

**DEVELOPMENT OF PROCESSING METHODS FOR EXTENDING
SHELF LIFE OF COCONUT NEERA**

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Submitted by

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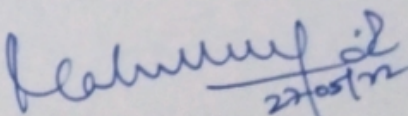
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CERTIFICATE

This is to certify that the thesis entitled “**Development of processing methods for extending shelf life of coconut neera**” submitted in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Biotechnology** to the Bharathidasan University, Tiruchirappalli is a record of bonafide research work carried out at *National Institute of Food Technology, Entrepreneurship and Management-Thanjavur (NIFTEM-T)* by **Ms. S. LATHA** (ID. No.: 201710DB005) under my supervision and guidance and that no part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes. However, some part of the research work has been published in peer-reviewed/ reputed scientific journals (copies enclosed).

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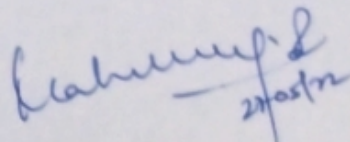
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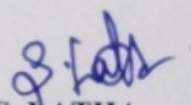
DECLARATION

I hereby declare that the work presented in this thesis entitled “**DEVELOPMENT OF PROCESSING METHODS FOR EXTENDING SHELF LIFE OF COCONUT NEERA**” submitted by me for the award of the degree of **Doctor of Philosophy in Biotechnology** to the Bharathidasan University, Tiruchirappalli is a record of the research work I have carried out under the supervision of **Dr. R. Mahendran**, Associate Professor and Head, Centre of Excellence in Non-Thermal Processing, National Institute of Food Technology, Entrepreneurship and Management -Thanjavur.

I further declare that this thesis is based on the original work done by me and has not been previously submitted to any other Institution/University for any degree/diploma by me or any other person. Research materials obtained from other sources have been duly acknowledged in this thesis.

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ABSTRACT

The natural sap from coconut tree (*Cocos nucifera*) is a rich source of sucrose, minerals and has low Glycemic Index. The spontaneous fermentation affects the physicochemical property, consumer acceptability and shelf life on atmospheric storage. Hence, it is important to reduce the microbial population to preserve the quality through the processing methods. In order to control rapid fermentation, freezing, preservatives, atmospheric pressure plasma jet and mild heat treatment were employed in neera. These methods were evaluated for microbial survival, physicochemical, and sensory properties on storage. Results indicated the reduced microbial survival for the method of freezing at -20°C. The frozen stored neera at -20°C exhibited the total viable and yeast survival as 6.26 ± 0.47 log CFU/ml, 3.18 ± 0.00 log CFU/ml and inhibited lactic acid bacteria (LAB) on 28 d. The microbial survival (28 d) were correspondent to the log reduction of 1.36 (6.26 ± 0.47 log CFU/ml) and 1.81 (3.18 ± 0.00 log CFU/ml) for total viable and yeast count respectively. The 50 ppm nisin and 3000 ppm calcium carbonate in N4 treatment, resulted log reduction of 0.48 for yeast and did not provide log reduction for total bacteria on 21 d. The plasma treatment at 35kV-7.5 min revealed the log reduction of 0.45 and 0.47 for total bacteria and yeast up to the storage period of 24 h. The double-stage heat treatment at 60°C-10 min resulted the log reduction of 1.16 ± 0.01 , and 0.86 ± 0.01 for total viable count and yeast on 21 d. Among the four methods, freezing at -20°C had highest microbial log reduction up to the storage period of 28 d, which had less changes in the physicochemical properties in neera (pH- 5.37 ± 0.02 and total soluble solids 14.0 ± 0.00 °Brix) and hence, it revealed better sensory attributes up to 21 d. Therefore freezing at -20°C extend shelf life for longer period (21 d) than the atmospheric storage of coconut neera.

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LIST OF ABBREVIATIONS AND SYMBOLS

LAB-----	Lactic Acid Bacteria
CFU-----	Colony Forming Units
TNTC-----	Too Numerous To Count
N1-----	50 ppm Nisin and 500 ppm Sodium Benzoate
N2-----	50 ppm Nisin and 1000 ppm Sodium Benzoate
N3-----	50 ppm Nisin and 2500 ppm Calcium Carbonate
N4-----	50 ppm Nisin and 3000 ppm Calcium Carbonate
AO-----	Acridine Orange
PI-----	Propidium Iodide
DNA-----	Deoxyribo Nucleic Acid
D-----	Decimal reduction time
ANOVA-----	Analysis of Variance
TEM-----	Transmission Electron Microscopy
FDA-----	Food and Drug Administration
FSSAI-----	Food Safety and Standards Authority of India
RONS-----	Reactive Oxygen and Nitrogen Species
g-----	Gram
mg-----	Milligram
L/l-----	Liter
ml-----	Milliliter
µg-----	Microgram
µl-----	Microliter

M-----	Molarity
mM-----	Millimolar
N-----	Normality
ppm-----	Parts Per Million
h-----	Hour(s)
d-----	Day(s)
min-----	Minute(s)
°C-----	Degree Celsius
kV-----	Kilovolt
%-----	Percentage
mPa s-----	Millipascal second
cm-----	Centimeter
mm-----	Millimeter
nm-----	Nanometer

CHAPTER I

I INTRODUCTION

1.1 The background of the research

Neera is a sweet, yellow coloured, inflorescence sap from the coconut palm tree (*Cocos nucifera*). In ancient literature, the tree is termed as 'Kalpavriksha'. Currently, the crop production is reported in 92 countries with a harvesting area of more than 10 million hectares. Moreover, the worldwide scenario by Robert et al., (2019) listed that, Philippines, India, and Indonesia were the primary producers who contributed 75 % of the total production. Srilanka, Mexico, Vietnam, Thailand, Brazil, and the Ivory Coast were other coconut-growing countries (Harshini and Samuel, 2019). According to the estimation of coconut development board in 2020, Kerala, Karnataka, Tamil Nadu, and Andhra Pradesh are the major producers with an area of 760.78, 624.03, 437.57 and 111.38 million hectares, respectively (CDB, 2020). In this region, the neera production and marketing are organized through the coconut producers' federations/society/companies. These organized bodies reduced the coconut growers' economic loss (Thamban et al., 2020).

The oozing sap from the coconut inflorescence is the rich source of sucrose (16.19 g/100 ml), potassium (168.4 mg/100 ml), sodium (90.6 mg/100 ml), phosphorous (3.9 mg/100 ml), and proven low glycemic index drink (GI-35) with a neutral pH (7) (Hebbar et al., 2018). Due to its vital nutrients, the rural people collected neera from inflorescence in a mud pot and consumed in the early morning (before the sunrise). The traditional tapping technique including stroking and slicing was executed on the selected inflorescence for the ooze out of neera (Francisco-ortega and Zona, 2013). The continuous process of tapping

yields neera, and the regular tapping in the morning and evening allowed the exudation up to 50 d from the inflorescence. The tapped single inflorescence can yield around 1-2 l/d of neera for the period of six months (Ghosh et al., 2018; Samsudeen et al., 2013). According to the neera act 2017 of Tamil Nadu, a maximum of 60 inflorescences per ha is allowed for neera tapping.

In addition to sugars and minerals, other nutritional benefits of vitamins (thiamine, riboflavin, niacin), antioxidants, amino acids, and volatile components are also present in neera (Flores-Gallegos et al., 2019). The volatile components 2-butanol, acetic acid, 2-methylcyclohexane, cyclohexilactone and the amino acids of glutamic acid, threonine, aspartic acid and serine were reported as major components in fresh neera (Purnomo, 2007). The nephroprotective activity, hepatoprotective activity, and few probiotics were the other benefits of neera (Asha et al., 2019; Somashekaraiah et al., 2019). However, all the nutritional benefits were affected by the growth of various microorganisms namely *Bacillus*, *Lactobacillus*, *Micrococcus*, *Enterobacter*, *Leuconostoc*, *Saccharomyces*, *Candida*, and *Pichia* (Atputharajah et al., 1986). These microorganisms exhibit spontaneous fermentation at atmospheric condition, which affect the physicochemical quality of neera. This undesirable changes make it unsuitable for consumption and spoil the neera in less than 24 h.

1.2 Definition of the problem

The atmospheric storage of neera immediately induces the fermentation by the indigenous microorganism. These microorganisms are the native flora of the palm tree, microbes from the atmosphere and the existing flora of the tapping receptacle (Atputharajah et al., 1986).

The heterogeneous microorganism is mainly from the community of the bacteria (39 isolates) and yeast (166 isolates). Among the microbes *Bacillus*, *Lactobacillus*, *Micrococcus*, *Enterobacter*, *Leuconostoc*, *Saccharomyces*, *Candida*, and *Pichia* were dominant genera involved in the fermentation. The fresh neera is a source for higher level of bacteria (10^6 cells/ml) and a lesser yeast (10^4 cells/ml). These microbial population and type of microorganisms varies with atmospheric condition (Singaravadivel et al., 2012). Among the microbes, the lactic acid bacteria (LAB) begins the hydrolysis of sugar by lactic acid fermentation, which converts the sugar into various acidic products. The conversion occurs rapidly within 9 h of storage (Shetty et al., 2017). At the end (20-24 h) the bacterial fermentation increased the total acidity of the neera due to the presence of lactic acid, acetic acid and citric acid (Borse et al., 2007). Moreover, the lactic acid fermentation drop down the pH to 4 (at 20 h) due to the developed acidity (2.5 mg/ L). Hence, the reduced rate of fermentation at refrigerated storage also affected the physicochemical properties of neera (Pandiselvam et al., 2021). The decline in the physicochemical property especially the pH to 4, which increases the concentration of invertase which promotes the yeast growth up to 10^8 cells/ml within 48 h of storage (Atputharajah et al., 1986). On alcoholic fermentation, the yeast causes an increase in alcohol content from 0.2 to 4.5 %. Overall, fermentation process affects quality of neera in terms of its colour, pH, acidity, taste, and flavour. The developed quality changes affect consumer acceptance and gives a huge loss to coconut neera producers.

The existing technology preserved the neera by combining various methods such as the addition of preservatives (nisin, acidifier and potassium salts), filtration, centrifugation and

pasteurization (Chinnamma et al., 2019). Another patented technology preserved the neera by adding preservatives (citric acid and nisin) and pasteurization (Ramalakshmi, et al., 2004). The conventional processing methods and the number of preservatives affect the flavour, nutritional composition, and other organoleptic qualities of neera (Ghosh et al., 2018). Even refrigerated storage, spoils neera quality within 2-3 d. In this regard, there is a need for developing processing methods to control the abundant growth of microorganisms in neera for shelf life extension. Based on the microbial spoilage and issues on the existing techniques, the current research aimed at controlling the bacteria and yeast growth in neera by applying four different processing methods such as freezing, the addition of preservatives, non-thermal atmospheric pressure plasma, and mild heat treatment. These methods were developed and carried out at National Institute of Food Technology, Entrepreneurship and Management-T, (Formerly-Indian Institute of Food Processing Technology) with the following objectives.

1.3 Objectives

- Effect of freezing on microbial and storage stability of coconut neera
- Impact of preservatives on natural fermentation of coconut neera
- To study the feasibility of thermal and non-thermal methods on coconut neera

1.4 Scope of the research work

The developed methods extended the shelf life of neera, which can be popularized as the nutritional drink among people. The preserved qualities of neera could be promoted as an acceptable drink among the consumer and its commercialization in the global market will reduce the economic loss of neera producers.

CHAPTER II

REVIEW OF LITERATURE

In this chapter, the literatures on the nutritional importance of neera, biology of the spontaneous fermentation and the existing methods for preservation of neera are discussed (Table 2.1). The developed methods for the inhibition of microorganisms, namely freezing, preservatives, non-thermal plasma and thermal treatment are also discussed in detail.

2.1 Nutritional importance of neera

The natural sap obtained from the tapping of coconut inflorescence (*Cocos nucifera* L) is widely called neera and it is a popular drink among rural people. The collected fresh neera is highly sweet due to the composition of 12-15 % sucrose and a trace amount of glucose and fructose (Ghosh et al.,2018). Neera has a neutral pH (7), colour of golden yellow and a diabetic-friendly drink (GI 35) (Hebbar et al., 2018). The 100 ml of neera is the abundant source of minerals which includes sodium (90.6 mg), potassium (168.4 mg), phosphorous (3.9 mg), zinc (0.020 mg), magnesium (0.012 mg) and copper (0.031 mg) (Hebbar et al., 2018). Asghar et al. (2020) proved the minerals and vitamin content of neera by their demonstrated results of 960.87 mg/L-potassium, 183.21 mg/L-sodium, and 116.19 µg/ml-vitamin C. It also contain vitamins such as inositol (127.70 mg), thiamine (77.00 mg), nicotinic acid (40.60 mg), and riboflavin (12.20 mg). Moreover, Xia et al., (2011) reported the phenolic and gallic acid content of 0.33 g/L and 350 µg/L respectively in the unfermented fresh neera. This drink helps in suppressing kidney toxicity through the nephroprotective effect of inhibited oxidative stress and lipid peroxidation. The mineral

content especially phosphorous present in neera aids in improving renal health by releasing waste from kidneys. Moreover, their consumption induced functional properties of modulated inflammatory markers, metalloproteinase and oxidative stress which inhibited the hepatic damage in humans (Asha et al., 2019). Due to the vital nutrients and collection method, various heterogeneous microbes were introduced to neera. The bacteria of the genus *Lactobacillus*, *Micrococcus*, *Enterobacter*, *Leuconostoc*, *Bacillus*, and yeast of the genus *Candida*, *Pichia*, *Saccharomyces* and acetic acid bacteria were the most common genera isolated during natural fermentation (Atputharajah et al., 1986). Among the existing LAB, seven isolates were identified as probiotics which has survival capability in low pH, and various gastric conditions (Somashekaraiah et al., 2019). Moreover, probiotics isolates exhibited antibacterial, antifungal, and hydroxyl-scavenging activity. Owing to the health benefits of neera in its natural form, requires a preservation technique to extend its shelf life.

2.2 Biology of spontaneous fermentation

The heterogeneous microbes of the genus *Bacillus*, *Lactobacillus*, *Micrococcus*, *Enterobacter*, *Leuconostoc*, *Saccharomyces*, *Candida*, *Pichia*, and *Acetobacter*, ferment the neera at atmospheric conditions. These microbes utilize the sugar content in three-stages of fermentation such as early-stage lactic acid fermentation, middle alcoholic, and finally acetic acid fermentation. At the initial stage of fermentation, the bacterial count of 10^6 cells/ml was reported in neera (Atputharajah et al., 1986). The bacterial population (10^6 cells/ml), hydrolysed the sugar by the sucrose hydrolyzing enzyme and produced glucose and fructose in neera (Shetty et al., 2017). This conversion was rapid within 9 h of harvest.

The reducing sugars were fermented up to 24 h by LAB. At the end of 24 h, the pH of neera dropped from 7 (Initial) to 4. The reduced pH (4) is evident for conversion of total sugar, and it accounted to 6 g/100 ml from 16.19 g/100 ml (initial). The reduction was due to the conversion of sucrose into reducing sugar, i.e. glucose and fructose (1-3 d) (Xia et al., 2011). In addition to sugar reduction, the bacterial fermentation also increased the acidity from 15 to 177 mg/L by their acidic end products and volatile acidity (Borse et al., 2007). Pandiselvam et al. (2021) reported the fermentation kinetics in atmospheric storage reduced the physicochemical properties like pH (3.74), total soluble solids (14.87 ° Brix), and total acidity (0.28 mg/L) within 6 h. In addition to that, refrigerated neera also reduced their physicochemical properties (6.57-pH, 15.03 °Brix-total soluble solids, and 0.05 mg/L-total acidity) during storage period. The changes in the total soluble solids and total acidity confirms the fermentation during the refrigerated storage (Hebbar et al., 2015 and 2018).

The changes induced the invertase activity in yeast, which promote the alcoholic fermentation in neera. The enzymatic activity in the presence of reducing sugar (2.1 %) enhanced the yeast count to 10^8 cells/ml at 48 h of storage (Shetty et al., 2017; Atputharajah et al., 1986). Finally, alcohol producing genera of *Saccharomyces* increases the alcohol level (0.2 to 4.5 %) in neera. The alcohol content in neera favours the growth of acetic acid bacteria and their fermentation. This acetic acid fermentation increased the acidity of neera to high level after 5 d of storage (Xia et al., 2011). Addition to the major fermentative microorganisms, the survival of *Bacillus*, *Staphylococcus*, and *Enterobacter* were reported during fermentation (Atputharajah et al., 1986). The spontaneous fermentation produced

12 different volatile acids, alcohols, esters and among this palmitoleic acid and dodecanoic acid greatly affect the palatability of neera (Borse et al., 2007).

2.3 Existing preservation methods

The traditional method of open pot collection initiates the fermentation within period of 12 h. In order to control the fermentation during collection process, the inner surface of the pot is coated with calcium carbonate by the neera tappers (Ghosh et al., 2018); (Somawiharja et al., 2018). In some other countries, the different plant portions such as jack fruitwood chips (*Artocarpus heterophyllus*), exocarp fruit of mangosteen (*Garciana mangosteen*), hall bark (*Artha acuminata*) were used to have preservation effect on neera (Kapilan, 2015). However, these traditional methods partially preserve fermentation and highly affect the original quality of the neera. For collecting neera without fermentation Hebbar et al., (2015) have developed portable coco sap chiller. Chinnamma et al., (2019) developed the technology for extending shelf life of neera by the collection of processing techniques from tapping to bottling. During collection, this method sprayed the sodium hypochlorite as a disinfectant and collected the neera with 10 ml of preservative solution (5 mM-acidifier and 2 mM-potassium salts). Then the collected neera further processed using microfiltration (100 microns), pasteurization (70-85°C for 10-15 min) and bottled. In another patented technology neera was filtered and then added with preservative (citric acid-0.045 %, nisin-10 ppm), pasteurized (95°C for 5 min) and bottled (Ramalakshmi, et al., 2004). The existing technologies preserved the neera by the addition of preservatives, various processing techniques and pasteurization at 85-95°C. Among these thermal method of pasteurization affects the flavour and nutritional composition of neera (Chia et al., 2012).

Leena et al. (2021) have developed the nano fibrous membrane for the removal of yeast from neera. This technology controlled the pH (6.78 ± 0.10), total soluble solid (15.07 ± 1.80), acidity (1.77 ± 0.04) and colour (6.51 ± 0.11) of the neera after filtration. The above mentioned technology did not include the shelf life of the neera filtrate. Hence, it is important to preserve the quality while storing to extend the shelf life of neera through new processing techniques.

2.4 Freezing

Freezing is another method of preservation, where it inhibited the microbial viability in many foods, which maintains the product's original flavour. However, their destruction mechanism depends broadly on the size and distribution of ice crystals (intracellular or extracellular). The fast freezing rate generates small ice crystals in the food material, whereas the slow cooling rate develops larger ice crystals. According to Charoenrein and Harnkarnsujarit., (2017) the freezing exhibited in two different phases in food components, i.e. aqueous in equilibrium phase (crystallization) and non-aqueous in non-equilibrium phase (amorphous). The food components especially carbohydrates, lipids, protein, and organic acids are in the amorphous phase affect the pH, titratable acidity, and ionic strength of the food material (Damiani et al., 2013). The stability of frozen food depends on the type, chemical composition, and microbial population. O'Brien et al. (2016) have reported the traditional kefir upon frozen storage (-14 to -8°C) reduced the LAB and yeast survival. Like kefir, the frozen storage could effectively inhibit the LAB and yeast in neera and extend the shelf life.

2.5 Preservatives

Preservatives were mainly used to prevent microbial deterioration in many food products by damaging its genetic material and cell membrane. Among the preservatives, nisin (E234) from *Lactococcus lactis*, one of the natural anti-microbial peptides is categorized as safe by the World Health Organization. The bio-preservative (nisin) was used to arrest the growth of gram-positive and spore producing microorganisms (Economou et al., 2009). Pei et al., (2017) were reported that 100 µg/ml of nisin were effectively inhibited the lactic acid fermentation in food. Hence, their anti-microbial mechanism were limited with the gram-positive microorganisms (Pokhrel et al., 2019). The synergistic effect of preservatives inhibits the growth of various bacteria and yeast in a food product and also reduced the concentration of preservatives (Kashani et al., 2012). Another preservative of alkaline additive (calcium carbonate-E170) is used as a nutritional supplement, curing agent, bulking agent, and modifier. Moreover, these calcium carbonate was coated in collection vessel to reduce the natural fermentation (Somawiharja et al., 2018). According to the FSSR 6.1.19., (2010) the concentration of 5000 ppm calcium carbonate were allowed for the food preservation. The calcium carbonate nanoparticles also exhibited the anti-microbial mechanism. The nanoparticles concentration of 125 µg/ml and 62.5 µg/ml were effective to control the growth of gram-positive bacteria and gram-negative bacteria respectively (Ataee et al., 2011). The chemical preservative of sodium benzoate (E211) widely added in juice, margarine, sweet products to control the growth of fungi (Stanojevic et al., 2009). The addition of sodium benzoate (1000 ppm) in black olive fermentation was reported to have reduced yeast growth (Turantaş et al., 1999). Therefore, the synergistic

effect of nisin with chemical preservatives (sodium benzoate and calcium carbonate) could effectively control heterogeneous microbial communities.

2.6 Non-thermal plasma

The non-thermal methods can preserve foods with fresh-like qualities without affecting their nutritional properties. The methods preserve the foods with the processing technologies like high-pressure processing, pulsed-electric field, ultrasound, ultraviolet light and cold plasma through different mechanisms. Among them atmospheric pressure cold plasma is one of the emerging method with an advantage of high reactivity and self-quenching reactivity of plasma species. Moreover, an existing study on neera reported that there was an 80 % log reduction on microbial population using the cold plasma bubbling system by the action of reactive species (Aparajhitha and Mahendran., 2019). The reactive species are high-energy electrons, ionized atoms or particles, UV radiation and they were involved in the inactivation of microorganisms (Bourke et al., 2018). These reactive species affect the proteins, nucleic acids, lipids, membranes, and other internal organelles of the microorganisms, thus leading to cell death (Redza-Dutordoir and Averill-Bates., 2016). Among the plasma species, O₂ is primarily responsible for initiating the membrane lipid peroxidation, which affected the cell membrane, oxidative stress, and result in leakage of intracellular components (Xu et al., 2020).

However, in a liquid medium, plasma is separated into three regions namely, gas-phase plasma, plasma-liquid interface, and bulk-liquid region. In the region of the plasma-liquid interface, the plasma species reacts with the water vapour content of the liquid sample. Further, their reactivity in liquid phase were achieved through the collisions, diffusion,

solvation, absorption, desorption, and chemical transfer mechanism (Perinban et al., 2019). These mechanisms are complex, and their inactivation rate differs based on its experimental settings, gases, and surrounding atmosphere. The previous study reported that the plasma jet system reduced gram-negative bacteria and yeast up to 3 log reduction in the food matrix (Surowsky et al., 2015). Therefore, non-thermal plasma processing could be effective against the heterogeneous microbial population in neera as well.

2.7 Thermal treatment

The increased health concern among consumers improved the acceptance and productivity of thermally processed foods worldwide. Heat treatments between 65 to 95°C are the most intensive procedure to destroy nearly all the microorganisms (Løvdaal et al., 2011). The techniques increased the food product's shelf life with the acceptable sensory properties. The application of heat on foods depends mainly on the temperature-time combination. The combination differs according to the thermal death-time of the heat-resisting microorganisms (Amit et al., 2017). The temperature treatment affects the growth rate of LAB by the enzymatic inactivation. Some of the resisting enzymes favour LAB growth after heat treatment (Malika et al., 2019). Several studies have indicated that thermal treatment at 50°C have a lethal effect on the vegetative yeast cells, and it depends on the sensitivity (López-Malo et al., 1999). Among the microorganism, *Bacillus* have more resistance against heat, and their vegetative cells produce heat-resistant spores. These spores may germinate into vegetative cells after the heat treatment, and produces toxins and spoilage enzymes which affect the product quality. Therefore, *Bacillus* reduction would be an important factor to controlling the quality deterioration in terms of flavour and

toxicity effect in neera (Zhuang et al., 2019). Another method of heating is the “double-stage heating technology”. It is a modified method of tyndallization. This method is generally effective against bacterial spore inactivation in foods by the sublethal injury or lethal effect (Cho et al., 1999). Basically it consists of two-stage heat treatment, the primary method or single-stage treatment inactivates the microorganisms followed by the incubation for the enhanced growth of resistant microbes and *Bacillus*. Finally, the double stage or a secondary treatment inactivates the resistant microbes in a treated food sample (Løvdal et al., 2011). This application reduced the resistant microbes at sublethal temperatures, could be one of an effective processing method for extending shelf life of neera.

Table 2.1: Review of literature on the existing studies in coconut neera

Research Findings	Author and year
The study identified the predominant genera of <i>Bacillus</i> , <i>Lactobacillus</i> , <i>Micrococcus</i> , <i>Enterobacter</i> , <i>Leuconostoc</i> , <i>Saccharomyces</i> , <i>Candida</i> , <i>Pichia</i> , and <i>Acetobacter</i> during natural fermentation of neera. The ambient storage of 20 h drops the pH from 7 to 4 due to the development of acids (2.5 g/L). The reduced pH enhances the invertase activity in yeast and initiate alcoholic fermentation in neera.	Atputharajah et al., 1986
In this study different volatile compounds from fermented neera were identified. Among the identified 12 different volatile compounds palmitoleic acid and dodecanoic acid are agents which cause astringency in neera.	Borse et al., 2007
The physicochemical changes during natural fermentation demonstrated the conversion of sucrose into reducing sugar,	Xia et al., 2011

i.e. glucose and fructose in neera was due to the early period (1-3 d) lactic acid fermentation. The converted reducing sugars utilized by yeast. Finally the highly rose acidity by the acetic acid fermentation occurred after 5 d of storage. Moreover, the study reported the phenolic and gallic acid content of 0.33 g/L and 350 µg/L in the unfermented fresh neera.	
For collecting neera without fermentation Hebbar et al. (2015) have developed portable coco sap chiller. The authors in 2018 reported that fresh neera has a neutral pH (7), yellow in colour, and a diabetic-friendly drink with a low glycemic index (GI 35). The 100 ml of neera is the abundant source of minerals which includes sodium (90.6 mg), potassium (168.4 mg), phosphorous (3.9 mg), zinc (0.020 mg), magnesium (0.012 mg) and copper (0.031 mg). It also contain vitamins such as inositol (127.70 mg), thiamine (77.00 mg), nicotinic acid (40.60 mg), and riboflavin (12.20 mg).	Hebbar et al., 2015 and Hebbar et al., 2018
The microorganism in neera hydrolysed the sugar content by the sucrose hydrolyzing enzymes and produced glucose and fructose (reducing sugar). In the presence of reducing sugar (2.1 %) yeast increased the invertase activity which promote the alcoholic fermentation in neera.	Shetty et al., 2017
The collected fresh neera is highly sweet due to the composition of 12-15 % sucrose and a trace amount of glucose and fructose. In order to control the fermentation during collection process, the inner surface of the pot is coated with lime (calcium carbonate) by the neera tappers.	Ghosh et al., 2018 and Somawiharja et al., 2018

Among the existing LAB, seven isolates were identified as probiotics which has survival capability at low pH of gastric conditions. Moreover, LAB exhibited antibacterial, antifungal activity and hydroxyl-scavenging activity.	Somashekaraiah et al., 2019
The neera drink helps in suppressing kidney toxicity through the nephroprotective effect of inhibited oxidative stress and lipid peroxidation. The mineral content especially phosphorous present in neera aids in improving renal health by releasing waste from kidneys. Moreover, their consumption induced functional properties of modulated inflammatory markers, metalloproteinase, and oxidative stress which inhibited the hepatic damage in humans.	Asha et al., 2019
The author developed the method for extending shelf life of neera by the collection of processing techniques from tapping to bottling. During collection, this method sprayed the sodium hypochlorite as a disinfectant and collected the neera with 10 ml of preservative solution (5 mM-acidifier and 2 mM-potassium salts). Then the collected neera further processed using microfiltration (100 microns), pasteurization (70-85°C for 10-15 min) and bottled.	Chinnamma et al., 2019
The study proved the minerals and vitamin content of neera by their demonstrated results of 960.87 mg/L-potassium, 183.21 mg/L-sodium, and 116.19 µg/ml-vitamin C.	Asghar et al., 2020
The study on neera reported that there was an 80 % log reduction on microbial population using the cold plasma bubbling system by the action of reactive species.	Aparajhitha and Mahendran., 2019

Fermentation kinetics in atmospheric storage reduced the physicochemical properties like pH (3.74), total soluble solids (14.87 ° Brix), and total acidity (0.28 mg/L) were reduced within 6 h. In addition to that, refrigerated neera also reduced their physicochemical properties (6.57-pH, 15.03 °Brix- total soluble solids, and 0.05 mg/L-total acidity) during storage period.	Pandiselvam et al., 2021
Developed the nano fibrous membrane for the removal of yeast from neera. This technology controlled the pH (6.78 ± 0.10), total soluble solids (15.07 ± 1.80 °Brix), titratable acidity (1.77 ± 0.04 mg/L) and colour (6.51 ± 0.11) of neera after filtration.	Leena et al., 2021

CHAPTER III

MATERIALS AND METHODS

The chapter briefly covers the collection of coconut inflorescence, tapping technique, detailed methodology employed in freezing, preservatives, non-thermal (atmospheric pressure plasma jet) and thermal method (mild heat treatment) in shelf life extension of neera. In addition, this chapter provides the procedure for quantitative estimation of viable microorganisms by standard serial dilution method with plate count analysis and different microscopic instruments used for the identification of microorganisms in neera. Finally, the technique for assessing physicochemical properties and sensory attributes are discussed in detail.

3.1 Collection of coconut neera from inflorescence

Matured coconut trees, having more coconuts are selected for tapping the inflorescence. The immature inflorescence or spathe with a total length of about 60 cm with the base swelling (about to burst stage) were identified in the campus of National Institute of Food Technology, Entrepreneurship and Management-T (Formerly-IIFPT), Thanjavur, Tamil Nadu. Then the spathe was prepared for oozing of sweet sap, known as exudation, and it's a stepwise process. The process begins with a tying of selected inflorescence with a rope or coir, beating it using a mallet, and hand massaged from bottom to tip in the morning and evening for a week and this overall process was termed as stroking (Hebbar et al., 2018). After the stroking, the tip of the spathe was sliced off about few mm regularly up to 15 d until the exudation starts from the inflorescence (Figure 3.1-a, b and c). Once the exudation initiated from an inflorescence, it extended for about six months.



Figure 3.1: Collection of coconut neera

(a) Stroking (b) Slicing on the selected inflorescence (c) Collected fresh neera

The drop-wise ooze out of neera yields about 1.5- 2 L after 12 h collection in the morning and the same quantity in the evening. Coco-sap chiller was used for collecting the neera, and it was procured from talltree ventures-pollachi, India. The collected sap was filtered to remove the contaminants such as pollen, ants and others. The filtered neera was stored at refrigerated temperature (2-6°C) for further treatments.

3.2 Methodology

The shelf life of neera was extended by four different methods, namely freezing, preservatives, atmospheric pressure plasma jet as a non-thermal and mild heat treatment (Figure 3.2). The freezing at two different temperatures (-6 and -20°C), the combination of bio preservative (nisin) and the chemical preservatives (sodium benzoate and calcium

carbonate), atmospheric pressure plasma jet at different input voltage (30, 35, and 40 kV, for 5, 7.5, and 10 min) and mild heat treatment (single and double-stage) at various temperature (50, 55 and 60 °C) are the processing conditions involved in this study.

