



**EFFECTS OF LACTIC ACID FERMENTATION ON
CITRULLINE CONTENT IN WATERMELON
(*Citrullus lanatus*) RIND**

SHANKAR NEUPANE

**MASTER OF SCIENCE
IN
INNOVATIVE FOOD SCIENCE AND TECHNOLOGY**

**SCHOOL OF AGRO-INDUSTRY
MAE FAH LUANG UNIVERSITY**

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**THIS THESIS IS A PARTIAL FULFILLMENT OF
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Thesis Title: Effects of Lactic Acid Fermentation on Citrulline Content in
Watermelon (*Citrullus lanatus*) Rind

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ABSTRACT

Watermelon rind, a major agricultural by-product of watermelon, is often discarded as waste despite its edibility. Fermentation *in situ* offers a sustainable approach to reintegrating such underutilized materials into the food value chain by enhancing preservation, safety, and bioactive compounds. This study aimed to optimize fermentation conditions to enhance L-citrulline, the predominant functional amino acid in watermelon rind with known health benefits. Natural fermentation was carried out by varying salt concentration (2%, 4%, and 6%), temperature (25, 30, 35, and 40 °C), and duration, and their effects on microbial dynamics, acidification, amino acid profile including citrulline and its metabolic flux, and other phytochemical properties were evaluated. Microbiological analysis demonstrated that 6% salt facilitated the highest lactic acid bacteria (LAB) growth, reaching peak populations across all temperatures. At 30°C, sustained LAB growth was observed with the minimum final pH (3.23 ± 0.01), establishing it as the optimal temperature for controlled fermentation. Enterobacteriaceae, initially present at high levels (7.35–7.72 log CFU/g), became undetectable in all samples by day 5, while yeast and mold populations were suppressed within 2 to 6 days. Subsequent fermentation at 30°C for 48 h demonstrated that amino acids involved in the arginine deiminase (ADI) pathway were strongly influenced by both salt concentration and fermentation time. While low-salt conditions (2% and 4%) led to citrulline depletion through its conversion to ornithine, 6% salt inhibited subsequent citrulline metabolism, resulting in significant accumulation reaching 737.12 ± 12.50 mg/kg at 12 hours, compared to 652.41 ± 7.77 mg/kg in the unfermented sample. Although citrulline levels declined thereafter, they remained significantly higher than the initial concentration throughout fermentation. These findings indicate that fermentation for 12 h at 6% salt concentration represents

the optimal condition for enhancing citrulline content. Total phenolic content (TPC) increased significantly across all treatments, with the 2% salt sample showing the highest relative increment (71.59%). DPPH radical scavenging activity more than doubled after 48 h, reaching 1.189–1.281 mg TE/g FW. Among free amino acids, glutamic acid and aspartic acid were more abundant at lower salt levels, suggesting greater umami character, while proline, threonine, histidine, lysine, and glutamine were elevated at higher salt concentrations. In case of color parameters, 6% salt better preserved rind lightness and yellowness, although greenness declined across treatments due to acid-induced chlorophyll degradation. These findings demonstrate that higher salt concentration not only promotes higher LAB counts but also drives citrulline overproduction, particularly at short fermentation durations. These findings support the valorization of watermelon rind by defining optimized fermentation conditions for citrulline enhancement, contributing to both agricultural waste reduction and the development of functional fermented products.

Keywords: Watermelon Rind, Fermentation, Citrulline, Lactic Acid Bacteria, Valorization, Amino Deiminase Pathway

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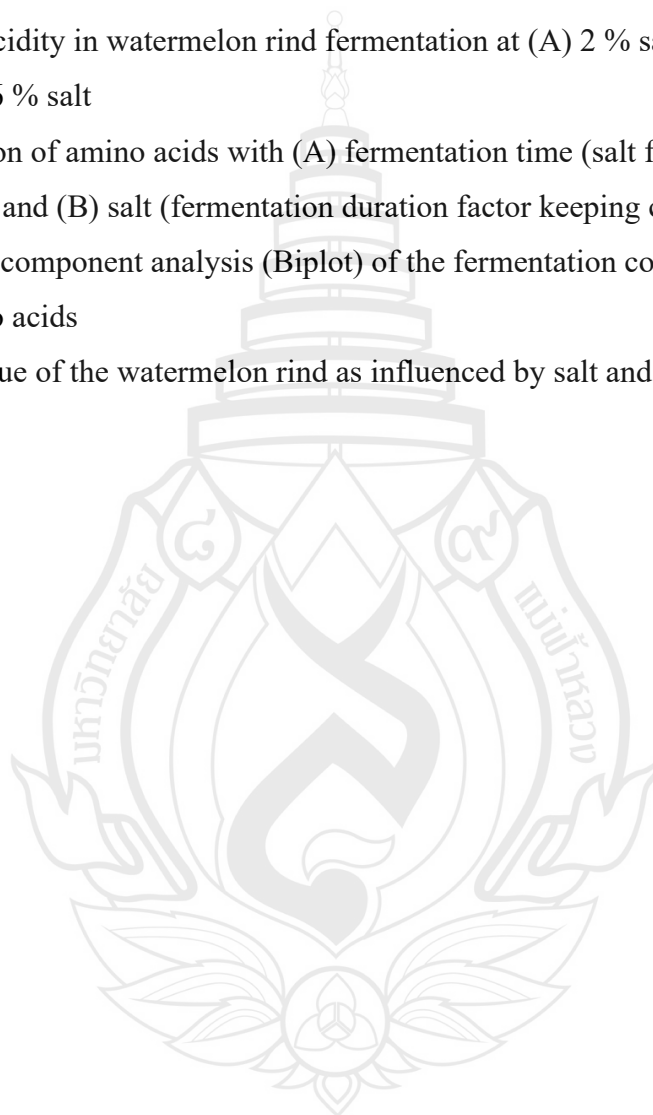
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ABBREVIATIONS AND SYMBOLS

ABA	Abscisic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADI	Arginine Deiminase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CK	Carbamate Kinase
CAS	Chemical Abstracts Service
CFU	Colony Forming Unit
cGMP	Cyclic Guanine Monophosphate
DAP	Diaminopimelate
DM	Dry Matter
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ED	Erectile Dysfunction
FAME	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organization
FeNO	Fractional Exhaled Nitric Oxide
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh Weight
GABA	γ -amino Butyric Acid
GAD	Glutamate Decarboxylase
GAE	Gallic acid Equivalent
GC/MS	Gas Chromatography-Mass Spectroscopy
HMDB	Human Metabolome Database
HRAC	Human Renal Cell Adenocarcinoma Cells
HPLC	High Performance Liquid Chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic Acid Bacteria

ABBREVIATIONS AND SYMBOLS

LC-QTOF-	Liquid Chromatography-Quadrupole Time of flight-Mass
MS	Spectroscopy
MRS agar	De Man–Rogosa–Sharpe Agar
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NO	Nitric Oxide
OTC	Ornithine Transcarbamylase
PDA	Potato Dextrose Agar
PPO	Polyphenol Oxidase
QE	Quercetin Equivalent
SDG	Sustainable Development Goals
TCA	Tricarboxylic Acid
TE	Trolox Equivalent
TPC	Total Phenolic Content
TTA	Total Titratable Acidity
UN	United Nations
VFCs	Volatile Flavoring Compounds
VRBGA	Volatile Red Bile Glucose Agar

CHAPTER 1

INTRODUCTION

1.1 Introduction to the Research Problem and Its Significance

Watermelon (*Citrullus lanatus*) is the second most produced fruit in the world after bananas. The total worldwide production accounts for more than 100 million tons, with approximately 80% concentrated in Asia as of 2022 data (FAO, 2024). The evidence of watermelon cultivation dates back over 5000 years in African regions but it is now celebrated worldwide for its juicy flesh as a thirst-quenching and heat-relieving drink especially in tropical regions (Paris, 2015). The rind is the firm and pale layer located between peel and flesh. It makes up about 30% of the total fruit weight and is usually discarded during consumption and processing. According to 2022, the rind alone accounts for more than 30 billion tons (FAO, 2024; Zia et al., 2021). This contributes substantially to urban waste and to missed opportunities for sustainable food use. The United Nations Sustainable Development Goals (SDG) for 2030 emphasize reducing food waste as a critical step towards eradicating hunger and promoting sustainability (Prokić et al., 2022). Efforts to incorporate food waste, like watermelon rind, into circular economy models could help alleviate environmental pressures while enriching food systems (Campos et al., 2020).

Previous studies indicate that watermelon rind is edible and a valuable source of nutrients like minerals, dietary fibers, phenolic compounds and other phytochemicals that contribute to its therapeutic properties; such as antioxidant (Neglo et al., 2021), anti-diabetic (Asari & Sugiyanta, 2020; Daughtry et al., 2023), antihypertensive (Abu-Hiamed, 2018), anti-carcinogenic (Reddy et al., 2023), and anti-inflammatory effects (Saiharini & Padmaja, 2022). Notably, watermelon is recognized as one of the richest natural sources of citrulline, a non-protein amino acid with significant physiological benefits. Interestingly, several studies have reported that citrulline concentration is higher in the rind than in the edible pulp (Du et al., 2022; El Gizawy et al., 2022; Neglo et al., 2021). Citrulline is a metabolic

intermediate in the urea cycle and serves as a precursor to L-arginine, a key substrate for nitric oxide synthesis. These roles give citrulline broad pharmacological and nutritional relevance, including potential in managing diabetes (Azizi et al., 2021; Bagheripour et al., 2023), obesity and associated complications (Daughtry et al., 2023; Holguin et al., 2019; Smeets et al., 2021) erectile dysfunction (Cormio et al., 2011; Sulyman et al., 2025), and improving exercise performance (Agarwal et al., 2017; Martínez-Sánchez et al., 2017). Despite the multiple benefits mentioned above, watermelon rind is not consumed due to its bland taste and other safety issues (Du et al., 2022).

Fermentation, particularly by lactic acid bacteria (LAB), is a traditional and cost-effective method widely used for food preservation, flavor development, and enhancement of nutritional quality (Sionek et al., 2023). Fermented products like kimchi, sauerkraut, miso, soy sauce, and fermented cucumber are widely accepted by consumers. In addition, scientific evidence has shown that fermented foods and their associated microorganisms offer various health benefits and safety enhancement, including enhanced bioactive compounds and antioxidants, improved bioavailability, and reduced pathogens and spoilage organisms (Rul et al., 2022; Tamang et al., 2020; Zapašnik et al., 2022). However, limited research has been conducted on the fermentation of watermelon rind, and existing studies have not adequately addressed the effects of fermentation and associated parameters on microbial dynamics, acidification, and physicochemical properties. Most of the available studies are restricted to evaluating changes in proximate composition (Ao & Es, 2016).

Beyond general fermentation benefits, LAB possess the arginine deiminase (ADI) pathway, which is of direct relevance to watermelon rind. In this pathway, arginine is first converted to citrulline by the enzyme arginine deiminase (encoded by *arcA*), and citrulline is subsequently converted to ornithine by ornithine transcarbamylase (encoded by *arcB*), with the concomitant generation of ATP and ammonia (Liu & Pilone, 1998). This pathway serves as an acid stress response mechanism in LAB, enabling cells to stabilize intracellular pH under acidic fermentation conditions (Zhang et al., 2016). A number of LAB species, including *Lactobacillus brevis*, *Lactobacillus hilgardii*, *Lactobacillus buchneri*, *Pediococcus pentosaceus*, *Lactobacillus fermentum*, *Weissella confusa*, *Pediococcus acidilactici*,

Leuconostoc mesenteroides, *Weissella koreensis* and *Lactobacillus plantarum* have been studied for their capacity to accumulate the citrulline from the arginine using different substrates, for example, soy sauce, wine and sourdough (Araque et al., 2011; Vrancken et al., 2009; Zhang et al., 2016). Nevertheless, these studies have largely been conducted using substrates with low natural levels of citrulline or arginine, often supplemented externally, and under controlled single-strain conditions. Watermelon rind is unique in that it naturally contains high concentrations of both arginine and citrulline, making it the first natural substrate to be investigated for citrulline enhancement through LAB fermentation under mixed-culture conditions. Critically, however, the ADI pathway presents a dual metabolic possibility: citrulline may either accumulate as an intermediate or be further converted to ornithine, depending on fermentation parameters and microbial dynamics (Zhou et al., 2021). It needs the systematic study of the fermentation condition, to enhance the accumulation of citrulline in natural condition, which remains unexplored.

Therefore, this study aims to systematically optimize lactic acid fermentation parameters including salt concentration, temperature, and fermentation duration to maximize citrulline content in watermelon rind, while evaluating their effects on microbial dynamics, acidification, amino acid composition, and phytochemical properties. By leveraging the rind's natural amino acid composition and exploring mixed LAB cultures, the research aims to transform an underutilized waste product into a palatable, nutrient-dense and safe food ingredient with enhanced citrulline. The findings will contribute to sustainable food innovation, waste reduction, and the development of health-promoting fermented products, bridging a critical gap in both food science and sustainability research.

1.2 Objectives

1.2.1 To study the effects of salt concentration, fermentation time and temperature on the chemical and microbial properties of watermelon rind during natural fermentation.

1.2.2 To optimize the salt concentration and fermentation duration regarding the citrulline content in fermented watermelon rind.

1.2.3 To study the effect of salt concentration and fermentation duration on the amino acids, color, phytochemical properties and metabolites of the watermelon rind.

1.3 Scope of Research

The goal of the study was to employ watermelon rind through natural fermentation with solid substrates to observe changes in its chemical, microbiological and physical characteristics as well as the generation of citrulline during fermentation process. The watermelon rind of *Kinnaree* variety was obtained from Triple J Food and Beverage Company Limited, Chiang Rai. The rind was washed, peeled and fermented *in situ* at different temperatures and salt concentrations. Total titratable acidity (TTA), pH, microbiology specifically LAB, yeast and mold and Enterobacteriaceae were observed over time for 10 days. Based on these results, the optimal fermentation temperature was determined and applied in subsequent experiments. The fermentation duration was then adjusted according to the growth of lactic acid bacteria and the pH of the substrate. Following this, the effect of salt concentration and fermentation duration was observed for the amino acids in ADI pathway; arginine, citrulline and ornithine with the aim of enhancing citrulline. In addition, variations in other amino acids, phytochemical properties, color, and metabolites were also assessed during the fermentation process. Due to resource issues, identification of organisms involved in fermentation could not be done.

CHAPTER 2

LITERATURE REVIEW

2.1 Watermelon

Watermelon is a famous fruit in tropical areas mainly consumed due to its refreshing taste, sweetness, appealing color, and thirst-quenching behavior (Meghwar et al., 2024). National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Government of Thailand defines the fresh watermelon fruit as “watermelon of genus *Citrullus* which is commonly referred to cultivar of the species *C. lanatus* (Thunb.) Matsum. & Nakai (synonym: *C. vulgaris* Schard) of the family Cucurbitaceae to be commercially produced for fresh consumption”. This definition limits the freshly consumed watermelon. The fruit is widely produced and consumed in the Asian region (FAO, 2024). However, archeological studies trace its origin in the African region. Archaeological research found seeds from around 5,000 years ago in northeastern Africa, which suggests that watermelons have been cultivated or consumed since early civilization. Additionally, Egyptian tomb art features an image of a large, striped, oblong fruit on a tray, which historians believe depicts as a watermelon and dates back 4,000 years (Paris, 2015).

The scientific classification of watermelon is as follows (Usman et al., 2023).

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Cucurbitales

Family: Cucurbitaceae

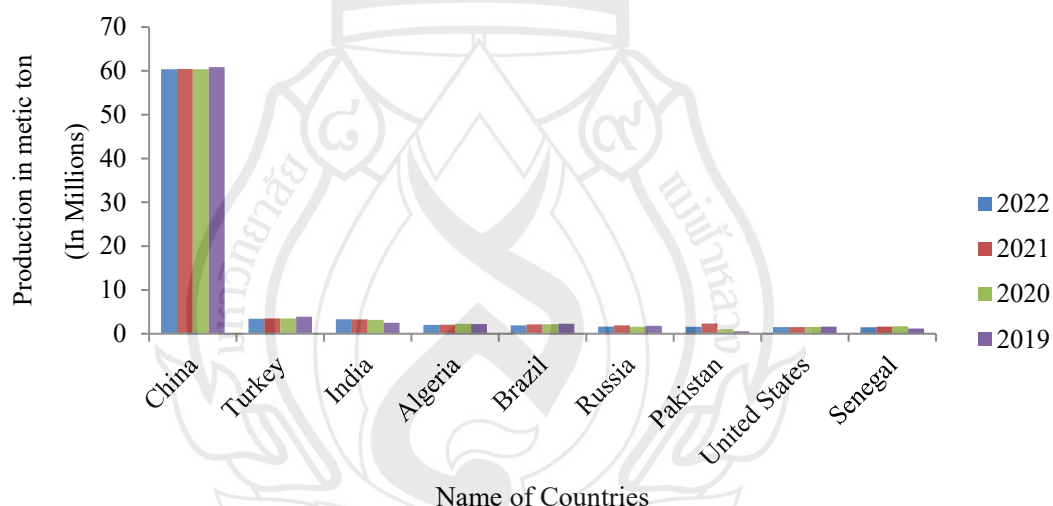
Genus: *Citrullus*

Species: *lanatus*

2.2 Watermelon Production

Watermelon is cultivated across tropical regions worldwide, with Asia accounting for 80% of global production. It is the second most produced fruit following bananas with global production of about 103 million tons as per 2022 data (FAO, 2024). China alone contributes around 60% of this total, followed by Turkey, India, and Algeria. The top ten watermelon producers, and their production volume from 2019-2022 are represented in Figure 2.1.

The Southeast Asian region is also a significant producer of watermelon due to favorable climatic conditions (Saediman et al., 2020). Within this region, Vietnam leads in production, with Indonesia following, while Laos, Malaysia, the Philippines, and Thailand produce nearly equal amounts (Figure 2.2).

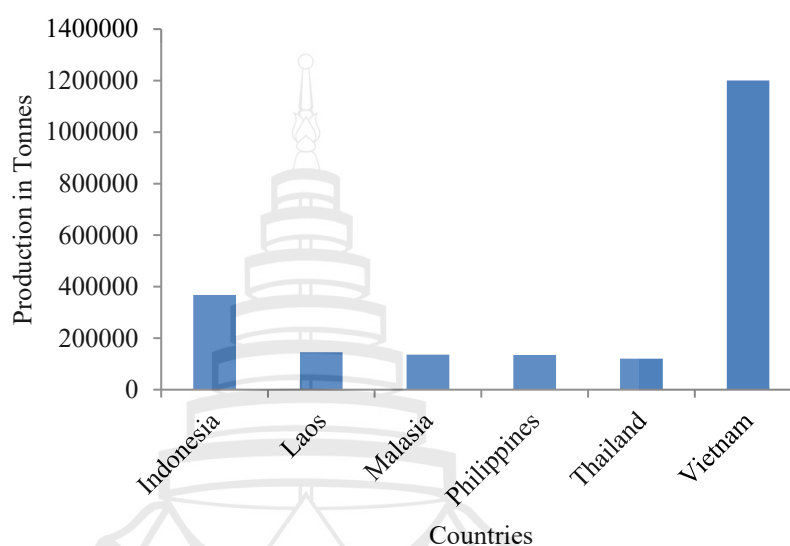


Source: FAO (2024)

Figure 2.1 Watermelon production by countries

Globally, Thailand ranks 55th in watermelon production, and in 2022, the total global production reached 166 thousand tons (FAOSTAT, 2024). In Thailand, watermelon has become an economically valuable crop, with cultivated areas expanding, especially in the northern provinces including Chiang Mai, Phayao, Kamphaeng Phet, Phichit, Sukhothai, and Phitsanulok. (Khuna et al., 2024). The country has two watermelon harvesting seasons, from January to May and mid-

October to December (Nuangmek, 2023). In 2023, Thailand's watermelon exports reached a value of over 2.47 million USD, with an export volume of 3.17 million kg (Tridge, 2026).



Source: FAO (2024)

Figure 2.2 Watermelon production in southeast Asia

Kinnaree variety is one of the famous varieties of watermelon consumed in Thailand due to its sweetness and refreshing taste (Petchsomrit, 2021; Wongkalasin et al., 2021). Most watermelon in Thailand is consumed fresh due to its refreshing taste and high-water content. In addition to fresh consumption, it is widely commercialized in processed forms such as juice, nectar, fruit cocktails, and concentrates. Value-added products, including dried watermelon and watermelon-flavored foods, have also gained popularity in recent years (Wanwimolruk et al., 2015). However, some industries, such as Triple J Food and Beverage Co. Ltd, in Mae Sai, Chiang Rai also produce processed products including fried Crispy Watermelon in Thailand (Figure 2.3).



Source Triple J Food and Beverage Co. (2026)

Figure 2.3 Crispy watermelon produced by Triple J Food and Beverage Co. Ltd.,
Chiang Rai, Thailand

2.3 Watermelon Waste

Food waste has become a critical challenge with significant implications for both society and the environment. On the one hand, millions face hunger, and available agricultural land is increasingly scarce for meeting the needs of a growing population. On the other hand, food loss and waste are on the rise, creating a stark contrast between scarcity and excess. The environmental impact of this problem is compounding, which has become a worldwide concern. Not only does wasted food represent a loss of valuable resources, but its decomposition releases greenhouse gases that contribute to climate change, amplifying environmental problems even further (FAO, 2023).

Considering this fact, the UN SDG has identified waste reduction and utilization as one of the goals. The SDG 12.3 states that “by 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses.” However, in 2022, 19% of global food was wasted, totaling 1.05 billion tons, with household waste accounting for 60%. This waste generates significant greenhouse gas emissions, costing over \$1 trillion annually, while 783 million people suffer from hunger (United Nations Secretary General, 2024). It is now well documented that reducing global food waste can create a ‘win-win’ situation for nutrition security and environmental sustainability (FAO, 2023).

Watermelon waste is a significant source of waste at household, retail and industrial levels. Watermelon itself comprises skin, rind, flesh, and seeds (Figure 2.4), with the flesh accounting for only about 60 % of the fruit's total mass. The edible watermelon rind makes up roughly 30 % of the mass but is frequently discarded as waste. This direct disposal of rind contributes to environmental issues, although some reuse approaches have been explored on a laboratory scale (Du & Ramirez, 2022). Given current watermelon production, rind waste alone amounts to approximately 30 billion tons globally (FAO, 2024; Zia et al., 2021). Unfortunately, over 90% of this rind is discarded directly into the environment, adding to environmental challenges. Presently, limited research has explored converting rind waste into valuable products, resulting in it being largely dumped as solid waste (Bellary et al., 2016).

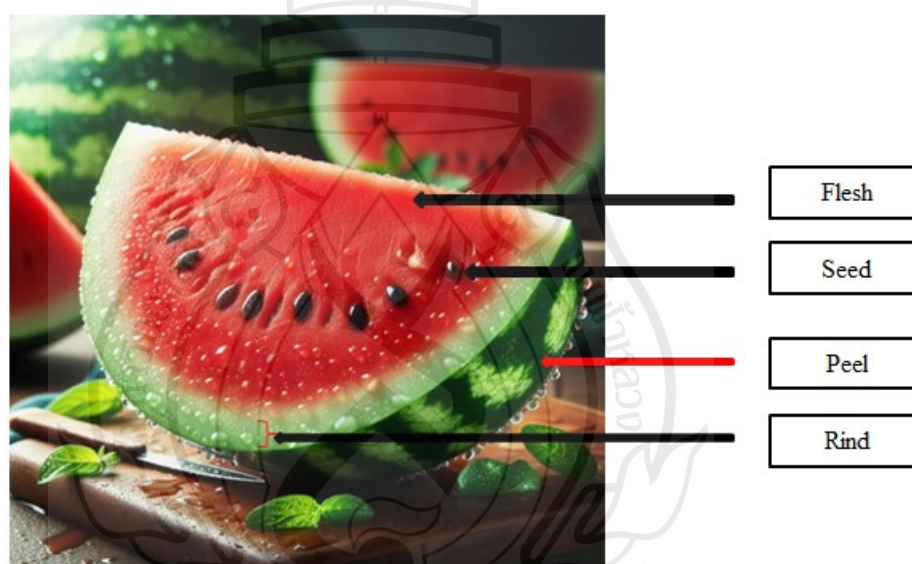


Figure 2.4 Different parts of watermelon (AI Image)

Further, watermelon rind contains significant amounts of water and nutrients, which makes it perishable and hosts large categories of pathogenic microorganisms. When watermelon rind is disposed improperly, it adds to the growing issue of organic waste accumulation in landfills. In such environments, the rind's high moisture content can accelerate anaerobic decomposition process, leading to the emission of methane, a potent greenhouse gas that contributes to climate change (Athanasiadis et al., 2023).

2.4 Watermelon Rind Chemical and Bioactive Compounds

Fresh watermelon is characterized by a high moisture content, typically exceeding 90%. Beyond its water content, the rind of watermelon is nutritionally significant, containing substantial amounts of carbohydrates, crude fiber, and protein as analyzed in rind powder (Table 2.1). Notably, it serves as a rich source of vitamins and minerals. Carbohydrates in the rind include starch, non-starch polysaccharides, sugars, resistant starch, oligosaccharides, and pectin (Meghwar et al., 2024). The mineral profile is marked by elevated levels of manganese, copper, iron, and zinc (El-Badry et al., 2014). Vitamins present in the rind (per 100 g) include vitamin A (52.13 µg), vitamin B1 (1.23 mg), vitamin B2 (2.71 mg), vitamin B3 (4.25 mg), vitamin B6 (5.34 mg), and vitamin C (8.46 mg). Notably, the concentrations of vitamins B1, B2, and B6 exceed the recommended daily intake (RDI) established for Thailand (Gladvin et al., 2017).

Watermelon rind is further distinguished by its abundance of bioactive compounds, including citrulline, flavones, saponins, sterols, terpenoids, and phenolic compounds, which confer antioxidant and health-promoting properties. Citrulline is a dominant bioactive compound linked to many of the rind's health benefits (Meghwar et al., 2024).

