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Evaluation of the Glucuronic Acid Production and Other Biological Activities of Fermented Sweeten-Black Tea by Kombucha Layer and the Co-Culture with Different Lactobacillus *Sp.* Strains

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Abstract: *The interactions between lactic acid bacteria (LAB) and acetic acid bacteria (AAB) from Kefir and Kombucha (KBC) have been concerned during the last decade since their positive stimulation on growth rate, biomass, and secondary metabolites. However, more study needs to be conducted to ascertain whether those enhancement can bring out actual benefits for human consumption. In this study, evaluation of three main healthy properties of KBC, which were glucuronic acid (GlcUA) concentration, antibacterial and antioxidant activities and under co-culture of LAB from Kefir is our main target. The combination of high-performance liquid chromatography (HPLC) and mass spectrophotometer (MS) detector was used to determine the GlcUA concentration. The agar-well diffusion method was used to test the antibacterial activities against three serious food borne pathogenic bacteria which were Escherichia coli ATCC 8739, Salmonella typhimurium ATCC 14028; Bacillus cereus ATCC 11778. 1, 1-Diphenyl-2picrylhydrazyl (DPPH) was applied to measure the radicals scavenging activity of KBC. The result shows that Lactobacillus casei can improve the GlcUA up to 39.6% while Lactobacillus platarum can enhance the antibacterial and antioxidant activities as a higher level comparing to the normal fermented tea and mixed culture with other LAB strains.*

***Keywords*:** *Antibacterial activities, DPPH, fermented tea, glucuronic acid, KBC.*

I. INTRODUCTION

Kombucha (KBC) known as "tea fungus" is a sour, tasty, and healthy drink obtained by the fermentation of sucrose-sweetened black tea. It has been consumed at least two thousand years in China BC and then spread out to Korea, Japan, Russia and now on over the world [1]. Many studies have shown that the microbial composition in KBC tea is symbiosis between acetic acid bacteria (AAB) and yeast[2]. They can be *Acetobacter* sp., *Gluconobacter* sp., *Bacterium* sp., *Saccharomyces* sp., *Zygosaccharomyces* sp., and *Pichia* sp., etc [3,4].

This fermented tea was considered as a healthy drink for human due to its detoxifying properties of glucuronic acid (GlcUA), inhibiting growth of pathogenic bacteria and antioxidant ability. The metabolites in KBC that mainly contribute to its value properties were identified as acetic, lactic, ethanol, glycerol, vitamin B complex, folic acid, D-saccharic acid 1,4 lactone (DSL), gluconic and GlcUA [5]. The GlcUA is the important key component found in KBC beverage which normally produces by a healthy liver [6]. It is well-known in an important process for detoxification and excretion of exogenous chemicals called glucuronidation [1] which was responsible for biotransformation of endogenous reactive metabolites, such as bilirubin, oxidized fatty acids and excess of steroid hormones. The conjugation of GlcUA with undesirable compounds, results in the decreased toxicity due to an increased solubility of them that further facilitates transport and elimination from the body. On the other hand, GlcUA can be converted in to glucosamine and associated with cartilage, collagen and the fluid which lubricate the joints [6]. GlcUA has many benefits to human health but its concentration level in KBC is not well documented and various from every culture since microbial symbiosis is depended on the different geographic and climatic conditions [7].

LAB presents a large amount in Kefir, a health benefit fermented milk produced by the symbiosis association mainly between LAB and yeast in Kefir grains [8]. On the other hand, Lactic acid bacteria (LAB) were also found in some certain KBC but in small amount and were not considered as the essential role in the symbiosis of tea fungus. Therefore it were not drawn much attentions in the past [9]. In recent years, the interest on bacterial co-operation between LAB from Kefir and AAB from KBC to improve the growth rate, biomass and their secondary metabolites has been concerned. In 2010, the symbiosis between LAB from Kefir and AAB

from KBC was investigated in improving D-saccharic acid 1,4 lactone (DSL) productions. The author has pointed out that the LAB can improve the survival of AAB and this combination would be an optimal mixed culture to enhance KBC function [9]. In 2011, LAB was applied as a core factor in a optimal conditions for KBC fermentation with AAB and yeast [10]. In 2013, Analy mentioned about the vitamin B complex which was secreted by AAB (*Acetobacter sp.*) may support a favorable environment for other growth of LAB and yeast in Kefir grains [8].

