

**INFUSION OF FRUIT'S NUTRITIONAL
CHARACTERISTICS AND FLAVOUR INTO CHOCOLATE
THROUGH DOUBLE FERMENTATION TECHNIQUE
PROJECT WORK**

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

CATEGORY -1 UNIVERSITY BY UGC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **ARAVINDAN S (41300005)**, **AMITHA C U (41300003)** who carried out the project entitled **"INFUSION OF FRUIT'S NUTRITIONAL CHARACTERISTICS AND FLAVOUR INTO CHOCOLATE THROUGH DOUBLE FERMENTATION TECHNIQUE"** under my supervision from January 2024 to April 2024.

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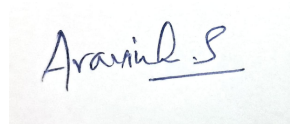
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DECLARATION

We, **ARAVINDAN S (41300005), AMITHA C U (41300003)** hereby declare that the Project Report entitled “**INFUSION OF FRUIT’S NUTRITIONAL CHARACTERISTICS AND FLAVOUR INTO CHOCOLATE THROUGH DOUBLE FERMENTATION TECHNIQUE**” done by us under the guidance of **Dr. Indumathi S M** and **Dr. Prakash P** at Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in **Microbiology**.

DATE: 26.04.2024

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SIGNATURE OF THE CANDIDATE

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ABSTRACT

The chocolate is made from the seeds of the *Theobroma cacao* L tree. The modern chocolate industry has created different types of chocolate and added new artificial fruit flavours to them. These artificial fruit flavours may impair human health by causing allergic reactions and affecting the quality of the food. To subdue this issue, the cocoa beans are fermented twice with the naturally occurring fruit pulps. This process is called double fermentation. Additionally, the cocoa bean's total antioxidant property is likely to be reduced due to the sun-drying process. These antioxidants have several health benefits, including being anti-inflammatory, lowering the risk of heart disease, and lowering blood pressure in humans. But because of the loss of antioxidants, their benefits cannot be obtained to their fullest. So, this study is done not only to infuse natural fruit flavours but also to impart the antioxidant and nutritional properties of fruits into chocolate. For the production of double-fermented dark chocolate, the cocoa beans are first fermented and dried to obtain their desirable chocolate flavour. Then fermented, sun-dried beans undergo a second fermentation with mixed fruit pulp. Further, the cocoa beans are sun-dried and processed (roasting, conching, and tempering) to produce a double-fermented dark chocolate with a mild fruit flavour. Later, the characteristic analysis of double-fermented dark chocolate is carried out.

Keywords: Double fermented chocolate, Mixed fruits, Nutritional characteristic, Antioxidant, Cocobiota, Artificial flavours.

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LIST OF ABBREVIATIONS

C	Celsius
LB Broth	Luria-Bertani Broth

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CHAPTER 1

INTRODUCTION

Chocolate is one of the leading flavours used in confectionaries such as cakes, brownies, and cookies, as well as in bars. Chocolate was first made from the cacao plant, which was found in Mesoamerica. Maya characterised chocolate as a hot water-prepared cocoa beverage that was frequently flavoured with pepper and cinnamon. Christopher Columbus became the first European to discover cocoa in 1502. When he seized a boat, it included cocoa beans, which Mesoamericans recognised as "mysterious-looking almonds" and used as a kind of money (Coe & Coe, 1996) & (Dillinger et al., 2000). Cocoa was first rejected in Europe due to its bitter characteristics. Carl Linnaeus, a Swedish scientist, named the plant *Theobroma cacao* L. in 1753. In Greek, *Theobroma* means 'food of gods,' where *Theo* means food and *broma* means god (Moramarco, S., & Nemi, L., 2016).

1.1 CHOCOLATE AND ITS TYPES

Chocolate can be solid, liquid, or paste, and it usually has some type of additional sweetness, which includes sugar or condensed milk, to balance the bitter flavour of cocoa. Chocolate is mostly made up of sugar and fat (cocoa butter), which melts at body temperature and adds to the delicious and satisfying feeling of eating chocolate (Hetherington & MacDiarmid, 1993). Based on the proportion of cocoa solids, chocolate industry has created different types of chocolate, including dark chocolate (55% - 90% cocoa beans and cocoa butter), milk chocolate (20-35% cocoa solids, cocoa butter, milk solids and sugar), white chocolate (cocoa butter, milk solids, and sugar) and gianduja chocolate (cocoa, hazelnuts, and sugar). Dark chocolate has higher cocoa content than other types and also, it has intense aroma and bitter taste. (Montagna et al., 2019).

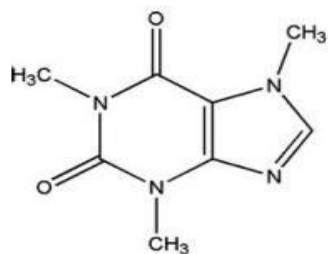
1.2 COCOA BEANS

The three primary materials needed to make chocolate are cocoa beans, sugar, and cocoa butter. Cocoa beans are produced from the seeds of the cocoa tree (*Theobroma cacao* L.). Cocoa beans are the major raw material in the production of

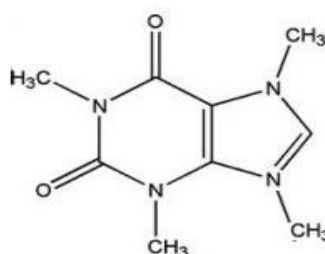
chocolate and by-products such as cocoa solids, cocoa butter, and other cocoa-based beverages. Around 20–60 beans are present in a single cocoa fruit. Each cocoa bean is covered by a white, sweet pulp that contains fermentable sugars, and they are surrounded by a thick outer rind (cocoa pod). Cocoa fruit comes in three different types. The most resilient and extensively grown variety is Forastero; the higher-quality but more costly kind is Criollo; and a mix of the other two is Trinitario (Danny, 2023). Lower levels of tropical evergreen rain forests are native to cocoa trees. With an annual maximum temperature of 30–32 °C and a minimum temperature of 18–21 °C, cocoa plants adapt well to both relatively high temperatures and typically high relative humidity, which can reach 100% during the day and drop to 70–80% at night (International Cocoa Organization, 2024).

1.3 SCIENCE BEHIND THE HEALTH BENEFITS OF COCOA BEANS

Cocoa contains around 300 volatile chemicals, including aliphatic esters, polyphenols, aromatic carbonyls, and theobromine. These compounds protect from rancidity of fat. In addition, amines, alkaloids theobromine, caffeine, theophylline, fatty acids, tyramine, trigonelline, magnesium, phenylethylamine, and N-acylethanolamines are also found in cocoa seeds and are pharmacologically active. A normal chocolate bar weighs 40–50 g and has 86–240 mg of theobromine and 9–31 mg of caffeine. 300–1200mg per ounce of theobromine are naturally present in cocoa beans (Haritha et al., 2014). Theobromine levels vary among on the type of chocolate. Theobromine content in dark chocolates is often greater (around 10 g/kg) than that of milk chocolates (1–5 g/kg) (Bruinsma K., & Taren DL., 1999).