Various studies emphasize its phytochemical richness, with research by Ho et al. (2018) reported total phenolic content values ranging from 127.93 to 218.39 mg GAE/100 g, alongside DPPH free radical scavenging activity, alongside DPPH free radical activity ranging from 23.49-80.88 % as ascorbic acid between different cultivar and extraction solvents. Furthermore, Priastomo et al. (2024) reported the TPC values between 1.63 to 3.57 mg GAE/g DM in the watermelon rind from different parts of Indonesia. Long et al. (2024) reported even higher values of phenolic content in the range of 7.13 to 8.20 mg GAE/g DM in watermelon rind powder of different particle sizes. They reported other phytochemical indices as total flavonoids (4433–4720 mg quercetin equivalents (QE)/kg), total anthocyanins (393–315 mg cyanidin-3-glucoside equivalents (CGE)/kg), DPPH scavenging activity (27,186–34,820 µmol Trolox equivalents (TE)/kg), and Fe(III) reducing power

(11,862–21,141 $\mu\text{mol TE/kg}$) However, Neglo et al. (2021) reported lower total phenolic content as 0.026 ± 0.006 mg GAE/g sample. They also reported that DPPH radical scavenging activity was 34.48 ± 1.63 % inhibition. However, they found that the phenolics and antioxidant activity in watermelon rind were significantly higher than that of flesh.

Table 2.1 Proximate composition of watermelon rind powder

Proximate composite		Percentage (%)			
Moisture	10.61	11.25	12.05	7.88	9.6
Ash	13.09	12.93	11.9	12.55	9.25
Fat	2.44	2.21	2.33	1.98	2.15
Protein	11.17	8.7	8.95	8.64	9.6
Crude fiber	17.28	16.8	15.85	15.98	13.7
Carbohydrate	56.02	76.16	76.82	60.85	65.3
Reference	(Al-Sayed & Ahmed, 2013)	(El-Badry et al., 2014)	(Saed, 2018)	(Saed et al., 2018)	(Hassan et al., 2017)

Phenolic compounds identified in watermelon rinds include gallic acid, synapic acid, hydroxycinnamic acid, quercetin, m-coumaric acid, chlorogenic acid, syringic acid, p-coumaric acid, myricetin, caffeic acid, vanillic acid, 4-hydroxybenzoic acid, and p-anisic acid (Zia et al., 2021). These findings collectively underscore the rind's potential as a functional food ingredient with significant antioxidant and nutraceutical value. The health effects of the watermelon rinds due to presence of bioactive compounds is summarized in Table 2.2.

Table 2.2 Health effects of watermelon rind

Part	Health Benefits	Health Effects	References
Rind powder	Cardiovascular health/ Rat experiment	Adding 10% watermelon rind to the diet of hypercholesterolemic rats significantly lowered their serum total cholesterol and low-density lipoproteins. The rats also showed improved liver tissue structure compared to untreated hypercholesterolemic rats.	(Abu-Hiamed, 2018)

Table 2.2 (continued)

Part	Health Benefits	Health Effects	References
Rind polysaccharide	Anticarcinogen/ In-vitro	Watermelon rind polysaccharide showed cytotoxic effects on human laryngeal carcinoma Hep-2 cells in a dose- and time-dependent manner, with a 25.93% inhibition rate at 2.5 µg/mL and 65.02% at 10 µg/mL.	(Dammak et al., 2019)
Rind water extract	Antidiabetic/ Rat experiment	Watermelon rind water extract can help lower blood glucose levels, with an optimal dose of 500 mg/kg body weight per day, while 1000 mg/kg bw/day was most effective in rats. NO formation induced by citrulline might have the effect.	(Asari & Sugiyanta, 2020)
Rind	Cardiovascular benefits/In vivo	Ingestion of Watermelon Rind increased the citrulline, arginine, and phenolic metabolites in human plasma, significance in cardiovascular benefits.	(Fan et al., 2020)
Rind aqueous extract	Anticarcinogenic/In-vitro	The rind aqueous extract significantly inhibited cell migration and induced apoptosis in HCT116 and Hep2 cells by causing cell accumulation in the S phase and increasing both caspase-3 activity and the BAX/BCL-2 ratio.	(El Gizawy et al., 2022)
Rind ethanol extract	Anticarcinogenic/ In vitro	Watermelon rind extract demonstrates a dose- and time-dependent reduction in human renal cell adenocarcinoma cells (HRAC-769-P) cell proliferation in vitro.	(Reddy et al., 2023)
Microencapsulated rind	Endothelial function/ In vivo	Watermelon rind increases the plasma citrulline and arginine level in plasma and improved flow-mediated dilation in young adult.	(Volino-Souza et al., 2023)

Table 2.2 (continued)

Part	Health Benefits	Health Effects	References
Rind extract	Antidiabetic & anti-inflammatory/in-vitro	Methanolic Extract of Watermelon rind shows inhibition effect on carbohydrate hydrolyzing enzymes, pancreatic lipase enzyme and hyaluronidase enzyme in vitro. The presence of dietary fiber, total phenolics, flavonoids and flavanols might have the effect.	(Saiharini & Padmaja, 2022)

2.5 Watermelon Rind Utilization

Due to the high volume of watermelon rind as waste and its health promoting properties, some initiatives are going on to align the watermelon rind in the food chain. Studies have shown the rind use in the preparation of salted pickle (Simonne et al., 2003), leather (Siskawardani et al., 2018), and candy (Rezagholizade-Shirvan et al., 2023). It has been used in pineapple pulp to increase the volume. Further, watermelon rind in powder form is used for the preparation of cookies (Ashoka et al., 2021; Naknaen et al., 2016), biscuits (Ogo et al., 2021), cakes (Hassan et al., 2017; Imoisi et al., 2023) and noodle products (Chakrabarty et al., 2020; Long et al., 2024). The details of relevant research are presented in Table 2.3.

Table 2.3 Products from the watermelon rind

Product	Part	Findings	References
Candy	Watermelon rind	Candy preparation with osmosis temperature of 40°C, with sugar concentration 70%, and immersion duration 5 h was found to be best on sensory score. However, antioxidant activity, phenolic and flavonoid content significantly decreased after osmotic dehydration	(Rezagholizade-Shirvan et al., 2023)
Pickle	Watermelon rind	Watermelon rind pickles prepared by differentiating the soaking pretreatment before brining and canning. Lime-soaked pickles were preferred over brine and water-soaked pickle	(Simonne et al., 2003)

Table 2.3 (continued)

Product	Part	Findings	References
Leather	Watermelon rind	Ten percent replacement of pineapple pulp had the best sensory quality	(Siskawardani et al., 2018)
Cake	Watermelon rind powder	Up to 40 % of replacement observed. Incorporation resulted in an increase in protein content and reduction in fat content.	(Imoisi et al., 2023)
Cake	Watermelon rind powder	Up to 40 % replacement is possible, no significant difference in sensory quality up to 20 % replacement. Incorporation improved the Ca, K and Mg, content and cake volume but decreased all textural parameters.	(Hassan et al., 2017)
Wheat bread	Watermelon rind powder, rind polysaccharide and rind hemicellulose	Incorporating rind, rind powder and rind polysaccharide and hemicellulose concentrations ranging from 0.5 to 1.5% into wheat flour enhanced the rheological characteristics of the dough. Enriching wheat flour with rind improved the bread's volume, texture and sensory quality in comparison to its derivatives.	(Ben Romdhane et al., 2024)
Cookies	Watermelon rind powder	Incorporating up to 20% rind powder in cookie production enhances antioxidant activity, boosts dietary fiber content, and lowers the predicted glycemic index.	(Naknaen et al., 2016)
Cookies	Watermelon rind powder	Thirty percent replacement has the highest sensory score, better than control. The final product was high in some essential minerals.	(Ashoka et al., 2021)
Biscuit	Watermelon rind powder	Biscuit samples were made using blends of wheat flour with watermelon rind and orange pomace flours, with up to 10% substitutions without negatively impacting their overall quality.	(Ogo et al., 2021)
Toast	Watermelon rind powder	About 9 % incorporation of rind powder positively affected water absorption, dough development, stability time, and extensibility values for the optimal sample compared to the control	(Shivapour et al., 2020)

Table 2.3 (continued)

Product	Part	Findings	References
Biscuit	Watermelon rind powder	Biscuit samples were made using blends of wheat flour with watermelon rind and orange pomace flours, with up to 10% substitutions without negatively impacting their overall quality.	(Ogo et al., 2021)
Toast	Watermelon rind powder	About 9 % incorporation of rind powder positively affected water absorption, dough development, stability time, and extensibility values for the optimal sample compared to the control	(Shivapour et al., 2020)
Noodle	Watermelon rind powder	The incorporation of rind powder improved cooking yield and cooking loss. Ten percent of rind-incorporated noodles were found best among the incorporated noodles; however, control was preferred more.	(Chakrabarty et al., 2020)
Pasta	Watermelon rind powder	Replacing 10% of durum semolina with finely ground watermelon rind powder increases soluble dietary fiber and total phenolic content, extends optimal cooking time, enhances water absorption, and reduces cooking loss in pasta, without impacting sensory acceptability.	(Long et al., 2024)
Pork patty	Rind extract	Ethanollic extract of watermelon rind incorporated at 0.10% level in pork patties showed a significantly ($P < 0.05$) lowering effect of TBARS, free fatty acids and peroxide value as compared to control.	(Kumar et al., 2018)
Beef patty	Watermelon rind powder	Replacing 6-9% of the burger patty with watermelon rind powder enhances fiber, ash, and soluble carbohydrate content while reducing fat. This addition also inhibits microbial growth, decreases lipid oxidation, and maintains good sensory quality in the beef patties.	(Saed et al., 2018)

Despite the nutritional, phytochemical, and health benefits of fruit peels, including watermelon rind, their utilization remains limited due to sensory issues, safety concerns, and technological challenges. Efforts to incorporate rind in food products, such as bread and cookies, have focused on partial substitution, typically up to 40% wheat flour replacement (Imoisi et al., 2023). However, higher substitution levels can negatively affect sensory and technological properties. Watermelon rind is more commonly used in products like jams and fruit leather, but broader market adoption is hindered by its bland taste and concerns over potential hazards, including microbial contamination, pesticide residues, and heavy metal accumulation. Public concerns about the safety of fruit peels further limit their consumption (Coderoni & Perito, 2021).

Watermelon rind's susceptibility to contamination by pathogenic microorganisms, heavy metals, and anti-nutritional factors poses significant challenges. Its direct contact with soil increases exposure to soil-borne pathogens and pollutants, such as pesticides and industrial waste (Scutarașu & Trincă, 2023; Steffan et al., 2020). Microbial contamination is exacerbated by poor field hygiene and handling, especially in developing countries, where studies have shown high levels of bacterial contamination in fresh produce (Balali et al., 2020). Fermentation has shown promise in addressing these issues, offering a low-cost processing method to reduce contaminants, mitigate antinutritional factors, and enhance sensory qualities, making watermelon rind a safer and more appealing ingredient (Tamang et al., 2015)

2.6 Citrulline

Citrulline (CAS: 372-75-8) is a non-protein amino acid with the chemical formula $C_6H_{13}N_3O_3$. The presence of the ureido group in the structure makes it different from other amino acids (Figure 2.5). This unique feature underpins its biological significance, as the chemical reactivity of the ureido group plays a central role in its function. Notably, citrulline is closely related to arginine, sharing a functional group connection that is critical to its role in metabolic pathways, particularly in the urea cycle and nitric oxide synthesis (Curis et al., 2005).

In the human body, the endogenous synthesis of L-citrulline primarily takes place in the enterocytes, the cells lining the small intestine. A variety of enzymes work to convert dietary amino acids into L-citrulline. A key example of this process occurs in the urea cycle, a vital pathway for detoxifying ammonia. During this cycle, the enzyme ornithine carbamoyltransferase catalyzes the conversion of L-ornithine into L-citrulline. Additionally, L-glutamine and L-arginine serve as indirect precursors for L-citrulline. L-arginine is converted into L-ornithine by the enzyme arginase, while ornithine aminotransferase utilizes L-glutamine to produce L-ornithine, which can then be further metabolized into L-citrulline (Figure 2.6). These interconnected pathways highlight the importance of L-citrulline in nitrogen metabolism and ammonia detoxification (Aguayo et al., 2021).



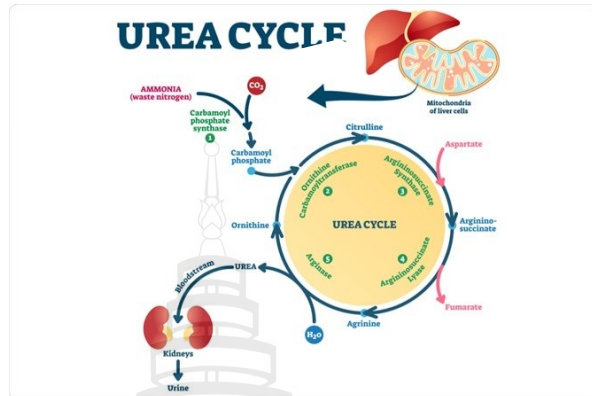
Source Curis et al. (2005)

Figure 2.5 Chemical structure of L-Citrulline

Citrulline plays a pivotal role in human health primarily through its ability to regenerate arginine, a precursor for nitric oxide (NO) synthesis. When arginine is metabolized by nitric oxide synthase (NOS), it produces NO and is reconverted into citrulline, creating a cyclic pathway known as the citrulline-arginine-NO cycle (Figure 2.7). This cycle underscores citrulline's unique value, oral supplementation effectively elevates plasma arginine levels, enhancing its bioavailability for NO production. Numerous bodily functions such as regulating vasodilatation, blood flow, mitochondrial respiration and platelet function are significantly influenced by NO (Aguayo et al., 2021).

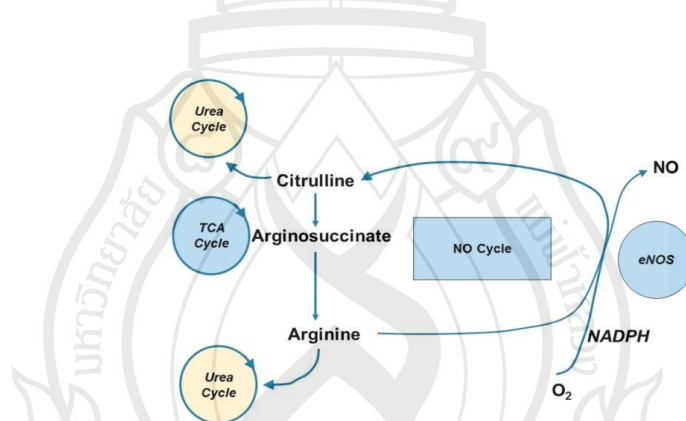
Due to its numerous health benefits, citrulline is sold as a dietary supplement, with its market steadily growing. Some of the leading brands are represented in Figure 2.8. In 2023, the global L-citrulline market generated revenue of USD 1,601.7 million

and is projected to reach USD 2,276.0 million by 2030, reflecting an expected CAGR of 5.1% from 2024 to 2030 (Grand View Research, 2024).



Source Yokoyama and Simmons (2021)

Figure 2.6 Citrulline metabolism in the human body



Source Allerton et al. (2018)

Figure 2.7 Citrulline-arginine-NO cycle



Source Cooperman (2026)

Figure 2.8 Citrulline supplements available in the market

2.7 Citrulline in Watermelon Rind

Citrulline is not widely distributed in nature. It is particularly found in the Cucurbitaceae family crops. Watermelon, cucumbers, pumpkins, bitter melons and some legumes are natural sources of citrulline. In fact, watermelon is the principal source of that amino acid in the diet; the name citrulline comes from *Citrullus lanatus* (Thunb.), the scientific name for watermelon (Hartman et al., 2019). Citrulline concentration in watermelon depends on the type of cultivar and usually ranges between 0.5 and 3.6 g/kg of fresh weight (Rimando & Perkins-Veazie, 2005). Several studies found that the citrulline content in watermelon is higher in rind than in pulp (Du et al., 2022; El Gizawy et al., 2022; Neglo et al., 2021). In contrast, Gu et al. (2023) found that citrulline content is higher in pulp ($1505 \pm 66.9 \mu\text{M}$) than in rind ($780.5 \pm 232 \mu\text{M}$). However, the study found that the citrulline from the watermelon rind showed the higher cytotoxicity against the Caco-2 cells as compared to that from pulp. Nguyen et al. (2019) measured the amino acids in watermelon rind powder and discovered that L-citrulline was found highest at 18.2 nmol/mg, followed by L-arginine at 2.72 nmol/mg. Other amino acids quantified were L-alanine, L-glutamine, L-valine, L-phenylalanine, L-isoleucine, L-serine, L-tyrosine, and γ -amino butyric acid (GABA). Similar amino acid profile was reported by Du et al. (2022). They found the concentration of citrulline as 61.43 mg/100 g fresh weight followed by arginine (53.77 mg/100 g FW), serine (18.02), aspartic acid (7.09) and alanine (6.78). The amino acid profile of watermelon rind found by different researchers is presented in Table 2.4.

Table 2.4 Amino acid profile of watermelon rind

Amino Acid	Fresh (mg/100 g fresh weight)	Powder (nmol/mg)	Powder (g/100g)
Phenylalanine	2.69	0.76±0.03	0.17
Isoleucine	2.37	0.47±0.02	0.35
Valine	3.24	0.78±0.03	0.42
Leucine	0.61	-	0.53
Methionine	Not detected	-	-
Histidine	Trace	-	0.22
Lysine	Trace	-	0.56

Table 2.4 (continued)

Amino Acid	Fresh (mg/100 g fresh weight)	Powder (nmol/mg)	Powder (g/100g)
Arginine	57.33	2.72±0.10	1.01
Citrulline	61.43	18.2±0.71	-
Alanine	6.78	0.97±0.04	0.69
Serine	18.02	0.37±0.01	0.23
Proline	3.31	-	0.12
Glycine	0.37	-	0.65
Aspartic Acid	7.09	-	-
Asparagine	2.71	-	-
Ornithine	1.71	-	-
Glutamine	-	0.84±0.03	1.13
Tyrosine	-	0.32±0.02	0.29
GABA	-	0.85±0.09	-
References	Du et al. (2022)	Nguyen et al. (2019)	Saed et al. (2018)

2.8 Health Effects of Citrulline

Citrulline plays a crucial role as an intermediate in the urea cycle, aiding in the elimination of ammonia through urea production (Burgos et al., 2022). In recent years, extensive research has focused on L-citrulline and its impact on exercise performance. Its ability to enhance NO production and facilitate waste removal during physical activity has drawn significant interest from researchers (Theodorou et al., 2021). As a result, both arginine and citrulline have gained popularity as supplements for promoting health and enhancing athletic performance. Interestingly, studies suggest that citrulline supplementation is more effective than arginine in increasing plasma arginine levels. Unlike direct arginine supplementation, citrulline bypasses hepatic metabolism, allowing more efficient systemic distribution and sustained arginine availability (Agarwal et al., 2017).

Furthermore, research indicates that citrulline-enriched watermelon extract can help delay fatigue during high-intensity exercise by promoting ammonia removal and reducing lactic acid buildup via the urea cycle. It also supports aerobic metabolism by

maintaining lower plasma lactate concentrations, ultimately contributing to improved recovery and exercise performance (Martínez-Sánchez et al., 2017).

Beyond its role in exercise, citrulline has been studied for its potential benefits in managing diabetes and related cardiovascular diseases. Reduced NO bioavailability is associated with the pathophysiology of Type II diabetes and its complications. Since citrulline serves as a precursor to NO production, it has been proposed as a promising therapeutic agent for Type II diabetes. Studies in both humans and animals suggest that citrulline supplementation can elevate circulating levels of citrulline and arginine while lowering blood glucose and free fatty acid levels, as well as improving dyslipidemia (Bagheripour et al., 2023). A clinical study on diabetic patients found that one month of citrulline supplementation reduced arginase activity by 21%, increased plasma NO levels by 38%, and led to modest improvements in HbA1c levels (Shatanawi et al., 2020). Similarly, another study on overweight and obese individuals with Type II diabetes reported improvements in glucose homeostasis, lipid profiles, and inflammatory markers following L-citrulline supplementation (Azizi et al., 2021). Animal studies have also demonstrated that citrulline supplementation may prevent or alleviate diabetes-induced kidney dysfunction more effectively than arginine (Persson et al., 2014). Additionally, in STZ-induced Type I diabetic rats, L-citrulline exhibited protective effects on the kidneys by significantly reducing urinary albumin excretion, tubulointerstitial fibrosis, and kidney hypertrophy (Romero et al., 2013).

Citrulline has also shown promise in addressing obesity and its associated complications. Short-term L-citrulline supplementation was found to improve asthma control and suppress fractional exhaled nitric oxide (FeNO) levels in obese individuals with asthma (Holguin et al., 2019). In healthy adults, long-term citrulline supplementation improved brachial artery flow-mediated vasodilation by 0.9 percentage points, while prolonged watermelon consumption reduced pulse wave velocity by 0.9 m/s and lowered postprandial glucose concentrations by 0.6 mmol/L, suggesting potential vascular benefits (Smeets et al., 2021). Additionally, a study conducted on overweight and obese adolescents found that consuming blended watermelon helped stabilize postprandial glucose levels and enhance satiety, with researchers attributing these effects to its citrulline content (Rasmussen et al., 2025).

Another study reported that watermelon consumption led to improvements in cardiometabolic risk factors, including BMI, BMIp, body fat percentage, and HbA1c, with citrulline-induced NO bioavailability likely playing a role (Daughtry et al., 2023).

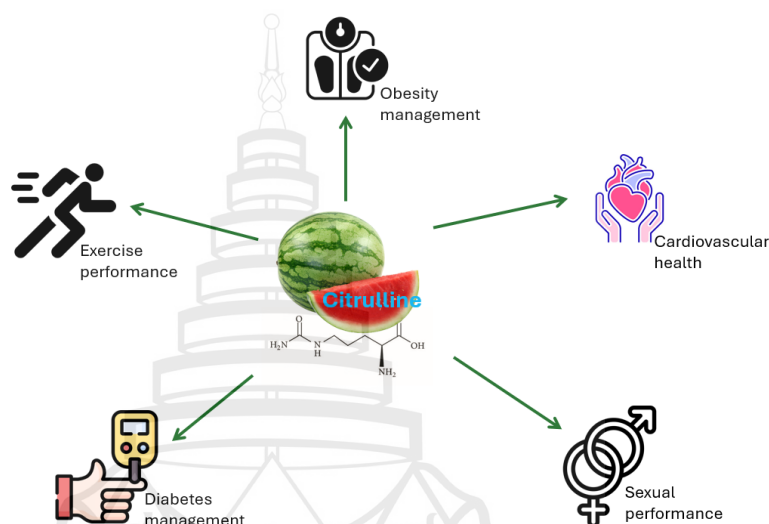


Figure 2.9 Health effects of L-Citrulline in humans

Moreover, L-citrulline has demonstrated potential in managing mild erectile dysfunction (ED). Its ability to enhance NO production facilitates the activation of soluble guanylate cyclase, leading to increased levels of cyclic guanosine monophosphate (cGMP), which promotes penile smooth muscle relaxation and improved erectile function. Studies show that short-term L-citrulline supplementation can enhance erection hardness scores from 3 (mild ED) to 4 (normal function) and increase the frequency of intercourse per month (Cormio et al., 2011). Another proposed mechanism suggests that citrulline, particularly from watermelon, may stabilize and inhibit PDE5, indicating its potential as an alternative or adjunct therapy for ED (Sulyman et al., 2025). Since PDE5 inhibition is crucial in ED management, this finding is particularly significant. Additionally, research suggests that NO-mediated increases in clitoral blood flow and velocity following the topical application of a gel containing L-arginine and L-citrulline could contribute to reducing female sexual dysfunction (Lovie & Marashi, 2022). The health effects of citrulline are summarized in Figure 2.9.

2.9 Food Fermentation

Food fermentation is one of humanity's oldest preservation techniques, with roots extending back thousands of years. Steinkraus (1995) gave an elaborate definition of fermented foods as "food substrates that are invaded or overgrown by edible microorganisms whose enzymes—such as amylases, proteases, and lipases—break down polysaccharides, proteins, and lipids into nontoxic products with flavors, aromas, and textures that appeal to human consumers." Historical records reveal that fermented beverages were consumed in Babylon around 5000 years ago, and bread was already common in Egypt by 1500 BCE. The practice of fermenting mixed vegetables likely originated in China. Scientific studies show evidence of fermented vegetable consumption dating back to the workers who constructed the Great Wall in the 3rd century BCE (Tamang et al., 2020).

Beyond preservation, fermentation improves taste and flavor, adding a unique appeal to traditional diets. As a result, diverse cultures continue to embrace fermented foods. Lactic acid fermentation has gained popularity across many regions, giving rise to distinctive products: *kimchi* in Korea, sauerkraut in Europe, *naw-mai-dong* and *hom-dong* in Thailand, *sunki* in Japan, *burong mustala* in Indonesia, and *gundruk*, *sinki*, and *khalpi* in Nepal and many others in different parts of the world (Tamang et al., 2015).

Lactic Acid Fermentation

Lactic acid fermentation is one of the common fermentations among food fermentations. In lactic fermentation, foods are overgrown with LAB mainly from the environment. LABs are Gram-positive, catalase-negative bacteria that produce large amounts of lactic acid (Mokoena, 2017). The bacterial groups that make up the LAB are among the most familiar to humans, because of their association with the human environment, and with a wide range of naturally fermented dairy products, grain crops, vegetables, and so on. Due to very long history of safe use of these microbial species, they have generally recognized as safe (GRAS) status (Wang et al., 2021). The LAB comprise a large bacterial group consisting of about 380 species in 40 genera of 6 families, belonging phylogenetically to the order *Lactobacillales* within

the phylum *Firmicutes* (Stiles & Holzapfel, 1997). Common genera of the LAB isolated from various fermented foods of the world are *Alkalibacterium*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Tamang et al., 2015).