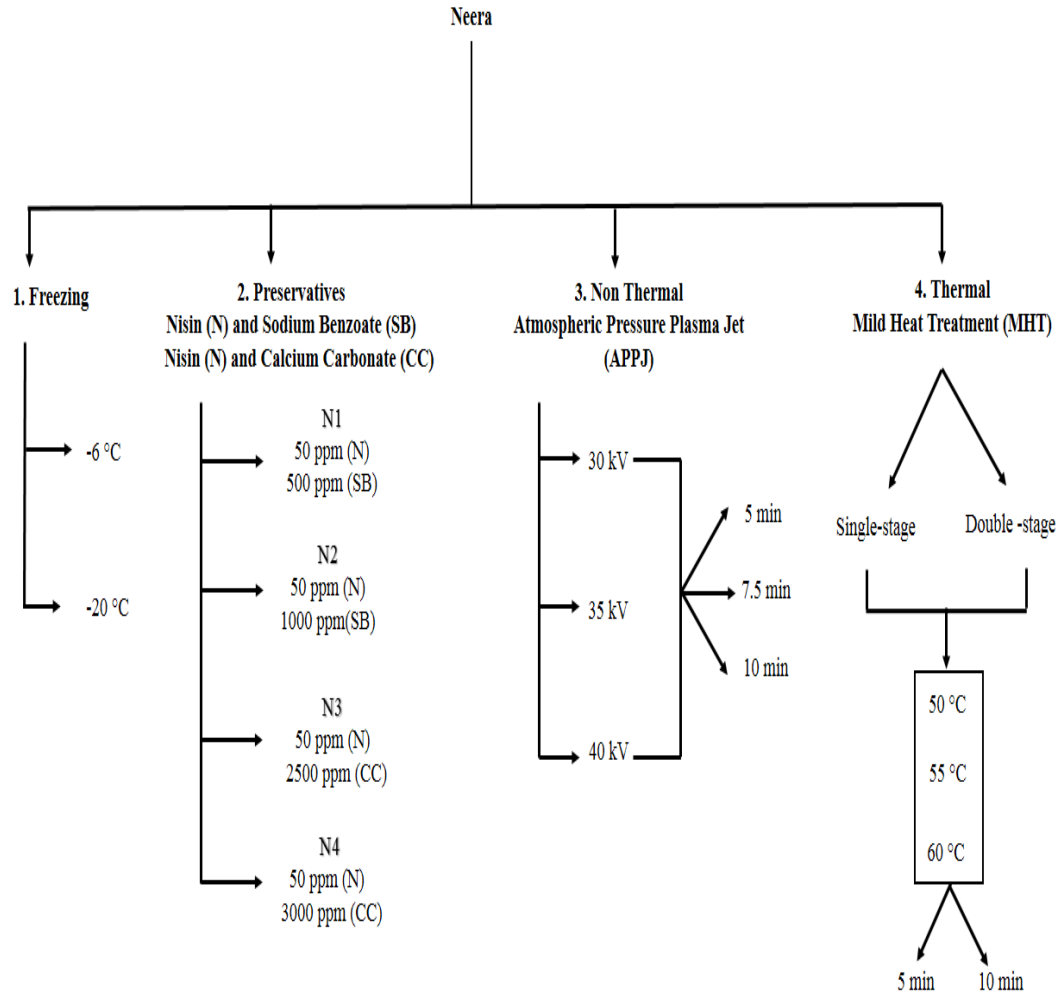


Figure 3.2 Flow process for the processing of neera

For the processing, the neera was selected based on the pH (6 ± 2) and total soluble solids ($16\pm2^\circ$ Brix). Then the neera sample was taken in sterile containers and exposed to various treatment conditions. The experiment was carried out in triplicates on the same day of sample collection. After the processing the treated neera samples were stored at the

temperature of $4\pm 1^{\circ}\text{C}$ and evaluated for the microbial survival, physicochemical and sensory properties over the storage period.

3.2.1 Freezing method

Under sterile conditions, 100 ml of neera sap was poured in a 250 ml stainless steel container for frozen storage. As a preliminary study, neera frozen from -20°C to 0°C such as 0, -2, -4, -6, -8, -10, -12, -14, -18 and -20°C using horizontal deep freezer (DF20, 0 to -26°C , sub-zero, India). The data logger was used to track the temperature of the deep freezer. The frozen neera were thawed at $28\pm 2^{\circ}\text{C}$ for the period of 1 h. Thereafter the quality changes in the frozen neera were compared with control neera (0 d) up to the storage period of 28 d. The frozen neera did not exhibit changes for the analyzed physicochemical properties in terms of pH and total soluble solids at -14 to 0°C and changed at -20 to -14°C . From the preliminary study, -6°C and -20°C were selected for further studies for evaluating the quality of neera.

3.2.2 Preservative method

According to Ramalakshmi et al. (2004), the bio-preservative nisin (N) (Bimal Pharma Pvt. Ltd, India) and chemical preservatives sodium benzoate (SB) (Ganesh Benzoplast, India) and calcium carbonate (CC) (Local market, Thanjavur) were selected for neera preservation. As a preliminary study, the selected nisin was added at the concentration of 10, 25 and 50 ppm in 1 L neera PET bottles (Pokhrel et al., 2019). In each neera bottle (10, 25 and 50 ppm-nisin), four different concentrations of sodium benzoate, i.e., 250, 500, 750 and 1000 ppm, or calcium carbonate (1500, 2000, 2500, and 3000 ppm), were added. These preliminary study results showed a pH of above 6 and total soluble solids $>16^{\circ}\text{Brix}$ for a

24 h storage period in the combination of 50 ppm nisin-2500, 3000 ppm of calcium carbonate and 500, 1000 ppm of sodium benzoate. Whereas the other treatments show reduced pH (<6) at 24 h. Therefore, the four combinations of N1 (50 ppm nisin and 500 ppm sodium benzoate), N2 (50 ppm nisin and 1000 ppm sodium benzoate), N3 (50 ppm nisin and 2500 ppm calcium carbonate) and N4 (50 ppm nisin and 3000 ppm calcium carbonate) were taken as treatments for further studies. The four combinations were gently mixed before being at $4\pm^{\circ}\text{C}$. The four treatments and control were tested on 3, 6, 9, 12, 15, 18, and 21 days of storage.

3.2.3 Atmospheric pressure plasma jet as a non-thermal method

25 ml of neera aliquots were taken in high-density polyethylene bags and kept in chilled condition until the treatment. The atmospheric pressure plasma jet used for the study, contains two rectangle metal electrodes separated by a 5 cm distance (Figure 3.3). One electrode is coupled to a high-voltage transformer, thus producing the tiny current filaments like micro discharge, while the other serves as the ground electrode (Lokeswari et al., 2021). The neera samples were placed between the electrode with the conveyor support and micro discharge breaks the available atmospheric gas in the operating zone, producing the various reactive species in neera. Neera samples were subjected to three different voltages, i.e. 30, 35, 40 kV for 5, 7.5, and 10 min at ambient conditions ($28\pm2^{\circ}\text{C}$). The treated neera was stored at refrigerated temperature ($4\pm2^{\circ}\text{C}$) and analyzed for pH. During the preliminary study at 30, 35, and 40 kV treatment, the pH of neera was reduced to 4-5 (indication of fermentation) within 24 h of storage. Due to this fermentation effect, samples were analyzed between 3 h intervals .i.e., 3, 6, 9, 12, 15, 18, 21, and 24 h.

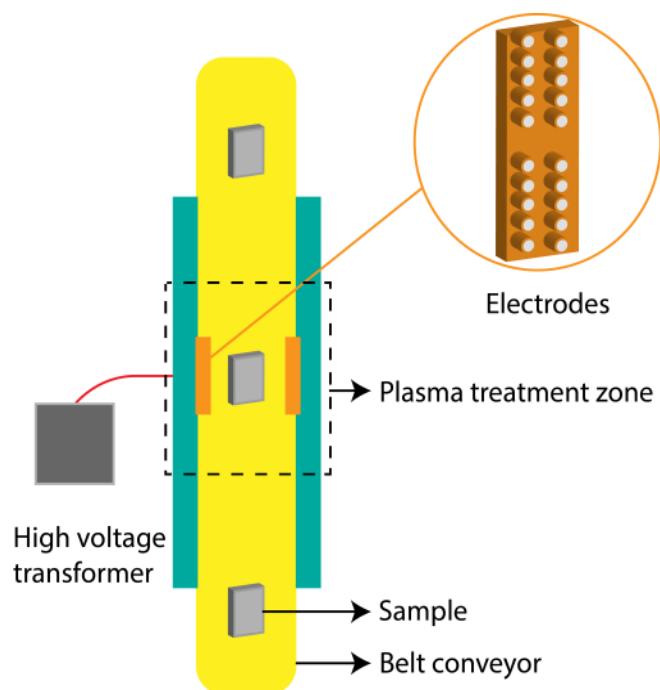


Figure 3.3: Experimental setup of plasma jet treatment used for coconut neera

3.2.4 Mild heat treatment as a thermal method

Under sterile conditions, 25 ml of collected fresh neera was transferred into a 125 ml capacity tube. For identifying suitable mild heat treatment, the preliminary study was carried out from 50-90°C at an interval of 5°C. Among the various heat treatments, at 50°C, 55°C, and 60°C the neera showed pH>6, total soluble solids >16 °Brix after 3 d of storage period. Therefore mild heat treatment at 50°C, 55°C, and 60°C was considered for both single-stage and double-stage methods.

In a single-stage treatment, neera was taken in 2 sets of tubes and treated at the temperature of 50°C, 55°C, and 60°C, for 5 and 10 min in a water bath electrified with a heating mantle (200 Watts, 230 A/C) (Figure 3.4). For double stage treatment, one set of tubes was cooled and incubated for the period of 60 to 90 min at 28±2°C. After that, the same tubes were re-

heated for 5 and 10 minutes at 50°C, 55°C and 60°C. Treated neera from single-stage and double-stage treatments were stored at 4±2°C up to 21 d and examined at 3 d intervals, i.e., 3, 6, 9, 12, 15, 18 and 21 d (Kim et al., 2012).

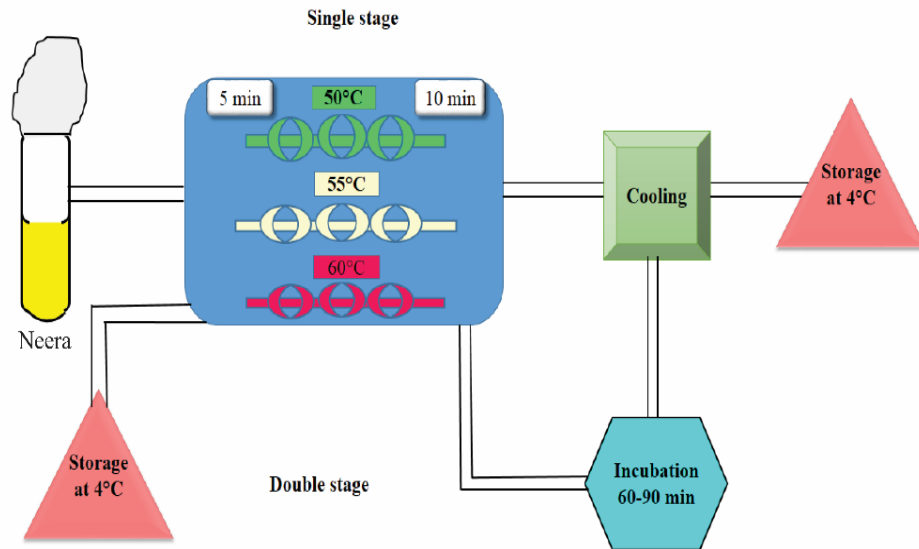


Figure 3.4 Representation of single-stage and double-stage heat treatment in neera

3.3 Microbiological analysis by standard plate count method

For the preparation of microbiological analysis, the neera samples were homogenized using a vortex for up to 2-3 min. The homogenized neera samples were enumerated for viable microorganisms by the standard plate count method. The homogenized sample was decimally diluted according to the ten-fold dilution (1:10) using 0.1 % peptone as a diluent. 0.1 ml of sample was added to various solidified agar media from the serially diluted samples. The overall protocol was carried out in the laminar flow cabinet (Kirloskar clean air equipment, KCH-B, India). Following the FSSAI., (2012) guidelines the

microorganisms were grown in an incubator (Remi CI-10, India) at a specific temperature (37°C-Bacteria and 28°C-Yeast) for total viable count, LAB, *Bacillus* and yeast. The above procedure was followed throughout the study for all treated and control neera samples.

3.3.1 Enumeration of total viable count

The total viable count indicated the aerobic mesophilic microorganisms from neera sample. The method used for fermented foods by Hariharan et al., (2014) was followed. For enumeration, the dilution with the concentration of 10^{-6} and 10^{-7} was chosen, transferred on plate count agar (M091, Hi-Media, Mumbai, India) and spread uniformly. After that, the plates were incubated in an inverted position in the dark at 37°C for 24 h.

3.3.2 Enumeration of Lactic Acid Bacteria (LAB)

For selective enumeration of LAB, all the treated samples were cultured on the Lactobacillus MRS agar (GM641, Hi-Media, Mumbai, India) with the adjusted pH of 6.2 (1 M HCl). Before pouring the media into the plates, 0.1 ml antibiotic solution of cycloheximide (FD311, Hi-Media, Mumbai, India) at the concentration of 2.0 mg in 10 ml were added at the temperature of 44°C to 47°C. From the dilution factor of 10^{-4} and 10^{-5} , the treated samples were taken for the spread plate method and incubated at 37°C for 48 h in an aerobic condition (Ortolani et al., 2007).

3.3.3 Enumeration of Yeast

The yeast growth in neera was enumerated from the aseptically drawn sample of 0.1 ml on the dilution of 10^{-3} and 10^{-4} . Then the samples were spread uniformly on the chloramphenicol yeast glucose agar-M1008 (Hi-Media, Mumbai, India). Finally the colonies were quantified after 72 h of incubation at $28 \pm 2^\circ\text{C}$ (Wang and Xu, 2019).

3.3.4 Enumeration of *Bacillus*

For the enumeration of *Bacillus*, 0.1 ml neera from the dilution 10^{-3} and 10^{-4} was spread over dried brain-heart Infusion Agar (M211, Hi-Media, India). The viable count of *Bacillus* was enumerated after the incubation period of 24 h at 37°C (Løvdalet al., 2011).

3.3.5 Expression of colony-forming units (CFU/ml)

According to the standard methods for examining food by the FDA, the colonies from the desired plates were enumerated and expressed as colony-forming unit (CFU) (Larry and James., 2001). The formula for a colony-forming unit (CFU) mentioned in equation 1.

$$N = \frac{\sum C}{(n_1 + 0.1n_2)d} \text{ --- (1)}$$

Where,

N= Number of counts per ml

$\sum C$ = Sum of the all counted colonies on the plates

n_1 = Counted number of plates in the first dilution

n_2 = Counted number of plates in the second dilution

d= Dilution of the first plate counted

When the count of CFU exceeded more than 250 in the plates, it was recorded as too numerous to count. Moreover, the concentrations of colony-forming units were expressed as logarithmic notation with the base value of 10 for the identification of efficient treatment or temperature (Parshionikar et al., 2009). The treatment count of CFU are compared to control, and the level of treatment effectiveness was calculated and expressed in Log reduction using equation 2 (Barria et al., 2013).

$$\text{Log Reduction} = \log_{10}(A) - \log_{10}(B) \text{-----} (2)$$

Where,

A = Number of viable microorganisms before heat treatment (control)

B = Number of viable microorganisms after heat treatment (treatment)

For identifying lethal temperature on mild heat treatment, the results were expressed as decimal reduction time (D). The calculated decimal reduction time for various temperatures (D) is denoted in the following formula (3) (Zhuang et al., 2019).

$$\left(\frac{t}{D_T}\right)^K = \log_{10}(A) - \log_{10}(B) \text{-----} (3)$$

Where,

B = Number of survival after heat treatment for t minutes

A= Number of survival before heat treatment

K= Slope curve between heating time and the number of survival

3.3.6 Optical cell density analysis

The concentration analysis of cells in treated neera samples was performed using a spectrophotometer Shimadzu UV-1800. The optical density at 660 nm was measured for 1 ml of neera suspension and the absorbance was taken between the storage days under aseptic conditions (Pan et al., 2014). The obtained absorbance of the treatment was subtracted from 0 d control neera absorbance and plotted against the treatment condition.

3.4 Microscopic analysis

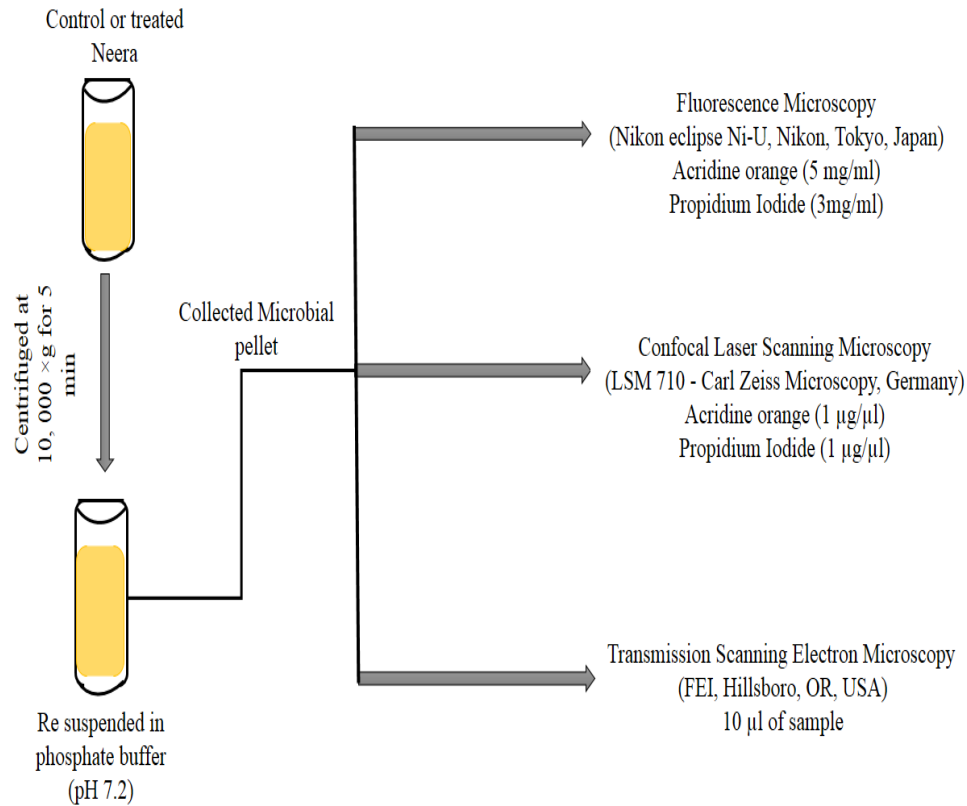


Figure 3.5 Sample preparation for various microscopic analysis

The image analysis of viable and non-viable cells was carried out in neera using the various microscopic techniques to evaluate the treatment effect. The fresh and treated neera samples were centrifuged for 10,000 g for 5 min to collect the microbial cells. The obtained pellet was prepared as resuspension using phosphate buffer saline solution at the pH of 7.2 to eliminate the pollutants according to the earlier method Zhang and Fang., (2004) (Figure 3.5). The test mixture was vortexed for 2-3 min and stained with the dye fusion .i.e., acridine orange (AO) and propidium iodide (PI). Thereafter the stained cells were incubated in a dark condition for 15 min and examined under a microscope.

3.4.1 Fluorescence Microscopy (FM)

FM technique was used in this study to compare the viable and non-viable cells from the frozen neera samples and control (fresh neera). Microbial cells were collected according to the sample preparation procedure of Figure 3.5 and analyzed using a fluorescent microscope (Ni-U, Nikon, Tokyo, Japan). The 100 μ l DNA binding dyes such as acridine orange (5 mg /ml) and propidium iodide (3 mg/ml) were added into the collected microbial cells and dark incubated for 15 min at room temperature. During the staining process, microbial cells with affected morphology emit the red fluorescence caused by the binding of PI. Whereas the other unaffected cells permeate the binding of AO and produce the green fluorescence. The fluorescence was dignified at the exciting wavelength and emission wavelength of 535 nm and 635 nm for AO and PI, respectively (Boulos et al., 1999).

3.4.2 Confocal Laser Scanning Microscopy (CLSM)

The effect of preservatives effect against microorganisms was identified with confocal laser scanning microscopy. CLSM functioned based on the pinhole mechanism to visualize fluorescence from microbial cells. The fluorescence image from the various region in the sample was focused by laser scanning. For imaging, the treated and control neera were centrifuged at 10,000 g for 5 min and the collected cells were re-suspended with phosphate buffer saline (pH 7.2) (Figure 3.5). After that, 10 μ l of collected cells were incubated with the equal volume of the staining components of AO and PI (1 μ g/1 μ l) for viable and non-viable cells identification. The incubated cells were analyzed under LSM 710-Carl Zeiss Microscopy GmbH, Germany. Cells were excited from 500 to 640 nm for obtaining the

images in Argon laser (488-nm laser excitation). The captured images were processed using Zen 2009 software (Auty et al., 2001).

3.4.3 Transmission Electron Microscopy (TEM)

Neera samples exposed to various heat treatments were examined using transmission scanning microscopy (Zhang and Fang., 2004). The samples from the single-stage and double stage heat treatment and control were prepared according to the sample preparation procedure Figure 3.5. The suspended sample of 10 μ l were prefixed for drying on a carbon-copper-coated sample grid for 10 min at $4\pm 1^\circ\text{C}$. Finally, the coated sample of single-stage and double-stage were examined using a transmission electron microscope (FEI, Hillsboro, ORUSA) for its effect on microbial morphology.

3.5 X-Ray Fluorescence spectrophotometric analysis (XRF)

XRF spectrophotometer work on the principle of wavelength-dispersive spectroscopy, where there is an interaction between the electron beam from the minerals in the sample and their spectrum. In this study, N4 treatment .i.e., added calcium carbonate of 3000 ppm was analyzed for its elemental analysis using an X-ray fluorescence spectrometer (XGT-5200, Horiba, Japan). The equipment fitted with the 50 kV X-ray tube source, filters, and detector of peltier silicon drift. Calcium microbe precipitation was confirmed from the calcium intensity peak of the treated sample of N4 and sediment portion of N4 (freeze-dried). The acquired spectra from the sample were converted into intensity in counts per second (cps) and plotted against the energy (keV) for the determination of Ca concentration (Chan and Palmer., 2013).

3.6 Physicochemical properties of neera

3.6.1 pH

The pH value indicates the free hydrogen ion in the neera sample. The pH of the neera were measured by a digital pH meter (ELICO -L1 120), and it was calibrated with pH of 4.0, 7.0 and 9.0 buffers.

3.6.2 Total soluble solids

The change in the total soluble solids content after treatment of neera were monitored by a hand-held refractometer (RHB - 55ATC), in the range of 0-85 °Brix at room temperature (30±2 °C). Before the analysis refractometer was calibrated using distilled water. After the calibration, a drop of neera was placed on a prism surface, and the results were recorded (FSSAI., 2016). The resulted °Brix indicates the sucrose content of the neera sample.

3.6.3 Total acidity

One ml of the treated neera was diluted into the 10 ml of distilled water (1:2 v/v) then 2 to 3 drops of phenolphthalein indicators were added. The burette solution of 0.1 N NaOH was titrated against the neera with constant shaking until the endpoint of pale pink colour appearance. The results were expressed using the following Equation (4).

$$\text{Acid value (mg /L)} = \frac{\text{Titer value} \times \text{Normality of NaOH} \times 90.08}{\text{Weight of the sample (ml)}} \text{---(4)}$$

3.6.4 Total colour difference

The colour changes were carried out using a hunter colorimeter (Colourflex EZ model: 45/0LAV). Before the analysis, using the white tile (X= 80.06, Y= 85.06, Z= 89.63) and a black tile the instrument was calibrated. The L*, a*, b* values (L*-indicates from lightness to darkness; a* indicates redness to greenness; b* indicates yellowness to blueness) of the

treated neera and L_0^* , a_0^* , b_0^* values of control neera sample were recorded. From the L^* , a^* , b^* values the total color difference (ΔE) were calculated by the following formula (5).

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad - - - - - (5)$$

3.6.5 Percentage of CO₂

The concentration of carbon dioxide (CO₂) in neera was monitored using a portable gas analyzer. For the analysis, neera was filled in glass bottles with the headspace and measured for CO₂ using PBI Dansensor (Denmark) by a needle through an adhesive septum.

3.6.6 Total protein

Lowry's method was adopted to quantify the protein in the neera samples based on the intensity of color. For estimation, the treated neera sample of 0.5 ml was made up to 1 ml using distilled water, then the alkaline copper solution (5 ml) and Folin's reagent of 0.5 ml were added. The added mixture was incubated under dark for 30 min. The sample absorbance was measured using a UV-spectrophotometer at 660 nm (Shimadzu UV-1800). All the reagents and standards were prepared, followed by Waterborg., (2003).

3.6.7 Ethanol content

The potassium dichromate method was used to determine the amount of ethanol in the neera sample. For the analysis, treated neera samples were centrifuged at the rpm of 5000 for 10 min and the collected supernatant (1 ml) taken in a volumetric flask. To the collected supernatant, potassium dichromate solution of 5 ml, acetate buffer of 5 ml (pH 4.3), and 6 N sulfuric acid of 25 ml were added. The standard stock solution of ethanol with the concentration of 1.6 mg/ml were further diluted into different concentration using distilled

water. The standard (ethanol) was added with a reagent similar to treatment and agitated for 1 min. After that 120 min incubation at room temperature, the absorbance (green color) was measured at 578 nm using a Shimadzu 1800 -UV spectrophotometer (Sumbhate et al., 2012). The concentration of the sample was determined using Equation no (6).

$$\text{Percentage of ethanol in sample (\%)} \equiv \left(\frac{CS}{CN} \right) \times \left(\frac{AS}{AN} \right) \times 100 \text{ --- (6)}$$

Where,

CS= Concentration of standard

CN = Concentration of neera

AS = Absorbance of standard

AN = Absorbance of neera

3.6.8 Viscosity

Viscosity (mPa s) of neera was measured using a rheometer (Anton Paar-MCR52) with the parallel plate PP50 at the temperature of 25°C. The analysis was performed at the given shear rate of 100 s⁻¹ for 200 seconds (Lachman et al., 2015).

3.7 Sensory evaluation of neera

Sensory attributes of neera were evaluated by the most common method 9-point hedonic scale and it's the popular method among consumers. A panel of eight members (five women and three men) were previously trained to evaluate coconut neera's sensory attributes. For scoring the values, neera (10-40 ml) were served in glass cups at chilled conditions between the intervals of 9.00 -11.30 h in the morning at the Centre of excellence in non-thermal processing, IIFPT, Thanjavur. In this method, the various degree of sensory attributes such as color, flavour, taste, after taste, consistency and overall acceptability was

scored as 1, 2, 3, 4, 5, 6, 7, 8 and 9 for the determinant of very poor, poor, fair, very fair, good, moderately, very good, extremely good and excellent (Mazzaglia and Lanza, 2018).

3.8 Statistical analysis

The experiments were performed in triplicates. The obtained results were analyzed using student's t-test. The analysis of variances such as Tukey's test and Duncan test was done utilizing the IBM SPSS statistical software version 23.0 (Armonk, NY: IBM Corp). The means were compared by least significant difference at significant level of 0.05.

CHAPTER IV

RESULTS AND DISCUSSIONS

This chapter deals with the results of the investigation carried out, for the preservation of neera using four different methods. Section 4.1 summarizes the effect of freezing at -6°C and -20°C on microbial survival through plate count, cell density and viable and non-viable fluorescence images. The chemical and sensory attributes of frozen neera for the determination of shelf life are also discussed. The results of the preservatives (section 4.2) describe the antimicrobial activity of nisin with sodium benzoate (N1-50 ppm + 500 ppm SB; N2-50 ppm N + 1000 ppm SB) and nisin with calcium carbonate (N3-50 ppm N + 2500 ppm CC; N4-50 ppm N + 1000 ppm CC) against the microorganisms of neera by plate count population, viable and non-viable cells and intensity of calcium. Its effect on physicochemical and sensory properties are also discussed in detail. The method of a non-thermal atmospheric pressure plasma jet in section 4.3 describes the reduction of microbial populations by plate count and its effect on the physicochemical properties. The mild heat treatment (section 4.4) describes the decimal reduction time at 50°C, 55°C and 60°C, microbial population reduction by single and double-stage heat treatment by plate count and TEM images. Finally, its effect on the physicochemical and sensory properties are discussed in detail.

4.1 Effect of freezing on shelf life extension of coconut neera

4.1.1 Effect of freezing on inhibition of microbes by standard plate count method

The plate count analysis revealed a reduction or inhibited microbial population for the total viable count, yeast, and lactic acid bacteria at two different freezing temperatures (-20°C

and -6°C) (Figure 4.1). Throughout the storage period, the viability of LAB at -20°C showed a gradual reduction from the initial population of 5.07 ± 0.47 (0 d) to 4.33 ± 0.00 log CFU/ml for the storage period of 14 d. After that, the frozen storage for 21 and 28 d inhibited the viability of LAB. However, this LAB survival at -6°C was reported till the end of the storage period of 28 d with the viability of 3.96 ± 0.00 log CFU/ml. The observed viability (3.96 ± 0.00 log CFU/ml) at -6°C was the highest population compared to the inhibited population at -20°C. The result of the inhibited LAB at a temperature of -20°C followed the findings of O'Brien et al., (2016) who reported the reduced number of *Lactococci* in frozen stored kefir. The inhibited LAB indicates the fast freezing at -20°C causes severe injury to the LAB community upon storage period.

The yeast count at the end of 28 d was found to be 4.44 ± 0.00 log CFU/ml and 3.18 ± 0.00 log CFU/ml at -6°C and -20°C, respectively. This lower number of survival (3.18 ± 0.00 log CFU/ml) and the high number of survival (4.4 ± 0.00 log CFU/ml) was due to the rapid (-20°C) and slow freezing (-6°C). The survival of alcohol-producing yeast at -6°C were correlated with the findings of Geiges., (1996) and the study reported the persistent growth of yeast during frozen storage.

The total viable count of 6.26 ± 0.47 log CFU/ml at -20°C was the highest number of reduction than the total viability of 7.12 ± 0.00 log CFU/ml at -6°C. The higher reduction of a total of viable count and yeast at -20°C indicated the presence of injured microbes and it entered into non-cultivable state during viability analysis (Speck and Ray, 1977). These findings suggested that the storage at -20°C affected the heterogeneous microbial survival and their spontaneous fermentation in neera. The rapid cooling at -20°C causes greater

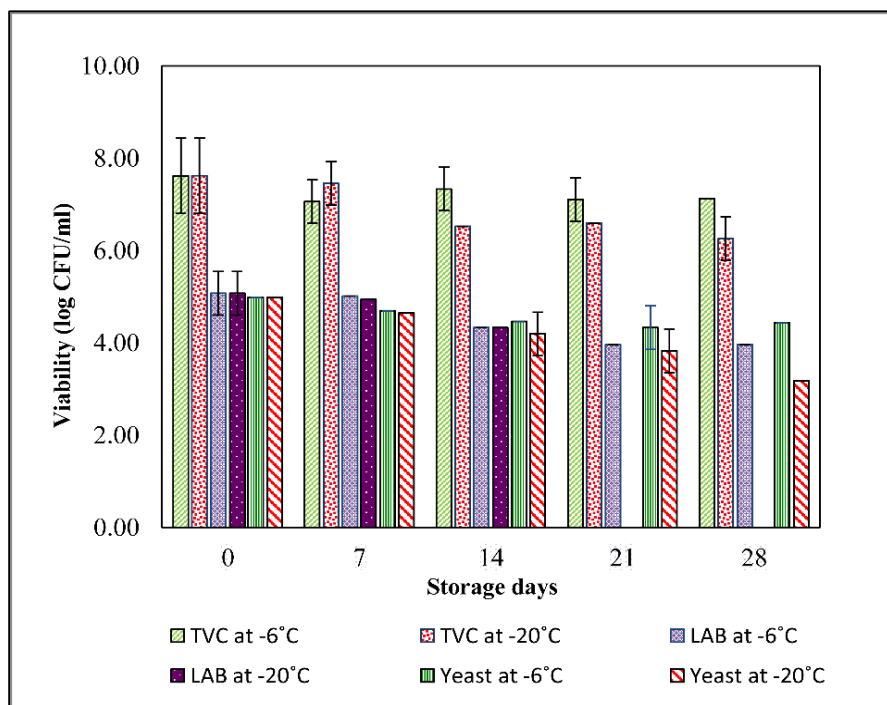


Figure 4.1: Effect of freezing on inhibition of microbial growth by standard plate count at -6°C and -20°C (Sukumaran, L., & Radhakrishnan, M. 2021a). TVC- Total viable count, LAB-Lactic acid bacteria, and Yeast.

intracellular ice production, which causes damage to microbial cells and reduces cell viability (Dumont et al., 2006). The low cooling rate resulted in the lowest microbial reduction at -6°C, where water content dispersed from the cytoplasm, which causes microbial cell dehydration. Moreover, the mesophilic bacteria and the yeast at this temperature (-6°C) exhibited spontaneous fermentation in neera (Golden and Arroyo-gallyoun, 1997; Tamang et al., 2016). Based on this result, frozen storage at -6°C did not control the mechanism of fermentation in neera, whereas the temperature at -20°C reduced their growth and metabolism.

4.1.2 Cell density analysis by spectrophotometric method

The spectrophotometric method of cell density analysis at two different freezing temperatures of -6°C and -20°C revealed the decreasing and increasing trend between storage periods (Figure 4.2). At -20°C cell density was decreased from the 0 d (0.45 ± 0.029) to 14 d (0.01 ± 0.005) after that the cell density was increased in 21 d (0.07 ± 0.002) and 28 d (0.10 ± 0.004). The reduced cell density of 0.10 ± 0.004 at -20°C which confirmed the result of the reduced viability in plate count analysis.

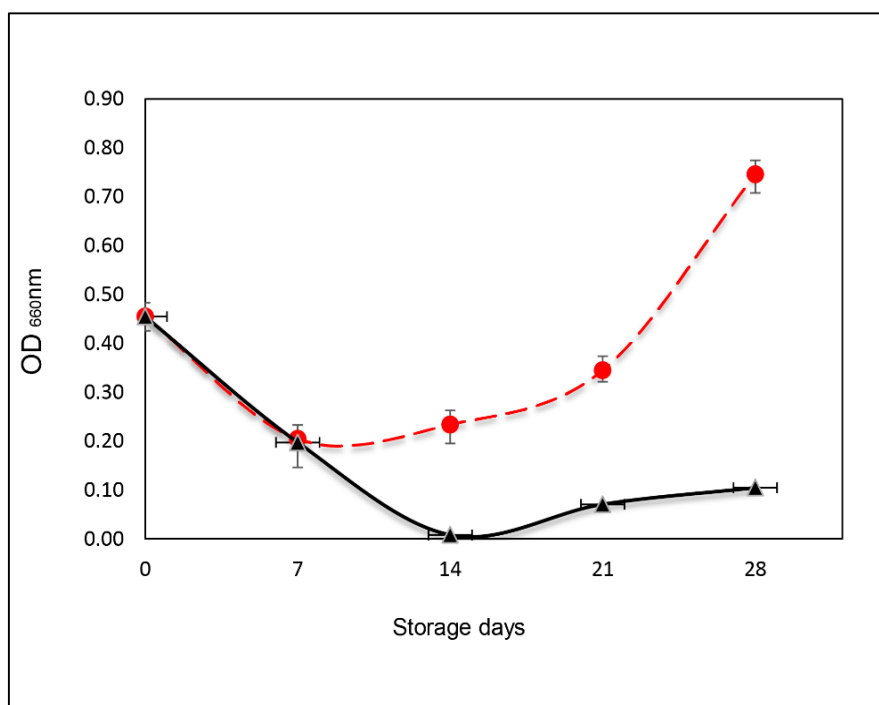


Figure 4.2: Optical cell density analysis of neera in storage period using a spectrophotometric method at 660 nm (Sukumaran, L., & Radhakrishnan, M. 2021a).

