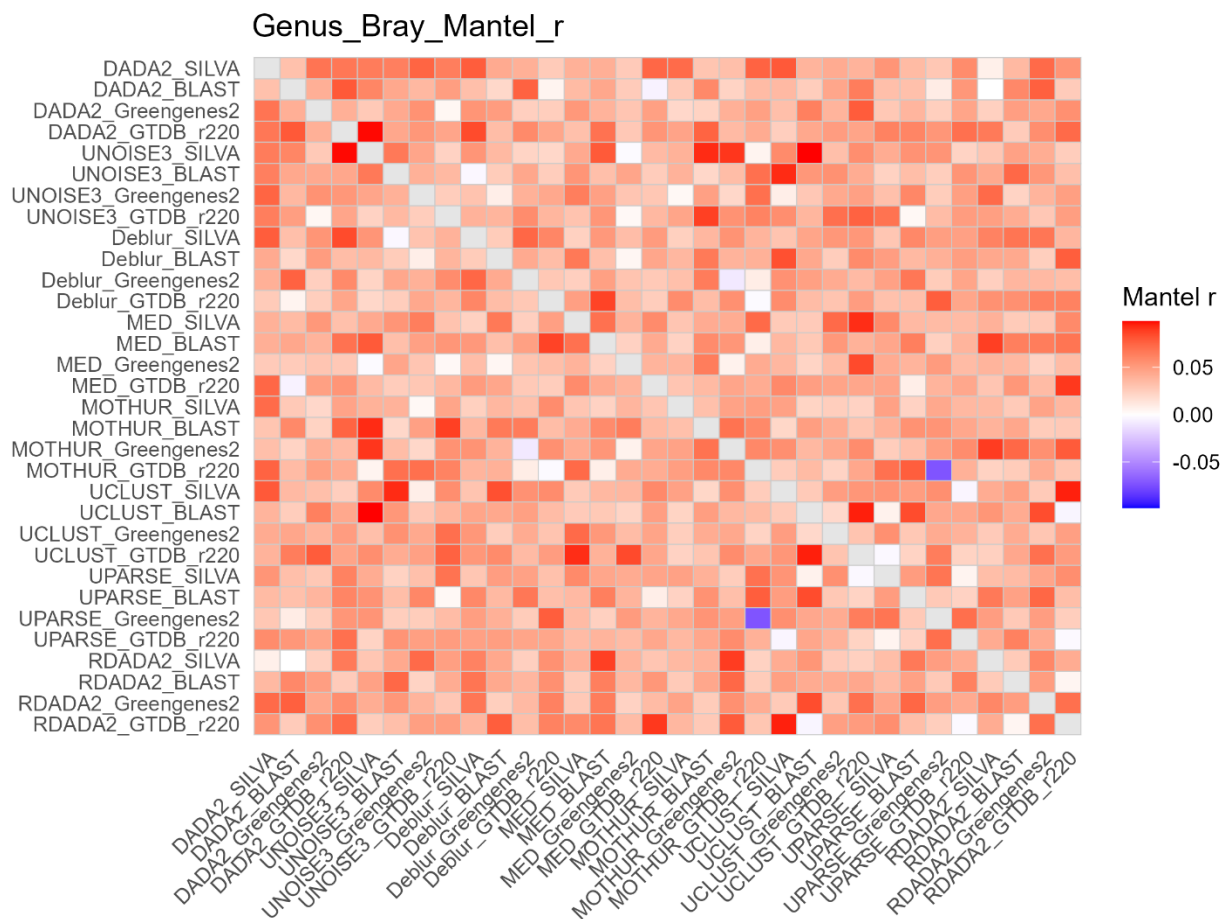


A: Family Bray–Curtis

Family_Jaccard_Mantel_r

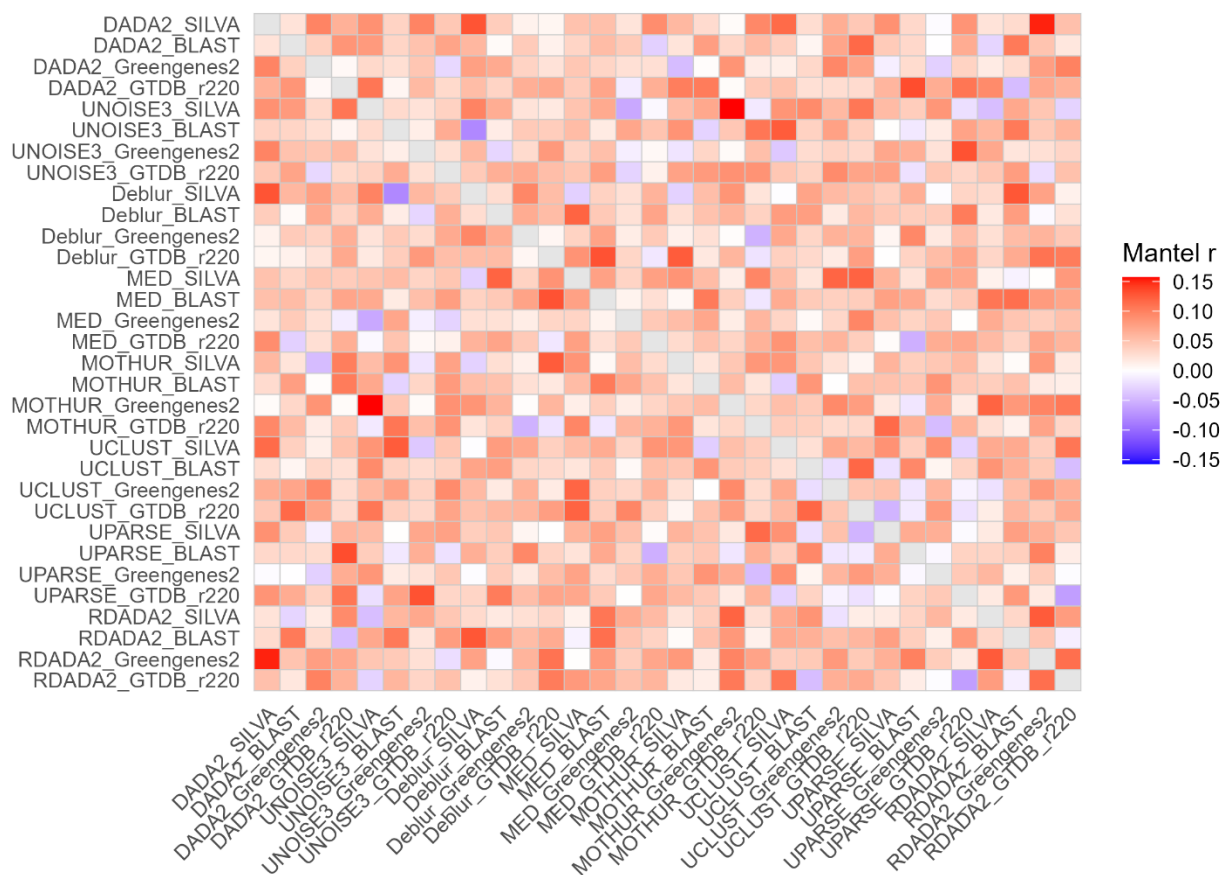


B: Family Jaccard



C: Genus Bray–Curtis

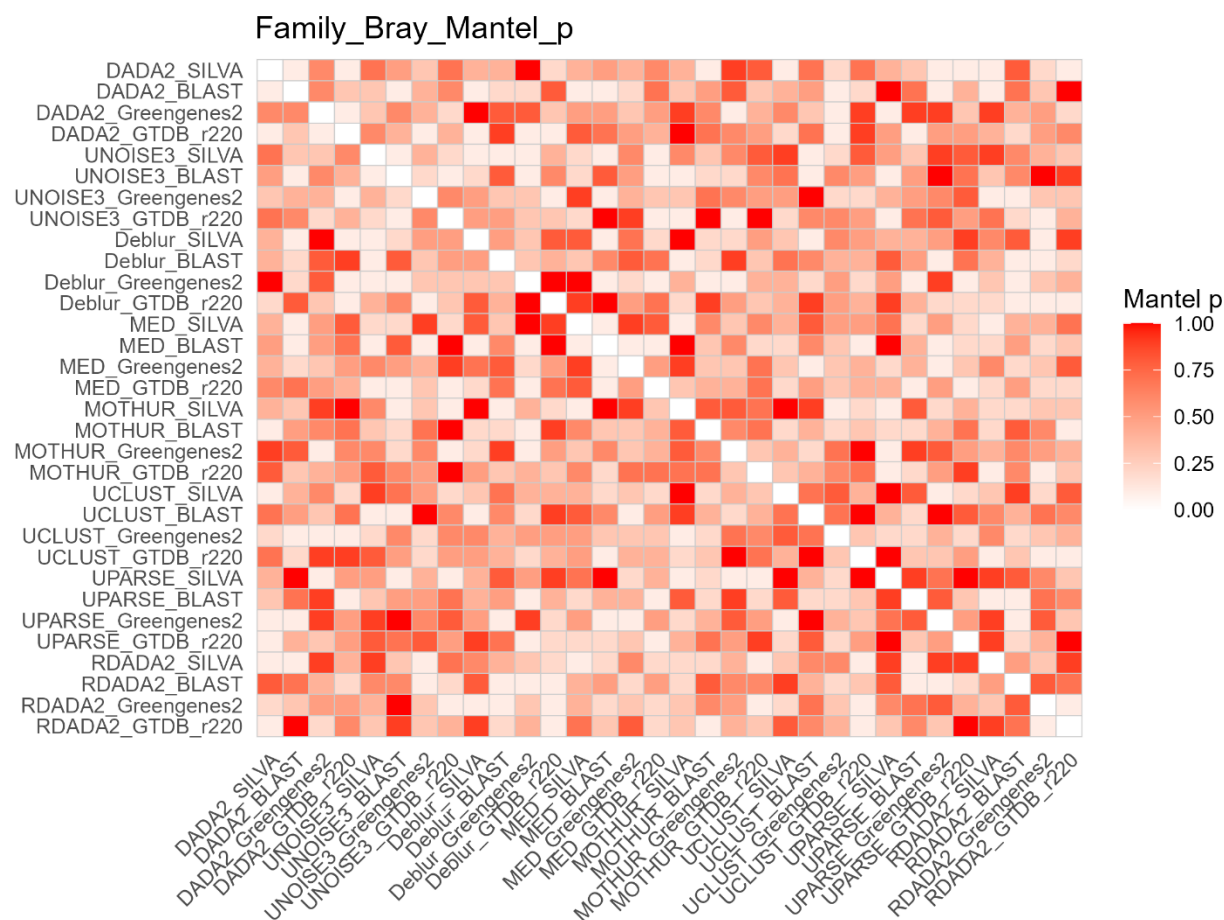
Genus_Jaccard_Mantel_r



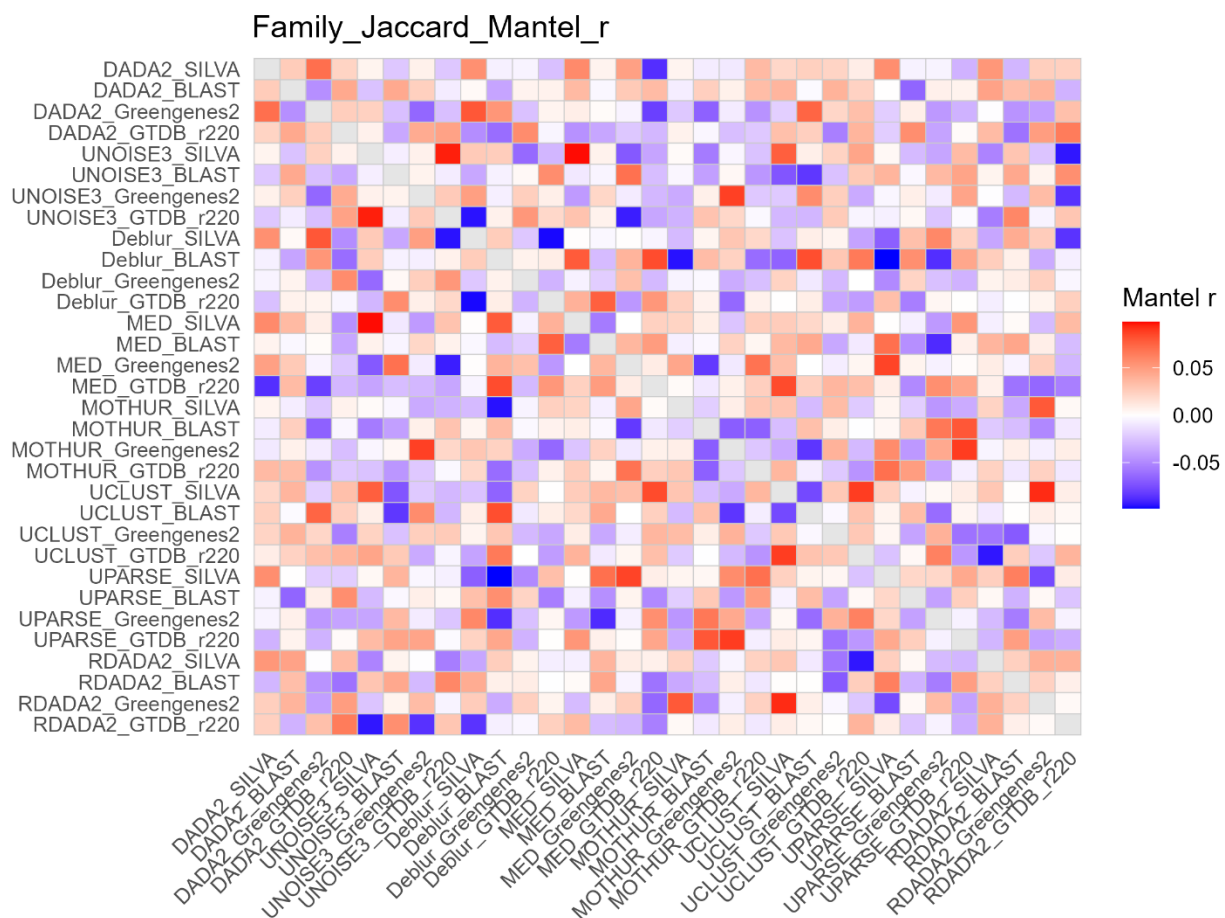
D: Genus Jaccard

Chapter 1 Supplementary Figure S3. 2

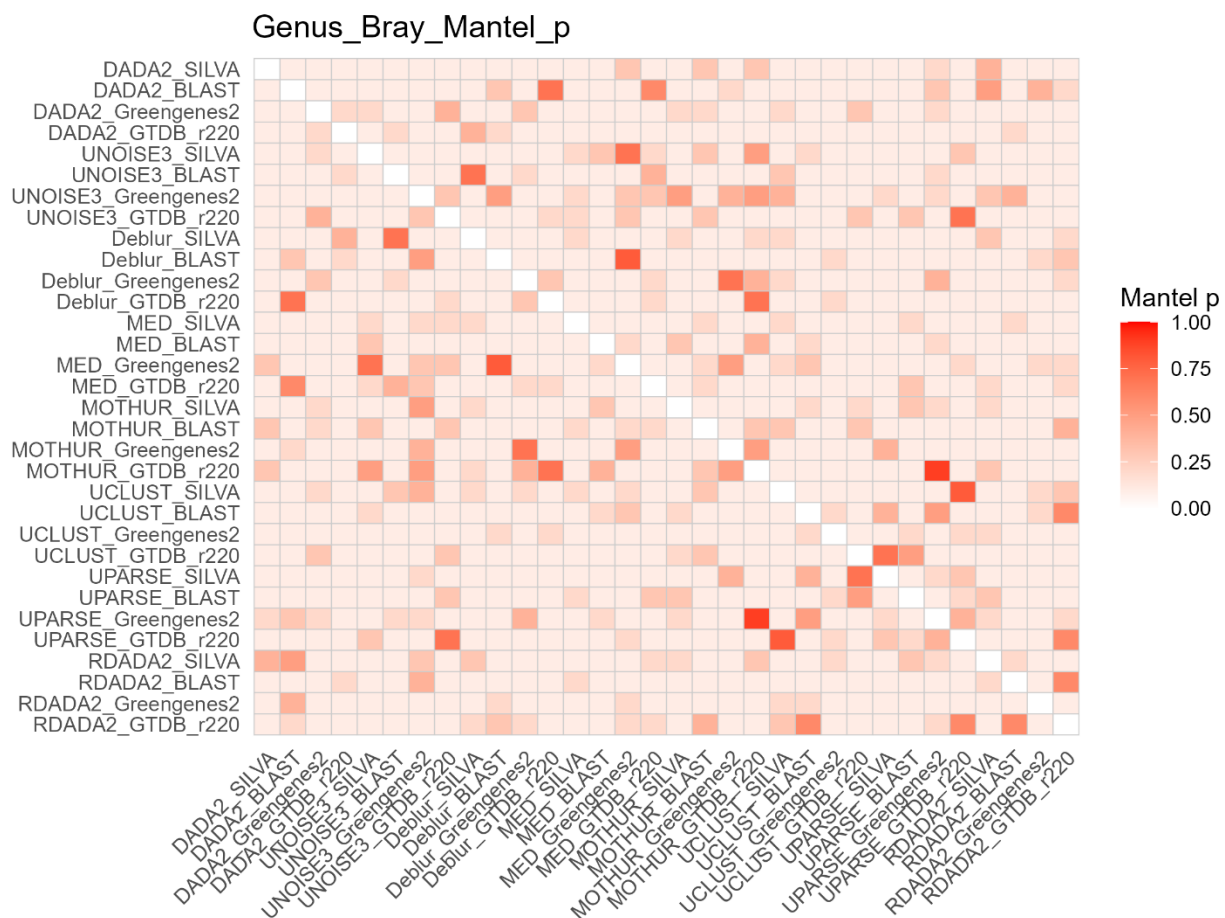
Corresponding Mantel p values for the pairwise beta-diversity comparisons shown in Supplementary Figure S1 across all 32 pipeline $\times$ reference-resource combinations. Heatmaps are shown for family-level Bray–Curtis (A), family-level Jaccard (B), genus-level Bray–Curtis (C), and genus-level Jaccard (D) comparisons. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.