The benefits of lactic acid fermentation extend beyond taste and preservation. According to Tamang et al. (2015), lactic acid fermentation in foods serves four primary purposes: (1) extending shelf life and enhancing safety, (2) improving nutritional and health benefits, (3) enriching sensory qualities, and (4) generating bioactive compounds of interest. These combined effects make lactic acid fermentation a valuable and versatile method of food processing across various cuisines and cultures.

2.10 General Physicochemical and Microbial Dynamics during Lactic Acid Fermentation

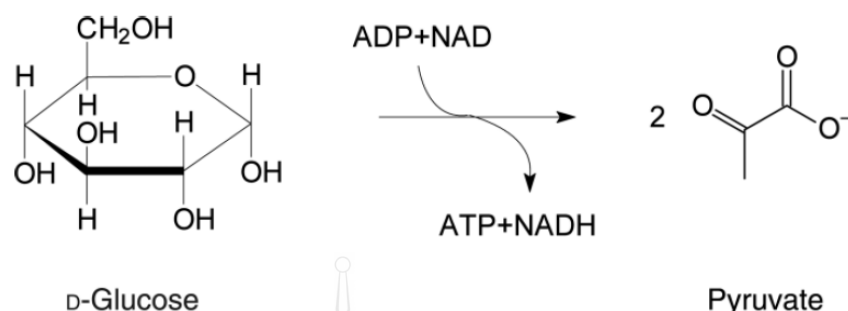
2.10.1 Acidification and Microbial Dynamics

Fermentation is a natural microbial process that has been utilized for centuries to preserve food and enhance its nutritional value. In spontaneous fermentation, microorganisms are selected based on the nature of the substrate or the dominance of naturally occurring microbes in the environment (Voidarou et al., 2021). Generally, in vegetable fermentation, the initial population of LAB is relatively low. Despite this, LAB gradually dominate the substrate due to their ability to produce acid. The acidification process, combined with the low buffering capacity typical of most vegetables, leads to a rapid decline in pH. Simultaneously, redox potential decreases due to microbial respiration, metabolic activity, and atmospheric changes driven by CO₂ production. These conditions create a selective advantage for LAB, making it difficult for other microorganisms to thrive. As a result, spoilage organisms such as aerobic bacteria, pathogenic enterobacteria, yeasts, and molds are effectively inhibited (Mäki, 2004).

The general pathogenic bacteria in food are categorized as Enterobacteriaceae. Enterobacteriaceae represent a large group of Gram-negative bacteria commonly associated with human diseases. They are predominantly found in the intestinal tract and include genera such as *Salmonella*, *Escherichia*, *Klebsiella*, and *Shigella*. Therefore, monitoring Enterobacteriaceae is a key indicator of food safety (Kang et al., 2018). These organisms are very sensitive to pH drop. Proliferation of these bacteria is significantly reduced with the presence of organic acids (Zhang et al., 2025a). Presence of unwanted yeast and mold is taken as the indicator of spoilage in vegetable fermentation (Pérez-Díaz et al., 2013). However, in the course of lactic acid fermentation, the acidic environment and specifically the anaerobic condition makes the environment harmful for survival of these strict aerobic microflorae (Mäki, 2004).

Carbohydrates, particularly simple sugars, serve as the primary energy source for fermentation. In lactic acid fermentation, lactic acid is the dominant byproduct, whereas yeast fermentation mainly produces alcohol and carbon dioxide. Other nutrients, including proteins, lipids, vitamins, nucleic acids, and minerals are essential for cellular synthesis. Since fermentative bacteria have limited biosynthetic capabilities, these nutrients must be supplied externally for proper growth. Fortunately, most food sources naturally contain adequate amounts of these essential components to support the active proliferation of fermentative microorganisms (Tamang et al., 2015).

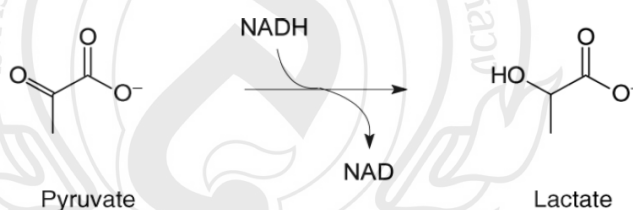
The conversion of sugars to lactic acid occurs in the absence of oxygen. Therefore, in vegetable fermentation, the substrate is often pressed to eliminate air and maintain anaerobic conditions (Abedi & Hashemi, 2020). In the energy generation process, sugars in microbial cells undergo enzymatic conversion to pyruvate, producing chemical energy in the form of adenosine triphosphate (ATP) and reduced equivalents such as NADH (Figure 2.10). In order to recycle these reduced equivalents, in anaerobic conditions, microorganisms convert the pyruvate into the more reduced lactic acid (Figure 2.11).



Source Pelczar et al. (2005)

Figure 2.10 General glycolysis process in cell

The chemical energy generated during this process is utilized for various cellular functions, including growth, maintenance, and, in some cases, motility. The end product of energy generating process is not the same. Based on the metabolite produced LAB are broadly classified into two types: homofermentative and heterofermentative (Figure 2.12). Homofermentative LAB primarily produce lactic acid as their main metabolic byproduct, whereas heterofermentative LAB generate a mix of lactic acid, acetic acid, ethanol, and carbon dioxide (Mokoena, 2017).

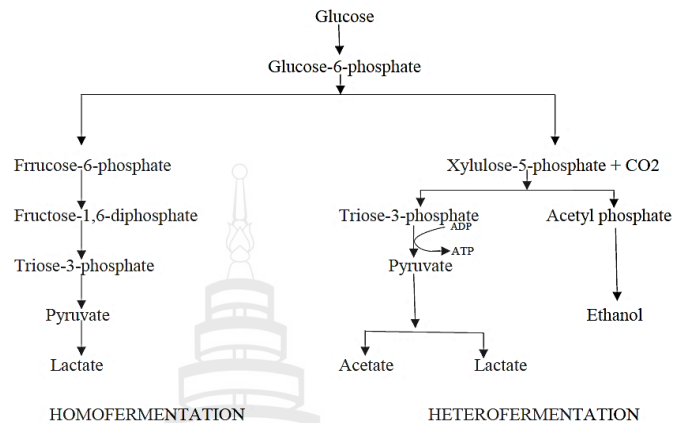


Source Pelczar et al. (2005)

Figure 2.11 Anaerobic conversion of pyruvate to lactate

The composition of LAB in a fermentation process varies depending on the substrate, available nutrients, and environmental conditions. Different LAB species emerge and disappear throughout the process, adapting to the changing fermentation environment. The composition of LAB in a fermentation process varies depending on the substrate, available nutrients, and environmental conditions. Different LAB

species emerge and disappear throughout the process, adapting to the changing fermentation environment.



Source Mora-Villalobos et al. (2020)

Figure 2.12 Glycolytic pathway for homofermentative and heterofermentative LAB

In sauerkraut fermentation, the process begins with heterofermentative LAB, predominantly *Leuconostoc mesenteroides*, which thrive due to their lower acid tolerance, microaerophilic nature, and shorter generation time compared to other LAB species. These bacteria metabolize the fermentable sugars in cabbage—mainly glucose and fructose—into lactic acid, mannitol, acetic acid, ethanol, and carbon dioxide (Zabat et al., 2018). The production of lactic and acetic acids lowers the pH, creating an anaerobic environment crucial for fermentation. Reducing oxygen levels is essential to prevent spoilage microorganisms from thriving while allowing LAB to continue their succession. As the acid concentration reaches 0.3–0.7% (w/w) and the pH drops below 4.0, *Leuconostoc* species are gradually replaced by more acid-tolerant homofermentative LAB, such as *Lactobacillus plantarum* and *Lactobacillus brevis*. These bacteria further ferment any remaining sugars, continuously producing lactic acid until a fully fermented and stable product is achieved (Fleming et al., 1988).

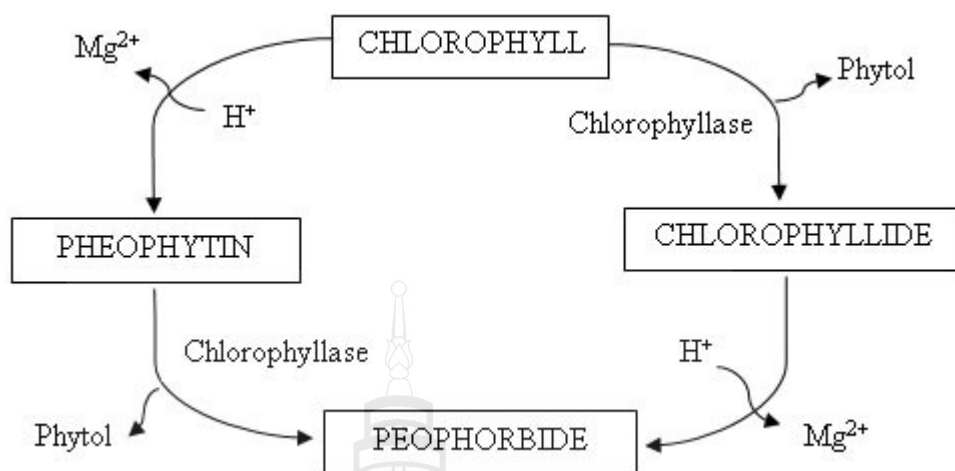
Similarly, in *gundruk* fermentation, a traditional fermented leafy vegetable product, the dominant LAB species change over time. During the initial stages, *Lactobacillus brevis* and *Pediococcus pentosaceus* are the primary fermenters. As fermentation progresses, the indigenous LAB community undergoes spontaneous shifts, and by the final stage, *P. pentosaceus* and *L. plantarum* become the

predominant species (Tamang & Tamang, 2010). Studies on watermelon fermentation are rare. A study done on fermentation of watermelon rind, particularly focusing on proximate composition change, reported the presence of *L. plantarum*, *L. acidophilus*, *L. fermentum*, *L. casei*, *L. bulgaricus*, *L. delbrueckii*, *S. epidermis*, *S. lactis*, *S. aureus*, *B. cereus*, *B. subtilis*, *Micrococcus luteus* on spontaneous fermentation of watermelon rind based on microscopic and biochemical observations (Ao & Es, 2016).

2.10.2 Color

Color is one of the most important quality attributes of vegetables, as it directly affects consumer perception and acceptability (Zhang et al., 2025b). During fermentation, a series of biochemical, enzymatic, and physicochemical changes occur, which can alter the natural pigments present in vegetables (Sawant et al., 2025). Vegetable color is primarily determined by natural pigments such as chlorophyll (green), carotenoids (yellow to orange), and anthocyanins (red to purple). These pigments are sensitive to environmental conditions like pH, temperature, oxygen availability, and enzymatic activity—all of which are affected during fermentation. One of the most significant factors in fermentation is the production of organic acids, particularly lactic acid, by lactic acid bacteria. This leads to a decrease in pH, creating an acidic environment that strongly influences pigment stability (Singh et al., 2023).

Chlorophyll, responsible for the green color in vegetables like cabbage, cucumber, watermelon rind and leafy greens, is highly sensitive to acidic conditions (Zhou et al., 2025). During fermentation, the drop in pH causes chlorophyll to convert into pheophytin, resulting in a dull olive-green or brownish color (Figure 2.13). The extent of this change depends on factors such as fermentation duration, salt concentration, and temperature. Longer fermentation duration enhances the color degradation due to higher acidification and pH drop (Koca et al., 2007). Generally, salt concentration resists the enzymatic degradation of the chlorophyll, which causes the slowing degradation process (Chung et al., 2020). Furthermore, salt also has preventive effect on enzymatic browning.



Source Indrasti et al. (2018)

Figure 2.13 Chlorophyll degradation pathway

In addition to pigment transformations, enzymatic activities also contribute to color changes. Enzymes such as polyphenol oxidase (PPO) can cause enzymatic browning in the presence of oxygen, leading to undesirable darkening. Salt concentration is a key factor influencing the extent of this browning in fruits and vegetables. Elevated salt levels inhibit PPO activity, thereby markedly reducing enzymatic browning. In addition, higher salt concentrations decrease oxygen solubility in the medium, further limiting the browning process (Ma et al., 2021).

2.10.3 Phytochemical Changes

Lactic acid fermentation is widely recognized not only for preserving vegetables but also for transforming their nutritional and functional properties. Among these changes, the modification of phytochemicals is of significant scientific and technological interest. These changes are strongly influenced by fermentation conditions, especially salt concentration and fermentation duration, which govern microbial activity, enzymatic reactions, and metabolite stability (Dissanayake et al., 2025).

Phenolic compounds are a diverse group of secondary metabolites naturally present in vegetables, including phenolic acids, flavonoids, and tannins. They are well known for their antioxidant properties, contributing to free radical scavenging and oxidative stability (Ahmad et al., 2025). During lactic acid fermentation, these

compounds undergo dynamic transformations due to microbial metabolism and endogenous enzymatic activity. LAB produce enzymes such as esterases, phenolic acid decarboxylases, and glycosidases, which hydrolyze complex phenolics into simpler and more bioavailable forms. As a result, bound phenolics are released from the plant matrix, often leading to an initial increase in detectable TPC. The mild acidic environment generated by lactic acid production further aids in solubilizing phenolic compounds (Dissanayake et al., 2025). However, as fermentation progresses, the trend may not remain linear. Prolonged fermentation can lead to degradation, transformation, or polymerization of phenolic compounds. Kao and Lin (2023) found that natural fermentation of cucumber led to the increase of total phenolic content up to day 8, thereafter it started to decline.

In parallel with phytochemical changes, significant modifications occur in the amino acid profile of fermented vegetables. Proteolytic enzymes from both plant tissues and LAB hydrolyze proteins into peptides and free amino acids. This results in an overall increase in total free amino acid content, particularly during the early and mid-stages of fermentation (Toe et al., 2019). Amino acids such as glutamic acid and aspartic acid, known for their contribution to umami taste, may increase and enhance the sensory quality of fermented products (Chen et al., 2021).

2.11 Factors Influencing Vegetable Fermentation

The fermentation of vegetables is a complex process influenced by various factors that directly impact on the quality, safety, and sensory characteristics of the final product. These factors can be broadly categorized into intrinsic and extrinsic factors. Intrinsic factors are inherent to the vegetables themselves, such as natural microflora, carbohydrate content, pH, and water activity. On the other hand, extrinsic factors include environmental and processing conditions like temperature, salt concentration, oxygen availability, and fermentation duration. Additionally, the presence of antimicrobial compounds, the type of starter cultures used, and hygienic conditions during fermentation play a crucial role in determining the overall success of the process (Rolfe & Daryaei, 2020).

2.11.1 Temperature

Among the extrinsic factors, temperature plays an important role in vegetable fermentation, significantly affecting microbial growth and metabolite production. Since microbial enzymes are highly sensitive to temperature changes, maintaining an optimal temperature range ensures efficient fermentation, desirable flavor development, and an ideal texture in the final product. Higher temperatures can accelerate microbial activity, leading to rapid lactic acid production, which in turn lowers the pH quickly. While this may speed up fermentation, excessive acidification at elevated temperatures can negatively impact the texture and taste of the final product (Rolfe & Daryaei, 2020). Furthermore, temperatures above the optimal growth range, LAB commonly experiences an excessive increase in cell membrane fluidity, as the lipid bilayer loses its structural integrity under heat stress, which might cause permanent damage of cell (Fonseca et al., 2019). In contrast, lower temperatures slow down fermentation, prolonging the process and potentially affecting product consistency. Therefore, maintaining fermentation within an optimal temperature range is essential for achieving high-quality fermented vegetables (Rolfe & Daryaei, 2020).

For vegetable fermentation, different temperature ranges yield different outcomes. Studies indicate that at 18–22°C, lactic acid production occurs steadily, promoting well-balanced flavors and a firm texture. However, at 30–37°C, fermentation speeds up, but the resulting product may have an overly sour taste and softer texture due to increased lactic acid production by thermotolerant microbes. These effects, however, vary depending on the specific vegetable being fermented (Battcock & Azam-Ali, 1998).

Temperature also influences the selection of dominant bacterial strains during natural fermentation. For instance, in the fermentation of Chinese Suancai, different bacterial species thrived at different temperatures: *Leuconostoc spp.* dominated at 10°C and 15°C, while *Weissella* and *Lactococcus* became predominant at 20°C and 25°C. Additionally, higher temperatures led to an increase in the variety of volatile flavor compounds (VFCs), with fermentation at 20°C accelerating maturation and improving sensory quality (He et al., 2020). Similarly, research on sauerkraut fermentation found that low-temperature fermentation (15–20°C) resulted in the

highest-quality product, characterized by optimal acidity, lactic acid, ascorbic acid content, and a superior texture. In contrast, fermentation at ambient (25–30°C) and high temperatures (35–40°C) led to faster fermentation but compromised quality, with lower ascorbic acid levels and poorer sensory scores (Thakur et al., 2017).

A study on *Khalpi*, a traditional fermented cucumber product from Nepal, found that fermentation at 20°C for 72 hours produced the most desirable aroma, taste, and overall acceptability. However, fermentation at a higher temperature (30°C) resulted in an overly acidic taste that was not well-received by consumers (Tamang & Tamang, 2010). However, in Asian countries most of the vegetable fermentation is carried out in room temperature (Swain et al., 2014).

Beyond microbial growth and sensory properties, temperature also influences the production of specific metabolites. The optimal temperature for maximizing certain metabolites may differ from the ideal growth conditions for microorganisms. Some metabolites are produced under stress conditions, requiring temperatures outside the typical optimal range. For example, in soy sauce fermentation, citrulline production by LAB varies with temperature. At lower temperatures (15°C), the conversion of arginine to citrulline was minimal. However, at higher salt concentrations, this conversion increased more than tenfold at 30°C (Zhou et al., 2021). On genetic study, they found that salt concentration was majorly responsible for the accumulation of citrulline and concluded temperature has the minimal effect. Another study found performed on the sourdough fermentation by *Lactobacillus fermentum* IMDO 130101 found that the arginine consumption started slowly at lower temperatures but was more pronounced after 5 hours at 25–30°C. However, they found that arginine to citrulline conversion was not affected significantly with the temperature. They also found that when temperature exceeds 30°C, citrulline further converts to ornithine, altering the final metabolic profile of the product (Vrancken et al., 2009).

2.11.2 Salt Concentration

Salt plays a crucial role in vegetable fermentation, influencing not only the preservation and sensory characteristics of the final product but also the microbiological aspects of the process. Beyond enhancing flavor and forming the fermentation brine, salt is essential for creating an environment that supports the

growth of lactic acid bacteria while inhibiting undesirable microorganisms. The formation of lactic acid by LAB is a key factor in ensuring safe and efficient acidification of the fermentation medium. The acidity, acid production rate, and pH of vegetable fermentations are significantly influenced by the type and concentration of salt used, with the initial salt levels determining the overall fermentation dynamics (Bautista-Gallego et al., 2013).

In anaerobic lacto-fermentation, salt selectively limits the growth of spoilage organisms such as *Pseudomonas*, *Flavobacterium*, coliforms, and various fungi, creating a favorable environment for LAB (Tamang et al., 2020). Sodium chloride (NaCl) lowers water activity (a_w) and increases ionic strength, both of which help inhibit the growth of undesirable microorganisms. Additionally, NaCl reduces oxygen solubility in water, thereby limiting the growth of aerobic microorganisms, which is desirable in lacto-fermented vegetables (Bautista-Gallego et al., 2013). In contrast, lactic acid bacteria (LAB) are well adapted to high-salt environments. They employ various tolerance mechanisms, including the accumulation of osmoprotectants, membrane remodeling, regulation of ion transport, synthesis of protective proteins, and metabolic reprogramming, all of which enable them to survive and remain metabolically active under salt-stressed fermentation conditions (He et al., 2025).

The optimal salt concentration varies depending on the specific fermented vegetable. In *sauerkraut*, for instance, salt levels are strictly maintained between 2% and 3%, with 2.25% being considered ideal. Salt facilitates osmosis, drawing water out of cabbage tissues to form a nutrient-rich brine that supports microbial activity. This brine serves as the fermentation medium. By the end of fermentation, acidity reaches approximately 1.7%, and the pH drops below 4.0, resulting in a stable, high-quality product (Hutkins, 2007).

Similarly, in Chinese fermented radish- *Paocai*, salt concentration is a key factor influencing product quality. Industrially produced *Paocai* is typically preserved in high-salt brines for long-term storage. However, studies indicate that low-salt brines are more conducive to LAB growth and maintain good microbial safety. In contrast, high-salt fermentations suppress acid production by LAB, leading to a higher final pH and lower TTA. Bacterial diversity analysis of *Paocai* samples suggests that increasing salt concentrations lead to greater microbial diversity. At the phylum level,

Firmicutes and *Proteobacteria* dominate. Moreover, salt concentration affects the volatile flavor compounds (VFCs) present in *Paocai*, with higher salt levels contributing to a greater variety of these compounds within a certain limit (Wang et al., 2021).

In *Suancai*, a Chinese fermented cabbage, salt concentration influences the production of VFCs and reduces isothiocyanates after fermentation. A study identified 79 volatile compounds strongly associated with *Lactobacillus* abundance during *Suancai* fermentation. The addition of 6% salt resulted in a higher *Lactobacillus* population and enhanced taste quality. Furthermore, fermentation with inoculated *Lactobacillus* strains improved the volatile compound profile, indicating that both salt concentration and microbial composition are key determinants of flavor development in *Suancai* (Liang et al., 2020). Tassou et al. (2002) reported that during the natural fermentation of black olives, a 6% salt concentration resulted in significantly greater growth of lactic acid bacteria compared to 4% and 10% salt conditions.

Salt is also an essential component in kimchi fermentation, serving both as a preservative and a fermentation regulator. According to the Codex Alimentarius Commission (2022), salt concentration in kimchi ranges between 1% and 4%, though household preparations typically use between 1.2% and 2.0%. Regional variations further influence optimal salt levels. Research have shown that in kimchi samples fermented at 5% salinity, *Leuconostoc mesenteroides* was more prevalent, while *Lactobacillus sakei* was less dominant after 50 days, indicating that higher salt concentrations slow down fermentation (Seo et al., 2018).

Salt concentration also plays a crucial role in shaping microbial composition and fermentation characteristics in radish *Paocai*. Different salt levels lead to variations in pH, TTA, nitrite content, biogenic amines, free amino acid profiles, and volatile organic compounds. Lower-salt brines accelerate *Paocai* maturation but result in more complex biogenic amine profiles and a delayed disappearance of *Enterobacteria* compared to high-salt brines. In contrast, high-salt fermentations slightly prolong maturation but yield better flavor and improved microbial safety (Mi et al., 2022).

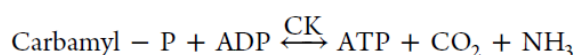
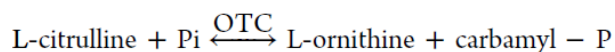
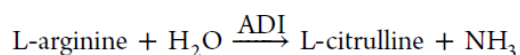
Salt concentration plays a vital role in metabolite accumulation during fermentation. In soy sauce fermentation, the presence of salt significantly influenced

the ability of *Weissella confusa* and *Pediococcus acidilactici* to accumulate citrulline. Research found that at a salt concentration of 9% NaCl (among 0, 9 and 18% NaCl concentration), these strains achieved the highest citrulline levels, with the arginine-to-citrulline conversion rate reaching approximately 60%. Interestingly, in *W. confusa*, the absence of salt led to ornithine as the final metabolite instead of citrulline (Zhou et al., 2021). Similarly, in sourdough fermentation involving *Lactobacillus fermentum* IMDO 130101, increasing NaCl levels from 1% to 6% showed that the conversion of arginine to citrulline was significantly enhanced at salt concentrations above 4% (Vrancken et al., 2009).

2.12 LAB for Citrulline Synthesis

Certain microorganisms, particularly LAB, are capable of producing citrulline through the metabolism of arginine via the arginine deiminase (ADI) pathway. In this pathway, arginine is first converted to citrulline and subsequently to ornithine, with the concomitant generation of energy in the form of ATP (Liu & Pilone, 1998). This makes arginine catabolism an important alternative energy-yielding mechanism for LAB, especially under carbohydrate-limited or stress conditions. In addition to ATP production, the ADI pathway results in the formation of ammonia. Specifically, the conversion of arginine leads to the release of two moles of ammonia, which plays a crucial physiological role. The production of ammonia increases the intracellular and extracellular pH by neutralizing excess protons, thereby counteracting the acidification caused by organic acid production during fermentation (Zhang et al., 2016).

As represented in Figure 2.14, ADI pathway is composed of three critical enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC), and carbamate kinase (CK), that are encoded by the arc operon arcA, arcB, arcC respectively. Accumulation of citrulline in fermented foods is mainly the consequence of microbial decomposing of arginine and synthesis of ornithine that catalyzed by ADI (arcA) and OTC (arcB), respectively (Noens & Lolkema, 2017).



Source Liu et al. (1996)

Figure 2.14 Arginine metabolic pathway

Most of the LAB are capable of arginine metabolism. However, the end product is not the same due to the lack of the genes and the respective enzymes. LAB having enzymes ADI, OTC and CK is presented in Table 2.5.