Theobromine



Caffeine

Fig 1.1: Structure of Theobromine and Caffeine

Saturated fatty acids, which are found in cocoa butter, aid with the body's cholesterol regulation (Munjal et al., 2019). Also, compared to other conventional foods that are high in antioxidants, chocolate has more antioxidants and is mainly rich in phenolic antioxidants. Catechins, anthocyanidins, and proanthocyanidins are the three groups of polyphenols found in cocoa beans (Meng et al., 2009) & (Magrone et al., 2017). These antioxidants provide a variety of health advantages, including protecting the body from damaging free radicals, having anti-inflammatory properties, lowering the risk of heart disease, boosting cognitive function, lowering blood pressure, elevating mood, and even reducing stress and anxiety levels. Moreover, the antioxidants in dark chocolate may benefit skin health by preventing UV damage and enhancing moisture (Katz et al., 2011). And the bitterness and astringent taste is mainly due to the polyphenol content in cocoa beans.

1.4 FROM BEAN TO BAR CHOCOLATE

Throughout the production of chocolate, cocoa beans go through a series of steps, including fermentation, drying, roasting, conching, and tempering. All these processes are important to obtain good-quality chocolate. The fermentation process helps to kill the beans and develop the chocolate flavour and floral notes from the raw cocoa bean, which has an intense bitter and astringent taste. In order to yield good quality and aroma, the beans undergo a fermentation process, which is a complex biochemical reaction that results in the death of seed embryos, the release of enzymes and substrates, protein and sugar hydrolysis, the diffusion of theobromine, caffeine, and polyphenolic compounds, and the accumulation of flavour precursors in the seed.

1.4.1 The Microbiology of cocoa fermentation

Since fermentation is a natural process, the microorganisms from the surface of the fruit, from the surrounding environment, the knives used to cut open the pod, and the hands of the workers contaminate the sterile cocoa bean and contribute to this process. The pH, temperature, oxygen, available nutrients, and moisture play an important role in determining the microbial diversity of the cocoa fermentation. In addition to this, different fermentation locations also determine the microbial diversity. Several studies revealed that in cocoa fermentation, yeast, lactic acid bacteria, and

acetic acid bacteria are the main contenders. It also shows that a vast variety of yeasts are involved in cocoa fermentation in different countries. The yeast acts first and undergoes anaerobic fermentation, followed by the lactic acid bacteria, and finally the acetic acid bacteria undergo aerobic fermentation (Ardhana & Fleet, 2003). Filamentous fungi and other spore-forming aerobes interfere with the process and produce off-flavours.

Once the fermentation is complete, the fermented beans are dried. Then the beans are roasted and ground to form a liquid, paste-like chocolate liquor. In most cases, the chocolate liquor is pressed to lower the cocoa butter content and pulverised to form cocoa powder. Finally, cocoa powder, cocoa butter, and sugar are standardised and mixed together to create sweet chocolate.

1.5 NEED FOR DOUBLE FERMENTATION

Additionally, added fruit flavours, preservatives, modifiers, and milk solids are included in contemporary commercial varieties of chocolate. The inclusion of these artificial fruit flavours renders the chocolate harmful for consumers by posing health risks. The side effects of synthetic flavour differ from person to person, but in most cases, it causes allergic reactions and food hypersensitivity. Even though they may not have an immediate and direct impact on your physical or mental well-being, they do alter your perception of nutritional value, flavour, and quality of food, making eating less pleasurable and healthier over time (Geoseph, 2023). Also, during the sun drying process, the polyphenol level of cocoa beans tends to reduce by around 44–77%, which in turn reduces the overall antioxidant level of the chocolate (Di Mattia et al., 2012). To overcome these problems, double fermentation is carried out. The double fermentation technique is where the cocoa beans are fermented twice, but the second fermentation is carried out with the addition of a new raw material that is natural fruit pulp. This second fermentation process is carried out for 4 days. After the second fermentation, the cocoa beans are dried again and further processed to transform into double-fermented chocolate. Furthermore, to enhance the rate of the second fermentation process, the indigenous microorganisms are isolated from the mixed fruits and cocoa fruit and inoculated in the cocoa fermentation. This technique helps to infuse

the mild fruit flavour with more antioxidants and other nutritional characteristics into the cocoa bean. Additionally, it may reduce the chocolate's overall fat and carbohydrate content, which raises the risk of obesity. This double fermentation process further enriches the overall nutritional aspect of the chocolate by infusing the mixed fruit pulp, making it a healthier option for consumers. This research is aimed at improving the flavour profile, aroma, texture, antioxidants, and other nutritional characteristics of chocolate through the double fermentation of cocoa beans with mixed fruit pulp.

CHAPTER 2

LITERATURE SURVEY

2.1 CHOCOLATE

Chocolate has been referred as ‘food of gods’ by ancient mayans. It was first prepared as a cocoa drink in hot water. Interest in chocolate has been grown and many types of chocolate were introduced by food industry. Among them, dark chocolate gained popularity for its potential health benefits such as regulation of blood pressure, lipid metabolism and vascular functions (Montagna et al., 2019).

2.2 COCOBIOTA

Cocobiota that are involved in the fermentation of beans in chocolate production, has a considerable value, contributing to the medicinal aspects of chocolate through its primary and secondary metabolites which are usually alcohols and acids (Petyaev & Bashmakov, 2016).

This Cocobiota mainly consist of indigenous microorganisms including lactic acid bacteria, acetic acid bacteria, yeasts, and rarely filamentous fungi (Sârbu & Csutak, 2019).

2.2.1 Yeast Culture

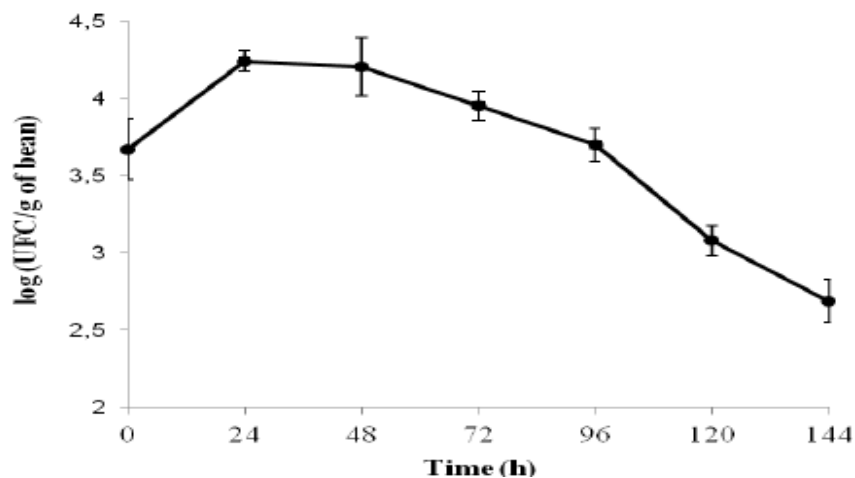


Fig 2.1: Growth curve of Yeast in Cocoa Fermentation

Yeasts cells dominate the first 24 hours of fermentation. During this period, they metabolize sugars in pulp of beans and produce ethanol and other volatile and non-volatile compounds. The population of yeasts remain constant in this period. This results in a semi aerobic condition in which lactic acid bacteria can predominate. Starter cultures of yeasts such as *Pichia manshurica*, *Zygosacharomyces bailli*, *Pichia fermentans* are used for controlled fermentation (Schwan et al., 2023).

