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Microbial Community, Phytochemical Profile of Indian Gooseberry Water Kefir, and Its Inhibition of lipogenesis in Adipocytes

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Abstract

Water kefir grains, a symbiotic culture of bacteria and yeast embedded in a polysaccharide matrix, play a crucial role in the fermentation process. The microbial diversity within water kefir grains is not only extensive but also variable, adapting to different environmental conditions and substrates. In this study, we aim to identify the microbial species present in the kefir grains and to investigate health benefit metabolites of Indian gooseberry water kefir fortified with inulin, which inulin is widely recognized as a prebiotic dietary fiber, serves as an alternative carbon source for the symbiotic microbial community. Our findings indicated that high yield of the kefir grain (140.8 g) was achieved after fermentation and the grain was abundance in *Lactobacillus* sp., *Bacillus* sp., *Schizosaccharomyces* sp., and *Saccharomyces* sp. The phytochemical profile revealed 65 distinct compounds of Indian gooseberry water kefir formulated with inulin. Many beneficial metabolites including phenolic compounds and choline were detected in the Indian gooseberry water, thereby highlight the potential of Indian gooseberry water kefir as a low-calorie functional beverage.

Keywords: Adipocytes, Indian gooseberry water kefir, Inulin, Microbiota, Water kefir grain

Introduction

Water kefir is a non-dairy, naturally fermented beverage made from a mixture of water, fruits, herbs, and sucrose, fermented with water kefir grains. These grains consist of a complex symbiotic community of lactic acid bacteria (LAB), such as *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides* and *Streptococcus lactis*, acetic acid bacteria (AAB), such as *Acetobacter*, and yeasts, *Zygosaccharomyces*, *Candida*, and *Kloekera* [1]. During fermentation, these microorganisms produce various metabolites such as ethanol, lactic acid, glycerol, acetic acid, and mannitol [3]. The microbial composition of water kefir grains varies significantly depending on geographical origin, environmental conditions, and the substrates used during fermentation [2,4]. Therefore, the metabolite profile of water kefir can differ considerably among different cultures and fermentation conditions.

Over recent decades, many studies have highlighted the nutritional and functional benefits of water kefir. Its bioactive metabolites contribute to a wide range of health-promoting properties, including antioxidant, antimicrobial, anticancer, anti-diabetic, cholesterol-lowering, and anti-lipogenic effects [5]. However, the growing popularity of water kefir is accompanied by concerns about its relatively high calorie content due to the use of sucrose as the main fermentation substrate. In response, this concern towards utilizing alternative low-calorie sweeteners in sugary beverages to reduce the energy content of sugar,

such as stevia, monk fruit extract, and inulin [6]. Among these, inulin, a group of fructans found in plant roots and rhizomes, is commonly used as a dietary fiber and prebiotic. It is a non-digestible oligosaccharide with a low glycemic index, making it an appealing ingredient for developing low-calorie functional beverages [7,8]. To further enhance the nutritional and function value of water kefir, alternative plant-based substrates have been investigated for their compatibility with the kefir fermentation process. One promising candidate is Indian gooseberry (*Phyllanthus emblica*), also known as amla. This fruit is rich in vitamin C, polyphenols, flavonoids, and tannins, and has long been recognized for its antioxidant, anti-inflammatory, and immunomodulatory properties. It contains a wide range of nutraceutical compounds such as gallic acid, ellagic acid, and chebulinic acid, and is also a good source of essential vitamins and minerals, including iron, calcium, phosphorus, vitamins A and E [9].

Given these attributes, the incorporation of Indian gooseberry into water kefir fermentation may enhance the functional characteristics of the beverage. Therefore, this study aimed to characterize the microbial composition of kefir grain starter, evaluate the physicochemical properties of Indian gooseberry water kefir produced with different carbohydrate sources, identified the bioactive compound profile of the formulation containing inulin, and assess its effect on intracellular lipid accumulation in 3T3-L1 adipocytes.

Materials and methods

Preparation of kefir grain starters

The water kefir grain was purchased from local shop in Thailand. The grain was stored at 4 °C to preserve microbial viability. Prior to use, the grain was activated to ensure microbial growth and functionality. The activation was performed by culturing 48 g of the kefir grain sample in a sterile 20% (w/v) brown sugar solution and incubating at 25 °C for 76 h. At the end of incubation, the grains were separated from the kefir liquor, and the weight was recorded.

Production of Indian gooseberry water kefir

Indian gooseberry was purchased from fresh markets around Chiang Mai City, Thailand. Subsequently, the fruits were washed thoroughly, deseeded, and dried in a hot-air oven at 60 °C. Once completely dry, they were ground using a blender. To prepare the kefir fermentation substrate, 3 g of Indian gooseberry powder was mixed with 100 mL of distilled water. The mixture was then boiled at 100 °C for 20 min to extract beneficial compounds. Finally, the solution was filtered to remove any remaining solids. The Indian gooseberry juice was mixed with each differing in sugar composition: 20% (w/v) brown sugar (20B), 10% (w/v) brown sugar (10B), and 10% (w/v) inulin (10I). All these mixtures underwent sterilization through autoclaving at 110 °C for 10 min. The sterilized mixtures were then inoculated with 2% (w/v) of the water kefir grain starter. The mixtures were covered with a muslin cloth then incubated at 25 °C in an incubator under aerobic condition for 48 h. Fermentation was carried out in triplicate using 200 mL of each formulation in Erlenmeyer flasks.

