

## Impact of *Lactobacillus* strains on free fatty acid profile in coconut neera

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### Article history:

Received: 5 December 2023

Received in revised form: 8 March 2024

Accepted: 20 May 2024

Available Online: 4 March 2025

### Keywords:

Fatty acids,

*Lactobacillus*,

Coconut neera,

Probiotic beverage,

Stearic acid,

Fatty acid biosynthesis

### DOI:

[https://doi.org/10.26656/fr.2017.9\(2\).384](https://doi.org/10.26656/fr.2017.9(2).384)

### Abstract

Coconut neera has been reported as a potential candidate for the development of a new probiotic beverage by inoculating it with *Lactobacillus casei* AP and *Lactobacillus plantarum*. This study aimed to determine the impact of *L. casei* AP and *L. plantarum* on the free fatty acid (FFA) content in inoculated coconut neera. The FFA were analyzed using gas-chromatography mass spectroscopy, and the biosynthetic pathways of stearic acid were elucidated by conducting a whole-genome BLAST search against seven databases. Palmitic acid was found to be the predominant fatty acid in coconut neera, constituting 49.85% of the total fatty acids. Inoculation of coconut neera with *L. casei* AP and *L. plantarum* resulted in a significant decrease in palmitic acid and an increase in stearic acid levels, reaching 8.93% and 3.42%, respectively. Both *L. casei* AP and *L. plantarum* utilized the fatty acid biosynthesis pathway of type 2 fatty acid synthase (FASII) to produce stearic acid. However, neither of them was able to synthesize conjugated linoleic acid (CLA) components in coconut neera. Consequently, neera fermented by *L. plantarum* and *L. casei* AP may have a more pronounced impact on the FFA profile compared to non-fermented neera.

## 1. Introduction

Lactic acid bacteria (LAB) are widely recognized as crucial microorganisms in food fermentation, medicinal applications, and specialized dietary uses (Zhang and Chai, 2014). The *Lactobacillus* genus represents the largest group of LAB and is extensively employed as a starter culture in fermented foods. *Lactobacillus plantarum* and *L. casei* are probiotic bacteria that naturally inhabit the intestinal tract. When used as probiotics, these species play essential roles in maintaining gut flora and supporting the immune system. Gut flora engages in a multitude of interactions with their host and is pivotal in upholding the host's well-being. Within the gut flora, lipid metabolism gives rise to a variety of fatty acid types, including conjugated fatty acids and trans-fatty acids, which have the potential to impact the host's lipid metabolism.

A fatty acid is a carboxylic acid with a long aliphatic tail (Kanchanamayoon and Kanenil, 2007). There are two types of fatty acids: saturated and unsaturated fatty acids. Saturated fatty acids can be further categorized into short-chain, medium-chain, long-chain, and very long-chain fatty acids based on their chain length (Papotti *et al.*, 2021). Saturated fatty acids with 12, 14, and 16 carbon atoms, namely lauric acid, myristic acid, and palmitic acid, have been identified as hypercholesterolemic saturated fatty acids. Consuming foods high in palmitic acid can lead to an increase in LDL cholesterol (often referred to as "bad" cholesterol) levels. High levels of LDL cholesterol are associated with an increased risk of cardiovascular diseases like heart disease and stroke (Rooijen and Mensink, 2020). The transformation and subsequent absorption of appropriate amounts of dietary fatty acids or lipids by

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intestinal epithelial cells are crucial for maintaining energy balance and metabolic health.

Coconut neera is known for its high nutritional value, low-fat content, and digestive benefits. Coconut neera is obtained by tapping the unopened spadix of the coconut palm (Borse *et al.*, 2007). On average, one spadix can yield approximately 2 L of neera per day (Mashud and Matana, 2014). Coconut neera is the naturally occurring sap from mature coconut palms, which is abundant in essential nutrients, minerals, and vitamins beneficial for human health (Chinnamma *et al.*, 2019). Moreover, due to its high sugar content, coconut neera undergoes rapid fermentation, making it a potential candidate for the production of a healthy probiotic beverage.

The growing consumer demand for low-cholesterol products has spurred the exploration of non-dairy alternatives. Fatty acid analysis has garnered significant attention due to its nutritional and health implications (Kanchanamayoon and Kanenil, 2007). Probiotics in food possess the potential to transform dietary components, such as fat, protein, and sugar, into healthier products. *Lactobacillus plantarum* (Kishino *et al.*, 2002) and *L. casei* AP (Widodo *et al.*, 2018) have the ability to produce conjugated linoleic acid (CLA) molecules. CLA has demonstrated benefits in improving body composition and reducing cardiometabolic risk factors (Lehnen *et al.*, 2015). *Lactobacillus* strains can also influence the fatty acid profile (Papotti *et al.*, 2021). Consequently, this study aims to investigate the changes in free fatty acid composition in coconut neera following fermentation by *L. plantarum* and *L. casei* AP.