—●— -6°C —▲— -20°C

The freezing temperature at -6°C observed a decreasing trend up to storage period of 7 d (0.20 ± 0.058) thereafter found to have a increasing cell density (0.25 ± 0.005) from storage period of 14 d. At the end of the 28 d resulted in the highest cell density of 0.75 ± 0.038 . The result of the cell density implies that most of the microorganism at -20°C was injured during the storage period (0-28 d). Hence, the microbial community of neera were resistant at -6°C , and it exhibited survival in the storage period. The results are in consistent with the results of Golden and Arroyo-gallyoun., (1997) who reported more number of survival at high temperature. The microbial survival favours the metabolic fermentation at this temperature (-6°C) and it was restricted at the freezing temperature of -20°C .

4.1.3 Identification of viable and non-viable cells by fluorescence microscopy

The results of the microbial survival and cell density absorbance were further confirmed by the viable (green-coloured) and non-viable (red-coloured) fluorescence microscopic analysis (Figure 4.3). The image of the control (0 d) shows a high number of DNA-AO coupled viable cells (Figure 4.3-a). Thus indicating survival of heterogeneous microbes in neera and it is responsible for natural fermentation (Atputharajah et al. 1986).

The image of frozen stored neera at -6°C depicts the presence of more viable and less non-viable cells, which means that the heterogeneous microbial viability were not affected during storage (Figure 4.3-b). The effect was similar to the control observation of more viable cells, therefore the frozen neera from this temperature (-6°C) fermented by microorganisms. The image analysis of frozen stored neera at -20°C shows more DNA-PI coupled non-viable cells than the viable cells (DNA-AO) (Figure 4.3-c). The results obtained from plate count and cell density analysis also revealed a better reduction of viable

cells at a freezing temperature of -20°C . The findings of the microbial analysis suggested that the freezing at -6°C was not effective in arresting the natural fermentative microorganism in neera, whereas the freezing at -20°C effectively restricted the microbial growth.

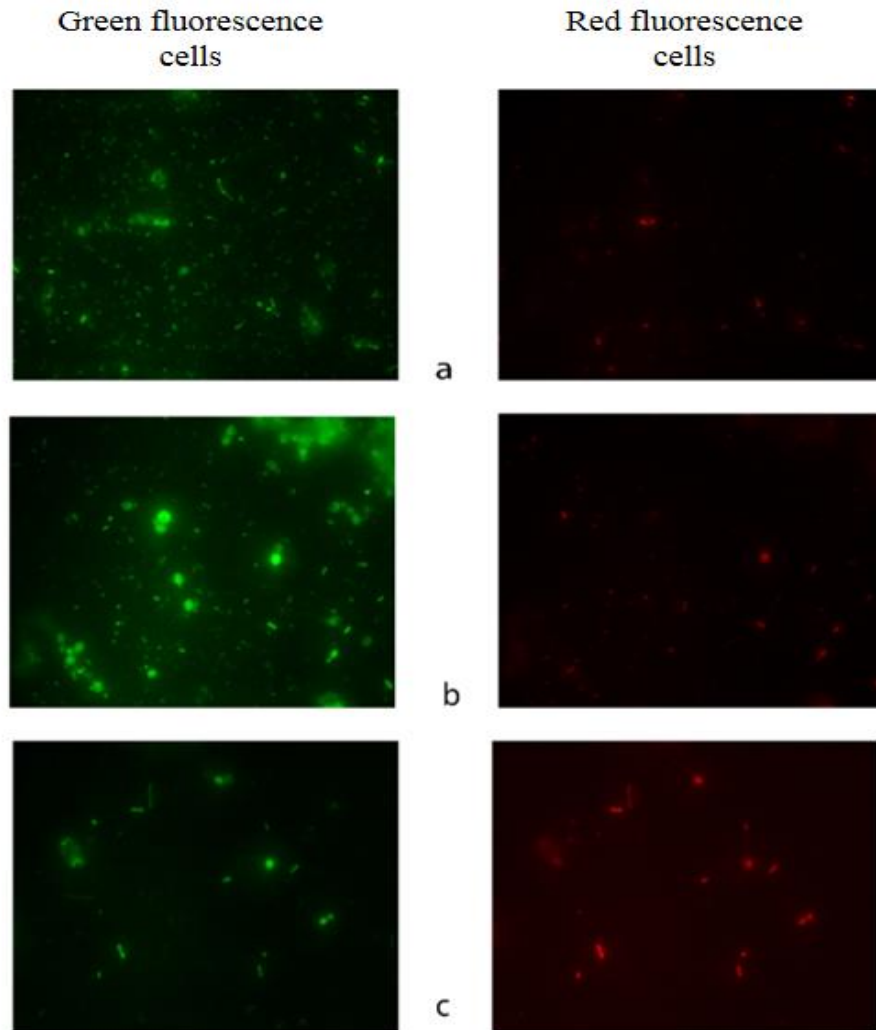


Figure 4.3: Fluorescence image analysis for viable and non-viable microbial cells (a) control (b) neera at -6°C and (c) neera at -20°C (Sukumaran, L., & Radhakrishnan, M. 2021a).

4.1.4 Chemical qualities of frozen neera on storage period

The observed changes in the chemical properties of frozen neera such as pH, total soluble solids, total acidity, colour, viscosity, total protein and ethanol among the two different temperatures were presented in Table 4.1. The analyzed chemical properties (pH, total soluble solids, total acidity and colour) exhibited a significant reduction ($P \leq 0.005$) in storage period. At the end of the storage period (28 d), the frozen neera at -20°C demonstrated the least chemical changes compared to the 0 d. The rapid freezing at the lower temperature (-20°C) froze the food components rapidly than at a higher temperature (-6°C) (Amato and Christner., 2009).

The fast freezing at -20°C affected the metabolism of fermentation by the mechanical disruption, oxidative damage and osmotic imbalance in the microbial cell. Due to the affected microbial cell at -20°C resulted a least change in pH from 5.85 ± 0.03 to 5.37 ± 0.02 . The pH of 5.37 ± 0.02 was comparatively least than the atmospherically stored neera pH of 4. At -6°C , some of the microorganisms had survived which affected the physicochemical quality of pH, and causes a reduction of pH to 4.97 ± 0.00 at the end of the storage (28 d) (Amato and Christner., 2009). The study at two frozen temperatures showed a decrease in total soluble solids of 13.17 ± 0.28 at -6°C and 14.0 ± 0.00 °Brix at -20°C . Furthermore, at -6°C total acidity increased to 0.96 ± 0.02 mg/L on 28 d than at -20°C (0.82 ± 0.02 mg/L). The increased total acidity at -6°C confirmed the lactic acid fermentation at this temperature (Xia et al. 2011). The amount of 0.82 ± 0.02 mg/L acidity at -20°C suggested the induced internal acidity from the stress adapted microorganism present in frozen storage (Park et al., 1997).

Table 4.1: Physicochemical properties of pH, total soluble solids, total acidity, and colour of frozen neera at -6°C and -20°C

Temperature (°C)	0 th day	7 th day	14 th day	21 st day	28 th day
pH					
-6	5.85±0.03	5.72±0.00 ^{a,1}	5.46±0.00 ^{b,1}	5.18±0.01 ^{c,1}	4.97±0.00 ^{d,1}
-20	5.85±0.03	5.82±0.01 ^{a,2}	5.76±0.00 ^{b,2}	5.53±0.06 ^{c,2}	5.37±0.02 ^{d,2}
Total soluble solids (°Brix)					
-6	16.50±0.00	15.67±0.28 ^{a,1}	15.00±0.00 ^{b,1}	14.50±0.00 ^{c,1}	13.17±0.28 ^{d,1}
-20	16.50±0.00	16.50±0.00 ^{a,2}	15.83±0.29 ^{b,2}	15.00±0.00 ^{c,2}	14.00±0.00 ^{d,2}
Total acidity (mg/L)					
-6	0.69±0.01	0.74±0.01 ^{a,2}	0.94±0.02 ^{b,2}	0.91±0.01 ^{b,2}	0.96±0.02 ^{c,2}
-20	0.69±0.01	0.73±0.01 ^{a,1}	0.79±0.02 ^{a,1}	0.82±0.01 ^{b,1}	0.82±0.02 ^{c,1}
Colour (ΔE)					
-6	*	5.43±0.01 ^{a,2}	6.04±0.05 ^{b,2}	6.07±0.05 ^{b,2}	6.49±0.06 ^{c,2}
-20	*	1.16±0.07 ^{a,1}	2.53±0.07 ^{b,1}	4.26±0.03 ^{c,1}	4.97±0.05 ^{d,1}

* Indicates there was nil total colour difference for the delta E value of fresh neera (L* 58.13 a* 0.36 and b* 9.26). The different alphabets in storage indicate significantly different at the 0.05 level from the control

The colour of neera was the most important property which defines the choice of purchase and customer acceptability. The significantly varied colour difference (ΔE) from control (0 d) was due to the change in the storage period L*, a*, and b* values (Table 4.1). The

disintegration of the food components in frozen storage mediated the release of pigments from neera and these effects accelerate the L^* , a^* , and b^* values (Oates and Alfred, 2002; Castro-López et al., 2016). Along the significant colour difference (6.49 ± 0.06) there was a noticeable visual change at -6°C , and these changes was less at -20°C ($4.97 \pm 0.05 \Delta E$), and the current study result was agreed with the report of Nowak et al., (2019).

Table 4.2: Physicochemical properties of viscosity, total protein, and ethanol content of frozen neera at -6°C and -20°C

Temperature ($^\circ\text{C}$)	0 th day	7 th day	14 th day	21 st day	28 th day
Viscosity (mPa s)					
-6	3.89 ± 0.10	$7.11 \pm 0.06^{a,2}$	$7.23 \pm 0.05^{b,2}$	$3.9 \pm 0.01^{c,2}$	$2.68 \pm 0.05^{d,2}$
-20	3.89 ± 0.10	$6.3 \pm 0.02^{a,1}$	$6.03 \pm 0.14^{b,1}$	$4.22 \pm 0.06^{c,1}$	$3.88 \pm 0.05^{d,1}$
Total protein (g /100 ml)					
-6	0.015 ± 0.02	$0.007 \pm 0.01^{a,2}$	$0.010 \pm 0.05^{b,2}$	$0.011 \pm 0.04^{b,2}$	$0.013 \pm 0.02^{c,2}$
-20	0.015 ± 0.02	$0.005 \pm 0.01^{a,1}$	$0.006 \pm 0.01^{b,1}$	$0.006 \pm 0.01^{b,1}$	$0.004 \pm 0.01^{c,1}$
Ethanol (%)					
-6	0.16 ± 0.001	ND	ND	$0.69 \pm 0.001^{a,1}$	$2.80 \pm 0.005^{b,2}$
-20	0.16 ± 0.001	ND	ND	ND	$0.79 \pm 0.001^{a,1}$

ND means not detected in storage days, and the different alphabets in storage indicate

significantly different at the 0.05 level from the control

The viscosity of frozen stored neera which was significantly affected, and it increased from 0 d to 7 d. After that, the viscosity was decreased in subsequent storage days (Table 4.2). The current results were similar to the report of Dan et al. (2019) who have reported increased viscosity from 0 d to 3 d and further decreased for milk due to presence of lactic acid bacteria. Finally, the viscosity were found as 2.68 ± 0.05 and 3.88 ± 0.05 mPa s at -6°C and -20°C at the end of the storage (28 d). The lowest viscosity between the temperature of -6°C and -20°C , such as 2.68 ± 0.05 mPa s (28 d) suggests the possibility of microbial metabolism at -6°C . Hence, the viscosity at -20°C was 3.88 ± 0.05 mPa s indicates that the reduced rate of microbial metabolism (Magala et al., 2015).

The protein content of frozen neera was reduced from 0.015 ± 0.02 g/100 ml (0 d) to 0.007 ± 0.01 (-6°C) and 0.005 ± 0.01 g/100ml (-20°C) on 7 d of storage (Table 4.2). The insolubilization and aggregation reaction in ice crystals decreased the initial content of protein on storage (Powrie, 1984; Twomey et al., 2013). There was an increase in protein concentration from the 7 d and it was extended up to 28 d of storage, which was due to the microbial cold shock protein (Wouters et al., 2001). Finally, the 28 d of frozen storage revealed the concentration of 0.013 ± 0.02 and 0.004 ± 0.01 g/100 ml at -6°C and -20°C respectively. Because of the microbial cold shock protein synthesis in frozen temperature resulted high protein content of 0.013 ± 0.02 at -6°C and least content of 0.004 ± 0.01 at -20°C . The results agreed with the findings of Cloutier et al., (1992) who have reported a similar difference due to cold shock protein.

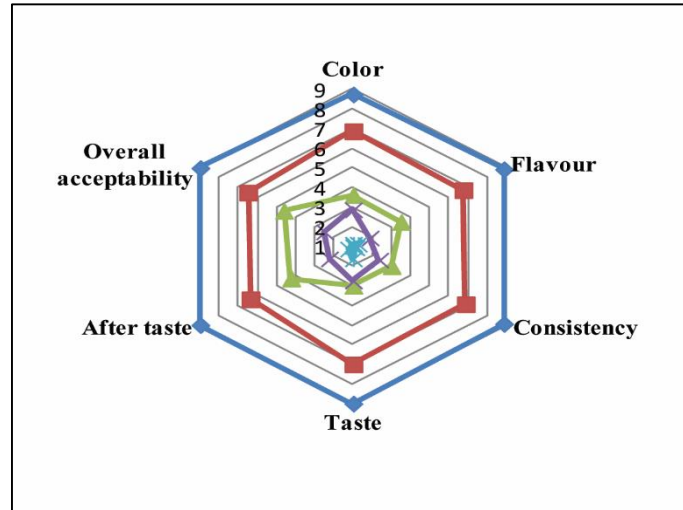
The ethanol production was not observed in frozen stored neera at -6°C from 7 to 14 d (Table 4.2). On successive storage of 21 and 28 d showed the ethanol production at -6°C .

At the end of the storage period (28 d), observed 2.80 % of ethanol content. The ethanol production and significant changes in total soluble solids content (13.17 °Brix) at -6°C confirm the glycolytic metabolism by microorganisms (Fan et al., 2005). This metabolism was induced by low temperatures aquaporins microbial gene expression, which triggered the growth and activity of anaerobic yeast (Tanghe et al., 2004), resulted in the production of ethanol in frozen conditions. However, at -20°C had a negligible ethanol production up to the storage period of 21 d, at the end of the 28 d it was only 0.79 %. The produced 0.79 % of ethanol was less than the 5-8 % concentration in ambient stored neera (Borse et al., 2007).

4.1.5 Sensory evaluation of frozen neera on storage period

The various sensory attributes of neera were deeply compared by panellists using a nine-point hedonic scale for the frozen stored neera (Figure 4.4 a and b). All the analysed attributes of frozen neera were gradually decreased from 0 d score. The frozen neera at -6°C was perceived as unacceptable and gave a least score (poor quality) by the panellists on 21 d. However, the frozen neera at -20°C retained its quality up to 21 d of storage and lost its quality on 28 d where observed a score of 2 (flavour), 3 (taste), 4 (after taste). Moreover, the ranked values of neera at -20°C were comparatively higher than the neera stored at -6°C. Similar scores were reported in prickly pear juice on frozen storage (Kgatla et al., 2010). Therefore, frozen storage at -20°C extend the shelf life of neera up to 21 d and preserves the original qualities.

(a)



(b)

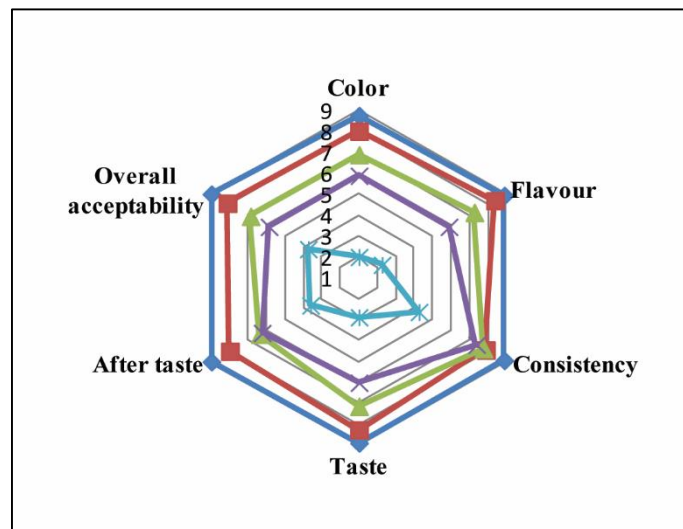


Figure 4.4: Sensory attributes of frozen neera at freezing temperatures versus storage days (a) -6°C (b) -20°C

◆ 0-day ■ 7-day ▲ 14-day ✖ 21-day * 28-day

4.2 Addition of preservatives for shelf life extension of coconut neera

4.2.1 Enumeration of microbial population using plate count method

From the plate count microbial analysis, the total bacterial count was found as 160×10^5 CFU/ml and 120×10^5 CFU/ml in N3 and N4 treatment, respectively (Table 4.3). The

treatments (N3 and N4) at the end of the 21 d revealed the yeast count of 260×10^2 CFU/ml and 143×10^2 CFU/ ml respectively. The other N1, N2, treatments had more than 250 CFU/ml from 6 d to 21 d for total bacteria and yeast population.

The combinations .i.e., N1 and N2 (nisin and sodium benzoate) was ineffective against the bacterial population in neera due to the addition of sodium benzoate. The sodium benzoate was generally effective against the yeast population in acidic foods and it affected the solubility of nisin (Glevitzky et al., 2009). The combination of calcium carbonate and nisin in N3 and N4 treatment reduced the microbial population due to its antimicrobial activity than N1 and N2 treatment. On 21 d of storage N4 treatment (50 ppm nisin and 3000 ppm calcium carbonate) decreases the total bacteria (120×10^5 CFU/ ml) and yeast count (143×10^2 CFU/ml) to higher number which confirms the effective inhibitory activity. However, the reduced concentration of calcium carbonate (2500 ppm) in N3 treatment have increased the growth to 160×10^5 and 260×10^2 CFU/ml for total bacteria and yeast than N4 treatment.

Table 4.3 Impact of preservatives on bacterial and yeast count (CFU/ml) on storage period at 4°C

Storage days	Total Bacteria (10^5)×CFU ml					Total yeast (10^2)×CFU/ml				
	C	N1	N2	N3	N4	C	N1	N2	N3	N4
0	120	130	120	3	1	160	67	63	7	10
3	*	270	230	10	3	*	247	203	70	10
6	*	*	*	30	8	*	*	*	130	30
9	*	*	*	60	17	*	*	*	220	47
12	*	*	*	100	37	*	*	*	233	67
15	*	*	*	110	73	*	*	*	243	107
18	*	*	*	130	100	*	*	*	250	123
21	*	*	*	160	120	*	*	*	260	143

Note * Represent more than 250 CFU/ml indicated as TNTC (Too Numerous To Count) colonies;

The values in the table represent the mean of triplicate. Abbreviation mentioned in the table throughout denotes: C - Control neera without treatment, N1 - Neera treated with 50 ppm nisin and 500 ppm of sodium benzoate, N2 - Neera treated with 50 ppm nisin and 1000 ppm of sodium benzoate, N3 - Neera treated with 50 ppm nisin and 2500 ppm of calcium carbonate, N4 - Neera treated with 50 ppm nisin and 3000 ppm of calcium carbonate.

4.2.2 Detection of viable and non-viable cells by confocal laser scanning microscopy

The viable and non-viable images (Figure 4.5) confirm the antimicrobial activity of N4 treatment. The N4 treatment had an increased number of non-viable bacterial cells (red fluorescence cells) which indicates the presence of less number of viable bacteria (Figure 4.5- c). The reduced viability arrest the lactic acid fermentation in N4 treatment. Moreover, the result confirms the solubility of nisin in neera and their inhibitory activity at N4. The C (control) (Figure 4.5-a) and N2 treatments (Figure 4.5-b) were observed with a large number of green fluorescence bacterial cells and it affects the coconut neera by lactic acid fermentation (Pandiselvam et al., 2021).

4.2.3 Determination of calcium intensity by X-Ray Fluorescence spectrophotometric analysis

The neera sap is a rich source of sugars (12 %) and minerals like potassium, sodium, magnesium, iron, calcium and zinc (Asghar et al., 2020). This sugar favours the microbe-calcium precipitation in N4 treatment. The precipitated calcium in the sediment revealed the calcium intensity of 65.34 % (Figure 4.7), in N4 treatment (Park et al., 2013). Hence, the whole treatment (N4) resulted the Ca as 79.96 % (Figure.4.6) on X-ray fluorescence spectrometric intensity analysis. The resulting percentage in the sediment evident maximum precipitation between the indigenous microorganisms and calcium, thus inhibited the spontaneous fermentation in N4 treatment (Kapilan, 2015).

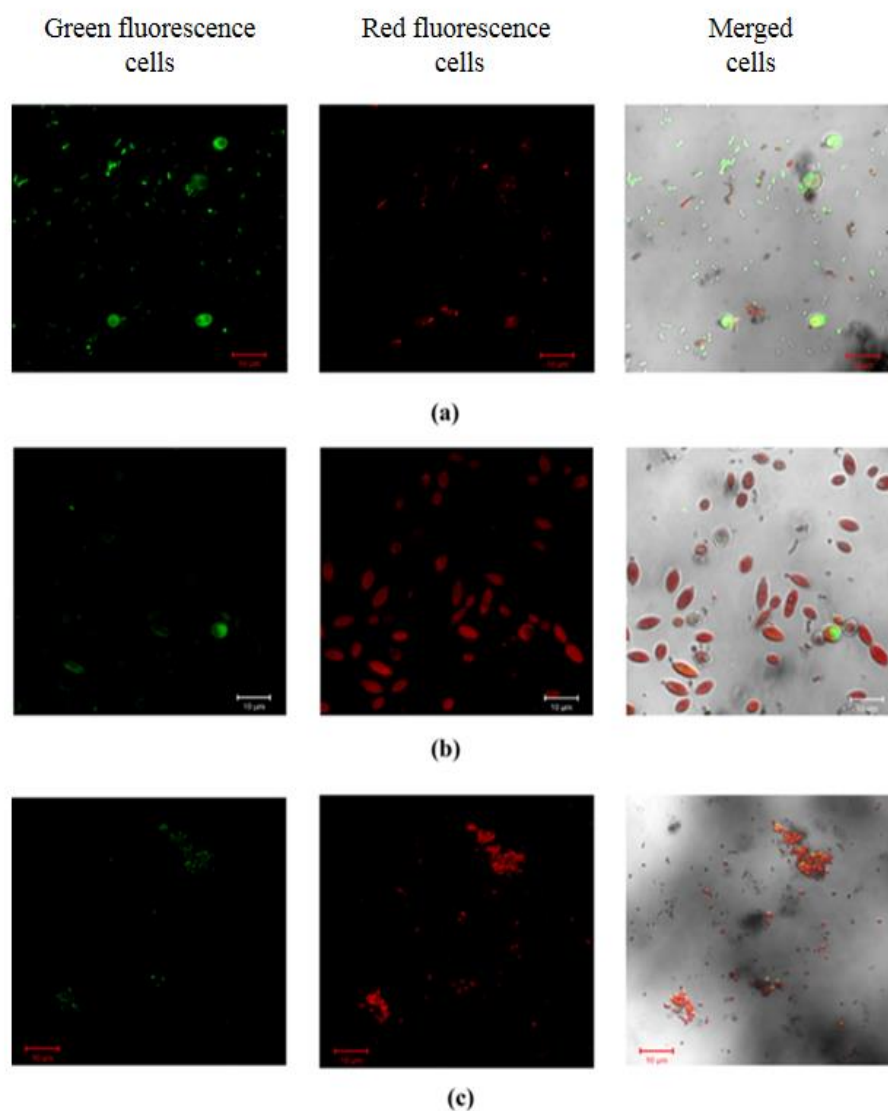


Figure 4.5: Detection of viable and non-viable cells in neera by confocal laser scanning microscopy. Observation of cells from neera (a) (control) without treatment (b) cells from nisin (50 ppm) and sodium benzoate (1000 ppm) added neera (c) cells from nisin (50 ppm) and calcium carbonate (3000 ppm) added neera. Green cells represent live microorganisms, while red cells represent dead microorganisms (Sukumaran L., & Radhakrishnan, M. 2021b).

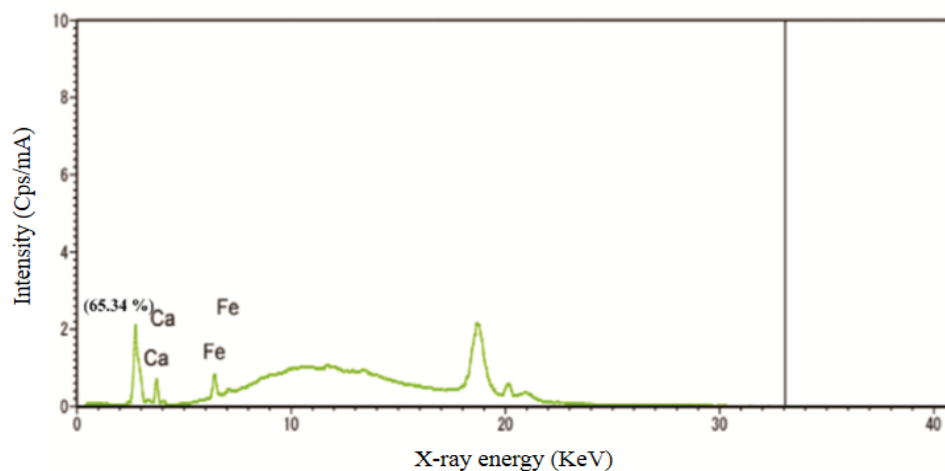


Figure 4.6: XRF patterns of minerals in N4 treatment (Nisin-50 ppm and Calcium carbonate-3000 ppm). The spectra explain the various mineral components (Sukumaran L., & Radhakrishnan, M. 2021b)

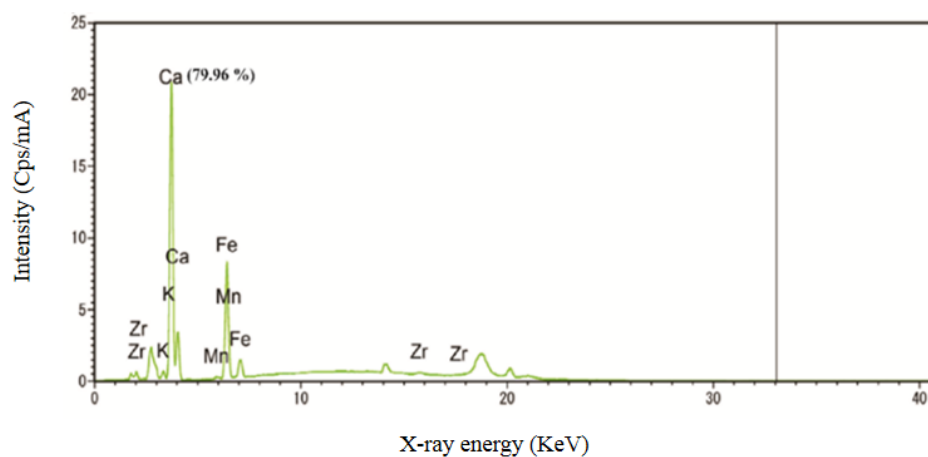


Figure 4.7: XRF patterns of minerals from sediment of N4 treatment (Nisin-50 ppm and Calcium carbonate-3000 ppm). The spectra explain the various mineral components (Sukumaran L., & Radhakrishnan, M. 2021b)

4.2.4 Effect of preservatives on the physicochemical characteristic of neera on storage period

The changes in physicochemical characteristic on neera due to preservative effect are presented in Table 4.4. The results reveal decreased pH in N1 (2.53 ± 0.03), N2 (3.48 ± 0.14) at the end of 21 d. The reduction in N1 and N2 were similar to control (C) pH (2.38 ± 0.01). The changes in pH value indicate the spontaneous fermentation in N1, N2 treatment and control. Furthermore, the development of acidic products such as lactic acid, acetic acid, and citric acid as a result of fermentation which affected the pH (Atputharajah et al., 1986). The N4 treatment at the end of 21 d showed a higher pH of 10.45 ± 0.05 from the 0 d (7.58 ± 0.01). In contrast, the N3 had a pH value of 5.62 ± 0.01 . Therefore added calcium carbonate increased the pH in N4 treatment and the concentration of 3000 ppm effectively controlled the rapid microbial fermentation in neera (Ataee et al., 2011).

In addition to pH, the CO₂ production in N4 treatment, i.e., 6.83 ± 0.06 %, was comparatively less than the 12.7 ± 0.12 % (N3), 25.67 ± 0.23 % (N2), 32.70 ± 0.00 % (N1), and 55.53 ± 0.21 % (C). The higher percentage of CO₂ production in C, N1, N2, and N3 affirmed the conversion of sucrose by yeast (Kapilan, 2015) and alkali-resistant *Bacillus* (Combet-Blanc et al., 1995). As a result of fermentation, the total soluble solids and total acidity were changed to 12.17 ± 0.06 °Brix, 12.27 ± 0.06 °Brix and 12.37 ± 0.06 °Brix and 11.83 ± 0.12 mg/L, 9.13 ± 0.02 mg/L and 10.07 ± 0.02 mg/L for C, N1, and N2 respectively (Xia et al., 2011). The reduced total soluble solids (15.43 ± 0.12 °Brix) and acidity (1.11 ± 0.04 mg/L) in the N4 treatment, was due to the synergistic effect of nisin and calcium (Pokhrel et al., 2019; Konopacka-Lyskawa et al., 2019).

Table 4.4 Physicochemical changes in fresh and preservatives added coconut neera during storage days

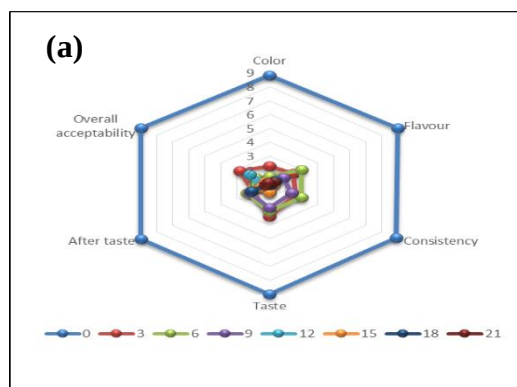
Treatment	pH		CO ₂ (%)		Total acidity (mg/L)		Total soluble solids (°Brix)		Color (ΔE)	
	0 d	21 d	0 d	21 d	0 d	21 d	0 d	21 d	0 d	21 d
C	7.58±0.01	2.38±0.01 ^{f,1}	ND	55.53±0.20 ^{h,5}	0.19±0.02	11.83±0.12 ^{h,4}	19.70±1.71	12.17±0.06 ^{f,1}	*	37.63±0.02 ^{h,5}
N1	7.16±0.03	2.53±0.03 ^{f,2}	ND	32.7±0.00 ^{h,4}	0.20±0.00	9.13±0.02 ^{h,2}	17.77±0.15	12.27±0.06 ^{f,1,2}	3.41±0.00	35.53±0.02 ^{h,4}
N2	7.27±0.00	3.48±0.14 ^{h,3}	ND	25.66±0.23 ^{h,3}	0.26±0.00	10.07±0.02 ^{h,3}	18.13±0.15	12.37±0.06 ^{h,2}	6.85±0.39	33.61±0.02 ^{h,3}
N3	9.67±0.01	5.62±0.01 ^{f,4}	ND	12.37±0.11 ^{h,2}	0.14±0.01	1.23±0.02 ^{h,1}	18.83±0.29	15.27±0.06 ^{g,3}	7.23±0.03	31.37±0.01 ^{h,2}
N4	10.82±0.03	10.45±0.05 ^{b,5}	ND	6.83±0.06 ^{g,1}	0.12±0.02	1.11±0.04 ^{g,1}	19.33±0.29	15.43±0.12 ^{g,3}	9.41±0.29	28.79±0.01 ^{h,1}

Note: ND means not detected. * Indicates no difference on the delta E value of fresh neera (L* 58.13 a* 0.36 and b* 9.26). The different alphabets in storage indicate significantly different at 0.05 level between the 0 d to 21 d. Abbreviation denotes: C - Control neera without treatment, N1-Neera treated with 50 ppm nisin and 500 ppm of sodium benzoate, N2-Neera treated with 50 ppm nisin and 1000 ppm of sodium benzoate, N3-Neera treated with 50 ppm nisin and 2500 ppm of calcium carbonate, N4-Neera treated with 50 ppm nisin and 3000 ppm of calcium carbonate (Sukumaran L., & Radhakrishnan, M. 2021b).

