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Enhancing ergogenic performance and antioxidant benefits of red sugarcane juice through probiotic fermentation

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Abstract
The potential of red sugarcane as a functional probiotic drink was investigated, with a focus on determining its physicochemical, ergogenic, and antioxidant activities. Three different variants of Malaysian red sugarcane, namely *Ragnar*, *Kapur*, and *Serai*, were selected for analysis. The concentrations of electrolytes (Na, K, Ca, Mg, Zn, and Fe) in sugarcane juice were determined using an inductively coupled plasma mass spectrophotometer (ICP-MS), while the antioxidant activity and Vitamin C were assessed through colorimetric assays. Additionally, the functionality of the optimal variant, *Ragnar*, was enhanced by inoculating it with lactic acid bacteria (LAB) *Lactobacillus plantarum* ATCC8014 and *Lactobacillus casei Shirota*. High antioxidative properties (TPC: 71.63 mg GAE/mL, FRAP: 2.76 mmol TE/mL, DPPH EC_{50}: 55.66 µg/mL, and Vitamin C: 0.72 mg/100 g) were observed in the *Ragnar*, which tends to exhibit an attractive blue-yellow hue. K exhibited the highest concentration in all samples (126.31 – 229.95 mg/mL), followed by Na, Mg, Fe, and Zn, which exceeded the commercial isotonic drink. The viability of LAB (above 10^7 CFU/mL) and the production of gamma-aminobutyric acid (GABA) were satisfactory while reducing sugars were generally lower after the fermentation. The findings present red sugarcane as a potential natural source for the development of functional drinks.

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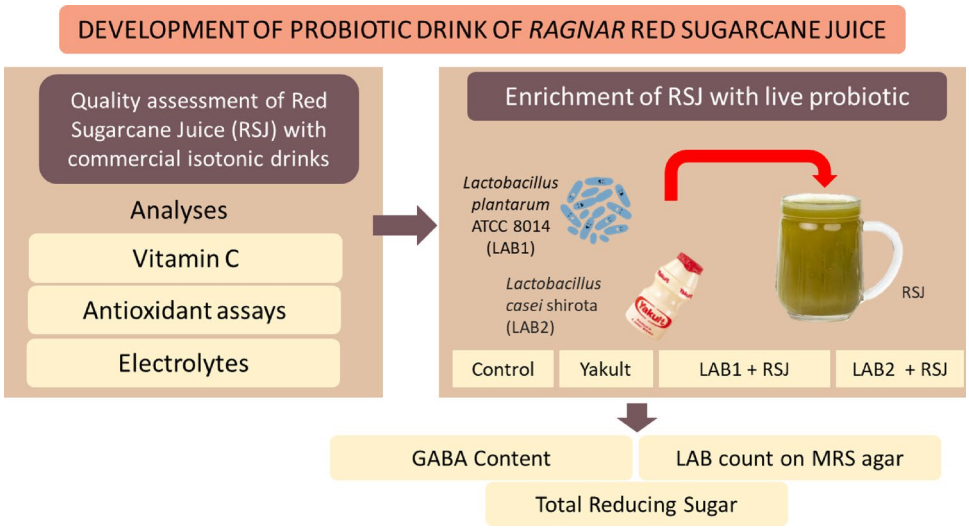
Graphical Abstract



Keywords Functional food · GABA · Ergogenic · Lactic acid bacteria · Antioxidant · *Lactobacillus*

1 Introduction

Sugarcane, belonging to the genus *Saccharum* and the *Gramineae* family, is an economically significant crop. In Malaysia, it has been cultivated since the nineteenth century primarily for sugar production. Certain sugarcane variants are known for their juice which possesses exceptional sensory characteristics, including taste, aroma, and flavor. The local identification of sugarcane is based on the color of the rind [1], with yellow being the most common type for juice production, followed by red or black sugarcane [2].

Consumption of sugarcane juice is often regarded as an instant energy booster due to its high content of simple sugars such as glucose, fructose, and sucrose, which are readily absorbed by the body [3]. Scientific studies have shown that sugarcane extract exhibits a wide range of biological effects, including immunostimulation, anti-thrombosis activity, anti-inflammatory activity, vaccine adjuvant properties, modulation of acetylcholine release, and anti-stress effects [4]. Moreover, sugarcane juice, being a natural plant-based source, contains significant antioxidant activities and compounds derived from phenolics and flavonoids [5, 6]. These antioxidants play a crucial role in neutralizing free radicals generated during physical activities, thereby reducing oxidative stress and protecting the cells [7, 8].

Furthermore, the presence of electrolytes in sugarcane juice contributes to its ergogenic properties, which aid in restoring and improving metabolic activity after strenuous catabolic processes [9, 10]. Well-known ergogenic aids such as calcium, sodium, and potassium are effective in replenishing lost metabolites in the body [10]. While artificial liquid energy drinks formulated with these metabolites are commonly used, they often lack the additional benefits derived from plant-derived biological compounds, such as phenolic compounds.