Microbial communities in the kefir grain using culture independent method

Metagenomic DNA was extracted from the water kefir grain starter used as the inoculum for all fermentation experiments by using DNeasy PowerSoil Pro DNA Kit (Qiagen, USA). This metagenomic DNA was used as a template to amplify 16S rRNA gene using 341F and 805R primers, targeting V3- V4 varible regions, and 2X sparQ HiFi PCR master Mix (QuantaBio, USA). The amplification condition included an initial denaturation step 2 min at 98 °C, followed by 30 cycles of 98 C for 20 s, 60 C for 30 s, and 72 °C for 60 s, followed by a single final extension step at 72 °C for 1 min. Subsequently, 16S amplicons were purified using sparQ Puremag Beads (QuantaBio, USA) and indexed using 5 µl of each Nextera XT index primer in a 50 µl PCR reaction, followed by 8 - 10 cycles of PCR using the same condition as described above. The final PCR products were cleaned, pooled, and diluted to final loading concentration at 4 pM. Cluster generation and 250-bp paired-end read sequencing were performed on an Illumina MiSeq at Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). For yeasts, the ITS region was sequenced using ITS5 and ITS4 primers. Additionally, 18S rRNA gene was sequenced using NS1, NS24 primers, and 26S rRNA gene was conducted using LROR, and LR7 primers. PCR cycle conditions for amplification were as follows, denaturation step 5 min

at 95 °C, followed by 31 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min, and 72 °C for 1.4 min, followed by a single step final extension step at 72 °C for 10 min.

LC-QTOF-MS analysis for bioactive compounds

Bioactive compounds in Indian gooseberry water kefir were determined using Liquid Chromatograph-Quadrupole Time-of-Flight Mass Spectrometer (LC-QTOF MS), 1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, USA with Zorbax eclipse plus C18 rapid resolution HD column (2.1×150 mm, particle size 1.8 µm. Agilent). Experimental conditions of the LC-QOF MS system were as follows: Flow rate of mobile phase A [water+0.1% (v/v) formic acid], and mobile phase B [acetonitrile+0.1% (v/v) formic acid] were set at 0.2 mL/min. Samples were dissolved in LC-MS grade water and the injection quantity of 2 µL was used. Samples were detected at 210, 280, and 360 nm. Bioactive compounds in the kefir were identified based on a comparison of their retention time (min), peak area, and mass spectral patterns with that spectral database of authentic compounds stored in the METLIN PCD (Personal Compound Database) and PCDL (Personal Compound Database and Library), version 8.

Cell culture, adipocyte differentiation, and treatment procedures

The procedures were modified from[10]. The 3T3-L1 mouse pre-adipocyte cell lines (IFO50416) were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB), National Institute of Biomedicine Innovation, Ibaraki, Osaka, Japan. These cells were cultivated in T25 flasks using Dulbecco's modified Eagle's medium (DMEM, Hyclone, Utah, USA), supplemented with 10% calf serum (CS, Hyclone, Utah, USA) and 10% penicillin/streptomycin (Caisson, Smithfield, USA). The cell cultures were maintained within a controlled incubation environment (37 °C, 5% CO_2). To initiate adipocyte differentiation, the cells were seeded at a density of 4×10^4 cells per well in 6-well plates, at a point known as 2-day-post-confluent (day 0). These cells were subsequently exposed to a differentiation-inducing medium (DMI medium). The DMI medium composition included 0.5 mmol of IBMX (Sigma-Aldrich, St. Louis, USA), 0.1 µg/mL of DEX (Sigma-Aldrich, St. Louis, USA), and 5 µg/mL of insulin. This medium was prepared by adding the specified concentrations of components to DMEM supplemented with 10% fetal bovine serum (FBS, HyClone, Utah, USA). Upon reaching day 3, the DMI medium was replaced with INS medium, comprised of DMEM supplemented with 10% FBS and 5 µg/mL insulin. Additionally, kefir samples, diluted 100-fold, were introduced to the INS medium. As the experiment progressed to day 6, the INS medium was substituted with DMEM containing 10% FBS and kefir samples, and this medium was consistently maintained throughout the incubation period until day 9.

Cell viability assessment

To evaluate cell viability, 3T3-L1 pre-adipocytes were cultured in 96-well tissue culture plates at a density of 1×10^4 cells per well for a duration of 2 days. Upon achieving cellular confluency exceeding 80% within the culture wells, the cells were subjected to various treatments involving kefir samples. These treatments encompassed different dilutions: 200-fold, 100-fold, 50-fold, and undiluted (0-fold), each administered over a 48-hour period. Concurrently, an untreated control group was maintained for comparison purposes. Upon completion of the kefir intervention, the culture medium was substituted with a solution of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, USA). The culture was allowed to continue incubating for an additional 4 h. Subsequently, the medium containing MTT was discarded, and 100 µl of dimethyl sulfoxide (DMSO, Labscan, Bangkok, Thailand) was introduced to extract the purple formazan crystals. The absorbance of the resultant solution was then measured at 540 nm using a microplate reader. The obtained results were calculated and subsequently presented as a percentage reflecting the cell viability.

Oil red O staining

The content of intracellular lipid accumulation was assessed through Oil Red O staining following the protocols of [11]. Initially, the cells were washed with PBS and fixed with 10% formaldehyde (Sigma-Aldrich) for 30 min at room temperature. After fixation, they were rinsed with distilled water, followed by treatment with 60% isopropanol (Labscan, Bangkok, Thailand) for 1 - 2 min each. Subsequently, the cells were stained with

a working Oil Red O solution for 20 min at 60 °C and an additional 20 min at room temperature. Following staining, the cells were washed with distilled water and examined under a microscope. For quantitative analysis of the staining content, Oil Red O was eluted with 100% isopropanol for 30 min. The dissolved Oil Red O content was then assessed using a microplate reader to measure the absorbance at 520 nm.

Statistical analysis

The results were generated from independent triplicate experiments and are expressed as the mean ± SD. ANOVA and Tukey's tests were performed using the Statistical Package for the Social Sciences (SPSS, version 17; SPSS, Chicago, IL, USA) and GraphPad Prism 10 (GraphPad Software). Statistically significant differences were considered at $p < 0.05$.

Results and discussion

Morphological and physicochemical changes in kefir grain starter

The grain biomass of water kefir grain was monitored during fermentation in a 20% (w/v) brown sugar (20B) solution over 72 h. During the fermentation, visual observations revealed a gradual lightening of the culture medium, beginning after 24 h of fermentation. Additionally, CO_2 production was observed through bubble formation on the surface of the medium, which became more pronounced between 24 and 72 h, as shown in **Figure 1**. The appearance of the kefir grain post-separation from the culture broth was illustrated in **Figure 2**. Grain biomass increased from 48 g to more than 140 g over the course of fermentation as depicted in **Figure 3**.

