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Isolation and Identification of Acid-Forming Bacteria in Arabica Coffee Fruit (*Coffea arabica*) During Wet Fermentation

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Abstract: The distinctive flavor and aroma possessed by Arabica coffee are an attraction for coffee enthusiasts. Post-harvest processing of coffee such as coffee fruit fermentation also contributes to the formation of the flavor and aroma of Arabica coffee. In this study, spontaneous fermentation was conducted by adding distilled water, with treatments on fermentation days 4 and 7 aimed at detecting acid-forming bacteria that played a role in the fermentation process. The bacterial isolates were characterized through macroscopic and microscopic observations, physiological, and biochemical tests. Identification of bacterial isolates was performed using the profile matching method based on Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and Axellson (2004) until the genus level. The identified bacterial genera were *Asaia, Gluconobacter, Neoasaia, Saccharibacter, and Lactobacillus.*

Keywords: Acetic acid bacteria; arabica coffee; fermentation; lactic acid bacteria

Introduction

Coffee is a plantation commodity that plays a role in economic growth and is a major export of Indonesia. Coffee is a plantation crop that has long been a cultivated plant. The coffee plant is a source of income for the people and also increases the country's foreign exchange through the export of raw and processed coffee beans. The coffee plant is a plant originating from Africa and South Asia, belonging to the Rubiaccea family with a height of up to 5 meters. The coffee plant has leaves 5-10 cm long and 5 cm (Halil et al., 2023). According to data from the Central Statistics Agency, coffee production in Indonesia increased by 2.75% in 2021 compared to the previous year. In 2021, total coffee exports in Indonesia also increased by 1.21% from the previous year (BPS, 2021). West Nusa Tenggara (NTB) is one of the coffee-forming regions with a total production of 4,865.14 tons spread across several districts (NTB Department of Agriculture and Plantation, 2017). In the community of Lombok, the types of coffee commonly found are robusta and arabica. The largest producer of arabica coffee on the island of Lombok is located in East Lombok regency, known for its quality and taste comparable to other coffees in Indonesia (Chandra et al., 2023). The increase in coffee production on the island of Lombok is partly attributed to the development of tourism. Coffee has become a tourism commodity in Lombok, commonly known as Lombok coffee.

Arabica coffee is known for its distinctive flavor and aroma, which attract coffee enthusiasts. The unique taste and aroma of Arabica coffee can be attributed to several factors, one of which is post-harvest coffee processing. Post-harvest processing of coffee fruits is carried out through various methods, including dry process, semi-dry process, and wet process. Different coffee processing methods result in variations in chemical characteristics, leading to differences in coffee flavor (Saputri et al., 2020). The wet processing method, despite its higher water consumption, stands out for its capacity to yield superior coffee, particularly suited for export. As coffee cherries ferment, they undergo a succession of microbial dominances, starting with bacteria in high-moisture settings, eventually giving

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way to yeast and fungi as the process concludes. During fermentation, each microbe introduces plenty of metabolites, and understanding the microbial dynamics in wet fermentation is pivotal to unlocking coffee's flavor secrets (Todhanakasem et al., 2024). Wet processing with fermentation aims to produce unique flavor in coffee. The fermentation process was one of the crucial steps in coffee processing (Muzaifa et al., 2016). Fermentation is one of the complex and essential stages of the post-harvest coffee process. Various bacteria and yeast play an important role during the fermentation process by producing various types of enzymes (pectinase) to break down the mucilage into acid and alcohol. Fermentation is pivotal in determining the quality of coffee and in turn, has an impact on the sensory quality of coffee. A non-standardized fermentation process will produce coffee with varying qualities (Silamba et al., 2024). During the fermentation process, physiological changes occurred in coffee fruits that could affect the development of aroma and flavor precursors in coffee. Naturally, the fermentation process was aided by enzymes and microorganisms such as fungi and bacteria. Microorganisms in the fermentation process produced various enzymes, alcohol, and acidic compounds (Haile et al., 2019). Fermentation of coffee beans by adding pectolytic bacteria is known to accelerate improves the fermentation process better than natural fermentation. A consortium of bacteria consisting of Saccharomyces cerevisiae (yeast), Lactobacillus plantarum (LAB), and Bacillus spaericus. A consortium of pectinolytic bacteria helps remove mucus from coffee beans and Improves the taste of coffee (Purwanto et al., 2024). Only mesophilic bacteria showed pectinolytic activities. The low pectinase production occurred due to several factors: (i) the bacteria were isolated from pulped coffee where there was not a large amount of pectic substrate to be degraded, (ii) pectinolytic enzymes could be suppressed by excess or lack of monosaccharides, (iii) lower availability of the nitrogen source, (iv) rapid lowering of pH value, which made the enzymatic activity difficult (pH 3.5–5.5) (Ribeiro et al., 2021).