An interesting approach to enhance the nutritional quality of sugarcane juice is the addition of probiotics, which can improve substrate composition compositions [11, 12] and contribute to a positive impact on human health via live bacteria [13, 14]. Additionally, certain LAB and fermented foods can lead to the production of gamma-aminobutyric acid (GABA), as demonstrated by the use of *Lb. plantarum* in a whey protein drink [15]. GABA is present in specific plant-based foods, such as sugarcane, and has demonstrated various exercise-related benefits [6]. These include promoting exercise-induced muscle hypertrophy, regulating cardiac arrhythmias, controlling blood pressure and lipids, and enhancing liver function [16]. Together with the ergogenic- and health-inducing properties of sugarcane juice, inoculating red sugarcane juice (RSJ) with probiotics is sensible as a functional drink to replace conventional probiotic drinks for both regular and actively exercising individuals.

Therefore, this study aims to assess the qualities of different variants of RSJ, exploring their potential as a natural functional drink. The physicochemical screening was conducted to determine the sugars and antioxidant content, while mineral content was measured using inductively coupled plasma mass spectrometry (ICP-MS). Lastly, two strains of Generally Recognised as Safe (GRAS) Lactic Acid Bacteria (LAB), namely *Lb. casei* subs *Shirota* and *Lb. plantarum* ATCC8014 was introduced to evaluate their impact on the RSJ. The assessment included measurements of LAB viability, reduction in sugar content, and the production of the bioactive neurotransmitter, GABA.

2 Materials and methods

2.1 Sample collection

The samples of red sugarcane juice (RSJ) with varieties *Ragnar, Kapur,* and *Serai* were obtained from a sugarcane farm in Kedah, Malaysia. The collection was performed in accordance with local and national guidelines. They were freshly harvested using a three-roller power crusher and filtered using a 6-layered muslin cloth. In this study, the different varieties were designated based on the species names. The subsequent transformation into a probiotic beverage involved using an established probiotic drink (Yakult) as the positive control, while non-inoculated *Ragnar* RSJ served as the negative control. The introduction of probiotics into *Ragnar* RSJ, with *Lb. plantarum* is referred to as LP, and with *Lb. casei Shirota* as YSJ.

2.2 Quality assessment of sugarcane juice

Various measurements were conducted on the RSJ, including Total Soluble Solids (TSS) using a refractometer, pH using a pH meter, and color analysis using a colorimeter (HunterLab, USA). Triplicate values were recorded and expressed in standard units (°Brix for total soluble solids and CIELAB color space for color analysis).

The reducing sugar content of the RSJ samples was determined using the dinitrosalicylic colorimetric (DNS) method. The DNS reagent was prepared by dissolving 1.5 g of DNS in 30 mL of 2M NaOH. A solution of sodium potassium tartrate (Rochelle salt) was prepared by dissolving 45 g in 75 mL of water. A standard glucose solution of 1 mg/mL was prepared. Samples were prepared by pipetting a standard sugar solution into test tubes, which were then filled to 3 mL with distilled water. Each sample was mixed with 1 mL of DNS reagent and incubated in a boiling water bath for 15 min. After cooling, 1 mL of a 40% Rochelle salt solution was added to stabilize the color. The absorbance was measured at 540 nm with a spectrophotometer, with all tubes cooled to room temperature before reading due to the temperature sensitivity of the absorbance.

2.3 High-performance liquid chromatography (HPLC)

The determination of gamma-aminobutyric acid (GABA) content was conducted using HPLC (Shimadzu LC 20AT, Shimadzu Corporation, Kyoto, Japan). The analysis utilized a Chromolith® RP-18 column (100 mm length × 4.6 mm internal diameter) provided by Merck KGaA (Darmstadt, Germany). In a nutshell, 1 g of RSJ underwent centrifugation, and 10 µL of the resulting supernatant was collected for vacuum evaporation (derivatization). The dried supernatant was reconstituted in a solution of ethanol, water, trimethylamine, and phenylisothiocyanate, followed by immediate vacuum evaporation. Subsequently, another solution comprising ethanol, water, triethylamine, and phenylisothiocyanate was added, and the sample was left for 20 min to facilitate phenylisothiocyanate-GABA formation. After vacuuming to remove excess solvent, the sample was diluted and subjected to HPLC analysis. Mobile phase A (pH 5.8) contained sodium acetate, trimethylamine, and acetic acid in deionized water, while mobile phase B consisted of acetonitrile and deionized water. Both mobile phases underwent filtration. The sample, injected at 5 µL, was eluted at a flow rate of 0.6 mL/min using an isocratic elution of 80% mobile phase A + 20% mobile phase B. Compound identification was performed at $\lambda = 254$ nm using a diode array detector, and GABA content was calculated by comparing the sample peak area with the GABA standard [17–19].