Table 2.5 LABs and their respective enzymes for arginine metabolism

Lactic Acid Bacteria	ADI	OTC	CK
<i>Leuconostoc mesenteroides</i>	•	•	
<i>Leuconostoc pseudomesenteroides</i>		•	
<i>Pediococcus pentosaceus</i>	•	•	•
<i>Streptococcus gallolyticus</i>	•	•	
<i>Weissella cibaria</i>	•	•	•
<i>Weissella confusa</i>	•	•	•
<i>Weissella hellenica</i>			
<i>Weissella paramesenteroides</i>			
<i>Weissella soli</i>			
<i>Weissella viridescens</i>	•		
<i>Weissella koreensis</i>	•	•	•
<i>Lactobacillus brevis</i>	•	•	•
<i>Lactobacillus curvatus</i>			
<i>Lactobacillus graminis</i>			
<i>Lactobacillus mali</i>		•	
<i>Lactobacillus parabrevis</i>		•	•
<i>Lactobacillus parabuchneri</i>	•	•	•
<i>Lactobacillus paraplantarum</i>		•	
<i>Lactobacillus pentosus</i>		•	
<i>Lactobacillus plantarum</i>	•	•	•

Table 2.5 (continued)

Lactic Acid Bacteria	ADI	OTC	CK
<i>Lactobacillus sakei</i>	●	●	●
<i>Lactococcus lactis</i>	●	●	●
<i>Leuconostoc citreum</i>		●	
<i>Leuconostoc lactis</i>		●	
<i>Leuconostoc carnosum</i>			
<i>Leuconostoc citreum</i>		●	
<i>Leuconostoc gelidum</i> subsp. <i>gasicomitatum</i>		●	
<i>Leuconostoc kimchii</i>		●	
<i>Leuconostoc lactis</i>		●	

Source Hwang and Lee (2018)

The end products of the ADI pathway in LAB are not determined solely by the three core enzymes and their corresponding genes but are also influenced by genes involved in these amino acid transport. The arginine/ornithine (A/O) antiporter system, encoded by *arcD*, is considered a putative transporter responsible for the uptake of arginine and the release of ornithine (Lee et al., 2021). However, detailed studies on microbial transporter genes have revealed that members of Lactobacillales also possess another related gene, *arcE*. Both genes function as the L-arginine/L-ornithine exchangers in the pathway that take up the substrate L-arginine and excrete the product L-ornithine. However, unlike *arcD*, *arcE* has high affinity towards citrulline, which causes efficient L-citrulline translocation in microbial cells. This gene facilitates the uptake of the citrulline from the medium to produce metabolic energy and ammonia rather than being converted from arginine. Furthermore, it also facilitates the excretion of citrulline as metabolic intermediate (Noens & Lolkema, 2017). This suggests that not only excretion but also uptake of citrulline is possible in the fermentation substrate.

Citrulline accumulation results from an imbalance between its formation and elimination (Liu & Pilone, 1998). In the presence of certain factors like salt concentration, the genes are differently expressed or inhibited causing the formation

of different outputs from ADI pathways. Several studies have explored the impact of LAB on citrulline accumulation through the ADI pathway, with particular focus on fermented foods such as soy sauce, wine, and other traditional products. However, these studies primarily aim to minimize citrulline levels, as citrulline reacts with alcohol to form ethyl carbamate (EC), a known carcinogen (Zhou et al., 2021).

Jung et al. (2022) investigated the ability of two LAB strains commonly found in kimchi fermentation to metabolize arginine. Their study demonstrated that in MRS media supplemented with arginine, *Weissella koreensis* significantly reduced arginine levels while increasing citrulline and ornithine concentrations by approximately 3.5- and 4.5-fold, respectively, within three hours of fermentation at 30°C. In contrast, *Leuconostoc mesenteroides* exhibited no significant changes in these compounds, indicating a strong ADI system in *W. koreensis* but a weak one in *L. mesenteroides*. Similar trends were observed in kimchi samples, where *W. koreensis* fermentation led to reduced arginine and increased ornithine levels, while *L. mesenteroides* showed minimal changes.

Further research by Zhou et al. (2021) highlighted that arginine metabolism is influenced by environmental factors such as pH, salt concentration, and temperature. They found that the citrulline accumulation capacity of *Weissella confusa* and *Pediococcus acidilactici* increased significantly with salt concentration, reaching its peak at 9% NaCl, where the arginine-to-citrulline conversion rate rose to approximately 60%. For *W. confusa*, the primary product of the ADI pathway shifted depending on salt levels: in the absence of NaCl, ornithine was predominant, whereas at 9% NaCl, extracellular citrulline levels increased while ornithine levels decreased. At this level of salt concentration, the expression of *arcB* was relatively lower than the *arcA* and *arcC*. Additionally, lower temperatures (15°C) resulted in minimal arginine conversion, whereas at higher salt concentrations, citrulline formation increased more than tenfold at 30°C. Gene expression analysis suggested that temperature had a minimal effect under high salt conditions.

Vrancken et al. (2009) explored sourdough fermentation by *Lactobacillus fermentum* IMDO 130101 and observed that at lower temperatures (20°C), arginine consumption began only after 35 hours, with citrulline as the primary end-product at a final concentration of 800 mM. Ornithine formation was moderate, reaching 400 mM.

However, at higher temperatures (25°C or above), arginine metabolism started after just five hours, yet citrulline levels remained similar. Their findings indicated that increasing NaCl concentration from 1% to 6% enhanced the arginine-to-citrulline conversion rate, confirming that salt concentration had a greater influence on citrulline accumulation than temperature. Additionally, at temperatures exceeding 30°C, citrulline-to-ornithine conversion was more pronounced, suggesting that high-stress conditions (osmotic and temperature stress) prompt energy generation via the ADI pathway.

Araque et al. (2011) studied four heterofermentative LAB strains (*Lactobacillus brevis*, *Lactobacillus hilgardii*, *Lactobacillus buchneri*, and *Pediococcus pentosaceus*) commonly found as contaminants in wine and beer. Their results showed that the arginine-to-citrulline conversion rate varied between 10% and 50%, depending on the strain. The highest citrulline accumulation occurred in the presence of ethanol (10–12%) and at a low pH (3.0), conditions that induced ammonia release via the ADI pathway to neutralize the environment. Among the tested LAB, *L. brevis* and *L. buchneri* exhibited the greatest citrulline accumulation.

Zhang et al. (2014) examined six soy sauce koji isolates, including *Weissella confusa*, *Weissella cibaria*, *Pediococcus acidilactici*, and three *Staphylococcus* species, to assess their ability to accumulate citrulline via the ADI pathway. *P. acidilactici* displayed the highest citrulline synthesis and accumulation under high-salt conditions. The study proposed two mechanisms for this phenomenon: first, NaCl inhibited the expression of *arcB* (OTC) and *arcC* (CK) genes, reducing enzyme activity and preventing further citrulline breakdown; second, NaCl suppressed the expression of citrulline/ornithine antiporter genes, limiting citrulline reuptake and promoting its extracellular accumulation. This effect was unique to *P. acidilactici* and was not observed in other bacterial strains.

Spano et al. (2007) investigated arginine metabolism in six strains of wine-derived *Lactobacillus plantarum* and found notable differences in citrulline and ornithine synthesis due to variations in enzyme activity. *L. plantarum* LP60 exhibited the highest ADI activity (8.51 ± 0.18 $\mu\text{mol citrulline/min}/\mu\text{g protein}$) and low OTC activity (1.89 ± 0.12 $\mu\text{mol ornithine/min}/\mu\text{g protein}$), leading to greater citrulline

accumulation. In contrast, LP90 and LP60 displayed moderate ADI activity but higher OTC activity, favoring ornithine accumulation.

Pols et al. (2021) conducted a comprehensive enzymological analysis of the ADI pathway in *Lactococcus lactis*. They found that the enzymes encoded by *arcA* (arginine deiminase), *arcB* (OTC), and *arcC1* (carbamate kinase) exhibit optimal activity at approximately pH 7.0. However, these enzymes differ in their tolerance to acidic conditions. When the pH was lowered from 7.0 to 5.8, the activity of *arcB* dropped by over 90%, whereas *arcA* showed only a 14% reduction. *arcC1*, by contrast, was completely inactivated below pH 5.8. Temperature also affected enzyme activity. The optimal temperatures for *arcA* and *arcB* were found to be 41 °C and 35 °C, respectively. Notably, reducing the temperature from 35 °C to 25 °C resulted in a 75% drop in *arcB* activity. Salt concentration had minimal impact on *arcA*, but higher concentrations of NaCl significantly inhibited *arcC1*. Specifically, while NaCl concentrations ranging from 50 mM to 300 mM had little effect on *arcA*, *arcC1* activity was inhibited by 70% at 300 mM NaCl. These findings collectively suggest that under conditions of high salt and low pH, *arcA* remains more stable and active compared to *arcB* and *arcC1*. Therefore, to selectively limit the activity of OTC (*ArcB*), environmental conditions such as pH below 5.8 and temperatures under 35 °C can be employed.

From these studies, it is evident that LAB are capable of metabolize arginine, with citrulline accumulation being highly dependent on bacterial species, population, and environmental factors such as temperature, salt concentration, and pH. However, existing research has primarily focused on arginine metabolism in controlled fermentation environments. Some studies have investigated naturally occurring arginine in food samples, while others have examined externally supplemented arginine. A promising direction for future research would be to investigate how LAB respond to substrates containing high levels of both arginine and citrulline, which may provide deeper insights into citrulline metabolism and its role in food fermentation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Watermelon (*Citrullus lanatus*) rind of *Kinnaree* variety was received from Triple J Food and Beverage Co., Ltd., Chiang Rai, Thailand. The rind, a byproduct during preparation of fried crispy watermelon processing, was collected from the company. The rind was transported in sterile plastic bags to the university, maintaining the cold chain and stored in the chilling room (4-7°C). The processing of the watermelon rind started within 24 h of storage at low temperature.

3.1.1 Chemicals and Equipment

The list of chemicals and equipment is given in Tables 3.1 and 3.2.

Table 3.1 Chemicals list

Parameters	Chemical Name	Brand/Manufacturer
Microbial Study	De Man-Rogosa-Sharpe (MRS) Agar, Violet red bile glucose (VRBG) Agar, Peptone, Tartaric acid, Potato Dextrose Agar (PDA)	Himedia Laboratories Pvt. Ltd., India
Amino Acid Analysis	L-Citrulline, L-Ornithine monohydrochloride, Amino acid mix standard	Sigma Aldrich
Phytochemical Analysis	Methanol, Acetonitrile, Trolox	RCI Labscan
	Folin-Ciocalteu reagent, Sodium carbonate (Na ₂ CO ₃)	Supelco KEMAUS
	Gallic Acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma Aldrich
Acidity	Sodium hydroxide (NaOH), Phenolphthalein	RCI Labscan Ltd.
Metabolite Analysis	Formic acid, acetonitrile	RCI Labscan

Table 3.2 Equipment list

Parameters	Equipment	Brand/Model
General Use	Electronic Balance	Mettler Toledo, ME 204
	pH meter	Mettler Toledo, F20 Five Easy
	pH probe	Mettler Toledo, LE438
Sample extraction	Blender	Problend Crush, Philips
	Sonicator	BRANSON 2800
	Low temperature centrifuge	Thermoscientific, Sorvalt Legend X1R
Amino acid analysis	Liquid chromatography-Mass spectrophotometry (LC-ESI-QQQ MS)	Shimadzu Nexera X2/ 8060 NX LCMSMS.
Metabolite analysis	Liquid Chromatography	Agilent Technologies
	Quadrupole Time-of-Flight Mass Spectrometer (LC-QTOF)	/1290 Infinity II LC/G6545B QTOF/MS
Phytochemical Analysis	UV-Visible spectrophotometer microplate reader	Thermoscientific Multiskan GO
Microbiological use	Stomacher	Seward Stomacher 400 circulator/ Scientific Promotion Co. Ltd., Thailand
	Autoclave	Bioplus Medical, GR110DA
	Water bath	Memmert WNB 29
	Biosafety Cabinet	LABGARD ES, Bioplus Medical Co. Lt.
	Incubator	SANYO Incubator, MIR 154
	Vortex Mixture	Vortex Genie 2, Scientific Industries
	Microscope	CX21FS1, Olympus Cooperation, Tokyo, Japan
	Colony Counter	Rocker Galaxy 230
Color Analysis	Colorimeter	Hunterlab, Color Quest XE

3.2 Methods

3.2.1 Effect of Salt Concentration and Temperature on Fermented Watermelon Rind Quality

Watermelon rind was fermented as the method mentioned by Dahal et al. (2005) for *Khalpi* (fermented cucumber product of Nepal) fermentation, with some modifications. In brief, watermelon rind was washed with tap water, peeled and cut into pieces (1 cm long). It was then mixed with salt at different concentrations (2%, 4 % and 6 %) and pressed in the glass bottles to minimize oxygen inside and kept for fermentation up to 10 days at different temperatures (25, 30 and 35 and 40°C). Samples of each temperature and salt concentration were taken every day for the analysis of the pH and acidity (as lactic acid) and microbial enumeration in terms of LAB, yeast and mold count and Enterobacteriaceae count. Factorial design was used for analysis. The general flow chart of the experiment is displayed in Figure 3.1.

3.2.2 Chemical and Microbiological Analysis

3.2.2.1 pH and Acidity

pH and acidity were assessed as described in AOAC (2005). Briefly, approximately 50 g of sample was collected from each glass jar. Samples were homogenized using a table blender (Problend Crush, Philips, Indonesia), and pH was determined in triplicate using a pH meter (Mettler Toledo™, F20 FiveEasy™, Switzerland). The flat surface pH probe (Mettler Toledo™, LE438, Switzerland) was rinsed with distilled water between measurements and calibrated beforehand using standard buffer solutions of pH 4.01, 7.00, and 10.01. The electrode was inserted directly into the homogenized fermented sample and left until a stable reading was achieved. Measurements were taken twice for each sample, and the mean pH value was calculated. The same samples were subsequently used for titratable acidity analysis.

For acidity measurement, about 10 g of homogenized sample was taken and diluted with distilled water up to appropriate dilution and titrated against 0.05 N NaOH with phenolphthalein indicator. The acidity was calculated by following formula.

$$\text{Acidity as lactic acid (\%)} = \frac{\text{Titer} \times \text{Strength of NaOH} \times \text{Vol make up} \times \text{Eq.wt of lactic acid (90.08)} \times 100}{\text{Wt. of sample taken} \times \text{Aliquot taken} \times 1000}$$

3.2.1.3 Microbial Enumeration

For microbial analysis, LAB count, yeast and mold count and Enterobacteriaceae count were performed. The LAB count was taken every day until 5 days of fermentation. Subsequently, it was measured in alternate days, specifically at 7 and 9 days. Yeast and mold count were taken at alternate days until the count became zero. The Enterobacteriaceae count was carried out on day zero and on day 5. Enumeration of LAB, yeast and mold and Enterobacteriaceae was carried out according to Tamang and Tamang (2010). Samples (10 g) were mixed with 90 ml of 0.10 % (w/v) sterile peptone water and homogenized in a Stomacher for 200 rpm for 2 min. A serial dilution up to appropriate dilution in the same diluents was made.

LAB Enumeration

One milliliter of appropriately diluted sample was transferred into a sterile Petri dish, followed by the addition of approximately 15 to 20 milliliters of molten MRS agar. The plates were then incubated at 35°C for 48 hours. After incubation, plates with 25 to 250 colonies were selected for counting, and the lactic acid bacteria (LAB) population was expressed as colony-forming units per gram (CFU/g). Confirmation of LAB was done by microscopic observation, Gram's reaction and catalase test. The cell morphology and biochemical tests were performed. For Gram staining, a loopful isolated colony from the MRS agar surface was taken on the glass slide. First, crystal violet dye was added to the microbial smear, stabilized for 1-2 min followed by the washing with Gram's Iodine. It was then decolorized with alcohol and counterstained by the saffron dye. The microscopic slide was then observed with microscope. Lactic acid bacteria retain the purple color of crystal violet. Additionally, the catalase test was performed by adding two drops of 3% hydrogen peroxide to cultures placed on a glass slide. A positive reaction was indicated by the appearance of oxygen bubbles, demonstrating that the test organism produces catalase enzyme. However, LAB shows negative catalase tests.

Yeast and Mold Count

Yeast and molds count was examined on potato dextrose agar (PDA) acidified with tartaric acid. Sterilized acidified PDA was poured in the sterile petri

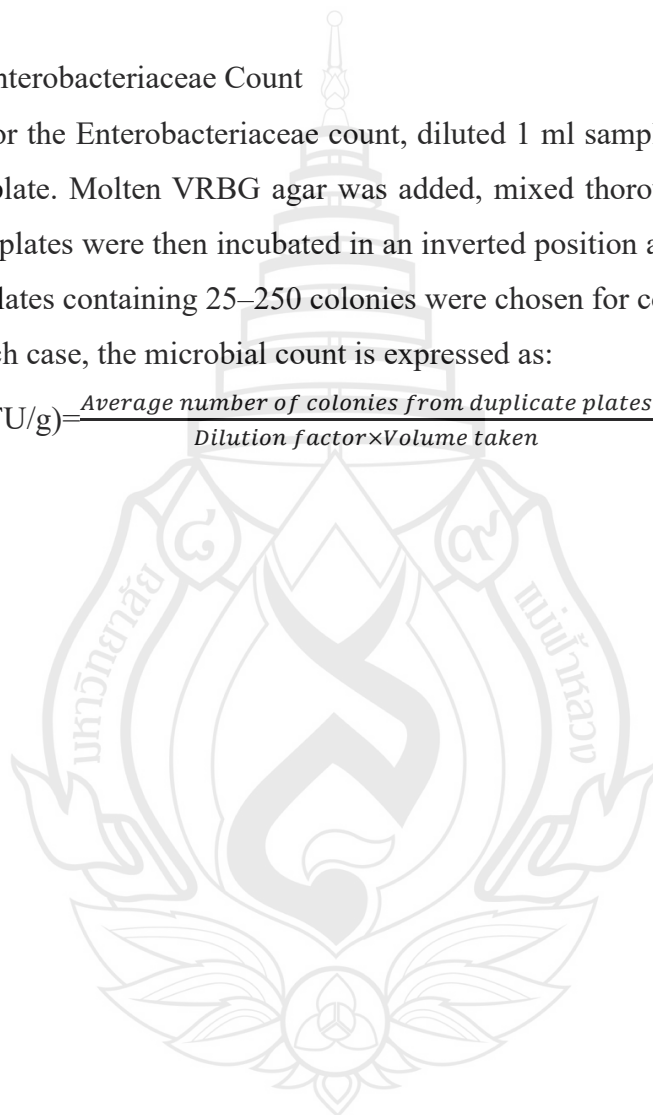
plate and allowed to set overnight in the refrigerator. Exactly 0.1 ml of diluted sample was put on the plated petri dish and was spread by the glass spreader. The plate was incubated at 28°C for 5 days. Colonies were enumerated from the plates with the highest dilution based on their morphological characteristics. To ensure purity, selected isolates were re-streaked and subcultured on fresh plates containing the same medium.

Enterobacteriaceae Count

For the Enterobacteriaceae count, diluted 1 ml sample was pipetted on the sterile petri plate. Molten VRBG agar was added, mixed thoroughly, and allowed to solidify. The plates were then incubated in an inverted position at 37°C for 24 h. After incubation, plates containing 25–250 colonies were chosen for counting.

In each case, the microbial count is expressed as:

$$\text{Count (CFU/g)} = \frac{\text{Average number of colonies from duplicate plates}}{\text{Dilution factor} \times \text{Volume taken}}$$



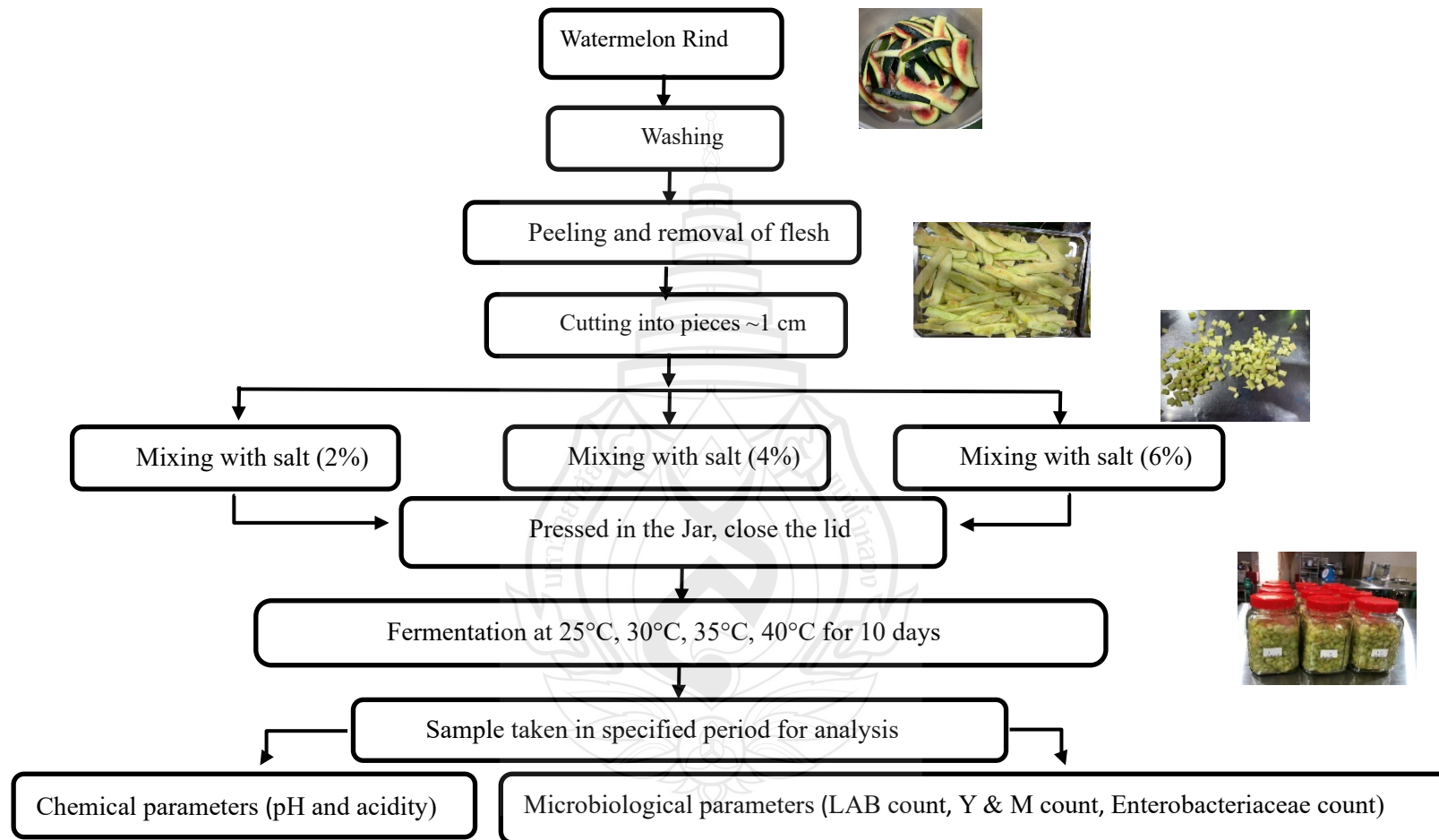


Figure 3.1 Fermentation of watermelon rind for general physical, chemical and microbial analysis

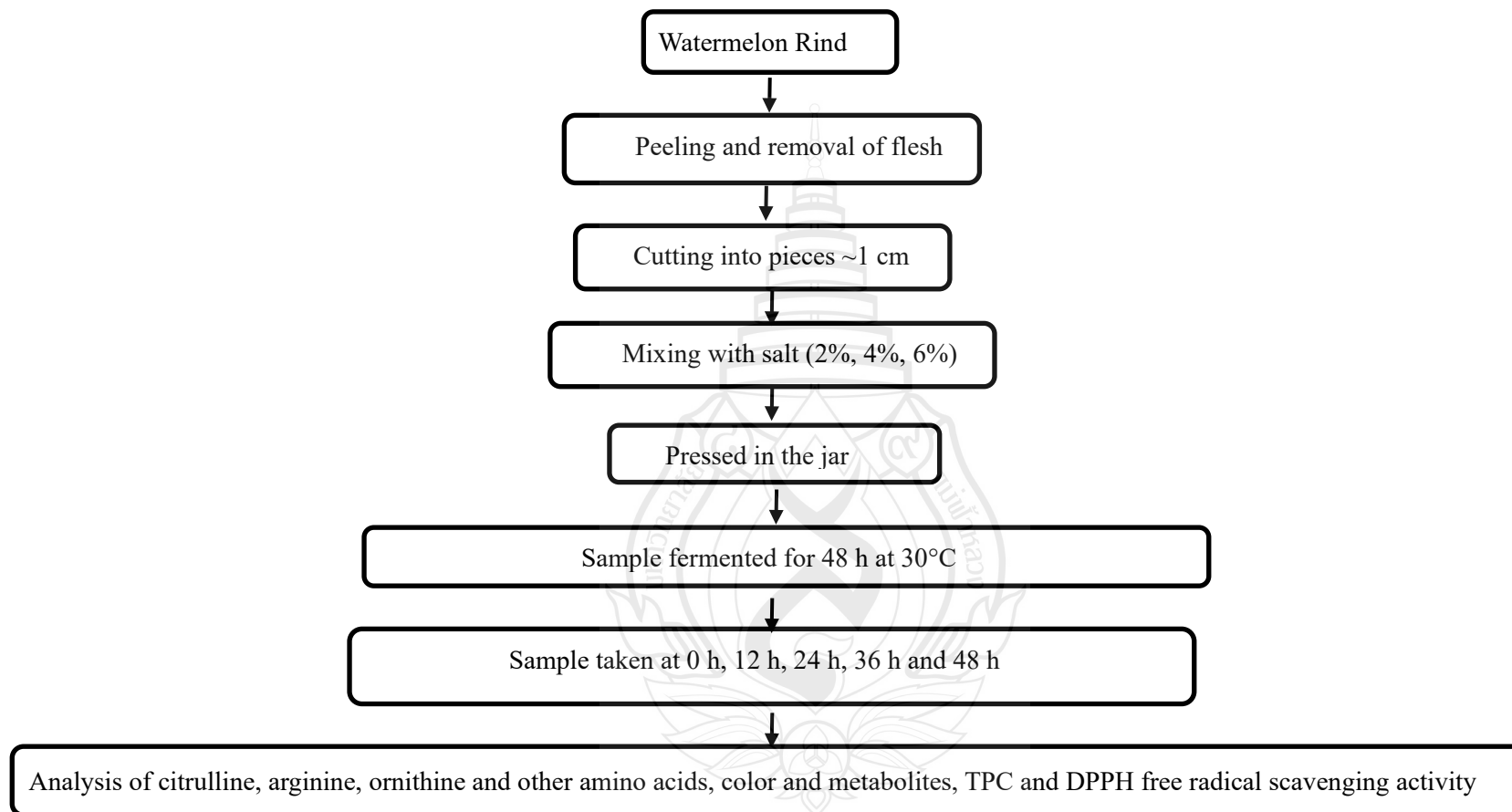


Figure 3.2 Flow chart of optimization of fermentation condition for citrulline enhancement

3.2.3 Optimization of Fermentation Parameters for Citrulline

Fermentation temperature and duration of the fermentation used in this step was chosen based on result 4.1 and 4.2. A temperature of 30°C was taken as the fermentation temperature for subsequent fermentation. Following this, fermentation time and salt concentration were selected as the key variables for optimizing citrulline production. Accordingly, watermelon rind with salt concentration of 2%, 4% and 6% were initially fermented for up to 5 days, and analyses were performed for arginine, citrulline, and ornithine, along with pH and titratable acidity. Samples were collected at day 0, day 2.5, and day 5. However, a significant reduction in citrulline content was observed by day 2.5 across all treatments.

