

Effects of *Lactobacillus plantarum* TAR4, TAR7 and TAR8 Isolated From *Tapai* On The Growth Of *Cutibacterium acnes*

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Abstract. Acne is a skin disease involving clogged hair follicles with dead skin cells secreted from the skin. It is a common skin condition and at least 85% of the people in the United States suffered from this problem (1). Despite not being a life threatening disease, acne may form permanent scars on skin and leading to emotional problems such as decreased self-esteem, social withdrawal, depression, and frustration. *Cutibacterium acnes* (*C. acnes*) is Gram-positive bacteria, slow-growing aerotolerant bacteria and involve in the pathogenesis of acne. Medication with benzoyl peroxide is often used to treat acne. Benzoyl peroxide has bactericidal effect which kills surface bacteria by the cleavage of peroxide bond of benzoyl peroxide to form 2 benzoyloxy radicals. The interaction of the radicals with bacterial proteins would affect the bacteria function, viability and lead to decreased production of sebum around follicles. However, the use of benzoyl peroxide is associated with adverse effect such as skin irritation. Besides that, oral antibiotics are also prescribed to control the growth of *C. acnes* and inflammation. There are potential harm of long term oral antibiotics treatment. Hence, this study aims to investigate the effect of probiotic *Lactobacillus plantarum* (*L. plantarum*) TAR4, TAR7 and TAR8 isolated from a fermented food, *Tapai* on the growth of *C. acnes* using co-culture technique with the purpose to offer a safe alternative solution to the use of benzoyl peroxide medication and oral antibiotics for acne treatment. The results shown that the *L. plantarum* TAR4, TAR7 and TAR8 exhibited significant ($P < 0.05$) inhibitory effects on the growth of *C. acnes*.

INTRODUCTION

Acne vulgaris is a common skin disease of the pilosebaceous units caused by the bacteria *Cutibacterium acnes* (*C. acnes*) (formerly *Propionibacterium acnes*) in adolescence and young adults (2). *C. acnes* is an anaerobic bacterium and the anaerobe promotes perifollicular inflammation. Proliferation of *C. acnes* increases the activity of sebaceous glands. An increase in sebum excretion is a major factor in the pathophysiology of acne vulgaris. *C. acnes* also activates the innate immunity by triggering the production of various cytokines, such as interleukins (IL-1, IL-8, IL-12), tumor necrosis factor (TNF) and interferon (INF) γ , as well as matrix metalloproteinases (MMPs) by keratinocytes leading to hyperkeratinization of the pilosebaceous unit (3). Benzoyl peroxide and antibiotics are commonly prescribed to treat acne by suppressing the growth of *C. acnes* by bactericidal mechanism and slow down the production of sebum. However, the use of benzoyl peroxide is associated with adverse effect such as skin irritation and more severe side effects such as dry skin, birth defects, and elevation of cholesterol level (4). Hence, a safe alternative to controlling acne problem would be desirable.

Tapai is a popular fermented traditional food in East and Southeast Asia particularly in Malaysia and Indonesia. *Tapai* is fermented rice product with sour and sweet taste and is often eaten directly as food. Starter culture is used for *Tapai* fermentation. Starter (*ragi*) is made from fresh plant material and spices. From the previous study, probiotic *Lactobacillus plantarum* (*L. plantarum*) TAR4, TAR7 and TAR8 strains have been isolated from *Tapai*. The probiotics have antimicrobial properties and *Tapai* has shown to exert cholesterol lowering effect on high cholesterol fed-rats (5). Probiotics which are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host,” are rising in popularity particularly for application in personal care industry. Studies have reported the effectiveness of *Lactobacillus* strains in promoting antimicrobial, balancing the skin microbiome, relief of atopic dermatitis (AD), improving the skin barrier, anti-aging, skin appearance enhancement and

moisturization (6). There is still a scarcity of information on the efficacy of *Lactobacillus* for acne control leading to acne treatment. The current report focused on controlling the proliferation of *C. acnes* which plays important role in the acne pathogenesis.

Hence, the inhibition effect of *L. plantarum* TAR4, TAR7 and TAR8 on the growth of *C. acnes* is being targeted in the current report. The study aims to determine the effect of *L. plantarum* TAR4, TAR7 and TAR8 on the growth of *C. acnes* using co-culture method as to provide an alternative approach for acne control. The specific objectives in this research include isolation of *C. acnes*, characterization of *C. acnes* by using biochemical and molecular methods, and investigation of co-culture interaction between *L. plantarum* strains and *C. acnes*.

EXPERIMENTAL

Materials

The microbiological media used includes Brain Heart Infusion (BHI) agar and broth (Oxoid, UK) and De Man, Rogosa and Sharpe (MRS) agar and broth (Oxoid, UK). Molecular biology reagents, kits and media include Presto™ genomic DNA bacteria kit (Geneaid, Taiwan), GoTaq® master mix (Promega, USA), ANT1F, 1392R oligonucleotide primers (Bio Basic, Canada).