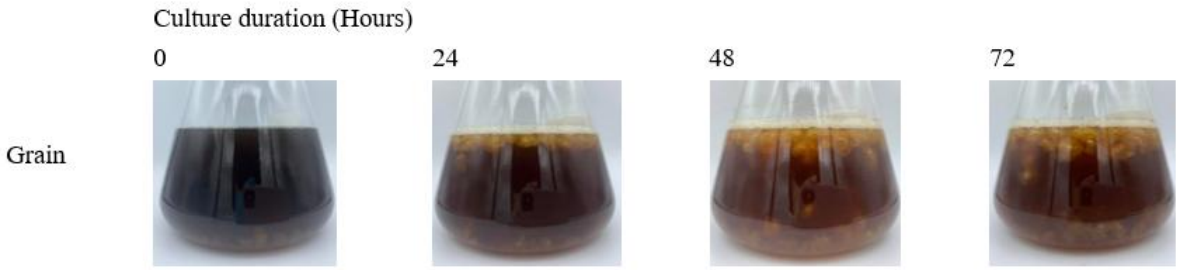


Figure 1 Changes in the appearance of kefir grains in 20% brown sugar (20BS) solution during 0 - 72 h of fermentation. The images illustrate progressive morphological changes, including increased grain size and CO_2 bubble formation, indicative of active microbial fermentation.



Figure 2 Representative visual characteristics of water kefir grains. The image shows the morphology, size, and translucent appearance of the kefir grains used as the starter culture in this study.

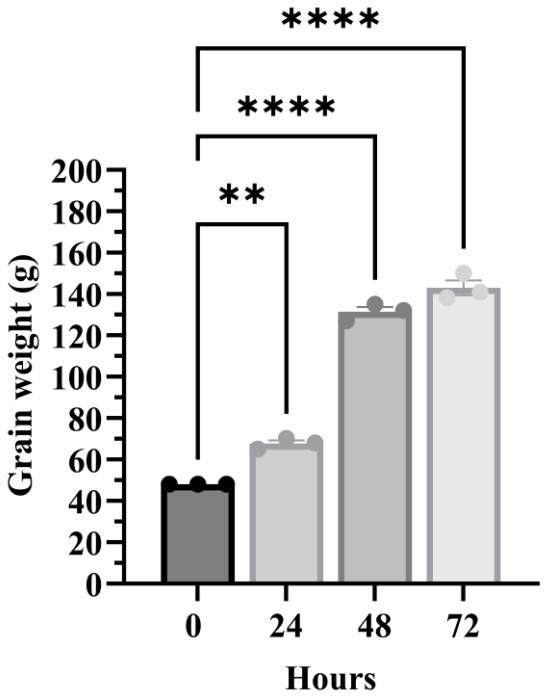


Figure 3 Weight of kefir grains from 0 to 72 h of fermentation. Bars represent mean ± SD and individual points indicate independent biological replicates. Asterisks indicate levels of statistical significance compared with the 0 h control group

Morphological and physicochemical production properties of Indian gooseberry water kefir

Three formulations of Indian gooseberry water kefir were produced using water kefir grains with different sugar compositions. During the fermentation process, all formulations displayed visible CO_2 bubbles on the surface after 48 h of fermentation, whereas no bubbles were observed in the corresponding unfermented control samples (0 h), as shown in **Figure 4**. The formation of CO_2 bubbles indicates active microbial fermentation and is likely associated with the metabolic activity of yeasts present in the kefir grains. After 48 h of fermentation, the weights of kefir grains increased from the initial inoculation weight of 2.00 ± 0.00 g. Overall, the grain biomass increased significantly ($p < 0.05$) in all sugar formulations (20B, 10B, and 10I), indicating microbial growth during fermentation. Among the formulations, the 20B kefir formula exhibited the highest grain weight (3.06 ± 0.21 g), followed by 10B (2.95 ± 0.11 g) and 10I (2.68 ± 0.24 g), as illustrated in **Figure 5**.

The final pH values and the change in pH values (ΔpH) during fermentation in kefir liquor were recorded. A consistent decrease in pH values was observed across all kefir samples post-fermentation. For all formulars, pH levels ranged between 2.69 ± 0.02 and 3.06 ± 0.01. The 20B showed the highest ΔpH at 0.07, followed by 10B. The 10I exhibited the lowest ΔpH at 0.02 as shown in **Table 1**. In terms of total soluble solids (TSS), a reduction was observed across all formulations. The 20B kefir showed the greatest change in TSS (ΔTSS = 1.50 °Brix), followed by 10B (ΔTSS = 0.83 °Brix), and 10I (ΔTSS = 0.50 °Brix), also detailed in **Table 1**. Although the 10I formulation showed lower grain growth and smaller changes in pH and TSS compared with sucrose-containing formulations, the observed fermentation activity suggests that inulin can still support microbial metabolism in water kefir fermentation.