Another physicochemical property i.e, colour on 21 d demonstrated the total colour difference in N1, and N2 were 35.53 ± 0.02 and 33.61 ± 0.02 ΔE , respectively. Hence, the N3 and N4 treatments have increased ΔE value on 0 d due to the undissolved and light scattering particles (calcium carbonate) which whiteness the colour of the neera (Hsu and Chiang, 2002). Moreover, at the end of the storage period (21 d), the treatment of N3 and N4 showed the further reduced total colour difference of 31.37 ± 0.01 and 28.79 ± 0.01 ΔE , due to the increased solubility on storage and it was non-significant changes than that of other treatments.

4.2.5 Effect of preservatives on sensory properties of neera on storage period

Sensory properties like colour, flavour, consistency, taste, after taste and overall acceptability are illustrated in Figure 4.8 and 4.9 for the treatments and control. Among the four treatments, the N4 depicted better sensory properties up to 21 d of storage (Figure 4.9-b). The N3 had a better sensory score till 15 d (Figure 4.9-a) which was due to the least concentration of 2500 ppm of calcium carbonate. After 15 d of the storage, the sample perceived undesirable flavours due to the physicochemical changes. The N1 and N2 samples lost their sensory properties on 3 d and their changes were similar to the control sample (Figure 4.8-a,b,c). Hence the treatment of N4 (calcium carbonate-3000 ppm with nisin-50 ppm) was preferred as effective according to sensory analysis (Asghar et al. 2020).



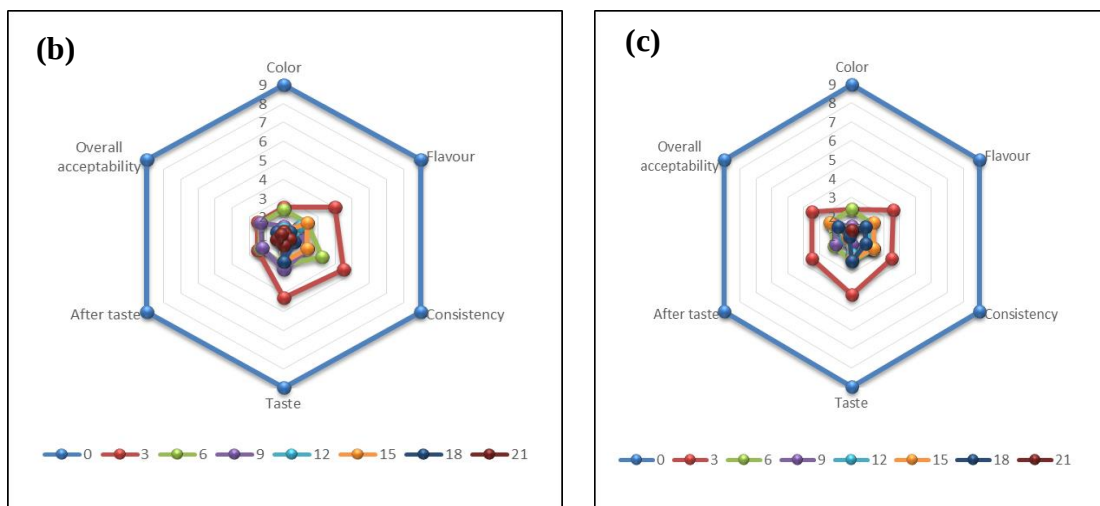


Figure 4.8: Sensory properties of fresh and preservatives added coconut neera during storage at 4 °C (a) Control neera (b) N1-Neera treated with nisin (50 ppm) and sodium benzoate (500 ppm), (c) N2-Neera treated with nisin (50 ppm) and sodium benzoate (1000 ppm) (Sukumaran L., & Radhakrishnan, M. 2021b)

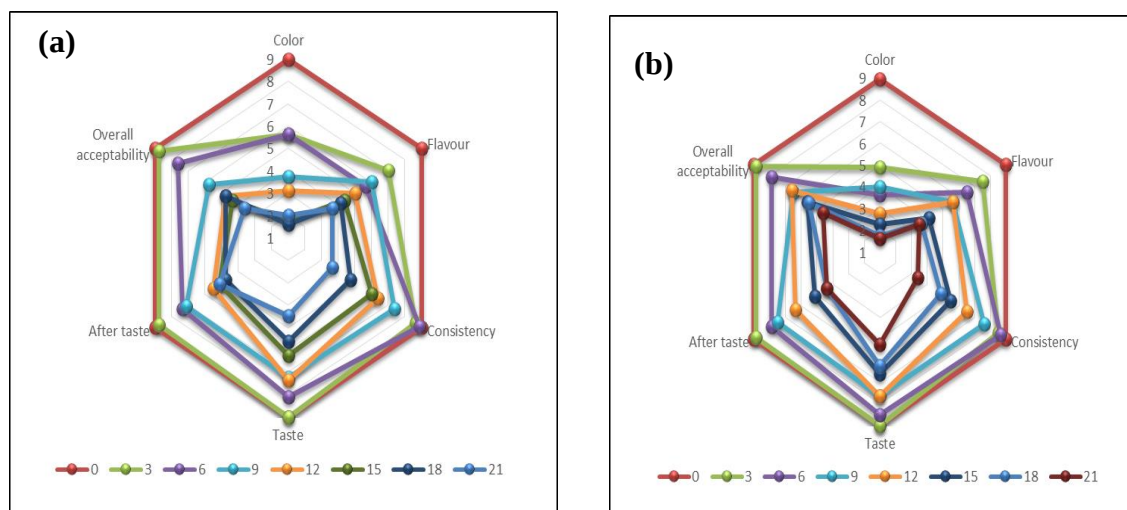


Figure 4.9: Sensory properties of preservatives added coconut neera during storage at 4 °C (a) N3-Neera treated with nisin (50 ppm) and calcium carbonate (2500 ppm), (b) N3-Neera treated with nisin (50 ppm) and calcium carbonate (3000 ppm)

4.3 Non-thermal atmospheric pressure plasma jet on coconut neera for shelf life extension

4.3.1 Effect of plasma on the reduction of the total bacterial population

The variation in the total bacterial log reduction during the storage period was significantly different between the function of voltage and treatment time (Table 4.5). The difference in results might be due to the emission spectrum of nitrogen, oxygen and other species in plasma treatment at various power levels (Lokeswari et al., 2021). The effective emission spectrum at 35 kV-7.5 min strongly affected the microorganism on the cell wall, plasma membrane and nucleic acids (Thana et al., 2019) and it significantly reduced the total bacterial log reduction (1.27 ± 0.05) on 3 h of storage than the other treatment of 30 kV-7.5 min (0.74 ± 0.06) and 40 kV-7.5 min (1.15 ± 0.12). The effective reduction (1.27 ± 0.05) resulted from the affected gram-negative bacteria and gram-positive bacterial cell wall (Han et al., 2016; Mai-Prochnow et al., 2016). Further the plasma species might have caused the leakage or damage of intracellular components (Ganesan et al., 2021). After destroying gram-negative bacteria, the plasma species could have affected the gram-positive bacteria in neera.

The antimicrobial mechanism of secondary plasma species extend the microbial reduction after 3 h storage. According to that, the plasma treatment at 35 kV-7.5 min exhibited log reduction of 1.13 ± 0.05 (6 h), 0.58 ± 0.03 (12 h), 0.47 ± 0.02 (18 h), and 0.45 ± 0.15 (24 h). However, the decreasing trend of log reduction are evident for the reduced concentration of plasma species in this treatment (30 kV-7.5 min) (Starek et al., 2020). The treatment of 40 kV-10 min gives an inconsistent log reduction 0.14 ± 0.02 , 0.72 ± 0.01 , 0.43 ± 0.03 and 0.35 ± 0.02 for 6, 12, 18 and 24 h respectively. At the longer treatment time of 10 min plasma

tuned their discharge and promoted water dissociation reaction, which produces H^+ ions in the plasma-water interface region. These H^+ ions react with the available H_2O and O_2 and form hydroxyls and then it converted to hydrogen peroxide in the presence of electrons and ions (Perinban et al. 2019; Sharkey et al., 2015). The multiple reactions resulted in the oxidation of food constituents such as carbohydrates, proteins, and amino acids (Surowsky et al., 2016). The greater number of H_2O_2 species in longer (10 min) treatment time affect the emission spectrum of secondary plasma and involves in other reactions through OH, peroxyxynitrite, and HO_2 species, thus affecting the antimicrobial metabolism (Surowsky et al., 2015; Thana et al., 2019). Han et al., (2016) who have reported the exposure of longer treatment lowered the intensity of plasma species during the direct treatment. Due to that the longer treatment time of 10 min at 35 kV revealed the reduction as 0.38 ± 0.02 (6 h), 0.84 ± 0.02 (12 h), 0.61 ± 0.01 (18 h) and 0.28 ± 0.08 (24 h). These reduction are inconsistent between the storage periods. By comparing the results the power level of 35 kV and treatment time of 7.5 min showed consistent log reduction from 3h to 24 h. Finally, the observed log reduction at 24 h (0.45 ± 0.15) on storage was the highest log reduction compared to other treatments.

Table 4.5: Effect of plasma jet treatment at different input voltage and exposure time on log reduction of total bacteria in coconut neera

Plasma treatment (kV)	Treatment time (min)	Log reduction during storage period (h)							
		3	6	9	12	15	18	21	24
30	5	0.33±0.03 ^{a,5}	0.27±0.06 ^{a,b,6}	0.22±0.06 ^{b,c,6}	0.19±0.03 ^{c,d,5}	0.14±0.06 ^{d,5}	0.07±0.02 ^{e,5}	0.05±0.01 ^{e,5}	0.07±0.01 ^{e,4}
	7.5	0.74±0.06 ^{a,3}	0.75±0.02 ^{a,3}	0.74±0.03 ^{a,1}	0.59±0.01 ^{b,3}	0.50±0.03 ^{c,3}	0.42±0.01 ^{d,3}	0.36±0.01 ^{e,3}	0.24±0.04 ^{f,2,3}
	10	0.06±0.18 ^{f,6}	0.39±0.13 ^{d,5}	0.63±0.10 ^{c,3}	0.81±0.06 ^{a,1}	0.71±0.07 ^{b,2}	0.83±0.03 ^{a,1}	0.33±0.02 ^{e,3,4}	0.33±0.03 ^{e,2}
35	5	0.48±0.02 ^{a,4}	0.45±0.04 ^{a,b,5,4}	0.41±0.02 ^{b,4}	0.36±0.02 ^{c,4}	0.36±0.02 ^{c,4}	0.29±0.03 ^{d,4}	0.26±0.01 ^{d,e,4}	0.23±0.02 ^{e,2,3}
	7.5	1.27±0.05 ^{a,1}	1.13±0.05 ^{b,1}	0.65±0.02 ^{c,2,3}	0.58±0.03 ^{c,d,3}	0.51±0.06 ^{d,e,3}	0.47±0.02 ^{d,e,3}	0.45±0.02 ^{e,1,2}	0.45±0.15 ^{e,1}
	10	0.40±0.04 ^{e,5}	0.38±0.02 ^{e,5}	0.72±0.01 ^{b,1,2}	0.84±0.02 ^{a,1}	0.90±0.04 ^{a,1}	0.61±0.01 ^{c,2}	0.49±0.06 ^{d,1}	0.28±0.08 ^{f,2,3}
40	5	0.52±0.08 ^{a,4}	0.48±0.09 ^{a,b,4}	0.46±0.08 ^{a,b,c,4}	0.39±0.06 ^{b,c,d,4}	0.35±0.04 ^{c,d,e,4}	0.29±0.04 ^{d,e,f,4}	0.26±0.01 ^{e,f,4}	0.21±0.03 ^{f,3}
	7.5	1.15±0.12 ^{a,2}	0.94±0.04 ^{b,2}	0.70±0.02 ^{c,1,2}	0.58±0.02 ^{d,3}	0.50±0.04 ^{e,3}	0.44±0.03 ^{e,f,3}	0.40±0.04 ^{f,g,2,3}	0.35±0.03 ^{g, 1,2}
	10	0.69±0.03 ^{a,3}	0.14±0.02 ^{d,7}	0.33±0.01 ^{c,5}	0.72±0.01 ^{a,2}	0.72±0.02 ^{a,2}	0.43±0.03 ^{b,3}	0.38±0.05 ^{c,2,3}	0.35±0.02 ^{c,1,2}

Note: The results are expressed as a mean ± standard error. Average values in the row and column were analyzed statistically and marked from highest mean to lowest mean. The different alphabets (a,b,c) in the superscripts are significantly different ($p < 0.05$) with respect to storage period and marked from highest mean to lowest mean. In addition, values having different 1, 2, 3 numbers in the superscripts are significantly different ($p < 0.05$) among the treatment and time.

4.3.2 Effect of plasma on inactivation of lactic acid bacteria

The log reduction of LAB at different treatment combination were shown in (Table 4.6). At 30 kV-5 min there was no log reduction from the storage period of 6 to 24 h due to the least exposure or treatment time (5min). A similar response was observed at 35 kV-5 min treatment. During 5 min of treatment, gram-positive bacteria resists the antimicrobial mechanism due to the complex assemblage of the cell wall (Niedzwiedz et al., 2019). The peptidoglycan of lactic acid bacteria consists of teichoic acids, polysaccharides, and proteins. It resists reactive oxygen species diffusion and peroxidation mechanism on lower treatment time of 5 min at 30 and 35 kV (Chapot-Chartier and Kulakauskas., 2014). The treatment time of 7.5 min at 35 kV exhibited a log reduction from 1.04 ± 0.01 (3 h) to 0.47 ± 0.06 (24 h). The effective log reduction at 35 kV of 7.5 min confirmed the diffusion of reactive oxygen species through the cell membrane via active transport of lipid bilayer or transient opening of the peptidoglycan layer (Han et al., 2016). The decreased log reduction confirms the bond breakage by oxygen species against the lactic acid bacteria cell wall (C-O, C-N, and C-C) (Mai-Prochnow et al., 2016). Moreover, the diffusion of plasma species affects the inner biomaterials (DNA) of the gram-positive lactic acid bacteria, which leads to cell death (Pignata et al., 2017). The plasma treatment at 40 kV-7.5 min showed the log reductions of 0.25 ± 0.04 at the end of the 24 h. The reduced log reduction at 40 kV was due to the effect of tuned discharge. In addition to that, the multiplication of unaffected lactic acid bacteria in storage reduced the log reduction at 40 kV (Starek et al., 2020). The survival of LAB ferment the neera within 24 h by lactic acid fermentation (Sharkey et al., 2015). According to this study, plasma treatment at 35 kV for 7.5 min had effectively reduced the LAB population up to 40 % within 24 h.

Table 4.6: Log reduction of lactic acid bacteria after plasma jet treatment at different input voltage and exposure time in coconut neera

Plasma treatment (kV)	Treatment time (min)	Log reduction during storage period (h)							
		3	6	9	12	15	18	21	24
30	5	0.14±0.03 ^{a,7}	0.05±0.01 ^{b,6}	-0.05±0.01 ^{c,7}	-0.11±0.01 ^{d,7}	-0.12±0.03 ^{d,e,5}	-0.14±0.04 ^{d,e,6}	-0.21±0.02 ^{e,4}	-0.20±0.07 ^{e,5}
	7.5	0.94±0.03 ^{a,2}	0.91±0.05 ^{a,1}	0.72±0.07 ^{b,2}	0.72±0.01 ^{b,2}	0.64±0.03 ^{b,1}	0.52±0.09 ^{c,2,3}	0.43±0.08 ^{c,d,2}	0.33±0.09 ^{d,2,3}
	10	0.61±0.10 ^{a,b,4}	0.43±0.04 ^{d,3}	0.70±0.02 ^{a,2}	0.46±0.05 ^{c,d,4}	0.62±0.07 ^{a,b,1}	0.54±0.03 ^{b,c,2}	0.13±0.03 ^{e,3}	0.40±0.01 ^{d,1,2}
35	5	0.48±0.06 ^{a,5}	0.31±0.03 ^{b,4}	0.24±0.03 ^{c,6}	-0.06±0.05 ^{d,7}	-0.11±0.05 ^{d,e,5}	-0.14±0.03 ^{e,f,6}	-0.20±0.01 ^{f,g,4}	-0.24±0.01 ^{g,5}
	7.5	1.04±0.01 ^{a,1}	0.88±0.07 ^{b,1}	0.81±0.06 ^{b,1}	0.79±0.04 ^{b,1}	0.63±0.08 ^{c,1}	0.63±0.04 ^{c,1}	0.52±0.01 ^{d,1}	0.47±0.06 ^{d,1}
	10	0.83±0.03 ^{a,3}	0.74±0.05 ^{b,2}	0.42±0.02 ^{e,4}	0.60±0.02 ^{c,3}	0.54±0.04 ^{c,d,2}	0.46±0.04 ^{d,e,3}	0.45±0.06 ^{d,e,1,2}	0.31±0.09 ^{f,2,3}
40	5	0.60±0.09 ^{a,4}	0.51±0.04 ^{b,3}	0.32±0.06 ^{c,5}	0.27±0.02 ^{c,d,5}	0.21±0.02 ^{d,4}	0.12±0.03 ^{e,5}	0.07±0.02 ^{e,3}	0.12±0.02 ^{e,4}
	7.5	0.84±0.02 ^{a,3}	0.74±0.03 ^{b,2}	0.52±0.09 ^{d,3}	0.58±0.08 ^{c,3}	0.41±0.05 ^{e,3}	0.34±0.04 ^{f,4}	0.33±0.03 ^{f,3}	0.25±0.04 ^{g,3}
	10	0.27±0.01 ^{a,6}	0.24±0.02 ^{a,5}	0.23±0.05 ^{a,6}	0.13±0.04 ^{b,6}	0.23±0.03 ^{a,4}	0.16±0.04 ^{b,5}	0.15±0.04 ^{b,3}	0.06±0.04 ^{c,4}

Note: The results are expressed as a mean ± standard error. Average values in the row and column were analyzed statistically and marked from highest mean to lowest mean. Values having different a,b,c alphabets in the superscripts are significantly different ($p < 0.05$) with respect to storage period and marked from highest mean to lowest mean. Values having different 1, 2, 3 numbers in the superscripts are significantly different ($p < 0.05$) with respect to treatment-time combination.

4.3.3 Effect of plasma on the inactivation of yeast population

At 35 kV-7.5 min, the 3 h stored neera detected with a log reduction of 0.87 ± 0.04 and it further reduced to 0.47 ± 0.01 on stored period of 24 h (Table 4.7). The other treatment at 24 h storage resulted in log reduction of 0.11 ± 0.09 (30 kV-7.5 min) and 0.33 ± 0.03 (40 kV-7.5 min). From the results, highest log reduction was achieved at 35 kV-7.5 min from the reactive species, i.e., $\cdot\text{OH}$, $\cdot\text{O}_2^-$, NO_2^- , NO_3^- , H_2O_2 , UV, and their antimicrobial mechanism on the eukaryotic yeast cell present in the liquid medium of the neera (Siadati et al., 2020). Among the plasma species O_2 initiate the membrane lipid peroxidation, which disrupts the cell membrane (Xu et al., 2020). The membrane damage favours the accumulation of reactive oxygen species on yeast cells. Whereas the species of $\text{HOO}\cdot$ induces the lipid peroxidation on the hydrophobic region of yeast genera. Therefore combined effect of reactive oxygen, nitrogen, and UV at 35 kV for 7.5 min plays an efficient role in yeast inactivation during treatment. The yeast inactivation in coconut neera is essential because it produces 6-7 % of ethanol by alcoholic fermentation during atmospheric storage (Asha et al., 2019). At 35 kV for 7.5 min, there was an increased log reduction (0.47 ± 0.01 -24 h) which confirms the apoptotic yeast cell death through various antimicrobial mechanisms. The mechanism of accumulation of intracellular reactive oxygen species, increased intracellular calcium, leakage of potassium ions, double strand breaks in DNA and mitochondrial dysfunction (Polčic and Machala., 2021).

Hence, the log reduction from 3 h (0.87 ± 0.04) to 24 h (0.47 ± 0.01) at 35 kV-7.5 min indicate the presence of unaffected yeast cells during storage period. At the end of the 24 h, the observed log reduction of 0.11 ± 0.09 (30 kV, 7.5 min) and 0.33 ± 0.03 (40 kV, 7.5 min) which indicates the occurrence of yeast fermentation. Finally, the present study

demonstrated a 40 % reduction of yeast by the plasma treatment at 35 kV for 7.5 min (Zhao et al., 2020). This log reduction was similar to the lactic acid bacteria. A better log reduction at 35 kV for 7.5 min confirmed the effective antimicrobial activity of plasma species than other treatment combination.

Table 4.7: Effect of plasma jet treatment at different input voltage and exposure time on log reduction of yeast in coconut

neera									
Plasma	Treatment	Log reduction during storage period (h)							
treatment	time	3	6	9	12	15	18	21	24
(kV)	(min)								
30	5	0.04±0.01 ^{a,5}	-0.04±0.01 ^{b,6}	-0.08±0.02 ^{c,5}	-0.05±0.01 ^{b,c,4}	-0.05±0.01 ^{b,c,3}	-0.16±0.03 ^{d,6}	-0.17±0.01 ^{d,4}	-0.22±0.02 ^{e,5}
	7.5	0.25±0.01 ^{a,3}	0.21±0.01 ^{a,b,4}	0.16±0.02 ^{b,c,4}	0.14±0.03 ^{b,c,3}	0.07±0.01 ^{d,e,2}	0.05±0.02 ^{d,e,3,4}	0.04±0.02 ^{e,3}	0.11±0.09 ^{c,d,4}
	10	0.28±0.10 ^{a,3}	0.24±0.04 ^{a,4}	0.13±0.02 ^{b,4}	0.13±0.01 ^{b,3}	0.12±0.03 ^{b,2}	0.08±0.01 ^{b,3}	0.06±0.01 ^{b,3}	0.06±0.01 ^{b,4}
35	5	0.26±0.06 ^{a,3}	0.17±0.05 ^{b,4,5}	0.14±0.02 ^{b,c,4}	0.13±0.02 ^{b,c,3}	0.07±0.02 ^{c,d,2}	0.04±0.02 ^{d,4}	0.04±0.01 ^{d,3}	0.04±0.05 ^{d,4}
	7.5	0.87±0.04 ^{a,1}	0.73±0.01 ^{b,2}	0.68±0.07 ^{b,1}	0.65±0.01 ^{b,1}	0.43±0.14 ^{c,1}	0.51±0.02 ^{c,1}	0.52±0.02 ^{c,1}	0.47±0.01 ^{c,1}
	10	0.31±0.01 ^{a,3}	0.22±0.02 ^{b,4}	0.14±0.05 ^{c,4}	0.15±0.02 ^{c,3}	0.09±0.01 ^{d,2}	0.08±0.01 ^{d,3,4}	0.07±0.01 ^{d,e,3}	0.04±0.01 ^{e,4}
40	5	0.28±0.10 ^{a,3}	0.14±0.01 ^{b,5}	-0.03±0.03 ^{d,5}	-0.08±0.04 ^{e,4}	0.04±0.05 ^{c,d,2,3}	-0.07±0.01 ^{e,5}	0.07±0.02 ^{b,c,3}	0.06±0.06 ^{b,c,4}
	7.5	0.14±0.04 ^{e,4}	0.88±0.08 ^{a,1}	0.52±0.10 ^{c,2}	0.64±0.03 ^{b,1}	0.53±0.06 ^{c,1}	0.36±0.03 ^{d,2}	0.26±0.02 ^{d,2}	0.33±0.03 ^{d,2}
	10	0.43±0.03 ^{b,2}	0.45±0.02 ^{b,3}	0.40±0.04 ^{b,c,3}	0.46±0.17 ^{b,2}	0.53±0.06 ^{a,1}	0.36±0.03 ^{c,2}	0.26±0.02 ^{d,2}	0.24±0.13 ^{d,3}

Note: The results are expressed as a mean ± standard error. Average values in the row and column were analyzed statistically and marked from highest mean to lowest mean. The different alphabets (a,b,c) in superscripts are significantly different ($p < 0.05$) with respect to storage period and marked from highest mean to lowest mean. In addition, values having different 1, 2, 3 numbers in the superscripts are significantly different ($p < 0.05$) with respect to treatment combination.

4.3.4 Estimation of physicochemical properties of plasma-treated neera

The unaffected microorganisms in neera cause a significant reduction in pH and total soluble solids of the treated sample (Figure 4.10 and 4.11). The treatment at 30 kV for 7.5 min have a pH of 5.90 ± 0.10 , 5.47 ± 0.06 and 4.60 ± 0.10 after 3, 12, and 24 h of storage. The treatment with the effective microbial reduction, i.e., 35 kV at 7.5 min have least physicochemical changes such as pH to 6.17 ± 0.15 for 3 h, 5.43 ± 0.21 for 12 h, and 5.07 ± 0.45 for 24 h. The exhibited pH of 5.07 (24 h) at 35 kV-7.5 min confirm the results of the log reduction and antimicrobial activity of plasma species (Aparajhitha and Mahendran, 2019). In addition to the microbial log reduction, degradation and oxidation of sucrose induced by the plasma species (ozone and hydrogen peroxide) may decrease the pH of neera. The highest input voltage of 40 kV had a significant pH changes at 5 min (4.37 ± 0.06), 7.5 min (4.40 ± 0.10), and 10 min (4.47 ± 0.06)) than the other input voltage due to the formation of carbonyl, carboxyl, formic acid, lactones, and CO_2 in neera (Prince et al., 2016). The produced acids and gas from the food plasma interaction have reduced the pH at the highest input voltage (40 kV). According to the food plasma interaction, the pH was observed at 40 kV-7.5 min were 6.17 ± 0.15 , 5.62 ± 0.38 , 4.40 ± 0.10 at 3, 12 and 24 h, respectively. From the results, 35 kV for 7.5 min has the potential to minimizing the change in pH.

The total soluble solids were changed from 18.03 (control) to 13.37 ± 0.06 °Brix (30 kV-5 min), 12.53 ± 0.25 (35 kV-5 min) and 13.43 ± 0.40 (40 kV-10 min) at the end of the 24 h (Figure 4.11). The gradual reduction of total soluble solids demonstrates the reproduction and fermentation mechanism of bacteria and yeast survival after cold plasma treatment

(Zhao et al., 2020; Shetty et al., 2017). The better microbial log reduction at 35 kV for 7.5 min revealed the total soluble solids as 15.46 ± 0.58 °Brix at 24 h. More hydroxyl species and scavenging activity of sucrose at 40 kV have reduced the antimicrobial activity, thus decrease the total soluble solids (Couée et al., 2006; Asha et al., 2019).

The total acidity of plasma-treated neera increased to 1.38 ± 0.06 (35 kV-7.5 min), 3.80 ± 0.42 mg/L (30 kV-5 min) at the end of 24 h storage (Figure 4.12). The increased acidity in neera was developed due to the lactic acid fermentation. The acidity of 1.38 ± 0.06 mg/L (24 h) from the treatment of 35 kV-7.5 min was the least acidity among the treatment. Moreover, the changes did not affected the palatability when compared to the fermented neera total acidity (177 mg/L) (Pandiselvam et al., 2021). Furthermore, nitrogenous acid sourced from reactive nitrogen species were also responsible for acidification of neera (Oehmigen et al., 2010).

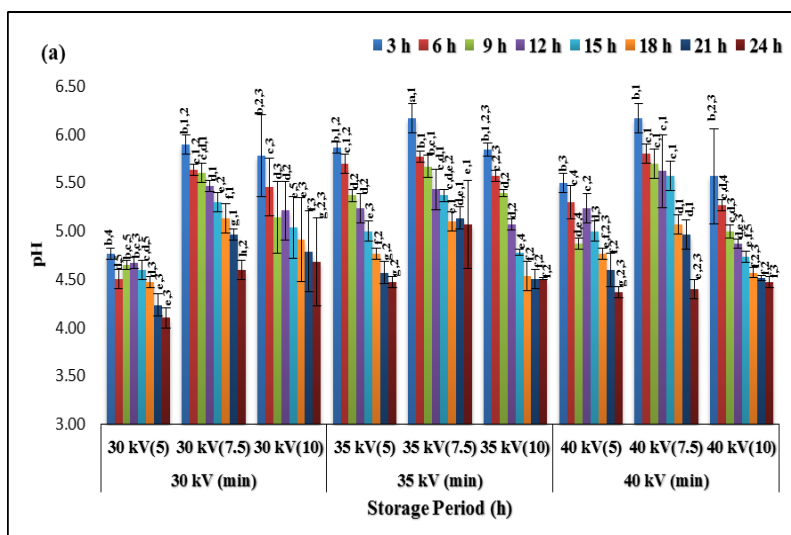


Figure 4.10: Effect of the plasma jet treatment on pH in coconut neera. The different alphabets and numbers in the graph indicate the significant difference from highest to lowest mean ($p < 0.05$). Control neera pH 6.27 ± 0.06 .

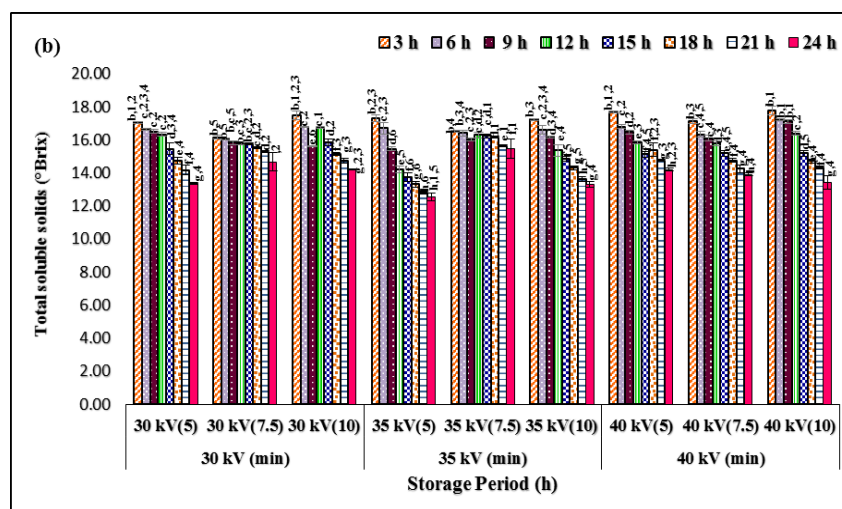


Figure 4.11: Effect of the plasma jet treatment on total soluble solids (°Brix) in coconut neera. Alphabets above the bar (a to h) and numbers (1,2,3) in the graph indicate the the significant difference from highest to lowest mean ($p < 0.05$). Control neera total soluble solids 18.03 ± 0.07 °Brix.

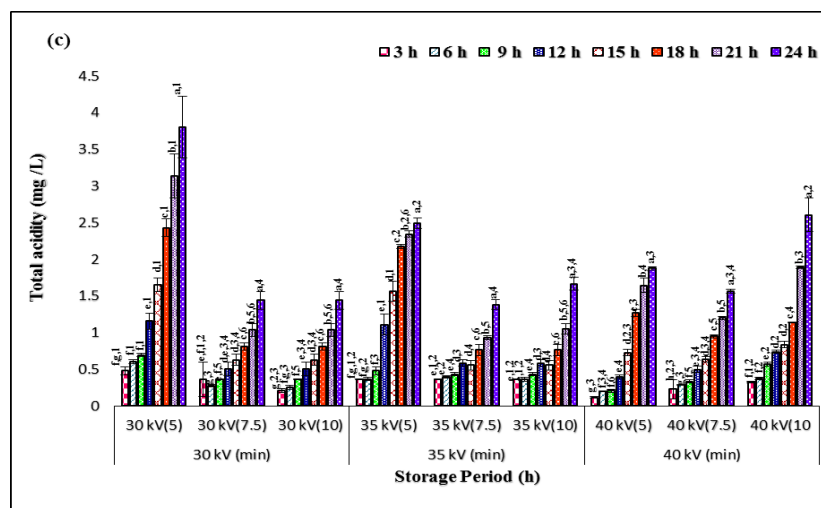


Figure 4.12: Effect of the plasma jet treatment on total acidity (mg/L) in coconut neera. Alphabets and numbers above the bar in the graph indicate the significant difference from highest to lowest mean ($p < 0.05$). Control neera total acidity 0.24 ± 0.03 (mg/L).

The total colour difference in Figure 4.13 revealed an increasing trend between the storage periods as a result of peroxidation and oxidation of pigments (Pankaj et al., 2018); (Aparajhitha and Mahendran, 2019). The oxidation reaction affects the golden yellow colour of neera and changes to pale white colour. The efficient concentration of free radicals and oxidation reaction at high input voltage 40 kV (10 min) resulted in the maximum colour difference of 5.93 ± 0.02 . However, at 35 kV for 7.5 min of plasma treatment showed remarkable decreased colour difference (ΔE) of 2.67 ± 0.19 in 24 h of storage. This value suggests that the treatment was efficient against the colour deterioration of neera.