Isolation and Characterization of *Cutibacterium acnes*

Isolation of bacteria was carried out by performing swab on the acne affected area from the face of subjects using sterile cotton swabs. The swabs were then streaked on BHI agar and subjected to incubation in anaerobic condition at 37 °C. After overnight incubation, bacteria colonies with different colony morphologies were sub-cultured until pure and single colonies obtained. Pure colonies were then cultured in BHI broth in anaerobic condition at 37 °C. The colonies were subjected to biochemical characterization which includes catalase test, Gram-staining and endospore staining. For molecular characterization, DNA extraction was carried out and DNA samples were subjected to Polymerase Chain Reaction (PCR) using primer pairs targeting *C. acnes*: forward primer (ANT1F: 5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer (1392R: 5'-ACG GGC GGT GTG TAC AAG-3') (7). The PCR amplicons were then subjected to agarose gel electrophoresis.

Investigation of Co-culture Interaction

Culture media preparation

MRS culture media and BHI culture media were used to culture *L. plantarum* TAR 4, TAR 7, and TAR 8 and *C. acnes* respectively. The pH of both MRS and BHI culture media was adjusted to pH7 prior sterilization via autoclave at 121°C for 15 minutes.

L. plantarum strains culture preparation

Characterized probiotic strains *L. plantarum* TAR 4, TAR 7 and TAR 8 isolated from *Tapai* were recovered from glycerol stocks and cultured on MRS plates. The plates were then subjected to incubation under anaerobic condition at 37 °C, overnight.

Investigation of co-culture interaction

C. acnes culture was grown on BHI plates under anaerobic condition at 37 °C, overnight. The *L. plantarum* cultures prepared earlier and the *C. acnes* cultures were then cultured on MRS and BHI broth cultures. The broth cultures were serially diluted to 10⁻⁸ and used for monoculture and co-culture experiments as per the combination of bacteria strains shown in Table 1. After several rounds of growth optimization, the volume of *C. acnes* and *L. plantarum* strains cultured on co-culture plates were 250 µl and 10 µl respectively. The growth of bacteria was determined by counting the colony forming unit (CFU/ml) in the monoculture and co-culture plates.

TABLE 1. Incubation of *C. acnes* and *L. plantarum* TAR 4, TAR 7, and TAR 8 in monoculture and co-culture media.

Bacterial culture	
Monoculture	
	<i>C. acnes</i>
	<i>L. plantarum</i> TAR 4
	<i>L. plantarum</i> TAR 7
	<i>L. plantarum</i> TAR 8
Co-culture	
	<i>C. acnes</i> with <i>L. plantarum</i> TAR 4
	<i>C. acnes</i> with <i>L. plantarum</i> TAR 7
	<i>C. acnes</i> with <i>L. plantarum</i> TAR 8

Statistical analysis

The co-culture growth determination between *C. acnes* and *L. plantarum* TAR 4, TAR 7, and TAR 8 were carried out in triplicates (n=3). Data were analyzed via one-way analysis of variance (ANOVA) and expressed as means. The significant differences were determined by applying Duncan's multiple range tests at 95% least significant difference ($P < 0.05$).

RESULTS AND DISCUSSIONS

Isolation of bacteria was performed by swabbing from acne infected areas of the face of three patients with acne problem. The BHI culture media which is general and rich in nutrient was used to culture *C. acnes* (8). Isolated bacteria colonies with different colony morphology were selected for subsequent sub-culturing on BHI plates until pure colonies were obtained. All incubation were carried out under anaerobic condition at 37 °C. Biochemical characterization showed that the isolated bacteria is catalase positive, Gram-positive and no formation of endospore (Figure 1). The results summarized in Table 2.

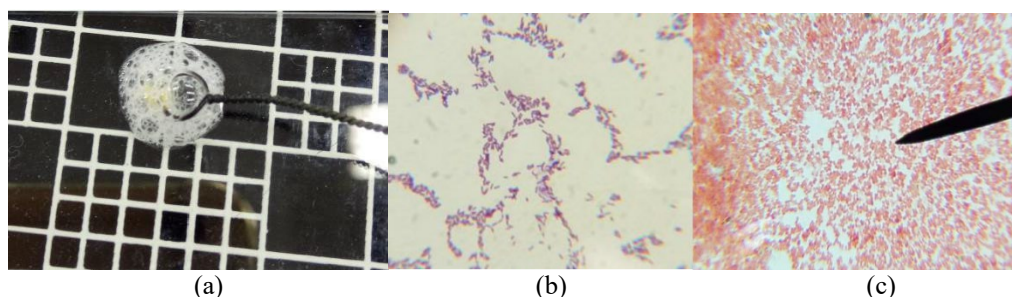


FIGURE 1. Representative diagram from biochemical tests; (a) Catalase test showed rapid elaboration of oxygen bubble (catalase positive); (b) Gram-positive, rod-shaped under magnification 1000x; (c) Endospore staining showed no endospore formation.