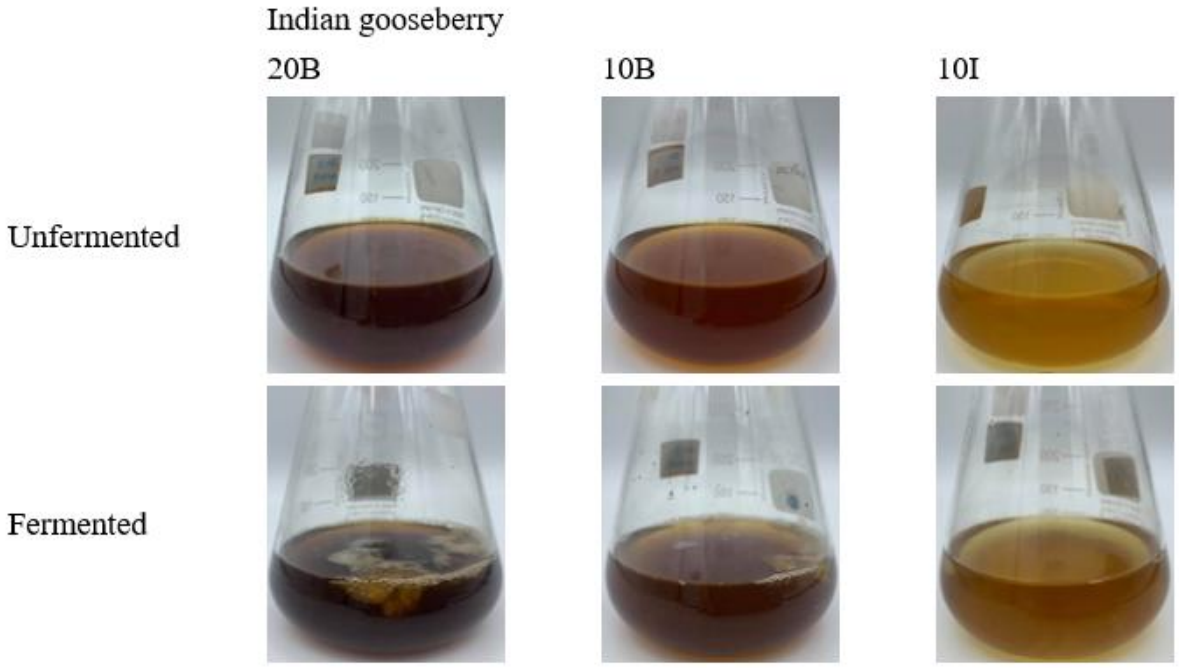


Figure 4 Appearance of Indian gooseberry water kefir produced using different sugar formulations (20B, 10B, and 10I). Images show the unfermented samples (0 h, control) and the fermented samples after 48 h. The unfermented samples for each formulation serve as the control groups for comparison with the corresponding fermented products. After fermentation, visible CO_2 bubble formation, slight turbidity, and minor color changes were observed, indicating microbial fermentation activity. These changes were more noticeable in the 20B formulation.

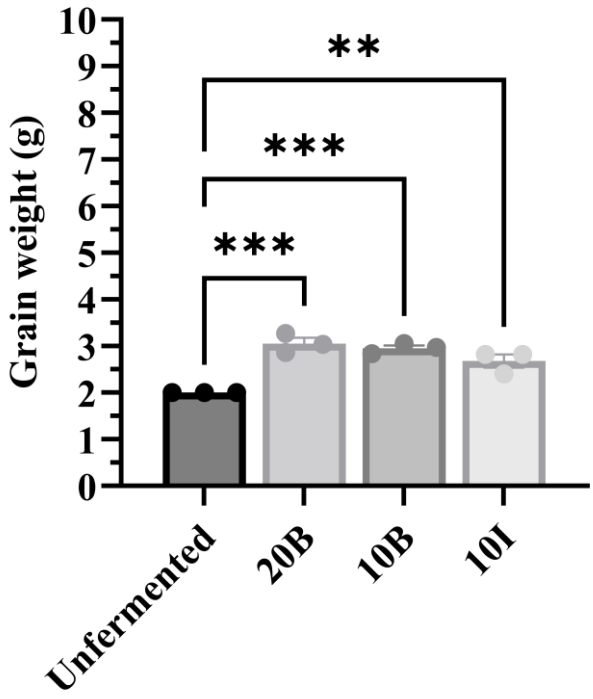


Figure 5 Weight of the grain in samples produced from Indian gooseberry juice with different sugar types after 48 h of fermentation. Bars represent mean ± SD and individual points indicate independent biological replicates. Asterisks indicate levels of statistical significance compared with the unfermented control group.

Table 1 The pH values and TSS of the water kefir in different formulars.

Sugar	pH $_{final}$	ΔpH	TSS $_{final}$ (°Brix)	ΔTSS (°Brix)
20B	3.06 ± 0.01	0.07	18.33 ± 0.29	1.50
10B	2.89 ± 0.01	0.05	10.00 ± 0.00	0.83
10I	2.69 ± 0.02	0.02	10.50 ± 0.00	0.50

Note: ΔpH and ΔTSS represent the decrease during fermentation, calculated as initial value – final value. The reduction in pH reflects the production of organic acids, while the decrease in TSS indicates microbial consumption of soluble sugars during fermentation.

Microbial communities in the kefir grain using culture independent method

The microbial composition of the kefir grain was assessed using 16S rRNA gene sequencing targeting various hypervariable regions (V4, V3, V3 - V4, and V4 - V5). The results revealed substantial microbial diversity and distinct bacterial profiles among the grain samples. Overall, the major bacterial species in the grain was lactic acid bacteria (LAB) with *Lactobacillus* accounted for more than 78% of the microbial population, followed by *Bacillus* at 2.23% and other minor indicating the presence of a diverse but unevenly distributed microbial consortium within the kefir grains. as shown in **Figure 6**. Fungal community profiling was performed through ITS region sequencing. Fungal community profiling was conducted using internal transcribed spacer (ITS) region sequencing. The results indicated that the yeast community in the the grain was highly dominated by *Schizosaccharomyces* sp. which accounted for 97.87% of the fungal population, while *Saccharomyces* sp. represented at 3.13% as presented in **Figure 7**. These yeasts are known to play important roles in sugar fermentation and carbon dioxide production, contributing to the characteristic effervescence and metabolic interactions within the kefir fermentation system. The coexistence of LAB and yeasts highlights the symbiotic microbial structure that supports the stability and functionality of kefir grain fermentation.

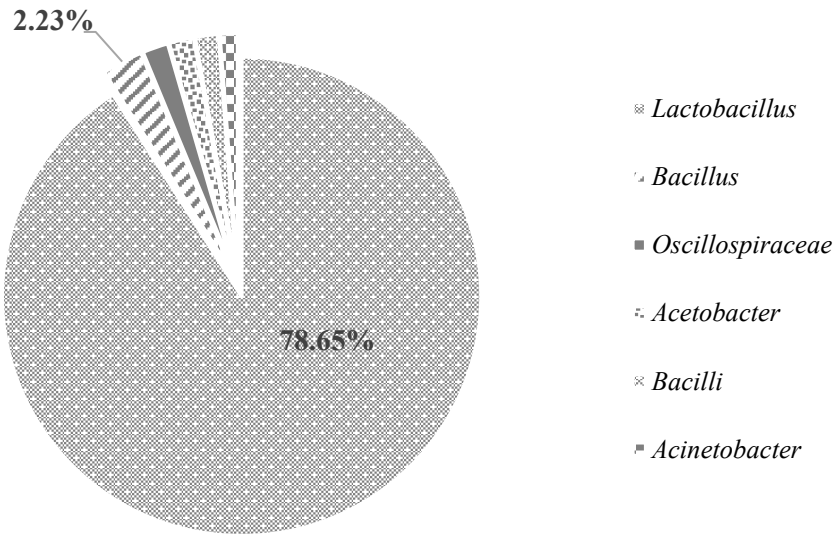


Figure 6 Distribution of microbial population in the water kefir grain starter using 16S rRNA gene sequencing analysis, highlighting the dominance of lactic acid bacteria, particularly *Lactobacillus*, along with other minor bacterial groups.