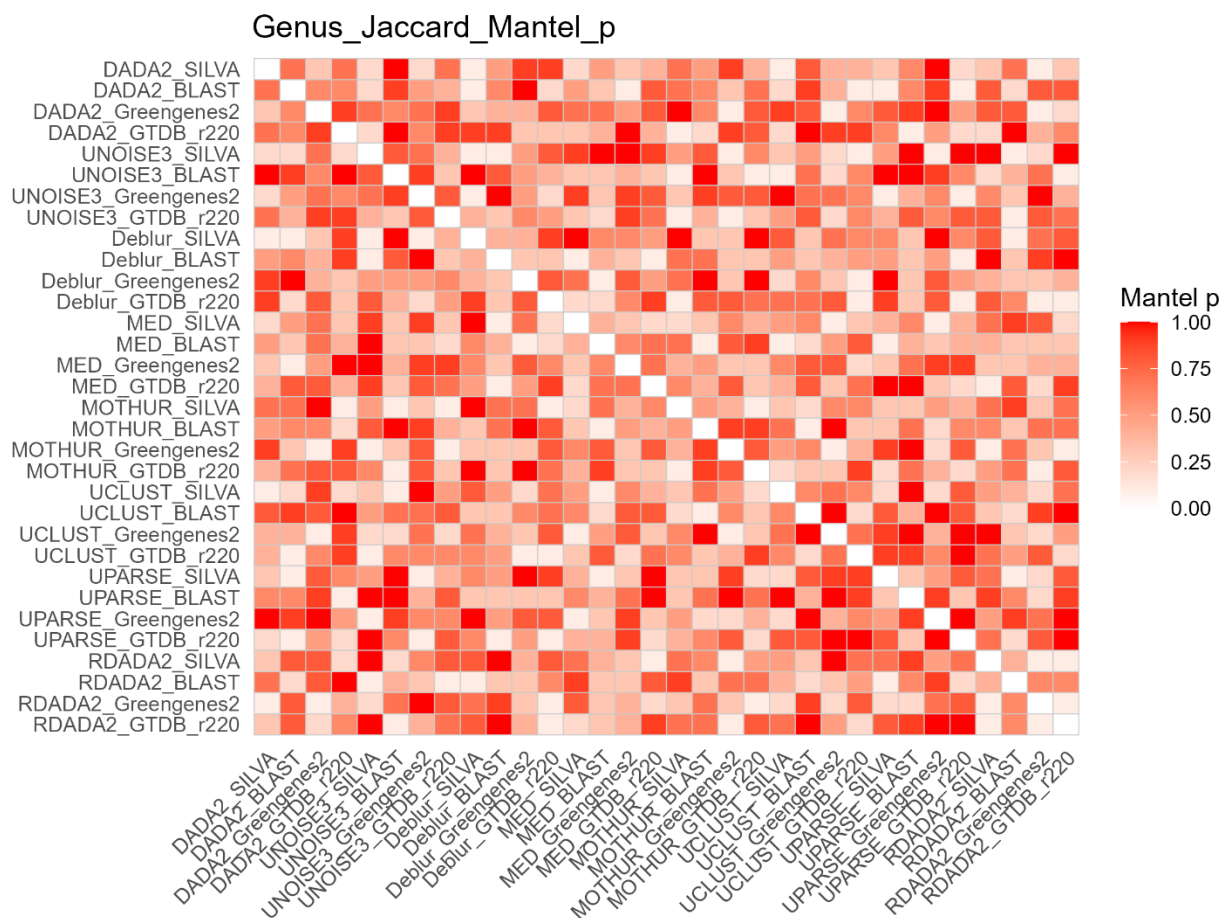
A: Family Bray–Curtis



B: Family Jaccard



C: Genus Bray–Curtis

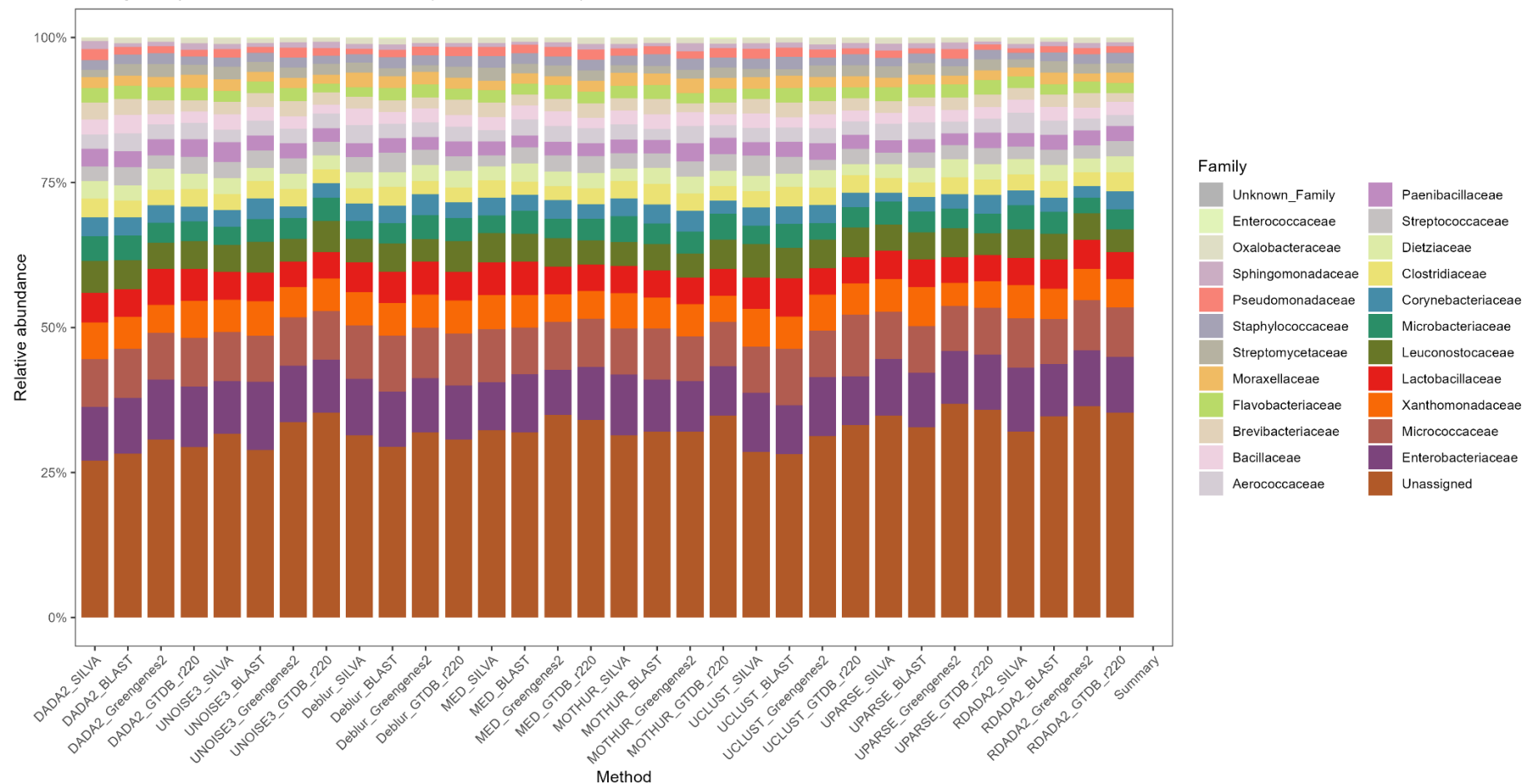


D: Genus Jaccard

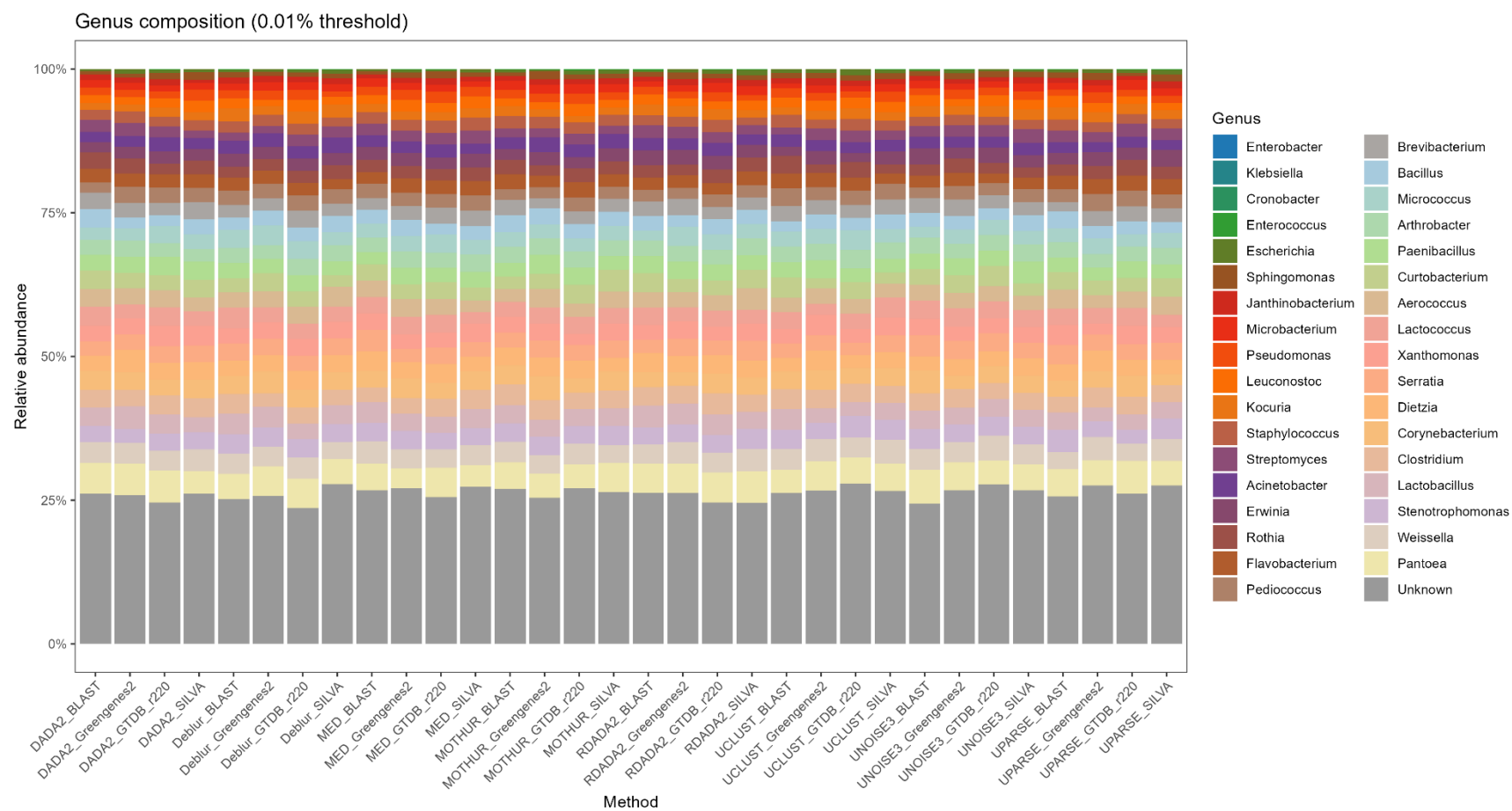
Chapter 1 Supplementary Figure S3. 3

Threshold sensitivity analysis of taxonomic composition across all 32 pipeline × reference-resource combinations using a lower presence threshold of $\geq 0.01\%$ relative abundance. Stacked bar plots show the mean relative abundance (%) of the dominant taxa at the **family level (A)** and **genus level (B)** across workflow combinations. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.

Family composition across 32 methods (0.01% threshold)



A. Family

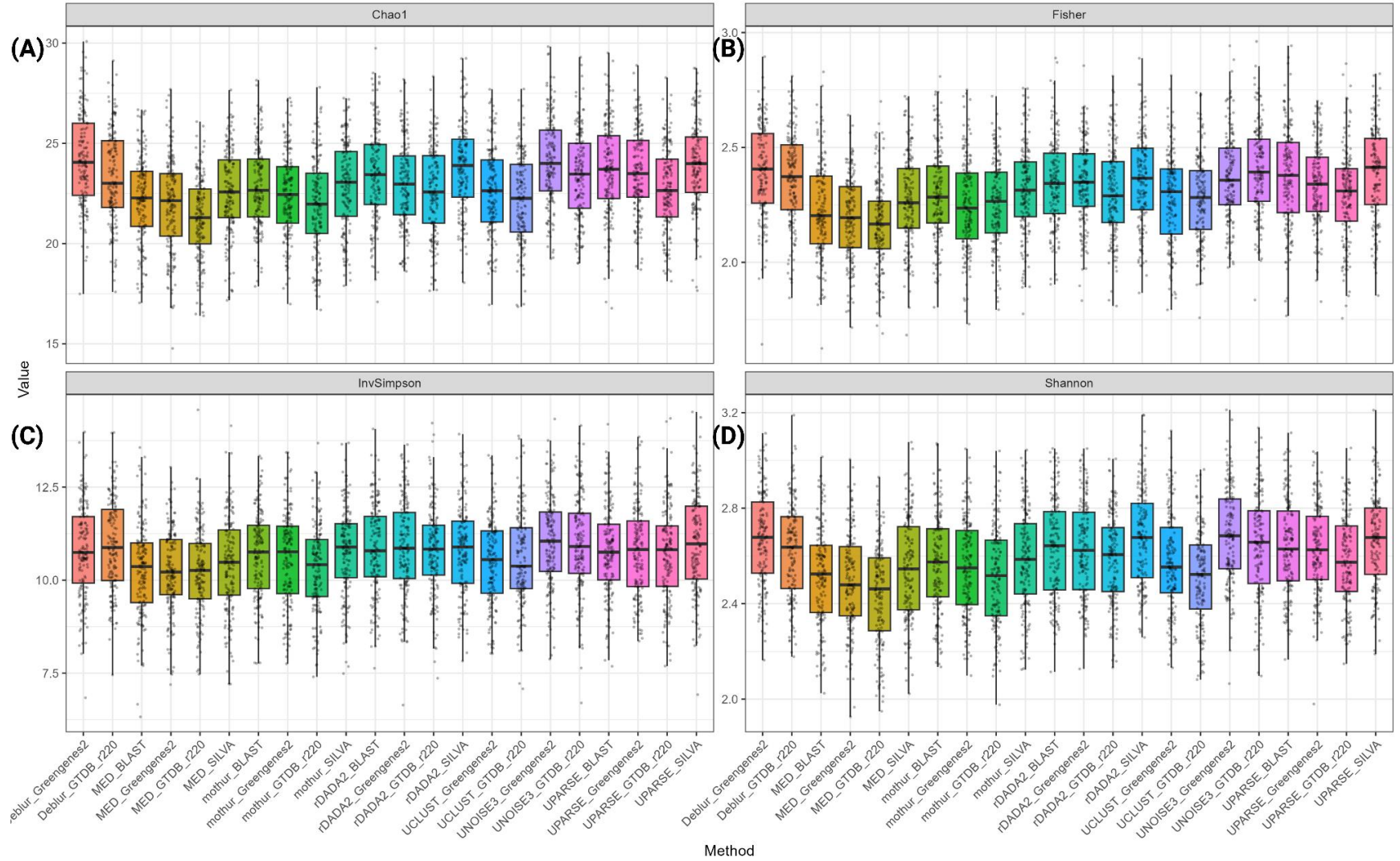


