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Review

Preparation and characterization of diacylglycerol via ultrasound-assisted enzyme-catalyzed transesterification of lard with glycerol monolaurate

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ABSTRACT

The study aimed to evaluate the effect of ultrasonic pretreatment on the transesterification of lard with glycerol monolaurate (GML) using Lipozyme TL IM to synthesize diacylglycerol (DAG), and the physicochemical properties of lard, GML, ultrasonic-treated diacylglycerol (named U-DAG), purified ultrasonic-treated diacylglycerol obtained by molecular distillation (named P-U-DAG), and without ultrasonic-treated diacylglycerol (named N-U-DAG) were analyzed. The optimized ultrasonic pretreatment conditions were: lard to GML mole ratio 3:1, enzyme dosage 6 %, ultrasonic temperature 80 °C, time 9 min, power 315 W. After ultrasonic pretreatment, the mixtures reacted for 4 h in a water bath at 60 °C, the content of DAG reached 40.59 %. No significant variations were observed between U-DAG and N-U-DAG in fatty acids compositions and iodine value, while P-U-DAG had lower unsaturated fatty acids than U-DAG. Differential scanning calorimetry analysis showed that the melting and crystallization properties of DAGs prepared by ultrasonic pretreatment significantly differed from lard. FTIR spectra noted transesterification reaction from lard and GML with and without ultrasonic pretreatment would not change the structure of lard. However, thermogravimetric analysis proved that N-U-DAG, U-DAG, and P-U-DAG had lower oxidation stability than lard. The higher the content of DAG, the faster the oxidation speed.

1. Introduction

The production of diglycerides (DAG) plays an essential role in food industry. As reported, DAG is used as an emulsifier because it has hydrophilic and lipophilic groups [1,2]. Shimada and Ohashi [3] also revealed that DAG had high surface activity and interfacial properties. In addition, DAG has certain functional characteristics, such as inhibiting the accumulation of abdominal and visceral fat [4], reducing the risk of cardiovascular disease [5], and reducing body weight [6]. In recent years, a variety of oils have been utilized to produce DAG oils, including palm oil [7], rapeseed oil [8], and soybean oil [9]. Our previous studies have found that lard can be converted into DAG through enzymatic glycerolysis [10]. However, lard-based DAG mainly consists of long-chain fatty acids [11]. Ye et al. [12] reported that compared with medium-chain fatty acids, the digestion products of long-chain fatty acids tended to accumulate at the oil–water interface, thereby restricting lipid digestion. Liang et al. [13] also showed the lipid digestion rate of long-chain triacylglycerols was lower than medium-chain triacylglycerols.

Glycerol monolaurate (GML) contains 12 carbon atoms and is composed of glycerol and lauric acid. As a derivative of medium-chain fatty acid, GML has broad-spectrum antimicrobial properties and can easily cross cell membranes without a carrier, quickly digest and deliver energy [14]. In recent years, GML has aroused great interest as an additive for many dietary foods such as bakery and meat products [15]. However, there are few studies on the preparation of DAG by GML.

Currently, DAG oils can be produced using enzymatic catalysis by direct esterification [16], transesterification [17], glycerolysis [10], and partial hydrolysis [18]. Among them, the transesterification method can introduce fatty acids with different chain lengths, and the acyl exchange or fatty acid rearrangement occurs under the action of catalyst [19]. Furthermore, enzymatic transesterification is one of the effective methods to improve the physical properties of oils. Compared with Novozym 435 and Lipozyme RM IM, Lipozyme TL IM is known for its low price and is commonly applied in interesterification [20]. Although the cost of Lipozyme TL IM used in the reaction is low, in order to increase the feasibility of commercialization, it is still a challenge to develop a strategy to improve the enzymatic activity and thus reduce production costs.

Ultrasonic pretreatment, an environmentally friendly method, can

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produce efficient cavitation energy and a significant mixing effect, which accelerates enzymatic reactions by reducing particle size, increasing substrate-enzyme interface area, and reducing mass-transfer resistance [21]. This method is especially suitable for multiphase enzymatic reactions using immiscible substrates and catalysts [22]. Furthermore, ultrasound irradiation can activate lipases and increase the active site by altering lipase conformations [23]. Liu et al. [24] found that the preparation of 1,3-dioleoyl-2-palmitoylglycerol from tripalmitin and oleic acid using lipases by ultrasonic pretreatment was more effective than traditional mechanical stirring due to ultrasound pretreatment enhancing the affinity of substrates to enzymes. Zhang et al. [25] also reported that compared with conventional mechanical stirring, the time required for the maximum conversion of ethyl ferulate with flaxseed oil by enzymatic transesterification was reduced approximately 5-fold. The ultrasonic irradiation technique has been recently identified as an excellent tool for the improvement of chemical, physical, and biological processes, particularly in highly viscous and immiscible reaction systems. However, the application of the ultrasound technique in the preparation of DAG by enzymatic transesterification reactions of GML and lard is less extensively studied.

Therefore, the objective of the current study is to investigate the effect of ultrasound pretreatment using a microtip probe in a solvent-free system on the preparation of DAG from lard with GML using enzymatic transesterification. A sequential experimental strategy was carried out to evaluate the effect of ultrasonic time, ultrasonic power, ultrasonic temperature, substrate molar ratio, enzyme dosage, and reaction time after ultrasonic pretreatment on the content of DAG. Additionally, the physicochemical properties such as fatty acid composition, iodine value, thermal properties, and FTIR spectra of DAG were analysed. The research may be useful for obtaining high yield of DAG by using this technology in the oils and fats industry.

2. Materials and methods

2.1. Materials

Pork backfat was obtained from a local commercial slaughterhouse (Jinzhou, China), and lard was prepared by heating the backfat at 120 °C. The obtained liquid lard was subsequently solidified and stored at 4 °C. Lipozyme TL IM from *Thermomyces lanuginosus* [lipase activity 360 Interesterase Units Novo (IUN)/g] was purchased from Novozymes A/S (Denmark). Dilauroyl-*rac*-glycerol standard was acquired from Sigma-Aldrich (USA). Glyceryl monolaurate (GML, food grade) was purchased from Jialishi Additives (Haian) Co., Ltd (Nantong, China). All other chemicals and reagents used were of analytical grades.