2.2.2 Lactic acid Bacteria

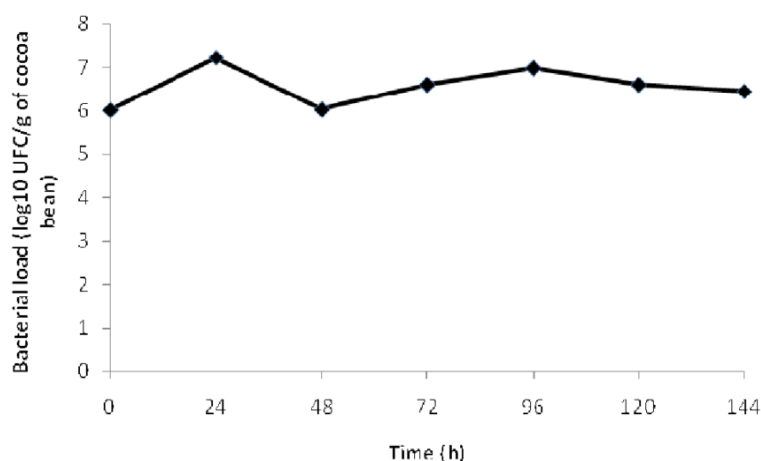


Fig 2.2: Growth curve of Lactic Acid Bacteria in cocoa fermentation

The lactic acid bacteria are present in fermentation even on first 24 hours of fermentation, but only becomes active during second phase of fermentation due to the semi aerobic conditions created by yeast cells. They remain active up to 96 hours of fermentation. Cultures including *Lactoplantibacillus*, *Leuconostoc* species has been used for controlled fermentation (Barišić et al., 2019).

2.2.3 Acetic acid Bacteria

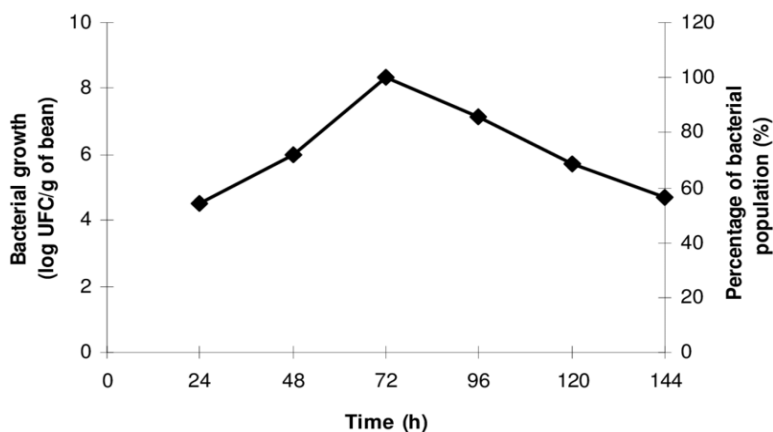


Figure 2.3: Growth Curve of Acetic Acid Bacteria in Cocoa fermentation

When aeration increases, the fermentation is dominated by acetic acid bacteria. This organism does an exothermic reaction which results in increase in temperature up to 50.c. Aldehydes, acetic acid and flavour inducing enzymes such as theobromine and flavour precursors are produced during acetic acid bacteria's activity period. Cultures including *Acetobacter pasteurians*, *Gluconoacetobacter* and *Acetobacter ghanensis* are employed for controlled fermentation (Barišić et al., 2019).

2.3 HEALTH ASPECTS OF DARK CHOCOLATE CONSUMPTION

Chocolate has higher phenolic antioxidant than any other food. Thus, studies proved that antioxidants in cocoa can reduce the risk of nerve injury and insulin resistance aiding diabetic management. They can also protect skin from oxidative damages due to UV irradiation, thus reducing the risk of skin cancer (Katz et al., 2011).

The bitterness of dark chocolate is also proportional to the antioxidants present in them (Zugravu & Oțelea, 2019).

It has been proved that although roasting of cocoa bean during chocolate production enhances flavour profile, it leads to significant drop of antioxidant levels, thus compromising the health benefits of the same (Barišić et al., 2019).

Researchers attempted fortification of dark chocolate with the help of fruits and spices. The end product has increased antioxidant levels and essential fatty acid contents. The antioxidants present in dark chocolate protects the consumer against various brain related disorders such as Alzheimer's disease and Parkinson's disease (Samanta et al., 2022).

Researchers conducted clinical trials and proved that antioxidants in dark chocolate have a role in switching important pathways such as toll-like receptor 4, signal transducer, activator of transcription etc. (Magrone et al., 2017).

Studies has been conducted by researchers further proved that, consumption of dark chocolate may lead to improvements in brain health and cognitive functions in middle aged adults (Nemoto et al., 2022).

Some researchers proved that intake of dark chocolate leads to reduced risk of venous thromboembolism and hypertension, indicating better heart health (Yang et al., 2024).

These researches prove that increase in antioxidant levels of dark chocolate through double fermentation may leads to various health benefits to the respective consumers, thus pressing the importance of the same.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To enhance nutritional characteristics, flavor profile and aroma of chocolate through double fermentation technique

3.2 OBJECTIVES

1. To collect the cocoa beans from the pod and carry out the first fermentation of cocoa beans to obtain natural flavour and aroma by native species
2. Isolation of native organisms from the cashew fruit and mixed fruits such as strawberry, apple, grape, kiwi, avocado and litchi
3. To carry out second fermentation with mixed fruits pulp using isolated organisms
4. To produce double fermented chocolate and analyse the characteristics

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

The materials required for the various methodology employed in the experiment are Cocoa fruit, Cocoa butter, Sugar, Honey, Mixed fruits (Apple, Avocado, Grapes, Kiwi, Litchi, and Strawberry), Knife, Bamboo baskets, Banana leaves, Aluminium foil, Drying cloth, Glass jar, Chocolate mould, Petri plate, Conical flask, LB broth, Potato dextrose broth, Potato dextrose agar, MacConkey agar, Inoculation loop, Gloves, Mask, Ethanol, Cotton, Spatula, Wet grinder, Mixer grinder, Cheese cloth, Rubber band, Kitchen Thermometer, Centrifuge tube 50ml, Gram's Staining kit, Glass slide and Distilled water.

4.2 METHODOLOGY

Production of chocolate from cocoa beans normally includes the following series of processes: selection of cocoa beans, fermentation, drying, roasting, conching, tempering, and moulding. During the drying process, the antioxidant levels of the final product are compromised. Hence, double fermentation of cocoa beans using mixed fruit pulp is experimented with, expecting to increase the antioxidant levels of the chocolate made as a product. After the first fermentation, the beans are sun-dried and submerged in mixed fruit pulp for the second fermentation. Native microorganisms that are isolated from the fruits used have been used to speed up the process further. The following process explains the production of chocolate using double fermentation method.