Although much works has been done to date to evaluate positive stimulation in growth rate, biomass, and secondary metabolites which were provided by the cooperation of *Lactobacillus sp.* from Kefir and *Acetobacter sp.* form KBC, actually human health benefiting properties form this combination remains unclear. Therefore, more studies need to be conducted to ascertain the significant role of LAB in mixed culture of KBC tea to enhance its three important functions for human consumption which were GlcUA concentration, antibacterial and antioxidant ability.

In this study, LAB strains were isolated form Kefir and cultured with KBC layer in the sweeten-black tea medium. The GlcUA production was determined by HPLC-MS, the antibacterial and antioxidant activities were also evaluated by agar-well diffusion method and DPPH radical scavenging capacity, respectively. Therefore, enhancement of three main functions of KBC tea by mixed culture of LAB and KBC layer is our main target. Moreover, this purpose is identified as being significance of the research in producing healthy and valuable fermented tea for the beverage industry since this kind of drink is so popular on over the world.

II. MATERIALS AND METHODS

2.1 Reagents, apparatus and medium preparation

Kefir grain, KBC layer was kindly provided by Biotechnology lab of University of Technology, Vietnam National University, HCMC and Food Technology lab of International University, Vietnam National University, HCMC. Black tea used in this study was Lipton black tea, a product of Unilever Company. Glucuronic acid (G5269-10G, Sigma), was used as standard in performance of HPLC-MS. The 1,1-diphenyl-2picrylhydrazyl (DPPH) (Sigma) was used in the antioxidant test. *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, and *Bacillus cereus* ATCC 11778 were used as the testing microorganism provided by Microbiologist USA Company. *Lactobacillus* de Man, Rogosa and Sharpe (MRS) medium culture and Trypton Soybean Agar (TSA) were provided by Himedia Company (India). The HPLC system (Agilent 1200) equipped with a MS detector (micrOTOF-Qll, Bruker) and ACE3 C-18 column (4.6 x 150 mm) was applied to determine the GlcUA concentration.

2.2 Prepare sweeten-black tea.

1000 mL of sweeten-black tea was prepared by adding Lipton black tea and sugar to fresh boiled water to the final concentration as 1 g/L and 100g/L, respectively. The mixture was autoclaved at 121 °C for 15 min.

2.3 Culture of KBC layer.

The KBC layers were weekly maintained in sweeten-black tea culture by adding 5g of wet layer in 100 mL of the total medium.

2.4 Fermented tea by single KBC layer and the mix culture with different LAB.

Pellet of 1 mL MRS broth culture of 8 x 10^8 different *Lactobacillus* strains were centrifuged at 10,000 rpm for 5 min at 4^0C, and then were added in sweeten-black tea. The mixed culture contained KBC layer and *Lactobacillus* pellet, the single culture contained only KBC layer. The fermentation was carried out in the following conditions: initial pH 5, 5 days, 30^0 C and the culture medium was 40% of the total volume vessel [10]. The unfermented sweeten-black tea was used as the negative control.

2.5 Isolation of *Lactobacillus spp.* strains from Kefir grain.

Kefir grain from Vietnam was maintained by serial subculture in defatted milk at 25^0C for 3 days and the bacterial strains were selected from the homogenous milk based on the growth habits and morphology. The single colony was exhibited on MRS after incubated at 37^0C, 72 hours under anaerobic condition.

The pure cultures were maintained and weekly transferred in MRS broth culture medium. Molecular sequences were identified by Nam Khoa Bioteck Company in HCMC. The nucleotide sequencing was analyzed by free BLAST soft -ware.

2.6 Quantification of glucuronic acid by HPLC-MS

The process was performed at Central Laboratory for Analysis in University of Science, Vietnam National University, HCMC. Tea sample was loaded through SPE C18 column then passed through Millipore filter (0.45 µm) before injected to HPLC vials. 20 µl of the filtrate was injected to a HPLC system equipped

with a MS detector and ACE3 C-18 column for the analysis. A mobile phase was 0.1% acid formic in deionized water, the stationary phase was 0.1% formic acid in methanol. The flow rate was maintained as 0.5 mL/min at room temperature (23-25^{0}C) of 210 nm wave-length. The resolution peaks were recorded on the HPLC chart according to the retention time of GlcUA as standards. The concentrations were quantified from standard curves and multiplied dilution factor.