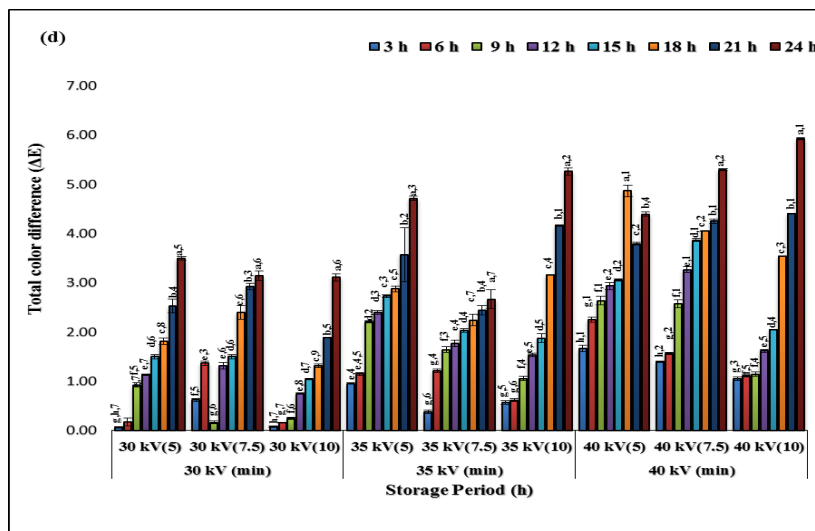


Figure 4.13. Effect of the plasma jet treatment on total color difference (ΔE) in coconut neera. Alphabets above the bar (a to h) in the graph indicate the significant difference from highest to lowest mean ($p < 0.05$).

4.4 Thermal method (mild heat treatment) on coconut neera for shelf life extension

4.4.1 Determination of microbial survival and decimal reduction time on heat treated neera

The microbial log value (CFU/ml) of single-stage and double-stage, at each temperature (50, 55, and 60°C) at the treatment time of 5 and 10 min presented in Table 4.8 Among the single and double-stage, the single-stage treatment at 60°C for 10 min exhibited higher microbial survival. It resulted in the microbial log values of 7.10 ± 0.01 log CFU/ml. Hence the double-stage treatment at 60°C-10 min demonstrated values of 6.68 ± 0.02 log CFU/ml, which was the least microbial survival than other treatment. The double-stage heat-treatment have reduced microbial log value which was due to the increased microbial lethality by tyndallization effect (Cho et al., 1999). The other combinations such as 60°C-5 min (8.24 ± 0.05), 55°C-10 min (7.15 ± 0.03), 55°C-5 min (8.41 ± 0.01), 50°C-10 min (7.78 ± 0.05), 50°C-5 min (9.04 ± 0.04) have increased microbial survival in double-stage treatment. In addition to the lethal effect of temperature, the incubation period (60-90 min) in the double-stage treatment induced survival of viable vegetative cells and *Bacilli* (Cho et al., 1999). The double-stage treatment inactivated these resistant microorganism. In comparison, the single-stage heat treatment has a lethal effect on microbes only by the temperature, which resulted in a high number of microbial survival in treatment (Løvdaal et al., 2011).

The D values of heat treatment against the microorganism in neera at various temperatures are summarized in Table 4.9. The more number of heat-resistant microorganisms at single-stage treatment of 50°C-5 min resulted the highest decimal

Table 4.8: Microbial survival of single and double-stage heat treated neera

Treatment	log (CFU/ml)		
	50°C	55°C	60°C
Single stage			
5 min	9.17±0.06 ^{b,1}	8.66±0.03 ^{a,2}	8.36±0.05 ^{a,3}
10 min	8.35±0.03 ^{d,1}	7.76±0.02 ^{d,2}	7.10±0.01 ^{d,3}
Incubation period			
5 min	9.48±0.02 ^{a,1}	8.35±0.02 ^{c,2}	7.63±0.05 ^{c,3}
10 min	8.34±0.03 ^{d,1}	7.31±0.01 ^{e,2}	7.04±0.05 ^{d,3}
Double stage			
5 min	9.04±0.04 ^{c,1}	8.41±0.01 ^{b,2}	8.24±0.05 ^{b,3}
10 min	7.78±0.05 ^{e,1}	7.15±0.03 ^{f,2}	6.68±0.02 ^{e,3}

Note: The results expressed as a mean ± standard error. Average values in the row and column were analyzed statistically and marked from highest mean to lowest mean. Values having different a,b,c alphabets in the superscripts are significantly different ($p < 0.05$) between single stage, double stage and incubation period in each temperature. In addition, values with different 1, 2, 3 numbers in the superscripts are significantly different ($p < 0.05$) with different treatment temperatures.

reduction time (D) of 14.99±0.10 min. The fewer heat resistance microorganisms in double stage heat treatment at 60°C-10 min resulted in the shortest D values of 3.50±0.04 min (Gabriel, 2012). At 55°C D value was found as 5.74±0.04 for 5 min and 4.19±0.03 for 10 min during single-stage and double stage treatment respectively. The double stage

treatment has a significant contribution to microbial reduction in neera (Kim et al., 2012). Cho et al., (1999) reported a similar trend of increased thermal inactivation mechanism of microorganisms using double stage heat treatment.

Table 4.9: Decimal reduction time at each temperature of single and double-stage heat treated neera

Treatment	D _T (min)		
	50°C	55°C	60°C
Single stage			
5 min	14.99±0.10 ^{a,1}	5.74±0.04 ^{a,2}	4.46±0.05 ^{a,3}
10 min	9.31 ±0.04 ^{c,1}	5.59±0.05 ^{b,2}	3.89±0.03 ^{b,3}
Double stage			
5 min	10.32±0.06 ^{b,1}	4.31±0.05 ^{c,2}	3.77±0.03 ^{c,3}
10 min	5.68±0.17 ^{d,1}	4.19±0.03 ^{d,2}	3.50±0.04 ^{d,3}

Note: The results expressed as a mean ± standard error. Average values in the row and column were analyzed statistically and marked from highest mean to lowest mean. Values having different a,b,c alphabets in the superscripts are significantly different ($p < 0.05$) between single stage, double stage and incubation period in each temperature. In addition, values with different 1, 2, 3 numbers in the superscripts are significantly different ($p < 0.05$) with different treatment temperatures.

4.4.2 Influences of temperature on the total viable count

The total viable log reduction after heat treatment is presented in Figure. 4.14. The three temperatures (50, 55, and 60°C) affect the growth of the microbes in neera during single-stage heat treatment. However, the log reduction at 50°C-5 min, and 60°C-10 min (single-stage) were 0.32 ± 0.02 and 2.57 ± 0.03 respectively (Figure. 4.4.1-a). This demonstrates that increased temperature-time combination causes effective microbial cell death (Gauvry et al., 2019). The heat treatment causes cell death by the affected or altered cellular membrane, peptidoglycan, and enzymes (Cebri, 2017). Moreover, the temperature-time combination (60°C-10 min) at double stage treatment shows the log reduction of 2.86 ± 0.01 after treatment which was the higher reduction than that of 55°C (2.41 ± 0.06) and 50°C (1.72 ± 0.02) (Figure. 4.14-b).

Storing at refrigerated temperature ($4 \pm 1^\circ\text{C}$) found to have significantly reduced log reduction in double stage heat treatment at 60°C-10 min and their value was 2.75 ± 0.01 (3 d), 1.46 ± 0.01 (12 d), and 1.16 ± 0.01 (21 d). The values suggested the few sub-lethally injured microorganisms and their growth during the storage period (Cebri, 2017). At 60°C 5 min of treatment time exhibited decreased microbial log reduction of 1.49 ± 0.01 (3 d), 1.02 ± 0.02 (12 d) and 0.81 ± 0.02 (21 d) than 10 min. The decreased log reduction in 5 min is evident to the proliferation of resistant and sub-lethally injured microorganisms (Machado et al., 2017). The heat resistant microbes in the single-stage heat treatment exhibited survival under refrigerated storage, which spoils the neera quality by the fermentation.

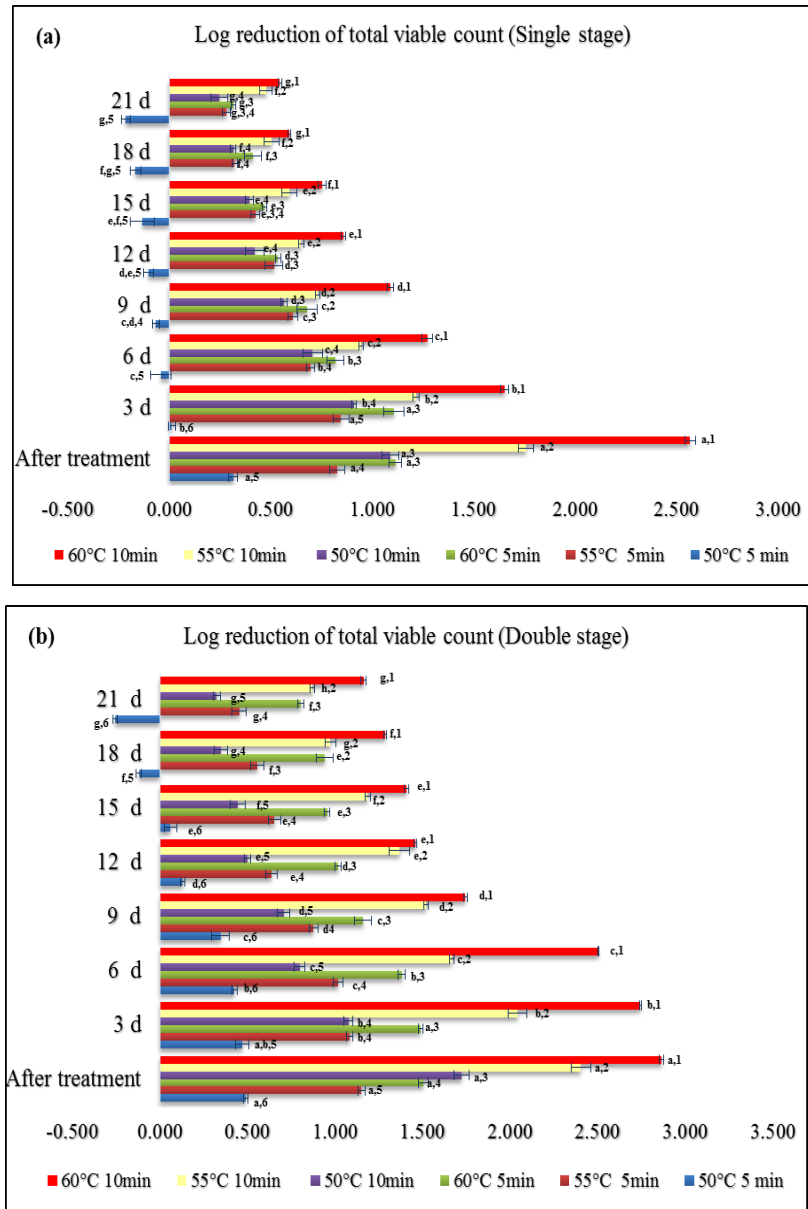
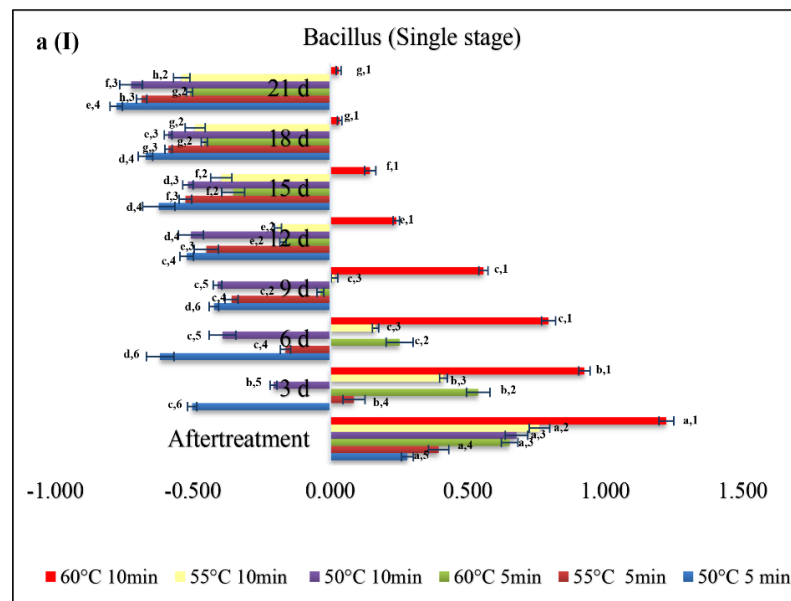


Figure 4.14: Total viable count of single and double stage heat treated neera at 50, 55 and 60°C with 5 and 10 min treatment time. (a) single-stage heat treatment (b) double stage heat treatment. The letters and numbers above the bars (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest mean to lowest mean.

4.4.3 Effect of heat treatment on inactivation of *Bacillus*

The overall log reduction of *Bacillus* after heat treatment at 60°C-10 min was greater for double stage treatment (1.94 ± 0.03) (Figure. 4.15-aII) than single-stage heat treatment (1.22 ± 0.03) (Figure. 4.15-aI). These variation in log reduction confirms the single-stage treatment has more heat-tolerant or resistant *Bacilli* than the double stage heat treatment (60°C-10 min). Zhuang et al. (2019) have indicated that heat tolerance of *Bacilli* after heat treatment due to the formation spores. Furthermore, the incubation period of 60-90 min in double stage treatment promoted the spore formation in *Bacilli* (Besten et al., 2010). These germinated spores, were inactivated during the double stage treatment, which increased the log reduction. The significant log reduction in double stage treatment (60°C-10 min) was similar to the Cho.et al. (1999), who reported that 1.1 log reduction of *Bacilli* after double stage heat treatment or tyndallization.



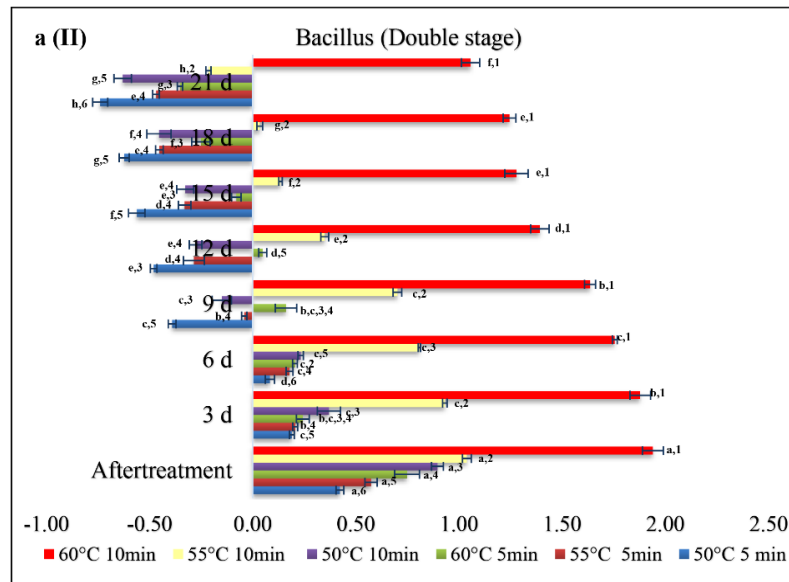


Figure 4.15: Effect of single and double stage heat treatment on log reduction of *Bacillus* (aI) single stage (aII) double stage. The letters and numbers above the bars (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest mean to lowest mean.

The storage period revealed the microbial log reduction up to 21 d at double stage treatment of 60°C-10 min. Due to the effective tyndallization at double stage treatment resulted the log reduction of 1.88 ± 0.01 (3 d), 1.39 ± 0.02 (12 d), 1.06 ± 0.01 (21 d) at 60°C-10 min. Moreover, the log reduction of 1.06 ± 0.01 has $<10^4$ CFU/ml at the end of the 21 d and the number of *Bacilli* are safe limit according to the Food Standards of Australia and New Zealand (Yu et al., 2020). The single-stage heat treatment affects one or more proteins of *Bacilli* during the treatment, and it recovers during the storage period (Warda et al., 2016). The increased rate of recovery at single-stage treatment did not show the log reduction from the storage period of 3 d, 6 d, and 12 d at 50, 55 and 60°C respectively. The more

affected *Bacilli* at 60°C-10 min (single-stage) extended log reduction up to 21 d (0.03 ± 0.01) of storage.

4.4.4 Effect of heat on inhibition of Lactic acid bacteria

After the single-stage and double-stage heat treatment at 60°C-10 min observed to have least lactic acid bacterial log reduction of 0.39 ± 0.01 and 0.66 ± 0.01 respectively (Fig. 4.16-bI and II). The heat treatment affects the lactic acid bacteria by the enzymatic inactivation (Malika et al., 2019). Due to reason most of the temperatures at single and double stage treatment not reflect the log reduction between the storage periods. At the end of storage (21 d) the treatment at 60°C-10 min demonstrated the log reduction of 0.06 ± 0.01 and 0.27 ± 0.01 for the single and double stage heat treatment, respectively. The lactic acid bacteria had resistance to heat treatment due to the thermo tolerance capacity up to the temperature of 70°C (Totosaus and Guerrero, 2008). And there is no report on the inactivation mechanism of lactic acid bacteria in tyndallized food products. However, the clinical study proved that the tyndallized probiotic lactic acid bacteria have functional properties. Hence the lactic acid bacteria have the metabolic conversion after heat treatment (Berlanga and Miñana-galbis., 2019).

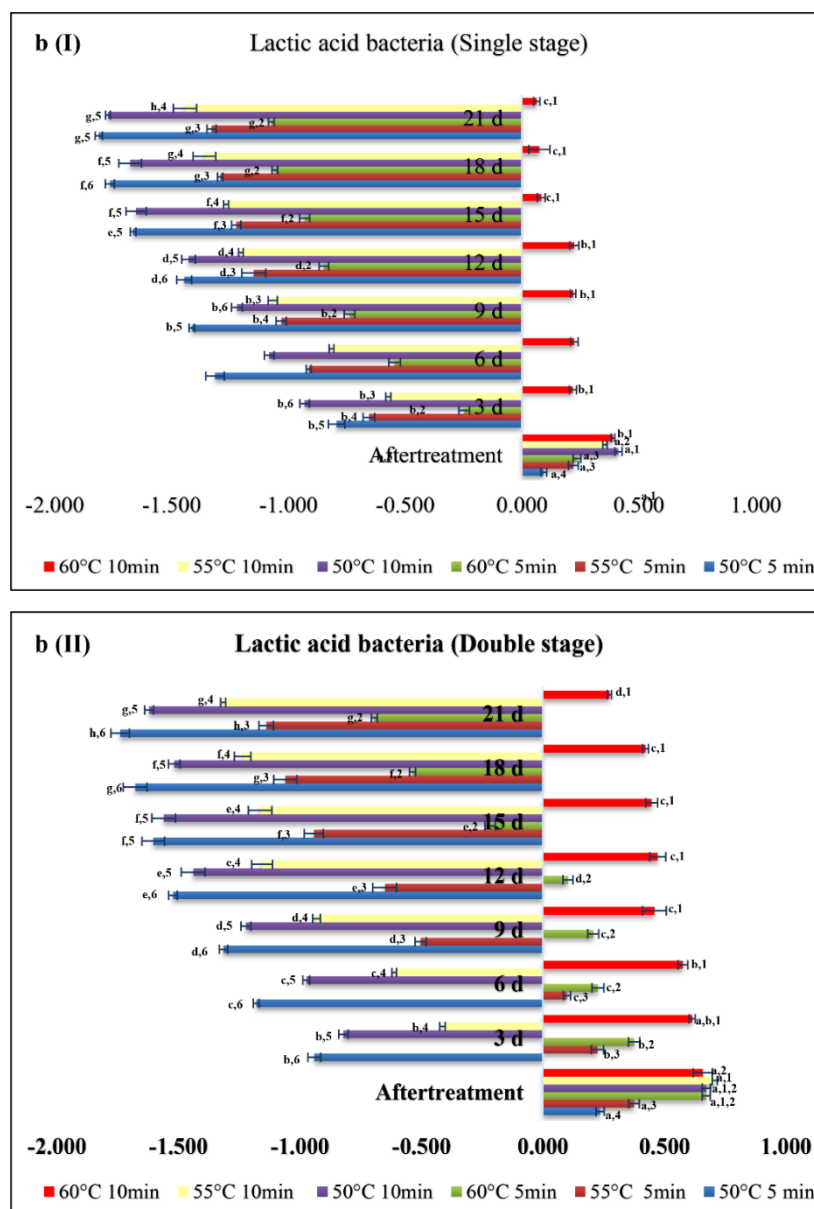
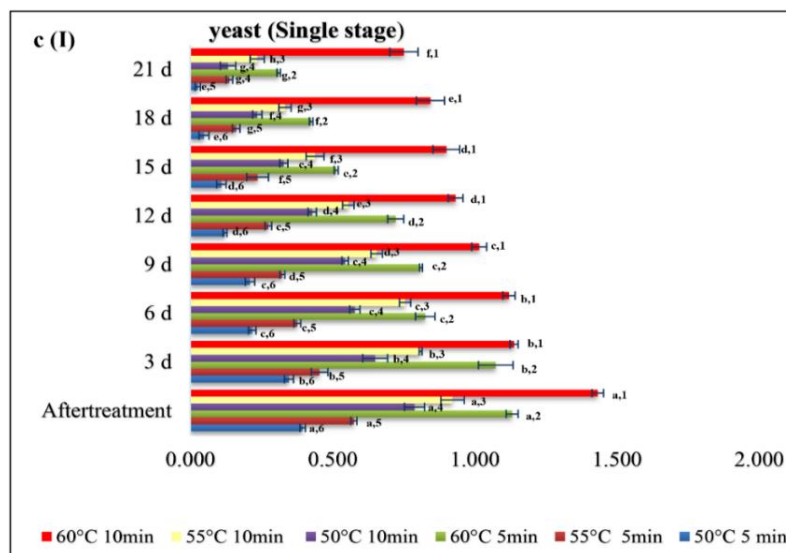


Figure 4.16: Effect of single and double stage heat treatment on log reduction of lactic acid bacteria (bI) single stage (bII) double stage. The letters and numbers above the bars (a,b,c, and 1,2,3) indicate a significant difference ($p < 0.05$) among the storage period and treatment temperature from highest mean to lowest mean.

4.4.5 Effect of heat treatment on inactivation of yeast

The log reduction of yeast at different temperatures in single and double-stage treatment is illustrated in Figure. 4.17-c I and II. The highest temperature (60°C for 10 min), demonstrated more lethality after treatment in double-stage treatment (1.77 ± 0.03) than single-stage treatment (1.43 ± 0.02). In a double-stage treatment, the temperatures of 50°C and 55°C resulted in much lower log reduction at 5 and 10 min. Because the membrane disruption of the yeast cells was achieved at high thermal stress, i.e., 60°C, whereas low thermal stress at 50°C and 55°C causes only cell damage in a few of the yeast cells (Guyot et al., 2015). The effective double stage treatment (60°C-10 min) resulted in the better reduction of yeast cells, were 1.50 ± 0.02 (3 d), 1.27 ± 0.01 (12 d), and 0.86 ± 0.01 (24 d) in storage period. At single-stage treatment, the same temperature-time combination (60°C-10 min) reduced to a log reduction of 1.14 ± 0.01 (3 d), 0.93 ± 0.03 (12 d), and 0.75 ± 0.05 (24 d). The lower reduction was due to yeast growth and alcoholic fermentation, which also impacted consumer acceptability (Borse et al., 2007).



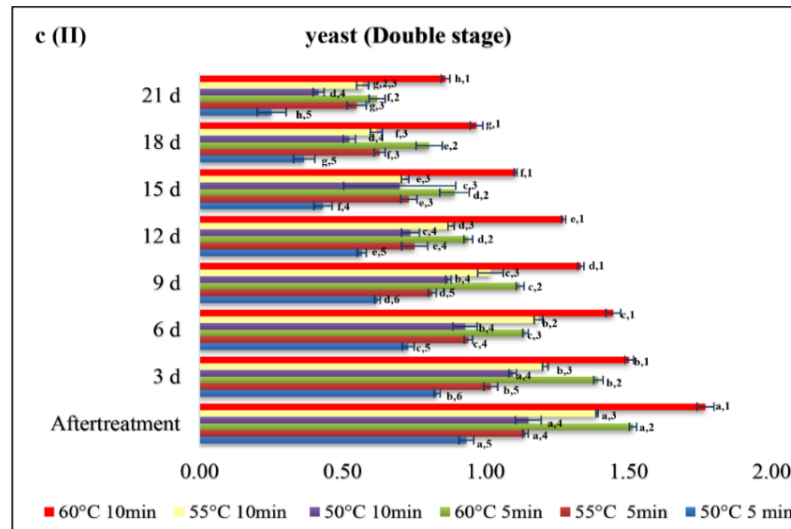


Figure 4.17: Effect of single and double stage heat treatment on log reduction of yeast (cI) single stage (cII) double stage. The letters and numbers above the bars (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

4.4.6 Investigation of microbial destruction using Transmission Electron Microscopy

TEM observation of *Bacillus* and lactic acid bacteria after heat treatment were shown in (Figure.4.18). The applied double stage heat treatment of tyndallization caused alterations in the morphology and integrity of *Bacillus* cells at 60°C-10 min. Furthermore, due to the influence of the tyndallization process, the morphology of lactic acid bacteria was lightly altered or shrunk at the same temperature (60°C-10 min). The ruptured cell membrane leads to the damage of cell wall, the release of cytoplasmic contents and DNA when compared with the control (a), and double-stage heat treatment of 55°C-10 min (b), 60°C-5 min (c), 60°C-10 min (d).

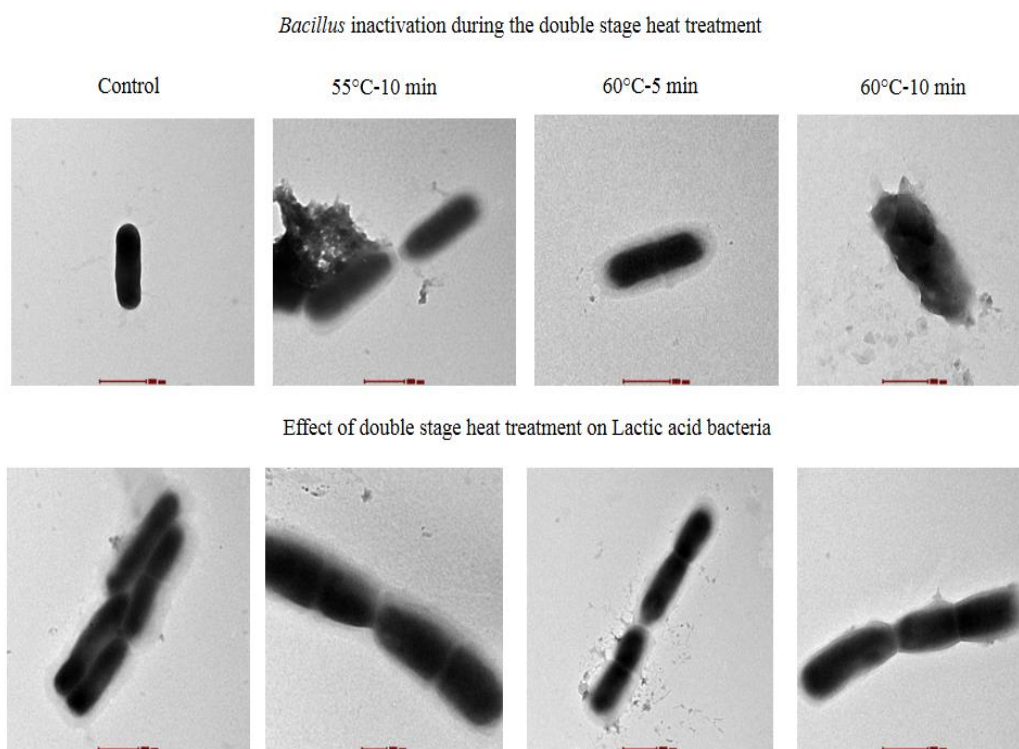


Figure 4.18: TEM Image of *Bacillus* and lactic acid bacteria from control neera and double stage heat treated neera

4.4.7 Effect of heat treatment on physicochemical characteristic of neera

The effect of heat on the various physicochemical properties like pH, total soluble solids (°Brix), total acidity (mg/L) and total colour difference (ΔE) were illustrated in Table 4.10-4.17. The method of single-stage treatment had significant *D* value (50°C-5 min) exhibited significant changes for pH (5.49 ± 0.04), total soluble solids (16.87 ± 0.05 °Brix), and total acidity 0.53 ± 0.03 mg/L after heat treatment. At double-stage treatment (60°C-10 min) the changes were 6.24 ± 0.05 , 17.97 ± 0.04 °Brix, and 0.24 ± 0.03 mg/L for pH, total soluble solids, and total acidity respectively. The results evident that the double-stage treated neera had non-significant changes for the analyzed physicochemical properties due

to the least decimal reduction time (D) of 3.50 ± 0.04 min. Gabriel., (2012) have also reported the treatment combination with shortest D values have least number of heat resistant microorganisms. The decimal reduction time of 3.50 ± 0.04 min suggests that double-stage treatment of 10 min had effective microbial inactivation than single-stage treatment.

Moreover, double-stage treatment of 60°C -10 min treatment at the end of storage period (21 d) revealed the least physicochemical changes such as pH- 5.77 ± 0.06 , total soluble solids- 16.02 ± 0.02 °Brix and total acidity- 0.72 ± 0.05 mg/L than the other treatment. In the single-stage treatment (60°C -10 min) had significant changes in the physicochemical properties (pH 5.08 ± 0.03 , 15.43 ± 0.06 °Brix, 1.01 ± 0.02 mg/L total acidity) at the end of the storage (21 d). The treatment with the decreased log reduction observed to have an increased physicochemical changes during storage period. This results were in agreement with earlier reports Naknean., (2013) who have reported a similar reduction in double stage treatment.

After the treatment, the double stage treatment at 10 min exhibited an increased colour difference (ΔE) of 0.07 ± 0.02 , 0.09 ± 0.01 , and 0.13 ± 0.03 at 50, 55 and 60°C respectively. Hence the single-stage treatment found to have the least colour value of 0.06 ± 0.01 (50°C -10 min), 0.08 ± 0.01 (55°C -10 min) and 0.09 ± 0.02 (60°C -10 min) after the heat treatment. The result suggests that an increasing temperature slightly degraded the colour of the neera in double stage treatment. The storage period of single-stage treated neera exhibited a higher colour difference (ΔE) on 21 d in the following sequence as 3.03 ± 0.04 (50°C), 2.83 ± 0.05 (55°C) and 2.19 ± 0.03 (60°C) for 5 min treatment time. However, at double

stage treatment of 50, 55, and 60°C for 10 min treatment time were observed with less colour difference of 1.15 ± 0.04 , 1.05 ± 0.03 , 1.01 ± 0.02 on 21 d. Compared to degradation reaction, the enzymatic reaction of polyphenol caused colour changes among single-stage treatment (Naknean, 2013). The decreased enzymatic reaction at double stage treatment revealed the less colour difference during storage period.