4.2.1 Selection of Cocoa Beans

Cocoa beans are seeds derived from the tropical *Theobroma cacao* L. tree. Generally, there are four types of cocoa beans: Criollo, Trinitario, Forastero, and Nacional. Among these, Forastero has been popularly used due to its higher phenolic content, antioxidants, and availability. (Di Mattia et al., 2017). Higher-grade cocoa beans that are handpicked from cocoa pods of the Forastero type have been used in the experiment.



Fig 4.1: Types of Cocoa Fruits

Forastero-type pods, weighing approximately 4 kg, have been locally sourced from Idukki, Kerala. The pods are checked further for any unwanted discoloration or physical damages that may impact the quality of the beans that are present inside the pod. Pods that have brown discoloration and a hard texture on them have been omitted as they indicate fungal contamination.

The knife to be used is sterilized with spirit. On a clean surface, the pods are cut using a sterile knife, and cocoa beans are removed from the pod. The beans are then transferred into a clean steel vessel. Cocoa beans that are broken, damaged, or look unappealing are removed, as a single damaged cocoa bean can hinder the whole fermentation process.

4.2.2 First Fermentation of Cocoa Beans

The materials required for first fermentation of cocoa beans are bamboo baskets, banana leaves, sterile spoon, sterile knife, and cocoa beans. Basket method of fermentation is employed due to smaller quantity of beans taken. Bamboo basket is used for first fermentation process. The basket is fully covered with banana leaves. Usage of banana leaves provides a natural aroma for the beans. The selected cocoa beans are transferred into the bamboo basket. The beans are then covered with banana leaves tightly to provide an anaerobic condition. The beans are allowed to ferment for 7 days in a shady, cool place.

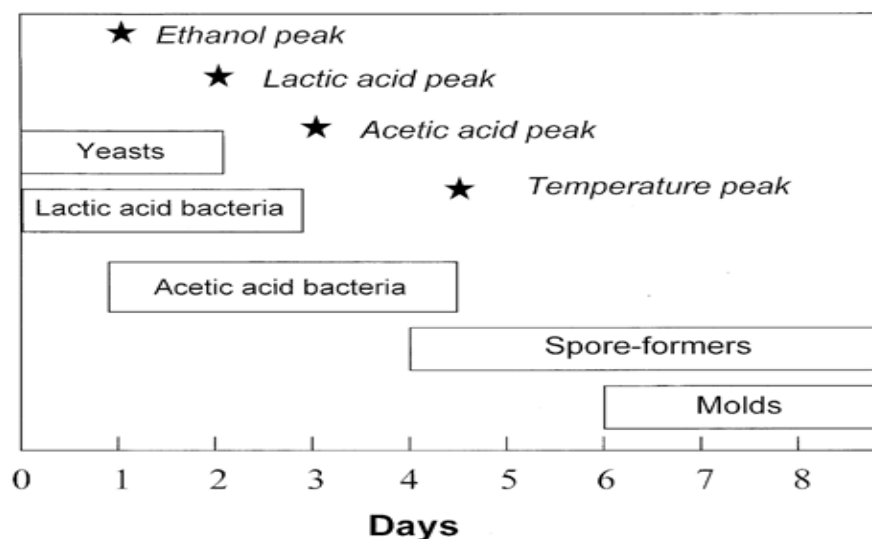


Fig 4.2: Succession of microorganisms in cocoa fermentation

The pulp surrounding the cocoa beans contains sugars such as glucose, sucrose and galactose which acts as an excellent source for the growth of yeasts. Hence, in first 24 hours of fermentation, yeast cells remain predominant due to anaerobic condition (Camu et al., 2008). The yeast cells involved in fermentation differs in locality. The commonly seen strains include *S. cerevisiae*, *C. krusei*, *C. tropicalis*, *P. kluyveri* (Sârbu & Csutak, 2019). These cells ferment the sugars present in the pulp and generates carbon di oxide , organic acids, and flavour precursors.

Followed by yeast, lactic acid bacteria start to ferment the pulp sugars present in the cocoa beans and produces lactic acid which helps in inhibition of spoilage

microorganisms. Commonly seen strains are heterofermentative *L. fermentum* and *L. plantarum* (Sârbu & Csutak, 2019). Ecological succession of acetic acid bacteria takes place after lactic acid fermentation. They ferment ethanol produced by yeast into acetic acid which actively diffuses into the cotyledon of the bean leading to the production of flavour precursors and cocoa colour, and also inhibits the spoilage organisms (Sârbu & Csutak, 2019).

4.2.2.1 Turning of Beans

The cocoa beans are turned every 48 hours of fermentation of better aeration and equal fermentation of beans.

4.2.2.2 Cut test



Fig 4.3: Fermented Cocoa beans

At the end of first fermentation, the inside of beans is expected to turn from dark brown colour to pale colour, which indicates the successful fermentation. Few beans are selected at random and cut horizontally across its axis to check the colour change.

4.2.3 Drying

The materials required for drying process are sterile absorbent cloth and fermented beans. After the first fermentation process, the cocoa beans are sundried. As the drying process removes the water from the cocoa beans, at the end of the sun-drying process, the moisture content of the cocoa beans is lowered below 7%. The fermented beans are placed on a clean moisture absorbing cloth and placed under direct sunlight for drying. Drying process may take up to 15 days depending on the

weather conditions and climate. Drying inhibits the microbiological process that takes place in the beans thus preventing over fermentation of beans. Over fermentation results in off flavours and taste which is undesirable (Líma et al., 2012).

4.2.4 Isolation of Native Microorganisms from Fruits

The materials required for isolation process are LB broth, potato dextrose broth, conical flask, petri dishes, forceps, gloves, centrifuge tube, distilled water, and fruits. Mixed fruits such as apple, litchi, grapes, strawberry, and avocado are taken for double fermentation process.

Using LB broth and potato dextrose broth, the microorganisms from the fruits taken are isolated, mainly yeast. The broths are prepared under sterile condition. Surface sterilization of fruits is done by dipping them on sterile water. This prevents the growth of soil microorganisms present on the surface of the fruits. Small pieces of each fruit are cut using sterile knife and introduced into the two conical flasks containing broths separately. A control broth for both LB broth and potato dextrose broth is used to ensure zero contamination. Broths are placed on shaker incubator at 32°C for 24 hours for the growth of microorganism.

Broths are then repeatedly centrifuged and washed for several times separately to obtain the pellet of microorganisms which is further used for second fermentation.

4.2.5 Identification of Native Microorganisms

The materials required for identification process are MacConkey agar, Potato dextrose agar, isolated culture from mixed fruits, inoculation loop, Bunsen burner, ethanol, Gram's staining kit, glass slide and sterile petri dishes. The microorganism pellet obtained from LB broth and potato dextrose broth are streaked on MacConkey agar plates and potato dextrose agar plates. MacConkey agar plates and potato dextrose agar plates are made under sterile conditions. Control plates are made to ensure that no contamination occurs. Plates are then incubated at 32°C for 24 hours. The colonies formed are observed for morphology.