2.7 Antibacterial activities by agar-well diffusion

Escherichia coli ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus* ATCC 11778 were used as the testing microorganism in agar diffusion method described [11]. 30 mL of TSA medium was poured in to 90mm diameter-Petri dish. A 5 day- fermented tea broth was obtained by 10,000 rpm centrifugation at 4^{0}C for 10 min, then the suspension was passed through 0,22 µm spore size filter papers. 100 µl of the sterilized tea samples were added into 9 mm diameter wells. The negative control well contained unfermented tea and the agar plate was incubated at 37^{0}C and the inhibited zone was observed at 18 hours for *E.coli* and *Bacillus*, 24 hours for *Salmonella.*

2.8 Determination antioxidant activities by DPPH

The antioxidant assay using DPPH were determined spectrophotometrically, according to the method described by [12] with slightly modification. 100 µM of concentration of Vitamin C solution was used as the positive control. The reaction contained of 0.5 mL sample was mixed with 1.5 mL of 250 µM ethanolic DPPH solution. The mixture of absolute ethanol 1.5 mL and sample 0.5 mL serve as blank. The absorbance was measured at 517 nm wave-length. The percentage of inhibition was calculated by following equations (1):

$$x\% = 100 - [\frac{(\mathrm{Abs}_{\mathrm{sample}} - \mathrm{Abs}_{\mathrm{blank}})\mathrm{x}\ 100}{\mathrm{Abs}_{\mathrm{control}}}$$

The experiment was performed in triplicate for each substance. The results were generated as percentage decrease with resp.ect to control values and compared by one-way ANOVA. A difference was considered statistically significant if $p \leq 0.05$.

III. RESULTS AND DISCUSSION

The symbiosis between yeast and AAB has long been known. The AAB support yeast to produce ethanol and then ethanol may sever as the substrate for the acetic acid production[5]. The combination of ethanol and acetic acid prevents competition from other pathogenic bacteria [3]. However, LAB does not draw much attention in KBC, and its roles in this kind of fermented tea have not been shown [9].

3.1 Isolation and identification of *Lactobacillus sp.* from Kefir grains.

The isolated bacterial strains showed morphology of rod shape, non-spore forming, gram positive under microscope, round and milky colonies on MRS agar. The results of nucleotide sequencing compared by BLAST software showed the three different bacterial strains was identified *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum.*

3.2 Evaluation the glucuronic acid production

The interest of GlcUA producing for food application has increased strongly during the last decade. Many studies have tried different methods to enhance the GlcUA producing by changing fermentation conditions and the culture medium as well, but this is the first report of using *Lactobacillus sp.* as the compositions of symbiosis in KBC.

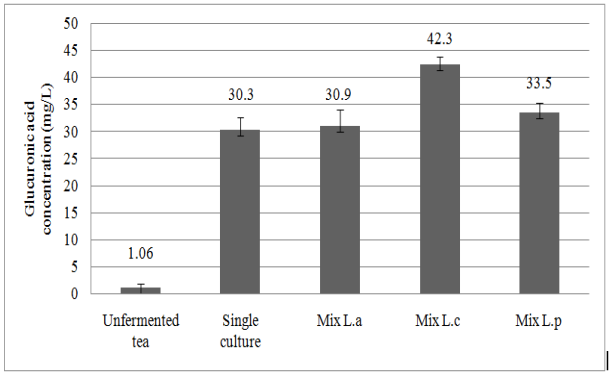

Fig. 1: The glucuronic acid production of KBC under the co-culture with each *Lactobacillus spp.* strain. Mix L.a: Mixed culture with *Lactobacillus acidophilus*, Mix L.c: Mixed culture with *Lactobacillus casei*, Mix L.p: Mixed culture with *Lactobacillus plantarum*, Single culture: The fermentation of sweeten black tea only by KBC layer. Unfermented tea: the sweeten-black tea.

At the 5th day of fermentation, the combination of *Lactobacillus casei* and KBC layer produced much more GlcUA about 39.6% than the single culture, which were 42.3 mg/L compared with 30.3 mg/L. In addition, *L.casei* can stimulate for the GlcUA production than the other LAB strains did.