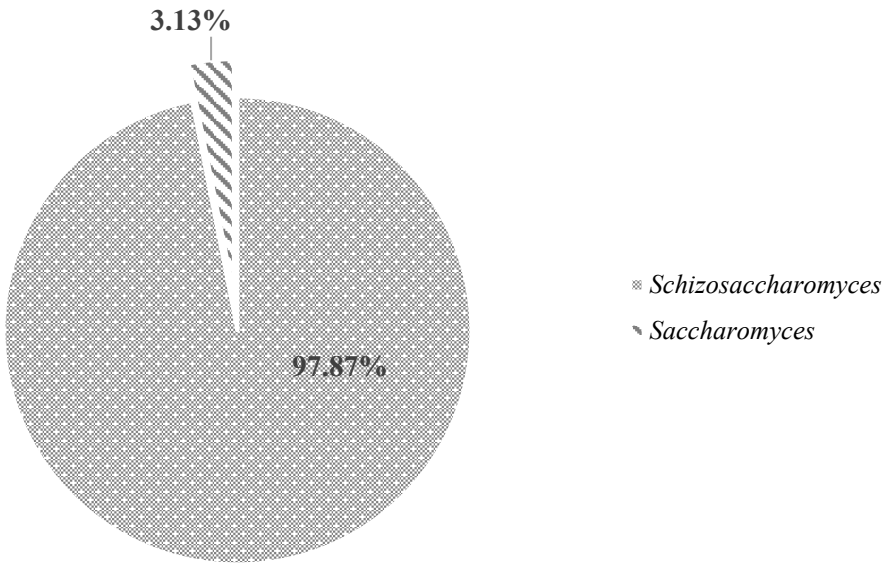


Figure 7 Distribution of yeast population in the water kefir grain starter using ITS regions sequencing. The results indicate the predominance of *Schizosaccharomyces* species, with a smaller proportion of *Saccharomyces* species.

Bioactive compounds in Indian gooseberry water kefir

The analysis of Indian gooseberry water kefir revealed a chemically diverse profile, comprising 65 distinct compounds which were systematically classified into 7 major categories: Aromatic and heterocyclic compounds, carboxylic acids and derivatives, lipids and fatty acid derivatives, organonitrogen compounds, organic hydroxy compound, phenolic compounds, sugars and sugar-derived compounds tannins, and other compounds. as shown in **Table 2**. Aromatic and heterocyclic compounds consist of eight compounds including 3,4-dihydro-4-[(5-methyl2-furanyl)methylene]-2hpyrrole, 2-acetylfuran, (z)-tamarindienal, 3-methyl-3-butenyl tetradecanoate, tetrahydropersin, 2-acetyl-5,8-dihydroxy-3- methoxy-1,4-naphthoquinone, 1,2,6,8-Tetrahydroxy-3- methylanthraquinone 2-O-b-dglucoside, and kojic Acid. Carboxylic acids and Derivatives included 1-pentadecanecarboxylic acid, 2-hydroxyhexadecanoic acid, galactaric acid, D-arabinono-1,4-lactone, glucaric acid lactone, citric acid, and piscidic A. For lipids and fatty acid derivatives, C16 sphinganine, phytosphingosine, and lauroyl diethanolamide were found. Organonitrogen compounds consisted of choline, 5-amino-6-(5'-phosphoribosylamino)uracil, 5-aminoimidazole, Citbismine C and ribonucleotide. Seventeen compounds belonging to phenolic compounds were presented in the sample such as ellagic acid, gallic acid, digallate, 6-C-methylkaempferol 3-glucoside, quercetin-3-galactoside. Additionally, tannin compounds eg. punicalin, 2-O-galloylgalactaric acid and 5-O-galloyl-1,4-galactarolactone were also identified, supporting the high phenolic content of the final product. These compounds are predominantly derived from Indian gooseberry. Sugars and sugar-derived compounds such as sucrose, alpha, beta-trehalose, D-fructofuranose 1,2':2,3'-dianhydride, and bis-D-fructose 2',1:2,1'-dianhydride were also found.

Table 2 Phytochemical contents in Indian gooseberry water kefir.

Classification	Name of compounds	RT	Mass	Molecular formulars
Aromatic and heterocyclic compounds	3,4-Dihydro-4-[(5-methyl2-furanyl)methylene]-2Hpyrrole	2.048	161.0842	C_{10} H_{11} NO
	2-Acetylfuran	9.628	110.0367	C_6 H_6 O_2