2.2. Preparation of DAG by optimizing ultrasonic conditions

DAG was prepared using a JRA-20CQ ultrasonic extraction instrument (25 kHz, 100–1500 W, Jieruian Instrument Equipment Co., Ltd., Wuxi, China) equipped with an ultrasonic probe (10 mm diameter) and a thermostatic water bath reactor (temperature accuracy ± 0.5 °C). The melted lard was mixed with GML in a certain molar ratio in a beaker, and then the Lipozyme TL IM was added. After the beaker was placed in an ultrasonic bath and the mixture reached a predetermined temperature, the ultrasonic probe was immersed into the mixture with more than a 5 mm displacement for ultrasonic treatment.

The influence of ultrasonic times (0, 3, 6, 9, 12, and 15 min), ultrasonic powers (105, 210, 315, 420, 525, and 630 W), ultrasonic temperatures (60, 65, 70, 75, 80, and 85 °C), molar ratios of melted lard to glyceryl monolaurate (4:1, 3:1, 2:1, 1:1, 1:2, and 1:3), enzyme dosage (3, 4, 5, 6, 7, and 8 wt%) were investigated. After ultrasonic pretreatment, the mixtures were transferred to a florence flask on a rotary evaporator with a vacuum pump and reacted for different times (2, 3, 4, 5, 6, and 7 h) at 60 °C with a constant speed of 200 r/min to prepare DAG. The vacuum degree was kept at −0.09 MPa to reduce lipid oxidant. After the reaction, Lipozyme TL IM was filtered utilizing six layers of cheesecloth, and samples were obtained. Lipozyme TL IM (immobilized lipase) was then washed-out three times with 20 mL of acetone to regenerate, dried at 37 °C for 3 h, and recycled for subsequent mass production [26].

Ultrasonic-treated diacylglycerol (named U-DAG) was obtained according to the above optimized reaction conditions (lard to GML mole ratio 3:1, enzyme dosage 6 %, ultrasonic temperature 80 °C, time 9 min, power 315 W). After ultrasonic pretreatment, the mixtures reacted for 4 h in a water bath at 60 °C with a constant speed of 200 r/min to prepare DAG. To obtain a higher purity of DAG, the U-DAG was purified according to the procedure of Diao et al. [10] using the two-step wiped film molecular distillation (SPE10, manufactured in Haiyuan Biochemical Equipment Co., Ltd. Wuxi, China) and labelled as purified ultrasonic-treated diacylglycerol (named P-U-DAG). The reaction conditions for the DAG obtained without ultrasonic treatment (named N-U-DAG) were the same as those presented in ultrasonic pretreatment method, but without ultrasonic pretreatment.

2.3. Quantitative analysis of DAG by TLC

The contents of triacylglycerol (TAG), DAG, and GML in samples were measured separately according to the method of Li et al. [27] by thin layer chromatography (TLC) combined with a spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan), and phosphomolybdic acid was used as a chromogenic reagent.

The samples were dissolved in developing solvent (petroleum ether, ether, and acetic acid, 40:10:1 v:v:v) to obtain a concentration of 50 mg/mL. Subsequently, the sample (5 μL) was spotted near the bottom of the TLC plate (20 × 20 cm, silica gel GF 254) using a micropipette. The sample was developed upward, dried normally, and coloured in iodine steam until the spots were entirely evident. The colour spots were completely covered with a phosphomolybdic acid ethanol solution (10 wt%), and the plate was placed in an oven at 105 °C until the spot colour switched to dark blue. Meanwhile, the plate was smoked with ammonia to reduce the effect of background colour. In reference to the retention factor values of pure GML, DAG, and TAG on a TLC plate, their corresponding blue spots were raked from the plate and dissolved in distilled water (3 mL). The supernatant obtained after centrifugation (6 000 × *g* for 10 min) was added with distilled water to 5 mL. The absorbance was measured at the wavelength of (700 ± 1) nm. A solution prepared by the previous method, in which no sample was spotted on the TLC plate, served as the blank. According to the above method, a series of TAG, DAG or GML standard solutions with different concentrations were analyzed to obtain standard curve equations. Their content (%) in the sample was separately expressed as a ratio of the amounts of them to the original sample and then multiplied by 100.

2.4. Fatty acid composition analysis

The fatty acids (FA) composition of lard, GML, U-DAG, P-U-DAG, and N-U- DAG were analysed by their fatty acid methyl esters according to the method of Li et al. [28] with some modification. Samples (200 mg) were mixed with 6 mL of n-hexane and 4 mL of KOH-CH_3OH (2 mol/L) and then shaken for 2 min. Subsequently, 6 mL of saturated NaCl solution and 2 mL of BF_3-CH_3OH were added to the mixture and centrifuged at 3,000 × *g* for 5 min. The supernatant was filtrated through a 0.22 μm organic filtration membrane to remove impurities. The clear supernatant (1 μL) was injected into an Agilent 7890A GC system (Agilent Technologies, CA, USA) equipped with an HP-5 capillary column (30 m length × 0.32 mm internal diameter × 0.25 μm film thickness) and a hydrogen flame ionization detector (FID). The nitrogen gas was used as a carrier gas with a flow rate of 1.0 mL/min and a split ratio of 20:1. The oven temperature was kept at 150 °C for 4 min, then increased to 210 °C at 15 °C/min, maintained for 5 min, and finally increased to 230 °C at 30 °C/min, hold for 5 min. The total analysis time was 25 min. The temperature of the injector and detector were kept at 250 °C and 300 °C,

respectively.

The FA in the sample was matched with the NIST-147 library spectrum by computer, and with greater than 90 % similarity was selected as the identification result. The content was expressed as a percentage of total fatty acid content.

2.5. Iodine value analysis

The iodine value of lard, GML, U-DAG, P-U-DAG, and N-U-DAG was measured using the Wijs method described by Chebet et al. [29]. The sample was dissolved in carbon tetrachloride, and the Wijs reagent (glacial acetic acid solution containing iodine monochloride, Shanghai Macklin Biochemical Co., Ltd. Shanghai, China) was added. The mixture was placed in the dark for 1 h, and then potassium iodide solution (10 mg/mL) and distilled water were added. The excess iodine was titrated with a sodium thiosulphate solution, and starch was used as an indicator. The blank test was conducted using the same procedure. Results were expressed in grams of iodine absorbed by 100 g of sample.

