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RESEARCH ARTICLE

The Influence of *Lactobacilli* in GABA and Amino Acid Profile of Fermented Mature Coconut Water

Izuddin Abdul Rahman, Mohd Izwan Mohd Lazim, Suhaiza Mohamad, Koh Soo Peng*, Muhammad Anas Othaman, Musaalbakri Abdul Manan and Mohd Azzammil Mohd Asri

Biotechnology and Nanotechnology Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia

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Abstract:

Objective:

Mature coconut water (MCW) is a waste product from the coconut milk industry. It is sour and unpalatable, yet it contains sufficient nutrients for microbial growth.

Methods:

Four Lactic Acid Bacteria (LAB), namely *L. acidophilus* B0258, *L. brevis* VM1, *L. casei* B0189, *and L. plantarum* B0103 were used to ferment MCW over 120 h. Among these LAB strains, only *L. casei* was capable to grow well with the highest viable bacteria count of 1 x 10^{11} colony forming unit (cfu)/ml. Although all LAB produced α-aminobutyric acid (GABA) after fermentation, *L. acidophilus* and *L. plantarum* produced the highest amount of GABA with the increment of 35.4%±7.9 and 38.9%±1.7, respectively. Other amino acid profiles of fermented MCW were also investigated, but most of them were consumed by the LAB. Both *L. acidophilus* and *L. plantarum* utilized the most essential amino acids. Within the first 24 h, GABA content was enhanced in all LAB strains when they were actively growing.

Result and Conclusion:

This study showed that both *L. acidophilus* and *L. plantarum* have great potentials to increase GABA content in MCW. Fermented coconut water can be formulated as a healthy functional drink as GABA is known to have therapeutic value in alleviating stress as reported by past research findings.

Keywords: Coconut water, Lactic acid bacteria, GABA, Fermentation, Amino acid profile, MCW, Stress.

1. INTRODUCTION

The demand for coconut water is increasing due to its unique and refreshing taste, rehydration potential [1], nutritional and health benefits [2 - 4]. Due to this high demand, sources for coconut water are more ubiquitous, and more land has being turned into coconut plantation. The coconut water for drinking is mostly taken from young coconuts, which have the most water. As the coconut mature, its water content decreases and turns sour, and eventually become less palatable for fresh consumption. Nevertheless, the natural aging process of coconut water produced other health-benefiting bioactive compounds, such as cytokinins [5].

Currently, the mature coconut is being used for the production of desiccated coconut product and coconut milk, and its water is wasted. Besides containing numerous compounds vital for human health, the water contains enough nutrients to support microbial growth. Lactic acid bacteria (LAB) can be used to ferment mature coconut water (MCW)

* Address correspondence to this author at the Biotechnology and Nanotechnology Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia, Tel: +60389536097; E-mails: karenkoh@mardi.gov.my; karenkoh999@gmail.com

and improve the bioactive compounds. One of the bioactive compounds that are widely studied is γ-aminobutyric acid (GABA), which is a by-product of several microbial fermentations including LAB [6]. GABA is the major inhibitory neurotransmitter that controls the central nervous system and plays a vital role in numerous human physiological processes. Abdou *et al.* conducted a study on human model reporting the function of GABA in promoting relaxation and improving body immunity during stress [7].

The LAB produce GABA in response to stress conditions, such as acidic environment. The presence of glutamate decarboxylase (GAD) system allows LAB to survive in low pH environment by producing GABA, which consumes protons they produced through fermentation to maintain pH homeostasis [8]. Most of the LAB strains also lack cell envelope proteinases, limiting their growth in protein-rich foods; therefore, LAB such as *L. brevis* have been shown to produce high amount of GABA as a strategy to grow in media with high protein content [9]. LAB are widely used for the fermentation of food products, such as cheese, *kimchi*, sourdough, sauerkraut, yogurt, and many other fermented foods [10]. Many studies have reported high amount of naturally produced GABA when LAB were used in the fermentation of food products [11, 12].

Therefore, LAB strains are considered as the suitable candidate for enhancing GABA in the fermentation of MCW. The main objective of this study was to screen the potential of four locally isolated LAB strains in enhancing GABA content in MCW. The amino acid profile of the fermented MCW was also examined throughout 120 h of fermentation. The results of this study allow us to formulate a fermented functional drink from MCW with naturally produced GABA by the selected LAB strains.