2.4 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay

DPPH free radical scavenging activity was assessed by adding varying concentrations of RSJ to a DPPH solution. Fresh DPPH reagent (3.94 mg in 100 mL methanol) was shielded from light. RSJ samples (6.25–100 µg/mL) were added to a 96-well microplate with DPPH reagent, incubated, and absorbance measured at 517 nm after 30 min. Results were obtained using a BIO-RAD 170–6930 Microplate Spectrophotometer. Controls included a negative control with distilled water and a sample blank. Radical scavenging activity was calculated as the percentage of inhibition, and the EC_{50} value was determined. All analyses were conducted in triplicate for accuracy and reproducibility. The EC_{50} value, representing the concentration required for 50% scavenging activity, was determined based on Eq. 1 [20].

$$Radical\ Scavenging\ Activity(\%) = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100 \quad (1)$$

2.5 Ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was conducted by adding diluted samples to a working FRAP reagent and incubating the mixture. In short, a fresh FRAP reagent was prepared by combining 300 mM acetate buffer (pH 3.6), a 10 mM solution of 4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and a 20 mM ferric chloride solution in a ratio of 10:1:1 (v:v:v). Each sample underwent a ten-fold dilution, with 20 µL added to a well in a 96-well microplate, followed by the addition of 180 µL of the prepared FRAP reagent. A reagent blank was established by mixing 20 µL of distilled water with 180 µL of the FRAP reagent. The absorbance of the reaction mixture was measured at 593 nm using the BIO-RAD 170–6930 Benchmark Plus Microplate Spectrophotometer after incubating the plate at 37 °C for 4 min. To create a standard calibration curve, the procedure was replicated using aqueous solutions of ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) at various concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM). FRAP assay results were expressed as antioxidant activity in millimoles of Trolox equivalent per milliliter (mmol TE/mL) of the sample, utilizing a standard calibration curve of the aqueous solution of $FeSO_4{\cdot}7H_2O$ [21, 22]. Each sample analysis was conducted in triplicate.

2.6 Total phenolic content (TPC) assay

TPC was determined using a modified Folin-Ciocalteu assay. A 10% Folin-Ciocalteu reagent (FCR) solution was prepared by diluting 1 mL of FCR with 9 mL of distilled water, while a 7.5% sodium carbonate solution was made by dissolving 0.75 g of sodium carbonate in 10 mL of distilled water. Subsequently, 20 µL of ten-fold diluted RSJ samples were added to 330 µL wells in a 96-well microplate, followed by the addition of 100 µL of the prepared 10% FCR to each well and a five-minute incubation at room temperature. Afterward, 80 µL of the 7.5% sodium carbonate solution was added to each well, mixed, and incubated for 30 min under aluminum foil cover to shield from light. Absorbance was measured at 750 nm using a microplate spectrophotometer, and readings were recorded with software. Standard calibration curves were generated using gallic acid solutions, and results were expressed as milligrams of gallic acid equivalent per milliliter (µg GAE/mL) of the sample calculated using Eq. 2 [23]. All experiments were performed in triplicate, and absorbance readings were taken using a microplate spectrophotometer.

$$Total\ phenol\ content\ (mg\ GAE/mL) = GAE\ (mg/mL)\ x\ DF \quad (2)$$

2.7 Vitamin C analysis

The Vitamin C analysis was conducted using the 2,6-dichlorophenolindophenol (DCPIP) dye method. Firstly, the DCPIP ($C_{12}H_7Cl_2NO_2$) dye solution is prepared by dissolving 26 mg of DCPIP dye and 21 mg of sodium bicarbonate in a 100 mL volumetric flask filled with distilled water. After filtering, the solution is stored in a dark-colored glass bottle. A Vitamin C (ascorbic acid) stock solution at a concentration of 0.1 mg/mL is prepared by dissolving 100 mg of Vitamin C in a 100 mL volumetric flask filled with 4% oxalic acid solution, followed by a serial dilution.

During titration, 5 mL of the Vitamin C working standard solution is titrated against the DCPIP solution using a burette. The DCPIP solution is added drop by drop until a pale pink color appears, indicating the endpoint of the titration. This

color change signifies the reaction between Vitamin C in the solution and the DCPIP dye, allowing for the determination of Vitamin C content in the sample (V1).