During coffee fruit processing, spontaneous fermentation was conducted by complex microorganisms such as fungi and bacteria. The addition of water during coffee fruit fermentation was known as wet fermentation (Cassimiro et al., 2022). During the fermentation process, microorganisms emerged naturally these fermented organisms utilize been pulp as a source of carbon and nitrogen which can produce large amounts of ethanol, lactic acid, secondary metabolites and cause a decrease in pH. In addition, some microbial metabolites are precursors of volatile compounds that help improve beverage flavor (de Carvalho Neto et al., 2018). The role of various consortiums of microorganisms in coffee fermentation has been discussed by Moreira et al. (2017), which stated that microbial activity in coffee pulp involves the succession of microorganisms, especially yeasts and bacteria (Tsaaqifah et al., 2023). Throughout fermentation, bacteria produced organic acids and other metabolites that influenced the resulting taste characteristics. The presence of organic acids generated during the fermentation process was caused by the breakdown of sugar components into acidic compounds with the assistance of acid-forming bacteria (Martati, 2007). During coffee fermentation yeast, lactic acid bacteria and acetic acid bacteria are present. Temperature and pH changes during Fermentation shows microbial activity during the day of coffee fermentation. Yeast It is proven to act as the dominant microorganism at the beginning and after 20 days of fermentation. Yeast metabolites promote the growth of other microorganisms such as LAB and AAB (Sulaiman et al., 2022). Based on the research by Rahmadani et al. (2020), lactic acid bacteria and acetic acid bacteria are typically found as acid-forming bacteria during the fermentation process.

Lactic acid, initially present in small quantities in coffee, emerges as the predominant acid during fermentation. This increase is attributed to the decarboxylation of malic acid by lactic acid bacteria (LAB), leading to a notable decrease in malic acid. Lactic acid production is strongly correlated (≥0.45) with microbes like Lactobacillus, Lactococcus, Candida, and Saccharomyces. Its production is crucial for fermentation, aiding in medium acidification and suppressing undesirable microorganisms. Fermentations increase concentrations of various compounds, including acetic acid. suggest that acetic acid contributes to coffee's acidity and can impart fruity or unpleasant flavors depending on its concentration. found that treating green coffee beans with acetic acid significantly enhances the final product's quality (Rocha et al., 2024).

Living organisms are always on board by microbes, both on the surface organism or in tissues. Microbe that are in the plant tissue called with endophytic microbes. Endophytic microbes can be bacteria or fungi (Zulkifli et al., 2018). The presence of acid-forming bacteria in the fermentation process contributed to a more complex flavor experienced in fermented coffee (Sari et al., 2021). It was essential to explore acid-forming bacteria to examine the flavor of Arabica coffee, involved in the Arabica coffee fermentation process to enhance the quality of flavor and aroma of Arabica coffee in Lombok.

In coffee cherries, a wide variety of bacterial species have been reported, a number of which are dominant and common across coffee cultivars and geographic origins, while others are rare and uncommon. Because of the vital role that bacteria play in coffee fermentation

and the impact it brings on coffee quality, understanding the indigenous bacterial ecology of coffee cherries is mandatory (Mahatmanto et al., n.d.). Limited exploration has been conducted to date regarding microbiological studies on the flavor of Arabica coffee. Therefore, the aim of this research was to analyze and identify acid-forming bacteria involved in the coffee fermentation process using the spontaneous wet fermentation method, relying on naturally occurring microbes present in coffee fruits.

Method

Research Time and Location

This research was conducted from April to November 2023. Coffee fruit sampling was carried out in coffee plantations located in Sapit Village, Suela District, East Lombok, West Nusa Tenggara. The coffee fruit fermentation process, isolation, and identification of microorganisms from coffee fruit were conducted at the Advanced Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Mataram.