The colonies are observed under microscope at 40x, and 100x after gram's staining. Identification of specific strains are not done, since no specific strains are

needed for second fermentation. Rather a group of native microorganisms of fruits are needed.

4.2.6 Second Fermentation of Cocoa Beans

The materials required for second fermentation process are isolated cells from mixed fruits, fermented beans, sterile glass container, cheese cloth and mixed fruit pulp. The sun-dried beans are transferred into sterile glass jar. The mixed fruits such as apple, grapes, kiwi, strawberry, and litchi are blended into puree using blender. The puree is poured on sundried beans until they are completely submerged. Inoculate the isolated microorganisms into jar. Using sterile spoon, mix the contents for uniform spreading of puree and beans. The glass jar containing puree and beans are covered using a sterile cheese cloth on the mouth of jar. Rubber bands are used to seal the cheese cloth on place. The beans submerged in the mixed fruit puree is allowed to ferment for 4 days in cool, shady place. Addition of isolated native species from fruits fasten up the fermentation process from 7 days to 4 days.

4.2.7 Drying

After second fermentation, the beans are again sun dried. The beans are placed on a moisture absorbing cloth under sun for drying. The beans are dried along with the pulp that covers them. This prevents the loss of antioxidants during drying. The process may take up to 15 days depending on weather and climatic conditions.

4.2.8 Roasting of Cocoa Beans

The materials required for roasting process are fermented beans, conventional stove, and pan. After drying, the beans are roasted conventionally on pan at high flame for 5 minutes and low flame for 25 minutes. Roasting helps in removal of excess moisture and any other undesirable volatile matter. Flavanol levels have been shown to decrease while roasting at temperatures between 120 - 150°C and for time between 5 and 120 minutes. This process is also essential for loosening of shells, colour development, aroma, and flavour production (Krysiak, 2011).

4.2.8.1 Removing of Shells

Roasted beans are allowed to cool for some time. The outer shell of beans is removed using hands and nibs are collected. Nibs are further used for the processing of chocolate. Nibs are then weighed.

4.2.9 Dry Conching

The materials required for dry conching process are blender and cocoa nibs. Nibs are made into semi powdered form by dry conching method using blender and transferred into a clean container.

4.2.10 Wet Conching

The materials required for wet conching process are wet grinder, semi powdered nibs, cocoa butter, and sweeteners such as sugar and honey. Wet conching is generally carried out in a wet grinder on small scale. Nibs along with other ingredients such as cocoa butter, sugar or honey as sweetener are added. First nibs are added and allowed to grind until they form into cocoa mass. Cocoa butter is added gradually in regular intervals, which ensures the smoothness of the end product. Lastly sweeteners such as honey or sugar are added and allowed to grind for approximately 3 to 4 hours. Depending upon the type of chocolate, the ratio of nibs, sugar, and cocoa butter changes. The 55% dark chocolate has nibs 55%, cocoa butter 15% and sugar 30%. The resultant cocoa mass is silky in consistency.

4.2.11 Moulding

The materials required for moulding are chocolate mould and cocoa mass. The cocoa mass is allowed to set on a desired shaped mould on cool temperature. The bar of chocolate is taken.

4.2.12 Tempering

The materials required for tempering are bar of chocolate and double boiler. Tempering of chocolate is done by subjecting them to 55°C and cooling it. This helps in crystallization of fats present in the chocolate. Tempering of chocolate provides an overall stability to the end product.

4.2.12.1 Remoulding

The chocolate is remoulded into the mould and set as cool temperature. The final bars of chocolate are obtained. A control chocolate is produced traditionally using single fermentation method for comparative analysis.

4.2.13 Characteristic Analysis of Chocolate

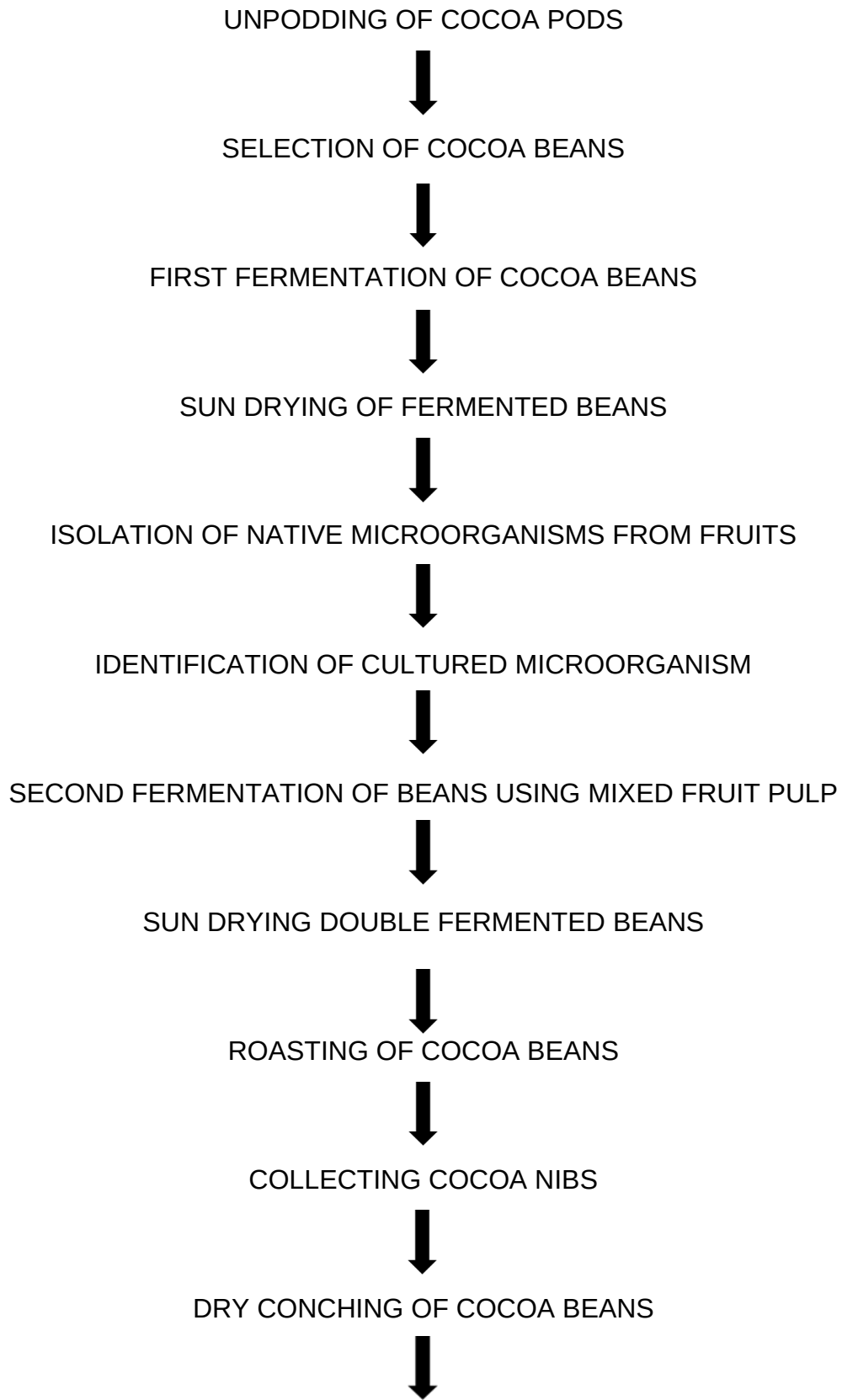
Characteristic analysis of chocolate includes certain parameters under physical and chemical analysis.