Table 1. Amino Acid Profiles of Coconut Water Fermented with Four Lactic Acid Bacteria Strains Throughout 5-Days Fermentation Time. (a) Total Essential Amino Acids (EAAs), (b) Total Non-essential Amino Acids (NEAAs), (c) Total Soluble Amino Acids (TAAs).

Total Essential Amino Acids (EAAs)				
Fermentation time (h)	**LA**	**LB**	**LC**	**LP**
0	**280±1**	**282±1**	**279±1**	**287±14**
24	**32±2**	**263±9**	**209±10**	**30±2**
48	**27±5**	**271±9**	**223±3**	**33±3**
72	**32±5**	**257±13**	**163±37**	**27±2**
96	**33±32**	**262±19**	**163±9**	**26±2**
120	**29±3**	**275±3**	**156±7**	**25±2**
Total Non-essential Amino Acids (NEAAs)				
Fermentation time (h)	**LA**	**LB**	**LC**	**LP**
0	**1283±7**	**1198±1**	**1265±29**	**1339±128**
24	**1015±58**	**970±35**	**1239±47**	**1066±113**
48	**869±46**	**986±66**	**1321±44**	**947±34**
72	**917±20**	**877±49**	**1339±524**	**713±30**
96	**1045±518**	**900±336**	**847±49**	**705±31**
120	**1017±424**	**1006±413**	**798±32**	**665±23**
Total Soluble Amino Acids (TAAs)				
Fermentation time (h)	**LA**	**LB**	**LC**	**LP**
0	**1562±6**	**1479±2**	**1543±29**	**1625±142**
24	**1047±59**	**1233±44**	**1448±57**	**1096±114**
48	**896±51**	**1257±75**	**1544±47**	**979±36**
72	**948±25**	**1134±61**	**1010±58**	**731±32**
96	**1078±549**	**1162±354**	**1010±58**	**731±32**
120	**1045±422**	**1281±416**	**954±38**	**690±25**

Values represent means and standard deviations of triplicate determinations. Values with different superscript letters (a-c) indicate significant different at (p<0.05).

Abbreviations: *L. acidophilus* (LA), *L. plantarum* (LP), *L. brevis* (LC), and *L. casei* (LC)

2. MATERIAL AND METHODS

2.1. Coconut Water Preparation

Fresh coconut water was obtained from mature coconut fruits (*Cocos nucifera L.*), which were harvested from the Malaysian Agricultural and Research Development Institute (MARDI) coconut plantation (Perak, Malaysia). The coconuts were thoroughly disinfected with alcohol to reduce the risk of contamination before collecting the coconut water. A pasteurization condition study was conducted previously on MCW, which took into account the sterility of the MCW before fermentation and the preservation of the existing bioactive compounds of interest in MCW. Based on that study, the coconut water was pasteurized at 105°C for 10 min before being subjected to microbial fermentation.

2.2. Bacterial Strains and Culturing

The starter culture was prepared according to a study with modifications [13]. Four locally isolated LAB strains, namely *Lactobacillus acidophilus* B0258, *Lactobacillus brevis* VM1, *Lactobacillus casei* B0189 and *Lactobacillus plantarum* B0103, were obtained from MARDI's Centre of Functional Food Cultures (CFFC). Fresh starter culture of each strain was prepared on de Man Rogosa and Sharp (MRS) medium and incubated at 37°C for 24 h. After 24 h, a single loop of pure culture colony of each LAB strains was inoculated into 15 mL of coconut water and incubated at 37 °C for 24 h, which was then used as inoculum stock.

2.3. Fermentation

The pasteurized coconut water was inoculated with 3% (v/v) starter culture and incubated at 37°C under static condition for 5 days. The growth profile of each LAB strain was observed from colony forming unit (cfu)/ml, and the pH of the media was determined at an interval time of 24 h.