Coffee Fruit Preparation and Fermentation Process

The harvesting process was carried out on ripe coffee fruits that were red in color. The washing process was conducted simultaneously with sorting or selecting coffee fruits of good quality. Sorting was done by soaking the coffee fruits so the floating and sinking coffee fruits could be observed. Sinking coffee fruits were considered to have good quality then were taken for further fermentation.

The fermentation process began by placing the sorted coffee fruits into sterilized sample bottles as fermentation containers. Sterilized distilled water was added, and the bottles were sealed with sterile corn paper and rubber bands. The fermentation process was carried out at intervals of 4th day and 7th day to observe the diversity of bacteria that grew until the 7th day.

Sample Preparation of Coffee Fruit and Dilution

Samples that had reached the fermentation time limit were taken, and their fermentation outcomes were measured for pH using pH meter.

The fermented sample was blended and its suspension was extracted. One milliliter of the coffee sample suspension was taken using a micropipette and placed into a test tube containing 9 mL of physiological NaCl solution. Each time the suspended sample was transferred to the next tube, it was homogenized using a micropipette until completely homogeneous. Dilutions used during isolation ranged from 10^{-5}, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13}, and 10^{-15}.

Isolation and Colony Observation

Isolation was conducted using the pour plate method. Samples diluted from 10-5 to 10-15 were inoculated onto GYC agar supplemented with 1% CaCO3 and then incubated for 24 to 48 hours. Subsequently, microbial colonies were observed. The growth of acid-forming bacteria colonies was indicated by the formation of clear zones around the colonies.

Bacterial Isolate Purification

Bacterial colonies forming clear zones were purified using the streak plate method, aiming to obtain colonies forming clear zones that are separated from others and showed different colony morphology. An isolate was considered pure if microscopic examination revealed bacterial cells with uniform shapes.

Characterization of Bacterial Morphology

Bacterial morphology characterization is based on both colony morphology and cell morphology. Macroscopic observation is conducted to evaluate colony morphology, while microscopic observation is conducted to examine bacterial cell morphology. Macroscopic parameters include color, shape, margin, and elevation. Microscopic examination entails using a microscope to observe the shape and color of bacterial cells.

Observation of cell morphology is achieved through Gram staining to differentiate between Gram-positive and Gram-negative bacteria. Gram staining is performed on 24-hour-old bacterial isolates streaked onto microscope slides and treated with the Gram staining components, consisting of Gram stain A (*crystal violet*), Gram stain B (*Lugol's solution*), Gram stain C (*acetone-alcohol*), and Gram stain D (*safranin*). The results of Gram staining are observed under a microscope at 100x magnification.

Biochemical and Physiological Characterization of Gram-Positive and Gram-Negative Bacterial Isolates

Catalase Test**,** 24-hour-old bacterial isolates were placed on a microscope slide using a needle and then 1-2 drops of 30% H2O2 were added. A positive result was indicated by the formation of bubbles after the addition of H2O2.

Motility Test, bacterial isolates were inoculated into semi-solid Sulfide Indole Motility (SIM) medium and incubated for 24-48 hours. Motile bacteria were characterized by bacterial growth spreading (moving out of the inoculation area). Non-motile bacteria were characterized by bacterial growth only within the inoculation area.

Carbohydrate Fermentation**,** bacterial isolates were inoculated into 100 mL of Nutrient Broth (NB) medium supplemented with 1 gram of each carbohydrate source (glucose, lactose, sucrose, fructose, maltose, and

mannitol) and 1 mL of bromocresol purple color indicator. A positive result was indicated by a color change to cloudy yellow.

TSIA Test, microbial isolates were obtained and inoculated onto Triple Sugar Iron Agar (TSIA) medium using the streak method on the slant agar and incubated at 37˚C for 24 hours. The observed changes were noted. If the color turned yellow in both the slant and butt portions, it indicated that the bacteria could ferment glucose, sucrose, and lactose (Muzaifa et al., 2016).

Carbohydrate Fermentation, the bacterial isolates were inoculated into 100 mL Nutrient Broth (NB) medium supplemented with 1 gram each of carbon sources (fructose, maltose, and mannitol), and 1 mL of bromocresol purple color indicator was added. A positive result was indicated by color change to cloudy color.

Biochemical and Physiological Characterization of Gram-Positive Bacterial Isolates

Fermentation Type, Bacterial isolates were taken and grown in MRSB medium in test tubes with inverted Durham tubes. They were incubated for 2-3 days at 30^{o}C. Heterofermentative fermentation was characterized by the formation of gas bubbles inside the Durham tube. Homofermentative fermentation was indicated by the absence of gas bubbles in the Durham tube.

