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The degree of esterification influences the bioactivity of pectic polysaccharides isolated from *Lithocarpus litseifolius*

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ABSTRACT

Pectic polysaccharides are the mainly bioactive components in *Lithocarpus litseifolius* (sweet tea) leaves. Nevertheless, owing to their diverse and complex chemical structures, the detailed structure-function relationships (SFR) of pectic polysaccharides from sweet tea (STP) are still unclear. Herein, the influence of STP's esterified degree on its diverse biological functions was uncovered. The results showed that the de-esterified STPs with a middle-esterified degree (33.53 %) and a low-esterified degree (7.66 %) were successfully prepared when compared with the original high-esterified STP (47.01 %), and their primary structural features were almost stable after the controllable de-esterification treatment. Furthermore, the findings inferred that STP's antioxidant, anti-diabetic, and immunostimulatory functions showed inverse correlations with the esterified degree. Moreover, the de-esterified STP with a lower-esterified degree could be more easily utilized by intestinal microorganisms to positively regulate the gut microbial composition. Overall, these findings can provide valuable insights for elucidating STP's precise SFR.

1. Introduction

Sweet tea (*Lithocarpus litseifolius*) is an evergreen tree that predominantly distributed in Southern China, and it is widely consumed as a daily beverage in China (Shang et al., 2020). Besides, it is also used as a folk medicine or an herbal tea to manage and prevent certain chronic diseases (*e.g.*, diabetes and hyperlipidemia) for a long history, because it contains a variety of bioactive ingredients, *e.g.*, polysaccharides, phenolic acids, and flavonoids (Lei et al., 2022; Shang et al., 2020; Wu et al., 2022). Notably, pectic polysaccharides represent the major bioactive polysaccharides in sweet tea, which are predominantly consisted of homogalacturonan (HG) and rhamnogalacturonan I (RG-I) pectin domains (Guo et al., 2021; Guo et al., 2022; Lei et al., 2022; Wu et al., 2022). Furthermore, pectic polysaccharides from sweet tea (STP) possess a variety of biological functions, *e.g.*, antioxidant, anti-diabetic, anti-glycation, prebiotic, and immunostimulatory functions (Guo et al., 2021; Guo et al., 2022; Lei et al., 2022; Wu et al., 2022), thereby attracting considerable attentions to be exploited as functional components in the health food, medicine, and cosmetic industries.

Pectic polysaccharides are a class of structurally complex and

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); Ara, arabinose; B/F, *Bacteroidetes* to *Firmicutes*; BLK group, blank group (negative control group); CDT, controllable de-esterification treatment; DES, deep eutectic solvent; DNA, *deoxyribonucleic acid*; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HPLC, high performance liquid chromatography; FRAP, ferric reducing antioxidant power; FT-IR, fourier transform infrared; Gal, galactose; GalA, galacturonic acid; HG, homogalacturonan; IL-6, interleukin-6; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; NO, nitric oxide; PCR, polymerase chain reaction; Rha, rhamnose; RG-I, rhamnogalacturonan I; rRNA, ribosomal ribonucleic acid; SCFAs, short chain fatty acids; SFR, structure-function relationships; STP, pectic polysaccharides from sweet tea; STP-HDE, the original high-esterified pectic polysaccharides from sweet tea; STP-LDE, the low-esterified pectic polysaccharides from sweet tea; STP-MDE, the middle-esterified pectic polysaccharides from sweet tea; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

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heterogeneous bioactive macromolecules (Jin et al., 2021), and their structural features always affect their processing characteristics, technological functions, and physiological benefits (Jin et al., 2021; Yue et al., 2023). For instance, the variations in their physicochemical structures (*e.g.*, different esterified degrees, various molecular mass distributions, different glycosidic bonds, and different sidechain types/lengths) can result in significant differences in their biological functions (*e.g.*, antioxidant, anti-obesity, anti-inflammatory, immunomodulatory, and prebiotic functions) (Fernandes & Coimbra, 2023; Jin et al., 2021; Lee et al., 2022). Notably, among these structural features, a plenty of studies have demonstrated that the esterified degree of pectic polysaccharides serves as a critical role in their various biological functions (Ferreira et al., 2023; Li, Feng, et al., 2024; Li, Lei, et al., 2024; Li, Wang, et al., 2024; Zhao et al., 2024). Nevertheless, although recent studies have inferred that STP's biological functions vary with its diversely chemical structures that caused by different sample preparation