## 2. Materials and methods

### 2.1 Coconut neera

Coconut neera was sourced from the Ngupoyo Bogo farmer group, located in Hargotirto village, Kokap sub-district, and Kulon Progo district. The neera extraction process took place in the afternoon and the collected neera was transported to the laboratory the following morning, using a cool box to ensure a temperature range of 4-10°C was maintained. Subsequently, it was stored at -20°C until it was ready for use (Mulyono *et al.*, 2021).

### 2.2 Fermentation process

Before the fermentation process, the neera underwent sterilization. This sterilization procedure involved centrifugation at 3000 rpm for 20 mins, followed by filtration using Whatman No. 1 filter paper, and finally, pasteurization at 84°C for 10 mins. The *Lactobacillus* strains (*L. plantarum* and *L. casei* AP) were then inoculated into 50 ml of sterile coconut neera

and incubated for 9 hrs at 37°C. Subsequently, samples were collected from the fermented neera for free fatty acid (FFA) analysis.

### 2.3 Free fatty acid analysis

Samples prepared using a combination of fat hexane and isopropanol extraction following the procedure outlined by Widodo *et al.* (2018), were subjected to GC-MS analysis. Fatty acid analysis was carried out using a Rtx 5 MS column (30 m × 0.25 mm i.d.) with helium serving as the carrier gas and electron ionization at 70 eV. The analysis was conducted under the following conditions: a column heating temperature of 50°C, an injection temperature of 300°C, a split injection model with a pressure of 13 kPa, a total flow rate of 79.3 mL/min, and a column flow rate of 0.55 mL/min, utilizing a split ratio of 139. Peak-free fatty acid identification was achieved by comparing their retention times.

### 2.4 Identification of pathways related to stearic acid as free fatty acids

The analysis of the fatty acid biosynthetic pathways involved conducting a comprehensive whole-genome BLAST search across seven distinct databases, which included Gene Ontology, the Kyoto Encyclopedia of Genes and Genomes (KEGG), Cluster Orthologous Groups, the Non-Redundant Protein Databases (NR), Swiss-Prot, TrEMBL UniProt, Brenda enzymes, and MetaCyc, as outlined by Widodo *et al.* (2021).

## 3. Results and discussion

### 3.1 Free fatty acid in neera

Borse *et al.* (2007) reported that palmitic acid and palmitoleic acid were the primary fatty acids found in neera. The findings regarding the free fatty acids in neera were presented in Figures 1, 2, and 3.

According to Figure 1, palmitic acid (C16:0) was identified as the predominant free fatty acid in coconut neera, constituting 49.85% of the total fatty acids. However, following fermentation by *L. casei* AP and *L. plantarum* for 9 hrs at 37°C (as depicted in Figures 2 and 3), palmitic acid was no longer detectable in coconut neera.

The fermentation process carried out by these *Lactobacillus* strains resulted in a reduction in the proportion of palmitic acid and brought about changes in the composition of other detected fatty acids, in contrast to non-fermented bean-based beverages (Ziarro *et al.*, 2020). A similar phenomenon was observed in fermented coconut neera, where palmitic acid became undetectable due to its minimal presence. This suggests that palmitic acid may serve as a carbon source in the fatty acid

biosynthesis process, leading to the production of stearic acid.

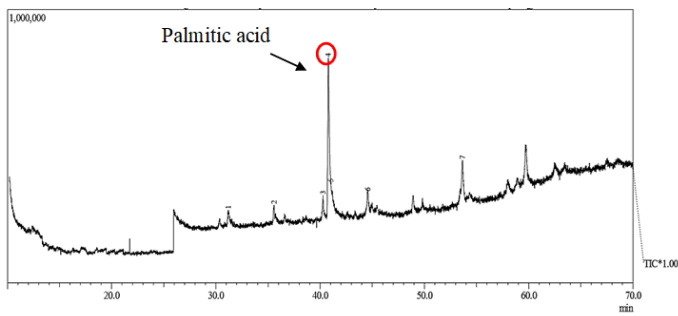


Figure 1. GC-MS analysis of coconut neera as a control without starter.