Bacterial Growth Test at Different Temperatures, one loopful of 24-hour-old bacterial culture was taken and inoculated into MRSB medium. Then, it was incubated at temperatures of 10^{o}C and 45^{o}C for 48 hours. Bacterial growth was indicated by the presence of turbidity in the MRSB medium.

Bacterial Growth Test at Different NaCl Concentrations, bacterial growth at NaCl concentrations of 6.5% and 18% was conducted on MRSB medium for 24 hours. Bacterial growth was indicated by turbidity in the MRSB medium.

Bacterial Growth Test at Different pH Levels, Isolates were grown on MRSB media with pH variations of 4.4 and 9.6. pH variations were achieved by adding NaOH and HCl to the samples, then incubating them for 24 hours at 30^{o}C. pH measurements were conducted using a pH meter. Bacterial growth was indicated by turbidity in the MRSB medium.

Biochemical and Physiological Characterization of Gram-Negative Bacterial Isolates

Bacterial Growth Test at Different Glucose Concentrations, bacterial isolates were grown on liquid GYC media containing 30% glucose in 250 mL of distilled water, then incubated for 24-48 hours. Bacterial growth was indicated by turbidity in the liquid GYC medium.

Bacterial Growth Test at Different Acetic Acid Concentrations, bacterial isolates were grown on liquid GYC media containing 35% acetic acid. Bacterial growth was indicated by turbidity in the liquid GYC medium.

Identification of Bacterial Isolates

To determine the genus of Acid Bacteria, the phenotypic characterization results were compared with its characteristics using profile matching methods based on *Axelsson's book* (2004) and *Bergey's Manual of Systematic Bacteriology* (Holt et al., 1994) as references in bacterial identification.

Result and Discussion

The isolation of bacteria on day 4th and 7th of fermentation resulted in the presence of two bacterial groups, namely non-acid-forming bacteria and acid-forming bacteria, characterized by the formation of clear zones around the bacterial colonies. The isolation results of bacterial isolates can be seen in Figure 1.

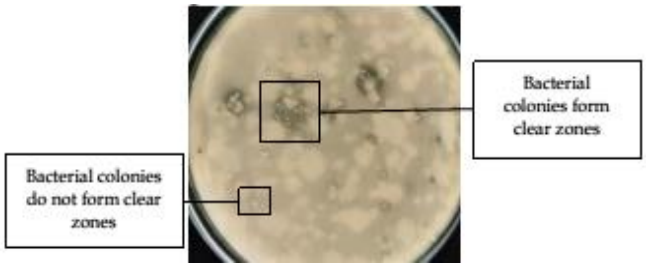

Figure 1. Acid-forming bacterial isolates

The growth of these two bacterial groups demonstrated that the presence of acid-forming bacteria could be distinguished by observing the formation of clear zones around bacterial colonies caused by the addition of $CaCO_3$ in the bacterial growth medium. Based on the research by Gordon et al. (2007), if acid-forming bacteria grew on a medium containing $CaCO_3$, the acid compounds produced would react with the $CaCO_3$ contained in the medium and produce Calcium acetate ($Ca(CH_3COO)_2$, H_2O, and CO_2. Calcium acetate is more readily soluble in water compared to $CaCO_3$, thus creating clear zones around acid-forming bacterial colonies.

After the isolation step, the purification of bacterial colonies was conducted. The purification process resulted in a total of 20 pure isolates of acid-forming bacteria, namely IBK 1, IBK 2, IBK 4, IBK 5, IBK 7, IBK 8, IBK 9, IBK 12, IBK 13, IBK 15, IBK 16, IBK 19, IBK 22, IBK 24, IBK 25, IBK 26, IBK 27, IBK 28, IBK 29, and IBK 30. These twenty isolates of acid-forming bacteria showed various colony shapes, colors, elevations, and margins (Table 1).