2.6. Differential scanning calorimetry analysis (DSC)

Melting and crystallization profiles of lard, GML, U-DAG, P-U-DAG, and N-U-DAG were analyzed using a Q2000 differential scanning calorimeter (TA Instruments, New Castle, USA), and nitrogen was used as the purge gas. Each sample (approximately 10 mg) was sealed in an aluminium pan and initially heated to 80 °C and hold for 5 min to destroy the previous crystal history [30]. Subsequently, the sample was immediately cooled from 80 °C at 8 °C /min to −50 °C to determine the crystallization profile. Afterwards, the sample was equilibrated for 5 min at −50 °C and heated again to 80 °C at the rate of 8 °C/min. During the heating process, the melting curve was recorded. An empty aluminium pan was used as a reference.

2.7. Thermogravimetric analysis

The thermogravimetry (TG) and derivative thermogravimetry (DTG) of lard, GML, U-DAG, P-U-DAG, and N-U-DAG were performed using a simultaneous thermal analyzer TG-DTA (STA8000, PerkinElmer, Inc. Massachusetts, USA). Each sample (approximately 10 mg) was placed in an aluminium crucible and heated from 25 °C to 600 °C at a heating rate of 10 °C/min. Nitrogen was used as the purge gas with a flow rate of 50 mL/min, and an empty aluminium pan was used as a reference.

2.8. Fourier transform infrared spectra qualitative analysis

Fourier transform infrared spectra (FTIR) of lard, GML, U-DAG, P-U-DAG, and N-U-DAG were acquired using an IRTracer-100 FTIR spectrophotometer (Shimadzu, Japan) according to the procedure of Vlachos et al. [31]. Different oil drops were deposited with a micropipette between two KBr disks to form a thin film. All spectra were recorded from 4000 to 500 cm^{-1} by taking 20 scans at a resolution of 4 cm^{-1}. Duplicate spectra were collected for the same sample.

2.9. Statistical analysis

All experiments were performed in triplicate and presented as mean ± standard deviation (SD). The significant difference of the main effects ($p < 0.05$) was evaluated by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test using SPSS version 22.0. Tbtools and Origin 2019 were used for drawing diagrams.

3. Results and discussion

3.1. Determination of reaction conditions for the production of DAG

3.1.1. Effect of ultrasonic time

The effect of ultrasonic pretreatment time (0, 3, 6, 9, 12, and 15 min) on the transesterification of lard and GML by Lipozyme TL IM was investigated under an ultrasonic temperature of 60 °C and power of 210 W. The other parameters, i.e., lard to GML molar ratio of 1:1, enzyme

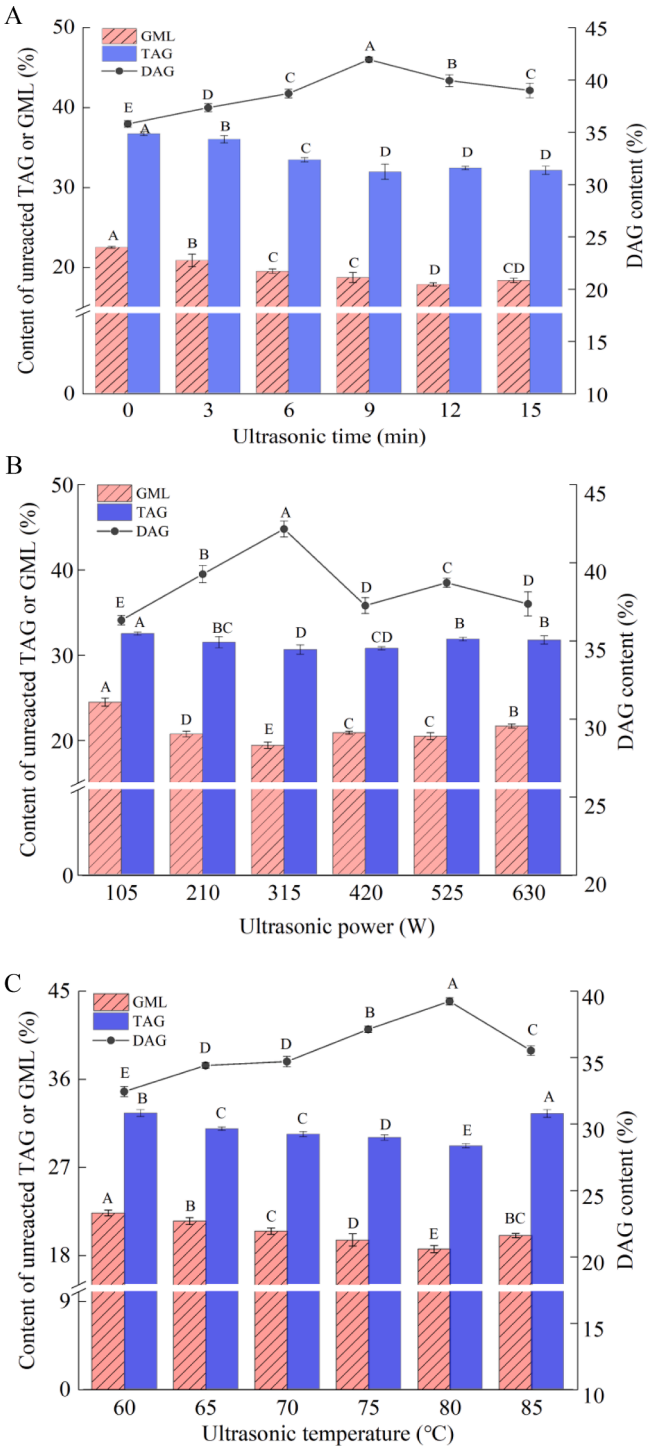

Fig. 1. Effect of ultrasonic time (A), power (B), and temperature (C) on the DAG content in transesterification reaction. Different letters (A-E) in the same indexes indicate significant differences ($p < 0.05$).