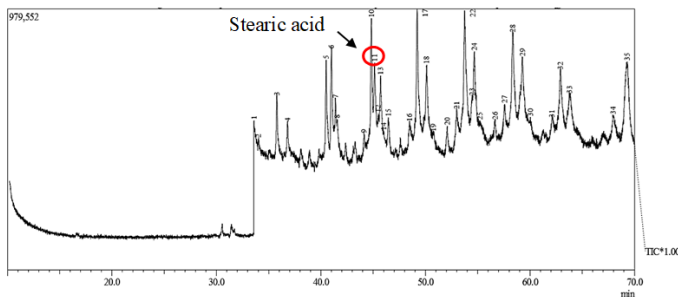


Figure 2. GC-MS analysis of coconut neera fermentation with starter *L. plantarum*.

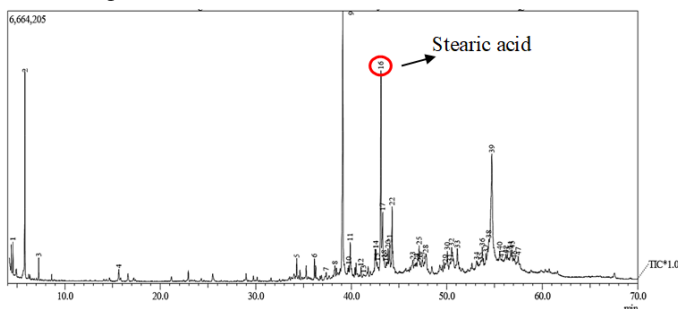


Figure 3. GC-MS analysis coconut neera fermentation with starter *L. casei* AP.

After fermentation by *L. casei* AP and *L. plantarum*, stearic acid emerged as the dominant fatty acid. Stearic acid is a saturated fatty acid that results from the conversion of dietary fat through lipolysis, leading to the formation of free fatty acids, which can then undergo processes such as isomerization and hydrogenation to form polyunsaturated fatty acids (Salsinha *et al.*, 2018). The proportion of stearic acid in coconut neera fermented by *L. casei* AP and *L. plantarum* was found to be 8.93% and 3.42%, respectively (as shown in Figures 2 and 3).

The fermentation results of *L. plantarum* and *L. casei* AP did not yield conjugated linoleic acid (CLA) but instead resulted in the production of stearic acid. The absence of CLA synthesis could be attributed to the lack of linoleic acid in coconut neera, which is required for its conversion to CLA (as depicted in Figures 1, 2, and 3). Kishino *et al.* (2002) noted that CLA can be generated through the isomerization of linoleic acid. Although CLA

was not produced, the presence of stearic acid appears to be neutral in terms of its effect on cholesterol levels, as stearic acid is rapidly converted into oleic acid (Papotti *et al.*, 2021). Consequently, neera fermented by *L. plantarum* and *L. casei* AP may exhibit a more favorable free fatty acid profile compared to non-fermented neera.

### 3.2 Identification of pathways related to stearic acid as free fatty acids

The synthesis of stearic acid in *L. plantarum* and *L. casei* AP involves the fatty acid biosynthesis pathway of type 2 fatty acid synthase (FASII). To identify all the genes responsible for the enzymatic reactions involved in stearic acid synthesis, a comprehensive search using the databases [www.brenda-enzyme.org](http://www.brenda-enzyme.org) was conducted (accessed on January 6, 2023) and <http://genome.jp/kegg/annotation/enzyme.html> (accessed on January 6, 2023). The KEGG annotation results of the genes potentially involved in FASII and unsaturated fatty acid metabolism in *L. casei* AP were presented in Table 1.

In this step, the gene enoyl-[acyl-carrier protein] reductase II (*fabK*) (locus = Scaffold8:80446:81420:+) and the gene acyl-CoA thioesterase (*tesA*) (locus = Scaffold10:48187:48978:-) were identified as listed Table 1. According to a previous study by Botta *et al.* (2017), type 2 fatty acid synthase (FASII) consists of initiation involving four separate subunits of the acetyl-CoA carboxylase enzyme, as well as fatty acid chain elongation. The *fabK* (or *fabI*) gene is involved in fatty acid chain elongation, while the *tesA* gene is involved in the unsaturated fatty acid pathway. However, a gene similar to *tesA* was not detected in *L. plantarum*. The KEGG annotation results of the genes potentially involved in FASII metabolism in *L. plantarum* are presented in Table 2.

In the genome of *L. plantarum*, the gene enoyl-[acyl-carrier protein] reductase I (*fabI*) (locus = Scaffold26:26398:27156:+) was observed, which is involved in fatty acid chain-elongation. According to a study by Dong and Cronan (2022), overexpression of either *fabI* or *fabK* leads to increased synthesis of saturated fatty acids rather than unsaturated fatty acids, with *fabK* exhibiting a significantly higher phenotype of saturated fatty acid overproduction compared to *fabI*. These findings are consistent with the observation in this study, which revealed that *L. casei* AP produced higher amounts of stearic acid from fermented neera compared to *L. plantarum*, involving the *fabK* gene. In summary, *L. casei* AP and *L. plantarum* employ different mechanisms in the FASII pathway for stearic acid synthesis.