Table 1. Macroscopic Characteristics of Acid-Forming Bacterial Isolates

Isolate Code	Colony morphology			
	Shape	Elevation	Margin	Colour
IBK 1	Circular	Raised	Undulate	Cream
IBK 2	Circular	Flat	Undulate	Cream
IBK 4	Circular	Flat	Entire	Cream
IBK 5	Circular	Raised	Entire	Cream
IBK 7	Circular	Raised	Undulate	White
IBK 8	Circular	Flat	Entire	White
IBK 9	Circular	Flat	Entire	White
IBK 12	Circular	Flat	Entire	Cream
IBK 13	Circular	Flat	Undulate	White
IBK 15	Circular	Flat	Undulate	White
IBK 16	Circular	Flat	Entire	White
IBK 19	Circular	Flat	Entire	Cream
IBK 22	Circular	Flat	Entire	Cream
IBK 24	Circular	Flat	Entire	White
IBK 25	Circular	Flat	Entire	Cream
IBK 26	Circular	Flat	Entire	White
IBK 27	Circular	Flat	Entire	White
IBK 28	Circular	Flat	Entire	White
IBK 29	Circular	Flat	Entire	White
IBK 30	Circular	Flat	Entire	Cream

The data provided in Table 1 indicate that in terms of elevation characteristics, three isolates displayed a raised elevation pattern, namely isolates IBK 1, IBK 5, and IBK 7, while the remaining 17 isolates showed a flat elevation. Regarding margin characteristics, five isolates exhibited an undulate margin shape, specifically isolates IBK 1, IBK 2, IBK 7, IBK 13, and IBK 15, while the other fifteen isolates had an entire margin. Colony color observations revealed two distinct colors: white colonies, found in 11 isolates, and cream-colored colonies, present in 9 bacterial isolates. The colony characteristics of the isolated bacteria generally were circular in shape, with entire margins, and raised elevations (Nurhasanah et al., 2019). Bacterial isolates were obtained from fermentation products had circular colony shapes, white to gray colors, flat elevations, and entire and undulate margins (Bansal et al., 2013).

Observations of the microscopic characteristics of acid-forming bacterial isolates obtained from fermented Arabica coffee fruits were conducted to determine the cell shape and gram properties of the bacteria (refer to Table 2 and Figure 2).

The Acidophilic bacteria group consisted of two groups of acid-forming bacteria, namely the acetic acid bacteria group and the lactic acid bacteria group. Lactic acid and acetic acid were produced during the fermentation process. The presence of lactic acid bacteria and acetic acid bacteria proved the contribution of these two bacterial groups during the fermentation process (Evangelista et al., 2015). The top ten family, genus, and species in abundance during the fermentation process were Acetobacteraceae, Lactobacillaceae, and Enterobacteriaceae for the family Acetobacter, Gluconobacter, and Leuconostoc for the genera; and the main bacteria were acetic acid bacteria and lactic acid bacteria (Lee et al., 2023).

Table 2. Microscopic Characteristics of Acid-forming Bacterial Isolates Obtained from Fermented Coffee, Based on Cell Shape and Gram Reaction

Isolate Code	Cell Morphology	
	Cell shape	Gram
IBK 1	Bacil	Negative
IBK 2	Bacil	Negative
IBK 4	Bacil	Positive
IBK 5	Bacil	Negative
IBK 7	Bacil	Negative
IBK 8	Bacil	Negative
IBK 9	Bacil	Positive
IBK 12	Bacil	Negative
IBK 13	Bacil	Negative
IBK 15	Bacil	Negative
IBK 16	Bacil	Negative
IBK 19	Bacil	Positive
IBK 22	Bacil	Negative
IBK 24	Bacil	Negative
IBK 25	Bacil	Negative
IBK 26	Bacil	Positive
IBK 27	Bacil	Positive
IBK 28	Bacil	Positive
IBK 29	Bacil	Positive
IBK 30	Bacil	Negative

Broadly, LAB has been widely used in the food world, where bacteria act as natural preservatives because they produce various metabolites such as organic acids, bacteriocins, primary metabolites, hydrogen peroxide, diacetyl, carbon dioxide, and acetaldehyde as a result of the fermentation process (Siahaan et al., 2023). The ability of lactic acid bacteria to preserve food because lactic acid bacteria have substances with the ability to inhibit the growth of spoilage microbes (pathogens) (Rahayu et al., 2023). Based on the study by Gomes et al. (2018), acetic acid bacteria (BAA) had rod-shaped cells or bacilli, while lactic acid bacteria (BAL) had bacillus-shaped cells (Finanda et al., 2021). According to the data in Table 2, 20 isolates of acid-forming bacteria isolated from spontaneously fermented Arabica coffee beans had bacillus-shaped cells, while the Gram stain characteristics of the bacteria revealed seven Gram-positive isolates and thirteen Gram-negative isolates. Based on these results, it was indicated that the Gram-positive bacterial isolates were indicated of the lactic acid bacteria group (BAL), while the Gram-negative bacterial isolates were indicated of the acetic acid bacteria group (BAA). This is consistent with the research by Gomez et al. (2018) and Finanda et al. (2021), which found that acetic acid and lactic acid bacteria have