Table 4.10: Effect of single-stage treatment on pH of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
Control	6.27±0.06 ^a					
After treatment	5.49±0.04 ^{b,5}	5.57±0.03 ^{b,4}	5.97±0.05 ^{b,3}	6.04±0.02 ^{b,2}	6.04±0.05 ^{b,2}	6.12±0.03 ^{b,1}
3 d	5.38±0.03 ^{c,4}	5.39±0.01 ^{c,4}	5.73±0.04 ^{c,3}	5.82±0.02 ^{c,2}	5.87±0.02 ^{c,2}	5.95±0.05 ^{c,1}
6 d	5.04±0.05 ^{d,5}	5.27±0.03 ^{d,4}	5.34±0.05 ^{d,3}	5.64±0.03 ^{d,2}	5.60±0.05 ^{d,2}	5.81±0.02 ^{d,1}
9 d	4.76±0.04 ^{e,6}	5.08±0.02 ^{e,5}	5.30±0.01 ^{d,3}	5.22±0.01 ^{e,4}	5.43±0.01 ^{e,2}	5.60±0.05 ^{e,1}
12 d	4.49±0.02 ^{f,5}	4.94±0.01 ^{f,4}	5.20±0.03 ^{e,3}	5.17±0.02 ^{f,3}	5.27±0.06 ^{f,2}	5.49±0.03 ^{f,1}
15 d	4.28±0.03 ^{g,5}	4.78±0.03 ^{g,4}	5.11±0.02 ^{f,2}	5.05±0.02 ^{g,3}	5.15±0.02 ^{g,2}	5.27±0.06 ^{g,1}
18 d	4.14±0.05 ^{h,5}	4.58±0.03 ^{h,4}	5.01±0.03 ^{g,2}	4.71±0.03 ^{h,3}	4.95±0.01 ^{h,2}	5.16±0.05 ^{h,1}
21 d	4.06±0.04 ^{i,5}	4.49±0.01 ^{i,4}	4.74±0.03 ^{h,3}	4.53±0.02 ^{i,4}	4.84±0.01 ^{i,2}	5.08±0.04 ^{i,1}

The letters and numbers above the mean value (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.11: Effect of double-stage treatment on pH of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
C	6.27±0.06 ^a					
After treatment	5.61±0.10 ^{b,4}	5.86±0.02 ^{b,3}	6.13±0.03 ^{b,2}	6.19±0.02 ^{b,1,2}	6.20±0.01 ^{b,1,2}	6.24±0.05 ^{a,1}
3 d	5.44±0.05 ^{c,5}	5.72±0.01 ^{c,4}	5.84±0.03 ^{c,3}	6.10±0.03 ^{c,2}	6.15±0.03 ^{b,1,2}	6.17±0.04 ^{a, b,1}
6 d	5.18±0.03 ^{d,5}	5.48±0.02 ^{d,4}	5.66±0.02 ^{d,3}	5.92±0.02 ^{d,2}	5.93±0.01 ^{c,2}	6.10±0.05 ^{b,c,1}
9 d	4.87±0.05 ^{e,5}	5.28±0.02 ^{e,4}	5.47±0.07 ^{e,3}	5.82±0.03 ^{e,2}	5.89±0.03 ^{c,2}	6.06±0.06 ^{c,1}
12 d	4.58±0.04 ^{f,6}	4.96±0.05 ^{f,5}	5.29±0.03 ^{f,4}	5.53±0.05 ^{f,3}	5.75±0.05 ^{d,2}	5.94±0.06 ^{d,1}
15 d	4.27±0.05 ^{g,6}	4.52±0.03 ^{g,5}	4.96±0.03 ^{g,4}	5.41±0.02 ^{g,3}	5.70±0.01 ^{d,2}	5.90±0.02 ^{d,e,1}
18 d	4.18±0.03 ^{g,6}	4.43±0.05 ^{h,5}	4.52±0.04 ^{h,4}	5.27±0.03 ^{h,3}	5.59±0.04 ^{e,2}	5.84±0.05 ^{e,f,1}
21 d	4.09±0.02 ^{h,6}	4.36±0.02 ^{i,5}	4.44±0.02 ^{i,4}	5.03±0.05 ^{i,3}	5.35±0.04 ^{f,2}	5.77±0.06 ^{f,1}

The above alphabet and numbers (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.12: Effect of single stage treatment on total soluble solids (°Brix) of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
Control	18.03±0.07 ^a					
After treatment	16.87±0.05 ^{b,3}	16.89±0.04 ^{b,3}	17.40±0.06 ^{b,2}	17.47±0.04 ^{b,2}	17.47±0.06 ^{b,2}	17.60±0.03 ^{b,1}
3 d	16.19±0.02 ^{c,5}	16.23±0.01 ^{c,5}	17.21±0.05 ^{c,2}	16.79±0.03 ^{c,4}	16.87±0.06 ^{c,3}	17.31±0.02 ^{c,1}
6 d	15.47±0.04 ^{d,5}	15.89±0.02 ^{d,4}	16.50±0.06 ^{d,3}	16.42±0.05 ^{d,3}	16.67±0.15 ^{d,2}	17.05±0.05 ^{d,1}
9 d	14.85±0.05 ^{e,6}	15.64±0.01 ^{e,5}	16.06±0.05 ^{e,4}	16.40±0.03 ^{d,3}	16.50±0.10 ^{e,2}	16.61±0.02 ^{e,1}
12 d	14.72±0.03 ^{f,5}	15.49±0.04 ^{f,4}	15.68±0.03 ^{f,3}	16.06±0.01 ^{e,2}	16.03±0.06 ^{f,2}	16.34±0.05 ^{f,1}
15 d	14.49±0.02 ^{g,6}	15.19±0.01 ^{g,5}	15.50±0.10 ^{g,4}	15.73±0.03 ^{f,3}	15.87±0.06 ^{g,2}	15.98±0.02 ^{g,1}
18 d	14.35±0.05 ^{h,5}	15.00±0.01 ^{h,4}	15.18±0.08 ^{h,3}	15.36±0.05 ^{g,2}	15.60±0.06 ^{h,1}	15.67±0.02 ^{h,1}
21 d	14.08±0.03 ^{i,4}	14.90±0.10 ^{i,3}	14.94±0.07 ^{i,3}	15.05±0.0 ^{h,2}	15.38±0.02 ^{i,1}	15.43±0.05 ^{i,1}

The letters and numbers above the mean value (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.13: Effect of double-stage treatment on total soluble solids (°Brix) of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
C	18.03±0.07 ^a					
After treatment	17.02±0.01 ^{b,6}	17.19±0.01 ^{b,5}	17.51±0.01 ^{b,4}	17.70±0.05 ^{b,3}	17.77±0.03 ^{b,2}	17.97±0.04 ^{a,1}
3 d	16.25±0.05 ^{c,5}	16.52±0.06 ^{c,4}	16.76±0.03 ^{c,3}	17.46±0.04 ^{c,2}	17.59±0.02 ^{c,1}	17.53±0.05 ^{b,1,2}
6 d	15.77±0.04 ^{d,6}	16.19±0.01 ^{d,5}	16.54±0.05 ^{d,4}	16.74±0.12 ^{d,3}	17.13±0.05 ^{d,2}	17.39±0.04 ^{c,1}
9 d	15.62±0.02 ^{e,6}	15.80±0.06 ^{e,5}	16.47±0.03 ^{e,3}	16.34±0.03 ^{e,4}	16.79±0.03 ^{e,1}	16.73±0.05 ^{d,2}
12 d	15.40±0.03 ^{f,6}	15.73±0.06 ^{f,5}	16.39±0.02 ^{f,3}	16.29±0.03 ^{e,4}	16.45±0.01 ^{f,2}	16.68±0.04 ^{d,1}
15 d	14.86±0.04 ^{g,6}	15.61±0.01 ^{g,4}	16.28±0.03 ^{g,2}	15.51±0.06 ^{f,5}	16.19±0.02 ^{g,3}	16.43±0.05 ^{e,1}
18 d	14.63±0.06 ^{h,5}	15.25±0.07 ^{h,4}	16.24±0.05 ^{g,2}	15.30±0.06 ^{g,4}	15.78±0.03 ^{h,3}	16.36±0.05 ^{e,1}
21 d	14.59±0.03 ^{h,6}	15.13±0.01 ^{i,4}	15.86±0.04 ^{h,2}	15.07±0.02 ^{h,5}	15.58±0.04 ^{i,3}	16.02±0.02 ^{f,1}

The above alphabet and numbers (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.14: Effect of single stage treatment on total acidity (mg/L) of coconut neera

Storge period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
Control	0.24±0.03 ⁱ					
After treatment	0.53±0.03 ^{f,1}	0.54±0.05 ^{g,1}	0.31±0.02 ^{h,2}	0.56±0.05 ^{f,1}	0.29±0.05 ^{f,2}	0.26±0.03 ^{e,f,2}
3 d	0.75±0.02 ^{e,1}	0.78±0.03 ^{f,1}	0.54±0.05 ^{g,3}	0.65±0.03 ^{e,2}	0.53±0.03 ^{e,3}	0.33±0.02 ^{e,4}
6 d	0.86±0.05 ^{d,1}	0.90±0.05 ^{e,1}	0.69±0.02 ^{f,2}	0.72±0.05 ^{d,2}	0.59±0.05 ^{e,3}	0.44±0.05 ^{d,4}
9 d	1.02±0.02 ^{c,1}	0.90±0.05 ^{e,2}	0.76±0.01 ^{e,3}	0.98±0.03 ^{c,1}	0.66±0.03 ^{d,4}	0.53±0.06 ^{c,5}
12 d	1.10±0.01 ^{c,1}	1.05±0.03 ^{d,1,2}	1.01±0.04 ^{d,2}	1.01±0.05 ^{c,2}	0.81±0.05 ^{c,3}	0.56±0.05 ^{c,4}
15 d	1.25±0.02 ^{b,1}	1.17±0.05 ^{c,2}	1.10±0.03 ^{c,3}	1.14±0.03 ^{b,2,3}	0.96±0.03 ^{b,4}	0.62±0.07 ^{c,5}
18 d	1.56±0.05 ^{a,1}	1.31±0.05 ^{b,2}	1.19±0.05 ^{b,3}	1.19±0.05 ^{b,3}	1.04±0.05 ^{a,4}	0.71±0.05 ^{b,5}
21 d	1.56±0.01 ^{a,1}	1.47±0.05 ^{a,2}	1.29±0.03 ^{a,3}	1.29±0.03 ^{a,3}	1.08±0.05 ^{a,4}	1.01±0.02 ^{a,5}

The letters and numbers above the mean value (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.15: Effect of double-stage treatment on total acidity (mg/L) of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
C	0.24±0.03 ⁱ					
After treatment	0.51±0.05 ^{e,1}	0.42±0.03 ^{e,2}	0.38±0.05 ^{g,2}	0.29±0.05 ^{g,3}	0.26±0.02 ^{g,3}	0.24±0.03 ^{d,3}
3 d	0.71±0.02 ^{d,1}	0.65±0.05 ^{d,1}	0.53±0.03 ^{f,2}	0.45±0.03 ^{f,3}	0.42±0.01 ^{f,3}	0.32±0.05 ^{d,4}
6 d	0.78±0.01 ^{d,1}	0.71±0.03 ^{d,2}	0.59±0.01 ^{f,3}	0.59±0.05 ^{e,3}	0.48±0.03 ^{e,4}	0.42±0.03 ^{c,5}
9 d	0.95±0.03 ^{c,1}	0.72±0.05 ^{d,2}	0.69±0.03 ^{e,2}	0.69±0.03 ^{d,2}	0.57±0.02 ^{d,3}	0.47±0.07 ^{b,c,4}
12 d	1.07±0.05 ^{b,1}	0.98±0.05 ^{c,2}	0.81±0.05 ^{d,3}	0.81±0.05 ^{c,3}	0.60±0.03 ^{d,4}	0.51±0.03 ^{b,5}
15 d	1.16±0.01 ^{b,1}	1.05±0.03 ^{c,2}	1.01±0.04 ^{c,2,3}	0.96±0.03 ^{b,3}	0.67±0.01 ^{c,4}	0.54±0.09 ^{b,5}
18 d	1.55±0.05 ^{a,1}	1.20±0.05 ^{b,2}	1.10±0.03 ^{b,2,3}	1.01±0.05 ^{b,3}	0.76±0.02 ^{b,4}	0.65±0.05 ^{a,4}
21 d	1.62±0.02 ^{a,1}	1.32±0.03 ^{a,2}	1.19±0.05 ^{a,3}	1.11±0.03 ^{a,4}	0.98±0.05 ^{a,5}	0.72±0.05 ^{a,6}

The above alphabet and numbers (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.16: Effect of single-stage treatment on total color difference (ΔE) of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
Control	0.03±0.01					
After treatment	0.05±0.01 ^{g,4}	0.07±0.02 ^{h,2,3}	0.07±0.02 ^{h,2,3}	0.06±0.01 ^{h,4}	0.08±0.01 ^{h,1,2}	0.09±0.02 ^{h,1}
3 d	0.95±0.03 ^{f,1}	0.74±0.01 ^{g,2}	0.64±0.01 ^{g,3}	0.31±0.02 ^{g,4}	0.28±0.02 ^{g,5}	0.25±0.01 ^{g,6}
6 d	1.11±0.01 ^{e,1}	1.06±0.02 ^{f,2}	0.96±0.04 ^{f,3}	0.76±0.02 ^{f,4}	0.43±0.03 ^{f,5}	0.36±0.02 ^{f,6}
9 d	1.43±0.03 ^{d,1}	1.36±0.03 ^{e,2}	1.02±0.02 ^{e,3}	0.87±0.03 ^{e,4}	0.62±0.06 ^{e,5}	0.49±0.09 ^{e,6}
12 d	2.15±0.01 ^{c,1}	1.66±0.01 ^{d,2}	1.29±0.05 ^{d,3}	1.32±0.03 ^{d,3}	1.18±0.05 ^{d,4}	1.04±0.02 ^{d,5}
15 d	2.76±0.02 ^{b,1}	2.54±0.03 ^{c,2}	1.70±0.05 ^{c,3}	1.68±0.02 ^{c,3}	1.55±0.06 ^{c,4}	1.35±0.01 ^{c,5}
18 d	2.72±0.05 ^{b,1}	2.61±0.04 ^{b,2}	2.04±0.01 ^{b,3}	2.03±0.01 ^{b,3}	1.95±0.04 ^{b,4}	1.80±0.03 ^{b,5}
21 d	3.03±0.04 ^{a,1}	2.83±0.05 ^{a,2}	2.19±0.03 ^{a,4}	2.33±0.02 ^{a,3}	2.05±0.05 ^{a,5}	1.93±0.04 ^{a,6}

The letters and numbers above the mean value (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

Table 4.17: Effect of double-stage treatment on total color difference (ΔE) of coconut neera

Storage period	50°C 5 min	55°C 5min	60°C 5min	50°C 10min	55°C 10min	60°C 10min
Control	0.03±0.01 ¹					
After treatment	0.06±0.01 ^{h,5}	0.09±0.01 ^{h,2,3}	0.11±0.02 ^{h,2}	0.07±0.02 ^{h,3,4}	0.09±0.01 ^{h,3}	0.13±0.03 ^{h,1}
3 d	0.65±0.02 ^{g,1}	0.54±0.05 ^{g,2}	0.44±0.03 ^{g,3}	0.21±0.03 ^{g,4}	0.18±0.02 ^{g,5}	0.15±0.01 ^{g,6}
6 d	0.98±0.01 ^{f,1}	0.76±0.03 ^{f,2}	0.66±0.01 ^{f,3}	0.56±0.04 ^{f,4}	0.33±0.01 ^{f,5}	0.26±0.03 ^{f,6}
9 d	1.13±0.03 ^{e,1}	0.83±0.02 ^{e,2}	0.71±0.04 ^{e,3}	0.67±0.02 ^{e,4}	0.42±0.04 ^{e,5}	0.39±0.01 ^{e,6}
12 d	1.46±0.06 ^{d,1}	1.16±0.01 ^{d,2}	0.98±0.01 ^{d,3}	0.74±0.04 ^{d,4}	0.68±0.01 ^{d,5}	0.52±0.02 ^{d,6}
15 d	1.72±0.02 ^{c,1}	1.34±0.02 ^{c,2}	1.14±0.05 ^{c,3}	0.98±0.06 ^{c,4}	0.74±0.02 ^{c,5}	0.67±0.04 ^{c,6}
18 d	2.08±0.06 ^{b,1}	1.61±0.04 ^{b,2}	1.21±0.04 ^{b,3}	1.04±0.03 ^{b,4}	0.95±0.04 ^{b,5}	0.80±0.02 ^{b,6}
21 d	2.23±0.03 ^{a,1}	1.83±0.05 ^{a,2}	1.39±0.05 ^{a,3}	1.15±0.04 ^{a,4}	1.05±0.03 ^{a,5}	1.01±0.02 ^{a,6}

The above alphabet and numbers (a,b,c, and 1,2,3) indicate a significant difference (p 0.05) among the storage period and treatment temperature from highest to lowest mean.

4.4.8 Sensory evaluation of heat treated neera

Sensory examination revealed that single-stage heat-treated neera samples depreciated on storage and were significantly different than double-stage heat-treated neera samples (Figure 4.19-4.24 a and b). On 12 and 15 d of storage, the flavour and taste score of single-stage treatment at 60°C for 10 minutes dropped to poor quality ($P < 0.05$). The poor score value confirms lactic acid, palmitoleic acid, and dodecanoic acid production (Borse et al., 2007). While the flavour and taste of double stage treated neera at 60°C-10 min did not perceive the poor quality up to a 21 d, it showed higher scores of 4.25 ± 0.46 and 4.63 ± 0.2 during this storage period due to tyndalization effect.

In single-stage heat treatment, the lowest temperature (50°C-5 min) on 21d storage resulted in a colour score of 1.13 ± 0.05 . In contrast, the highest temperature of 60°C-5 min at the double stage treated neera sample resulted in a colour score of 3.63 ± 0.52 (Figure 4.21-a and b). In the double stage heat treatment, the increased rate of log reduction may slow the microbial fermentation, results in lower changes on 21 d. Due to the decreased log reduction the attributes of consistency, after taste and overall acceptability were significantly different after 3, 6, 9, 12, 15, 18 and 21 d of storage at a single-stage heat-treated neera. The results of tyndallization and their reduced microbial metabolism at 60°C for 10 min were perceived to have higher results, with overall acceptability of 4.50 ± 0.52 .

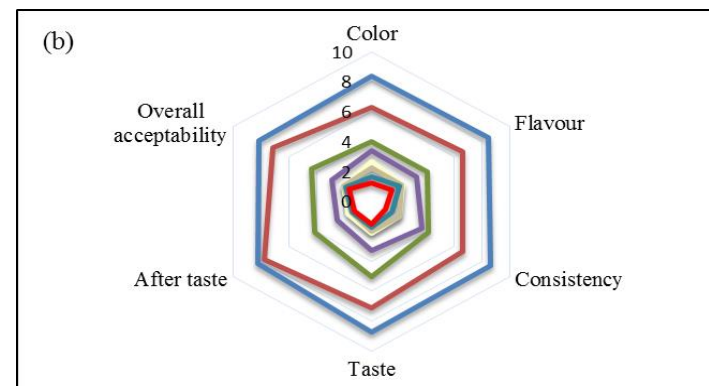
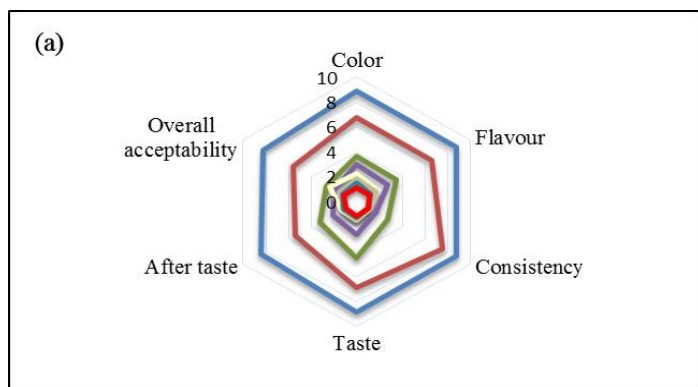


Figure 4.19: Sensory attributes of neera after heat treatment at 50°C-5 min (a) single-stage and (b) double-stage

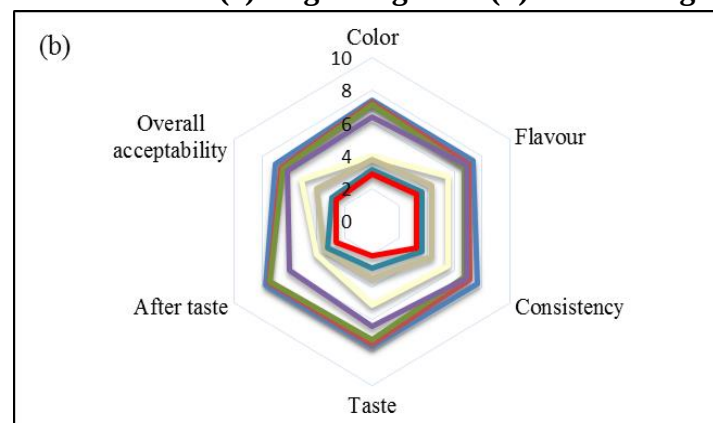
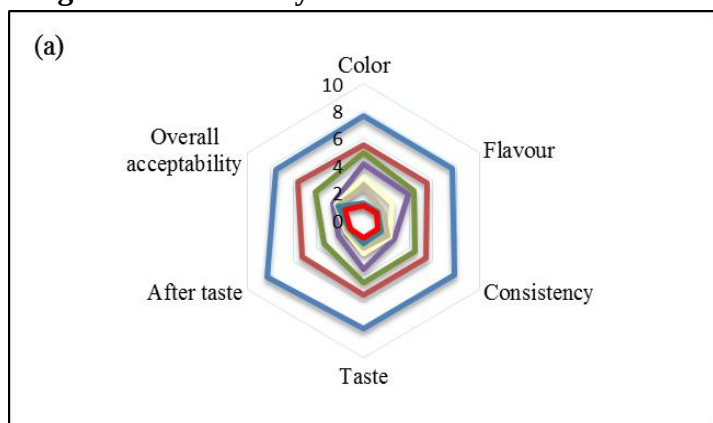
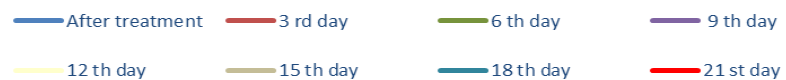


Figure 4.20: Sensory attributes of neera after heat treatment at 55°C-5 min (a) single-stage and (b) double-stage



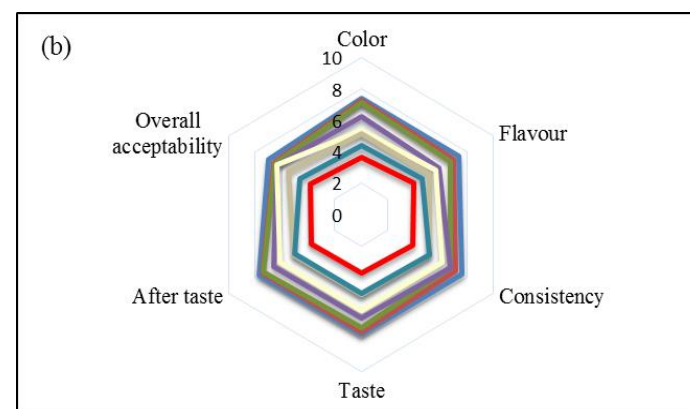
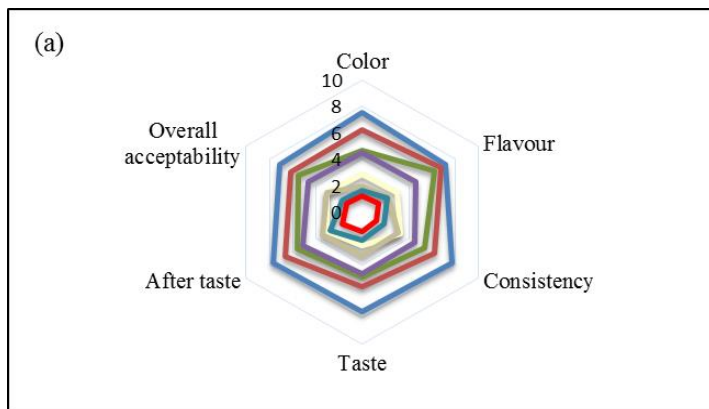


Figure 4.21: Sensory attributes of neera after heat treatment at 60°C-5 min (a) single-stage and (b) double-stage

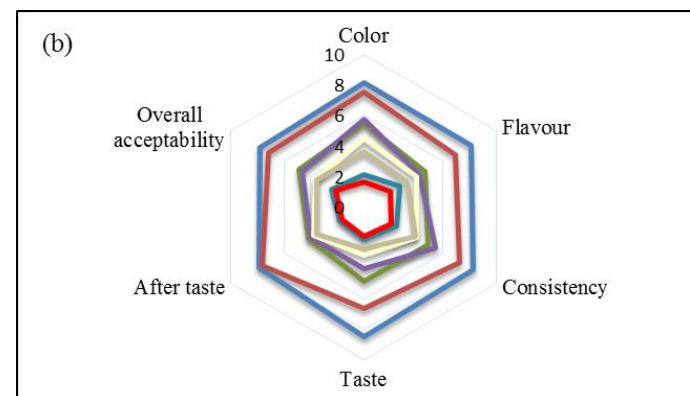
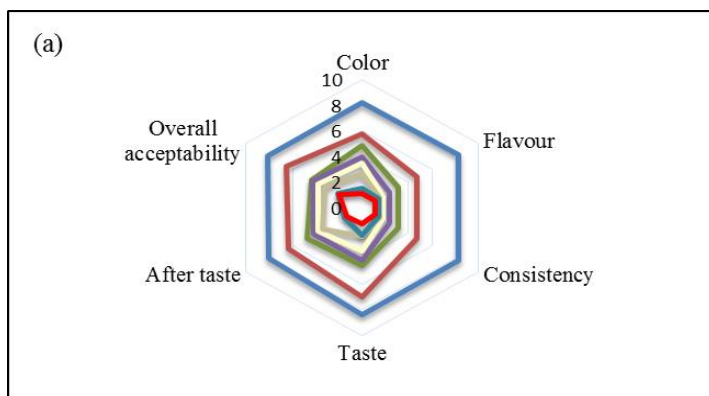


Figure 4.22: Sensory attributes of neera after heat treatment at 50°C-10 min (a) single-stage and (b) double-stage



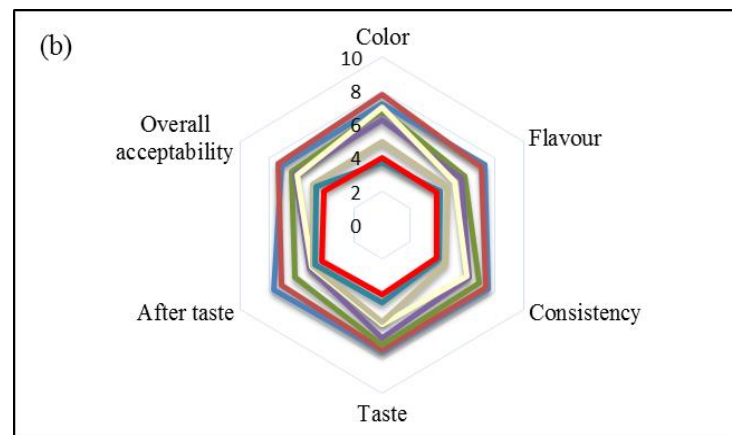
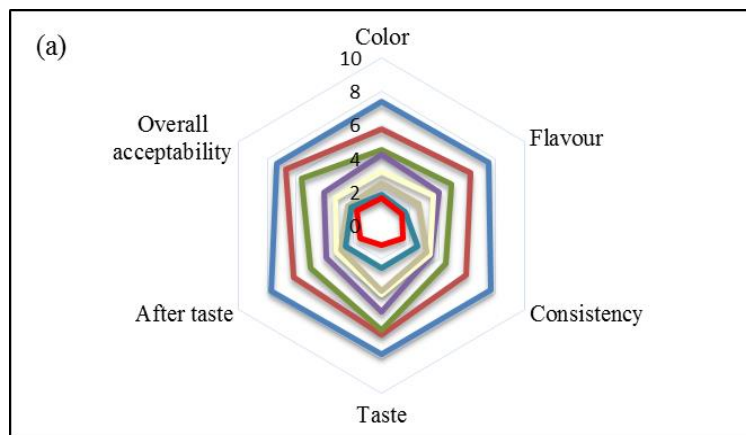


Figure 4.23: Sensory attributes of neera after heat treatment at 55°C-10 min (a) single-stage and (b) double-stage

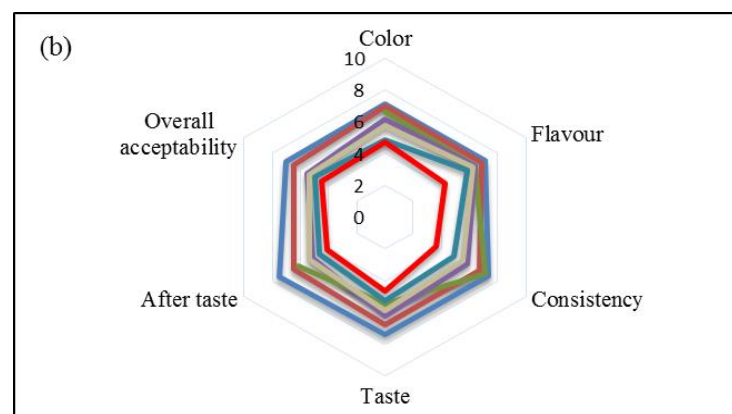
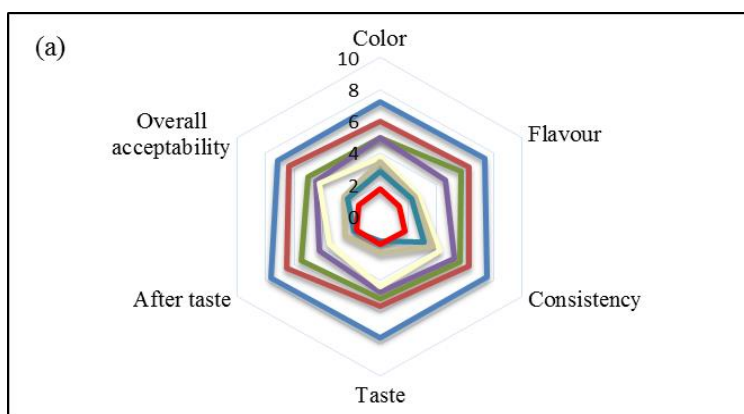


Figure 4.24: Sensory attributes of neera after heat treatment at 60°C-10 min (a) single-stage and (b) double-stage



CHAPTER V

SUMMARY AND FUTURE PROSPECTS

This chapter summarizes the research findings from the present work and subsequent conclusions inferred from it. The main objective of the study was to develop a method for the shelf life extension of neera based on the microbial survival, physicochemical and sensory properties. The results of each method are summarized below.

5.1 Method of freezing at the temperature of -6°C and -20°C

The microbial survival on the plate count analysis represents heterogeneous microbes of neera. The microbial reduction is the evidence for the effectiveness of the method against fermentation. The slow freezing at -6°C demonstrated the survival as 7.12 ± 0.00 log CFU/ml, 4.44 ± 0.00 log CFU/ml and 3.96 ± 0.00 log CFU/ml for total viable count, yeast and lactic acid bacteria, respectively on 28 d. The survival i.e., 6.26 ± 0.47 log CFU/ml (total viable count), 3.18 ± 0.00 log CFU/ml (yeast) and 0 log CFU/ml (LAB) on 28 d, were confirmed at -20°C frozen storage. The survival of 6.26 ± 0.47 log CFU/ml in total viable count and 3.18 ± 0.00 log CFU/ml in yeast were represents the log reduction of 1.36 and 1.81 respectively (Table 5.1). The log reduction confirms fast freezing with intracellular ice crystals at -20°C affects most of the microbes in neera through mechanical disruption, oxidative damage, and osmotic imbalance. The microbial destruction mechanism affects the entire LAB and injured other existing bacteria and yeast in neera. The fluorescence images have confirmed the injury by emitted DNA-PI coupled (red fluorescence) non-viable cells at -20°C. The slow freezing at -6°C retains more population of DNA-AO coupled (green cells) viable bacterial cells. The viable bacterial cells at -6°C favored the

lactic acid fermentation in neera. Therefore least count of microbes at -20°C reduces the rate of fermentation which control the physicochemical changes of pH (5.37 ± 0.02), total soluble solids (14.0 ± 0.00 °Brix), total acidity (0.82 ± 0.02 mg/L) and color (4.97 ± 0.05 ΔE) on 28 d. In the course of storage, frozen neera (-20°C) had an acceptable sensory attribute up to 21 d, with overall acceptability score of 4. On 28 d storage, the neera was not acceptable by the panelist due to the odd flavour/ taste generated from the growth of cold-resistant microorganisms. The frozen storage at -20°C reduced microbial population, preserved the physicochemical and sensory properties up to 21 d. Therefore freezing at -20°C extends the shelf life of neera for a longer period (21 d) than the atmospheric storage.

Table 5.1: Log reduction of total viable count and yeast for the developed methods

Name of the method	Storage period	Log reduction for total viable count	Log reduction for yeast
Freezing (at -20°C)	28 d	1.36	1.81
Preservatives (N4)	21 d	Not enumerated	0.04
Atmospheric pressure plasma jet (35 kV-7.5 min)	24 h	0.45	0.47
Tyndallization (60°C-10 min)	21 d	1.16	0.86

5.2 Preservation using a combination of nisin with chemical preservatives

The combination effect of nisin with chemical preservative on neera was identified from the total bacterial and yeast population. Among the four different combinations, the N4 treatment (50 ppm nisin with 3000 ppm calcium carbonate) suppressed the microbial

growth in highest number as 120×10^5 and 143×10^2 CFU/ml for total bacteria and yeast respectively on 21 d of the storage. The synergistic effect of nisin and calcium carbonate in N4 reduced the indigenous bacterial and yeast growth in neera. The number of non-viable (DNA-PI coupled) bacterial cells confirms the reduction, solubility of nisin and their antimicrobial activity. The precipitation reaction of calcium carbonate with microorganisms was proved by the 65.34 % intensity of calcium on the sediment portion of N4 treatment. The lower concentration (2500 ppm) of calcium carbonate in N3 treatment revealed the increased microbial growth as 160×10^5 CFU/ml (total bacteria) and 260×10^2 CFU/ml (yeast). The other treatments .i.e., N1 (50 ppm nisin with sodium benzoate 500 ppm) and N2 (50 ppm nisin with sodium benzoate 1000 ppm), had more than 250 CFU/ml for the microbial population on 21 d storage. The treatment ineffectiveness was due to the insolubility of nisin by the developed acidic pH by sodium benzoate which promoted the lactic acid fermentation in neera. The effective microbial reduction, in N4 treatment, showed the pH of 10.45 ± 0.05 , total soluble solids of 15.43 ± 0.12 °Brix and total acidity of 1.11 ± 0.04 mg/L at the end of 21 d. While calcium carbonate addition increased pH from 0 d due to the alkalinity nature of calcium carbonate and their effect extended up to 21 d (10.45 ± 0.05). The increased alkaline pH was another reason for the reduction of microbial survival in neera. Hence, the calculated log reduction (Table 5.1) from the CFU/ml of 21 d did not provide any reduction for bacteria and it resulted 0.04 for yeast at 21 (d), which evident that existence fermentative microbes in neera.

5.3 Non-thermal using atmospheric pressure plasma jet

The antimicrobial activity of a non-thermal atmospheric pressure plasma jet on neera was evaluated by the microbial log reduction. The treatment at 35 kV for 7.5 min caused a better log reduction of 0.45, 0.47 and 0.47 for total bacteria, lactic acid bacteria and yeast on the storage period of 24 h. More importantly, the treatment (35 kV-7.5 min) with 40 % reduction, which might be due to the diffusion of reactive oxygen species via lipid bilayer or transient opening through peptidoglycan layer. Their diffusion affects the inner biomaterials (DNA) of the gram-positive LAB which leads to cell death. The efficient antimicrobial activity in 35 kV-7.5 min controlled the changes in physiochemical properties up to 24 h. The physicochemical changes in 35 kV 7.5 such as pH (5.07 ± 0.45), total soluble solid (15.46 ± 0.58 °Brix), total acidity (1.38 ± 0.06 mg/ L) and color difference (2.67 ± 0.19 ΔE) suggested the plasma jet treatment was preserved the quality up to 24 h.