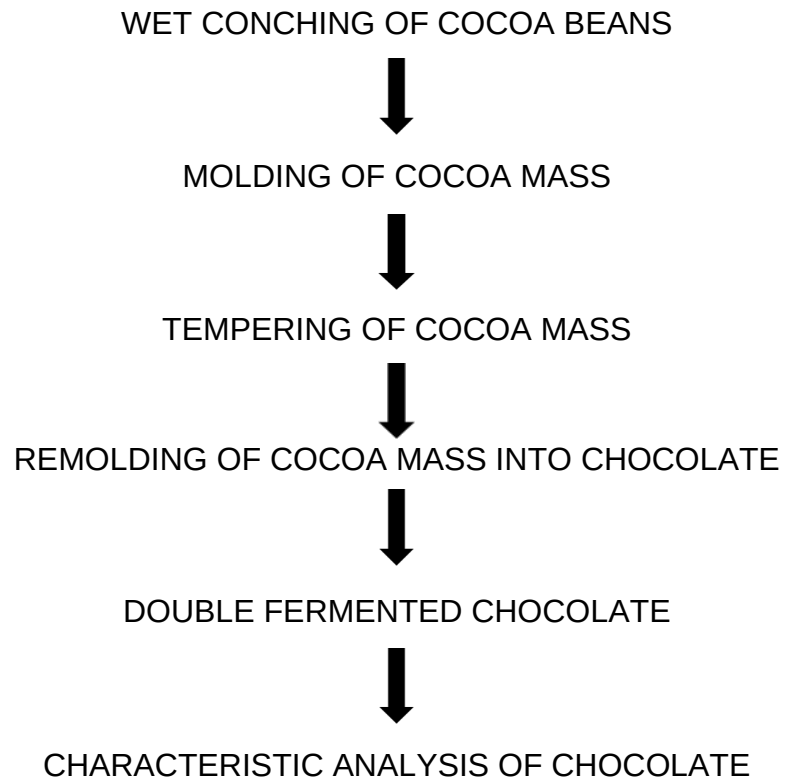
4.2.13.1 Physical Characteristics

Physical characteristics such as glossiness, smooth texture and reaction to temperature ensures the quality of the chocolate (Shahanas et al., 2020). A snap test is done by snapping the bar, indicating a good quality chocolate.

4.2.13.2 Chemical Characteristics

Nutritional analysis and antioxidant testing is done for control chocolate and double fermented chocolate by a certified food laboratory. Nutritional analysis includes parameters such as energy, carbohydrates, total fat, protein, and total sugars. Antioxidant capacity assay is done using Phosphomolybdenum method. A comparative analysis is done between control chocolate and double fermented chocolate to interrupt the result of the experiment.





4.1.14 Flow chart of the Double fermentation process

CHAPTER 5

RESULTS AND DISCUSSION

5.1 SELECTION OF COCOA FRUITS



Fig 5.1: Cocoa fruit

The cocoa fruits have been locally imported from Idukki, Kerala. The fruits received were in good condition, with minimal to no discoloration. The pods are further processed within 2 days of arrival.



Fig 5.2: Unpodding cocoa beans

The selection of Forastero cocoa fruits and the removal of damaged fruits. After the selection of cocoa fruits, the opening of cocoa pods using a knife is done. The

removal of raw cocoa beans covered with sweet-white pulp from cocoa beans is done and transferred to a sterile vessel.

5.2 FIRST FERMENTATION OF COCOA BEANS



Fig 5.3: Day 1 of Cocoa fermentation

On the first day, the raw beans were transferred to the bamboo basket covered with banana leaves and allowed for first fermentation. No external microorganisms were added.



Fig 5.4: Day 4 of Cocoa Fermentation

The surrounding sweet pulp was reduced to 50%, and yeast colonies were observed on the surface of the cocoa beans on the fourth day. 'Alcohol smell with pleasant floral notes' was observed. The liberation of heat during cocoa fermentation has been observed by using a kitchen thermometer. The temperature of cocoa fermentation was raised to 50°C.



Fig 5.5: Day 7 of Cocoa fermentation

On the seventh day, the first fermentation was stopped to avoid over fermentation. The desired 'fruity odour' is noted, and the pulp content is completely reduced at the end of the fermentation. A reddish-brown fluid oozed out of the fermented cocoa beans. Also, the temperature of the beans was reduced, and a cut test was done.

5.2.1 Cacao Cut Test

The cacao cut test shows the cocoa bean's colour changed from dark purple to pale. This indicates that the beans are well fermented.



Fig 5.6: Well fermented cocoa bean

5.3. SUN DRYING



Fig 5.7: Sun drying of cocoa beans

The fermented beans were washed with distilled water, spread out on a clean cloth, and sun-dried uniformly using the traditional method for 15 days. The cocoa beans were turned every two hours, and the moisture of the cocoa beans was reduced.

5.4. ISOLATION AND IDENTIFICATION OF INDIGENOUS MICROORGANISMS FROM MIXED FRUITS (APPLE, AVOCADO, GRAPES, LITCHI, KIWI, AND STRAWBERRY)

5.4.1 Screening of Indigenous Microorganisms

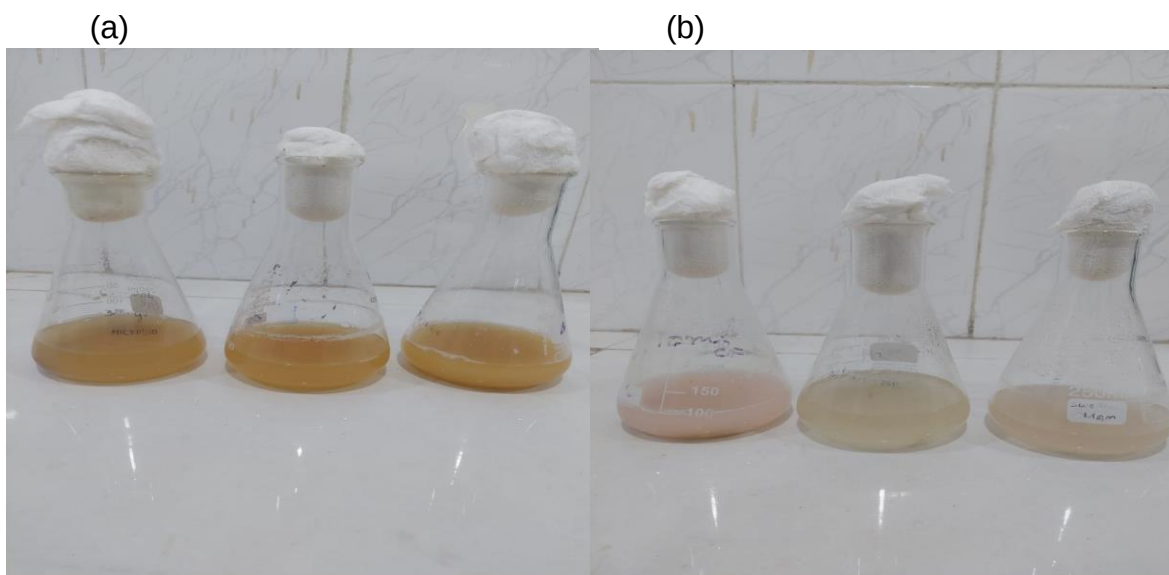


Fig 5.8: Isolation of indigenous microorganisms from the mixed fruits. LB Broth (a) and Potato dextrose broth (b).