B: Genus

Chapter 1 Supplementary Figure S3. 4

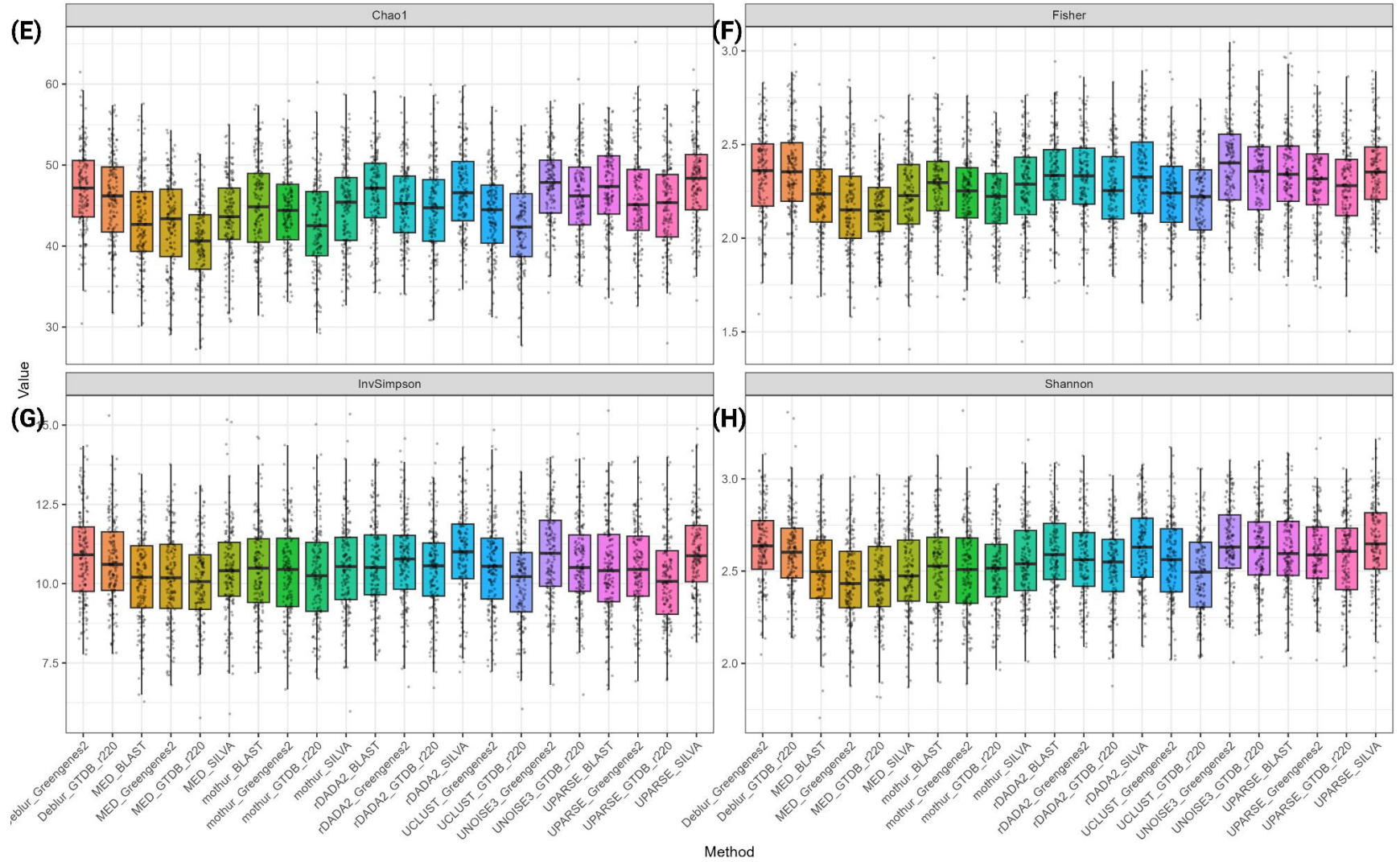
Alpha-diversity distributions across all **32 pipeline × reference-resource combinations** at the **family level (A–D)** and **genus level (E–H)**. Box-and-whisker plots show **Chao1 richness**, **Fisher’s alpha**, **Inverse Simpson diversity**, and **Shannon diversity** calculated from rarefied feature tables. Panels **A–D** summarize family-level alpha diversity, and panels **E–H** summarize genus-level alpha diversity. Boxes represent the interquartile range, center lines indicate the median, whiskers extend to $1.5 \times \text{IQR}$, and points represent individual samples. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.