To prepare the test sample, 10 g of sample (RSJ) is placed into a 100 mL beaker, transferred to a 100 mL volumetric flask, and diluted to 100 mL with 4% oxalic acid solution. 5 mL of test sample (RSJ) is titrated against DCPIP solution until a similar pale pink colour is achieved and the volume was recorded (V2).The Vitamin C content was calculated based on Eq. 3.

$$Vitamin\,C\left(\frac{mg}{100\,g}\right)=\frac{0.5\,mg}{V2\,mL}x\frac{2\,mL(V1)}{5\,mL}x\frac{100\,mL}{10\,g(Sample\,Weight)}x100 \quad (3)$$

V1 = Volume of the DCPIP used for standard vitamin C (mL).V2 = Volume of DCPIP used for RSJ (mL).

2.8 Reducing sugar estimation

The dinitrosalicylic colorimetric (DNS) method was utilized to assess the reducing sugar content in RSJ samples based on the previous publication [24]. Preparation of the DNS reagent involved dissolving 5 g of dinitrosalicylic acid in 250 mL of distilled water at 80 °C ("DNS solution"). To this solution, 100 mL of sodium hydroxide (2 N) and 150 g of potassium sodium tartrate-4-hydrate were added at 27 °C, with the volume adjusted to 500 mL using distilled water (all reagents sourced from Sigma-Aldrich, Missouri, USA). Standard calibration curves were generated with glucose concentrations ranging from 0.0625% to 1%. Sample dilution was carried out at a ratio of 1:10 by adding 10 µL of the sample to 990 µL of distilled water. Subsequently, 1 mL of the DNS solution was added and thoroughly mixed. The mixture was incubated at boiling temperature (100 °C) for 5 min, followed by immediate cooling. Then, 10 mL of distilled water was added, and the absorbance was measured at 540 nm using a spectrophotometer (Thermo Spectro, USA).

2.9 Determination of electrolytes by inductively coupled plasma mass spectrophotometer (ICP-MS)

The electrolyte content of RSJ was determined using an ICP-MS. The RSJ samples and standards were subjected to ICP-MS (Perkin Elmer, USA) for the quantification of electrolytes in sugarcane juice with the following conditions: peak processing mode: average- signal profile processing mode: average- detector mode: dual- dead time (ns): 55- calibration type: external calibration- the number of replicates- 3. Prior to analysis, all glassware and centrifuge tubes were treated with 15% nitric acid and dried in an oven at 60 °C (Memmert, USA) for 24 h. Sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) in samples were determined after dilution with Milli-Q water at a ratio of 1:2000, while iron (Fe) and zinc (Zn) were assessed at a 1:200 dilution. After dilution, the samples underwent filtration through a 0.45 µm nylon filter. Calibration curves for each element were established within the range of 0.01–0.20 µg/mL [9].

2.10 Microbiological inoculation and determination

The De Man–Rogosa–Sharpe (MRS) agar, obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), was used in the study. The LAB strain *Lb. plantarum* ATCC8014 from the Lab Bioprocessing, Faculty of Food Science and Technology UPM Culture Collection, and the LAB isolated from commercial probiotic drink Yakult (*Lb. casei* strain *Shirota*) were used in this study. Initially, Yakult (150 mL) was inoculated into MRS broth and allowed to incubate for 48 h. Following two washes with peptone water and centrifugation, a stock solution was created. To achieve a bacterial inoculum of 10^7, a 3% LAB culture was initiated by transferring 300 µL from the stock solution into 10 mL of MRS broth. After a 48-h incubation, the culture underwent washing, followed by resuspension in phosphate buffer. Dilution was performed using the equation $M_1V_1 = M_2V_2$. Subsequently, LABs isolated from the stock solution (1%) were introduced into pasteurised RSJ (using double boiler at 72 °C for 15 s), with 1 mL of LAB from the stock sample used for every 100 mL of RSJ. The storage temperature is maintained at 4 °C. To determine the viability of LAB, a serial dilution was performed and inoculated on the MRS agar under anaerobic condition (Anaerocult™, Sigma-Aldrich), and the formation of the colonies were counted.

2.11 Statistical analysis

All the data were reported in the form of graphic images of triplicate measurements. One-way analysis of variance (ANOVA) and Tukey's test were performed to detect differences among the juices for each parameter considered using Minitab 16 (Minitab Inc. State College, Pa. U.S.A).

Table 1 Quality attributes of red sugarcane juice from different varieties

Red Sugarcane Species	pH	Colour			Total Soluble Solid (°Brix)
		*L**	*a**	*b**	
Kapur	5.39 ± 0.01^{a}	24.70 ± 0.15^{c}	1.42 ± 0.12^{a}	1.73 ± 0.03^{c}	11.40 ± 0.20^{b}
Ragnar	5.30 ± 0.01^{c}	25.43 ± 0.12^{b}	0.71 ± 0.06^{c}	2.34 ± 0.06^{b}	14.33 ± 0.12^{a}
Serai	5.39 ± 0.01^{a}	24.58 ± 0.13^{c}	1.01 ± 0.08^{b}	1.44 ± 0.44^{d}	11.73 ± 0.31^{b}

