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Antioxidant activities of postbiotics produced by several *Lactobacillus plantarum* strains using reconstituted media

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الملخص

أجريت الدراسة الحالية لتقدير نشاط مضادات الأكسدة لنواتج أيض منتجة من سلالات مختلفة لبكتريا *Lactobacillus plantarum* النامية في بيئة غذائية مركبة. كذلك، لتحديد أي من السلالات أكثر نشاطاً كمضاد للأكسدة وفقاً للالتهامها الشقوق الحرة. باستخدام البيئة الغذائية المركبة، ستة سلالات من *L. plantarum* (RG11 ، RG14، RI11، UL4، TL1 RS5) استخدم لإنتاج نواتج الأيض. قيس نشاط مضاد الأكسدة للنواتج بواسطة أختباري {2,2-Diphenyl-1-Picryl-hydrazyl} & { 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)}. لوحظ نشاطاً أعلى معنوياً كمضاد للأكسدة في سلالة RI11 مقارنة بالسلالات الأخرى، في حين كانت UL4 و RS5 أعلى معنوياً (P<0.05)مقارنةً بسلالات RG11 ، RG14، TL1. لكن، لم تلاحظ اختلافات معنوية بين RG11، RG14 وTL1 في اختبار نشاط الالتهام لنواتج الأيض. كلا الطريقتين أوضحت امتلاك السلالات البكتيرية المستخدمة في الدراسة نشاطاً مضاداً للأكسدة.

الكلمات المفتاحية:

اكتوباكيللوس. أخمصي. بوستبيوتيكس. النشاط المضاد للأكسدة.

Abstract

The current study was conducted to determine the antioxidant activity of postbiotics produced by various strains of *Lactobacillus plantarum* grown in reconstituted media, as well as to identify the one with the best antioxidant activity based on scavenging free radicals. By using reconstituted media**,** six strains of *L. plantarum* (RG11, RG14, RI11, UL4, TL1, and RS5) were used to produce the postbiotics. The antioxidant activity of the postbiotics was determined using the 2,2-Diphenyl-1-Picryl-hydrazyl (DPPH) assay and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. A significantly higher antioxidant activity of postbiotics was observed in RI11 compared to other postbiotics, whereas UL4 and RS5 were significantly higher (P<0.05) compared with RG11, RG14, and TL1. However, no significant difference was recorded between RG11, RG14, and TL1 in the postbiotics scavenging activity. Both the DPPH assay and ABTS radical cation scavenging potential showed that the various strains possess antioxidant activity.

Keywords: *lactobacillus; plantarum*; postbiotics; antioxidant activity.

1. INTRODUCTION

Several studies have investigated the effects and efficacy of postbiotics on essential aspects of animal health and production. However, heat stress remains a vital cause of suboptimal production in poultry, especially in the tropics [1]. Heat stress is likely to induce oxidative changes and increase free radicals in the cell [2], reducing feed intake, live weight gain, feed efficiency [3], immune suppression [4]. Thus, the growth performance of poultry will be negatively affected. Nevertheless, no study has been conducted to evaluate the postbiotics as a potent antioxidant activity in broiler under heat stress conditions. Therefore, the objectives of this study were to determine the antioxidant and inhibitory activity of postbiotics obtained from different strains of *Lactobacillus plantarum* in order to be applied in the broiler feed under heat stress.

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2. MATERIALS AND METHODS

2.1. Medium preparation

The research was conducted in the Microbiology Laboratory at the Institute of Bioscience at the Universiti Putra Malaysia. The strains of *L. plantarum* were sub-cultured and cultivated using de Man-Rogosa-Sharpe (MRS) medium (Merck, Darmstadt, Germany). The postbiotics (RG11, RI11, UL4, RG14, RS5, and TL1) were prepared using the reconstituted media of *L. plantarum* in accordance with their respective composition.