Family-level alpha diversity across remaining 22 pipeline × reference combinations



A Family Chao1; B Family Fisher; C Family Inverse Simpson; D Family Shannon

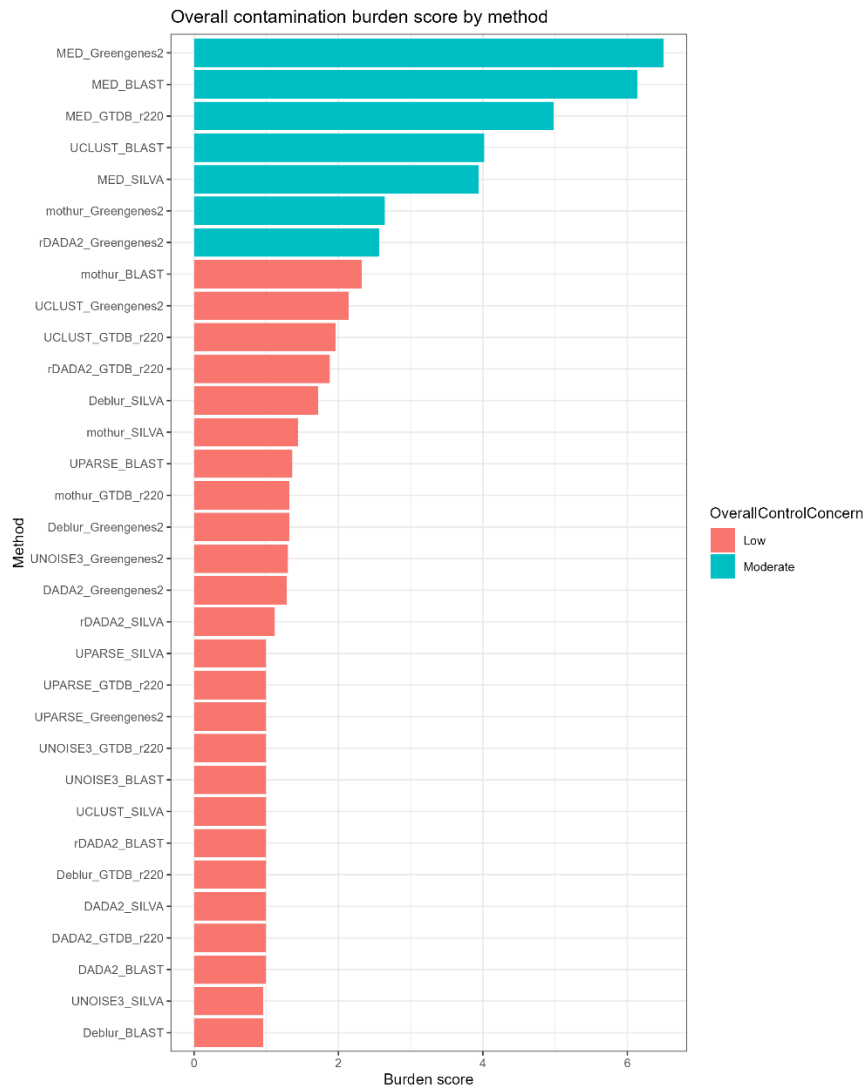
Genus-level alpha diversity across remaining 22 pipeline × reference combinations



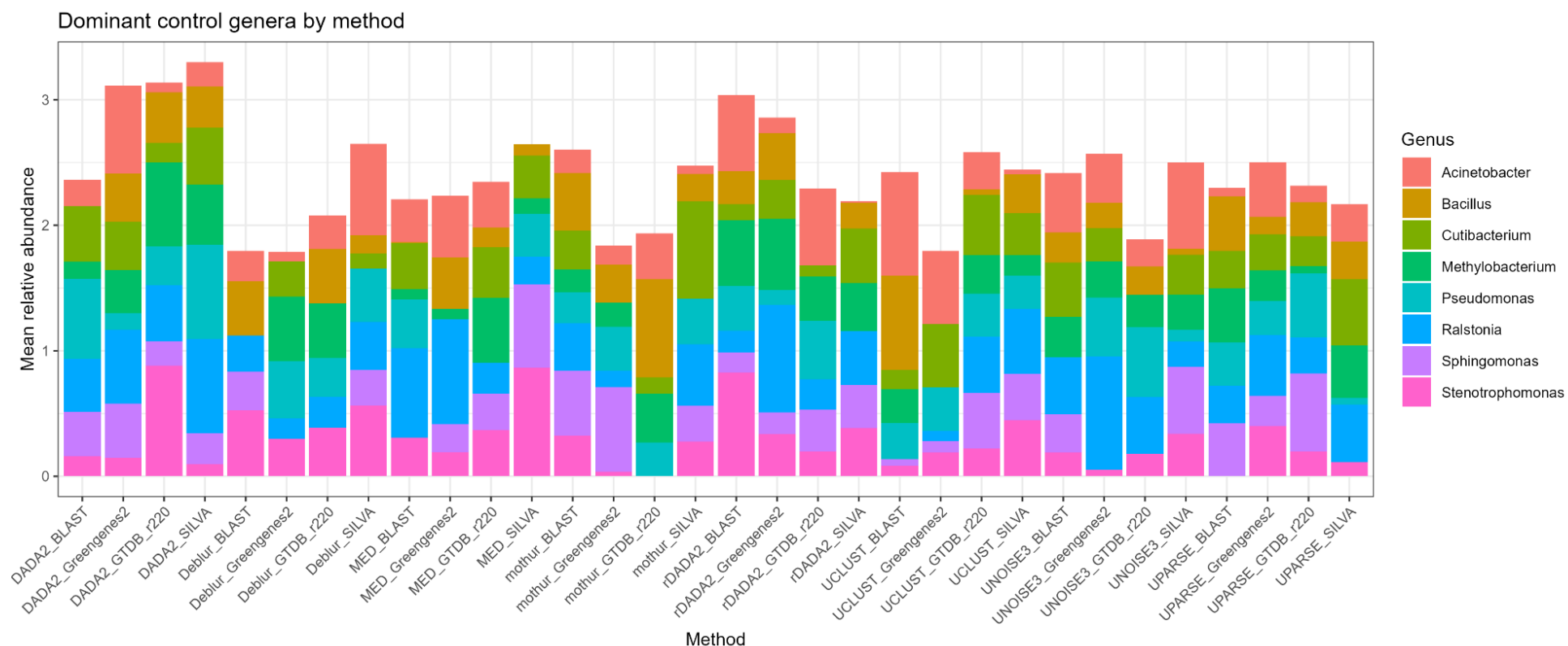
E Genus Chao1; **F** Genus Fisher; **G** Genus Inverse Simpson; **H** Genus Shannon

Chapter 1 Supplementary Figure S3. 5

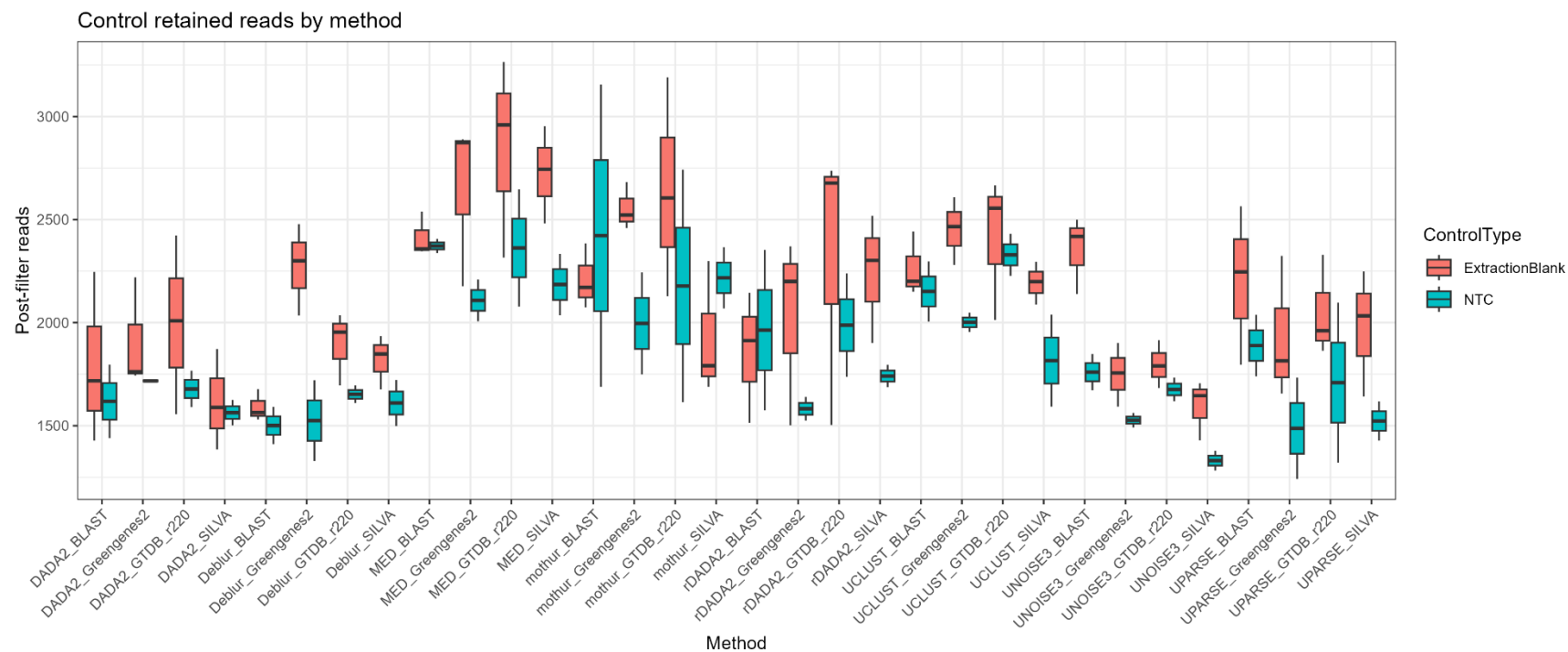
Workflow-level summaries of negative-control performance across all 32 pipeline $\times$ reference-resource combinations. **(A)** Overall contamination burden score by method, where workflows are ranked according to the cumulative burden associated with retained control reads, assigned control signal, and recurring control-associated taxa. **(B)** Dominant genera detected in extraction blanks and no-template controls across workflows, shown as mean relative abundance of recurrent low-biomass background genera. **(C)** Post-filter retained read counts in DNA extraction blanks and no-template controls (NTCs) across workflow combinations. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.