TABLE 2. Biochemical characterization of bacteria isolate.

Biochemical test	Results
Catalase test	Catalase positive
Gram staining	Rod-shaped, Gram-positive
Endospore staining	No endospore was found

DNA extracted from the single colony culture was subjected to PCR and the amplicon size of approximately 1300 base pairs was obtained and this indicated that the targeted gene was amplified (Figure 2). Hence, it showed that *C. acnes* has been isolated.



FIGURE 2. Agarose gel electrophoresis of PCR amplicons. Lane 1 and 8: 1kb DNA ladder; Lane 2 and 3: PCR amplicons amplified using the primer pair.

The growth of *C. acnes* in co-culture media with *L. plantarum* TAR 4, TAR 7 and TAR 8 respectively was determined by comparing the plate count (CFU/ml) with the respective monoculture bacteria plates. Figure 3 shows the means (average) growth (CFU/ml) of *C. acnes* in monoculture media as compared to its growth in co-culture media with the probiotic strains *L. plantarum* TAR 4, TAR 7 and TAR 8 respectively.

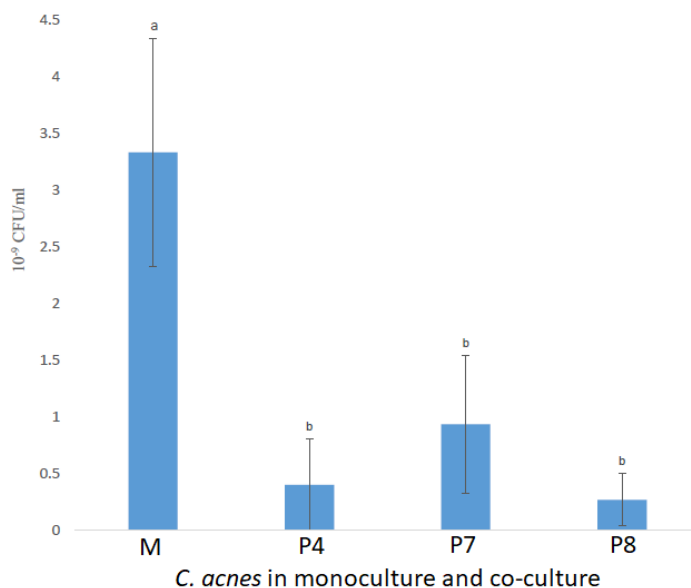


FIGURE 3. The growth (CFU/ml) of *C. acnes* in monoculture media (M) and in co-culture media with *L. plantarum* TAR 4 (P4), TAR 7 (P7), and TAR 8 (P8) respectively. The values in the bar chart represent the means of triplicate whereas different superscripts labelled indicated the values were significantly different ($P < 0.05$).

From the results (Figure 3), the CFU/ml of *C. acnes* decreased in co-culture media with *L. plantarum* TAR 4 (P4), TAR 7 (P7), and TAR 8 (P8) as compared to the CFU/ml of its monoculture media. The results showed that *L. plantarum* strains used in this study exhibited inhibitory effect on the growth of *C. acnes*. Growth suppression of *C. acnes* by the probiotic strains were possibly due to the antimicrobial substances production (organic acids) by *L. plantarum* strains which lower the pH of the culture environment. Organic acids produced exhibits antimicrobial effects. Their ability to pass through cell membrane is due to their undissociated form with lipophilic nature, which further allows them to modify the protons and associated anion concentrations present in the cytoplasm of the bacterial cells, leading to unfavorable growth condition (9).

Several studies conducted have reported the ability of *Lactobacillus* strains to suppress the growth of pathogens in co-culture conditions. Probiotics such as *Lactobacillus reuteri* displayed inhibitory effects against *Staphylococcus*

epidermidis and *C. acnes* (10) while other *L. plantarum* strains could inhibit the growth of some common human pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Listeria monocytogenes* (11). Besides, *L. plantarum* UM55 isolated from milk is capable of reducing the growth of pathogenic fungus *Aspergillus flavus* via its production of organic acid with anti-alfatoxigenic effect (12). From the studies, the inhibitory effects on pathogens were due to the similar mechanism of action, which involves the secretion of organic acid by *Lactobacillus* strains. Other antimicrobial mechanism include the production of bacteriocin by *L. plantarum*, such as plantaricin SLG1 (13) and plantaricin JY22 (14).

CONCLUSION

In this study, the isolation of *C. acnes* from acne infected area of face of the patients was successful. Probiotics strains *L. plantarum* TAR 4, TAR 7, and TAR 8 exhibited significant ($P < 0.05$) inhibitory effect on the growth of *C. acnes* in co-culture condition with *L. plantarum* strains as compared to monoculture media. The current results pave path to the potential use of probiotics as an alternative control measure for acne infection.

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