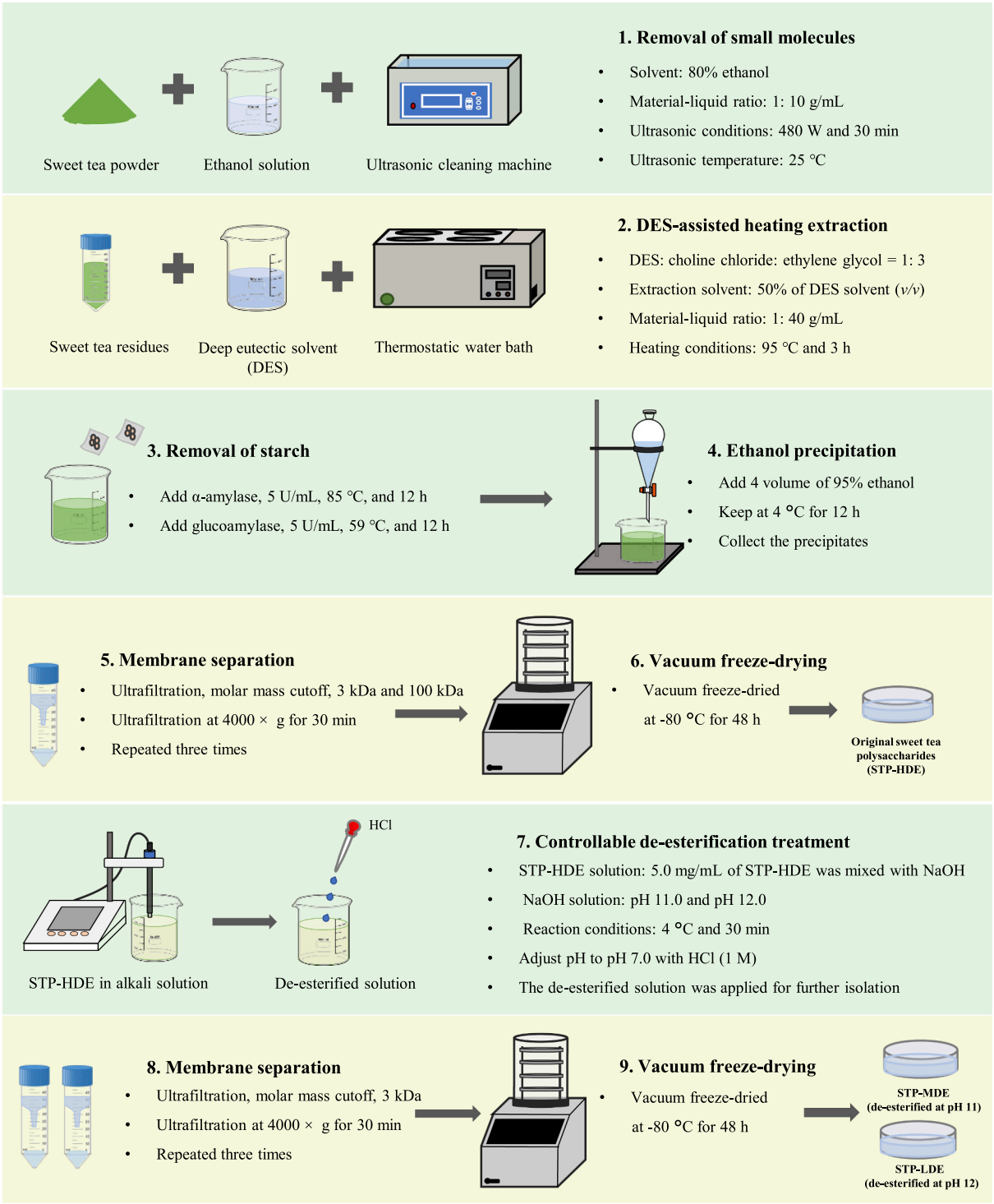

Fig. 1. The flowchart for the extraction, isolation, and de-esterification of pectic polysaccharides from sweet tea (STP).
STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively.

methods (Guo et al., 2021; Guo et al., 2022), the detailed structure-function relationships (SFR) of STP are still unclear and remain to be fully elucidated.

Therefore, in order to understand STP's precise SFR, the impact of esterified degree on its *in vitro* antioxidant, anti-diabetic, and immunostimulatory functions as well as *in vitro* fermented characteristics was systematically studied. The present findings can provide crucial insights for elucidating STP's precise SFR, and are also helpful for facilitating its development as a functional ingredient in nutraceutical, pharmaceutical, and cosmeceutical applications.

2. Materials and methods

2.1. Materials and chemicals

Dried leaves of sweet tea were commercially sourced from a Chengdu-based supermarket in China, and then were pulverized using an ultrafine pulverizer (RS-FS553, Royalstar, Hefei, China) and sieved *via* an eighty-mesh sieve to obtain a homogeneous powder. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), heat-stable α-amylase (20,000–60,000 U/mL), glucoamylase (40,000 U/g solid), α-amylase (300–1500 U/mg protein), α-glucosidase (100 U/mg protein), and standard short chain fatty acids (acetic, propionic, *n*-butyric, and *i*-butyric acids) were acquired from Sigma-Aldrich (St. Louis, MO, USA), Aladdin-E (Shanghai, China), and Solarbio® (Beijing, China). Acarbose tablets were acquired from Bayer (China) Co., Ltd. (Shanghai, China). ELISA kits, resatorvid (TAK-242), and *deoxyribonucleic acid* (DNA) extraction kit were purchased from Elabscience, MedChemExpress China, and Qiagen, respectively.