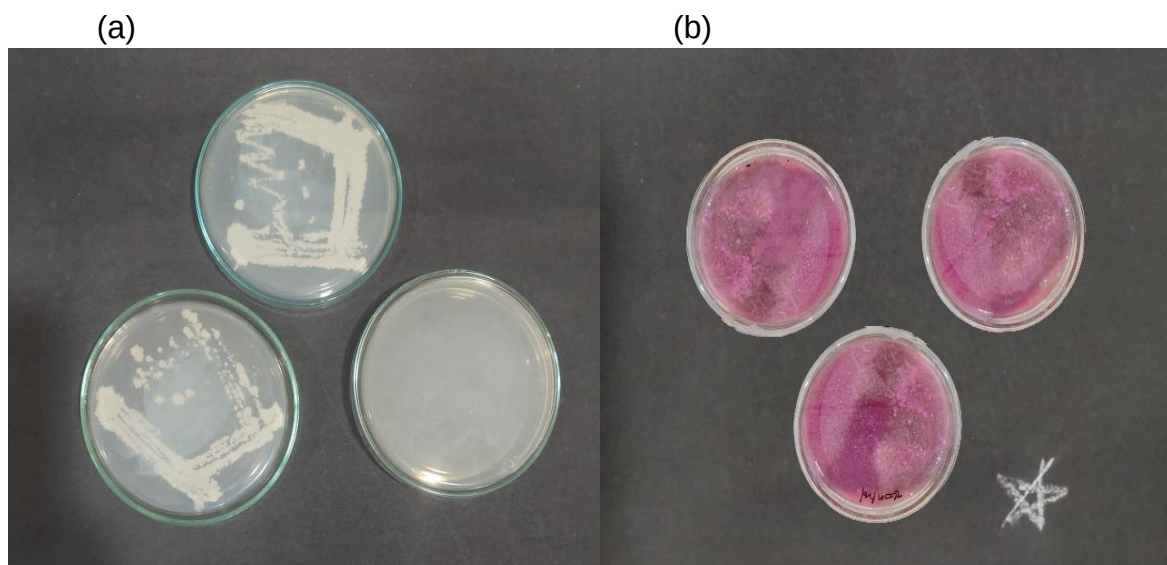


Fig 5.9: Serial dilution of 10^{-1} & 10^{-2} .

(a) Potato dextrose agar (b) Mac Conkey agar

The microorganisms were isolated from the broth and serially diluted on Potato dextrose agar and MacConkey agar plates. Creamy-white coloured colonies on Potato dextrose agar were observed. MacConkey agar did not support the colony growth. No colonies were observed. A colony from the Potato dextrose plate was selected for identification.

5.4.2 Gram's Staining

The Gram staining procedure was successfully performed and the results revealed the presence of long and short gram-positive bacilli. Based on these findings, it has been confirmed that the species belongs to the lactobacillus genus and also contains yeast cells.

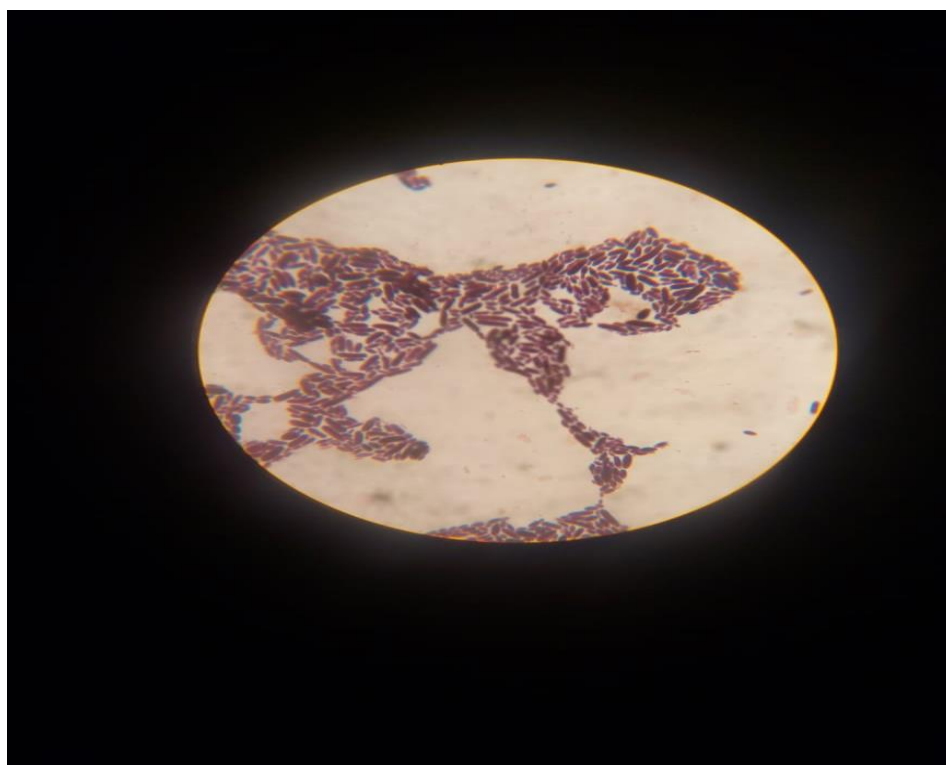


Fig 5.10: Gram's staining

5.5 INOCULATION OF INDIGENOUS MICROORGANISMS

The medium containing the sample was centrifuged repeatedly at 3000 rpm for 1 minute, and the pellet was collected and suspended in distilled water. The pellet was repeatedly washed to remove debris by centrifugation, and the collected cells were inoculated into the second fermentation of cocoa beans along with mixed fruit pulp.



Fig 5.11: Centrifuge tube containing cells

5.6 DOUBLE FERMENTATION OF COCOA BEANS WITH MIXED FRUIT PULP



Fig 5.12: Double fermentation of cocoa beans

The cocoa beans were soaked in the mixed fruit puree, and the isolated microorganisms were added to the second fermentation, which hastened the rate of the second fermentation.



Fig 5.13: Day 2 of the Second Fermentation of Cocoa Beans with Mixed Fruit Pulp

On the second day of the second fermentation of cocoa, yeast colonies were observed on the surface of the fruit pulp, and while turning over the cocoa beans, a 'pleasant sweet wine odour' was noted. The second fermentation lasts up to 4 days. After completing the fermentation, the double-fermented cocoa beans were coated with the mixed fruit pulp and sun-dried without washing.