2.4. Quantitative Analysis

Amino acid and GABA contents were analyzed using ultra performance liquid chromatography (UPLC) according to a method described by Danial *et al.* with slight modifications [14]. Briefly, 10 µL of sample was derivatized using 70 µL of AccQ-Tag™ Ultra and completely dissolved in borate buffer. A total of 20 µL of AccQ-Tag™ Fluor agent was added, and the mixture was heated to 55 °C for 10 min. A volume of 1 µL mixture solution was injected into the UPLC system. An AccQ-Tag™ Ultra column (2.1 mm x 100 mm, 1.7 µm) was used with a flow rate of 0.7 mL/min. The column temperature was set to 55 °C and the UV spectra were read at 260 nm. The elution solvents consisted of mobile phase A: ACN:formic acid:ammonium (10:6:84) and mobile phase B: ACN:formic acid (98:2) were prepared. The bi-elution strategy was performed as follows: 99.9% A (0.01% B) was maintained from 0.00 to 0.54 min; followed by linear gradient of A from 99.9 to 90.9% at 0.54 to 5.74 min; linear gradient of A from 90.9 to 78.8% at 5.74 to 7.74 min; linear gradient of A from 78.8 to 40.4% from 7.74 to 8.50 min; constant flow of A at 40.4% for 0.30 min; linear gradient of A from 40.4 to 99.9% at 8.80 to 8.90 min; and finally a constant flow of A at 99.9% for 2.10 min. The amino acid content was quantified from the standard curve of alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. The total essential amino acids were calculated based on the sum of histidine, phenylalanine, threonine, methionine, leucine, isoleucine, tryptophan, lysine and valine. All analyses were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Bacterial Growth

The advantage of LAB in the coconut water fermentation is their ability to survive at low pH environment. Fig. (**1A**) shows that all LAB strains were able to reach their viable counts above 10^9 cfu/ml after 24 h of fermentation but later, showed a slight decline in the bacterial viability rate after 5 days of fermentation. However, the viable count of all LAB strains still remained above 7.5 ($\log_{10}$ cfu/ml). Among the four selected LAB strains, only *L. casei* was observed to be actively growing within the first 24 h with the highest bacterial viable count of 10^{11} cfu/ml. However, the viability rate of *L. casei* declined significantly after 48 h and remained constant in its growth profile throughout 5 days fermentation time. The slight decreased in the bacterial viability rate might be caused by the highly acidic growth media condition; thus, retarding the rapid growth of the bacteria [15].

Fig. (**1B**) shows a dramatic decline in pH in all fermented coconut water samples after 24 h of incubation and

remained constant throughout 5 days of fermentation period. Among all LAB strains, only coconut water fermented with *L. brevis* showed slightly higher pH value of 3.8 even though subjected to prolonged fermentation up to 5 days. A very low, acidic pH condition might cause a decrease in bacterial viability rate. Nevertheless, the viability rate of *L. brevis* showed a slight decline after day 3 of fermentation Fig. (**1A**). The higher pH was probably the reason why *L. brevis* was able to sustain its growth within 48 h. Moreover, many studies have reported *L. brevis* as the predominant microbe that can continue spoiling food despite the hostile conditions after 24 h of fermentations [16]. Most of these studies showed the spoilage of beer by *L. brevis*, which was due to its hop resistance [17].