dosage of 5 %, water-bath of 60 °C for the reaction time of 5 h. Results were depicted in Fig. 1A. It can be seen from the plot that the content of DAG significantly increased from 0 min (35.77 %) to 9 min (41.92 %) of ultrasonic pretreatment. Meanwhile, the content of unreacted TAG showed the lowest at 9 min of ultrasonic pretreatment. This may be due to ultrasonic pretreatment reducing particle size and increasing catalytic surface area substrate enzyme interface area, thereby improving the efficiency of the esterification reaction [21]. Another reason may be that the appropriate ultrasonic treatment altered lipase conformation and thus affected its activity [32]. However, as the reaction processes further, the content of DAG significantly decreased after 9 min, which may be attributed to the fact that long ultrasonication times might generate more heat, leading to partially inactivating of the enzyme [24]. Therefore, the optimum ultrasonic pretreatment time of 9 min was selected in follow-up experiments.

3.1.2. Effect of ultrasonic power

The effect of ultrasonic power (105, 210, 315, 420, 525, and 630 W) on the transesterification of lard and GML by Lipozyme TL IM was studied. The reaction was carried out with an ultrasonic pretreatment time of 9 min, and other parameters were kept constant. As shown in Fig. 1B, the content of DAG increased rapidly with the increase of ultrasonic power from 105 W to 315 W ($p < 0.05$) and then decreased with the further increase of ultrasonic power, which indicated a positive role of an appropriate ultrasonic power in the transesterification reaction. One possible explanation for the finding was that ultrasonic pretreatment could enhance the strength of the reaction because of the cavitation phenomenon [33]. On the other hand, an appropriate ultrasonic power can increase the sustainability and catalytic activity of the enzyme [34]. However, too high ultrasonic power may cause enzyme inactivation, and the reaction rate would be greatly reduced or even halted. Awadallak et al. [35] also reported that the proper ultrasonic pretreatment power could improve the production of diacylglycerol by enzymatic glycerolysis. Therefore, 315 W of ultrasonic power was chosen in follow-up experiments.

3.1.3. Effect of ultrasonic temperature

The temperature has a great effect on the thermodynamic equilibrium of a reaction. The transesterification of lard and GML by Lipozyme TL IM was performed by varying the ultrasonic temperature from 60 to 85 °C, keeping fixed the other variables. Fig. 1C showed that the content of DAG significantly increased from 32.42 % to 39.23 % ($p < 0.05$) with an increase of ultrasonic temperature from 60 to 80 °C. This was probably due to the fact that the viscosity of the reaction mixture could be reduced at a higher temperature because of the increased cavitation effect, thus improving the mass transfer and increasing the formation of DAG [36]. However, when the temperature was over 80 °C, lipase tended to be inactive, leading to a decrease in the reaction rate. Meanwhile, the contents of unreacted TAG and GML increased correspondingly. Similar results also were found by Li et al. [37], who noted that the high temperature increased the interaction of the algal oil with the lauric acid mixture and ensured the activity of the enzyme, and then the highest content of lauric acid in medium and long-chain triacylglycerols was obtained. Hence, in the current research, 80 °C was set as the reaction temperature for the following investigation.

3.1.4. Effect of lard to GML molar ratio

The transesterification was conducted with different molar ratios of lard to GML ranging from 4:1 to 1:3, and the results depicted in Fig. 2. Results displayed that with increasing GML concentration, the DAG content showed a trend of increasing at first and then decreasing. This may be explained that the frequency of collisions among lard, GML, and enzyme heightened with increasing GML concentration, which promoted the forward reaction for DAG [38]. When the molar ratio of lard to GML was 3:1, the content of DAG reached the highest level. A lower GML concentration could result in an incomplete transesterification

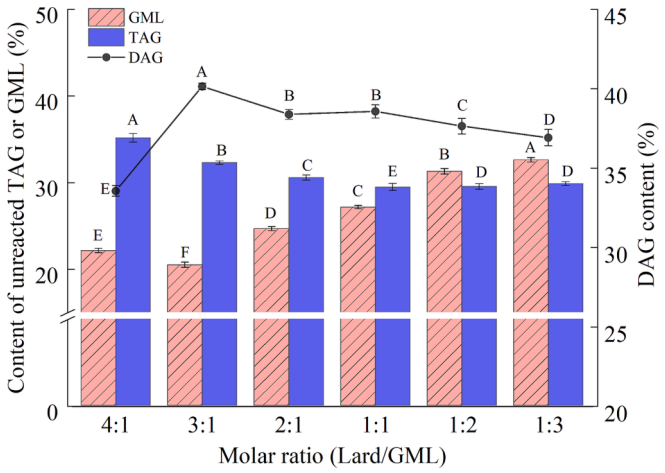

Fig. 2. Effect of molar ratio of lard to GML on the DAG content in the transesterification reaction. Different letters (A-E) in the same indexes indicate significant differences ($p < 0.05$).

reaction, while a higher GML concentration could cause a higher viscosity of the reaction mixture [39], which may lead to considerable mass transfer resistance and reduce the reaction rate. Chang et al. [40] also indicated that the system formed by chitosan and GML had higher viscosity with an increase in GML content. Additionally, varying the molar ratios of lard to GML from 4:1 to 1:3, unreacted lard showed a downward trend, while GML indicated an upward trend. This may be related to the decrease of lard and the increase of GML added to the reaction mixture. Thus, a lard to GML mole ratio of 3:1 was used for further investigation.