5.7. PROCESSING OF DOUBLE FERMENTED CHOCOLATE

5.7.1 Roasting



Fig 5.14: Roasted Cocoa beans

The cocoa beans were roasted on a pan at high heat for 5 minutes, then at low heat for 25 minutes. The temperature reached around 120–150 °C. The roasted cocoa beans developed the dark brown colour and produced the desired ‘chocolate aroma’ and bitter taste.

5.7.2 Wet Conching



Fig 5.15: Wet conching of cocoa beans

The shells were removed. Nibs, cocoa butter, and sugar were added based on the composition of the resultant chocolate. At the end of wet conching, a smooth, silky paste like cocoa mass has been produced.

5.7.3 Moulding and Tempering



Fig 5.16: Tempered double fermented chocolate in chocolate mould

The glossiness, desired texture, and aroma have been obtained after the tempering process. A 'snap' sound was observed when splitting the bar.

5.8. DOUBLE-FERMENTED CHOCOLATE



Fig 5.17: Double fermented 55% dark chocolate

Two bars of double-fermented 55% dark chocolate with mild fruit flavour and enhanced nutritional and antioxidant properties were produced, and each bar weighed around 100g.



Fig 5.18: Double fermented chocolate made using honey instead of sugar

Usage of honey instead of sugar resulted in a crumbling texture which is not a satisfactory texture. The taste of this chocolate remains acceptable.



Fig 5.19: Control-Single fermented 55% dark chocolate

Control chocolate was produced using traditional single fermentation and compared with the Double-fermented 55% dark chocolate. The results were observed.

5.9. CHARACTERISTIC ANALYSIS

The chocolate samples were given to the certified food laboratory for the characteristic analysis of chocolate.

5.9.1 Nutritional Profile

Table 5.1: NUTRITIONAL PROFILE OF DOUBLE FERMENTED 55% DARK CHOCOLATE

S. NO	PARAMETERS	METHOD	UNITS	RESULTS
1	Energy (By Calculation)	FAO Method	kcal/100g	559
2	Carbohydrates (By difference)	CTL/SOP/FOOD/262 - 2014	g/100g	43.0
3	Total Fat	AOAC 20 th Edn.2016, 954.02	g/100g	40.0
4	Protein (Nx 6.25)	AOAC 20 th Edn.2016, 986.25	g/100g	6.75
5	Total Sugars	FSSAI Manual 2015- Beverages Sugar and confectioneries	g/100g	19.2

Table 5.2: NUTRITIONAL PROFILE OF CONTROL 55% DARK CHOCOLATE (SINGLE FERMENTED)

S. NO	PARAMETERS	METHOD	UNITS	RESULTS
1	Energy (By Calculation)	FAO Method	kcal/100g	593
2	Carbohydrates (By difference)	CTL/SOP/FOOD/262 - 2014	g/100g	43.7
3	Total Fat	AOAC 20 th Edn.2016, 954.02	g/100g	43.2
4	Protein (Nx 6.25)	AOAC 20 th Edn.2016, 986.25	g/100g	7.46
5	Total Sugars	FSSAI Manual 2015- Beverages Sugar and confectioneries	g/100g	22.8

Tables 5.1 and 5.2 show the nutritional characteristics of both chocolate samples. Compared to the control 55% dark chocolate, the double-fermented 55% dark chocolate contains less total fat, sugar, and carbohydrate, as well as fewer calories per serving.

5.9.2 Antioxidant Profile

Table 5.3: Total Antioxidant Capacity Assay

S. No	Parameter	Method	Sample 1 (Double fermented 55% dark chocolate) mg/g	Sample 2 (Control - single fermented 55% dark chocolate) mg/g
1	Antioxidant	Total antioxidant capacity assay (Phosphomolybdenum method)	74.87	68.08

The data presented in the above table clearly illustrates the outcomes of the total antioxidant capacity assay performed on both chocolate samples. The findings reveal that the double-fermented 55% dark chocolate infused with mixed fruits exhibits a significantly higher level of antioxidant properties compared to the single-fermented 55% dark chocolate used as a control. This indicates that the addition of mixed fruits during the fermentation process has a positive impact on enhancing the overall antioxidant content of dark chocolate, further highlighting its potential health benefits.

CHAPTER 6

SUMMARY AND CONCLUSION

The production of chocolate from cocoa beans involves the selection of cocoa beans, fermentation, drying, roasting, conching, temper and mould processes. Double fermentation of cocoa beans using mixed fruit pulp is being developed to elevate the antioxidant levels. The use of native microorganisms isolates from the mixed fruits can be beneficial to accelerate the process of fermentation. The process includes handpicking of Forastero-type cocoa beans, fermenting in the bamboo basket covered with banana leaves, drying the beans, isolating the microorganisms from the mixed fruits, and second fermentation by adding the fruit pulp. Finally, the cocoa beans are dried again, roasted, conched, temped, moulded to make double fermented chocolate with mild fruit flavour. As previously mentioned, the first process involves the growth of yeast cells followed by lactic acid bacteria and then acetic acid bacteria. These bacteria produce flavour precursors and inhibit the growth of spoilage microorganisms. The beans are turned every 48 hours to ensure aeration. At the end of fermentation, a cut test is conducted to ascertain the success of the process. Sun-drying is immediately done afterwards to stop the fermentation process to avert over-fermentation and off flavours. The native microorganisms are isolated from mixed fruits using LB broth and potato dextrose broth, and the species identified are used for the second fermentation. The second process includes submerging the sun-dried beans into blended mixed fruit puree and inoculation. The mixture is left to ferment in a cool, dark place for 4 days. The beans are dried for the second time, roasted, and made into cocoa mass using dry and wet conching techniques. The cocoa mass is then moulded into chocolate bars and went through a tempering process for the purpose of stability. The bars are assessed in terms of their physical properties, such as smoothness and gloss, as well as their chemical properties, including their nutritional composition and antioxidant levels.

The analysis of the single-fermented and double-fermented chocolates in comparison shows that latter has a lower total fat, sugar, and carbohydrate content and

fewer calories per serving. The double-fermented chocolate shows strong evidence of the fruit infusion effect on increasing the antioxidant properties, which are good to health. This research indicates the possibility of incorporating fruit pulp and local microorganisms in chocolate production so as to enrich nutrition, antioxidant concentration, and flavour.

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ANNEXURE

PLAGIARISM REPORT

ORIGINALITY REPORT			
13%	9%	8%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS
PRIMARY SOURCES			
1	rajafarms.com Internet Source	1%	
2	d-nb.info Internet Source	1%	
3	www.mdpi.com Internet Source	1%	
4	R Kamalesh, S Gokula Narayanan, S Thiruvengadam, Ivo Romauld. " The Influence of Chocolate Fortification with and on the content of biologically active substances ", Journal of Food Processing and Preservation, 2021 Publication	1%	
5	www.frontiersin.org Internet Source	<1%	
6	www.researchgate.net Internet Source	<1%	
7	Wiston Javier Morales-Rodriguez, Jaime Morante-Carriel, Robinson J. Herrera-Feijoo, María Concepción Ayuso-Yuste et al. "Effect of	<1%	