The data were expressed as mean ± SD (n = 3). values in the same column followed by different lower-case letters differed significantly ($p < 0.05$)

3 Result and discussion

3.1 Quality assessment of red sugarcane juice – physicochemical, minerals, and antioxidants

In order to create healthy and tasty plant-based functional drinks, pH is an important factor to be considered. pH also plays a vital role in these drinks' stability and functional properties. Based on Table 1, the pH range of all the RSJ variants is 5.30 to 5.39, which is consistent with the findings of a previous study that reported a pH of around 5.29 for sugarcane juice [25]. This pH range indicates that consuming the juice could provide a healthier alternative to energy drinks, which typically have a pH below 4 [26]. Lower pH levels in energy drinks can lead to dental erosion and various health issues, including gastrointestinal discomforts like acid reflux or heartburn [26]. However, when converting fruit juice into a functional drink via fermentation, a pH below 4.5 is often targeted. This lower pH is because of organic acid production by LAB, which is essential for preservation purposes [27]. Overall, the pH range of 5.30 to 5.39 observed in the juice variants suggests a favorable balance between taste and potential health implications. It indicates that the juice can serve as a healthier option compared to highly acidic energy drinks, while still maintaining a pH level suitable for functional drink conversion.

The color of RSJ, such as observed in the variant *Ragnar* from Table 1, tends to have a higher blue-yellow color and a lower green–red color hue, aligning with the typical yellow hue of sugarcane juice. This color characteristic, influenced by compounds like xanthophyll and anthocyanin, can be an important factor in attracting consumers and enhancing their preference for the product [28]. In the context of sports isotonic drinks, which often come in various attractive colors [29], the natural color of sugarcane juice can serve as an indicator of its phenolic compound content. Normally, anthocyanins contributed to red-blue color, while xanthophyll and carotene are responsible for yellow and orange, respectively [28]. Understanding the relationship between color variations and specific phenolic compounds in sugarcane juice can provide valuable insights into its nutritional value and consumer perception [29].

To select an ideal candidate for the development of a functional drink, the Total Soluble Solids (TSS) value of RSJ was assessed. TSS serves as a rough measure of sugar content and is commonly used in the beverage industry to evaluate sweetness. RSJ typically contains higher sugar content compared to the yellow variant [25]. Opting for sugarcane juice with higher TSS levels may be advantageous for probiotic growth, as it provides a plentiful carbon source and increased energy value. As probiotics consume sugar during fermentation, it can lead to a reduction in sugar content while simultaneously producing beneficial bioactive compounds like GABA, thereby enhancing its nutritional significance [17]. In this study, *Ragnar* variety exhibited a significantly higher TSS value (Table 1), making it optimal for probiotic inoculation. It is important to consider that TSS measurement encompasses not only traditional sugars but also other dissolved components, including "functional" sugars such as D-tagatose, D-allose, D-talose, and D-psicose, organic acids, minerals, and various substances [30]. As a result, TSS can only provide an estimation of the sugar content rather than an absolute value.

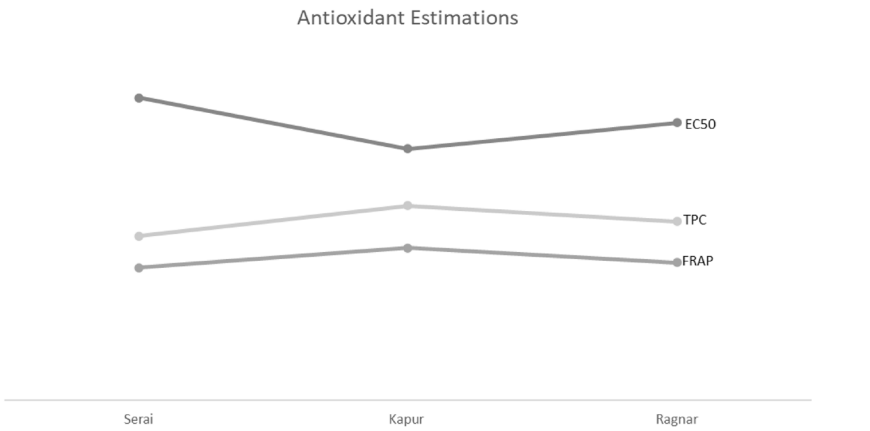
The presence of electrolytes is considered crucial as an ergogenic aid on top of sugars, as they help replenish lost electrolytes and enhance energy production [9]. Since our bodies are unable to produce electrolytes, we rely on obtaining them from food sources. Table 2 shows that *Serai* exhibited the highest mineral content in five out of the six minerals tested, while *Kapur* demonstrated the highest potassium content. In comparison to commercial isotonic drinks, all variants of RSJ demonstrated significantly higher levels of electrolytes, except for sodium. This is advantageous since sodium is often consumed in excess in our daily diets, and dietary guidelines often recommend limiting sodium intake. Therefore, the higher electrolyte content found in RSJ variants can be highly beneficial for individuals engaged in physical activity.