3.1.5. Effect of enzyme dosage

Generally, with the increase in enzyme loading, the reaction conversion rate will increase until an overload point comes [41]. Thus, to choose the optimal lipase dosage for obtaining DAG, the transesterification of GML with lard was carried out using a different dosage of Lipozyme TL IM (3, 4, 5, 6, 7, and 8 %). As illustrated in Fig. 3, an increase in enzyme dosage from 3 to 6 % resulted in an increase in the content of DAG from 31.36 % to 38.39 %. Ultrasonic treatment can modify the structure of the enzyme and substrate or by increasing the

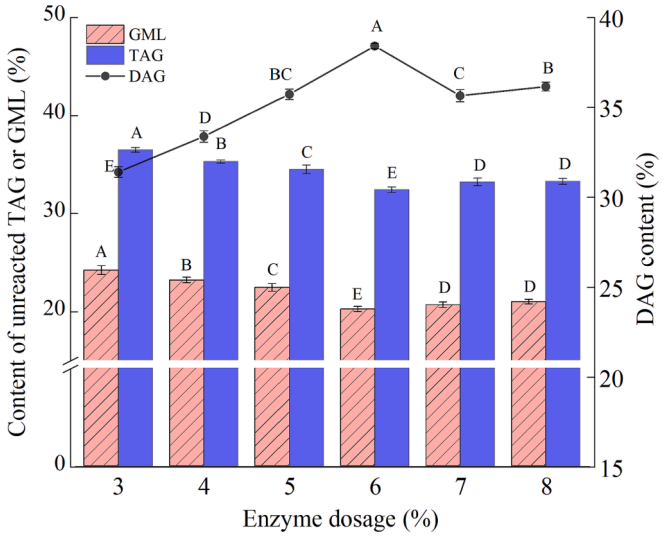

Fig. 3. Effect of enzyme dosage on the DAG content in the transesterification reaction. Different letters (A-E) in the same indexes indicate significant differences ($p < 0.05$).

mass transfer rate of the substrate in the reaction system to accelerate the enzyme reaction [42]. Furthermore, higher enzyme loading could make the enzyme-substrate complex form rapidly and then accelerate the reaction rate. However, the DAG content showed a downward trend when the enzyme addition was over 6 %. Meng et al. [43] reported that the excess enzyme caused the biocatalyst agglomeration, which resulted in a poor blend of substrates and enzymes, and ultimately inhibited mass transfer. Another reason was likely that the excessive enzyme contacted directly with the ultrasonic probe, interfering with the reaction process to some extent [21]. Overall, for enzyme dosage, 6 % was chosen as the optimum for the following investigation.

3.1.6. *Effect of reaction time after ultrasonic pretreatment*

After ultrasonic pretreatment, the mixtures were transferred to a florence flask on a rotary evaporator, and the effect of reaction times (2, 3, 4, 5, 6, and 7 h) on the transesterification was investigated at 60 °C with a constant speed of 200 r/min. As depicted in Fig. 4, the content of DAG increased with the increasing reaction time. Liu et al. [44] studied the impact of reaction times on the esterification reaction and pointed out that when lipase and reaction substrate at the interface attained a saturation state, reaction time had a considerable effect on the generation of DAGs. However, after 4 h, there was a decrease in the content of DAG, and the reaction systems reached steady levels. This was most likely due to the fact that the accumulation of reaction products on the enzyme resulted in a reduction of the surface area used for the reaction [33]. Hence, 4 h was considered to be the best reaction time after ultrasonic pretreatment.

3.1.7. *Comparison of DAG production between ultrasonic pretreatment and without ultrasonic pretreatment*

Experiments were conducted to compare the DAG content among the U-DAG, P-U-DAG, and N-U-DAG. The content of DAG depended strongly on the reaction conditions, and under the optimum conditions (enzyme dosage 6 %, lard to GML mole ratio 3:1, ultrasonic temperature 80 °C, time 9 min, power 315 W, and water bath reaction time 4 h), the DAG content reached 40.59 % in U-DAG and was further increased to 77.08 % in P-U-DAG by molecular distillation. However, under the same enzyme dosage and the mole ratio of lard to GML, the content of DAG in N-U-DAG obtained by a water bath reaction for 4 h was only 28.47 %. The results suggest that ultrasonic pretreatment could be applied to enhance the conversion of enzymatic transersterification. The result may be explained by the fact that ultrasonic treatment effectively improved the mass transfer and consequently enhanced the rate of transesterification [45]. Another reason may be that ultrasonic treatment could enhance enzyme activity [19].

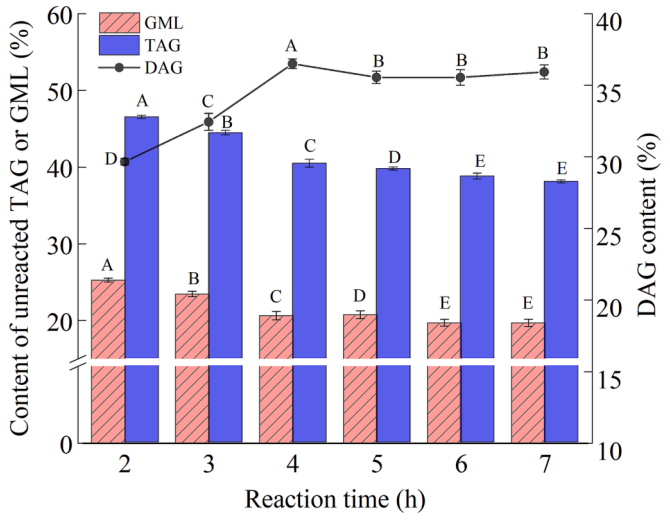

Fig. 4. Effect of reaction time on the DAG content in the transesterification reaction. Different letters (A-E) in the same indexes indicate significant differences ($p < 0.05$).

3.2. *Fatty acids and iodine value analysis*

Heat map is a statistical method widely used in recent years. It can aggregate many data and display the results as gradual colour bands to illustrate the density and frequency of the data. In order to verify the possible effects of ultrasonic pretreatment on fatty acids composition and content in the diacylglycerol oils, a heatmap analysis was performed. The deeper the red, the higher the content, and the deeper the blue, the lower the content. As shown in Fig. 5A, 14 kinds of fatty acids were identified from lard, GML, N-U-DAG, U-DAG, and P-U-DAG, including 6 kinds of saturated fatty acids (SFA) and 8 kinds of unsaturated fatty acids (UFA). The main fatty acids of lard were palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1), and the content of C18:1 was the highest. GML was predominantly composed of C16:0 and C18:0. The obtained by transesterification of lard and GML with and without ultrasonic pretreatment had a similar fatty acid composition and content, which meant ultrasonic pretreatment did not influence fatty acid composition in U-DAG. Compared with lard, SFA from U-DAG and N-U-DAG increased, whereas their UFA decreased, which was likely due to the adding GML. Additionally, the heat map (Fig. 5A) indicated that P-U-DAG had lower UFA than U-DAG, which may be attributed to a part of antioxidants such as tocopherols were removed during the molecular distillation, leading to the oxidation of UFA. Šimon and Cvengroš [46] also reported that the oxidative stability of vegetable oils decreased after molecular distillation.