According to Zhiwei, the co-culture of LAB with AAB in KBC tea can also stimulate the D-saccharic acid 1,4 lacton (DSL) a compound related to indirect anticancer activities, from 4,86% up to 86.7%. Being parts of glucuronate pathway, the presence of DSL can determine the existence of GlcUA and allow it to repel toxicant easier include carcinogen, some tumor promoters, and hepatotoxin [9]. So LAB in Kefir can promote the DSL production in KBC, they may also improve the GlcUA production.

The amount of GlcUA produced by the single culture and by the mixed culture with *L. casei* in this study were 30.3 mg/L and 42.3 mg/L, higher than those reported by Blanc [13] and Loncar [14] were only 10 mg/L and 3,39 mg/L, respectively. In those researches, there are very similar fermented conditions such as: black tea, concentration of sucrose, temperature and even longer duration (days) so, the GlcUA concentration may different since the various symbiotic culture [7].

The more study needs to be conducted to ascertain *L. casei*, a potential LAB, that can be applied for KBC tea to improve many other health benefits for human.

3.3 Evaluation the antibacterial activities

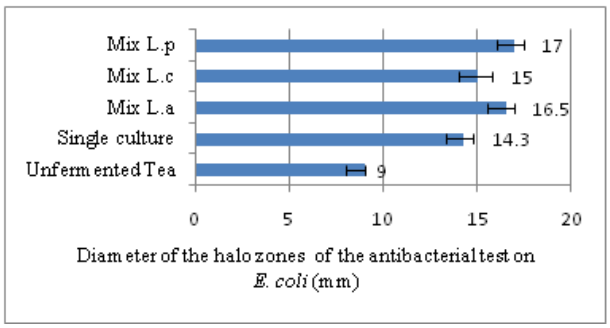

Fig. 2a**:** The antibacterial activities of KBC tea on *E. coli*

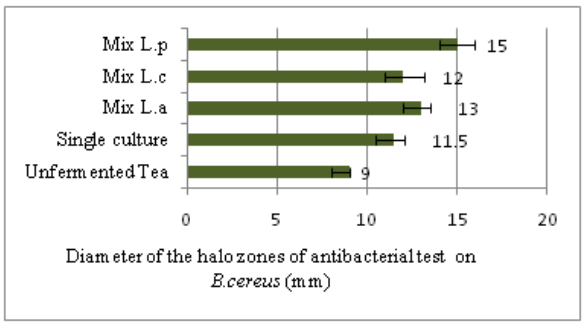

Fig. 2b: The antibacterial activities of KBC tea on *B. cereus*

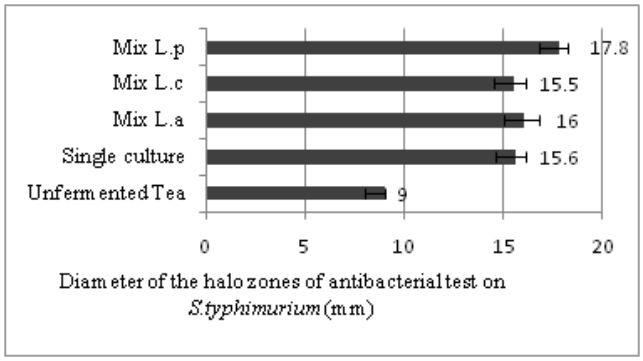

Fig. 2c**:** The antibacterial activities of KBC tea on *S. typhimurium*

Fig2a, 2b, 2c: The antibacterial test of KBC tea under the co-culture of different *Lactobacillus sp.* Mix L.a: Mixed culture with *Lactobacillus acidophilus*, Mix L.c: Mixed culture with *Lactobacillus casei*, Mix L.p: Mixed culture with *Lactobacillus plantarum*, Single culture: The fermentation of sweeten black tea only by KBC layer. Unfermented tea: the sweeten-black tea. The diameter of the well: 9 mm.