In response to this decline, the fermentation duration was shortened to a duration of 0–48 h, and samples were collected at 12 h intervals (0, 12, 24, 36, and 48 h). Fermentation was carried out at three salt concentrations: 2%, 4%, and 6% (Figure 3.2). All samples were analyzed for arginine, citrulline, ornithine, and other amino acids. In addition, pH and titratable acidity were measured at each sampling point. Full factorial design was used for the design of experiments.

Furthermore, the samples were evaluated for metabolite profiles, total phenolic content (TPC), and antioxidant activity using the DPPH free radical scavenging assay. Color parameters were also determined.

3.2.4 Analysis of Citrulline, Arginine, Ornithine and Free Amino Acids

Watermelon rind samples fermented according to Section 3.2.2.1 were analyzed for the amino acids citrulline, arginine, and ornithine and other free amino acids following the method described by Jung et al. (2022), with slight modifications. For the extraction of free amino acids, fermented samples were homogenized using a hand-held blender. A 10 g portion of the homogenized sample was mixed with an equal volume of distilled water and centrifuged at 10,000 rpm for 20 min at 4 °C. The resulting clear supernatant was collected, appropriately diluted, and filtered through a 0.22 µm syringe filter prior to analysis.

Standard solutions of citrulline and ornithine monohydrochloride were initially prepared as 1,000 ppm stock solutions and subsequently diluted to concentrations of 0.01, 0.05, 0.1, 0.5, and 1 ppm to construct calibration curves. For arginine and other amino acids, a mixed standard solution was used. A stock solution of 2,500 nM was

first prepared and then serially diluted to obtain the required concentrations for standard curve preparation.

An aliquot of 1 μ L from each prepared extract was injected into an ultra-high-performance liquid chromatography system coupled with triple quadrupole electrospray ionization mass spectrometry (LC-QQQ-ESI-MS) for analysis. Separation of amino acids was carried out using an Agilent Poroshell EC-C18 column (2.1 $\times$ 150 mm, 2.7 μ m particle size). The column temperature was kept at 40 $^{\circ}$ C. A two-solvent mobile phase was applied, comprising water with 0.1% (v/v) formic acid as eluent A and acetonitrile containing 0.1% (v/v) formic acid as eluent B. The analysis was performed at a flow rate of 0.20 mL/min using a gradient program. Initially, the composition was kept at 1% solvent B up to 4.5 min. It was then gradually increased to 10% at 9.0 min, followed by a rapid rise to 90% at 13.0 min, which was maintained until 16.0 min. After that, the system was returned to the starting condition (1% B) at 17.0 min and allowed to equilibrate until 22.0 min. The total run time was 22 min. Mass spectrometric analysis was conducted using an electrospray ionization source, operated in both positive and negative ionization modes according to analyte characteristics. Detection and quantification were achieved using multiple reaction monitoring (MRM), enabling selective measurement through defined precursor-to-fragment ion transitions. Instrumental parameters were set as follows: interface temperature at 300 $^{\circ}$ C, desolvation temperature at 526 $^{\circ}$ C, and heat block temperature at 400 $^{\circ}$ C. The nebulizing gas was supplied at 3.0 L/min, whereas both drying and heating gases were maintained at 10.0 L/min. Collision-induced dissociation was carried out under a gas pressure of 270 kPa.

Multiple reaction monitoring (MRM) MS generated the following transitions: arginine, m/z 175.10>70.10; ornithine, m/z 133.15>70.10; citrulline, m/z 174.05>131.10; aspartic acid, m/z 134>74.05; Glycine, m/z 75.90>30.15; Glutamine, m/z 147.10>84.15; Threonine, m/z 120.10>74.15; Glutamic acid m/z 147.90>84.10; Proline, m/z 116.10>70.15; Lysine, m/z 147.10>84.10; Histidine, m/z 155.90>110.10; Leucine, m/z 132.10>86.05; Phenylalanine, m/z 166.10>120.10.

3.2.5 Metabolite Analysis

The sample fermented under optimal conditions was selected for metabolite extraction, following the procedure outlined in Section 3.2.3.1 with slight

modifications. In brief, 5 g of the homogenized sample was mixed with 50 mL of distilled water and subjected to centrifugation. The resulting supernatant was collected, filtered, and subsequently subjected to metabolite profiling using liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF/MS).

Untargeted metabolomic analysis was performed using an ultra-high-performance liquid chromatography quadrupole time-of-flight tandem mass spectrometer (UHPLC-QTOF/MS; 1290 Infinity II LC system with a 6545B QTOF/MS, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved using a Poroshell 120 EC-C18 column (2.1×150 mm, $2.7 \mu\text{m}$), with the column temperature maintained at 35°C throughout the analysis. Separation of metabolites was achieved using a reversed-phase C18 column maintained at 35°C . The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was set at 0.2 mL/min. A gradient elution program was applied as follows: initial conditions of 5% B (0–1 min), increased to 17% B (1–10 min), maintained at 17% B until 13 min, then ramped to 95% B at 20 min and held until 25 min. The composition was subsequently returned to initial conditions (5% B) at 27 min and equilibrated until 33 min. The total run time was 33 min. The injection volume was 1 μL , and samples were introduced using an autosampler equipped with a multi-wash system to minimize carryover.

Mass spectrometric detection was carried out in negative ionization mode. Data acquisition was performed in MS1 scan mode over a mass range of m/z 100–1100 at a scan rate of 2 spectra per second. The acquired mass spectrometric data were processed using Agilent Mass Hunter Profinder (version 10.0) to detect and extract molecular features based on predefined parameters. Feature selection criteria included a minimum peak intensity threshold of 400 counts per second, a retention time window of ± 0.30 minutes, and a mass accuracy tolerance within 20 ppm. The isotope distribution model was specified as “organic molecule,” and batch recursive molecular feature extraction (BRMFE) was applied to improve feature detection consistency across samples. Putative metabolite identification was achieved by matching the detected features with reference entries from publicly available databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the

Human Metabolome Database (HMDB), from which relevant compound information was obtained.

3.2.6 Total Phenolic Content (TPC), DPPH-free Radical Scavenging Capacity Sample Extraction

The extraction and analysis methods for the TPC and DPPH radical scavenging activity were conducted as reported by Konsue et al. (2023). Briefly, 1 g of blended sample was combined with 10 mL of 80% (v/v) methanol in a centrifuge tube. The mixture was subjected to ultrasonication (30 minutes, room temperature) using a sonicator (Branson™ 2800), followed by centrifugation (Thermo Fisher Scientific, Multiskan GO, Germany) at 14,000 rpm for 15 minutes at 4°C. The supernatant was carefully collected, while the residue underwent second extraction under identical conditions to ensure maximum recovery of soluble compounds. The combined supernatants were filtered and volume made up to 25 mL with 80% methanol for analysis.

Determination of Total Phenolic Content

The phenolic content of the extracts was measured via the Folin–Ciocalteu method. An aliquot of 30 µL of the extract was dispensed into a 96-well microplate, followed by 150 µL of Folin–Ciocalteu reagent (10%, v/v) and 120 µL of sodium carbonate solution (7.5%, w/v). Distilled water served as the blank. After mixing, the reaction mixture was incubated for 60 minutes. Absorbance was subsequently recorded at 765 nm using a microplate reader (Thermo Fisher Scientific Multiskan GO). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh sample.

Determination of DPPH- radical Scavenging Capacity

The antioxidant activity of the extracts was evaluated based on their ability to scavenge DPPH radicals. A volume of 50 µL of the extract was added to 1.950 mL of DPPH solution (60 µM), incubated in the dark for 30 minutes. Following incubation, the decrease in absorbance was measured at 517 nm using a microplate reader (Thermo Fisher Scientific Multiskan GO). The radical scavenging capacity was calculated and expressed as milligrams of Trolox equivalents (TE) per gram of fresh sample.

3.2.7 Color Analysis

Color values of fermented watermelon rind bean were analyzed according to Aung Moon et al. (2024) using a Hunter Lab/ Color Quest XE colorimeter basis of CIE L^*a^* and b^* Color Scale. The colorimeter was adjusted with a white standard tile and obtains the coordinates for the illuminant D65: $L^*=94.64$, $a^*=-0.80$, $b^*=0.07$. The color space was selected to achieve the results conveyed in the chromaticity coordinates $L^* a^* b^*$ samples. Color measurements were recorded in terms of L^* , a^* , and b^* coordinates, where L^* represents lightness on a scale from black (0) to white (100), a^* reflects the red–green axis (positive values indicating redness and negative values indicating greenness), and b^* corresponds to the yellow–blue axis (positive values for yellowness and negative values for blueness).

3.3 Statistical Analysis

All the physicochemical measurements were carried out in triplicate. Mean values of different samples were compared by one-way Analysis of Variance (ANOVA). Duncan's HSD test was used to separate means in SPSS software version 23 (IBM Corporation, Marlborough, USA). Calculations and chart design were performed in Microsoft Office Excel 365. OriginPro® 2024 (Originlab Corporation, USA) generated PCA for amino acids, while MetaboAnalyst 6.0 created correlation charts.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microbiological Change During the Fermentation

4.1.1 Lactic Acid Bacteria

The lactic acid bacteria count was observed for 10 days in three salt concentrations (2 %, 4 % and 6 %) at different fermentation temperatures (25°C, 30°C, 35°C and 40°C). The LAB count at 2 %, 4 % and 6 % salt concentration watermelon at day 0 was 6.50 ± 0.07 , 6.37 ± 0.05 and 6.30 ± 0.07 log CFU/g respectively (Figure 4.1). On this day, LAB count at 6 % salt concentration was significantly lower ($p \leq 0.05$) than that of the 2 % salt concentration sample, however it is not significantly different when compared to the 4 % salt sample. As expected, the higher salt concentration provides the osmotic shock to the surface lactic acid bacteria (Wierzbicka & Janiszewska-Turak, 2024).

At 25°C, the highest LAB growth at 2% salt was observed on the 5th day of fermentation (7.64 ± 0.09 log CFU/g), after which it remained stable. At 4% salt, the maximum growth occurred one day earlier. For the 6% salt sample, the highest count (8.40 ± 0.03 log CFU/g) was recorded on day 3, which was significantly higher than those at 2% and 4% salt. With an increase in fermentation temperature at 30°C, LAB growth was also highest at 6% salt, reaching its peak (8.35 ± 0.05 log CFU/g) on day 3, staying stable until day 4, and then gradually declining. At 35°C, the 6% salt sample again showed the highest count, with maximum growth on the 4th day, however, the count at this salt concentration was not significantly different from days 2 and 3. A comparable trend was observed at 40 °C, where growth peaked on all salt levels on day 2, but the 6% salt sample still showed the highest count (8.80 ± 0.01 log CFU/g). However, after the second day, LAB growth decreased sharply, which can be attributed to the simultaneous stress of high temperature and rapid acidification. At 40°C, the elevated temperature accelerated LAB metabolism, resulting in a faster and more intense accumulation of lactic acid compared to lower temperatures. This rapid

acid production caused an abrupt decline in pH. Concurrently, at temperatures above the optimal growth range, LAB commonly experiences excessive increase in cell membrane fluidity, as the lipid bilayer loses its structural integrity under heat stress. This loss of membrane integrity increases its permeability to protons, disrupting the transmembrane pH gradient that is essential for energy generation and ion homeostasis (Fonseca et al., 2019). When this occurred alongside a low external pH, the already high extracellular proton concentration drives accelerated proton influx into the cytoplasm, causing intracellular acidification. This in turn leads to denaturation of key metabolic enzymes and ribosomal structures, inhibition of DNA replication, and energy-generating mechanism of the cell (Papadimitriou et al., 2016). The convergence of these two stresses, auto-acidification and thermal, membrane disruption likely caused the decline of LAB count and thus ultimate pH at 40°C observed after day 2.

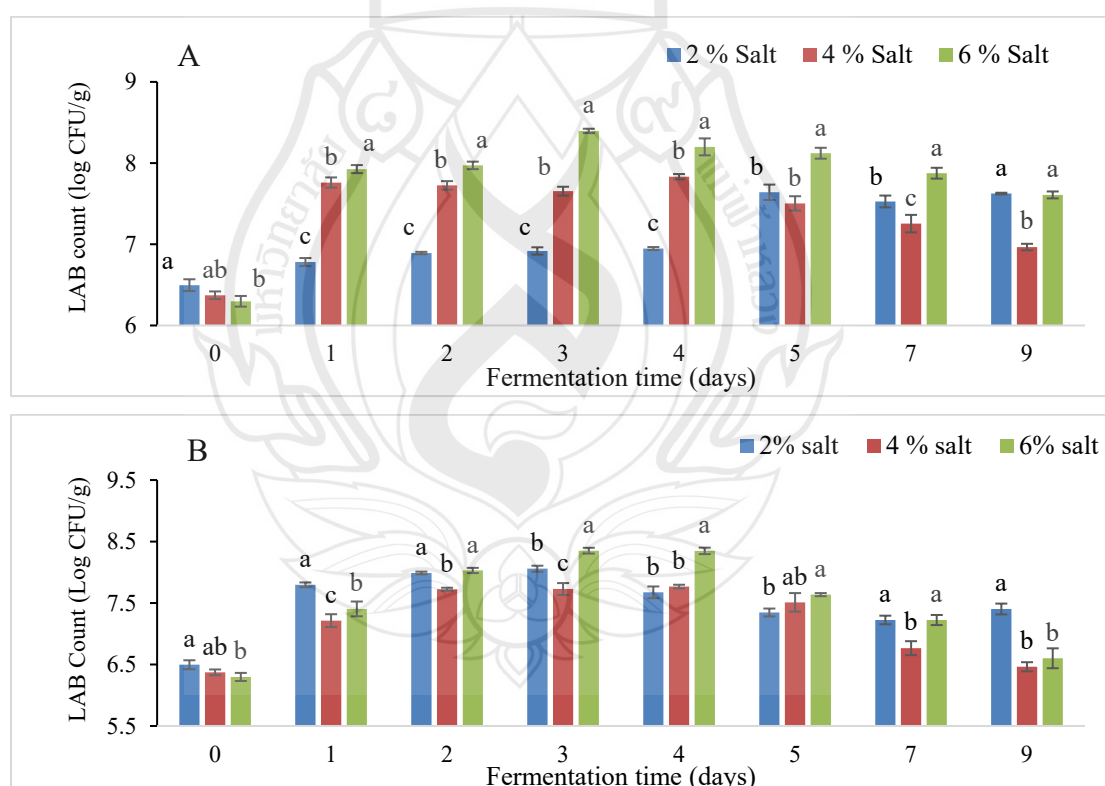


Figure 4.1 Effect of fermentation time and salt concentration on LAB count (log CFU/g) at different temperatures (A) 25°C, (B) 30°C, (C) 35°C, and (D) 40°C. Different lowercase letters indicate a significant difference among salt concentrations (2%, 4%, 6%) on the same day.

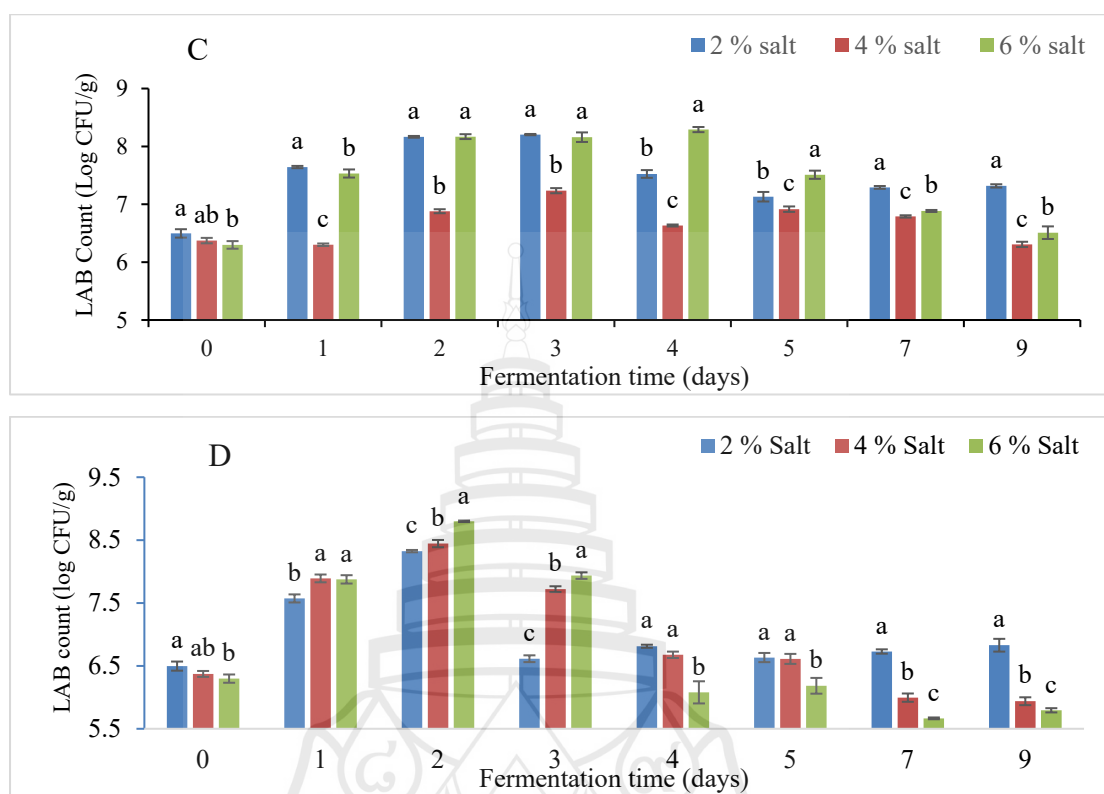


Figure 4.1 (continued)

Across all tested temperatures, the highest microbial growth was observed at 6% salt concentration. Temperature shown to have significant effect on the growth kinetics. While compared between temperatures, maximum count at 40°C was found significantly higher ($p < 0.05$) than other lower temperatures. Maximum count was not found significantly different between 25 and 30°C (Table 4.1). However, the stability period, growth and death kinetics differed between temperatures.

Generally, LAB can withstand relatively high levels of salt. In commercial fermentations, the salt level typically exceeds 2%, depending on the product; cucumbers are fermented at 6–10%, olives at 5–8%, and cabbage (sauerkraut) at 2–3% (Pérez-Díaz et al., 2013). Another study reported a similar trend to our results, observing that during the natural fermentation of black olives with 4%, 6%, and 10% salt, the greatest LAB growth occurred at 6% under both high and low temperatures (Tassou et al., 2002). Likewise, in Suancai fermentation conducted with 0%, 3%, 6%, and 9% salt, a 6% concentration yielded the highest abundance of *Lactobacillus* and

lactic acid production (Liang et al., 2020). Conversely, previous research reported that salt concentration (2.5%, 5%, and 10%) did not significantly affect LAB counts during natural cucumber fermentation (Stoll et al., 2020). In contrast, Paocai (Chinese radish) fermentation showed that the lowest salt concentration (1%) resulted in the highest microbial counts among the treatments tested (1%, 3%, 5%, and 7%) (Mi et al., 2022). These findings suggest that LAB responses to salt concentration vary across different food matrices. Nonetheless, LAB are known to tolerate high salinity through mechanisms such as osmo-protectant accumulation, membrane remodeling, ion transport regulation, protective protein synthesis, and metabolic reprogramming. All of which support their survival and activity under salt-stressed fermentation conditions (He et al., 2025).

Table 4.1 Maximum LAB count observed at different fermentation temperatures

No.	Fermentation Temperature (°C)	Maximum count observed (log CFU/g)	Respective salt concentration (%)
1	25	8.40±0.03 ^b	6
2	30	8.35±0.05 ^{bc}	6
3	35	8.29±0.04 ^c	6
4	40	8.80±0.01 ^a	6

Note Values are presented as mean ± standard deviation (SD). Different subscript letters within the same column indicate statistically significant differences between the treatments ($p \leq 0.05$).

Furthermore, higher salt content might have destroyed harmful undesirable microorganisms, leaving the nutrients for LAB only. Some studies reported that for some strains, salt promotes the growth of LAB (Sionek et al., 2024). In addition, the sample with higher salt concentration likely promoted greater extraction of sugars into the fermentation medium (Battcock & Azam-Ali, 1998), which could have provided more readily available substrates for LAB metabolism, allowing them to grow and ferment more actively than in samples with lower salt concentrations.

4.1.2 Yeast and Mold

At day 0, yeast and mold counts were 5.68 ± 0.03 , 5.54 ± 0.14 , and 5.41 ± 0.16 log CFU/g for samples containing 2%, 4%, and 6% salt, respectively (Figure 4.2). The count observed at 2% salt concentration was significantly higher than that of 6%, but not significantly different from the 4% sample. The reduction in microbial load at higher salt concentrations may be attributed to the osmotic stress or cell lysis induced by salt, which could have inactivated or destroyed some surface yeast and mold cells (Serrazanetti et al., 2013). At 25 °C of fermentation, complete elimination of yeast and mold occurred after 6 days in the 2% salt sample, 4 days in the 4% sample, and 2 days in the 6% salt sample. At higher fermentation temperatures, only the 2% salt sample required 6 days for elimination, while yeast and mold disappeared within 2 days in all other treatments. The rapid decline of fungal populations in the high-salt and higher-temperature fermentations can be attributed to the enhanced growth of lactic acid bacteria, particularly in the 6% salt sample, which significantly reduced pH and created a more anaerobic environment unfavorable for fungal survival. The accelerating effect of temperature aligns with reports that the optimal growth range of most mesophilic fungi is 25–30 °C, beyond which their activity declines (Awuchi et al., 2021). The initial yeast and mold counts in this study were within the general range reported for raw vegetables (5–7 log CFU/g) (Di Cagno & Coda, 2014), but higher than those observed in cucumber fermentation (3.39 ± 0.42 log CFU/g) (Swider et al., 2021) and raw cabbage used for sauerkraut fermentation (3.75–4.00 log CFU/g) (Xiong et al., 2016). The comparatively higher fungal counts in samples may be attributed to the use of an industrial byproduct as the fermentation substrate. Similar patterns of fungal reduction have been documented in vegetable fermentations such as sauerkraut, where yeast and mold inhibition occurred after 96 h in 5% brine or 108 h in 2% brine (Xiong et al., 2016), supporting the present observation that both higher salt concentration and fermentation temperature effectively suppress fungal growth. Some researchers have reported that a yeast and mold count of 10^3 log CFU/g is the maximum acceptable limit for fermented pickles (Lozer et al., 2025). Accordingly, our samples containing 4% and 6% salt reached values within the acceptable standard by the second day of fermentation, whereas the 2% salt sample required 6 days to fall within the standard range.