The iodine value is used to measure the unsaturation of oil and fat [10]. It can be seen from Fig. 5B, the iodine value of lard was the highest while that of GML was the lowest. Furthermore, compared with lard, the iodine value of N-U-DAG, U-DAG, and P-U-DAG decreased, which was possible because of the transesteration reaction increasing the saturation of the three fats. In addition, consistent with the fatty acid results, there was no significant difference in the iodine values between N-U-DAG and U-DAG ($p < 0.05$).

3.3. *Crystallization and melting profiles*

DSC is a relatively rapid and straightforward physical technique, which is generally employed to assess the thermotropic phase behavior of oils [47]. The crystallization and melting profiles of lard, GML, U-DAG, P-U-DAG, and N-U-DAG were investigated by DSC. As evident from Fig. 6A, GML contained two distinct crystallization peaks, lard contained three crystallization peaks, but N-U-DAG, U-DAG, and P-U-DAG contained four crystallization peaks. A possible explanation for this phenomenon was that the exothermic thermogram was affected by the chemical composition of different fats. The fourth peak was possibly due to the recrystallization of DAG in N-U-DAG, U-DAG, and P-U-DAG. Silva et al. [48] also reported that an extra crystallization peak appeared in glyceryl trioleate upon the addition of diolein or dipalmitin. GML had a sharp crystallization peak, which was likely related to the large crystal structure in the GML [49]. Additionally, the large crystal structure suggested the GML may have the highest melting temperature, which was clearly reflected in the DSC melting curves (Fig. 6B). Moreover, compared with lard, the crystallization onsets of N-U-DAG, U-DAG and P-U-DAG were significantly shifted to a higher temperature, which indicated that DAG promoted the crystallization of lard. Meanwhile, a sharper crystallization peak was observed in P-U-DAG, which may be attributed to the fact that purified glycerolized lard has a high concentration of DAG, promoting nucleation and crystal growth. Huang et al. [50] also proved that the influence of diacylglycerol on the crystallization behaviour of palm olein fractions depended on its concentration. Nevertheless, similar crystallization curves were found in N-U-DAG and U-DAG, which showed they had similar chemical compositions and

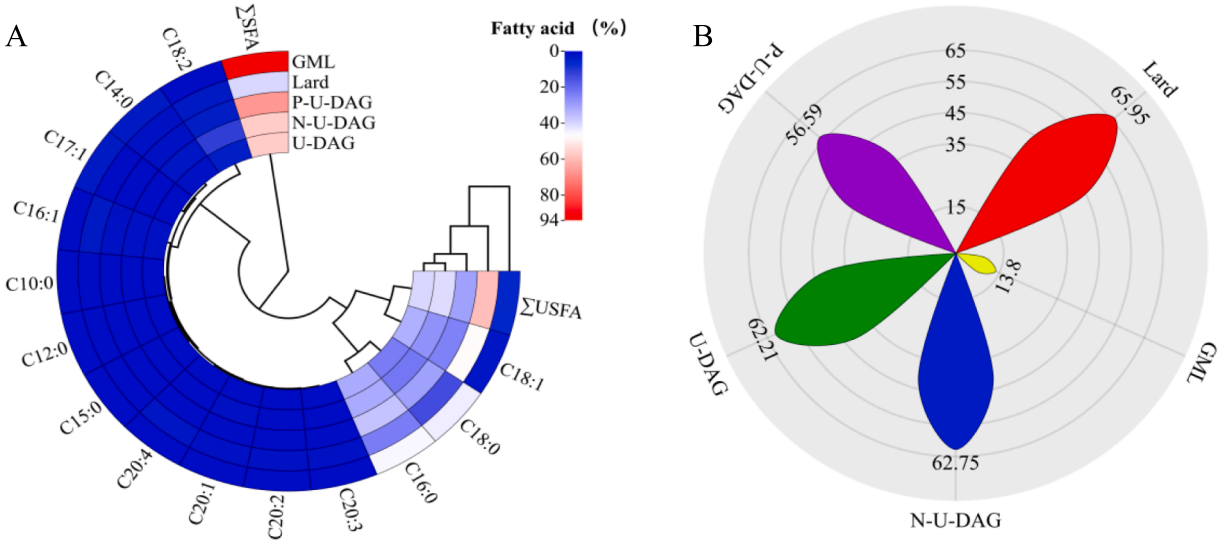

Fig. 5. Heat map of fatty acids composition (A) and iodine value (B) of lard, GML, N-U-DAG, U-DAG, and P-U-DAG.

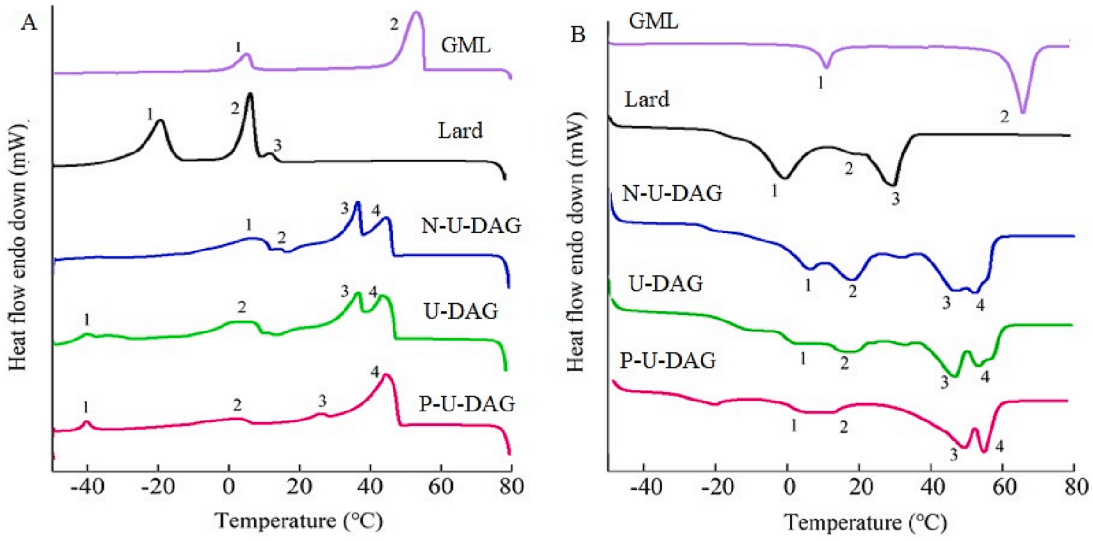

Fig. 6. DSC crystallization curves (A) and DSC melting curves (B) of lard, GML, N-U-DAG, U-DAG and P-U-DAG.