The observation on antibacterial activities of KBC tea shows the clear inhibition on three pathogenic bacteria. The longest diameter of the halo zones recorded on *E. coli, B. cereus, S. typhimurium* were 17, 15, 17.8 (mm) respectively, by the fermented tea of KBC layer and *L. plantarum.* The halo zone of the unfermented tea sample was not recognized, so the data were presented in 9 mm- diameter of the well. In some reports, the tea extract or unfermented tea can against *S. tiphimurium* but does not effect on *E. coli* [15]. It is different from our study the unfermented tea sample inhibits none of these strains. However, this results contributed to the significant antibacterial abilities of fermented tea in control the growth of *E. coli, B. cereus* and *S .tiphimurium* because these microorganisms are responsible for diarrheal disease, food-borne illness, gastroenteritis and enteric fever, which are still the most serious and important public health problems in many developing countries [16,17]. Findings more about the effect of fermented tea are so helpful because KBC is popular drinks

on over the world. The results can use for producing an oriented valuable drink that improve the gastrointestinal systems. Moreover, the combination of *Lactobacillus* in this research also provides a healthy, functional and potential probiotic property for human consumption [18].

3.4 Evaluation the antioxidant activities

This systematic study determines the direct antioxidant potential of KBC tea, fermented by single culture and mix culture with different *Lactobacillus spp.*, against a spectrum of oxidant 1,1-diphenyl-2picrylhydrazyl (DPPH). The mechanism of antioxidant properties on DPPH radical scavenging may because of their hydrogen-donating ability. DPPH scavenger capacity of tea sample was compared with the known antioxidant substance as vitamin C.

As the results, the level of DPPH decolonization of KBC sample shows no significant differences between the mixed culture of KBC layer with *lactobacillus spp.*, and the single KBC layer, which were 80.28% to 88.56%. The mix culture of *L. platarum* and KBC layer shows the higher antioxidant activities compared to other combinations. The positive control, vitamin C showed the highest percentage of 91.15 % and the lowest 63.63% belong to the unfermented tea. The antioxidant properties of KBC tea may high because of vitamin C, Vitamin B, DSL synthesized during the fermentation. This result is lower than those reported in lemon-blam KBC, which the antioxidant percentage was about 95.5% [19]. The reason may because of the difference in amount used and antioxidant compounds of black and lemon-blam tea.

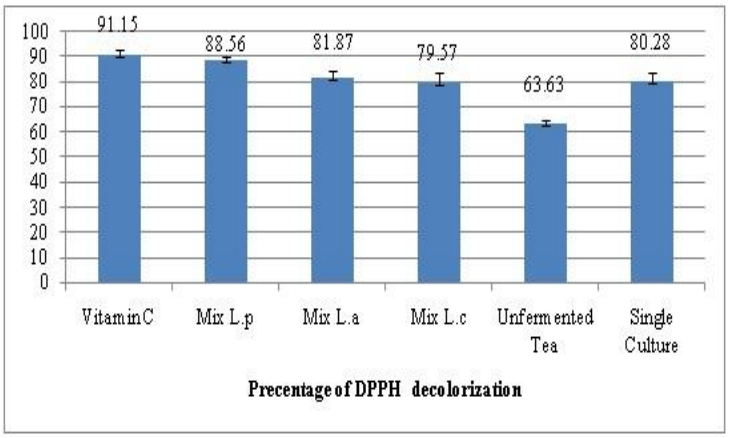

Fig 3: The DPPH antioxidant test of KBC tea under the co-culture of *Lactobacillus* sp. Mix L.a: Mixed culture with *Lactobacillus acidophilus*, Mix L.c: Mixed culture with *Lactobacillus casei*, Mix L.p: Mixed culture with *Lactobacillus plantarum*, vitamin C: a standard vitamin C served as the positive concentration. Single culture: The fermentation of sweeten black tea only by KBC layer. Unfermented tea: the sweeten-black tea.

IV. CONCLUSION

In this study, *Lactobacillus casei* and *Lactobacillus plantarum* have advantages in improving the glucuronic acid concentration and antibacterial, antioxidant activities in the original KBC tea. These enhancements of biological functions of KBC tea may contribute to the orientation of fermented drink production for human. However, more study need to be investigated about the customer's taste, order, and overall appearance of the mixed culture compared to the original KBC tea. On the other hand, this study is identified as being significance of the research in producing healthy and valuable fermented tea for the beverage industry since this kind of drink is well-known through many countries on over the world.

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