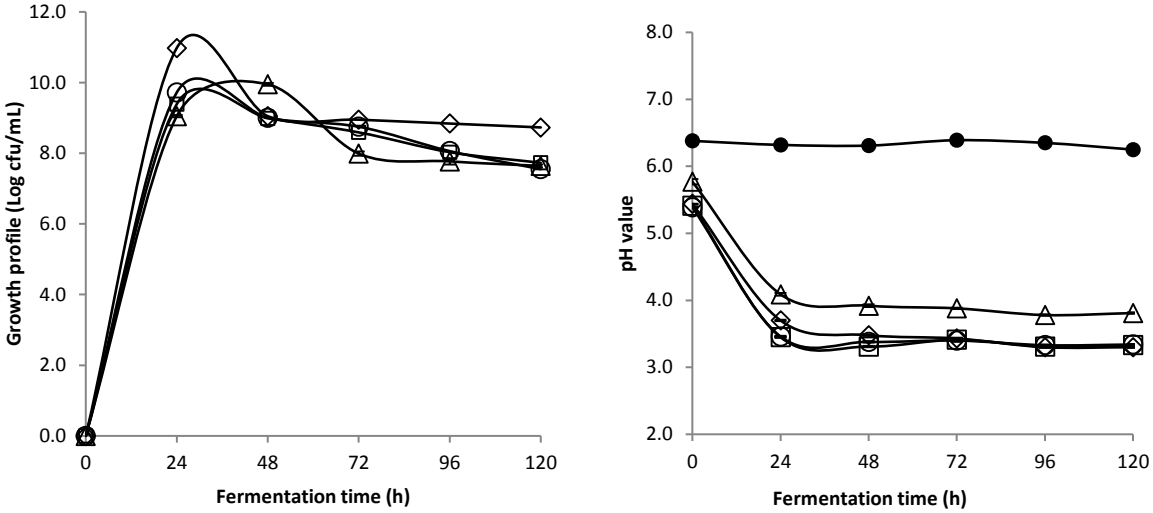

Fig. (1). Growth profile **(A)** and pH **(B)** of *L. acidophilus* (□), *L. brevis* (Δ), *L. casei* (◊), *L. plantarum* (°), and negative control (●) for 120 h of fermentation in mature coconut water at 37 °C. The results are presented as the mean of triplicates with standard deviations.

3.2. GABA Production

At the interval time of 24 h, all coconut water samples fermented with four LAB strains were collected to examine their amino acids profile throughout 5 days of fermentation as shown in Fig. (**2**). Generally, *L. acidophilus, L. plantarum, L. brevis* and *L. casei* showed an improvement in GABA content with increments of 35.4%±7.9, 38.9%±1.7, 5.8%±3.1 and 9.7%±2.9, respectively, after 24 h of fermentation. It is well known that the major pathway for GABA production involves the utilization of glutamic acid and pyridoxal 5'–phosphate (PLP) as its co-factor [18]. Therefore, *L. acidophilus* and *L. plantarum* strains showed an inverse trend of glutamic acid level, implying that these bacteria consumed glutamic acid to produce GABA during fermentation.

On the other hand, the glutamic acid content of both *L. brevis* and *L. casei* showed a parallel trend of GABA profile change, indicating that both LAB strains might produce glutamic acid during fermentation, and part of the glutamic acid produced was consumed to produce GABA when the fermented coconut water became acidic after 24 h. A fermentation study without the addition of L-MSG also showed similar result whereby glutamine was converted into glutamic acid during the later stage of fermentation. Eventually, the glutamic acid was converted into small amount of GABA [19], implying that both glutamic acid and GABA were being produced in small quantity.

When exposed to the prolonged fermentation period under extreme conditions, GABA was metabolized in fermented coconut water to sustain the LAB growth under stress condition, resulting in the decline trend of GABA content as observed in all fermented coconut water. Zhuang *et. al.* also hypothesized that after 48 h of fermentation, GABA transaminase activity had to be restored to replenish pyruvate within the bacterial cells, leading to conversion of GABA to succinate semialdehyde and finally to succinate before entering the citric cycle [20].

3.3. Amino Acid Profiles

Table **1**. summarizes the changes in amino acid profiles of all LAB strains at the interval time of 24 h over 5 days fermentation period. Generally, most of the essential amino acids (EAAs) were consumed significantly after 24 h, particularly for *L. acidophilus* and *L. plantarum*. It was worth noting that *both L. acidophilus* and *L. plantarum*

exhibited similar pattern changes of EAAs metabolism. In contrast, *L. brevis* and *L. casei* did not consume EAAs as compared with the other two counterparts but still showed a decreasing trend of EAAs content. This finding was supported by past studies, which reported a reduction in EAAs after fermentation with LAB, specifically *L. acidophilus* [21]. It was initially thought that the reduction in EAAs by *L. acidophilus* and *L. plantarum* was used for the production of GABA; however, it was learned that the major pathway for GABA production utilizes glutamic acid, which is a non-essential amino acid (NEAA) [18]. Among all LAB fermented coconut samples, coconut water fermented with *L. brevis* remained with the highest amount of total soluble amino acids, especially for essential amino acids. This finding indicated that *L. brevis*-fermented coconut water could provide more beneficial effect for human consumption, as our body cannot synthesize essential amino acids. Whereas, *L.acidophilus*–fermented coconut water exhibited the highest content of GABA, indicating a great potential for functional beverage marketed as anti-stress coconut beverage.