bacillus-shaped cells. Similarly, in the studies by Holt et al. (1994) and Williams et al. (1989), it was noted that the lactic acid bacteria group exhibits characteristics of Gram-positive bacteria, whereas the acetic acid bacteria group displays characteristics of Gram-negative bacteria. The formation of a purple color is caused by the main component of the cell wall of Gram-positive bacteria, which is peptidoglycan, capable of binding to crystal violet dye (Tjahjaningsih et al., 2016).

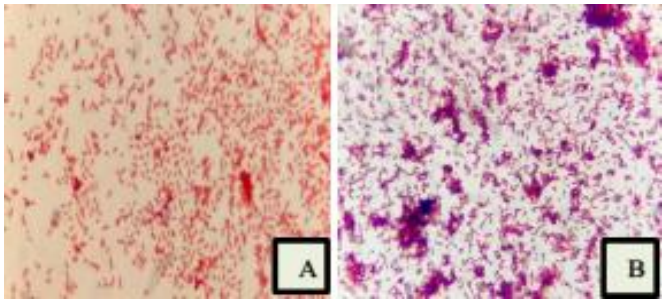

Figure 2. Representation of the results of the Gram staining test on acid-forming bacterial isolates: (A) Gram-negative reaction (isolates IBK 1, IBK 2, IBK 5, IBK 7, IBK 8, IBK 12, IBK 13, IBK 15, IBK 16, IBK 22, IBK 24, IBK 25, IBK 30) and (B) Gram-positive reaction (isolates IBK 4, IBK 9, IBK 19, IBK 26, IBK 27, IBK 28, IBK 29)

Physiological and biochemical tests were conducted to obtain data on the characteristics of acid-forming bacteria from spontaneous fermentation of Arabica coffee beans. The results of physiological and biochemical tests on the 20 acid bacteria isolates can be seen in Table 3. Based on the data in Table 3, all Gram-negative bacterial isolates showed positive catalase results with the presence of bubbles after being dripped with 3% H_2O_2. These results were consistent with the characteristics of acetic acid bacteria in Bergey's Manual of Determinative Bacteriology, which states that acetic acid bacteria were catalase-positive. Furthermore, previous studies have also stated that acetic acid bacteria were catalase-positive (Abubakar et al., 2020; Gualliman et al., 2011; Kadere et al., 2008; Ley et al., 1989; Yamada et al., 2008; Yunita et al., 2017). Gram-positive bacterial isolates showed negative results with no bubble formation after being dripped with 3% H_2O_2. These results were consistent with the characteristics of lactic acid bacteria (BAL), which were Gram-positive, and nearly all strains were unable to produce catalase enzyme (Widodo, 2019). The presence of the catalase enzyme indicated that the bacteria were capable of forming catalase enzyme that broke down hydrogen peroxide (H_2O_2) into water and oxygen (forming bubbles) (Tjahjaningsih et al., 2016). The catalase enzyme functioned to protect bacteria from the accumulation of hydrogen peroxide at the end of aerobic metabolism (Khatoon et al., 2022).

Bacterial motility tests were conducted to observe the spread of bacterial growth caused by the active and passive movement of bacterial isolates (Kosasi et al., 2019). Based on the data in Table 3, several Gram-negative bacterial isolates showed non-motile characteristics, while others were motile with bacterial growth was spreading not only in the stab area. Isolates with codes IBK7, IBK8, IBK12, IBK15, and IBK25 showed non-motile results. Meanwhile, all Gram-positive bacterial isolates showed non-motile results because the bacteria only grew in the stab area.

This was consistent with the findings of Permana et al. (2021) which indicated that BAA was motile, while BAL was non-motile (Finanda et al., 2021). The overall morphological, biochemical, and physiological characteristics were presented in Table 3.