2.2. Bacterial reviving and postbiotic preparation

The strains of *L. plantarum* used in this study were the previously isolated strains obtained from Malaysians fermented *tapioca*, *Tapai Ubi* (TL1, UL4, RG11, RI11, RG14, RS5) [5, 6]. In addition, the reviving and postbiotic preparation was carried out based on the method of Foo et al., [5, 6]. The strains were kept at - 20°C in MRS broth containing 20% (v/v) glycerol. One per cent of stock culture was revived in 10 mL MRS broth,

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incubated at 48 h and sub-cultured in the same media for another 24 h. Thereafter, the revived cultures were spread on a plate and incubated at 30°C for 48 h. A single colony was picked from the plate, inoculated twice into MRS broth (10 mL) and incubated for 48 h and 24 h, respectively. To produce the postbiotics, the active culture of the *L. plantarum* strains was sub-cultured in MRS broth [5]. Each of the respective reconstituted media was inoculated with the *L. plantarum* strain (1%; v/v) and incubated at 30°C for 24 h. Finally, following the separation of the bacterial cells using centrifugation at 10,000 ×*g* for 15 min at 4°C, the postbiotics were collected and used for the antioxidant and inhibitory activity assay.

2.3. Antioxidant activity assay

The 2,2-Diphenyl-1-Picryl-hydrazyl (DPPH) assay and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) assay were used to determine the antioxidant activity of all the 6 types of postbiotics obtained from the six *L. plantarum* strains (RG14, RG11, RI11, TL1, RS5, and UL4) as described by Chan et al. [7].

2.4. DPPH free radical scavenging assay

With slight adjustments, the procedure described by Chan et al. [7] was used in measuring the free radical scavenging activity of the various postbiotics. Briefly, 10 µL of each sample and 390 µL of freshly prepared 0.2 mM DPPH solution in methanol were evenly reacted. Thereafter, the mixture was incubated for 60 minutes in a dark chamber and centrifuged at 7500 rpm for 10 minutes at 15°C. The supernatant (200 µL) was loaded into a 96-well plate, while the absorbance of the sample was measured at 517 nm (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). Overall, the average absorbance value was estimated after conducting the assay in triplicates. The reference antioxidant used was ascorbic acid. The equation shown below was used in calculating the percentage of the scavenging activity of the DPPH free radical:

Scavenging percentage = $[(Ac - A_s / A_c] \times 100$

Where A_c = absorbance of control, A_s = absorbance of the sample.

2.5. ABTS radical cation scavenging assay

Determination of the scavenging activity of the ABTS radical cation was conducted as described by Chan et al. [7] with slight adjustments. The preparation of the ABTS radical cation comprised a reaction between 50 mL of 7 mmol/L ABTS stock solution and 50 mL of 2.45 mmol/L potassium persulfate at ambient temperature for 2h in the dark. Dilution of the ABTS solution was done using ultrapure water with an absorbance of 0.70 ± 0.02 at 734 nm. Also, in a 96-well, a reaction was done using 190 µL of the adjusted solution and 10 µL of sample in the dark for 10 minutes and maintained at ambient temperature. Lastly, using a spectrometer (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan), the absorbance of the reaction mixture was noted at 734 nm. The standard antioxidant used was ascorbic acid and all the determinations were conducted in triplicates. The following equation shows the calculation of the ABTS scavenging ability (%):

ABTS scavenging ability percentage = $[(A_C - A_S / A_c] \times 100$

Where A_c = Absorbance of control, A_s = Absorbance of sample.

2.6. Statistical Analysis

Each of the samples was measured in triplicates. All the statistical analyses were carried out using SAS program version (9.4). The mean and the corresponding standard error (SE) were used in the data presentation. One-way ANOVA with General Linear Model procedure was applied as a specific inferential statistical test, while Duncan multiple range tests were used to compare the means of treatments at 5% probability.

3. RESULTS

Antioxidant activity of postbiotics

The result of the DPPH scavenging potential of cell-free extracts is presented in Figure 1.