Table 1. KEGG annotation of the genes involved in FASII and unsaturated fatty acids pathways in *L. casei* AP.

KO	Abbreviation	Gene Names	Enzyme	Putative Gene
K02371	<i>fabK</i>	enoyl-[acyl-carrier protein] reductase II	1.3.1.9	LCAP_GM001318
K02372	<i>fabZ</i>	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	4.2.1.59	LCAP_GM001314, LCAP_GM001323
K09458	<i>fabF</i>	3-oxoacyl-[acyl-carrier-protein] synthase II	2.3.1.179	LCAP_GM001321
K00059	<i>fabG</i>	3-oxoacyl-[acyl-carrier protein] reductase	1.1.1.100	LCAP_GM001320, LCAP_GM001969, LCAP_GM002093
K00645	<i>fabD</i>	[acyl-carrier-protein] S-malonyltransferase	2.3.1.39	LCAP_GM001319
K01962	<i>accA</i>	acetyl-CoA carboxylase carboxyl transferase subunit alpha	6.4.1.2	LCAP_GM001326
K02160	<i>accB, bccP</i>	acetyl-CoA carboxylase biotin carboxyl carrier protein	--	LCAP_GM001322
K01961	<i>accC</i>	acetyl-CoA carboxylase, biotin carboxylase subunit	6.4.1.2 6.3.4.14	LCAP_GM001324
K01963	<i>accD</i>	acetyl-CoA carboxylase carboxyl transferase subunit beta	6.4.1.2	LCAP_GM001325
K00648	<i>fabH</i>	3-oxoacyl-[acyl-carrier-protein] synthase III	2.3.1.180	LCAP_GM001316
K10804	<i>tesA</i>	acyl-CoA thioesterase I	3.1.2.- 3.1.1.5	LCAP_GM001486

Table 2. KEGG annotation of the genes involved in FASII pathways in *L. plantarum*.

KO	Abbreviation	Gene Names	Enzyme	Putative Gene
K00208	<i>fabI</i>	enoyl-[acyl-carrier protein] reductase I	1.3.1.9 1.3.1.10	LPDR_GM002661
K02372	<i>fabZ</i>	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	4.2.1.59	LPDR_GM002650, LPDR_GM002657
K09458	<i>fabF</i>	3-oxoacyl-[acyl-carrier-protein] synthase II	2.3.1.179	LPDR_GM002655
K00059	<i>fabG</i>	3-oxoacyl-[acyl-carrier protein] reductase	1.1.1.100	LPDR_GM000931, LPDR_GM002654
K00645	<i>fabD</i>	[acyl-carrier-protein] S-malonyltransferase	2.3.1.39	LPDR_GM002653
K01962	<i>accA</i>	acetyl-CoA carboxylase carboxyl transferase subunit alpha	6.4.1.2	LPDR_GM001437, LPDR_GM002660
K02160	<i>accB, bccP</i>	acetyl-CoA carboxylase biotin carboxyl carrier protein	--	LPDR_GM001440, LPDR_GM002011, LPDR_GM002656
K01961	<i>accC</i>	acetyl-CoA carboxylase, biotin carboxylase subunit	6.4.1.2 6.3.4.14	LPDR_GM001439,
K01963	<i>accD</i>	acetyl-CoA carboxylase carboxyl transferase subunit beta	6.4.1.2	LPDR_GM001438, LPDR_GM002659
K00648	<i>fabH</i>	3-oxoacyl-[acyl-carrier-protein] synthase III	2.3.1.180	LPDR_GM001441, LPDR_GM002651

#### 4. Conclusion

This study demonstrates that both *L. casei* AP and *L. plantarum* have the ability to convert palmitic acid into stearic acid. Fermentation of coconut neera by *L. casei* AP resulted in a stearic acid content of 8.93%, while *L. plantarum* fermentation produced 3.42% stearic acid. Both *Lactobacillus* strains utilized the type 2 fatty acid synthase (FASII) pathway for stearic acid synthesis. The fermentation of neera generates stearic acid, known for its neutral effect on blood cholesterol levels. This was in contrast to palmitic acid in non-fermented neera, which tends to raise cholesterol levels. Given its potential health advantages, fermented neera can be considered as an alternative to probiotic beverages.

#### Conflict of interest

The authors declare no conflict of interest.

#### Acknowledgments

This research work was supported by Hibah Penelitian Terapan Unggulan Perguruan Tinggi 2019 (Grant No. 2754/UNI.DITLIT/DIT-LIT/LT/2019).

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