According to Table 3, the physiological test results on Gram-negative bacterial isolates indicated that 4 isolates had the ability to grow in medium containing 30% glucose. Meanwhile, 9 other isolates couldn't grow in medium containing 30% glucose. The acetic acid bacteria group from the genera Asaia and Saccharibacter had the ability to grow in media with a glucose concentration of 30% (Lisdiyanti et al., 2002). In addition to the genera Asaia and Saccharibacter, the genus Neoasaia also had the ability to grow in media with 30% glucose (Yukphan et al., 2005). Observations on the growth test of bacterial isolates on medium containing 35% acetic acid showed that some bacterial isolates could grow in medium containing 35% acetic acid. However, isolates with codes IBK1, IBK2, and IBK15 couldn't grow in medium with 35% acetic acid.

The acetic acid bacteria group from the genera Asaia and Sacharibacter did not have the ability to grow in medium with a 35% acetic acid content (Lisdiyanti et al., 2002). Research by Yunita et al. (2017) stated that acetic acid bacteria were bacteria that could thrive in liquid media containing glucose and alcohol. According to Bergey's Manual of Determinative Bacteriology, acetic acid bacteria had physiological characteristics that enabled them to survive in media containing a glucose concentration of 30% and acetic acid concentration of 35%. This was because acetic acid bacteria were overoxidizers and were capable of oxidizing the produced acetic acid into energy, allowing them to survive in high concentrations of acetic acid.

The identification of physiological characteristics of Gram-positive bacterial isolates was conducted by culturing the bacterial isolates on liquid GYC medium under different pH, temperature, and salt concentration conditions to observe the growth ability of the isolates under these physiological conditions. According to Axelsson's identification book (2004), the physiological test results on Gram-positive bacteria with the growth of isolates at different pH levels, temperatures, NaCl

concentrations, and fermentation types of bacterial isolates indicated that, under different pH conditions, seven Gram-positive bacterial isolates indicative of BAL were able to grow at pH 4.4. However, under pH 9.6 conditions, seven isolates were unable to grow in media with pH 9.6. According to Axelsson's identification book (2004), some BAL bacteria are capable of growing in acidic conditions while others are not, depending on the species. The results of the growth ability test at different temperatures showed that seven bacterial isolates were unable to grow at temperatures of 10˚C and 45˚C. According to Axelsson (2004), some genera of *Lactobacillus* and *Pediococcus* were capable of growing at temperatures of 10˚C and 45˚C, while others were not, depending on the species. In the physiological test for salt concentration, it was found that three out of the seven bacterial isolates with codes IBK 9, IBK 19, and IBK 27 were able to grow in media with a salt concentration of 6.5%. However, in media with a salt concentration of 18%, all seven bacterial isolates were unable to grow. According to Axelsson (2004), some genera such as *Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus* were capable of and incapable of growing at a salt concentration of 6.5%, depending on the species.

Table 3. The Biochemical and Physiological Characteristics of Acid-Forming Bacterial Isolates Isolated from Fermented Arabica Coffee are Crucial for Identification Purposes Using Profile Matching Methods Based on Two Reference Books, Namely Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and Axellson (2004), up to the Genus Level

Characteristic	IBK 1	IBK 2	IBK 4	IBK 5	IBK 7	IBK 8	IBK 9	IBK 12	IBK 13	IBK 15	IBK 16	IBK 19	IBK 22	IBK 24	IBK 25	IBK 26	IBK 27	IBK 28	IBK 29	IBK 30
Motility	Motile	Motile	Non-motile	Motile	Non-Motile	Non-Motile	Non-motile	Non-Motile	Motile	Non-Motile	Motile	Non-Motile	Motile	Motile	Non-Motile	Non-Motile	Non-Motile	Non-Motile	Non-Motile	Motile
Catalase test	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	+
Growth in the presence of:	+	+		-	-	-		+	-	+	-		-	-	-					-
Glucose 30%																				
Acetic acid 35%	-	-		+	+	+		+	+	-	+		+	+	+					+
Growth in pH:			+				+					+				+	+	+	+	
pH 4.4																				
pH 9.6			-				-					-				-	-	-	-	
Growth in temperature:																				
10˚C			-				-					-				-	-	-	-	
45 ˚C			-				-					-				-	-	-	-	
Growth in the presence of:																				
6.5% NaCl			-				+					+				-	+	-	-	
18% NaCl			-				-					-				-	-	-	-	
Type of fermentation			Ho				Ho					Ht				Ho	Ho	Ho	Ho	
Acid production from:																				
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Genus of isolate	Asaia	Asaia	Lactobacillus	Gluconobacter	Gluconobacter	Gluconobacter	Lactobacillus	Neoasaia	Gluconobacter	Sacharibacter	Gluconobacter	Lactobacillus	Gluconobacter	Gluconobacter	Gluconobacter	Lactobacillus	Lactobacillus	Lactobacillus	Lactobacillus	Gluconobacter