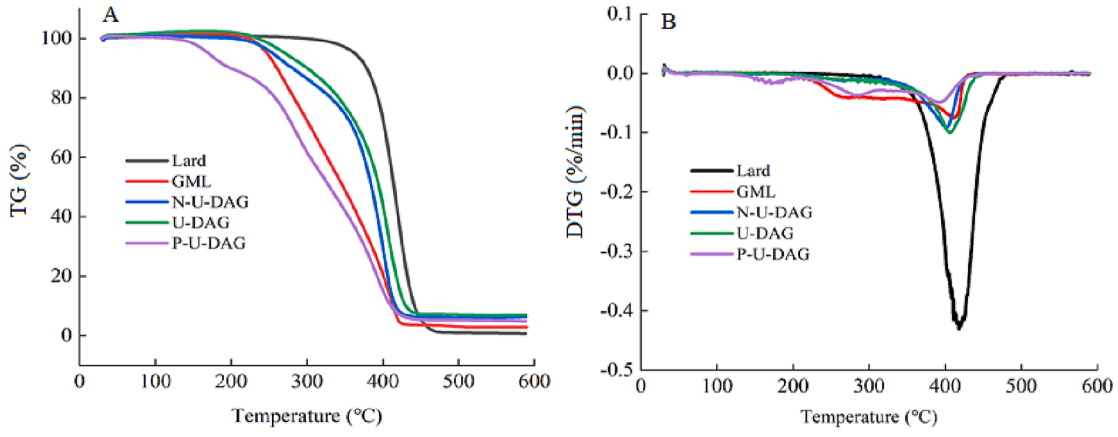

Fig. 7. TG (A) and DTG (B) curves of lard, GML, N-U-DAG, U-DAG and P-U-DAG.

structures.

Similar observations were found in the DSC melting curves in which an onset melting peak of lard was shown at approximately 0.54 °C, but the melting onsets of N-U-DAG, U-DAG, and P-U-DAG also exhibited a shift to a higher temperature, respectively (Fig. 6B), which suggested they had higher melting points compared to lard. The finding was in accordance with the results of Miklos et al. [51], who noted that the melting peaks and onset points generally shifted slightly towards higher temperatures as the content of DAGs increased. In conclusion, the melting and crystallization properties of DAGs prepared by ultrasonic pretreatment were significantly different from lard.

3.4. Thermogravimetric analysis

The TG and DTG curves represent the thermal behaviour of oil and fat, and thermal stability is expressed by the temperature range in which the mass remains constant [52]. Mass change occurs when the physical and chemical bonds are formed and broken at high temperatures [53]. As can be seen from the TG curve (Fig. 7A), the extrapolated onset decomposition temperatures of lard, GML, U-DAG, N-U-DAG, and P-U-DAG were 335.28, 222.86, 204.73, 199.64, and 135.02 °C, respectively. The discovery showed that lard was not susceptible to thermal deterioration compared to other samples, which may be due to their different molecular structures. In general, the higher the unsaturated degree of fatty acids in oil and fat, the lower the thermal stability. Santos et al. [54] also reported that the thermal stability of oils was dependent on fatty acid composition. Although lard has a high content of unsaturated fatty acids (Fig. 5A), it is formed by the esterification of a glycerol backbone and three fatty acids. While DAG has two fatty acids esterified with glycerol, leading to less steric hindrance [55]. Therefore, U-DAG, N-U-DAG, and P-U-DAG were more easily oxidized than lard. The P-U-DAG especially contains high concentrations of DAG (77.08 %), which made it had lower stability. Additionally, Huang et al. [17] suggested that different conditions, including molecular weight, viscosity, intermolecular forces, conjugated double bonds, and fatty acids distribution, might randomly affect the oxidative stability of oil and fat.

Analyzing the behaviour of all samples on DTG curves (Fig. 7B), it was observed that lard had the maximum mass loss rate at 419.47 °C. Compared with lard, although GML, U-DAG, N-U-DAG, and P-U-DAG showed a displacement of the largest DTG peak to low temperature, their oxidation and decomposition rate slowed down. This phenomenon may be attributed to the fact that the high content of unsaturated fatty acids in lard makes oxidation proceed quickly once it occurs. Liu et al. [56] also suggested that the unsaturated fat fraction in minced pork promoted the increase of oxidative stability of both lipids and proteins.