5.4 Thermal method using mild heat treatment

The microbial log reduction and decimal reduction time identified the effective temperature time combination from the single and double-stage heat treatment. The microbial survival between the single and double-stage (tyndallization) heat treatment demonstrated the least survival (6.68 ± 0.02 log CFU/ml) at 60°C-10 min due to the lethal effect of temperature. The lethal effect at 60°C-10 min was sensitive to most of the existing microbes of neera which decreased the decimal reduction time as 3.50 ± 0.04 . The treatment (60°C-10 min) on 21d causes better log reduction for total viable count (1.16 ± 0.01), *Bacillus* (1.06 ± 0.01), Lactic acid bacteria (0.27 ± 0.01) and yeast (0.86 ± 0.01) which might due to the affected

cellular membrane or peptidoglycan, inactivated spores, inactivated enzymes and high thermal stress. The log reduction at 60°C-10 min controlled the spontaneous fermentation in neera which limited physicochemical changes into 5.77 ± 0.06 , 16.02 ± 0.02 °Brix, 0.72 ± 0.05 mg/L and 1.01 ± 0.02 ΔE for pH, total soluble solids, total acidity and colour (21 d). And the method of tyndallization at 60°C-10 min preserved sensory attributes up to 21 d.

5.5 Summary of the developed methods

The storage period microbial log reduction were important to identify the effective method for shelf life extension of neera. The log reduction trend was freezing (1.36- total bacteria, 1.81- yeast) > mild heat treatment (1.16- total bacteria, 0.86- yeast) > atmospheric pressure plasma jet (0.45- total bacteria, 0.47- yeast) > preservatives (0- total bacteria, 0.04- yeast) (Table 5.1). Among the four method the freezing at -20°C exhibited the better log reduction for total viable count (1.36) and yeast (1.81) than the other methods. The reduced microbial survival preserved the physicochemical properties of neera as 5.37 ± 0.02 , 14.0 ± 0.00 °Brix, 0.82 ± 0.02 mg/L and 4.97 ± 0.0 ΔE for pH, total soluble solids, total acidity, and color on 28 d. Hence the method had acceptable sensory score up to the storage period of 21 d. Therefore the study conclude that the method of freezing at -20°C could extend the shelf life of neera up to 21 d.

5.6 Key findings

- The preservative combination of N4 (Nisin 50 ppm and Calcium Carbonate 3000 ppm) in neera did not provided any microbial log reduction for bacteria and it resulted 0.04 for yeast on 21 (d).

- The atmospheric pressure plasma jet treatment in neera had a better reduction up to the storage period of 24 h. The effective log reduction of total bacteria (0.45) and yeast (0.47) at 35 kV-7.5 min preserved the physicochemical qualities up to 24 h.
- The double-stage heat treatment at 60°C-10 min in neera causes better log reduction for total viable count (1.16 ± 0.01) and yeast (0.86 ± 0.01) up to the storage period of 21 d. The lethal effect limited physicochemical changes and sensory attributes up to 21 d.
- The freezing at -20°C effectively reduced the log reduction as 1.36 and 1.81 for total viable and yeast count respectively. The reduced survival at -20°C provide the neera with reduced physicochemical and sensory changes up to 21 d.
- Among the four method the method of freezing at -20°C which extend the shelf life of neera for a longer period of 21 d by the effective microbial log reduction. The method can be recommended for the shelf life extension of natural sap of neera with the cold chain distribution system.

5.7 Scope for the future work

- The study on the temperature resistant microorganisms and its gene expression system for fermentation could be helpful for the development of effective method.
- The isolation, identification and application of suitable bacteriophage as a preservative can be explored for the early stage lactic acid fermentation and retention of neera quality.

- The emerging non-thermal technology with combination of gas effectively arrest the enzyme activity of microbes and control the natural fermentation in neera. Other emerging processing techniques can also be studied in neera for shelf life extension.
- The combination of thermal and non-thermal technology may work against heterogeneous microbes of neera and extend its shelf life, which could also be an area of investigation.

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LIST OF PUBLICATIONS

1. Sukumaran, L., & Radhakrishnan, M. (2021a). Effect of frozen storage on the inhibition of microbial population, chemical and sensory characteristics of coconut neera. *Journal of Applied Microbiology*, 131(4), 1830-1839. <https://doi.org/10.1111/jam.15068>
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ORIGINAL ARTICLE

Effect of frozen storage on the inhibition of microbial population, chemical and sensory characteristics of coconut neera

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Keywords

chemical quality, coconut neera, frozen storage, microbial population, sensory characteristics.

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Abstract

Aim: In this study, the effect of frozen storage on the inhibition of microbial population in coconut neera for shelf life extension was analysed.

Methods and Results: The aliquots of fresh neera were frozen at -6 and -20°C . The microbial reduction and chemical qualities were analysed during storage days, and were compared with the control. The highest reduction in microbes was obtained at -20°C for lactic acid bacteria, yeast and total viable count. Moreover, the fluorescence cell staining and cell density analysis exhibited a decline at -20°C , however it showed higher at -6°C . At the end of 28 days, neera exhibited a pH of 5.37, total soluble solid of 14.0 °Brix and total acidity of 0.82 mg l^{-1} at -20°C . Besides, color, viscosity, total protein and ethanol showed a less difference with control and better sensory attribute up to 21 days at -20°C .

Conclusions: Frozen storage at -20°C resulted in a better inhibition of lactic acid bacteria, which preserves neera from the metabolic conversion. The physicochemical qualities of neera were preserved for a longer period when stored at -20°C than at atmospheric storage.

Significance and Impact of the study: Frozen storage reduced microbial population and preserves the chemical properties with acceptable sensory attributes and increases the shelf life of coconut neera, making it fit for consumption.

Introduction

Neera is a sweet, brown-coloured, flavoured sap from the coconut palm tree (*Cocos nucifera*). The tree of coconut is depicted as 'Kalpavriksha' in ancient literature; currently, the crop is spread over Asia, the Pacific, Africa and the America. Among these regions, India (31.02%), Indonesia (23.41%) and the Philippines (21.04%) are the major producers. Moreover, Sri Lanka, Mexico, Vietnam, Thailand, Brazil and the Ivory Coast are other producers in the world (Harshini and Samuel 2019). The rural people in these regions collect the inflorescence sap and consume it before the sunrise. The traditional tapping of the unopened matured inflorescence allows the sap to exude into the collection vessel (Francisco-Ortega and Zona 2013). The regular tapping process increased the flow of these sugary sap up to 50 days (Samsudeen *et al.* 2013).

A recent report depicted that the total sugar content of 16.19 g 100 ml^{-1} with least amount of glucose and fructose (1.0 g 100 ml^{-1}) in neera, indicating high concentration of sucrose. Moreover, the sweet 'sap' has a low glycaemic index (GI 35) and hence is diabetic friendly. The fresh sap is more nutritious than fruit juices due to the presence of 13 minerals, including K (168.4 mg 100 ml^{-1}), Na (90.6 mg 100 ml^{-1}), P (3.9 mg 100 ml^{-1}), and phenolic compounds (5.10 mg 100 ml^{-1}) (Hebbar *et al.* 2018). In addition to sugars and minerals, other nutrients like vitamins (e.g. thiamine, riboflavin, niacin) are also present in neera like a fermented beverage (Flores-Gallegos *et al.* 2019). Vitamins E and C and antioxidants (antioxidant activity of 0.321 m MTE) are also reported in neera. The rural drink also contains about 12 volatile compounds and various amino acids. In fresh sap, the major volatile compounds reported are

Impact of Nisin in Combination with Sodium Benzoate and Calcium Carbonate on the Bacterial and Yeast Population of Coconut Neera (Coconut Inflorescence sap)

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Abstract

A natural sap from mature coconut palm known as coconut neera is enriched with essential minerals and vitamins. Rapid microbial fermentation affects neera processing industries because it spoils the physicochemical properties. There are various methods in preservation which extend the shelf life of coconut neera. The addition of nisin is one of the methods which protect neera against fermentation. Therefore, the study is focused to identify the effective combination of nisin (50 ppm) with preservatives like sodium benzoate (500, and 1000 ppm), and calcium carbonate (2500, and 3000 ppm) at two different combinations in neera. At the end of 21 d, 3000 ppm calcium carbonate with 50 ppm nisin in N4 treatment had an effective reduction of 120×10^5 CFU/ml and 143×10^2 CFU/ml for total bacteria and total yeast count. The reduced microbial survival resulted in the pH of 10.45 ± 0.05 , total soluble solids of 15.43 ± 0.12 °Brix, and total acidity of 1.11 ± 0.04 mg/L, at this combination. The treatment of nisin with 3000 ppm calcium carbonate demonstrated the high red fluorescence bacterial cells than the treatment of nisin with 1000 ppm sodium benzoate. Additionally, the microorganisms in N4 treatment precipitated 65.34% Ca^{2+} from 79.96% in XRF intensity analysis. The synergistic effect of nisin and calcium carbonate explored their antimicrobial activity against the heterogeneous microbial population in coconut neera. The concentration of 3000 ppm calcium carbonate and nisin 50 ppm preserves the physicochemical and sensory qualities, up to 21 d at 4°C, and offer hope for the industrial-scale implementation.

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A recent report depicted that the total sugar content of $16.19\text{ g }100\text{ ml}^{-1}$ with least amount of glucose and fructose ($1.0\text{ g }100\text{ ml}^{-1}$) in neera, indicating high concentration of sucrose. Moreover, the sweet 'sap' has a low glycaemic index (GI 35) and hence is diabetic friendly. The fresh sap is more nutritious than fruit juices due to the presence of 13 minerals, including K ($168.4\text{ mg }100\text{ ml}^{-1}$), Na ($90.6\text{ mg }100\text{ ml}^{-1}$), P ($3.9\text{ mg }100\text{ ml}^{-1}$), and phenolic compounds ($5.10\text{ mg }100\text{ ml}^{-1}$) (Hebbbar *et al.* 2018). In addition to sugars and minerals, other nutrients like vitamins (e.g. thiamine, riboflavin, niacin) are also present in neera like a fermented beverage (Flores-Gallegos *et al.* 2019). Vitamins E and C and antioxidants (antioxidant activity of 0.321 m MTE) are also reported in neera. The rural drink also contains about 12 volatile compounds and various amino acids. In fresh sap, the major volatile compounds reported are

2-butanol, acetic acid, 2 methylcyclohexane and cyclohexiloctane, and the major amino acids reported are glutamic acid, threonine, aspartic acid and serine (Purnomo 2007). Generally, the volatile compounds exhibit antimicrobial, antioxidant, anticarcinogenic and immune-boosting activities (Goff and Klee 2006). In addition, the nephroprotective and hepatoprotective activities, and the presence of few lactic acid bacteria with probiotic properties were reported in neera (Asha *et al.* 2019; Somashekaraiah *et al.* 2019). Due to the vital benefits, neera has been stated as a superior drink than tender coconut water by the food research institutions. The potential benefit towards the human health increased the demand of neera in the global market.

However, spontaneous fermentation by various micro-organisms at ambient storage converts the coconut neera into an alcoholic beverage. Different micro-organisms, such as *Bacillus*, *Lactobacillus*, *Micrococcus*, *Enterobacter*, *Leuconostoc*, *Saccharomyces*, *Candida* and *Pichia*, were reported in coconut neera. Among these, the lactic acid bacteria and yeast highly utilize the sugar substrate that changes the total acidity and produces a higher concentration of alcohol in neera (Atputharajah *et al.* 1986). Moreover, the microbial metabolism formulates dodecanoic acid and palmitoleic acid which induce the astringency in neera, making the sap unfit for consumption (Borse *et al.* 2007). Generally, neera is preserved using a combination of processing methods such as pasteurization, filtration, chemical or biopreservatives, clarifying agents, centrifugation and carbonation. However, various technologies did not arrest the growth of micro-organisms in neera and it changes the organoleptic qualities (Hebbar *et al.* 2018). Even at refrigerated temperatures, the microbes maintain their viability and spoil the quality of neera within 3 days; the number of days may vary according to the initial microbial population. The survival of microbes at these low temperatures is due to the expression of various proteins, such as cold-inducible protein, cold shock protein and aquaporin, which extensively regulates the metabolic function of micro-organisms (Aguilera *et al.* 2007). Hence, the freezing preservation technique can control the microbial viability and maintain the original flavour in various food. However, the mechanism of microbial cell destruction is based on the size of ice crystals and its type of distribution either intracellular or extracellular. Generally, the fast freezing rate generates small ice crystals; on the contrary, the slow cooling rate develops the large ice crystals. During freezing, various food components exist in two different phases: aqueous in equilibrium phase (crystallization) and aqueous compounds or amorphous in non-equilibrium phase (Charoenrein and Harnkarnsujarit 2017). The non-aqueous compounds, such as carbohydrates, lipids, protein and organic acids, change during storage and affect the pH,

titratable acidity and ionic strength of the food (Damiani *et al.* 2013). Moreover, the stability depends on the type, chemical composition and microbial population of food. The recent study in kefir reported that lactic acid bacteria and yeast in frozen storage was reduced between -8 to -14°C (O'Brien *et al.* 2016). Therefore, the current study aimed to evaluate the inhibition of micro-organisms during frozen storage, and their impact on chemical characteristics and sensory quality of neera.

Materials and methods

Coconut neera treatments

The preliminary study was conducted in neera (collected using a coco sap chiller) according to the method reported by O'Brien *et al.* (2016) at three different freezing temperatures. The filled neera of 100 ml aliquots in the sterilized stainless container was placed at -6 , -10 and -20°C with data loggers. The frozen sample was investigated on 7, 14, 21 and 28 days of storage after thawing at 28°C for 1 h. The analysed chemical properties did not exhibit discernible change on storage at -6 and -10°C . Based on the preliminary observation, the storage study was conducted at -6°C and -20°C to determine the quality of neera. The fresh neera values before freezing were presented in "0 d" and their results were compared with frozen neera.

Enumeration of the microbial population by colony forming units

Ten millilitres of the sample was withdrawn from the frozen neera and subsequently serially diluted using 0.1% peptone water. The aliquots of 0.1 ml from the appropriate dilution were plated into plate count agar (M091, Hi-Media, Mumbai, India) to determine the total viable count. After 42 h incubation at 32°C , the colonies on the plates were counted to determine the number of colony forming units (CFU; Alrabadi 2015). *Lactobacillus* MRS agar (GM641, Hi-Media) and chloramphenicol yeast glucose agar (M1008, Hi-Media) were incubated at 28°C for 48 h to determine the population of lactic acid bacteria and yeast (Wang and Xu 2019).

Optical cell density measurement

The cell density in coconut neera was assayed by measuring the optical density in a spectrophotometer at 660 nm (Shimadzu UV-1800). During storage, 1 ml of neera suspension was withdrawn and their absorbance was measured. The absorbance of the experimental values was subtracted from the control values (Pan *et al.* 2014).

Fluorescence viability imaging

The viable and non-viable cells in response to frozen storage were evaluated using a fluorescent microscope (Nikon eclipse Ni-U, Nikon, Tokyo, Japan). For imaging the microbial cells, the frozen neera samples were centrifuged at 10,000 g for 5 min and the collected microbial pellet was re-suspended in phosphate buffer saline solution of pH 7.2 (Zhang and Fang 2004; Zhang and McCarthy 2016). For staining the cells, the dye mixture of 100 μ l acridine orange (5 mg ml⁻¹) and propidium iodide (3 mg l⁻¹) was added into the sample. The cells were incubated at room temperature in the dark for 15 min. For each sample, the green fluorescent (535 nm) and red fluorescent (635 nm) intensities were measured for viable and non-viable cells.

Chemical analysis

The pH was measured using a LAQUA (PH1100) pH meter with 50 ml of sample and the total soluble solids were identified by a handheld refractometer (RHB-55ATC); the results are expressed in the degree of Brix. The total acidity of neera was analysed against 0.1 nor l⁻¹ NaOH using phenolphthalein indicator and expressed as lactic acid equivalent. The colour of the sample was determined using a Hunter color lab (Color Flex EZ—45/0LAV). The multiple measurements of frozen neera, L^* (lightness), a^* (redness/greenness) and b^* (yellowness/ blueness) values, were converted into total colour difference (ΔE^*) using control neera (L_0^* , a_0^* and b_0^*) values during storage days.

$$\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

Rheology and total protein

The viscosity (mPa s) of neera samples were analysed at a constant shear rate of 100 s⁻¹ using a parallel plate PP50 (Anton Paar -MCR52) at 25°C for 200 s. The total protein content was measured according to Lowry's method and the absorbance was read at 660 nm (Sadasivam 1996) using a spectrophotometer (Shimadzu UV-1800).

Estimation of ethanol

The ethanol content in the sample was estimated using potassium dichromate and sulphuric acid. One microlitre of neera supernatant was added with 5 ml of K₂Cr₂O₇ solution (40 mg ml⁻¹), 25 ml of 6 nor l⁻¹ H₂SO₄ solution and 5 ml of acetate buffer (pH 4.3). The intensity of the green colour was read at 578 nm in a Shimadzu UV-1800 spectrophotometer (Sumbhate *et al.* 2012). The

blank was prepared in the same manner without ethanol and assayed by comparing it with a standard graph.

Sensory analysis

A panel of eight judges (five women and three men with different age groups) were selected and trained for various sensory characteristics of coconut neera for a week. The trained judges evaluated the frozen neera samples along with reference (fresh) sample using a 9-point hedonic scale (Mazzaglia and Lanza 2018). The score value of various attributes are defined as 9—excellent, 8—extremely good, 7—very good, 6—moderately good, 5—good, 4—very fair, 3—fair, 2—poor and 1—very poor. Various attributes, such as appearance, colour, flavour, taste, after taste, consistency and overall acceptability, of neera were analysed by the panellist.

Statistical analysis

The mean comparison of control and frozen neera as a function of temperature was evaluated using Tukey's test using SPSS.v.23.

Results

Evaluation of microbial reduction using plate count method, cell density analysis and fluorescence analysis

Figure 1 shows the reduction of various microbes in coconut neera during frozen storage. This indicated that freezing affected the microbial viability during the storage period. The results of the plate count method showed a

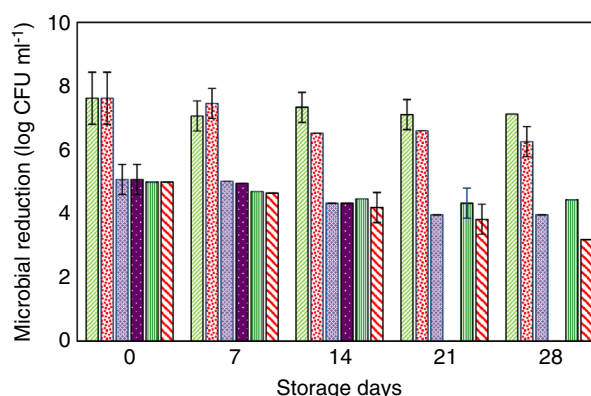


Figure 1 Effect of frozen storage on the reduction of the total viable count, lactic acid bacteria and yeast in coconut neera by the plate count method at -6 and -20°C. Data are recorded as the mean $\pm$ standard deviation from 3 replications. (▨) TVC at -6°C; (▤) TVC at -20°C; (▥) LAB at -6°C; (▦) LAB at -20°C; (▧) Yeast at -6°C; (▨) Yeast at -20°C.

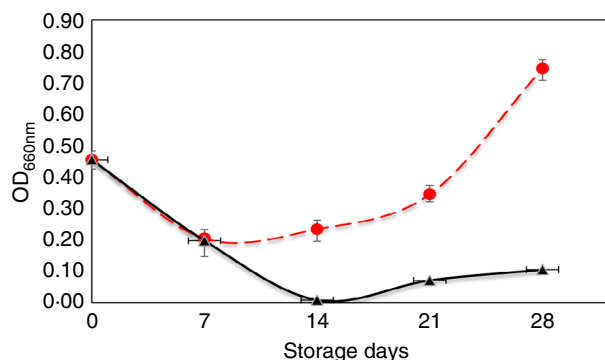


Figure 2 Microbial cell density of coconut neera quantified during the storage period. Data are shown as means $\pm$ standard deviation of $n = 3$ independent experiments. (—•—) -6°C ; (—▲—) -20°C .

higher reduction of lactic acid bacteria, followed by yeast and total viable counts. At -20°C , the count of lactic acid bacteria showed a better reduction from its initial population of 5.07 ± 0.47 to 4.33 ± 0.00 log CFU per ml on day 14; thereafter, the growth was completely inhibited on days 21 and 28. While their viability extended up to 28 days at -6°C , the survival number of 3.96 ± 0.00 log CFU per ml was the highest during the storage period. During the 21-day storage period, the count of yeast was 4.46 ± 0.00 log CFU per ml and 4.19 ± 0.47 log CFU per ml at -6 and -20°C respectively. At the end of the 28 days, the yeast count of 4.44 ± 0.00 log CFU per ml was observed at -6°C and better reduced viability of 3.18 ± 0.00 log CFU per ml was exhibited at -20°C . Although the reduction of 7.12 ± 0.00 log CFU per ml was enumerated for the total viable count at -6°C , it was the least reduced population when compared to the other temperatures. However, neera at -20°C declined the total viability into 6.26 ± 0.47 log CFU per ml at the end of the storage. These findings suggested that the storage at -20°C affected the spontaneous fermentation in neera than the storage at -6°C . Moreover, the spectrophotometric method of microbial analysis at -20°C showed a declined cell density of 0.01 ± 0.005 up to 14 days of storage but it increased afterwards in neera (Fig. 2). The optical absorbance at 28 days was reported as 0.10 ± 0.004 in -20°C , which exhibits the least cell density during storage. However, the decreasing trend was found up to 7 days (0.20 ± 0.058) at -6°C thereafter optical density increase to 0.75 ± 0.038 on 28 days. The results of the cell density were further supported by observation of live (green-coloured) and dead (red/yellow-coloured) cells using fluorescence microscopic analysis (Fig. 3). Frozen storage at -20°C caused a reduction in viable or cultivable population; therefore, it possesses much higher DNA—PI

coupled red cells than the green stained cells. Whereas at -6°C (Fig. 3b) the cells are stained with more green cells. Moreover, the effect coincides with the control implying that frozen storage at -20°C affects most of the microbial community in neera. The microbial viability on plate count and chloramphenicol yeast glucose agar revealed the least population at -20°C compared to -6°C . Moreover, the live/dead cells from fluorescent microscopy correlated with the finding of the plate count technique thus demonstrating the freezing temperature of -20°C had a better impact on the microbial reduction in neera.

Effect of frozen storage on chemical qualities of coconut neera

The effect of freezing on various chemical qualities (pH, total soluble solids, total acidity, colour, viscosity, total protein and ethanol content) are presented in Table 1^{1–7}. A significant reduction of pH from 5.85 ± 0.03 to 4.97 ± 0.00 and 5.37 ± 0.02 at -6 and -20°C was observed. The changes in pH during frozen storage were found to be lower at -20°C when compared to -6°C . This study displayed a decrease in total soluble solids of 13.17 ± 0.28 at -6°C and 14.0 ± 0.00 °Brix at -20°C . The changes were significant from control. The total acidity significantly ($P \leq 0.005$) increased from 7 to 28 days. At the end of the storage period, the acid content was found to be 0.96 ± 0.02 and 0.82 ± 0.02 mg l^{-1} at -6 and -20°C respectively.

Colour is one of the most important attributes of neera, as it defines the choice of purchase and acceptability among customers. The total colour difference (ΔE^*) between the control and frozen neera samples increased significantly during the storage period (Table 1⁴). The higher ΔE^* value suggested that the frozen sample had the highest difference in their L^* , a^* and b^* values when compared to the control (0 days). In this study, the difference was more at -6°C (6.49 ± 0.06 ΔE^*) and it was less at -20°C (4.97 ± 0.05 ΔE^*) when compared to the control.

The viscosity of neera was significantly (Table 1⁵) affected by freezing temperature. While the viscosity of neera increased from 7 days, later it decreased in subsequent days. At the end of the storage, the viscosities were 2.68 ± 0.05 and 3.88 ± 0.05 mPa s at -6 and -20°C respectively. The protein content of frozen neera was found to reduce from the control concentration of 0.015 ± 0.02 to 0.013 ± 0.02 and 0.007 ± 0.01 g 100 ml^{-1} on 28 days at -6 and -20°C respectively (Table 1⁶). However, the increased concentration of protein was occurred between 7 to 28 days. At the end of the study, the identified protein content at -20°C was least changed concentration from control. However, frozen storage at -6°C not showed ethanol production from the 7 to 14 days (Table 1⁷). But the

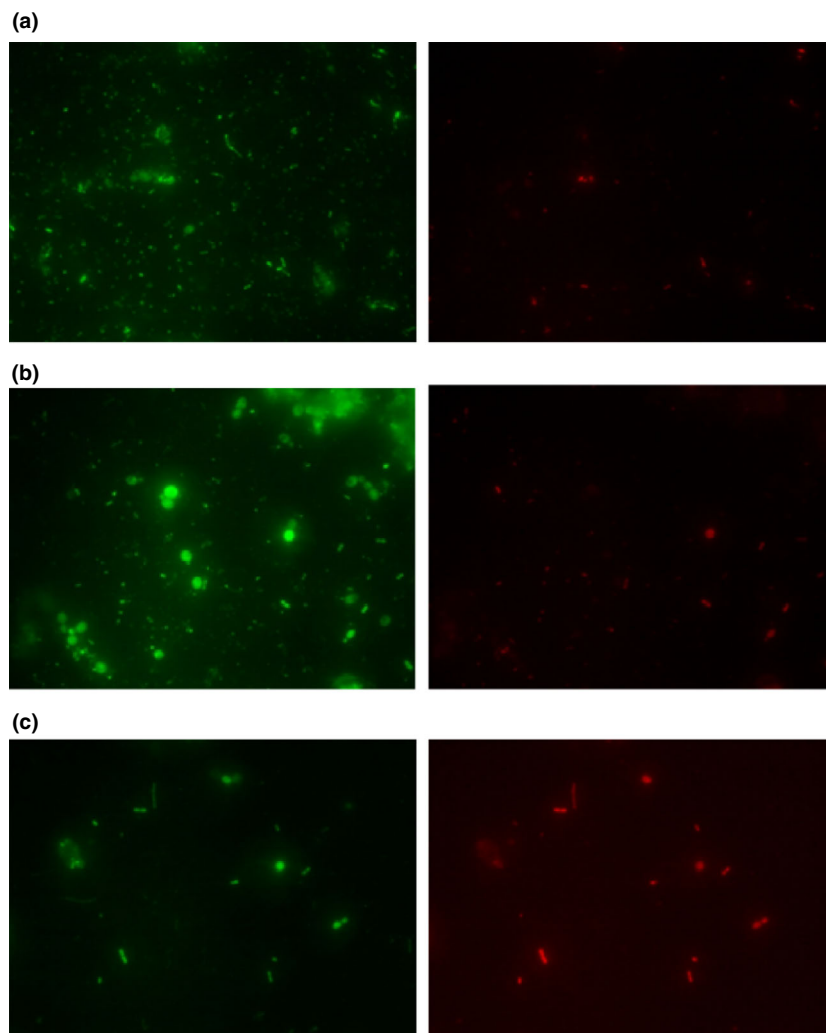


Figure 3 Images of viable and non-viable cells on fresh and frozen neera stained by fluorescence microscopy FM ($\times 100$). Green and red fluorescence indicate live and dead cells (a) control, (b) -6°C and (c) -20°C .

subsequent storage days showed the ethanol concentration at -6°C . Therefore, neera at this storage temperature produced the ethanol content of 2.80% on the 28 days and it was the highest concentration. The negligible production of ethanol was extended up to the 21 days at -20°C , at the end of the 28 days the ethanol content of 0.79% was exhibited at this storage temperature.

Effect on sensory

The sensory attributes of flavour, taste, and after taste are important parameters for neera, and it was deeply compared by panellists throughout the evaluation. The freezing temperatures induced undesirable flavour in neera during the storage period (Fig. 4a,b). In the course of frozen storage, all the analysed attributes in frozen neera samples were decreased significantly. The assigned score values of 1, 2, 1

on 28 days for the attributes of flavour, taste and after taste at -6°C were significantly different ($P \leq 0.005$) than control neera. Moreover, these samples perceived unacceptability (poor quality) by the panellists after the 14 days, these two divergences indicated that the freezing temperature -6°C caused more changes in neera. Whereas the changes on 28 days were lower (2—flavour, 3—taste and 4—after taste) at -20°C , and this storage temperature did not alter the acceptability up to the 21 days.

Discussion

Effect of freezing temperatures on microbial viability

The findings of the microbial reduction in neera revealed the frozen storage temperature has affected the survival of the heterogeneous microbial population. Among

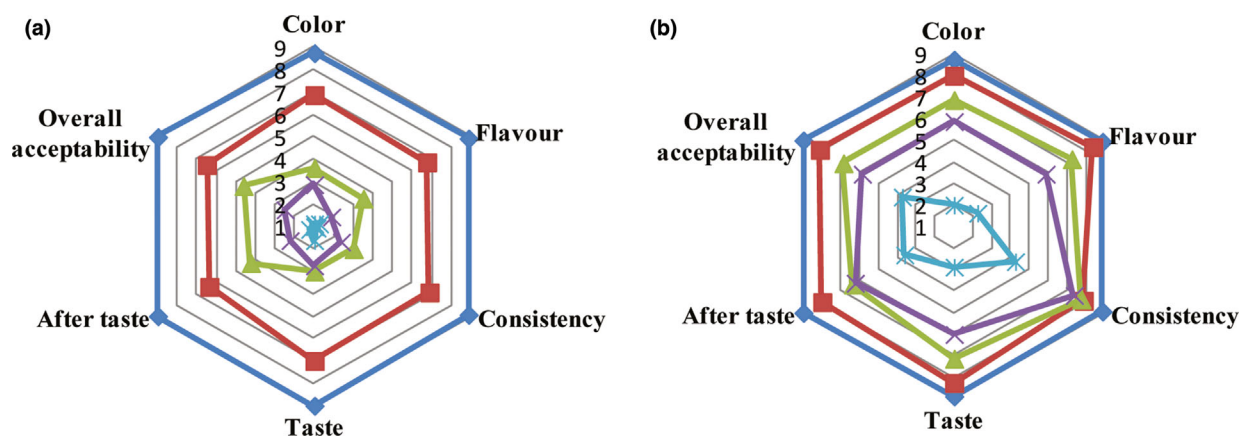
Table 1 Effect of freezing on pH, total acidity, total soluble solids, colour, viscosity, total protein and ethanol content of coconut neera

Temperature (°C)	0th day	7th day	14th day	21st day	28th day
pH ¹					
–6	5.85 ± 0.03	5.72 ± 0.00 ^a	5.46 ± 0.00 ^b	5.18 ± 0.01 ^c	4.97 ± 0.00 ^d
–20	5.85 ± 0.03	5.82 ± 0.01 ^a	5.76 ± 0.00 ^b	5.53 ± 0.06 ^c	5.37 ± 0.02 ^d
Total soluble solids (°Brix) ²					
–6	16.50 ± 0.00	15.67 ± 0.28 ^a	15.00 ± 0.00 ^b	14.50 ± 0.00 ^c	13.17 ± 0.28 ^d
–20	16.50 ± 0.00	16.50 ± 0.00 ^a	15.83 ± 0.29 ^b	15.00 ± 0.00 ^c	14.00 ± 0.00 ^d
Total acidity (mg l ^{–1}) ³					
–6	0.69 ± 0.01	0.74 ± 0.01 ^a	0.94 ± 0.02 ^b	0.91 ± 0.01 ^c	0.96 ± 0.02 ^d
–20	0.69 ± 0.01	0.73 ± 0.01 ^a	0.79 ± 0.02 ^b	0.82 ± 0.01 ^c	0.82 ± 0.02 ^d
Colour (ΔE*) ⁴					
–6	*	5.43 ± 0.01 ^a	6.04 ± 0.05 ^b	6.07 ± 0.05 ^c	6.49 ± 0.06 ^d
–20	*	1.16 ± 0.07 ^a	2.53 ± 0.07 ^b	4.26 ± 0.03 ^c	4.97 ± 0.05 ^d
Viscosity (mPa s) ⁵					
–6	3.89 ± 0.10	7.11 ± 0.06 ^a	7.23 ± 0.05 ^b	3.9 ± 0.01 ^c	2.68 ± 0.05 ^d
–20	3.89 ± 0.10	6.3 ± 0.02 ^a	6.03 ± 0.14 ^b	4.22 ± 0.06 ^c	3.88 ± 0.05 ^d
Total protein (g 100 ml ^{–1}) ⁶					
–6	0.015 ± 0.02	0.007 ± 0.01 ^a	0.010 ± 0.05 ^b	0.011 ± 0.04 ^c	0.013 ± 0.02 ^d
–20	0.015 ± 0.02	0.005 ± 0.01 ^a	0.006 ± 0.01 ^b	0.006 ± 0.01 ^c	0.004 ± 0.01 ^d
Ethanol (%) ⁷					
–6	0.16 ± 0.001	ND	ND	0.69 ± 0.001 ^a	2.80 ± 0.005 ^b
–20	0.16 ± 0.001	ND	ND	ND	0.79 ± 0.001 ^b

The different alphabets in storage indicate significant differences at 0.05 level from the control.

ND, Not detected during the storage days.

Indicates no difference for the delta E value of fresh coconut neera (L 58.13; a* 0.36; and b* 9.26).