Classification	Name of compounds	RT	Mass	Molecular formulars
	(Z)-Tamarindienal	10.481	126.0317	$C_6 H_6 O_3$
	3-Methyl-3-butenyl tetradecanoate	40.297	298.2875	$C_{19} H_{38} O_2$
	Tetrahydropersin	41.276	384.3243	$C_{23} H_{44} O_4$
	2-Acetyl-5,8-dihydroxy-3- methoxy-1,4-naphthoquinone	25.639	262.0482	$C_{13} H_{10} O_6$
	1,2,6,8-Tetrahydroxy-3-methylanthraquinone 2-O-b-Dglucoside	26.392	448.1003	$C_{21} H_{20} O_{11}$
	Kojic Acid	12.64	142.0267	$C_6 H_6 O_4$
Carboxylic acids and derivatives	1-Pentadecanecarboxylic acid	24.637	310.1061	$C_{15} H_{18} O_7$
	2-Hydroxyhexadecanoic acid	38.829	272.2356	$C_{16} H_{32} O_3$
	Galactaric acid	2.147	210.0381	$C_6 H_{10} O_8$
	D-Arabinono-1,4-lactone	2.373	148.037	$C_5 H_8 O_5$
	Glucaric acid lactone	2.473	192.0274	$C_6 H_8 O_7$
	Citric acid	6.665	192.027	$C_6 H_8 O_7$
	Piscidic Acid	10.63	256.058	$C_{11} H_{12} O_7$
Lipids and fatty acid derivatives	Decyl isobutyrate	34.926	228.2093	$C_{14} H_{28} O_2$
	C16 Sphinganine	38.565	273.2674	$C_{16} H_{35} N O_2$
	Phytosphingosine	38.691	317.2934	$C_{18} H_{39} N O_3$
	Xestoaminol C	38.728	229.2414	$C_{14} H_{31} N O$
	C17 Sphinganine	39.143	287.2825	$C_{17} H_{37} N O_2$
	Lauroyl diethanolamide	41.376	287.2463	$C_{16} H_{33} N O_3$
	PG(10:0/10:0)[U]	45.567	554.322	$C_{26} H_{51} O_{10} P$
	13E-Docosenamide	46.195	337.3348	$C_{22} H_{43} N O$
	Repenol	20.656	388.0425	$C_{18} H_{12} O_{10}$
Organonitrogen compounds	Choline	1.998	104.1081	$C_5 H_{14} NO$
	5-Amino-6-(5'-phosphoribosylamino)uracil	8.135	354.0574	$C_9 H_{15} N_4 O_9 P$
	5-Aminoimidazole ribonucleotide	9.126	295.0562	$C_8 H_{14} N_3 O_7 P$
	Citbismine C	2.988	684.2321	$C_{37} H_{36} N_2 O_{11}$
	Trp Met Cys	3.427	438.1383	$C_{19} H_{26} N_4 O_4 S_2$
Phenolic compounds	2-O-Galloylgalactaric acid	3.705	362.049	$C_{13} H_{14} O_{12}$
	(+)-Chebulic acid	5.173	356.0384	$C_{14} H_{12} O_{11}$
	Ellagic acid	23.783	302.0069	$C_{14} H_6 O_8$
	4-Glucogallic acid	4.707	332.0739	$C_{13} H_{16} O_{10}$
	5-O-Galloyl-1,4-galactarolactone	6.74	344.0377	$C_{13} H_{12} O_{11}$
	Gallic acid	8.158	170.0216	$C_7 H_6 O_5$

Classification	Name of compounds	RT	Mass	Molecular formulars
	6-O-Galloylsucrose	9.274	494.1261	C_{19} H_{26} O_{15}
	Digallate	15.9	322.0321	C_{14} H_{10} O_9
	7-Deshydroxypyrogallin-4- Carboxylic Acid	17.758	248.0322	C12 H_8 O_6
	Sanguiin H11	19.314	952.0794	C_{41} H_{28} O_{27}
	Shoyuflavone C	19.59	418.0535	C_{19} H_{14} O_{11}
	m-Trigallic acid	22.125	474.0428	C_{21} H_{14} O_{13}
	Isoterchebin	22.276	954.0969	C_{41} H_{30} O_{27}
	Shoyuflavone B	22.727	402.0587	C_{19} H_{14} O_{10}
	Quercetin 3-galactoside	24.183	464.0953	C_{21} H_{20} O_{12}
	6-C-Methylkaempferol 3- glucoside	31.537	462.1153	C_{22} H_{22} O_{11}
	Leonuriside A	11.535	332.1111	C_{14} H_{20} O_9
Sugars and sugar-derived compounds	Myo-Inositol	2.111	180.0641	C_6 H_{12} O_6
	Sucrose	2.212	342.1174	C_{12} H_{22} O_{11}
	alpha,beta-Trehalose	3.053	342.1177	C_{12} H_{22} O_{11}
	D-Fructofuranose 1,2':2,3'- dianhydride	3.806	324.1066	C_{12} H_{20} O_{10}
	3-O-alpha-D Glucopyranuronosyl-D-xylose	2.222	326.0851	C_{11} H_{18} O_{11}
	6-Acetyl-D-glucose	2.586	222.0736	C_8 H_{14} O_7
	4-O-Methyl-a-D-glucosyl-(1->2)- b-D-xylosyl-(1->4)-D-xylose	3.904	472.1415	C_{17} H_{28} O_{15}
	2-Deoxy-D-Ribose	4.431	134.0577	C_5 H_{10} O_4
	2-Keto-3-deoxyoctonate (KDO)	4.694	238.0686	C_8 H_{14} O_8
Tannins	Punicalin	19.228	782.06	C_{34} H_{22} O_{22}
	2-O-Galloylpunicalin	19.314	934.0704	C_{41} H_{26} O_{26}
	3-O-Galloylhamamelitannin	19.816	636.0949	C_{27} H_{24} O_{18}
Other compounds	1,2'-Di-Ogalloylhamamelofuranose	15.977	484.0863	C_{20} H_{20} O_{14}
	2-Acetyl-5,8-dihydroxy-3- methoxy-1,4- naphthoquinone	28.501	262.048	C_{13} H_{10} O_6
	2,3-Dihydro-3-hydroxy-6- methoxy-2,2-dimethyl-4H1-benzopyran-4-one	40.473	222.0893	C_{12} H_{14} O_4
	Citreoviridinol A1	23.331	420.1785	C_{22} H_{28} O_8
	Dambonitol	5.688	208.0949	C_8 H_{16} O_6
	2-(beta-D-Glucosyl)-sn-glycerol 3-phosphate	2.021	334.0659	C_9 H_{19} O_{11} P
	10-Formyltetrahydrofolyl Lglutamate	3.327	602.2063	C_{25} H_{30} N_8 O_{10}

Effects of Indian gooseberry water kefir on cell viability of 3T3-L1 pre-adipocytes

The cytotoxicity of kefir on 3T3-L1 pre-adipocyte cells was evaluated using a cell viability assay. Specifically, the MTT assay was performed to assess the viability of 3T3-L1 pre-adipocytes following 48 h of exposure to four different kefir concentrations (0, 50-, 100-, and 200-fold dilutions). Results are presented as the percentage of viable cells relative to the untreated control group. All tested kefir samples demonstrated a dose-dependent effect on cell viability (**Figure 8**). Exposure to undiluted kefir (the highest concentration tested) resulted in a marked reduction in cell viability. In contrast, no cytotoxic effects were observed at the 100-fold dilution, where cell viability remained above 80%. Based on these results, Indian gooseberry water kefir at a 100-fold dilution was considered safe and was therefore selected for use in all subsequent experiments.