Syimbols: + : positive, - : negative, Ho : Homofermentatif, Ht: Heterofermentat

Based on its fermentation type, Gram-positive bacteria indicated as BAL were distinguished into 2 types: homofermentative which fermented glucose into lactic acid, and heterofermentative which fermented

glucose into lactic acid, ethanol, acetic acid, and CO_2 gas (Wang et al., 2021). The fermentation process found in lactic acid bacteria is a group of homofermentative and heterofermentative lactic acid bacteria. Homofermentative lactic acid bacteria only produce lactic acid, while heterofermentative lactic acid bacteria produce various other compounds, namely acetate, ethanol, carbon dioxide, and lactic acid (Siahaan et al., 2023). The fermentation type test results on bacterial isolates revealed that 6 isolates with codes IBK4, IBK9, IBK26, IBK27, IBK28, and IBK29 exhibited homofermentative fermentation type. Meanwhile, the isolate with code IBK19 exhibited heterofermentative fermentation type.

Fermentation ability tests using different carbon sources, as presented in Table 3, indicated that most acid-forming bacterial isolates could ferment various types of sugars such as glucose, lactose, sucrose, mannitol, fructose, and maltose. According to Malimas et al. (2007), not all genera of *Gluconobacter* could ferment maltose sugars. The acetic acid bacteria group from the genus *Neoasaia* couldn't ferment maltose sugars (Yukphan et al., 2005). Bacterial isolate with code IBK 15 couldn't ferment fructose sugars. According to Jojima et al. (2004), not all genera of Saccharibacter had the ability to grow on fructose. Lactic acid bacteria from the genus *Lactobacillus* mostly could ferment several types of sugars (Gebreselassie et al., 2016). In the study by Sayuti et al. (2017), some genera of *Lactobacillus* had the ability to ferment carbon sources such as glucose, mannitol, lactose, maltose, while others couldn't ferment those carbon sources.

Based on the conducted tests, the acid-forming bacterial isolates were divided into two groups: acetic acid bacteria and lactic acid bacteria. Identification was carried out based on reference books such as *Bergey's Manual of Determinative Bacteriology*, Axellson (2004), and previous research journals. Bacterial isolates with codes IBK 1 and IBK 2 were identified as belonging to the genus *Asaia*. IBK 5, IBK 7, IBK 8, IBK 13, IBK 16, IBK 22, IBK 24, IBK 25, IBK 30 were identified as belonging to the genus *Gluconobacter*. IBK 12 was identified as belonging to the genus *Neoasaia*, and the isolate with code IBK 15 was identified as belonging to the genus *Sacharibacter*. The BAL group identified with isolate codes IBK 4, IBK 9, IBK 19, IBK 26, IBK 27, IBK 28, IBK 29 were classified into the genus *Lactobacillus*.

Conclusion

In this study, fermentation was conducted spontaneously with the addition of distilled water on fermentation days 4 and 7, aiming to detect acid-forming bacteria involved in the fermentation process. Characterization of bacterial isolates was performed based on macroscopic and microscopic observations, physiological and biochemical tests. Identification of bacterial isolates was carried out using profile matching method based on Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and Axellson (2004) up to the genus level. The identification results of the genus for the 30 isolates revealed that 7 isolates were lactic acid bacteria, namely IBK 4, IBK 9, IBK 19, IBK 26, IBK 27, IBK 28, and IBK 29, belonging to the genus Lactobacillus. Another 13 isolates were identified as acetic acid bacteria, which were divided into 4 genera: Asaia (IBK1 and IBK2), Neoasaia (IBK12), Sacharibacter (IBK15), and Gluconobacter (IBK5, IBK7, IBK8, IBK13, IBK16, IBK22, IBK24, IBK25, IBK30).

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Author Contributions

Conceptualization, S, A. M; methodology, S, Q. A. S; funding acquisition, A. M; data processing, Q. A. S; article writing, Q. A. S; validation, S; review, S, A. M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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