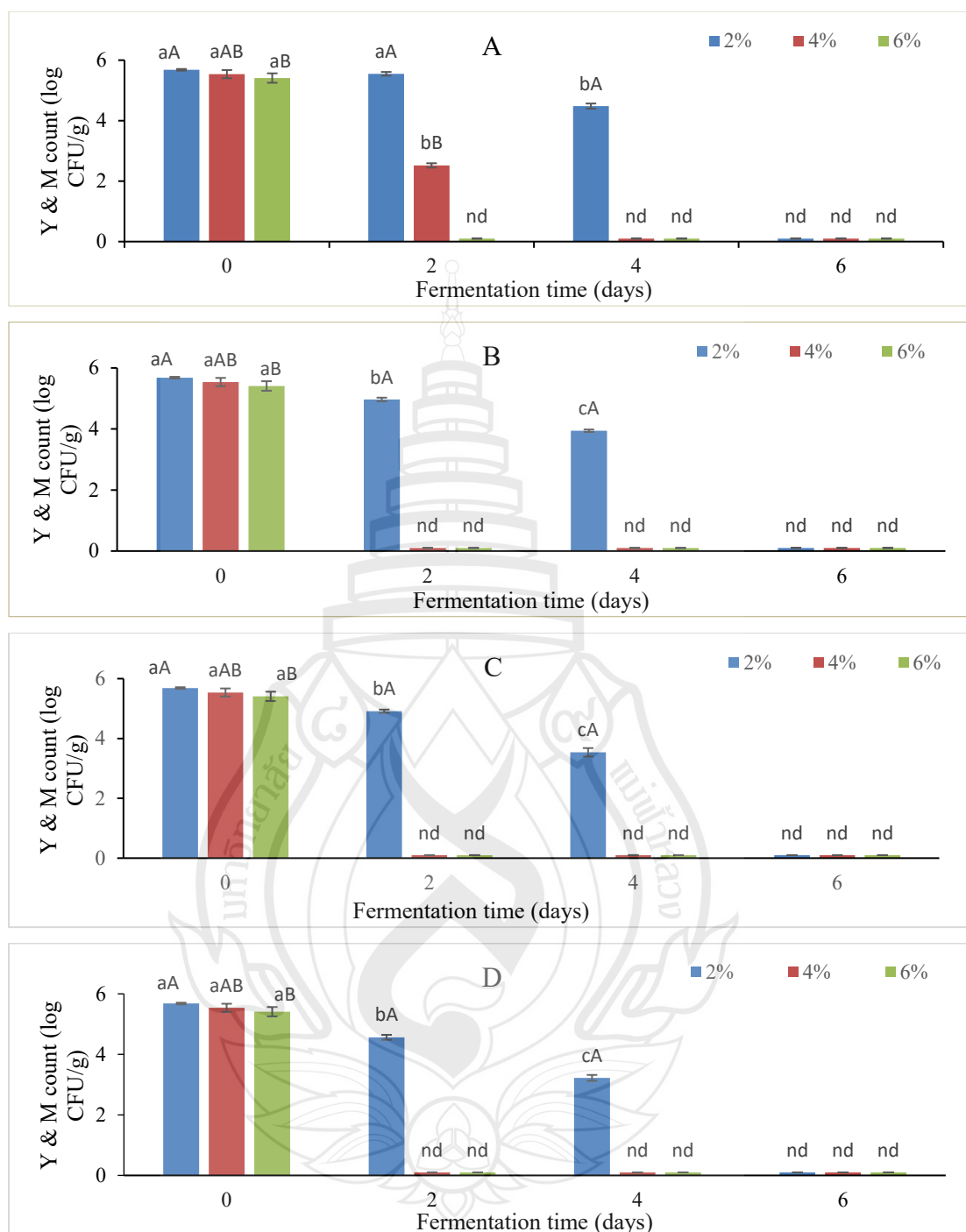


Figure 4.2 Effect of fermentation time and salt concentration on yeast and mold count (log CFU/g) at different temperatures (A) 25°C, (B) 30°C, (C) 35°C, (D) 40°C. Different lowercase letters indicate a significant difference among fermentation days within the same salt concentration. Different uppercase letters indicate a significant difference among salt concentrations (2%, 4%, 6%) on the same day. nd = not detected.

4.1.3 Enterobacteriaceae

Enterobacteriaceae represent a large group of gram-negative bacteria commonly associated with human diseases. They are predominantly found in the intestinal tract and include genera such as *Salmonella*, *Escherichia*, *Klebsiella*, and *Shigella*. Therefore, monitoring Enterobacteriaceae is a key indicator of food safety (Kang et al., 2018). In this study, the initial Enterobacteriaceae counts at day 0 were 7.72 ± 0.07 , 7.61 ± 0.03 , and 7.35 ± 0.09 log CFU/g for samples containing 2%, 4%, and 6% salt, respectively. The significantly lower ($p \leq 0.05$) counts observed on this day at higher salt (6%) concentrations could be attributed to the inhibitory effect of salt on surface microflora (Bautista-Gallego et al., 2013; Osaili et al., 2018). By the 5th day of fermentation, Enterobacteriaceae were no longer detected in any of the samples, regardless of salt concentration or fermentation temperature, indicating effective microbial suppression during fermentation. The initial Enterobacteriaceae counts observed in this study were somewhat higher than those reported in other fermented vegetables, which may be related to the use of an industrial byproduct as the substrate. For instance, one study reported counts of 5.0 ± 0.9 log CFU/g in cucumber fermentation (Pérez-Díaz et al., 2019), while another research observed 6.62 ± 0.07 log CFU/ml (Yang et al., 2020). In Chinese *Paocai* (fermented radish), study reported 4 log CFU/ml, with complete disappearance of Enterobacteriaceae by day 6 (Xiong et al., 2016). Similarly, in traditional Nepali fermented vegetables such as *gundruk* and *khalpi*, reported that Enterobacteriaceae became undetectable after 8 days and 18 hours of fermentation, respectively (Tamang & Tamang, 2010).

4.2 Effect of Fermentation on Chemical Parameters

4.2.1 pH

During fermentation, the pH showed a declining trend across all salt concentrations and fermentation temperatures. The initial pH of sample was 5.17 ± 0.03 , 5.19 ± 0.03 and 5.05 ± 0.02 for 2 %, 4 % and 6 % salt concentration sample, respectively. As fermentation progressed, the rate of pH decline was more pronounced at higher temperature compared to low temperature. Across all

temperatures range, 6 % salt sample had the lowest ultimate pH. It might be due to higher LAB growth at this salt concentration (Figure 4.1) and subsequent acidification in high salt concentration sample. The influence of salt concentration and fermentation temperature on pH is displayed in Figure 4.3.

Salt concentration, fermentation temperature, and their interaction ($T \times S$) all had significant effects on pH ($p < 0.05$). At 25 °C, the pH gradually decreased throughout fermentation and stabilized around day 7, irrespective of salt concentration, reaching final values between 3.31 and 3.56. This slower decline can be attributed to the sustained activity of LAB over a longer period. In contrast, at 30 °C and 35 °C, the pH dropped more rapidly and became relatively stable after day 4, coinciding with the peak LAB population. At 30 °C, the minimum pH values recorded for 2%, 4%, and 6% salt concentrations were 3.67 ± 0.02 , 3.33 ± 0.02 , and 3.23 ± 0.01 , respectively, while at 35 °C, the corresponding values were 3.71 ± 0.03 , 3.42 ± 0.00 , and 3.28 ± 0.01 . A rapid pH drop was also observed at 40 °C up to day 2; however, the final pH remained higher than at lower temperatures. This pattern corresponds well with the sharp initial increase in LAB count followed by a rapid decline at 40 °C, indicating thermal and acidic stress on microbial survival. At this temperature, the minimum pH for the 6% salt sample was 3.65 ± 0.02 , compared to 4.01 ± 0.02 for the 2% salt sample. The comparison of minimum pH values across different fermentation temperatures revealed that the sample fermented at 40°C exhibited the highest minimum pH, followed by those fermented at 25°C and 35°C, between which no significant difference was observed (Table 4.2). In contrast, fermentation at 30°C produced the lowest minimum pH among all conditions tested, suggesting optimal conditions for acidification. Except at 25 °C, all treatments reached $\text{pH} < 4$ within the first three days of fermentation, whereas at 25 °C, this level was attained by day 4. Similar trends have been reported in vegetable fermentation; for example, Xiong et al. (2016) observed a pH drop to 4 up to 3.5 days in sauerkraut, while Liang et al. (2020) reported a minimum pH in 6% salt *Suancai* after up to days, with pH falling below 4 within 5 days. These observations align closely with the acidification pattern observed in the present study.

Table 4.2 Effect of fermentation temperature on minimum pH value and maximum acidity

No.	Temperature (°C)	Minimum pH observed	Maximum acidity observed (% lactic acid)
1.	25	3.31±0.03 ^b	1.14±0.03 ^a
2.	30	3.23±0.01 ^c	1.07±0.04 ^a
3.	35	3.28±0.01 ^b	1.08±0.07 ^a
4.	40	3.65±0.02 ^a	0.80±0.03 ^b

Note Values are presented as mean ± standard deviation (SD). Different subscript letters within the same column indicate statistically significant difference between the treatments ($p \leq 0.05$).

4.2.2 Acidity

Overall, titratable acidity showed an increasing trend and eventually stabilized, indicating an inverse pattern to pH, which decreased and reached a stable point. The stabilization period of acidity closely corresponded to the point at which the pH value became stable (Figure 4.3). The initial acidity values were $0.06 \pm 0.01\%$, $0.05 \pm 0.00\%$, and $0.06 \pm 0.02\%$ as lactic acid at 2 %, 4 % and 6% salt, respectively. Except at 40 °C, all fermentation temperatures showed acidity levels exceeding 1%. When comparing the maximum acidity values obtained at different fermentation temperatures, no significant differences were observed among samples fermented at 25°C, 30°C, and 35°C (Table 4.2). In contrast, fermentation at 40°C produced a significantly lower acidity value ($0.80 \pm 0.03\%$). This reduction in acidity at 40°C is likely attributed to reduced acid production caused by the rapid decline of LAB, which also corresponded with the highest pH values. Regarding salt concentration, at 25 °C, samples with 2% salt consistently showed the highest acidity throughout fermentation. At 30 °C and 35 °C, the 2% salt samples also had higher acidity up to the 4th day compared to other salt levels. On the 4th day, the acidity values for the 2% salt samples were $0.97 \pm 0.05\%$ at 30 °C and $0.94 \pm 0.02\%$ at 35 °C. Even at 40 °C, the highest acidity was observed on the 4th day with 2% salt, though the overall acidity was lower than at other temperatures.

Mi et al. (2022) reported a lower range of acidity in radish *paocai* compared to the present study. However, similar to our findings, they observed that lower salt concentrations resulted in higher acidity, with a maximum of about 3.75 g/L as lactic acid. However, in their study, acidity continued to increase throughout the 20-day fermentation period. Likewise, a study on fermentation of sauerkraut found that at a low salt concentration (0.5%), both pH and acidity values were the highest in most of their observations, supporting our results (Yang et al., 2020). Similarly, another recent study investigated the fermentation of *Brassica napus* at salt concentrations ranging from 4% to 9% and found the highest total acidity ($2.01 \pm 0.01\%$) at 8% salt, followed by 6% ($1.59 \pm 0.24\%$), with the lowest acidity ($0.35 \pm 0.01\%$) recorded at 7% (Zhang et al., 2023b). A study on table olive fermentation reported that both temperature and salt concentration significantly affected the pH and acidity during fermentation. At 4% salt, the titratable acidity reached 1.2 g at 22°C but only 0.59 g at 30°C. Nevertheless, the final acidity was found to be strongly influenced by the salt concentration (Issaoui et al., 2022).

The results of the microbial and chemical analyses demonstrated that both salt concentration and fermentation temperature play crucial roles in shaping the microbiological and chemical characteristics of the final product. Among the tested conditions, a 6% salt concentration proved most effective in promoting vigorous lactic acid bacteria (LAB) growth and rapid acidification, particularly at a fermentation temperature of 30 °C, which resulted in the lowest final pH. All fermentation conditions evaluated were effective in ensuring food safety, as evidenced by the elimination of Enterobacteriaceae within five days and the suppression of yeast and mold populations, with higher salt concentrations and temperatures accelerating their reduction. These kinds of products are majorly consumed as pickle as the side dish in main course. Product was comparable with fermented cucumber pickles in terms of taste and texture.

Based on these findings, subsequent experiments were conducted at a fermentation temperature of 30 °C. Additionally, fermentation time was selected as a key variable, with a duration ranging from 0 to 5 days.

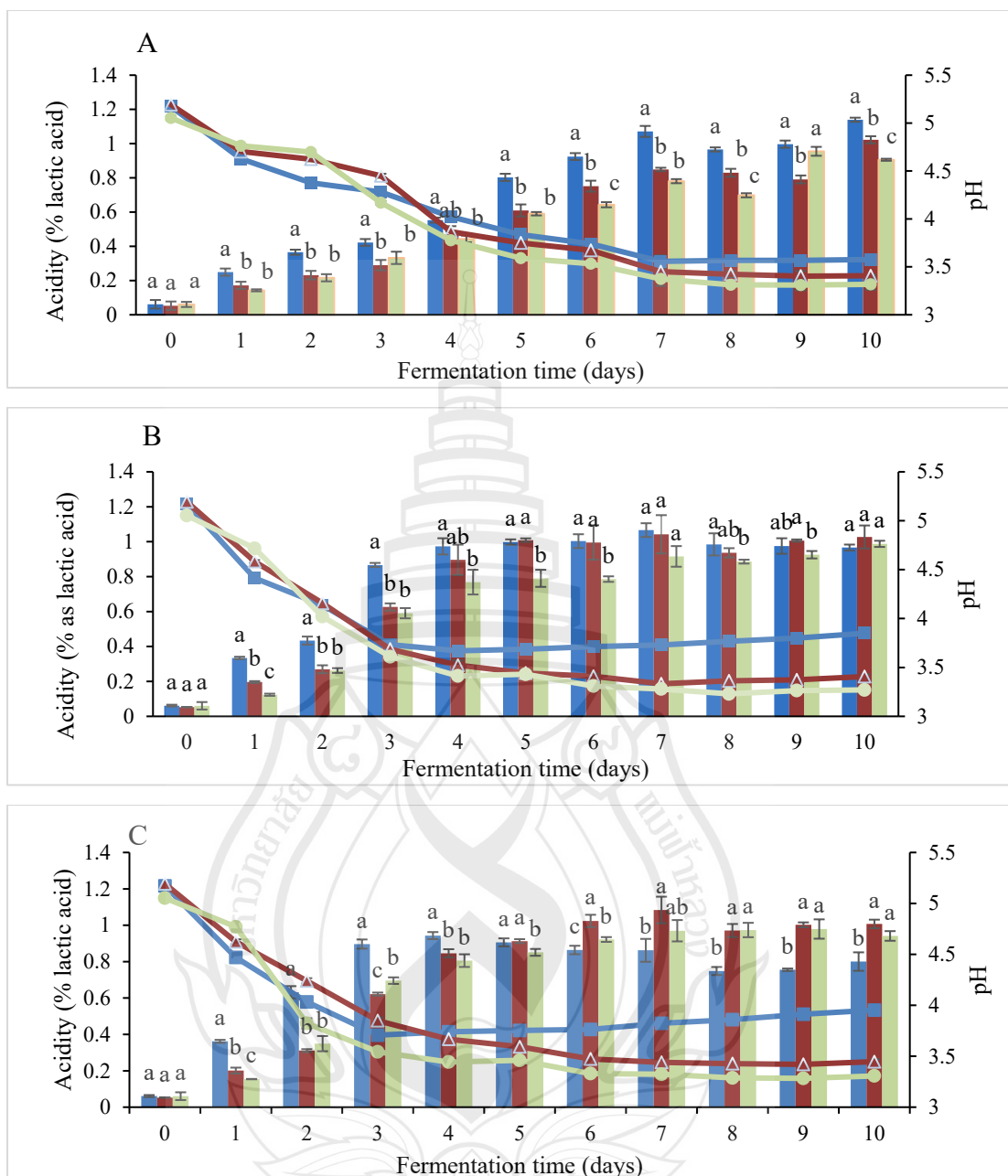


Figure 4.3 Effects of fermentation time and salt concentration on titratable acidity and pH at different temperatures (A) 25°C, (B) 30°C, (C) 35°C, (D) 40°C. Different lower-case letters indicate a significant difference between three salt concentration (2 %, 4%, 6%) on the same day.

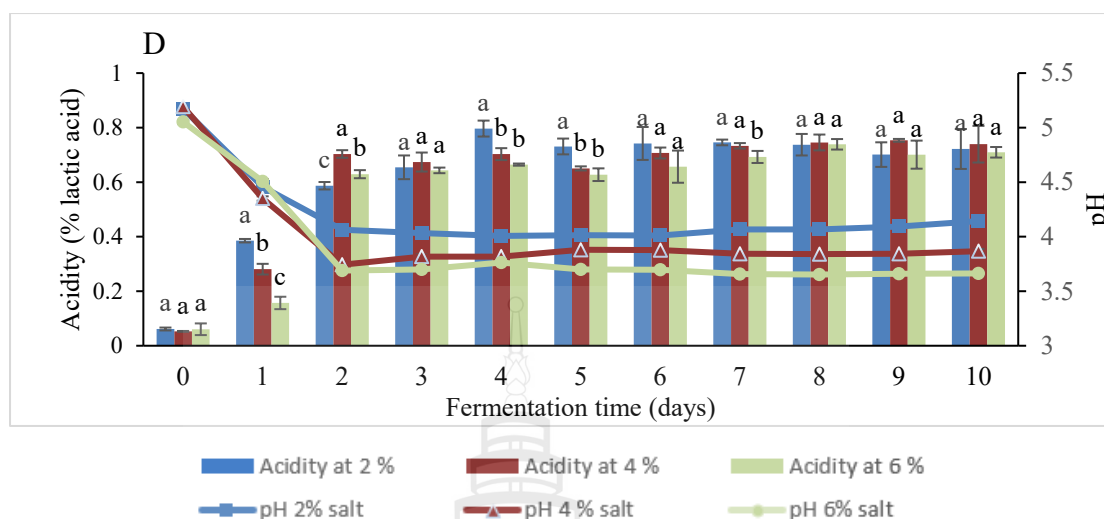


Figure 4.3 (continued)

4.3 Effect of Salt Concentration and Fermentation Time on Arginine, Citrulline and Ornithine

Arginine, citrulline, and ornithine are three key amino acids involved in the ADI pathway, which is the primary route of arginine metabolism in many lactic acid bacteria. In this process, arginine presented in the substrate is first converted into citrulline as an intermediate, which is then further transformed into ornithine as the final product of the pathway. (Zhang et al., 2016).

To observe the effects of salt concentration and duration of fermentation on the amino acids involved in the ADI pathway, watermelon rind was fermented using three salt levels (2%, 4%, and 6%) for up to five days at 30 °C. As presented in Sections 4.1 and 4.2, lactic acid bacteria exhibited optimal growth within this five-day period, and 30 °C was identified as the most suitable temperature for maintaining both microbial growth and acidification. Samples were taken and analyzed at day 0, day 2.5 and day 5. On day 0, initial pH was in the range of 4.88 to 4.97. At day 2.5, 2% salt sample reached the pH of 3.83 ± 0.02 , while 4 % and 6 % reached the pH of 4.04 ± 0.02 and 3.77 ± 0.02 respectively. On day 5, pH values of all samples reached below 3.5. While tracking the arginine, citrulline, and ornithine content, it was found that both arginine and citrulline decreased rapidly within the first 2.5 days across all salt concentrations. Meanwhile, the ornithine value increased. This suggests the ADI

pathway is highly sensitive to fermentation time and pH levels. The decline in both arginine and citrulline may be due to the long fermentation time and the consistent drop in pH, which likely converted the generated citrulline into ornithine. This observation is consistent with literature indicating that the ADI pathway favors the conversion of citrulline to ornithine under low pH conditions, whereas lactic acid bacteria (LAB) tend to excrete citrulline at moderately higher pH levels (Rimaux et al., 2012; Vrancken et al., 2009). Based on these findings, to maximize citrulline content, the fermentation duration was adjusted to 48 hours with a higher sampling interval of 12-hour (0, 12, 24, 36, and 48 h) at 30°C. These samples were subsequently analyzed for arginine, citrulline, and ornithine to map the metabolic changes more precisely.

The result showed that concentration of salt and the duration of fermentation significantly influenced the metabolic pathways of watermelon rind, particularly regarding the concentrations of arginine, citrulline, and ornithine. In unfermented rind the arginine, citrulline and ornithine content were in the range of 163.95-183.29 mg/kg, 620.25-669.90 mg/kg and 1.18-1.27 mg/kg respectively. The arginine value was found lower but citrulline value was comparable to the value reported by Du et al. (2022).

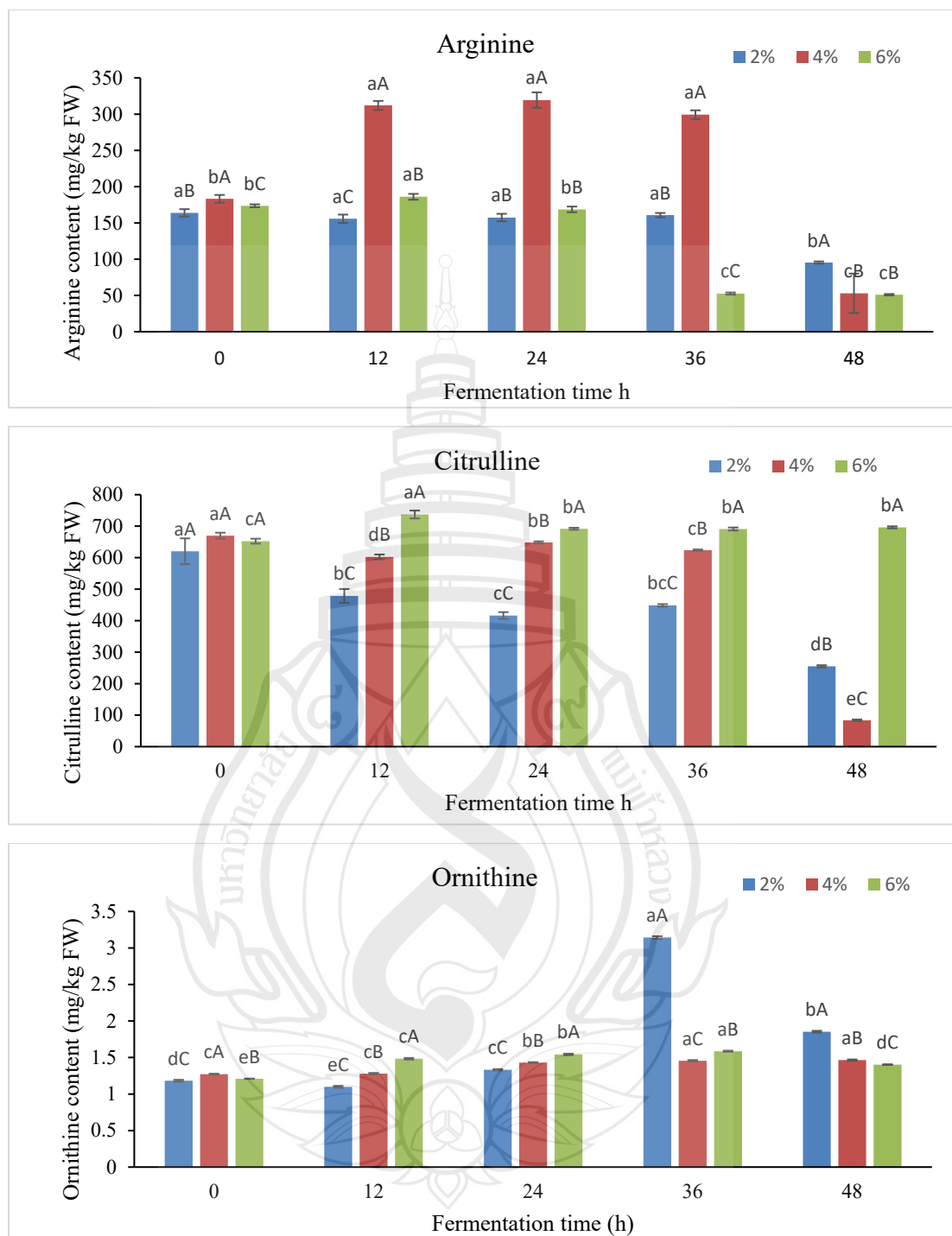


Figure 4.4 Effects of salt concentration and fermentation time on the arginine, citrulline and ornithine content in fermented watermelon rind. Different uppercase letters indicate a significant difference between three salt concentration (2 %, 4%, 6%) on the fermentation time and different lower-case letters indicate a significant difference among different fermentation time on same salt concentration

Rimando and Perkins-Veazie (2005) reported the citrulline content in the range of 0.5 to 3.5 mg/g fresh weight in different cultivars of watermelon rind. Our value fell within but at lower side of that range. Studies showed that the content of these amino acids content not only varies with cultivar, but also with the cultivation condition (Fish, 2014).

In unfermented sample (0 h) at 2 % salt, arginine, citrulline and ornithine content were 163 ± 5.06 , 620.25 ± 41.09 and 1.18 ± 0.01 mg/kg fresh weight (FW), respectively. At this salt concentration, the arginine and citrulline concentration tended to decrease as fermentation progressed (Figure 4.4). Arginine became stable up to 36 h and declined to 95.36 ± 1.59 mg/kg after 48 h. While citrulline decreased consistently and reached 255.10 ± 3.33 mg/kg at the end of fermentation. In contrast, ornithine content increased from baseline (1.18 ± 0.01 mg/kg) to its maximum at 3.14 ± 0.02 mg/kg at 36 h of fermentation. This accumulation suggests the activation of ADI pathway. In a typical ADI pathway, arginine is converted to citrulline and ammonia by arginine deiminase enzyme which is encoded by *arcA* gene. Further, citrulline is subsequently converted into ornithine and carbamoyl phosphate by transcarbamylase encoded by *arcB*. This pathway provides two physiological advantages: production of ATP and ammonia. The generated ammonia serves to stabilize intracellular pH against the rapidly acidifying external environment (Zhang et al., 2014). In our fermentation system, in 2 % salt sample, pH dropped rapidly, from an initial 4.85 to 3.85 (Figure 4.5A), which makes ADI pathway an obvious coping mechanism for pH stabilization.

The subsequent decline in ornithine to 1.85 ± 0.01 mg/kg after 36 h, though still significantly higher than the unfermented sample, may be attributed to the activation of the ornithine decarboxylation pathway. This is often a response to extreme acidic stress, occurring as the pH levels fall below 4.0 (Romano et al., 2014).