Findings showed that radical scavenging activity from both assays was at effective levels for most of the culture filtrates. Specifically, RI11 showed the maximum antioxidant activity (62.4%), followed by UL4 and RS5 corresponding to 58.6% and 48.7%, respectively. A significantly higher antioxidant activity was observed for RI11 compared to the other metabolites. Also, the antioxidant activity of UL4 (58.63%) and RS5 (48.67%) was higher ($P<0.05$) compared with RG11, RG14, and TL1 (29.98, 28.74 and 27.80%, respectively). However, there were no significant differences in the scavenging activity between the later three postbiotics.

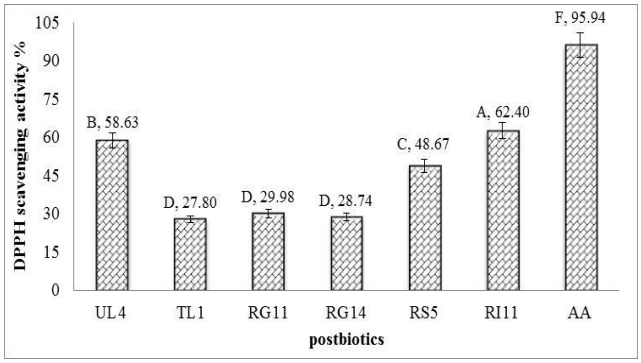

Figure 1. DPPH radical scavenging activities percentage of six postbiotics (UL4, TL1, RG11, RG14, RS5, and RI11) produced from *Lactobacillus plantarum* strains.
AA (ascorbic acid) as a positive standard
Common letters are significantly different (P<0.05).

Figure 2 shows the ABTS radical cation scavenging potential of the cell-free extract of various strains of *L. plantarum*. The scavenging potential was present in all the postbiotics and ranged from 18.1 to 38.6%. In this regard, RI11 recorded the highest activity followed by UL4, RS5, RG14, RG11 and TL1 corresponding to 38.6, 34.4, 30.9, 24.6, 18.3, and 18.1%, respectively. However, there was no significant difference in the antioxidant activity between RG11 and TL1. The recorded antioxidant activity for RS5 (30.9%) was significantly higher compared with RG11, RG14, and TL1. Also, that of RG14 (24.6%) was significantly higher than RG11 (18.11%) and TL1 (18.25%), but lower when compared with RI11, UL4, and RS5 corresponding to 38.64, 34.42 and 30.85%, respectively. The

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postbiotics TL1 and RG11 recorded the lowest antioxidant values (18.3%).

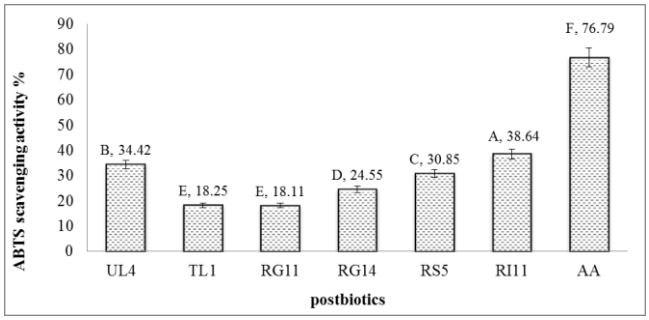

Figure 2. ABTS radical scavenging activities percentage of six postbiotics (UL4, TL1, RG11, RG14, RS5, and RI11) produced from *Lactobacillus plantarum* strains.
AA= ascorbic acid (positive standard)
Common letters are significantly different (P<0.05).

4. DISCUSSION

Oxidation is a vital mechanism for energy provision and supporting biological processes in living organisms. Nevertheless, oxidative stress occurs when the concentration of oxygen exceeds the normal level [8]. As such, the anti-oxidant supplies from the body lag behind the required level to annul free radicals [9]. Reactive oxygen species (ROS) such as peroxides and oxygen ions produced either exogenously or endogenously are often associated with oxidative stress [10]. Hence, these products are very reactive and could damage the biological molecules including DNA, proteins, lipids, and other oxygen species [11].

The aforementioned effects are prevented by antioxidants and therefore, their development and utilization remain an aspect receiving much attention. For instance, the DPPH assay is based on the principle that hydrogen donors have antioxidant properties. The contents of postbiotics such as organic acid are electron donors due to the presence of hydroxyl groups (electron-donating substituents), which enhances the scavenging activity of free radicals. For example, LAB has been demonstrated to resist ROS, such as hydroxyl and peroxide radicals [11].