(A) Overall contamination burden score by method



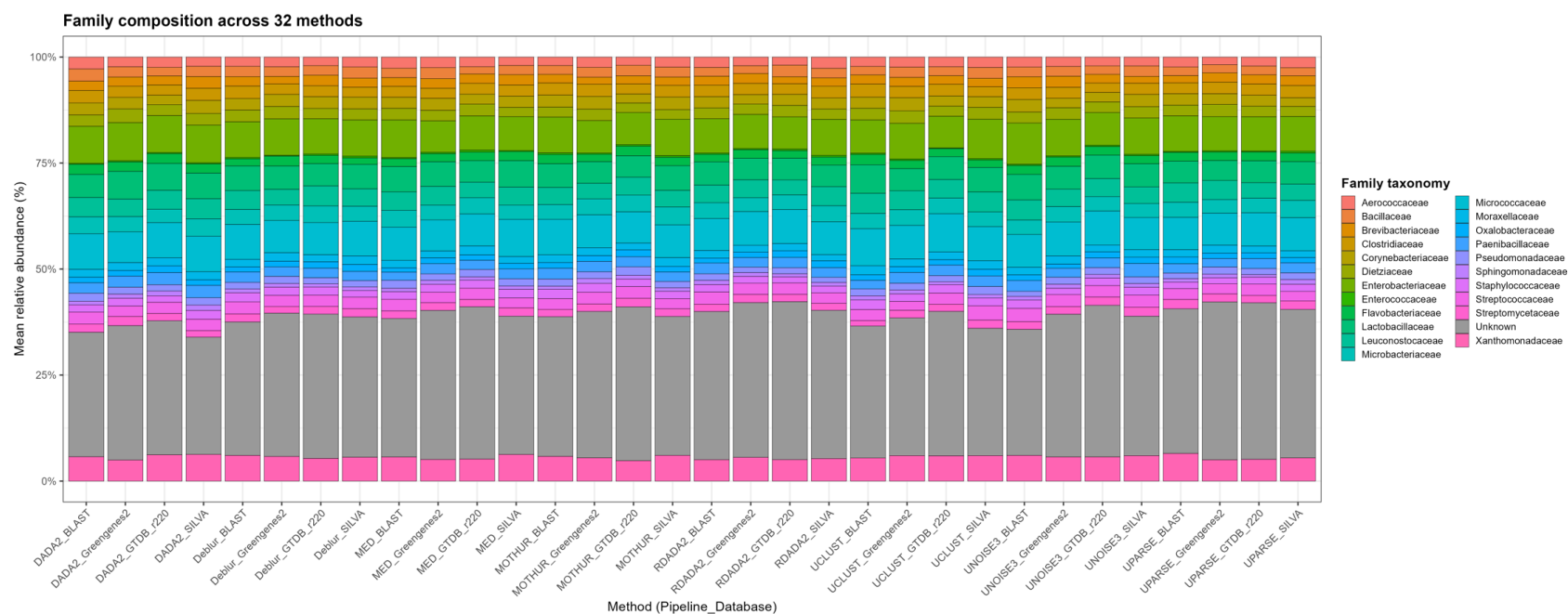
(B) Dominant genera detected in extraction blanks and no-template controls



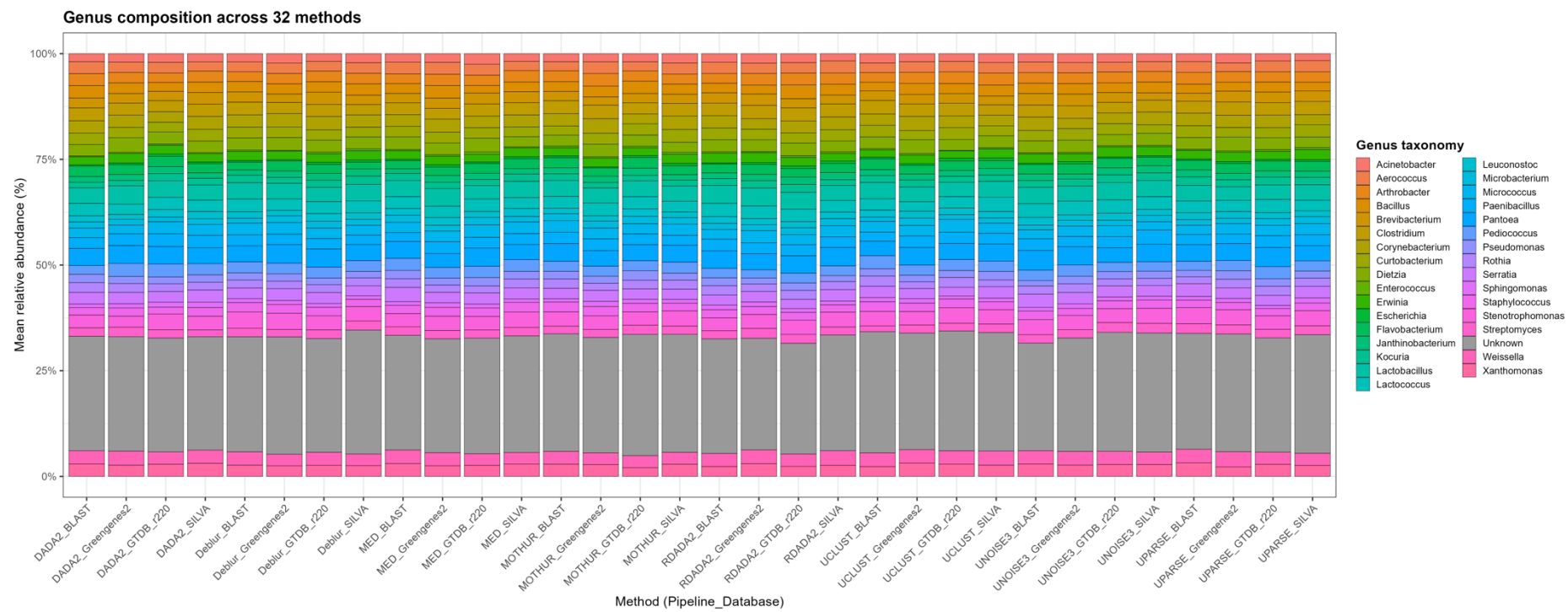
(C) Post-filter retained read counts in DNA extraction blanks and no-template controls (NTCs)

Chapter 1 Supplementary Figure S3. 6

Descriptive taxonomic composition across all 32 pipeline × reference-resource combinations at the family level (A) and genus level (B). Stacked bar plots show the mean relative abundance (%) of the dominant bacterial taxa recovered across workflows. Each bar represents one pipeline_database combination. Workflow combinations were generated using different amplicon-processing pipelines together with SILVA v138 SSU NR99, Greengenes2 (2022), GTDB r220, or BLAST-based taxonomic assignment. Taxonomy for SILVA, Greengenes2, and GTDB were assigned using pretrained Naive Bayes classifiers, whereas BLAST taxonomy was assigned using a consensus alignment-based workflow.