Sugarcane juices are known to be rich in flavonoid and phenolic compounds, which possess natural antioxidant properties and can effectively scavenge free radicals [31]. In the context of energy isotonic drinks, antioxidants play a crucial

Table 2 Electrolytes in red sugarcane juice from different varieties, with two controls from commercial isotonic drinks, as detected by ICP-MS

Sample	mg/100 mL				µg/100 mL	
	Na	K	Ca	Mg	Zn	Fe
Kapur	3.45 ± 0.04^{d}	229.95 ± 21.20^{a}	4.25 ± 0.33^{c}	5.11 ± 0.09^{d}	499.85 ± 12.53^{c}	377.20 ± 46.00^{b}
Ragnar	5.14 ± 0.05^{c}	208.16 ± 4.53^{b}	8.56 ± 0.20^{b}	7.33 ± 0.04^{b}	1149.02 ± 14.76^{b}	375.70 ± 35.70^{b}
Serai	17.84 ± 0.32^{a}	126.31 ± 0.20^{d}	30.48 ± 0.28^{a}	14.25 ± 0.14^{a}	2527.90 ± 21.40^{a}	1030.40 ± 31.30^{a}
Isotonic A	48.92 ± 4.35^{a}	14.15 ± 0.99^{b}	5.60 ± 0.95^{b}	–	–	–
Isotonic B	46.00 ± 2.71^{a}	–	1.50 ± 0.13^{c}	–	10.00 ± 1.73^{b}	30.00 ± 4.16^{b}

The data were expressed as mean ± SD (n = 3). values in the same column followed by different lower-case letters differed significantly ($p < 0.05$)



Variants	EC_{50} (DPPH)	mmol TE/mL (FRAP)	µg GAE/mL (TPC)
Serai	60.89 ± 5.39^{a}	2.66 ± 0.90^{b}	328.96 ± 14.41^{b}
Kapur	50.44 ± 1.41^{b}	3.05 ± 0.13^{a}	390.21 ± 19.13^{a}
Ragnar	55.66 ± 1.85^{ab}	2.76 ± 0.21^{ab}	358.13 ± 12.57^{ab}

Fig. 1 The antioxidant capacities of three different variants of RSJ (*Serai*, *Kapur*, and *Ragnar*) were assessed using three representative values: EC_{50} (DPPH), TPC (total phenolic content), and FRAP (ferric-reducing antioxidant power). Significant differences between RSJ varieties (column) were observed based on the results of Tukey's test ($p < 0.05$). The line graph is not drawn to scale; its purpose is to illustrate trends rather than provide accurate proportional representation

role in neutralizing free radicals generated during intense energy production in the mitochondria's electron transport chain. Based on the results depicted in Fig. 1, all RSJ exhibited significant antioxidant activity, with *Kapur* displaying the lowest EC_{50} value. However, when considering other antioxidant values such as TPC and FRAP, there was no significant difference in antioxidant capacity between *Kapur* and *Ragnar* varieties, indicating comparable antioxidant prowess. Taking into account the superior physicochemical properties of *Ragnar*, this particular variant was chosen for further development as a functional drink. Most importantly, *Ragnar* RSJ is among the most popular red sugarcane cultivated in Malaysia, owing to its excellent sensory properties [5].

Prior to the inoculation of these probiotics into the *Ragnar* RSJ, an independent Vitamin C analysis was conducted in comparison with the commercial isotonic drinks A and B. Our finding demonstrated that *Ragnar* RSJ contains significantly higher Vitamin C compared to commercial Isotonic A and B. The Vitamin C content in RSJ was measured at 0.72 mg/100 g, whereas Isotonic A and B contained only 0.12 mg/100 g and 0.00 mg/100 g, respectively. Typically, active individuals possess greater number of mitochondria, hence experience rapid energy generation and leading to increased free radicals and inflammation. Therefore, Vitamin C and other neutralising antioxidants are crucial for post-recovery treatment in athletes [32].