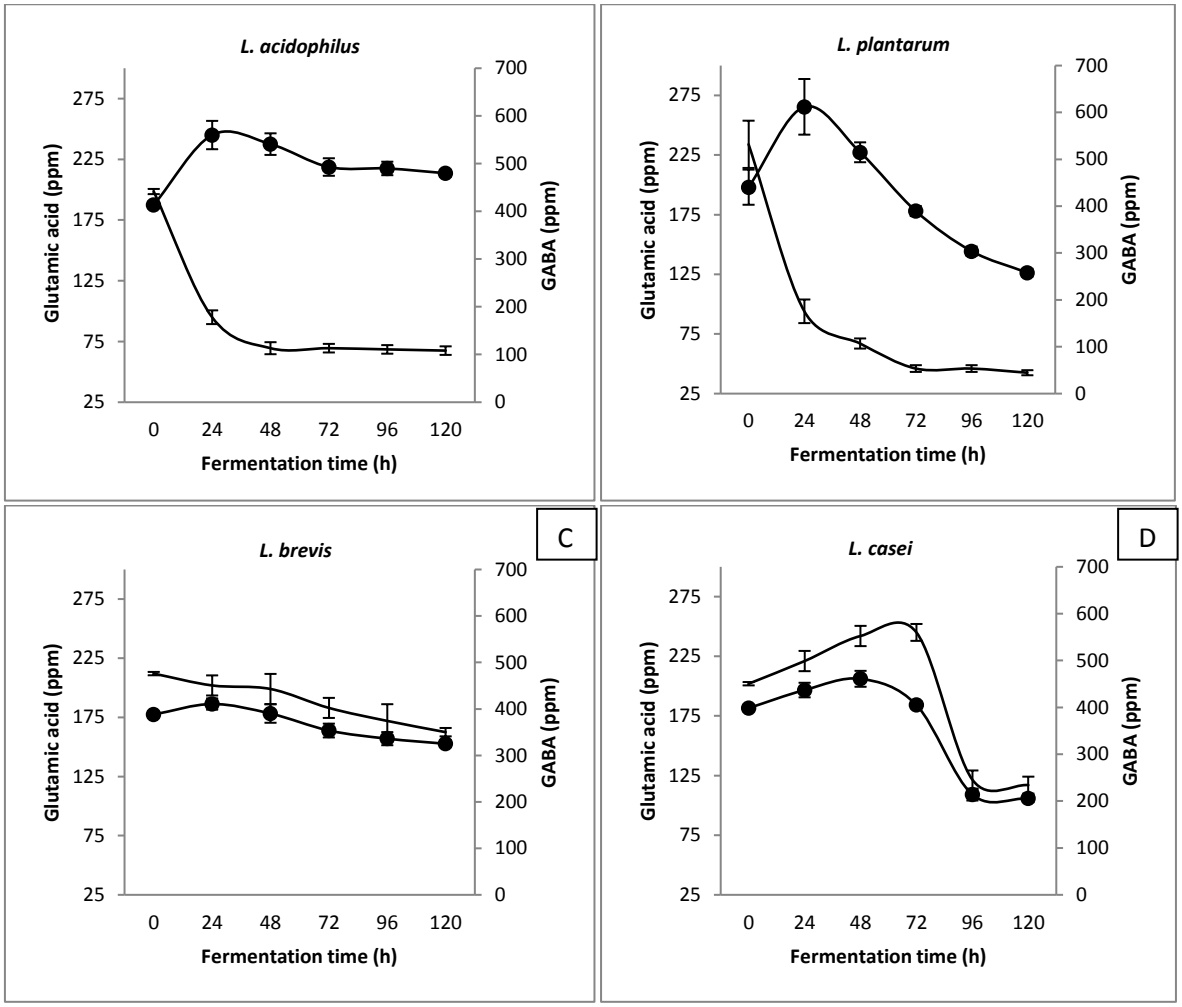

Fig. (2). Glutamic acid metabolism (–) *vs* GABA (●) production by *L. acidophilus***(A)**, *L. plantarum***(B)**, *L. brevis***(C)**, and *L. casei***(D)**. The results are presented as the mean of triplicates with standard deviations.