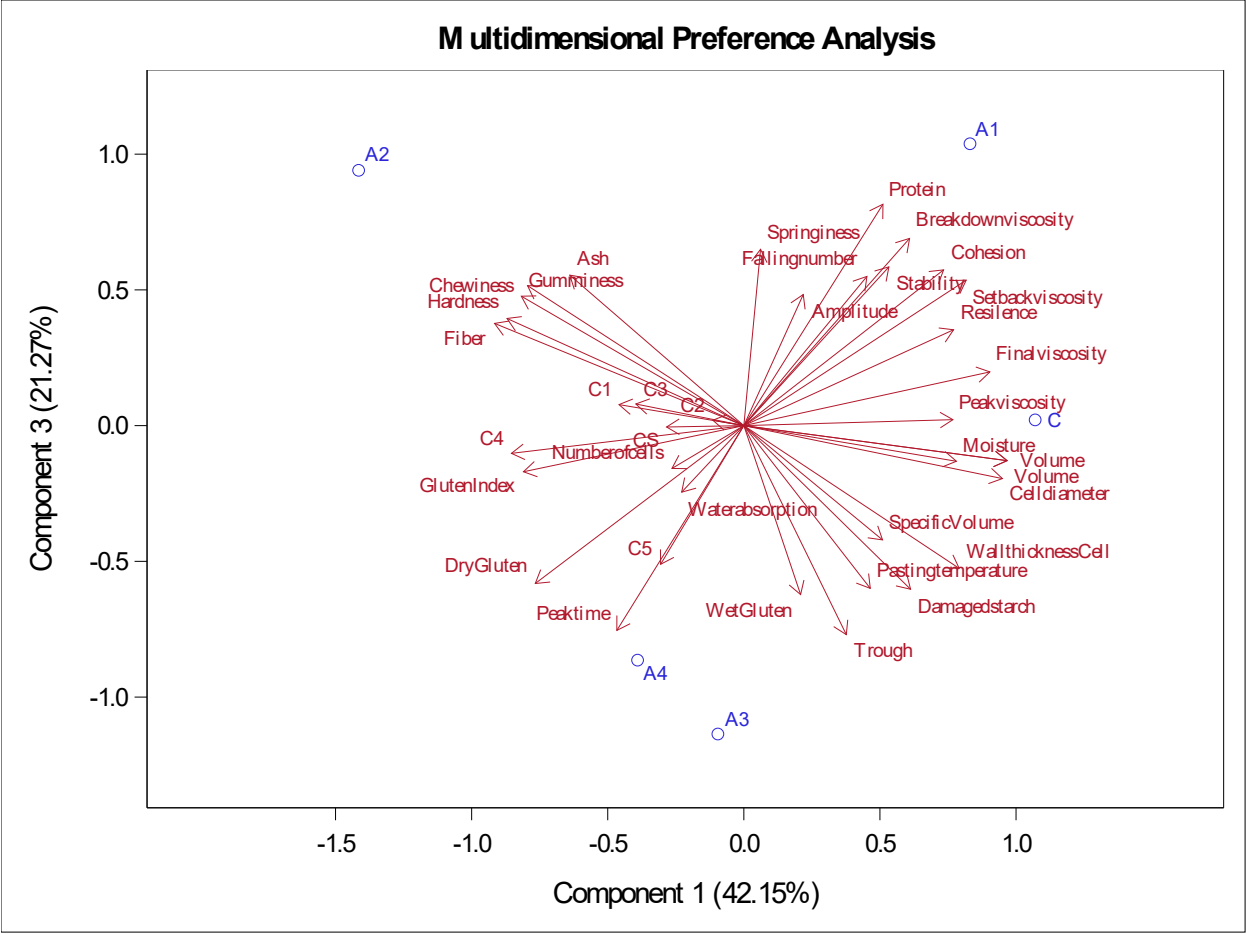
A: Family



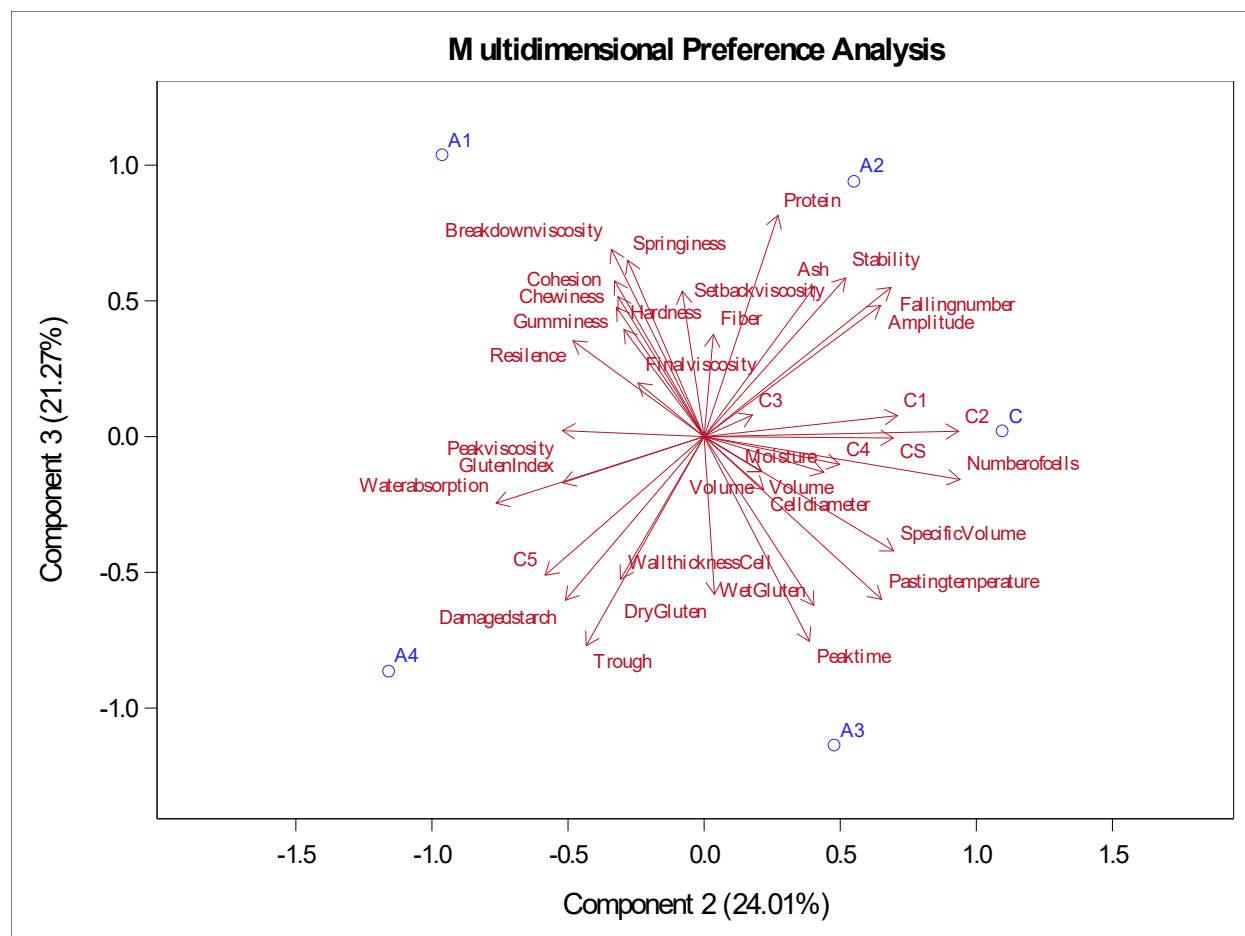
B: Genus

Chapter 6 Supplementary Figure S6. 1

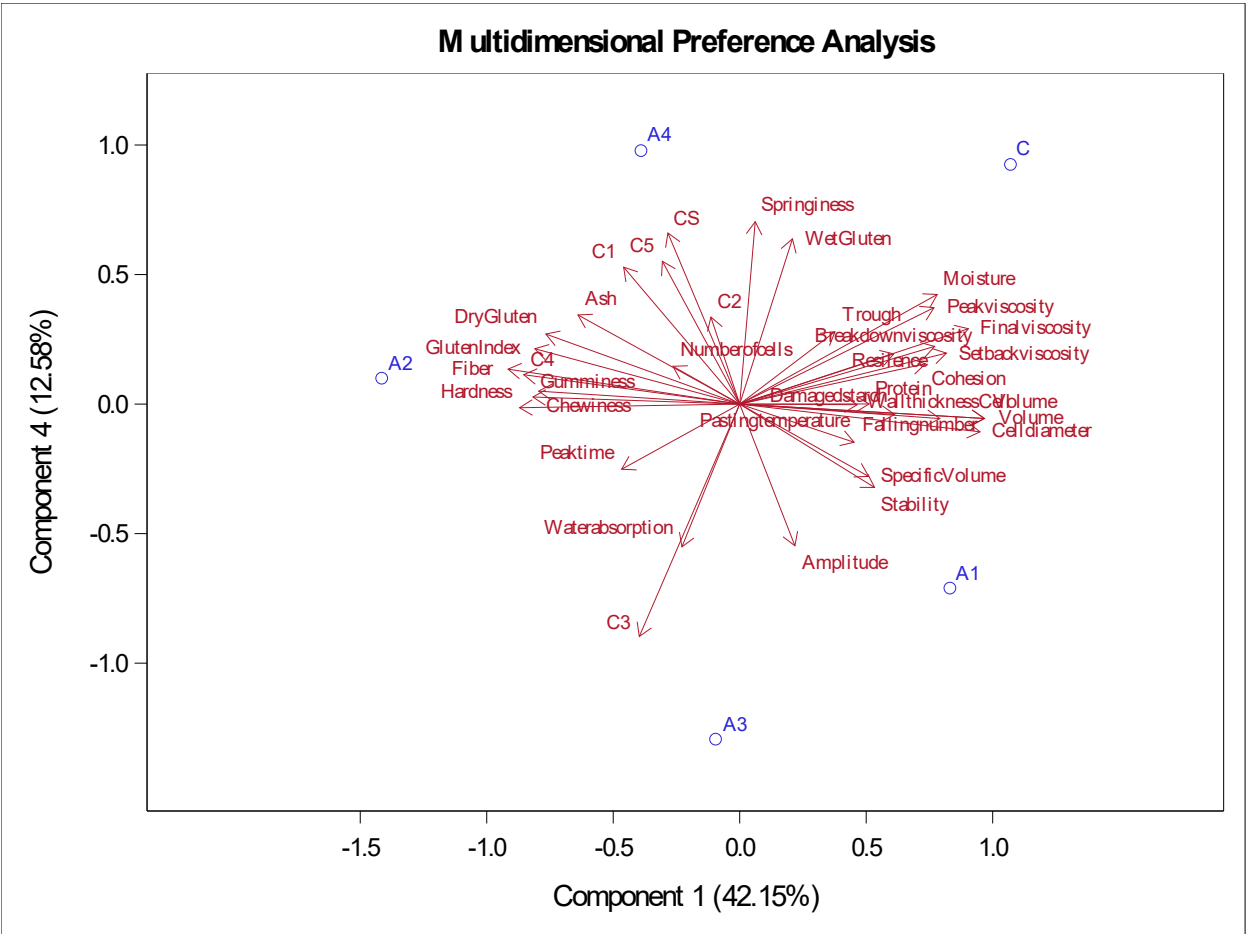
Principal component analysis (PCA) plots showing additional combinations of principal components (e.g., PC3–PC1, PC3–PC2, PC4–PC1; PC4-PC2; PC4-PC3) derived from flour quality parameters under different tempering conditions. While the main manuscript presents PC1–PC2 (66.16% variance explained), these supplementary plots illustrate remaining component relationships contributing to the cumulative 99.99% variance explained by the first four principal components. The biplots depict variable loadings and sample distributions, where proximity indicates positive correlation and opposing directions indicate negative correlation, providing further insight into treatment-driven variation among samples