**Figure 4** Sensory attributes of coconut neera at freezing temperatures versus storage days. (a) –6°C and (b) –20°C.

various communities, the better reduction of lactic acid bacteria was in agreement with the study of O'Brien *et al.* (2016). The investigation reported that the decreased trend of *Lactococci* in kefir on frozen storage. However, their absence in the current study from 21 days at –20°C suggests that the high freezing rate injured the entire lactic acid bacterial community. The alcohol producing yeast trend at –6°C throughout the study was similar to the findings of Geiges (1996) and the results showed the constant count of yeast on frozen storage. Although the

higher reduction in the total viable count, and yeast at –20°C suggested that there was an evolution of injured microbes in these populations and it enters into the non-cultivable state in viability analysis (Speck and Ray 1977; Rault *et al.* 2007). From the view of microbial colony forming units, the freezing temperatures used in this study have an impact on the microbial reduction in neera. However, the least microbial reduction at –6°C occurred due to the low cooling rate diffused water content from the cytoplasm which causes cell dehydration on

the microbial cell. Comparably the high cooling rate at -20°C induces the more intracellular ice formation that creates injury on microbial cells which affects the more cell viability (Dumont *et al.* 2006). Hence, the result of our study suggested most of the microbes were injured at -20°C . However, at -6°C , the more resistant microbes have existed in storage which was mainly from the mesophilic microbial community (Golden and Arroyo-Gallyoun 1997). Generally, these mesophilic have symbiotic associations with yeast and produce alcohol as the end product through natural fermentation (Tamang *et al.* 2016). Considering these findings, frozen storage at -6°C did not arrest the growth of the fermentative organism in neera and it favors the metabolic activity whereas growth was comparably restricted at -20°C .

Chemical qualities of coconut neera

Generally, the lower temperature froze the food components more rapidly than the higher temperature. The temperature at -20°C with the fast freezing restricted the microbial cells by mechanical disruption, oxidative damage and osmotic imbalance that affected the metabolic function of fermentation at this temperature. Although, some of the microbes able to function within the ice and it may cause a reduction in pH (Amato and Christner 2009). Such microbes affected the physicochemical quality of neera in frozen storage. Although the ambient storage reduced the pH up to 4 within 24 h due to the natural fermentation (Hebbar *et al.* 2015). By comparing the ambient and frozen storage temperatures, the pH changes were quite less at frozen storage. The total soluble solids content of neera determines the freshness and taste. The present study resulted in the lower total soluble solids at -6°C which indicated the more conversion of sucrose by the micro-organisms than frozen storage -20°C . Moreover, the investigation of Xia *et al.* (2011) reported, the increased amount of total acidity in fermented neera than the fresh. Besides these, the microbes induce the internal acidity through the stress adaptive mechanism that may also increase the acidity in frozen storage (Park *et al.* 1997). The changes are consistent with the finding of Gou *et al.* (2019) who reported the temperature of -1.5°C causes significant changes in total acidity and total soluble solids than a lower temperature at -18°C in apple juice.

Changes in colour, rheology, total protein and ethanol content

The release of pigments from the stored neera due to disintegration of components causes either oxidative changes or degradation of neera. These effects accelerate the

colour changes in the stored neera (Lee and Coates 2002; Castro-López *et al.* 2016). Accordingly significant reduction of colour with a noticeable visual difference was reported at -6°C . However, the colour difference was the least at -20°C when compared to the control which was similar to the results of Nowak *et al.* (2019). The viscosity and their trend in this study was similar to the results of Dan *et al.* (2018). The reported result for milk with lactic acid bacteria increased the viscosity from 0 to 3 days and it decreased the values in the remaining days. The decrease in viscosity on the end of storage may suggest the possibilities of microbial metabolism at -6°C . Hence, the reduced microbial metabolism at -20°C maintains better viscosity in storage (Magala *et al.* 2015).

In earlier studies, reported $1.13 \text{ g } 100 \text{ ml}^{-1}$ of protein and it was as maximum protein content in unfermented sap (Kaur and Goswami 2020). These proteins under frozen storage were affected due to the insolubilization and aggregation with ice crystals which decreased the total protein from control to end of the storage (Powrie 1984; Twomey *et al.* 2013). But the reported increased concentration between the storage days may due to the microbial cold shock protein (Wouters *et al.* 2001). This cold shock protein resulted in the high protein content at -6°C and the least concentration at -20°C . These result of the protein was similar to the finding of (Cloutier *et al.* 1992).

At -20°C , the quantification of ethanol resulted in the negligible concentration in all the storage days expect in the 28 days. Whereas the ambient storage of neera induces the ethanol concentration from 5 to 8% (Borse *et al.* 2007). Moreover, ethanol production in food was associated with glycolysis and oxygen concentration (Fan *et al.* 2005). In our study, the significant changes in total soluble solids content (13.17°Brix) from the control were reported at -6°C . Thus indicates the more glycolytic metabolism by the viable micro-organism. Moreover, the induced aquaporins microbial gene expression at these low temperatures mediated the growth and activity of anaerobic yeast (Tanghe *et al.* 2004) and it may induce the production of ethanol in frozen storage. However, this gene expression and their further metabolic functions were restricted at -20°C which favours lower production at the end of the storage.

Sensory findings

The 9-point hedonic scale and their data by the panellists suggested the highest satisfactory values for the frozen neera sample was at -20°C . Moreover, the ranked values at this temperature were higher than the storage day value at -6°C . The qualities of fresh neera were preserved at -20°C for 21 days, whereas the storage at -6°C decreased their quality after 14 days of storage. A similar

least score values were reported in frozen prickly pear juice (Kgatla *et al.* 2010). The score values and comments of the panellist on storage days suggested that better sensory attributes were preserved at -20°C up to 21 days. Thereafter, the attributes were not acceptable by the panellist. At -6°C , the quality was not acceptable on 21st day, hence shelf life was only 14 days. Therefore, frozen storage at -20°C can extend the shelf life of neera and preserve the original qualities during the extended period than that of ambient storage.

In conclusion, frozen storage can preserve the qualities and can extend the shelf life of neera. At both frozen temperatures, the total viable count, yeast and lactic acid bacteria were reduced from the original population. The evaluation of injured microbes in frozen storage reduced the total number of the viable microbial community in neera. Consequently, frozen storage with a high freezing rate (-20°C) completely inhibits the survival of lactic acid bacteria that affects the fermentation in neera. Therefore, frozen storage at -20°C favours better reduction of 6.26 log CFU per ml for total viable count and 3 log CFU per ml for yeast, but inhibits lactic acid bacterial growth. The resistant microbes from this community and their respective changes in neera need to be studied further. Moreover, live/dead cell fluorescence staining analysis confirmed that injury to the viable and non-viable cells during frozen storage would be effective in differentiating the injured and resistant microbes in frozen foods. Moreover, the least microbial count at -20°C limits the chemical changes in pH (5.37), total soluble solids (14.0°Brix) and total acidity (0.82 mg l^{-1}). Furthermore, the total colour difference (4.97 ΔE^*), viscosity (3.88 mPa s), total protein (0.007 ± 0.01 g 100 ml^{-1}) and ethanol content (0.79%) indicate that the high freezing rate of -20°C less affected the quality than at atmospheric storage. At this frozen storage, the sensory attributes extended (21 days) with acceptable qualities. Overall, our result indicates that neera quality can be preserved at -20°C with the highest similarity to fresh neera for a longer period. Frozen storage reduced microbial population, which causes quality changes, preserves the chemical properties with acceptable sensory attributes and increases the shelf life of coconut neera making it fit for consumption.

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Conflict of interest

The authors have no conflict of interest to declare.

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Impact of Nisin in Combination with Sodium Benzoate and Calcium Carbonate on the Bacterial and Yeast Population of Coconut Neera (Coconut Inflorescence sap)

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Abstract

A natural sap from mature coconut palm known as coconut neera is enriched with essential minerals and vitamins. Rapid microbial fermentation affects neera processing industries because it spoils the physicochemical properties. There are various methods in preservation which extend the shelf life of coconut neera. The addition of nisin is one of the methods which protect neera against fermentation. Therefore, the study is focused to identify the effective combination of nisin (50 ppm) with preservatives like sodium benzoate (500, and 1000 ppm), and calcium carbonate (2500, and 3000 ppm) at two different combinations in neera. At the end of 21 d, 3000 ppm calcium carbonate with 50 ppm nisin in N4 treatment had an effective reduction of 120×10^5 CFU/ml and 143×10^2 CFU/ml for total bacteria and total yeast count. The reduced microbial survival resulted in the pH of 10.45 ± 0.05 , total soluble solids of 15.43 ± 0.12 °Brix, and total acidity of 1.11 ± 0.04 mg/ L, at this combination. The treatment of nisin with 3000 ppm calcium carbonate demonstrated the high red fluorescence bacterial cells than the treatment of nisin with 1000 ppm sodium benzoate. Additionally, the microorganisms in N4 treatment precipitated 65.34% Ca^{2+} from 79.96% in XRF intensity analysis. The synergistic effect of nisin and calcium carbonate explored their antimicrobial activity against the heterogeneous microbial population in coconut neera. The concentration of 3000 ppm calcium carbonate and nisin 50 ppm preserves the physicochemical and sensory qualities, up to 21 d at 4°C, and offer hope for the industrial-scale implementation.

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INTRODUCTION

The inflorescence sap of coconut neera is the abundant source of sucrose (16.19 g/ 100 mL), minerals such as K-68.4 mg/ 100 mL, Na-90.6 mg/ 100 mL, P-3.9 mg/ 100 mL, vitamins and proven low glycemic index drink (GI-35) with added medicinal benefits of nephroprotective, and hepatoprotective activity.¹ In addition, the natural sap is the source of indigenous microorganisms such as *Bacillus*, *Lactobacilli*, *Micrococci*, *Enterobacter*, *Leuconostoc*, *Saccharomyces*, *Candida*, and *Pichia*.² These microbes ferment the neera in a rapid way, and affected the palatability by producing the astringency, unpleasant volatile, cloudy appearance which gives economic loss to the neera entrepreneurs.^{3,4} However, the natural sap preserved by the existing technology through the combination of preservatives (citric acid and sodium metabisulphite), processing methods such as centrifugation, filtration and pasteurization.⁵ Another patented technology preserved the neera by acidification, preservatives such as nisin and pasteurization.⁶ Though the developed technologies bestow towards effective shelf life enhancement of neera, they might deplete the quality attributes due to thermal pasteurization.⁷ To overcome these disadvantages, the current study is designed to minimize the processing methods by adding antimicrobial agents for retaining the physicochemical and sensory properties.

The bio-preservative nisin (E234) from *Lactococcus lactis* classified as "Generally Recognized as Safe" for the food industry by World Health Organization has antimicrobial peptides that are widely used against gram-positive organisms and spores.⁸ Previous study reported that the application of nisin at a maximum concentration of 100 µg/ mL in wine effectively inhibited lactic acid fermentation.⁹ However, the microbial inactivation mechanism of ion-permeable pores in the cytoplasm is reported mainly for gram-positive bacteria than gram-negative bacteria.¹⁰ Hence, their combination with chemical preservatives can affect the heterogeneous microbial (gram-positive, gram-negative bacteria, yeast) growth in neera. Calcium carbonate (E170) is the widely used alkaline additive with a varied role in industries; nutritional supplements, curing agent, bulking agent, and modifiers. Traditionally, calcium

carbonate is coated on the neera collection vessel to extend the storage period.¹¹ As per the Food Safety and Standard Regulations 6.1.19 (FSSR, 2010), the maximum ppm of 5000 allowed for calcium carbonate in food preservation. Earlier studies reported that nanoparticles of calcium carbonate showed antimicrobial activity for gram-positive and gram-negative bacteria. The minimum inhibitory concentration (MIC) of 125 µg/ mL and 62.5 µg/ mL were reported against the gram-positive and gram-negative bacteria.¹² Likewise, Sodium benzoate (E211) is another preservative used in fresh juice, margarine, sweet products against the fungi. The study in black olive fermentation reported that 1000 ppm of sodium benzoate reduced the yeast count upon storage.¹³ Hence, the current study is aimed to control heterogeneous microbial communities of neera by nisin in combination with preservatives (sodium benzoate, and calcium carbonate), and assess the quality changes during storage.

MATERIALS AND METHODS

Addition of preservatives to the coconut sap

The collected fresh coconut neera was screened for pH and total soluble solids (≥ 6 and 15 °Brix), and then the sample was taken for further treatment. Based on the preliminary study, four different combinations (N1, N2, N3, and N4) were selected for the study. N1 and N2 contain sodium benzoate (Ganesh Benzoplast, India) of 500 ppm and 1000 ppm, respectively, while calcium carbonate (Local market, Thanjavur) of 2500 ppm and 3000 ppm were ascertained for N3 and N4 along with 50 ppm of nisin (Bimal Pharma Pvt. Ltd, India). Fresh coconut neera without preservatives was taken as control (C). The control and treated samples were stored at 4°C and analyzed for physicochemical, microbial, and sensory changes during 0, 3, 6, 9, 12, 15, 18, and 21 d of storage.

Microbiological analysis

The total viable bacteria and yeast in the control (C) and treated (N1, N2, N3, N4) samples were analyzed using a serial dilution method. For the enumeration of bacteria and yeast, plate count agar and oxytetra glucose yeast agar base (Hi-Media, Mumbai, India) were used. After spreading the diluted sample, plates were incubated at 30 ± 2°C for 24 and 72 h, respectively.¹⁴ The observed colonies were expressed in the colony-forming unit

(CFU), and more than 300 CFU/ml in storage days were denoted as too numerous to count.¹⁵

Physicochemical analysis

Aseptically drawn samples were analyzed for pH through the ELICO pH meter (L1 120 - model). The percentage of CO₂ was measured using PBI Dansensor. Hunter color parameters (Lightness, redness, and yellowness) of the neera samples were measured using a colorimeter (Color Flex EZ - 45/OLAV). The total soluble solids of the neera sample were measured by a hand-held refractometer (RHB - 55ATC), and results were expressed in °Brix. The total acidity of neera was analyzed against 0.1 N NaOH with the addition of a phenolphthalein indicator.

Microscopic analysis

The treatment effect on the microbial population of neera was evaluated by the live/dead cells staining using confocal laser scanning microscopy (model LSM 710 - Carl Zeiss Microscopy GmbH, Germany). For differentiating the viable and non-viable cells, the fluorescent dyes mixture of acridine orange and propidium iodide were added (1 µg in 1 µL) into the cells. Before the addition, the treated coconut neera sample was centrifuged at 10,000 × g for 5 m, and the collected pellet was washed with phosphate buffer solution.¹⁶ The stained cells were excited and detected using Argon laser (488-nm laser excitation) with a long pass from 500 to 640 nm. The image analysis was performed using Zen 2009 image software.

XRF analysis

The freeze-dried treated whole sample and sediment mineralogy was analyzed using an X-ray fluorescence spectrometer (XGT-5200, Horiba, Japan) equipped with an X-ray tube of 50 kV and a peltier cooled silicon drift detector.¹⁷

The Sensory evaluation

A trained panelist of eight judges from the Indian Institute of Food Processing Technology has scored the values using the nine point scale. The coded samples were presented in white cups, and their intensity of appearance, color, flavor, taste, after taste, consistency, and overall acceptability were scored on the respective storage days.

Statistical analysis

The experiments were carried out in triplicates. The values were evaluated statistically from 0 to 21 d by Student's t-test (SPSS.23). The p-value < 0.05 denotes the significant difference between the storage days.

RESULTS AND DISCUSSION

Addition of preservatives against the microbial growth in neera

The microbial population analysis in plate count revealed that the reduced total bacteria, and yeast count in N4 treatment (nisin 50 ppm with calcium carbonate 3000 ppm) than the N3 (nisin 50 ppm with calcium carbonate 2500 ppm) treatment (Table 1). Whereas, other treatments such as N1, N2, and control sample (C) had more than 300 CFU/ml for the bacteria and yeast population upon

Table 1. Total bacterial and yeast count (CFU/ml) of fresh and treated coconut neera during the storage days at 4°C

Storage days	Total Bacteria (10 ⁵)					Total yeast (10 ²)				
	C	N1	N2	N3	N4	C	N1	N2	N3	N4
0	120	130	120	3	1	160	67	63	7	10
3	*	270	230	10	3	*	247	203	70	10
6	*	*	*	30	8	*	*	*	130	30
9	*	*	*	60	17	*	*	*	220	47
12	*	*	*	100	37	*	*	*	233	67
15	*	*	*	110	73	*	*	*	243	107
18	*	*	*	130	100	*	*	*	250	123
21	*	*	*	160	120	*	*	*	260	143

* Represent TNTC (Too Numerous To Count) colonies on storage days.

Abbreviation mentioned in the table throughout denotes: C - Control neera without treatment, N1 - Neera treated with 50 ppm nisin and 500 ppm of sodium benzoate, N2 - Neera treated with 50 ppm nisin and 1000 ppm of sodium benzoate, N3 - Neera treated with 50 ppm nisin and 2500 ppm of calcium carbonate, N4 - Neera treated with 50 ppm nisin and 3000 ppm of calcium carbonate. The values in the table represent the mean of triplicate.

storage days. The enhanced antimicrobial activity of nisin with calcium carbonate significantly reduced microbial growth in coconut neera than the nisin with sodium benzoate treatment.¹⁸ The added concentration of 3000 ppm calcium carbonate in N4 treatment suppressed the microbial growth in highest count such as 120×10^5 CFU/ml for total bacteria and 143×10^2 CFU/ml for yeast at the end of the storage period (21 d). Whereas, the lowest ppm of 2500 calcium carbonate in N3 treatment reduced the growth as

160×10^5 and 260×10^2 CFU/ml for total bacteria and yeast.

Detection of viable and non-viable cells by microscopy

The live and dead cells between treatment demonstrated the antimicrobial activity in neera. In Fig. 1(a) the control sample (C) without preservatives exhibited large number of green fluorescence bacterial cells and it affect the coconut neera by lactic acid fermentation.¹⁹ The treatment of N2 expressed more non-viable yeast

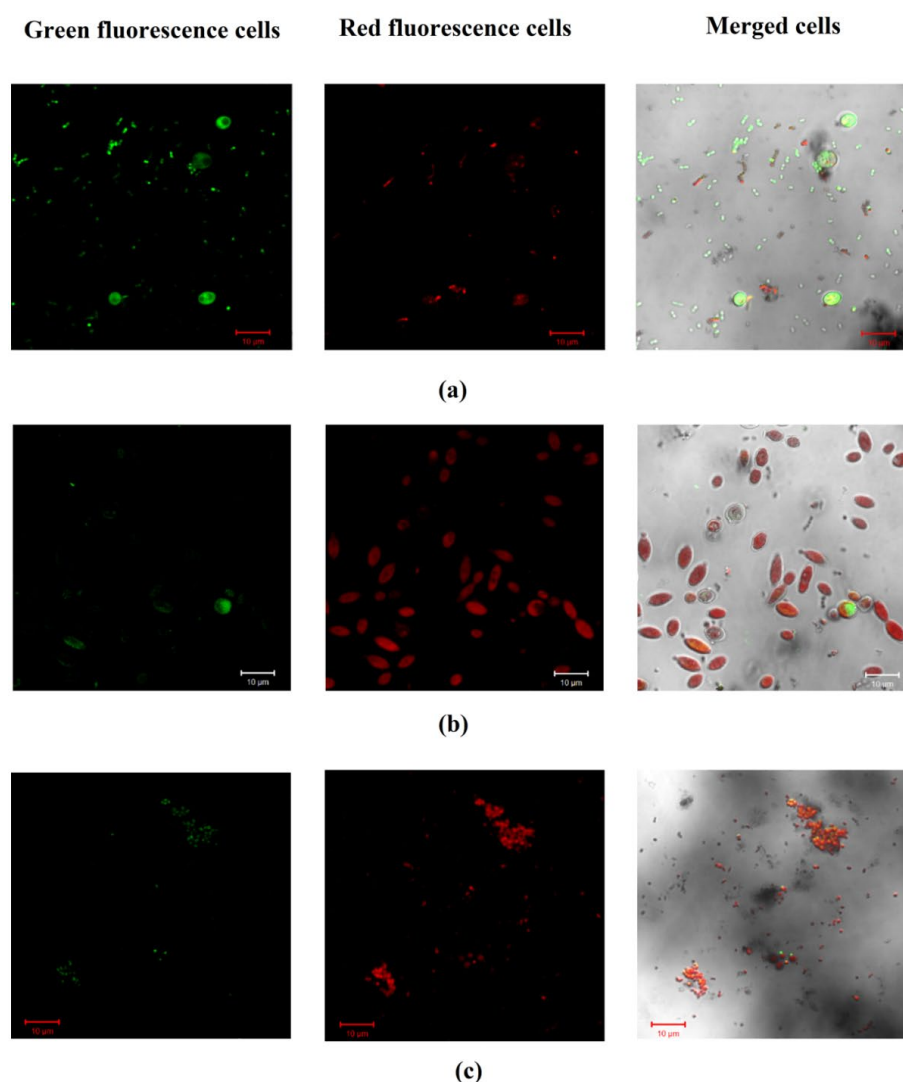


Fig. 1. Detection of viable and non-viable cells in coconut neera by confocal laser scanning microscopy. Observation of cells from neera (control) without treatment (a) cells from nisin (50 ppm) and sodium benzoate (1000 ppm) added neera (b) cells from nisin (50 ppm) and calcium carbonate (3000 ppm) added neera (c). Green cells represent live microorganisms, while red cells represent dead microorganisms.

cells (red fluorescence cells) than the bacterial cells (Fig. 1b). This combination was ineffective against the bacteria because the added preservative of sodium benzoate developed acidic pH which affected the solubility of nisin, and it generally effective towards acidic foods.²⁰ In the presence of nisin and calcium carbonate, there was an increase in the non-viable bacterial cells (red fluorescence cells) which arrest the lactic acid fermentation (Fig. 1c). The reduced bacterial viability confirms the solubility of nisin and their inhibitory activity at this combination.

Calcium microbe precipitation by XRF analysis

The calcium microbe precipitation in N4 (nisin 50 ppm with calcium carbonate 3000 ppm) treatment was identified through XRF intensity analysis. The intensity of the total treatment was observed with the 79.96% of calcium and sediment portion of the same treatment revealed the percentage of 65.34% (Fig. 2a, b). The resulting percentage in the sediment evident that the sugar rich medium of neera favors the mechanism of precipitation between microbes and minerals.²¹ The intensity of zirconium (Zr) indicated synthesis of new bio mineral from the precipitation reaction than other minerals of fresh coconut neera (K, Na, Mg, Fe, Ca, and Zn).²² These findings suggest that the precipitation of calcium with indigenous microorganisms had a better inhibition against the spontaneous fermentation.²³

Physicochemical properties of treated neera

The combination effect of preservatives with nisin on the physicochemical qualities of coconut neera were presented in Table 2, and their significant changes between three days interval were tabulated in supporting document. The results revealed that C and N1, and N2 treatment had significantly reduced the pH. However, N4 treatment at the end of 21 d showed a higher pH of 10.45 ± 0.05 from the 0 d control. The pH reduction in sodium benzoate treatments might be due to the production of lactic acid, acetic acid, and citric acid in neera at a temperature of 4°C. Moreover, the pH changes in the C, N1, and N2 were similar to the pH of fermented neera.² Addition of calcium carbonate in N3 and N4 increased the pH than other treatments. Along with pH, the physicochemical property of CO₂ increased in storage days with the greater percentage of 55.53 ± 0.21 , 32.70 ± 0.00 , and 25.67 ± 0.23 in C, N1, and

Table 2. Physicochemical changes in fresh and treated coconut neera during storage days at 4°C

Treatment	pH		CO ₂ (%)		Total acidity (mg/L)		Total solids soluble (°Brix)		Color (ΔE)	
	0 d	21 d	0 d	21 d	0 d	21 d	0 d	21 d	0 d	21 d
C	7.58±0.01	2.38±0.01 ^f	ND	55.53±0.20 ^h	0.19±0.02	11.83±0.12 ^h	19.70±1.71	12.17±0.06 ^f	*	37.63±0.02 ^h
N1	7.16±0.03	2.53±0.03 ^f	ND	32.7±0.00 ^h	0.20±0.00	9.13±0.02 ^h	17.77±0.15	12.27±0.06 ^f	3.41±0.00	35.53±0.02 ^h
N2	7.27±0.00	3.48±0.14 ^h	ND	25.66±0.23 ^h	0.26±0.00	10.07±0.02 ^h	18.13±0.15	12.37±0.06 ^h	6.85±0.39	33.61±0.02 ^h
N3	9.67±0.01	5.62±0.01 ^f	ND	12.37±0.11 ^h	0.14±0.01	1.23±0.02 ^h	18.83±0.29	15.27±0.06 ^g	7.23±0.03	31.37±0.01 ^h
N4	110.82±0.03	10.45±0.05 ^b	ND	6.83±0.06 ^g	0.12±0.02	1.11±0.04 ^g	19.33±0.29	15.43±0.12 ^g	9.41±0.29	28.79±0.01 ^h

Note: ND means not detected. * Indicates no difference on the delta E value of fresh neera (L* 58.13 a* 0.36 and b* 9.26). Data are shown as means ± standard deviation of n = 3 independent experiments. The different alphabets in storage indicate significantly different at 0.05 level between the 0 d to 21 d. Abbreviation mentioned in the table denotes: C - Control neera without treatment, N1 - Neera treated with 50 ppm nisin and 500 ppm of sodium benzoate, N2 - Neera treated with 50 ppm nisin and 1000 ppm of sodium benzoate, N3 - Neera treated with 50 ppm nisin and 2500 ppm of calcium carbonate, N4 - Neera treated with 50 ppm nisin and 3000 ppm of calcium carbonate. The values in the table represent the mean of triplicate.

N2 respectively. This changes in storage indicates the conversion of sucrose by vigorous microbial action of the yeast in N1, N2, and C.²⁴ Whereas, N3 and N4 treatments exhibited less production of CO₂ due to the alkali-resistant *Bacillus* and yeast cells.²⁵

According to the fermentation phenomenon, C, N1, and N2 treatment had changes in total soluble solids and total acidity. Their quantification in storage days were similar to the trend of fermented neera than treatment of N3 and N4.²⁶ The acidity from lactic acid fermentation was increased as 1.23 ± 0.02 and 1.11 ± 0.04 mg/L in N3 and N4 respectively which was least acidity in storage. Addition of sodium benzoate in N1 and N2 treatments were ineffective in controlling two-stage fermentation by the bacteria and yeast.²⁷ The

better total soluble solids and acidity in N3 and N4 might be due to the precipitation reaction of microbes-calcium.²⁸

The treatment effect on the color of the neera was depicted as color difference (ΔE) from the L, a, b values (Table 2). The combination of nisin and sodium benzoate quickly increased the ΔE values between the storage period, and it causes a significant difference from the 0 d control. The addition of calcium carbonate in N3 and N4 had a high impact on whitening the neera color compared to sodium benzoate treatment.²⁹ The undissolved and light scattering calcium carbonate particles in the treatment-induced the 0 d color of neera in N3 and N4. At the end of storage studies, the increased solubility of calcium carbonate produced the least color difference of $31.37 \pm$

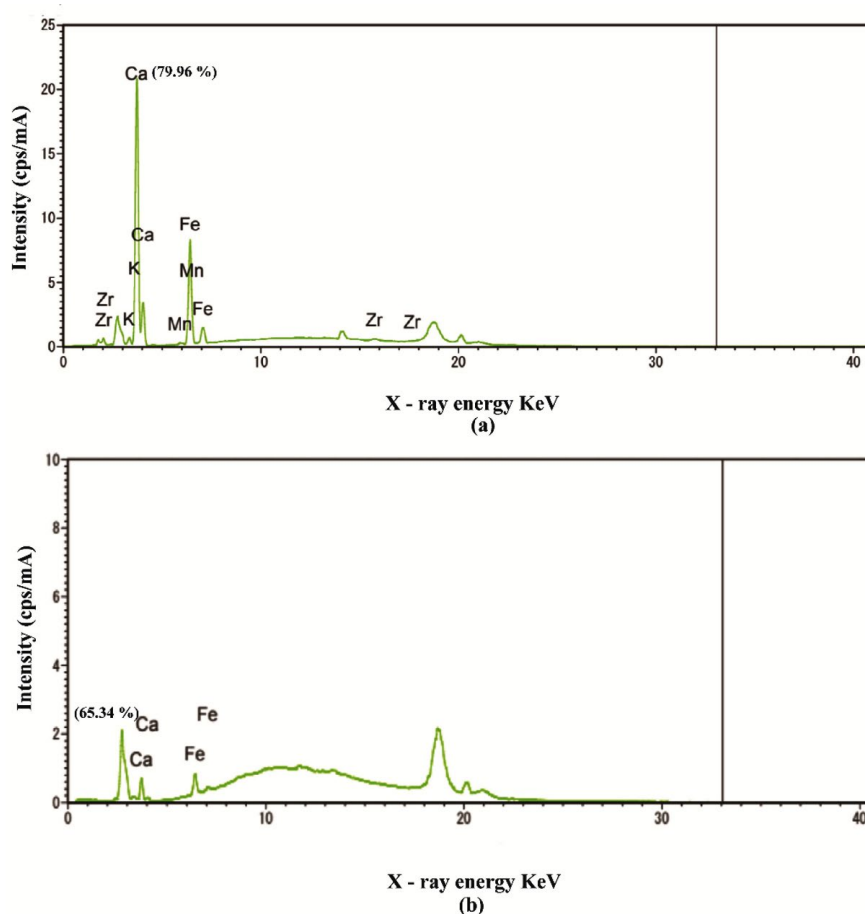


Fig. 2. XRF patterns of minerals from nisin (50 ppm) and calcium carbonate (3000 ppm) treated coconut neera (a) whole treated sample includes the sediment (b) sediment of treated sample. The spectra explains the various mineral determinants.

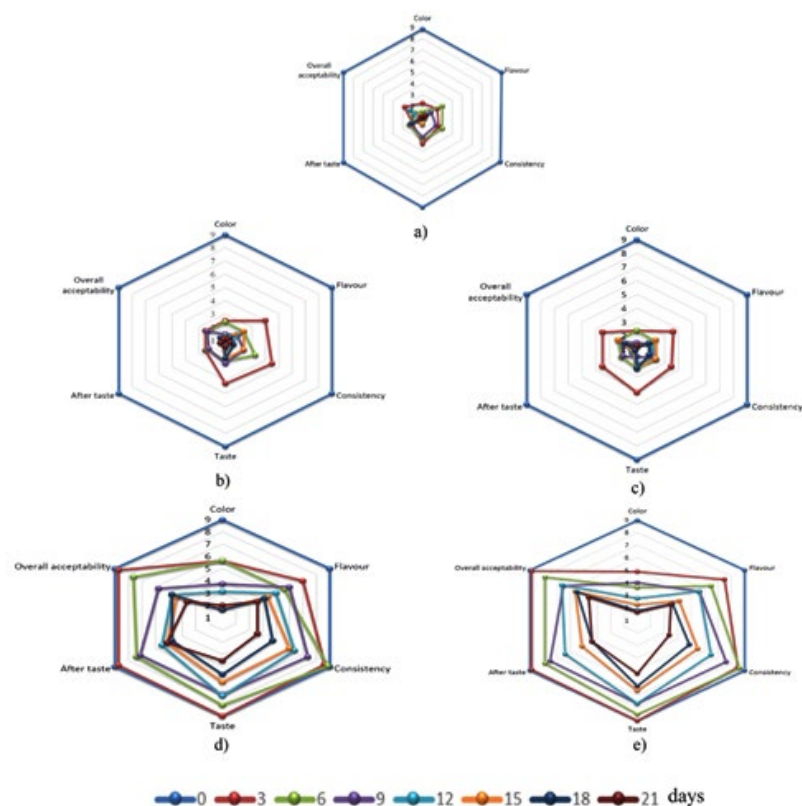


Fig. 3. Sensory properties of coconut neera during storage at 4 °C. (a) Control neera (b) Neera treated with nisin (50 ppm) and sodium benzoate (500 ppm) (c) Neera treated with nisin (50 ppm) and sodium benzoate (1000 ppm) (d) Neera treated with nisin (50 ppm) and calcium carbonate (2500 ppm) (e) Neera treated with nisin (50 ppm) and calcium carbonate (3000 ppm).

0.01 and $28.79 \pm 0.01 \Delta E$ in N3 and N4 treatments respectively.

Evaluation of sensory attributes in treated neera

The treatment of N4 (Fig. 3e) exhibited better sensory properties for a longer period of 21 d as compared to other treatments. Whereas calcium carbonate concentration of 2500 ppm in N3 lost its sensorial properties on 15 d (Fig. 3d). The sample of C, N1 and N2 samples lost their sensory properties with a shorter period of 3 d (Fig. 3a, b & c). The decreased trend of sensorial properties in increasing storage days was due to the physicochemical changes. The treatment of N4 (nisin 50 ppm with 3000 ppm) with calcium carbonate had better sensory characteristics for more extended period than N3 treated sample. Finally, added 50 ppm nisin did not

affect the sensorial properties in any of the neera combinations.⁹

CONCLUSION

The addition of sodium benzoate and nisin in neera was ineffective against the microbial growth and resulted the similar physicochemical and sensory quality of fermented neera. The enhanced antimicrobial activity of calcium carbonate with nisin against the bacteria and yeast preserved the quality of coconut neera. However, 3000 ppm of calcium carbonate with 50 ppm nisin was found to be effective to control high number of indigenous microorganisms that preserve the physicochemical, and sensory qualities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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