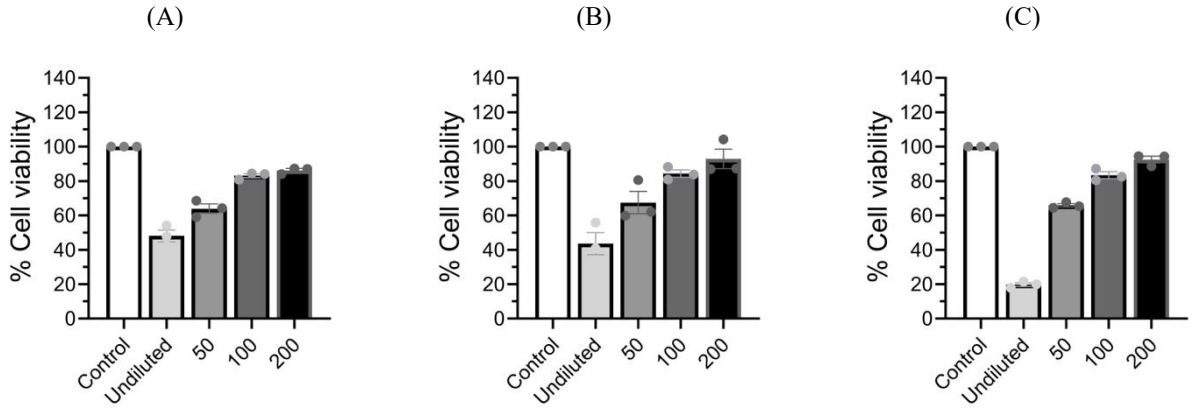


Figure 8 Effects of Indian gooseberry water kefir on cell viability in 3T3-L1 pre-adipocytes. Cell viability of 3T3-L1 pre-adipocytes after treatment of kefir samples with types of sugar: 20B (A), 10B (B), and 10I (C) at the dilution of undiluted, 50-fold (50), 100-fold (100), and 200-fold (200). The results are presented as the mean ± SD of the experiment, which was conducted in triplicate (n = 3).

Effect of Indian gooseberry water kefir on intracellular lipid accumulation

The effects of the Indian gooseberry water kefir on lipid accumulation in 3T3-L1 mature adipocytes were determined using the Oil Red O staining method, serving as an indicator of the degree of lipid droplets in the cells. According to quantitative analysis results of the Oil Red O staining, the highest efficiency was observed in the Indian gooseberry water kefir treated cells which significantly lowered the percentage lipid accumulation to 79.70 ± 1.81% ($p < 0.05$) (**Figure 9**). Consistent with the Oil Red O staining, 3T3-L1 cells treated with G310I showed a remarkable reduction of intracellular oil droplets compared to untreated control cells, as shown in **Figure 10**.

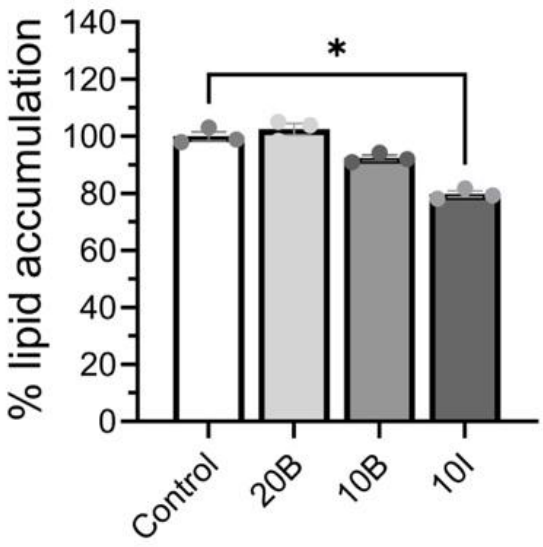
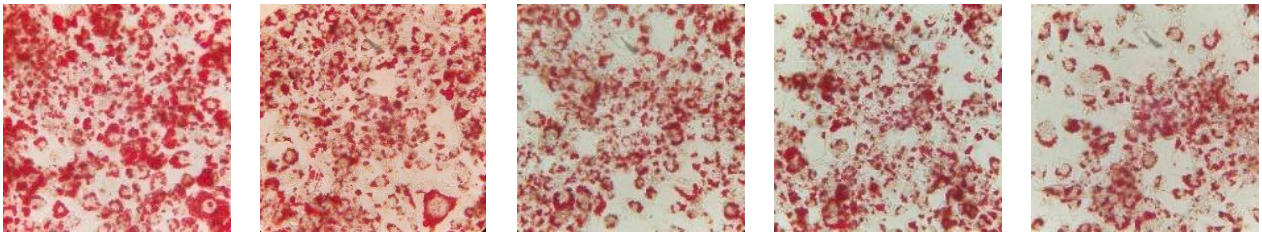


Figure 9 Inhibition effect of Indian gooseberry water kefir (at a 100-fold dilution) on the accumulation of intracellular lipid in 3T3-L1 mature adipocytes, along with three types of sugars.



Control D0 20B 10B 10I

Figure 10 Oil Red O staining of intracellular lipid accumulated in 3T3-L1 adipocytes after treatment with Indian gooseberry water kefir compared to untreated control, observed under a microscope at a magnification of ×100.

Discussion

This study investigated the microbial communities and phytochemical profile of water kefir grain, as well as the physicochemical and health-related properties of water kefir fortified with Indian gooseberry and inulin. Our findings provide the microbial composition of water kefir grains, the bioactive compound composition of Indian gooseberry-fortified kefir, and its potential effects on adipocyte biology.