Table 3 LAB count on MRS agar of different samples over 30-day at 4 °C

	Log CFU/mL			
Days	Control	Yakult	YS	LP
Day 1	1.03 ± 0.03^{a}	9.41 ± 0.11^{a}	8.45 ± 0.03^{b}	9.31 ± 0.43^{ab}
Day 3	1.85 ± 0.11^{a}	10.66 ± 0.04^{a}	8.13 ± 0.04^{c}	9.07 ± 0.02^{b}
Day 7	1.78 ± 0.09^{a}	9.11 ± 0.11^{a}	8.21 ± 0.04^{b}	9.22 ± 0.04^{a}
Day 30	ND	9.02 ± 0.02^{b}	9.36 ± 0.04^{a}	9.29 ± 0.11^{ab}

Values are mean ± SD of three replicates. Different superscript letters within the same row indicate statistical differences ($p < 0.05$) among different samples. ND- not detectable. Control—Sugarcane juice without probiotics; YS – *Lb. casei Shirota* and sugarcane juice; LP – *Lb. plantarum* in sugarcane juice

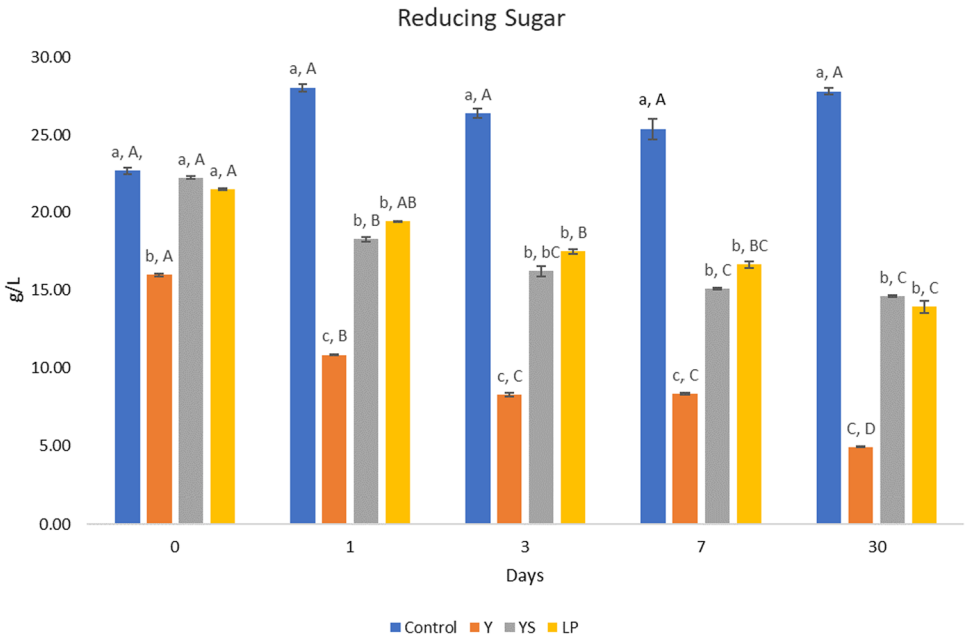


Fig. 2 The total reducing sugar in the sugarcane juice enriched with probiotics over a 30-days storage period at 4 °C. Each value from the table represents mean ± standard deviation (n = 3). Lowercase letters denote significance ($p < 0.05$) within the same time interval, whereas uppercase letters signify significance between the same treatments (as indicated by similar bar colors)

3.2 Inoculation of probiotics into *Ragnar* red sugarcane juice (RSJ)

Lactic acid bacteria (LAB) play a crucial role in the food industry and are classified into homofermentative and heterofermentative types. Homofermentative LAB, such as *Streptococcus thermophilus* and *Lactococcus lactis* ssp. *lactis*, are commonly used, while heterofermenters like *Lb. acidophilus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Leuconostoc mesenteroides* are also important [33]. Probiotics offer numerous health benefits, including metabolic support for active individuals, gut and brain health through the gut-brain axis mechanism, reduction of sugar content, production of bioactive compounds, and more [12, 34].

Probiotic bacteria produce a diverse range of metabolites that have positive effects on human health. These include bacteriocins, metabolic enzymes, amino acids, peptides, short-chain fatty acids, vitamins, antioxidants, anti-inflammatory and immune-modulating agents, and exopolysaccharides. Considering the potential of *Ragnar* sugarcane juice as an isotonic drink, further development into a probiotic drink was carried out using the generally recognized as safe (GRAS) strain *Lb. plantarum* (abbreviation: LP) and *Lb. casei Shirota* strain (abbreviation: YSJ). Meanwhile, negative control experiment was RSJ without probiotics and commercial Yakult sample as the positive control.