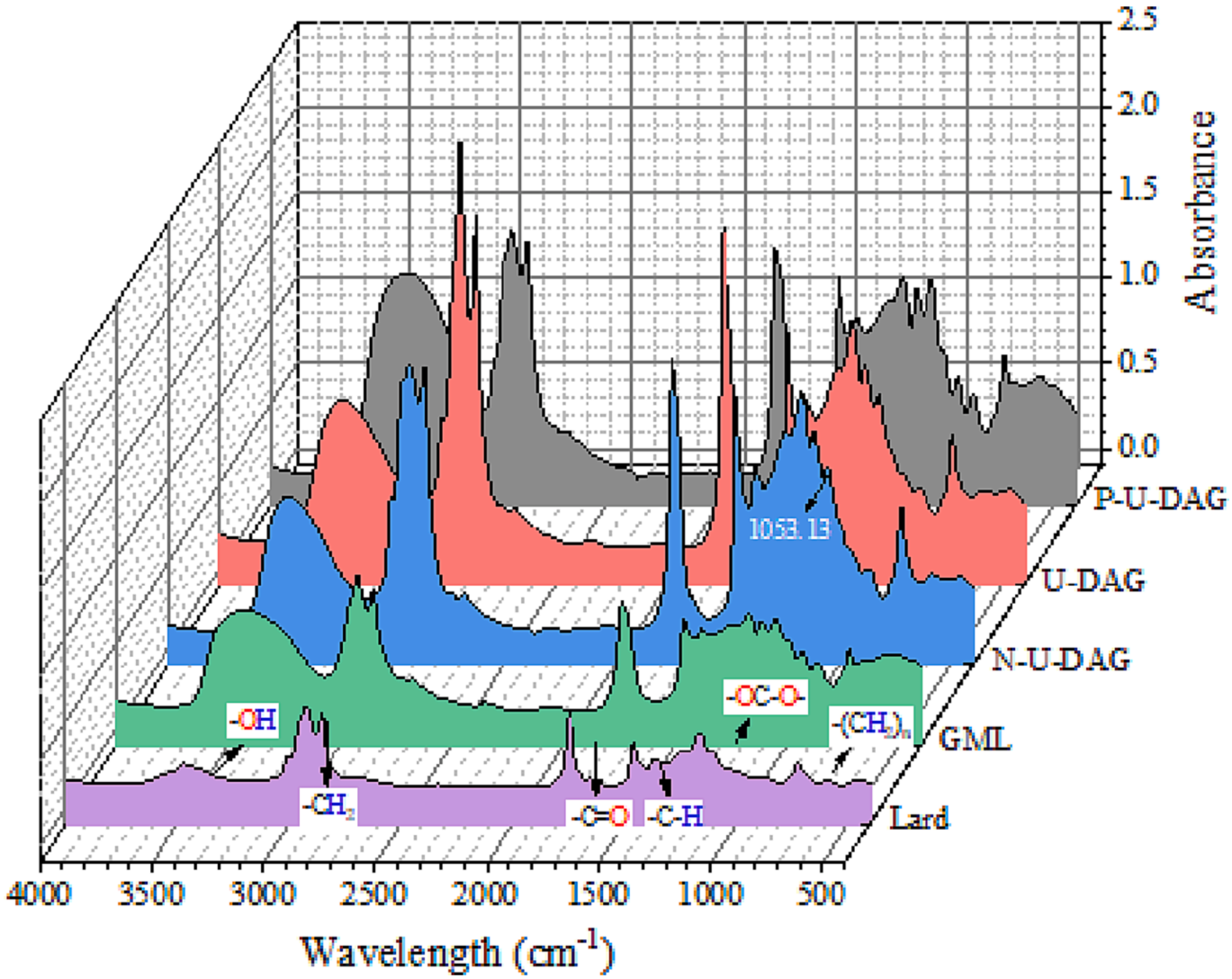

Fig. 8. Fourier-transform infrared spectra of lard, GML, N-U-DAG, U-DAG and P-U-DAG from 4000 to 500 cm^{-1}.

3.5. FTIR analysis

The absorption bands in the FTIR spectra can provide information about the functional groups [57]. According to Gumel et al. [58] and Lerma- Garcia [59], the band at 3499 cm^{-1} indicates the presence of a hydroxyl group, the band at 2995 cm^{-1} corresponds to the aliphatic CH_2 symmetric stretching vibrations, the band at 1731 cm^{-1} is attributed to the stretching vibrations of C = O, the band at 1466 cm^{-1} is due to the plane flexural vibration of -C-H, the band at 1258 cm^{-1} is linked to the C-O stretching vibration in OC-O, and the band at 726 cm^{-1} represented the swing vibration of $–(CH_2)_n$. Fig. 8 compares the FTIR spectrum of lard, GML, U-DAG, N-U-DAG, and P-U-DAG. As can be observed, the five types of fats contained these functional groups, and the major absorption bands were similar. Minor differences in the FTIR spectra were observed. Compared with N-U-DAG, U-DAG, and P-U-DAG, the height and width of the 3466.08 cm^{-1} (–OH), 2922.16 cm^{-1}($–CH_2$), 1741.72 cm^{-1}(-C = O), 1240.23 cm^{-1}(–OC-O), and 721.38 cm^{-1} [-$(CH_2)_n$] for the lard were small. The phenomenon may be explained by the fact that the formation of DAG in the transesterification reaction between lard and GML, and the higher the content of DAG, the higher the band. Another reason may be that the intensity of the peaks is related to the size of the carbon chain of the fatty acids [60]. Additionally, GML, N-U-DAG, U-DAG, and P-U-DAG had an absorption peak at 1053.13 cm^{-1}, whereas lard had no absorption peak in the wavelength, which was attributed to the GML added in the transesterification reaction. The results noted transesterification reaction from lard and GML with and without ultrasonic pretreatment would not change the structure of lard.

4. Conclusions

The present work has reported the transesterification of lard with GML under the influence of ultrasonic pretreatment using Lipozyme TL IM. Results showed that at the optimized ultrasonic pretreatment conditions, the content of DAG reached 40.59 % in U-DAG. However, under the same water bath reaction conditions without ultrasonic pretreatment, the content of DAG in N-U-DAG obtained by transesterification was only 28.47 %. Furthermore, no significant variations were observed between U-DAG and N-DAG in fatty acids compositions, iodine value, and FTIR spectra. DSC analysis indicated that the DAG could promote the crystallization of lard. However, the TG and DTG curves showed that DAG was more easily oxidized than lard. In conclusion, ultrasonic-assisted enzyme transesterification is a feasible process for high yield of DAG, and is considered bo be more environmentally friendly and economical. Thus, the present study will provide a theoretical reference for the DAG production in the oils and fats industry.

CRediT authorship contribution statement

Xiaoqin Diao: Conceptualization, Methodology, Writing – original draft. **Weiting Sun:** Visualization, Formal analysis. **Ruixin Jia:** Software, Data curation. **Ying Wang:** Formal analysis, Investigation. **Dengyong Liu:** Funding acquisition, Conceptualization, Supervision. **Haining Guan:** Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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