We observed a substantial increase in kefir grain starter biomass and pronounced CO_2 production during fermentation, indicative of active microbial metabolism. The observed increase in the grain starter containing 20% brown sugar is consistent with previous reports that sugar enhance the proliferation of kefir grains due to the availability of fermentable substrates and increased yeast and LAB activity. In water kefir fermentation, yeasts typically hydrolyze sucrose into glucose and fructose through invertase activity, which subsequently becomes available for bacterial fermentation. These fermentable sugars are then metabolized by LAB, leading to the production of lactic acid and other metabolites that contribute to acidification and microbial stability within the kefir matrix [1].

The microbiota analysis of the kefir starter revealed that *Lactobacillus* was a major species in the kefir grain, followed by *Bacillus, Acetobacter* and other minor species. This finding aligned with the predominance of LAB, particularly *Lactobacillus* spp. in the grain. Consistent with findings by Magalhães, the predominant LAB. Some LAB (*Lactobacilli* and *Leuconostocs*) species were commonly found in water kefir and are associated with producing grain exopolysaccharide such as *L. nagelii, L. hilgardii, L. satumensis, L. mesenteroides, L. citreum* [12], with specific species such as *L. nagelii* and *L. hilgardii*

reported to facilitate exopolysaccharide production [13]. Additionally, the occurrence of Acetobacter and Gluconobacter further aligns with literature describing their role in ethanol and sugar alcohol oxidation, yielding organic acids that contribute to beverage acidity and flavor development [14]. In this study, the yeast community was predominantly comprised of *Schizosaccharomyces* and *Saccharomyces* species. Yeasts are essential in the initial stages of fermentation, contributing to pH modulation and excretion of biologically active substances (e.g., vitamins, enzymes, amino acids) that support bacterial partners. The composition of the kefir microbiome is governed by the interplay of the fermentation environment, sugar source, and co-cultivation practices [13].

Among the Indian gooseberry water kefir formulation, the inclusion of inulin provided an additional fermentable substrate for LAB proliferation while also serving as a low-calorie alternative sweetener for health-conscious formulations. Inulin has been widely reported as a fermentable prebiotic fiber that capable of selectively supporting the growth of LAB while offering a lower-calories carbohydrate source. During fermentation, probiotic bacteria, particularly *Lactobacillus* species, can metabolize inulin and produce short-chain fatty acids (SCFAs) and other bioactive metabolites that may contribute to the potential health-promoting properties of fermented beverages [15]. Consistent with previous findings, inulin supplementation may enhanced microbial fermentative activity, resulting in increased short-chain fatty acid (SCFA) production and the biosynthesis of beneficial exopolysaccharides [16]. In addition, inulin may promote the release or microbial transformation of polyphenols from Indian gooseberry, including gallic acid, ellagic acid, and punicalin [17], suggesting a potential synergistic interaction between substrate and fermenting microbes.

In present study, Indian gooseberry water kefir formulated with 10% inulin demonstrated a chemically diverse profile, with 66 diverse bioactive compounds identified, of with phenolic compounds represented the dominant class. Among these, ellagic acid, gallic acid, and quercetin-3-galactoside were detected and are known constituents of Indian gooseberry. These phenolic compounds have been widely reported to exhibit various biological activities, including antioxidant [18-20], anti-inflammatory [21-23], antimutagenic [24,25], and antitumor properties [26,27]. Quercetin-3-O-galactoside, in particular, is a common flavonoid glycoside present in many medicinal plants and contributes to antioxidant and anti-inflammatory activities.

In addition to phenolic compounds, choline, was also detected in Indian gooseberry water kefir formulated with 10% inulin. Choline is an essential nutrient involved in maintaining cell membrane integrity, neurotransmission, and liver function, and its presence may contribute to the nutritional value of the fermented beverage. A similar observation was reported by Maekawa *et al*. (2024), who detected choline in fermented brown rice and rice bran with *Aspergillus oryzae*, where its concentration increased during fermentation [30]. These findings suggest that microbial fermentation may contribute to the formation or enhanced availability of certain bioactive metabolites.

Functionally, the Indian gooseberry water kefir containing 10% inulin showed a greater capacity to reduce intracellular lipid droplet accumulation compared with the brown sugar formulation. Although the magnitude of this effect was modest, the result suggests that the combination of fermentation-derived metabolites and polyphenolic compounds may contribute to potential functional properties of the beverage. Collectively, these findings highlight the potential of Indian gooseberry water kefir as a functional fermented beverage with bioactive compound diversity and possible health-promoting effects.

Despite these findings, there are some limitations should be considered. First, the microbial community analysis was performed on kefir grains and may not fully represent the dynamic microbial changes occurring throughout the fermentation process. Second, the functional effects observed in the 3T3-L1 adipocyte model were based on an *in vitro* system, which may not fully reflect physiological responses *in vivo*.

Conclusions

This study examined microbial composition of kefir grain starter, the physicochemical characteristics of various Indian gooseberry water kefir formulations, and bioactive compound profile of Indian gooseberry water kefir produced with 10% inulin. The inclusion of inulin appeared to support the proliferation of lactic acid

bacteria (LAB) and was associated with the presence of diverse bioactive metabolites, including phenolic compounds with antioxidant properties. In addition, the detection of choline contributes to the nutritional profile of the fermented beverage. Functional assays indicated that Indian gooseberry water kefir formulated with 10% inulin showed a reduction in intracellular lipid accumulation in 3T3-L1 adipocytes compared with the brown sugar formulation.

Taken together, these findings suggest that Indian gooseberry water kefir, particularly when formulated with 10% inulin, may serve fermented beverage containing diverse bioactive compounds.

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Declaration of Generative AI in Scientific Writing

Only ChatGPT was used to improve the readability and language of a manuscript. All scientific content, interpretation, and conclusions were developed independently by the authors.

CRediT Author Statement

Natsinee U-on: Data curation, Formal analysis, Investigation, Methodology, Software and Writing – original draft. **Yingmanee Tragoolpua**: Conceptualization, and Supervision. **Wasu Pathom A-Ree**: Conceptualization, and Supervision. **ltthayakorn Promputtha**: Conceptualization, Funding administration, Project administration, Resources, Supervision, Validation, Visualization, Writing -review & editing.

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