From Table 3, it is shown that the samples inoculated with either *Lb. plantarum* (LP) and *Lb. casei Shirota* (YS) have reached the minimum limit of probiotic count that is above $\log_{10}7$ (7.25–9.36 log CFU/mL), to confer its beneficial effects on human health [35]. In the control experiment, the LAB levels were relatively low, and in some instances, they were undetectable. This can be attributed to the pasteurization process and immediate refrigeration of the RSJ (4 °C) during the investigation. In contrast, all other findings indicated a stable population of LAB within the range of 8 to 10 log CFU/

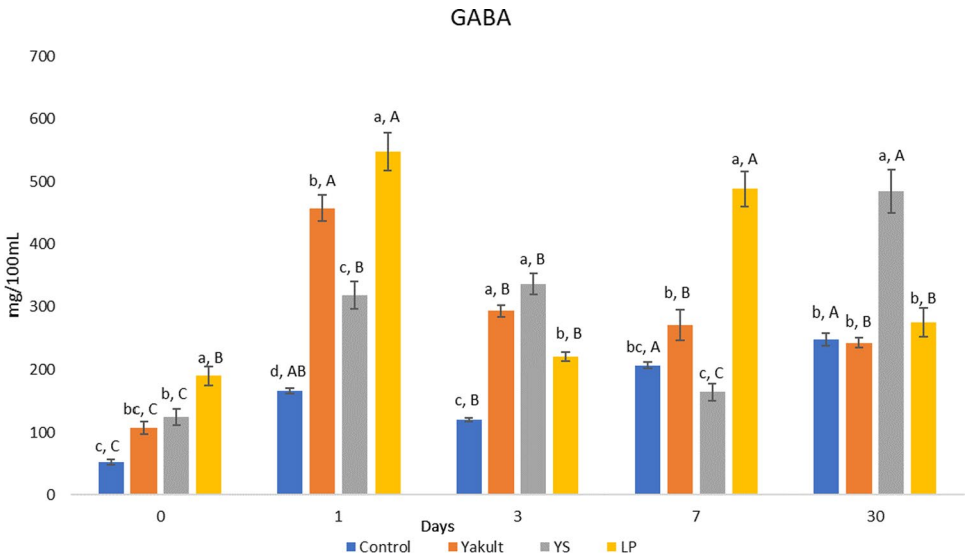


Fig. 3 GABA content of different *Ragnar* RSJ treatments and its associated controls. Each value from the table represents mean ± standard deviation (n = 3). Lowercase letters denote significance ($p < 0.05$) within the same time interval, whereas uppercase letters signify significance between the same treatments (as indicated by similar bar colors). Control—Sugarcane juice without probiotics; YS – *Lb. casei Shirota* and sugarcane juice; LP – *Lb. plantarum* in sugarcane juice

mL within 30 days-period, showcasing the substrates' suitability for LAB maintenance. Since simple sugars in RSJ are often the preferred carbon source for LAB, the culture should remain stable as long as the carbon does not become a limiting nutrient [36].

Moreover, throughout the fermentation process, there is a decrease in the quantity of reducing sugars (Fig. 2) attributed to their consumption by LAB. However, the reduction occurs at a slower rate in RSJ compared to the control probiotic drink, Yakult. Despite the slower reduction rate, the total reducing sugar content in RSJ samples still decreased over time, indicating the potential of *L. plantarum* as a probiotic strain to a healthier version of probiotic drink [37]. Yakult's lower sugar content than other sugarcane drink samples suggests that Yakult's commercially prepared nature may involve sugar reduction during production [37].

Control—Sugarcane juice without probiotics; YS – *Lb. casei Shirota* and sugarcane juice; LP – *Lb. plantarum* in sugarcane juice.

3.3 Assessing the functionality of probiotic-enriched *Ragnar* red sugarcane juice (RSJ)

As a next step in evaluating the functionality of this probiotic-enriched drink, the analysis of gamma-aminobutyric acid (GABA) was conducted on the probiotic drinks to investigate its functionality and potential health benefits. Based on Fig. 3, all samples of RSJ possess at least an initial amount of GABA, whereby the control sample had the lowest GABA content (52.22 mg/100 mL), while the Yakult (107.80 mg/100 mL), YS (324.70 mg/100 mL), and LP (190.85 mg/100 mL) samples displayed higher GABA. The fermentation process appeared to influence GABA production in the RSJ samples. Notably, on day one, the Yakult and LP samples exhibited a significant increase in GABA content, with values of 457.15 mg/100 mL and 547.20 mg/100 mL, respectively. This suggests that the probiotic strains present in Yakult and YS played a role in enhancing GABA synthesis during fermentation. These findings are consistent with previous studies, which demonstrated the production of GABA through the use of *Lb. plantarum* in a whey protein drink [15]. However, GABA content fluctuates in all the samples over the 30 days of storage. GABA may be unstable, thus random degradation and reformation can occur during storage [38].

4 Conclusion

Among the three varieties of red sugarcane juice (RSJ) investigated, *Ragnar* exhibited the highest potential for the development of a functional drink. This is attributed to its remarkable antioxidant, vitamin C, and essential mineral content, particularly beneficial for highly active individuals. The fortification of *Ragnar* RSJ with suitable probiotics, like *Lactobacillus*, not only enhances its functionality by promoting GABA production and reducing sugars but also ensures optimal growth of beneficial probiotics, imparting health benefits.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Competing interests The authors declare no competing interests.

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