LAB; *L. plantarum* have been identified in Malaysian fermented tapioca Tapai Ubi foods [5, 6] and the isolated strains of postbiotics were used in the present study. Similarly, Ji et al. [12] isolated several strains from Kimchi; a Korean traditional fermented food. Several studies have investigated the scavenging activity of *L. plantarum* strains isolated from various food sources [13-16]. For instance, Li et al[13] reported antioxidant activity from the metabolites produced by *L. plantarum* strains from Chinese fermented foods. Similar radical-scavenging activity was observed in the majority of the *Lactobacillus* strains investigated by Mikelsaar and colleagues [17]. While using DPPH assay, Gao [18] successfully investigated the antioxidant activity of *L. rhamanosus.* Hence, these elaborated findings were confirmed in our study.

In the present study, antioxidant activities occurred in various degrees (28% - 62.4%) amongst the isolated *L. plantarum* strains. The outcome is typical of previous studies reporting strain-specificity in antioxidant activity among *Lactobacillus* spp. [15,16]. Likewise, others reported that the antioxidant activity of Lactobacilli was high and strain-dependent especially among the facultative and obligatory hetero-fermentative group [19]. For instance, Khalil et al.[20] found antioxidant activities ranging from 32.3 to 74% amongst the evaluated *Lactobacillus* strains obtained from food sources. As described by Wang et al.[11] such observation could be attributed to enzyme production or cell surface molecules.

The bacteria enzymes observed to be beneficial in terms of antioxidant properties include glutathione peroxidase, nicotinamide adenine dinucleotide (NADH)-oxidase, superoxide dismutase, and NADH-peroxidase [13]. However, there are indications of both enzymatic and non-enzymatic roles in antioxidant capacity. Such events were demonstrated in the studies where cell-free extracts of *Lactobacillus* spp had higher antioxidant capacity compared with the whole cell structure [21]. ROS and reactive nitrogen species scavenging features were also suggested as the attribute for the antioxidant activity of non-enzymatic probiotics [22].

L. plantarum strains are well-recognised producers of exopolysaccharide (EPS). Antioxidant activity has been demonstrated in EPS containing bacteria strains [23, 24]. Using ABTS, *L. plantarum* strains obtained from camel milk were found to increase the scavenging rate in the EPS culture + cheese by >60% [25]. Using the same assay, similar findings were obtained in the current study based on the varying scavenging activity amongst the *L. plantarum* strains. These antioxidant activities, for instance, decreased and increased levels of malondialdehyde and serum glutathione peroxidase, respectively [15] and were related to the ability of the bacteria to colonize and survive in the intestinal tract [14]. Other authors attribute the activity to the increased level of uronic acid [23, 24]. Ji et al [12] also reported antioxidant activity following ABTS assay of culture filtrates of 11 strains (A1, A2, A3, A4, E1, E2, E3, E4, S1, S2, and S3) from *Lactobacillus* spp. The ABTS scavenging activity observed in our study (18-37%) was lower compared to the latter study (around 50%). Nevertheless, our results were consistently lower based on the antioxidant activity compared with the control (ascorbic acid, 95.94% and 76.79% Figure 1 and 2, respectively).

The postbiotics derived from different strains in the current study have significant antioxidant activity and could be relevant to the development of health-related products and functional foods [11]. As suggested by other authors, antioxidant effects found in *Lactobacillus* spp. strains are potential preventive measures against oxidative stress-related diseases [12].

5. CONCLUSIONS

The study established that postbiotics derived from *L. plantarum* strains have antioxidant properties and inhibitory effects against the evaluated pathogenic microorganisms. Nevertheless, the antioxidant activity was strain-dependent. The postbiotics; RI11, UL4, and RS5 showed the highest antioxidant activity. Therefore, RI11, UL4, and RS5 could be used as alternatives for antioxidants and antibiotics.

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