4. RESULTS

Among non-essential amino acids, it was noted that aspartic acid was heavily consumed by *L. acidophilus* and *L. plantarum* Fig. (**3A**). These fermented coconut water samples recorded higher increment in GABA content Fig. (**2**). It is unknown whether aspartic acid was consumed for the production of GABA or not. Nevertheless, aspartic acid was reported to be involved in stress response mechanism in LAB fermentation [22]. The conversion of aspartic acid to alanine and carbon dioxide through enzyme aspartic acid decarboxylase (AspD) was demonstrated in *L. acidophilus* [23] and *L. plantarum* [24]. On the other hand, Fig. (**3B**) shows a dramatic reduction in arginine content by *L. brevis* when compared to other strains of LAB. De Angelis's study reported the involvement of arginine in adapting environmental stress through the arginine deiminase (ADI) pathway [25]. Enzyme ADI helps to generate ATP from

arginine, allowing the LAB to cope with acidic stress condition. This phenomenon indicated that *L brevis* might consume arginine to adapt the stress of acidic environment, which displayed another stress response mechanism that was slightly different from other LAB strains. Based on the amino acid profile changes for *L. casei*-fermented coconut water, *L. casei* might utilize both glutamic acid and aspartic acids in its stress response system. Generally, it was concluded that different strains of LAB showed various trends of reductions in specific types of amino acids, indicating different stress response mechanisms in order to survive in acidic growth environment.

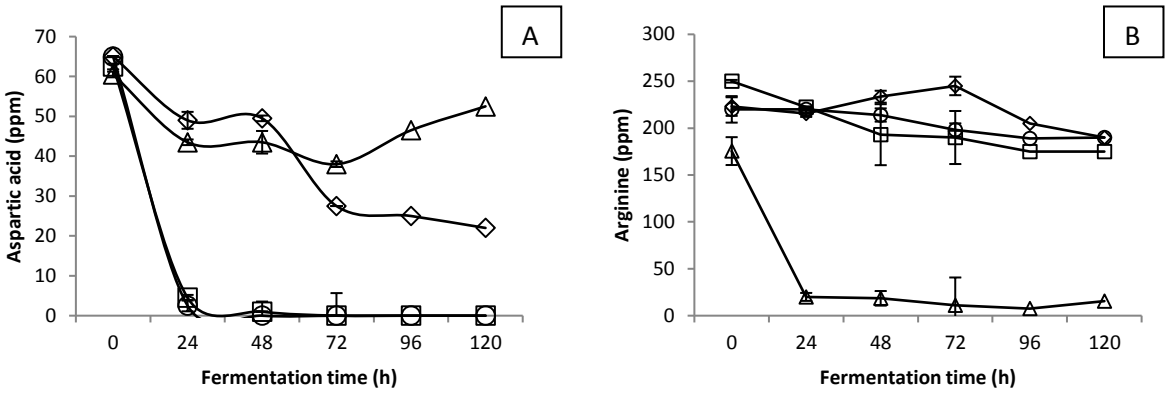

Fig. (3). Metabolism of aspartic acid **(A)** and arginine **(B)** by *L. acidophilus* (Ũ), *L. brevis* (Δ), *L. casei* (◊), and *L. plantarum* (°). The results are presented as the mean of triplicates with standard deviations.

CONCLUSION

Most mature coconut water fermentation with different strains of LAB produced GABA as a stress response mechanism in surviving under extreme acidic environment. Among four LAB strains, both *L. acidophilus and L. plantarum* produced higher GABA content with the increment of 35.4% and 38.9%, respectively. On the other hand, *L. brevis* utilized arginine as its best strategy in coping with acidic stress growth environment. The main contributing role of these LAB fermentations was to enhance the nutrient of MCW, particularly GABA content. Therefore, both *L. acidophilus and L. plantarum* were selected for further functionality study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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