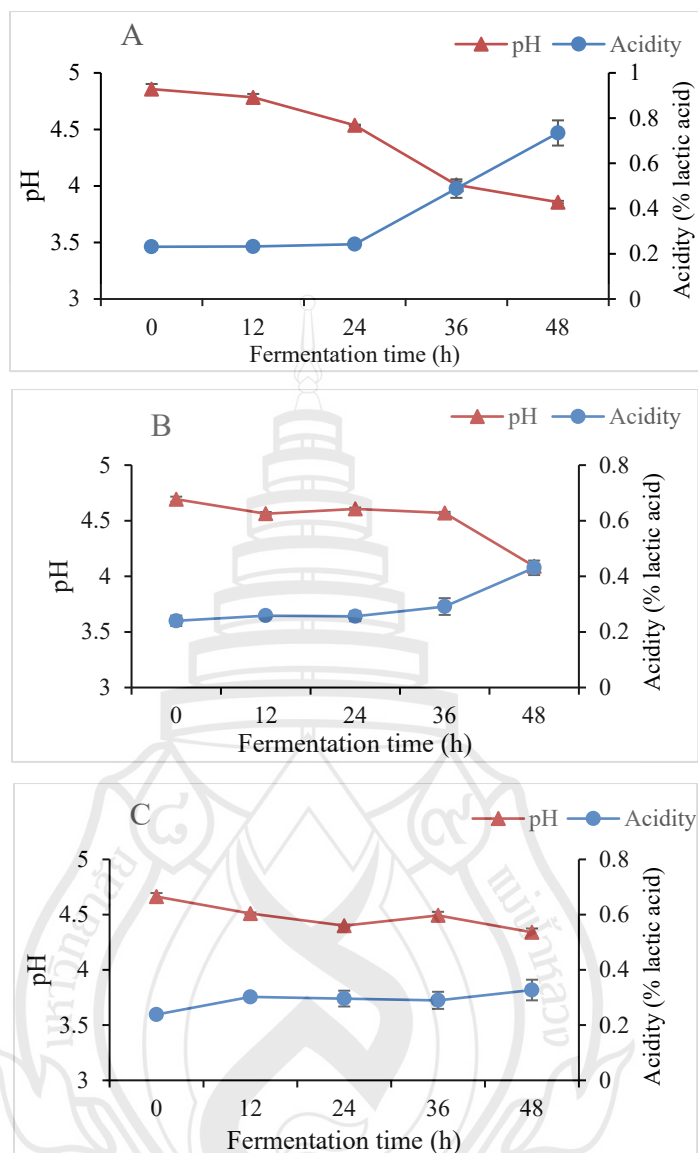


Figure 4.5 pH and acidity in watermelon rind fermentation at (A) 2 % salt, (B) 4 % salt, (C) 6 % salt.

At the 4% salt, initial values were 183.29 ± 5.37 mg/kg for arginine, 669.90 ± 9.36 mg/kg for citrulline, and 1.27 ± 0.01 mg/kg for ornithine. Interestingly, arginine levels increased to 312.08 ± 6.18 mg/kg within the first 12 h and remained stable until 36 h, after which they declined to 51 ± 0.99 mg/kg. This early increase in arginine suggests potential endogenous arginine generation by the microflora. Previous studies have indicated that approximately 52% of lactic acid bacteria possess arginine synthesis capacity (Karapetyan et al., 2022). In contrast, a similar peak was not observed in the 2% salt sample. This could be explained by the more rapid drop in pH

under lower salt conditions, which likely stimulated the ADI pathway for acid stress regulation. As a result, any arginine formed by microorganism may have been quickly converted into downstream metabolites, particularly ornithine. It is well established that under acidic conditions, the ADI pathway becomes more active, with ornithine accumulating as the primary end product (Leroy & Charlier, 2017; Rimaux et al., 2011; Vrancken et al., 2009). Citrulline followed an overall decreasing trend similar to the 2% sample reaching 82.73 ± 2.35 mg/kg, while ornithine reached its maximum (1.46 ± 0.01 mg/kg) at 48 h of fermentation. These shifts confirm that the ADI pathway remained active at this salt concentration.

In the 6% salt samples, initial levels were 173.66 ± 1.99 mg/kg for arginine, 652.41 ± 7.77 mg/kg for citrulline, and 1.21 ± 0.002 mg/kg for ornithine. While arginine followed a decreasing trend as seen in the lower salt groups, citrulline exhibited an increasing trend. From an initial 652.41 ± 7.77 mg/kg, citrulline levels rose significantly ($p < 0.05$) to 737.12 ± 12.50 mg/kg at 12 h, before slightly decreasing to 691.76 ± 3.33 mg/kg at 24 h and stabilizing thereafter. However, this value is also significantly higher ($p < 0.05$) than the unfermented sample. Ornithine showed an increasing trend.

The enhancement of citrulline in 6 % salt sample can be explained by salt stress. Zhou et al. (2021) reported in *Pediococcus acidilactici* and *Weissella confusa* that elevated NaCl concentrations have been shown to specifically increase the conversion of arginine to citrulline while simultaneously decreasing the subsequent conversion of citrulline to ornithine. They found that high salt concentration, at about 9 %, the expression of *arcB* was found relatively lower than that of *arcA*, this led to accumulation of citrulline in fermentation media. In their investigation with *Lactobacillus fermentum* 130101, Vrancken et al. (2009) also noted that raising the salt content over 4% promotes the conversion of arginine to citrulline but disrupts the conversion of citrulline to ornithine. Further, less pH drop might be a factor for accumulation of citrulline in the 6 % sample. At higher pH, microorganisms tend to avoid the accumulation of the ammonia, causing excretion of intermediate citrulline from the cell (Leroy & Charlier, 2017). Inoue et al. (2022) during fermentation of pineapple juice with *Lactococcus lactis* found that citrulline accumulation was lower at lower pH. Citrulline accumulation at pH 4.0 was about 3 times lower than that at

pH 4.5. Therefore, 6 % sample in this study, with higher pH, might have impacted citrulline accumulation in that sample. Moore et al. (2022) also found significant higher citrulline content in 6 % salt fermentation as compared to 2 % and 4 % salt sample.

The consistently lower citrulline levels observed in the low-salt samples can be explained by the native transport and metabolic dynamics of arginine metabolites in microbial cells. ADI pathway is an intracellular process, and during the conversion of arginine to citrulline and subsequently to ornithine, citrulline is merely a transient intercellular intermediate. Under normal stress conditions, citrulline is rapidly converted to ornithine to sustain ATP generation. In many microorganisms, the arginine–ornithine antiporter encoded by *arcD* plays a central role in this process, allowing arginine uptake into the cell while exporting ornithine into the extracellular environment with ammonia and ATP generation (Pols et al., 2021). As a result, citrulline did not accumulate under low-salt conditions.

However, Noens and Lolkema (2017) reported that, in addition to *arcD*, lactic acid bacteria also possess *arcE* transporters, which exhibit a higher affinity for citrulline and enable efficient L-citrulline translocation across the cell membrane. These transport systems can also facilitate the excretion of citrulline as a metabolic intermediate. Taken together, these findings suggest that similar transport and stress-response mechanisms may operate under high-salt conditions in our samples, which could explain the observed differences in citrulline profiles.

4.4 Effect of Fermentation on Amino Acid Profile

Free amino acids are crucial flavor compounds in fermented foods (Shang et al., 2022). The changes in free amino acids in fermented watermelon rind with different salt concentrations and fermentation time are summarized in Table 4.3. Apart from arginine, citrulline and ornithine; we were able to quantify 8 other amino acids including aspartic acid, leucine, glutamic acid, threonine, histidine, glutamine, lysine and proline. It should be noted that other amino acids were also found but the content was below detection and quantification limit. While comparing the amino acids,

citrulline was the most prevalent amino acid in watermelon rind followed by aspartic acid. Arginine appeared as the third-largest amino acid. However, Du et al. (2022) identified aspartic acid as the third largest amino acids after citrulline and arginine. The discrepancy might be due to a difference in the variety of watermelons. Supporting this explanation, Fish (2014) reported that the levels of these amino acids can vary by up to 100%, even within the same cultivar.

Aspartic acid was found in the range of 217.96-233.37 mg/kg in unfermented watermelon rind. This value is higher than the value (70.9 mg/kg) reported by Du et al. (2022). Both fermentation time and salt concentration affected the aspartic acid value. In 2 % salt concentration, an increase in aspartic acid level at 36 h was observed up to the maximum level (236.25 ± 3.46 mg/kg), which later reduced at the end of fermentation, while in 4 % and 6 % salt samples, reduction from initial aspartic acid content was observed during fermentation. In general, aspartate is heavily utilized in the diaminopimelate (DAP) pathway for the biosynthesis of cell wall components and other essential amino acids such as lysine and threonine (Liu et al., 2021). Higher microbial activity and subsequent proteolysis may have accelerated the cellular metabolic production at lower salt concentrations. Lactic acid bacteria possess both endopeptidases and exopeptidases that hydrolyze proteins and oligopeptides, producing smaller peptides and liberating terminal amino acids (Moore et al., 2022).

In general, at 2 % and 6 % salt concentration, leucine level did not increase during the course of fermentation, while at 4 %, it increased significantly and reached a maximum value at 24 h of fermentation, before reduction to the lowest level at 48 h. The subsequent reduction in leucine over time can be attributed to its utilization by lactic acid bacteria. According to Kleerebezem et al. (2003), some LAB such as *Lactobacillus plantarum* lacks the genetic capacity to synthesize branched-chain amino acids like leucine and therefore relies on external sources. Moreover, many lactic acid bacteria are known to catabolize leucine into aroma-active compounds, including 3-methylbutanal, 3-methylbutanol, and 3-methylbutyric acid (Afzal et al., 2017), which likely contributed to the observed decline in leucine during fermentation. Correlation analysis showed that leucine level was slightly positively correlated with salt and negatively correlated with fermentation time (Figure 4.6). The halotolerant species such as *L. plantarum*, which is discovered to have strong

proteolytic activity but lack the synthesizing capacity for leucine, may have been chosen by higher salt samples (Duan et al., 2019; Kleerebezem et al., 2003).

Glutamic acid is widely known for its umami flavor. Glutamic acid was found to be 21.62-22.59 mg/kg FW in the unfermented sample; the value initially decreased at 12 h in all salt concentrations. Subsequently, it continued to rise eventually attaining its peak at the end of fermentation. The initial decline is attributed to the utilization of glutamic acid as a carbon and nitrogen source by the rapidly growing microbial population, or its conversion into γ -aminobutyric acid (GABA) via glutamate decarboxylase (GAD), an enzyme frequently upregulated in the early acidic phase of fermentation to consume intracellular protons (Zhao et al., 2015). This conversion is facilitated in a pH range of 4.5 to 5.0 (Peng et al., 2013). Low salt concentration seems to favor glutamic acid. On the other hand, with lowered salt concentrations, glutamine appears to be reduced throughout the fermentation process. It might be due to the conversion of glutamine to glutamate by glutaminase. However, at higher salt concentrations, glutamine seems to be accumulated. The enzyme glutaminase is inhibited by higher salt concentrations (Liu et al., 2022). The correlation study indicated that salt has a positive association with glutamine, while demonstrating a negative correlation with glutamic acid (Figure 4.6).

Threonine and histidine both showed positive effects with higher salt concentration throughout the fermentation period. Both amino acids increased overall during the course of fermentation but decreased at the end of 48 h. However, except for 2 % salt concentration, the value was not significantly lower than the initial content. Both amino acids showed positive correlation with salt and fermentation time. Swider et al. (2021) also reported that fermenting cucumber with 5 % salt resulted in a significantly higher histidine content than fermentation with 1.5 % salt after 48 h of fermentation. Lysine showed different trends for different salt concentrations. In the 2% salt sample, lysine content exhibited a general decline over the fermentation period. Conversely, samples containing 4% and 6% salt showed a consistent increase in lysine levels. This pattern may be attributed to the activity of *Enterobacteriaceae*, which is known to decarboxylate free lysine (Lau et al., 2022). In our experiment, the lower salt sample contained higher *Enterobacteriaceae* count

compared to the higher salt treatments, which could explain the observed reduction in lysine at lower salt concentration.

Proline content in the 6% salt treatment remained statistically higher to both the 2% and 4% treatments across all time points during fermentation. In the context of LAB stress physiology, proline is a preferred osmolyte because its chemical structure allows it to accumulate to high intracellular concentrations without denaturing proteins or disrupting enzymatic cycles (Luo et al., 2025; Tian et al., 2016). Zhang et al. (2023a) also reported high proline content while fermenting rape stalk in high salt concentration sample. The reduction of proline content in low salt sample might be due to conversion of proline to glutamate by proline dehydrogenase enzyme present in most of the bacteria (Tanner, 2008). This also validates the specific rise of glutamate in low salt concentration samples in our observation.

Except for glutamic acid and aspartic acid, higher salt concentrations promoted the accumulation of all other amino acids. Glutamic acid and aspartic acid are well recognized for contributing umami flavor in fermented foods; therefore, their greater abundance in the 2% salt sample suggests a stronger umami flavor in this treatment. In contrast, proline and threonine were favored at higher salt levels. Since these amino acids are associated with a sweet taste (Bachmanov et al., 2016), a mild sweetness may be anticipated in the sample containing 6% salt. Leucine, lysine, and histidine are considered bitter-tasting amino acids (Harmon et al., 2024). Their reduction in the 2% salt sample toward the end of fermentation may contribute to decreased bitterness, whereas their higher levels under increased salt concentrations may enhance the bitter taste.

Table 4.3 Effect of salt concentration and fermentation time on amino acid content in watermelon rind

Amino Acids (mg/kg FW)	Fermentation time (h)	Salt concentration (%)		
		2	4	6
Aspartic acid	0	217.96±1.00 ^{Ab}	218.12±15.34 ^{Aa}	233.37±27.39 ^{Aa}
	12	186.27±7.85 ^{Bc}	184.39±2.55 ^{Bb}	215.24±16.19 ^{Aa}
	24	168.10±7.76 ^{Bd}	205.91±11.21 ^{Aab}	198.67±24.40 ^{ABa}
	36	236.25±3.46 ^{Aa}	191.30±17.56 ^{Bb}	126.28±28.07 ^{Cb}
	48	182.48±3.95 ^{Ac}	90.66±11.59 ^{ca}	212.66±10.02 ^{Ba}

Table 4.3 (continued)

Amino Acids (mg/kg FW)	Fermentation time (h)	Salt concentration (%)		
		2	4	6
Leucine	0	27.93±1.97 ^{Aa}	22.47±10.03 ^{Ab}	31.62±1.22 ^{Aab}
	12	24.73±0.42 ^{Bb}	33.58±1.66 ^{Aa}	33.43±0.62 ^{Aa}
	24	15.41±0.55 ^{Cc}	37.72±1.38 ^{Aa}	29.92±1.46 ^{Bab}
	36	27.21±0.43 ^{Ba}	32.53±1.10 ^{Aa}	11.54±0.41 ^{Cc}
	48	16.17±0.91 ^{Ac}	14.43±3.35 ^{Ab}	24.77±9.53 ^{Ab}
Glutamic acid	0	21.62±0.43 ^{Ac}	22.59±0.30 ^{Ac}	22.44±0.95 ^{Ac}
	12	14.34±0.02 ^{Bd}	11.98±0.20 ^{Cc}	14.60±0.10 ^{Ac}
	24	23.17±0.14 ^{Ab}	13.03±0.18 ^{Cd}	16.94±0.19 ^{Bd}
	36	29.79±0.79 ^{Aa}	25.26±0.04 ^{Cb}	27.71±0.99 ^{Ba}
	48	30.48±0.43 ^{Aa}	28.83±0.33 ^{Ba}	26.15±0.91 ^{Cb}
Threonine	0	10.61±0.18 ^{Bb}	11.10±0.24 ^{Bc}	12.51±0.62 ^{Ac}
	12	13.58±0.17 ^{Ba}	13.22±0.27 ^{Bc}	16.52±0.07 ^{Ab}
	24	10.48±0.07 ^{Cb}	15.62±0.17 ^{Ba}	18.35±0.12 ^{Aa}
	36	10.72±0.57 ^{Cb}	12.02±0.17 ^{Bd}	16.00±0.65 ^{Ab}
	48	9.90±0.31 ^{Cc}	15.21±0.08 ^{Bb}	18.50±0.27 ^{Aa}
Histidine	0	4.40±0.23 ^{ABa}	4.17±0.48 ^{Bb}	4.93±0.09 ^{Ab}
	12	4.65±0.14 ^{Ba}	4.73±0.24 ^{Ba}	6.21±0.31 ^{Aa}
	24	4.71±0.18 ^{Ba}	4.97±0.09 ^{Ba}	6.55±0.21 ^{Aa}
	36	4.70±0.20 ^{Ba}	4.85±0.14 ^{Ba}	6.67±0.31 ^{Aa}
	48	3.66±0.14 ^{Cb}	4.95±0.06 ^{Ba}	6.21±0.33 ^{Aa}
Glutamine	0	2.01±0.05 ^{Ab}	1.85±0.05 ^{Bc}	1.84±0.06 ^{dB}
	12	2.50±0.05 ^{Aa}	2.32±0.01 ^{Cc}	2.39±0.02 ^{Bc}
	24	0.93±0.02 ^{Cc}	2.96±0.06 ^{Aa}	2.77±0.03 ^{Bab}
	36	0.92±0.02 ^{Cc}	1.96±0.04 ^{Bd}	2.83±0.04 ^{Aa}
	48	0.91±0.01 ^{Bc}	2.74±0.03 ^{Ab}	2.68±0.12 ^{Ab}
Lysine	0	1.34±0.02 ^{Ab}	1.40±0.05 ^{Ac}	1.42±0.05 ^{Ac}
	12	1.73±0.03 ^{Ca}	1.85±0.02 ^{Bc}	2.11±0.06 ^{Ab}
	24	0.52±0.01 ^{Bc}	2.41±0.04 ^{Aa}	2.49±0.10 ^{Aa}
	36	0.53±0.01 ^{Cc}	1.51±0.02 ^{Bd}	2.49±0.07 ^{Aa}
	48	0.52±0.01 ^{Bc}	2.19±0.02 ^{Ab}	2.33±0.13 ^{Aa}
Proline	0	0.82±0.01 ^{Ba}	0.82±0.01 ^{Ba}	0.97±0.01 ^{Ac}
	12	0.74±0.00 ^{Bc}	0.74±0.01 ^{Bcd}	1.02±0.02 ^{Aab}
	24	0.75±0.00 ^{Bb}	0.77±0.00 ^{Bb}	1.05±0.02 ^{Aa}
	36	0.76±0.01 ^{Bb}	0.75±0.00 ^{Bc}	1.03±0.01 ^{Aab}
	48	0.52±0.01 ^{Cd}	0.73±0.01 ^{Bd}	1.01±0.01 ^{Ab}

Note Values are expressed as mean $\pm$ standard deviation. Different uppercase letters indicate significant differences among salt concentrations at the same fermentation duration, while different lowercase letters indicate significant differences across fermentation times at the same salt concentration for each amino acid.

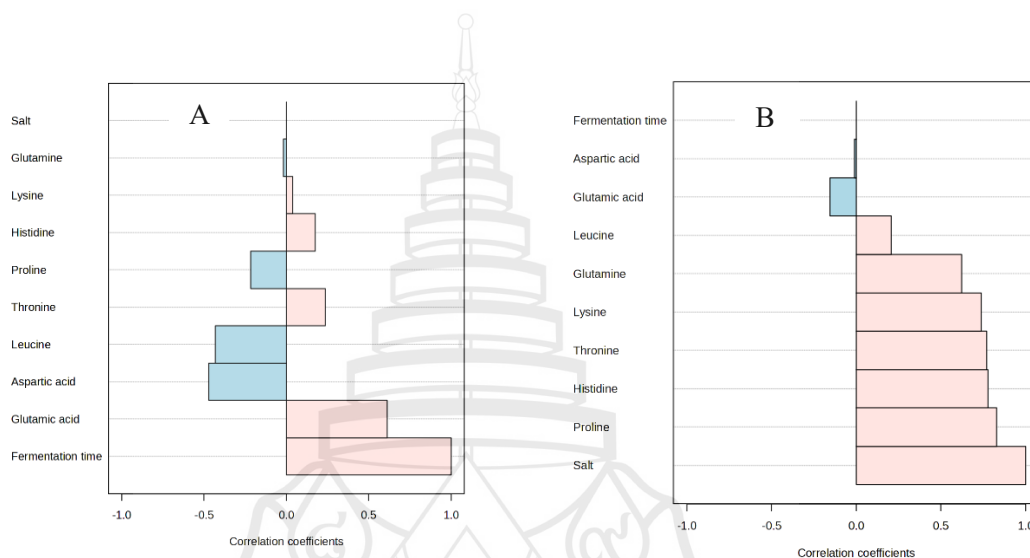


Figure 4.6 Correlation of amino acids with (A) fermentation time (salt factor keeping constant) and (B) salt (fermentation duration factor keeping constant)

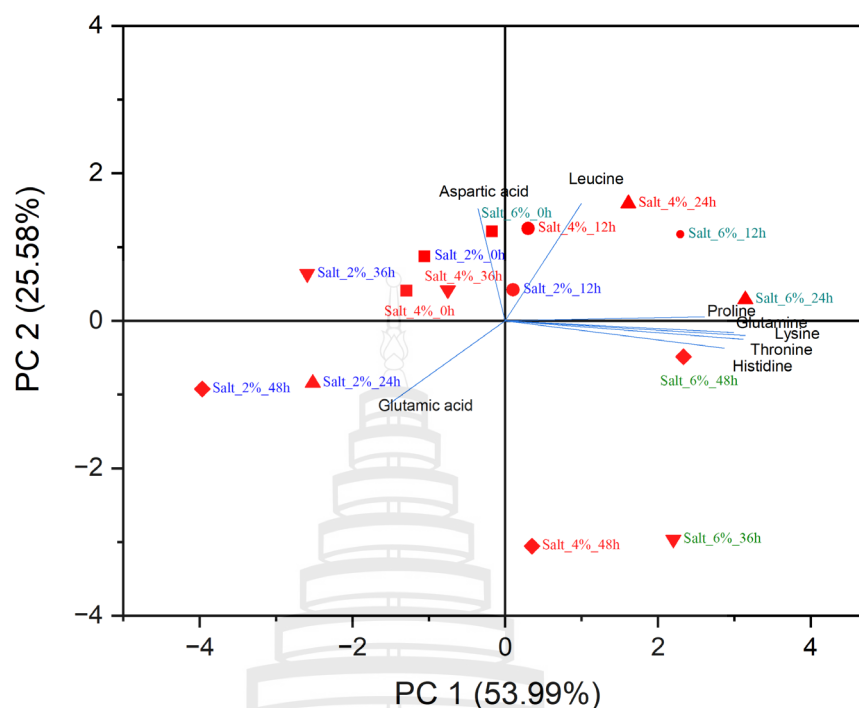


Figure 4.7 Principal component analysis (Biplot) of the fermentation condition and the amino acids.

Principal component analysis shows that two principal components PC1 and PC2 explained 79.57 % of the variations (Figure 4.7). Amino acids such as proline, glutamine, lysine, threonine and histidine are clustered with 6% salt concentration signifying these amino acids are favored with higher salt concentration. In contrast, 2% salt samples were located on the negative side of PC1 and were strongly associated with glutamic acid, suggesting improved umami potential at lower salt concentrations as explained above.

4.5 Color Changes during Fermentation

Both fermentation time and salt concentration significantly affected color value ($p < 0.05$) in the fermented watermelon rind. At 2 % salt concentration, the lightness value (L^*) increased and reached maximum at 48 h of fermentation. For 4 % salt concentration, it reached maximum value at 24 h of fermentation and became

stable thereafter. At 6 % salt concentration, the lightness value did not change significantly, which means color is preserved. At higher salt concentration, the NaCl might have inhibited the polyphenol oxidase and other enzymes, which otherwise were responsible for the degradation of color compounds causing fruits and vegetables to turn pale (Li et al., 2015). Furthermore, at 6% salt concentration, the pH drop was limited as compared to lower salt sample, which might have caused minimum changes on the color compounds during fermentation.

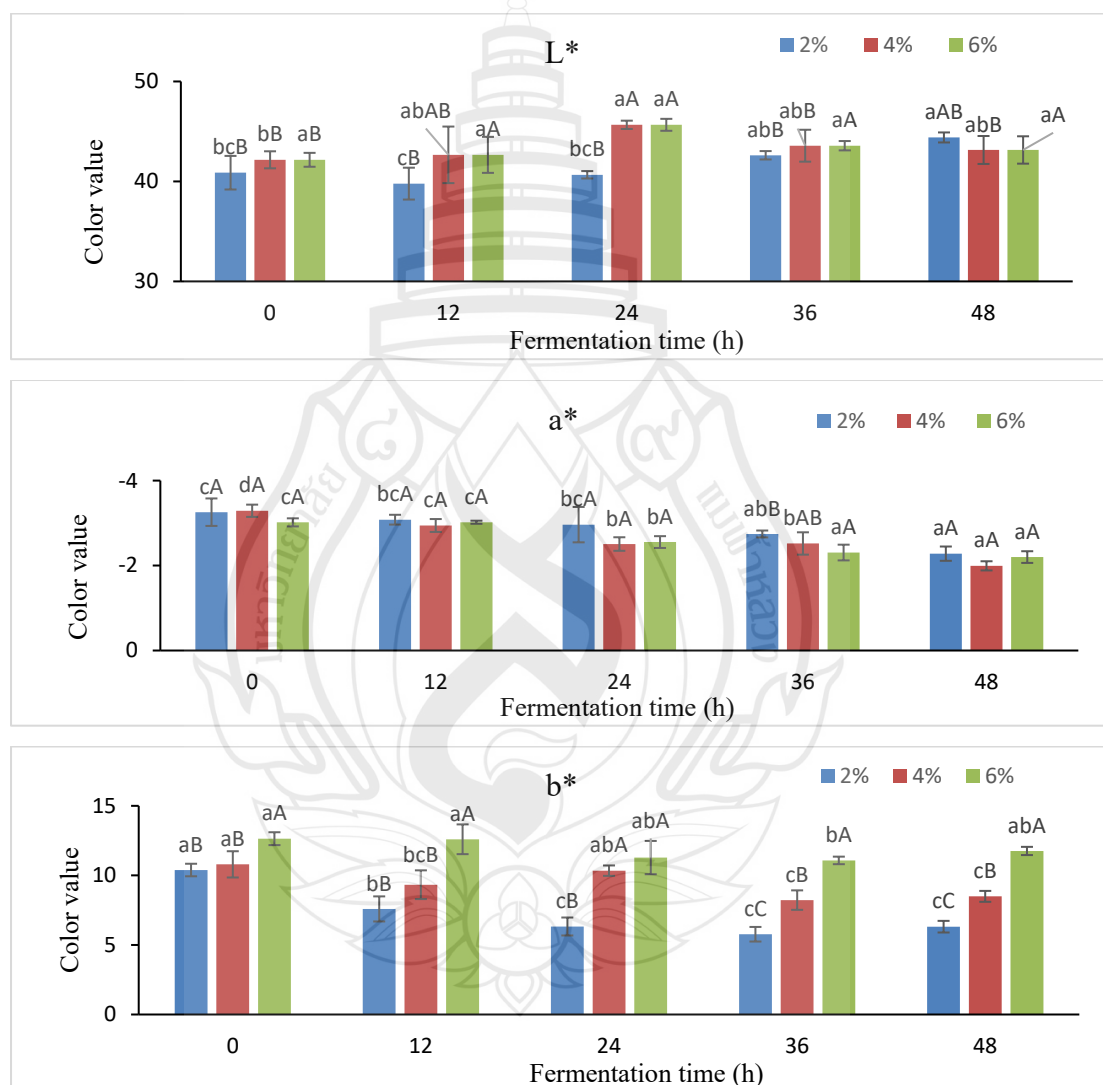


Figure 4.8 Color value of the watermelon rind as influenced by salt and fermentation time. Different uppercase letters indicate significant differences among salt concentrations at the same fermentation duration, while different lowercase letters indicate significant differences across fermentation times at the same salt concentration.