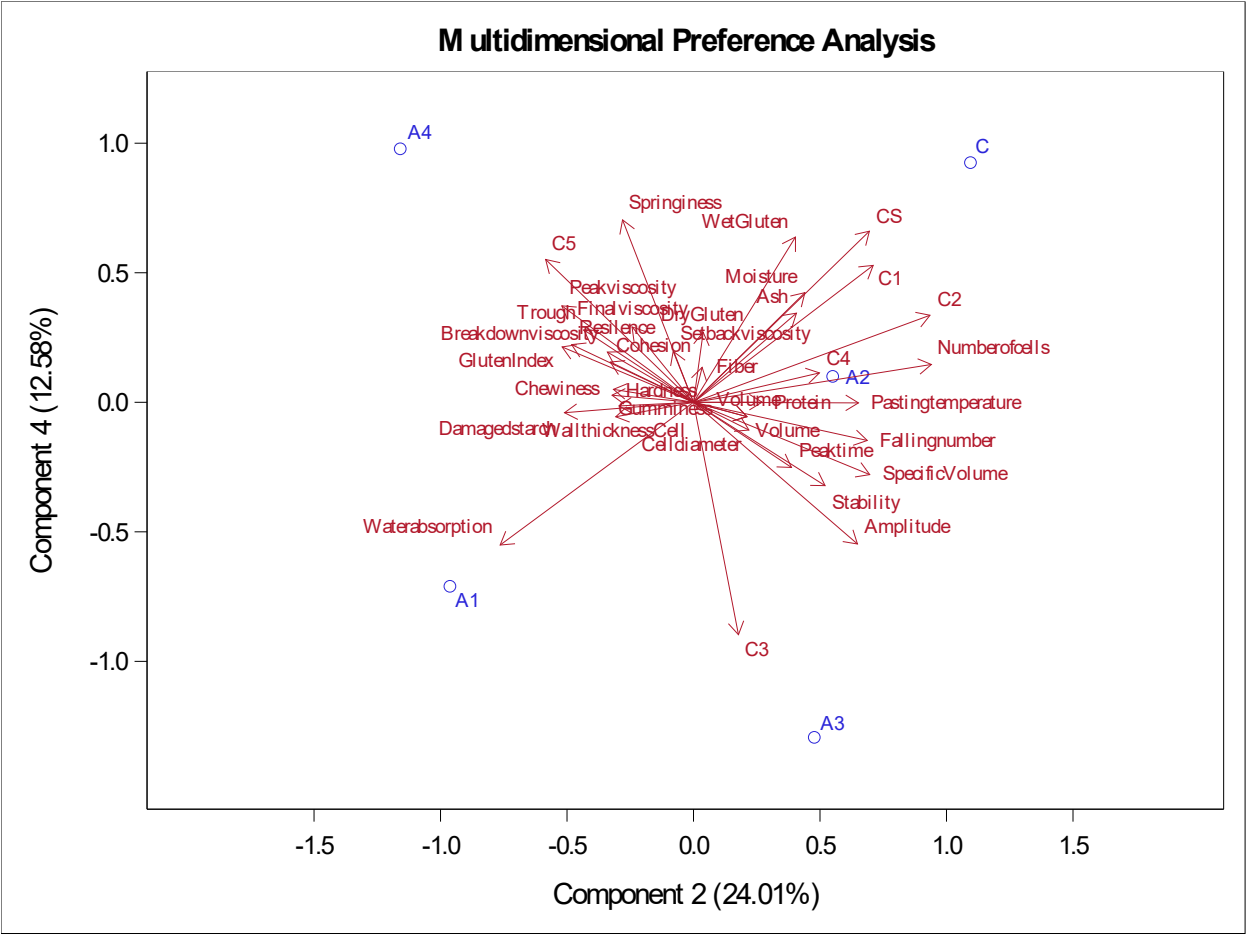
PC3 vs PC1



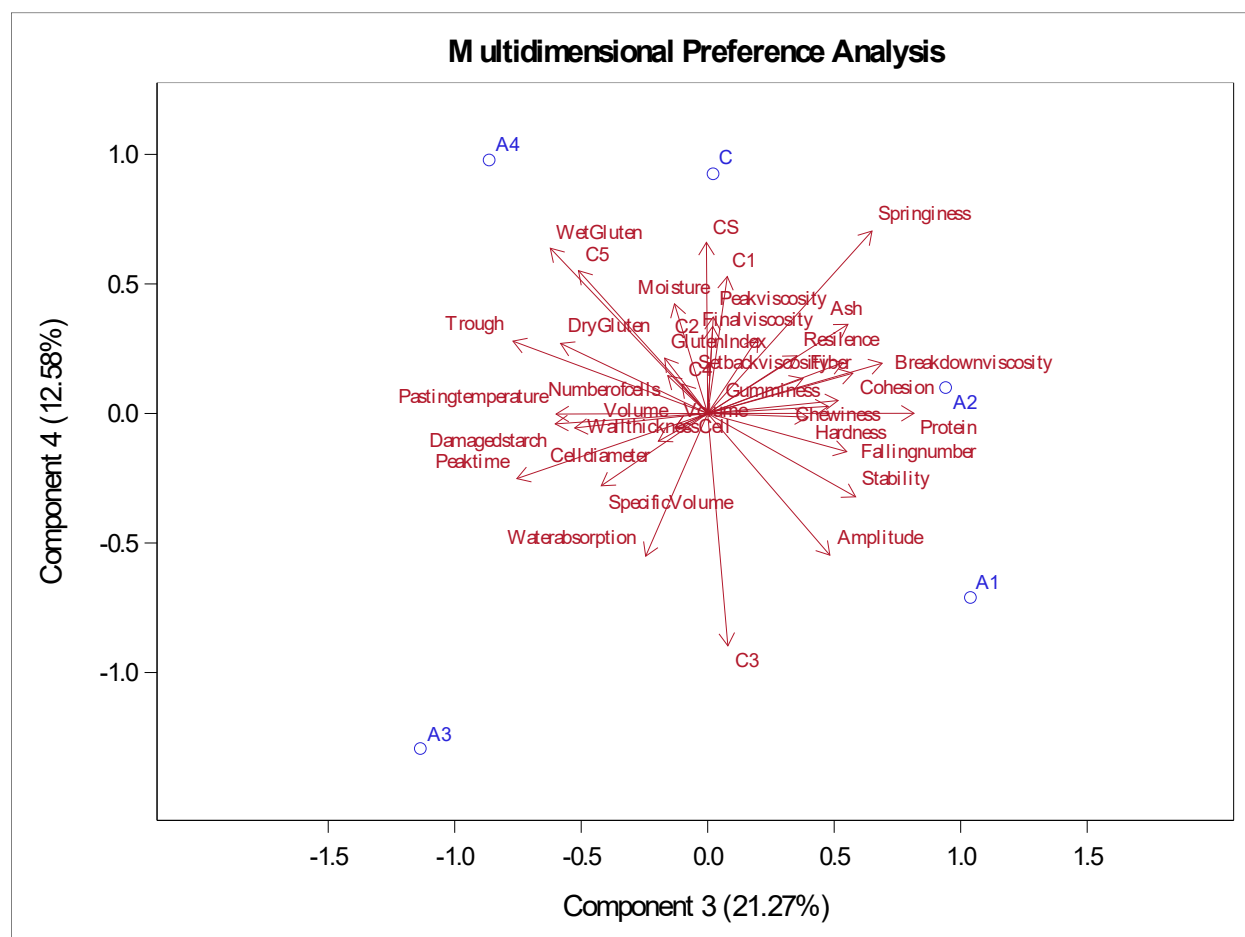
PC3 vs PC2



PC4 vs PC1



PC4 vs PC2



PC4 vs PC3