The unfermented watermelon rind sample exhibited an a^* value ranging from -3.26 to -3.02 , indicating a greenish hue. This green coloration of the watermelon rind is attributed to the presence of chlorophyll (Zhou et al., 2025). During fermentation, regardless of salt concentration, the a^* value decreased significantly ($p < 0.05$), reaching its lowest level at 48 h. Chlorophyll is highly sensitive to acidic conditions; under low pH, the central Mg^{2+} ion of chlorophyll is displaced, resulting in the formation of olive-brown pheophytin (Santra et al., 2021). As fermentation progressed, the pH of the watermelon rind decreased significantly (Figure 4.5), which likely contributed to the marked decline in a^* values. A similar reduction in a^* values during fermentation has also been reported for green bell pepper (Janiszewska et al., 2022).

The b^* value of watermelon rind ranged from 10.39 to 12.64 in the unfermented sample. Positive b^* corresponds to the yellowish nature of watermelon rind. Significant higher b^* value in the unfermented 6 % sample corresponds to protection from enzymatic browning by the salt. Usually, enzymatic browning causes the reduction in yellowness and an increase in brown tone causing the reduction in b^* value. On fermentation, high salt concentration preserved the yellow color.

4.6 Phenolic and DPPH Scavenging Activities

Salt and fermentation time and their interaction had significant effects ($p < 0.05$) on the total phenolic content of watermelon rind. In the unfermented watermelon rind, the TPC ranged from 0.088 to 0.115 mg GAE/g of sample (Table 4.4). These values were higher than those reported by Neglo et al. (2021); but were comparable to the findings of Priastomo et al. (2024), who reported the TPC values between 1.63 to 3.57 mg GAE/g DM for the watermelon rind collected from different parts of Indonesia. Similarly, Ho et al. (2018) reported TPC values ranging from 1.63 to 1.94 mg GAE/g DM in methanol-extracted watermelon rind powder. In contrast, Long et al. (2024) reported higher phenolic contents (7.13–8.20 mg GAE/g DM) in watermelon rind powder of varying particle sizes. These variations indicate that TPC is strongly influenced by factors such as extraction method, cultivation location, and

varietal differences. Supporting this, Tlili (2010) demonstrated that bioactive compounds, antioxidant activity, and phenolic content vary significantly with cultivar and growing conditions.

In general, TPC was higher at higher salt concentration in all fermentation time. Except for day 0, no significant difference was observed between 4 % and 6 % salt samples. Higher concentrations of NaCl create a substantial osmotic pressure gradient across the membranes of the rind cells. This pressure forces the rapid efflux of intracellular water and soluble solutes with free phenolic acids into the surrounding liquid phase. Addition of salt and other osmolyte showed an increased phenolic content in apple, banana and potato (Rózek et al., 2010). Zubaidah et al. (2020) also reported higher TPC on increasing the salt concentration during 5-day fermentation of sauerkraut.

Table 4.4 TPC and DPPH free radical scavenging activity of fermented watermelon rind at different salt concentrations and fermentation times

Time (h)	TPC (mg GAE/g FW)			DPPH scavenging activity (mg TE/g FW)		
	2% salt	4% salt	6% salt	2% salt	4% salt	6% salt
0	0.088±0.008 ^{Bd}	0.096±0.014 ^{ABc}	0.115±0.011 ^{Ac}	0.513±0.023 ^{Bd}	0.621±0.024 ^{Ac}	0.637±0.015 ^{Ad}
12	0.112±0.005 ^{Bc}	0.142±0.002 ^{Ab}	0.140±0.004 ^{Ab}	0.547±0.034 ^{cd}	0.723±0.032 ^{Bd}	0.830±0.022 ^{Ac}
24	0.127±0.006 ^{Bb}	0.156±0.002 ^{Aab}	0.159±0.002 ^{Aa}	1.009±0.054 ^{Bc}	1.024±0.033 ^{ABc}	1.101±0.026 ^{Ab}
36	0.127±0.005 ^{Bb}	0.145±0.012 ^{Aab}	0.144±0.004 ^{Ab}	1.089±0.020 ^{Bb}	1.116±0.025 ^{ABb}	1.140±0.019 ^{Ab}
48	0.151±0.004 ^{Aa}	0.161±0.010 ^{Aa}	0.157±0.005 ^{Aa}	1.189±0.009 ^{Ba}	1.242±0.027 ^{Aa}	1.281±0.025 ^{Aa}

Note Values are expressed as mean ± standard deviation. Different uppercase letters indicate significant differences among salt concentrations at the same fermentation duration, while different lowercase letters indicate significant differences across fermentation times at the same salt concentration. Time= fermentation time.

As the fermentation progressed to 12 and 24 h, the TPC increased significantly across all salt concentrations. In the 6% salt treatment, the TPC peaked at 24 h (0.159 mg GAE/g FW) and stabilized at 48 h. For the 2% and 4% treatments, the increase was more gradual but persistent, reaching their highest values of 0.151 mg GAE/g FW and 0.161 mg GAE/g FW, respectively, at the 48-h mark. Overall, samples with 6 % salt exhibited a little increase of 36.5 %, but those with 2 % salt showed a substantial

increase of 71.59 % in TPC. In lower salt concentration, diverse microflora, including bacteria and various yeast and mold, might have hydrolyzed the plant cell in greater extent to cause significant increase in phenolic content (Đorđević et.al., 2010). In fermentation, hydrolytic enzymes elaborated by microorganisms, for e.g. amylases, xylanases and proteases might break down the fruit cell wall or other compounds that bind to the phenolic compounds. During fermentation, enzymes from the fruit and microorganisms can facilitate the release of bound phenolics by enzymatic treatment of samples prior to extraction (Kumar et.al., 2025). Furthermore, lactic acid bacteria themselves can produce phenolic compounds in fermentation substrate (Kao and Lin, 2023).

Đorđević et.al. (2010) reported a significant increase in TPC after fermentation with *L. ramnosus* and *S. cerevisiae* on fermentation of various cereals and pseudocereals. However, they observed that TPC value is always higher for LAB than the yeast. In blueberry pomace, dramatic increase in TPC value from 1066 to 4269 µg GAE/ml after fermentation with *L. ramnosus* and *L. plantarum* (Yan et al., 2019). Kao and Lin (2023) found that natural fermentation of cucumber led to an increase of TPC up to day 8, thereafter it started to decline. Generally, fermentation increases the phenolic content by releasing bound phenolic compounds. However, after long fermentation, phenolic compounds might be oxidized, bound with other constituents, degraded by microbial enzymes (Adebo and Gabriela Medina-Meza, 2020). In our sample, the fermentation period was limited, which might be the cause of consistent increase in total phenolic content.

The antioxidant capacity of the fermented rind, assessed using the DPPH radical scavenging assay, exhibited a trend similar to that observed for TPC. Phenolic compounds are known to contribute significantly to the antioxidant activity of plant-based samples. The antioxidant activity was found to be 0.513 to 0.637 mg TE/g FW sample. The value corresponds to Neglo et al. (2021), who reported the DPPH radical scavenging activity as 27186-34820 µmol TE/kg (6.80-8.72 mg TE/g DW). During the 48-hour fermentation period, the DPPH radical scavenging activity increased more than two-fold, corresponding closely with the observed rise in total phenolic content. A similar increasing trend in antioxidant activity during fermentation has

been reported by Hashemi and Jafarpour (2020) in bergamot juice fermented with mixed strains of *Lactobacillus plantarum* over 72 h.

Specifically, in the 6% salt treatment, antioxidant activity increased from 0.637 mg TE/g FW at 0 h to 1.281 mg TE/g at 48 h. Although the TPC in this treatment stabilized after 24 h, antioxidant activity continued to increase until 48 h. This suggests the contribution of non-phenolic antioxidants, such as citrulline. Citrulline is known to be an effective hydroxyl radical scavenger with strong antioxidant potential (Zia et al., 2021). Consistent with this, the higher citrulline concentration observed at 6% salt in the present study may have contributed to the continued enhancement of antioxidant activity even after phenolic compounds reached a plateau.

4.7 Metabolites of Watermelon Rind Fermented in Optimal Condition as Determined by LC-QTOF-MS/MS

The untargeted metabolite profile of the optimally fermented (6% salt and 12 h fermentation time) watermelon rind sample was determined using liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS), a high-resolution analytical technique capable of identifying a broad spectrum of low-abundance bioactive compounds in complex food matrices (Aslam et al., 2021). A range of metabolites was tentatively identified across six major chemical classes: amino acids and peptides, lipids, nucleosides/nucleotides, organic acid derivatives, phenolic compounds, and sugar derivatives (Table 4.5).

Gamma-aminobutyric acid (GABA) is a non-protein, four-carbon amino acid that functions as the primary inhibitory neurotransmitter in the mammalian central nervous system. In fermented foods, it is biosynthesized by LAB through the irreversible α -decarboxylation of L-glutamic acid, a reaction catalyzed by the enzyme glutamate decarboxylase (GAD) (Yogeswara et al., 2020). Among the amino acids identified, GABA was detected in the form of its ethyl ester (gamma-aminobutyric acid ethyl ester, m/z 131.0943). The detection of L-citrulline (m/z 175.0976) in the fermented rind is consistent with our result as the most abundant amino acid in the

watermelon rind. Furthermore, Abu-Reidah et al. (2013) also reported citrulline detection in this m/z range in watermelon rind extract. The compounds 4-oxoornithine (m/z 146.0694) and (2R,4S)-2,4-diaminopentanoate (m/z 132.0915) were also identified and are recognized as oxidative catabolites of ornithine, an amino acid closely related to the urea cycle. These compounds are likely formed through microbial enzymatic action on ornithine present in the substrate, as described by Barker (1981) in the context of amino acid degradation by anaerobic bacteria. Several oligopeptides were identified in the fermented rind, including Glu-Pro (m/z 244.1073), Asn-Gly-Pro (m/z 286.1332), Lys-Gly-His (m/z 340.1892), Ala-Asp-Pro-Ser (m/z 388.1613), Ala-Ala-His-Val (m/z 396.212), Ala-Leu-Ser-Leu (m/z 402.2479), and Ala-His-Pro-Val (m/z 422.2321). Additionally, N-succinyl-L-diaminopimelic acid (m/z 290.1127) and L-hydroxylysine (m/z 208.1097) were also detected as amino acid derivatives. The generation of these peptides is attributable to the proteolytic activity of fermentation microorganisms, particularly LAB, which possess cell-envelope proteinases and intracellular peptidases capable of hydrolyzing proteins into bioactive peptide fragments (Peres Fabbri et al., 2024).

Three classes of lipid-related compounds were identified in the fermented rind: a hydroxy fatty acid, an acylcarnitine, and three phospholipids. DL-2-hydroxy stearic acid (m/z 300.2652) is a hydroxylated derivative of stearic acid, and its presence is consistent with the well-documented capacity of LAB to hydroxylate long-chain fatty acids via hydratase and oxidoreductase enzymes (Fiorino et al., 2023). Linoleyl carnitine (m/z 423.3344) was identified as a significant lipid metabolite. This acylcarnitine is formed when linoleic acid, which is the most abundant polyunsaturated fatty acid in watermelon rind, is conjugated with L-carnitine to facilitate mitochondrial beta-oxidation (Zia et al., 2024). Three phospholipids were tentatively identified: phosphatidylserine [PS(P-16:0/18:3(9Z,12Z,15Z)), m/z 741.5003], phosphatidylglycerol [PG(P-20:0/18:3(9Z,12Z,15Z)), m/z 830.5663], and phosphatidylinositol [PI(20:0/22:0), m/z 950.6773]. These phospholipids are essential amphiphilic constituents of biological membrane bilayers in both plant and microbial cells. The detection of these phospholipids in the fermented sample likely reflects both the structural integrity of

plant cell membranes and the de novo phospholipid biosynthesis or turnover by the fermenting microorganisms (Aslam et al., 2021; Sohlenkamp & Geiger, 2016).

Table 4.5 Metabolites in fermented watermelon rind obtaining from optimal fermentation condition

Proposed compound	Empirical formula	Mass (m/z)	Retention Time	Intensity (in Log ₂)
Amino acid and peptides				
gamma-Aminobutyric acid ethyl ester	C ₆ H ₁₃ NO ₂	131.0943	2.6574	19.20
(2R,4S)-2,4-Diaminopentanoate	C ₅ H ₁₂ N ₂ O ₂	132.0915	1.7029	20.99
4-Oxoornithine	C ₅ H ₁₂ N ₂ O ₃	146.0694	1.6733	17.33
Citrulline	C ₆ H ₁₃ N ₃ O ₃	175.0976	1.7028	20.49
L-Hydroxylysine	C ₆ H ₁₄ N ₂ O ₃	208.1097	19.2400	19.14
DL-Histidine	C ₆ H ₉ N ₃ O ₂	155.0730	20.2100	15.69
Lysine butyrate	C ₁₀ H ₂₂ N ₂ O ₄	234.1615	21.4446	19.32
Glu Pro	C ₁₀ H ₁₆ N ₂ O ₅	244.1073	19.1187	17.10
Asn Gly Pro	C ₁₁ H ₁₈ N ₄ O ₅	286.1332	18.8078	18.30
N-Succinyl-L-diaminopimelic acid	C ₁₁ H ₁₈ N ₂ O ₇	290.1127	19.1189	18.60
Lys Gly His	C ₁₄ H ₂₄ N ₆ O ₄	340.1892	18.3203	17.93
Amino acid and peptides				
Ala Asp Pro Ser	C ₁₅ H ₂₄ N ₄ O ₈	388.1613	22.3891	16.96
Ala Ala His Val	C ₁₇ H ₂₈ N ₆ O ₇	396.2120	18.1681	15.83
Thr Met Thr	C ₁₃ H ₂₅ N ₃ O ₆ S	397.1513	22.8744	18.65
Ala Leu Ser Leu	C ₁₈ H ₃₄ N ₄ O ₆	402.2479	10.2033	17.07
Ala His Pro Val	C ₁₉ H ₃₀ N ₆ O ₅	422.2321	23.2433	19.80
Lipids				
DL-2-hydroxy stearic acid	C ₁₈ H ₃₆ O ₃	300.2652	22.1953	17.31
Linoleyl carnitine	C ₂₅ H ₄₅ NO ₄	423.3344	20.8619	14.99
PS(P-16:0/18:3(9Z,12Z,15Z))	C ₄₂ H ₇₈ NO ₈ P	741.5003	16.5283	20.97
PG(P-20:0/18:3(9Z,12Z,15Z))	C ₄₄ H ₈₉ O ₉ P	830.5663	16.7102	16.09
PI(20:0/22:0)	C ₅₁ H ₉₉ O ₁₃ P	950.6773	16.8192	16.19
Organic acids derivatives				
L-(+)-Lactic acid	C ₃ H ₆ O ₃	112.0137	1.9470	18.70
DL-Malic acid	C ₄ H ₆ O ₅	134.0190	1.8430	23.16
Citric acid	C ₆ H ₈ O ₇	192.0266	2.3340	22.94

Table 4.5 (continued)

Proposed compound	Empirical formula	Mass (m/z)	Retention Time	Intensity (in Log₂)
Gluconic acid	C ₆ H ₁₂ O ₇	196.0565	1.7050	18.93
Nonanedioic acid	C ₉ H ₁₆ O ₄	188.1046	16.9767	18.43
Orthothymotinic acid	C ₁₁ H ₁₄ O ₃	194.0941	19.3389	20.70
Phloionolic acid	C ₁₈ H ₃₆ O ₅	332.2559	19.4588	18.28
L-ascorbic acid	C ₆ H ₈ O ₆	176.0274	1.8270	14.18
Phenolic compounds				
cis-Cinnamic acid	C ₉ H ₈ O ₂	148.0525	4.4633	17.08
3,4-Dichloroisocoumarin	C ₉ H ₄ Cl ₂ O ₂	213.9586	1.8350	19.53
4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.0312	8.1090	17.70
Methyl 3,4,5-trimethoxycinnamate	C ₁₃ H ₁₆ O ₅	252.0996	19.2320	19.75
Butyl 3-O-caffeoylquininate	C ₂₀ H ₂₆ O ₉	456.1630	16.4052	15.01
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.0898	1.9229	15.88
Quercetin hexoside	C ₂₁ H ₂₀ O ₁₂	464.1087	1.9389	15.24
Sinapic acid hexoside	C ₁₇ H ₂₁ O ₁₀	385.1208	1.7730	15.21
Sugar derivatives				
Trehalose	C ₁₂ H ₂₂ O ₁₁	354.0898	1.6380	15.67
L-Sorbose	C ₆ H ₁₂ O ₆	180.0608	1.7859	17.27
2,3-Dioxogulonic acid	C ₆ H ₈ O ₇	192.0278	2.3377	21.81
Salicylic acid glucoside	C ₁₃ H ₁₆ O ₈	300.0847	5.8609	18.54
1-O-E-Cinnamoyl-(6-arabinosylglucose)	C ₂₀ H ₂₆ O ₁₁	442.1475	13.4262	17.58
dihydrophaseic acid 4-O-beta-D-glucoside	C ₂₁ H ₃₂ O ₁₀	444.1995	8.0126	17.63

Several organic acids were detected in the sample including lactic acid (m/z 112.0137), malic acid (m/z 134.019), citric acid (m/z 192.0266) and gluconic acid (m/z 196.0565), among them citric acid and malic acid was found dominant. Other research also reported malic and citric acid as the predominant organic acids in watermelon and are key intermediates of carbon metabolism (Aslam et al., 2021). Their presence in watermelon rind has also been previously reported by Reddy et al. (2023). The detection of lactic acid can be attributed to the metabolic activity of LAB, which ferment sugars present in the rind. Nonanedioic acid (azelaic acid, m/z 188.1046) was identified as a dicarboxylic acid, consistent with its reported presence as one of the major organic acids in watermelon (Aslam et al., 2021). Orthothymotinic

acid (m/z 194.0941) and phloionolic acid (m/z 332.2559) were also detected; these compounds have previously been reported by Moukamnerd et al. (2025) during fermentation of seagrass (*Caulerpa lentillifera*) and coffee cherry pulp by *L. plantarum*, suggesting that these metabolites may be common microbial metabolic products during LAB-mediated plant fermentation. Furthermore, L-ascorbic acid (m/z 176.0274) was detected at an early retention time, consistent with its polar nature. This compound is well known for its strong antioxidant capacity, and its presence in watermelon rind has been confirmed by Acun et al. (2025).

In addition to organic acids, several phenolic compounds were identified. Cinnamic acid in its cis-form (m/z 148.0525) was detected along with its methoxylated derivative, methyl 3,4,5-trimethoxycinnamate (m/z 252.0996). Other phenolic acids and their derivatives, including 4-hydroxybenzoic acid, coumaric acid derivatives (e.g., 3,4-dichloroisocoumarin), chlorogenic acid, and its ester form (butyl 3-O-caffeoylquininate), were also present. Additionally, two important phenolic conjugates, sinapic acid hexoside and quercetin hexoside were also identified. Most of these phenolic compounds were reported by Acun et al. (2025) and El Gizawy et al. (2022) in watermelon rind sample.

Several sugar-related compounds were detected, most of which appeared as conjugates with other phytochemical classes. Trehalose (m/z 354.0898), a disaccharide composed of two glucose units, was observed at an early retention time; its presence has also been reported by El Gizawy et al. (2022). L-sorbose (m/z 180.0608) is a monosaccharide that may arise through the microbial oxidation of L-sorbitol or the isomerization of fructose by LAB enzymes during fermentation. 2,3-Dioxogulonic acid (m/z 192.0278) is a well-characterized intermediate in the catabolism of ascorbic acid (Vitamin C), arising through the oxidation of L-dehydroascorbic acid, and its presence might be due to non-enzymatic and enzymatic degradation of ascorbic acid in the fermented substrate (Wechtersbach et al., 2011). Two phytohormone-derived glycoside conjugates were detected. Salicylic acid glucoside (m/z 300.0847) is the inactive vacuolar storage form of salicylic acid. In plants, Salicylic acid is an important phenolic phytohormone involved in systemic acquired resistance, growth regulation, and defense signaling against pathogens (Vlot et al., 2021). The detection of salicylic acid glucoside in the fermented sample is

consistent with earlier phytochemical profiling of watermelon by Abu-Reidah et al. (2013), who reported its presence in watermelon extracts using HPLC-ESI-QTOF-MS. Dihydrophaseic acid 4-O-beta-D-glucoside (DPAGS, m/z 444.1995) was also identified. Dihydrophaseic acid (DPA) is a principal catabolite of another plant hormone, abscisic acid (ABA), formed via the sequential 8'-hydroxylation of ABA to phaseic acid and its subsequent reduction to DPA by phaseic acid reductase (Milborrow & Vaughan, 1982).

The LC-QTOF-MS/MS analysis identified a diverse range of compounds in the fermented watermelon rind across several chemical classes. The result shows that the sample contains both original plant metabolites, such as L-citrulline, citric acid, malic acid, and some phenolic acids, and metabolites formed during fermentation, including GABA, lactic acid, and various oligopeptides. This indicates that while the microorganisms preserved many of the rind's natural components, they also converted existing metabolites into various other derivatives. Specifically, the detection of peptides and organic acid derivatives suggests that microbial enzymatic activity modified the protein and carbohydrate structures of the substrate.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

This study demonstrated that temperature, salt concentration and fermentation duration impacted the microbial dynamics and biochemical parameters during natural fermentation of watermelon rind. A fermentation temperature of 30°C and salt concentration of 6 % was identified as optimal, providing the most effective balance for sustained LAB growth and rapid acidification (minimum pH 3.23). Furthermore, the fermentation process effectively ensured food safety by eliminating Enterobacteriaceae within 5 days and suppressing yeast and mold populations within 2 to 6 days depending on the salt concentration. The metabolic flux of the ADI pathway was found to be strongly salt-dependent. While low-salt conditions (2% and 4%) led to the depletion of citrulline to ornithine while a 6% salt concentration successfully inhibited its further metabolism therefore causing accumulation of citrulline in the fermented sample. A salt concentration of 6% and a fermentation period of 12 h were identified as optimal conditions for enhancing citrulline content. Notably, even after 12 h of fermentation, citrulline levels remained significantly higher than in the unfermented sample. Other free amino acids were differentially influenced by salt concentration and fermentation duration. Total phenolic content (TPC) and antioxidant activity (DPPH radical scavenging) increased significantly across all fermentation conditions. Additionally, higher salt concentrations helped preserve the natural color attributes, particularly lightness and yellowness. At the optimum condition for citrulline (6% salt and 12 h of fermentation), various metabolites were identified, including amino acids, peptides, sugar derivatives, phenolic acids, and organic acids and their derivatives Overall, this study highlights the potential of fermentation *in situ* as an effective valorization strategy for watermelon rind, a commonly discarded agro-industrial by-product, by transforming it into a safe, nutritionally enriched, and functionally enhanced food ingredient. The

improvement in bioactive compounds, including citrulline, phenolics, and antioxidant capacity, along with the development of desirable umami-related amino acids, contributes to enhanced functional quality and palatability. Moreover, rapid acidification and microbial suppression ensure product safety and shelf stability. These findings support the integration of fermented watermelon rind into sustainable food systems, promoting waste reduction, value addition, and the development of novel functional foods.

5.2 Recommendations

1. Future studies should focus on the detailed characterization of microbial succession during fermentation, including the identification and quantification of specific LAB strains as well as the monitoring of particular foodborne pathogenic microorganisms to further validate safety aspects.

2. The risk of pesticide and agrochemical residues in watermelon rind is high, being the outer layer of the fruit. Future studies should focus on this aspect

3. As the optimum condition for citrulline enhancement is in the early phase of fermentation, there is high chance of presence of pathogenic microorganisms in that condition, as shown in Enterobacteriaceae count. Future studies should focus on identification, quantification and their reduction strategy of pathogenic microorganisms specifically *S. aureus*, *Salmonella*, *Bacillus cereus* and *Clostridium perfringens*.

4. The role of individual LAB strains in citrulline biosynthesis could be investigated to determine strain-specific capabilities for enhancing functional compounds during fermentation.

5. The inclusion of pH as an independent experimental factor may provide deeper insight into its influence on microbial dynamics, metabolic pathways, and overall fermentation performance. Therefore, controlled pH fermentation systems could be explored in this context.

6. Advanced molecular approaches (e.g., 16S rRNA sequencing or metagenomics) could be employed to identify the microbial communities associated with citrulline accumulation and their functional contributions.

7. Further exploration of enzymatic activities and gene expression, particularly those involved in the Arginine Deiminase (ADI) pathway in this substrate may help elucidate the biochemical mechanisms underlying citrulline production.

8. Sensory evaluation is fundamental part of valorization therefore studies could be incorporated to assess consumer acceptability, especially in terms of flavor, aroma, and texture development during watermelon fermentation.



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