2.2. Isolation and chemical modification of pectic polysaccharides from sweet tea (STP)

Deep eutectic solvent (DES)-assisted heating extraction has been considered as a powerful technique for extracting pectic polysaccharides from edible plants (Wu et al., 2022; Wu et al., 2024). Therefore, STP was prepared by DES-assisted heating extraction following an established protocol (Wu et al., 2024). The detailed steps and conditions for the extraction and isolation of STP, including 1) removal of small molecules, 2) DES-assisted heating extraction, 3) removal of starch, 4) ethanol precipitation, 5) membrane separation, and 6) vacuum freeze-drying, are shown in Fig. 1. Finally, the partially purified STP was obtained and named as STP-HDE (the original high-esterified STP). In addition, the precise de-esterification of STP-HDE was carried out by the controllable de-esterification treatment (CDT) according to a previously established procedure (Li, Feng, et al., 2024), and the detailed steps (steps 7–9) and conditions for the de-esterification of STP-HDE are also displayed in Fig. 1. Finally, the de-esterified STPs were obtained and named as STP-MDE (the middle-esterified STP) and STP-LDE (the low-esterified STP), respectively.

2.3. Chemical characterization of original and de-esterified STPs

To confirm the influence of the CDT treatment on the chemical structure of STP, the chemical compositions, molecular masses, rheological properties, compositional monosaccharides, esterified degrees, and glycosidic bonds of original and de-esterified STPs were systematically investigated according to a range of established analytical techniques (Li, Feng, et al., 2024; Li, Wang, et al., 2024; Wu et al., 2024). More specifically, the chemical compositions, including the level of total polysaccharides, level of total uronic acids, level of total proteins, and level of total bound polyphenols, were measured by the phenol-sulfuric acid method, *m*-hydroxyphenyl method, Bradford's method, and Folin-Ciocalteu assay, respectively. The molecular masses were determined by size exclusion chromatography (Agilent 1260, Agilent Technologies, Palo Alto, CA, USA) combined with laser light scattering detector (HELEOS II, Wyatt Technology Co., Santa Barbara, CA, USA) and refractive index detector (DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA). The rheological properties were measured by a compact rheometer (MCR302e, Anton Paar GmbH, Graz, Austria). The compositional monosaccharides were detected by high performance liquid chromatography (HPLC, L-20 A, Shimadzu, Tokyo, Japan). The chemical groups were detected by fourier transform infrared (FT-IR) spectroscopy (Spectrum Two spectrometer, PerkinElmer, Waltham, MA, USA) and the esterified degrees were calculated based on the characteristic signals at 1744.1 cm^{-1} and 1627.7 cm^{-1}. The glycosidic bonds were measured by nuclear magnetic resonance (NMR) spectroscopy (Ascend 600 MHz spectrometer, Bruker, Rheinstetten, Germany).

2.4. Comparison of biological functions of original and de-esterified STPs

To reveal the precise SFR of STP, the *in vitro* biological functions of original and de-esterified STPs were systematically studied. In detail, for the comparison of antioxidant effects of original and de-esterified STPs, their ABTS, DPPH, and nitric oxide (NO) free radical scavenging abilities as well as ferric reducing antioxidant power (FRAP) were investigated based on previous studies (Li, Feng, et al., 2024; Li, Wang, et al., 2024). Besides, for the comparison of *in vitro* anti-diabetic effects of original and de-esterified STPs, their inhibitory effects against the digestive enzymes, namely α-amylase and α-glucosidase, were investigated according to previous methods (Wu et al., 2022; Yuan et al., 2019). Furthermore, for the comparison of *in vitro* immunostimulatory effects of original and de-esterified STPs, their influences on the secretion of pro-inflammatory mediators and cytokines from RAW 264.7 macrophages were evaluated based on previous methods (Li, Feng, et al., 2024). Besides, the Toll-like receptor 4 (TLR4) inhibitor (TAK-242) was utilized to explore whether the TLR4-mediated signaling pathway was involved in the activation of RAW 264.7 macrophages by original and de-esterified STPs (Li, Feng, et al., 2024). At last, for the comparison of *in vitro* fermented characteristics of original and de-esterified STPs, their potential microbial metabolic behaviors and influences on the intestinal microbial composition were investigated based on previous studies (Lei et al., 2022; Wu et al., 2021). The variations in contents of reducing sugars and total carbohydrates in the fermented broth during the *in vitro* fermentation period were detected according to previously reported methods (Lei et al., 2022; Wu et al., 2021). The variations in pH values and contents of short chain fatty acids (SCFAs) in the fermented broths during the *in vitro* fermentation period were also determined based on previously reported methods (Lei et al., 2022; Wu et al., 2021). Besides, to reveal the impact of STP's esterified degree on its gut microbial regulation effect, the DNA extraction, polymerase chain reaction (PCR) amplification, and 16S ribosomal ribonucleic acid (16S rRNA) gene sequencing were also conducted based on previous studies (Lei et al., 2022; Wu et al., 2021).

2.5. Statistical analysis

Statistical analysis was conducted using Origin software (Origin 9.0, OriginLab Corporation, Northampton, Mass., USA). One-way analysis of variance (ANOVA) plus *post hoc* Duncan's test and Student's *t*-test were carried out for significance determination, and values of $p < 0.05$ were regarded as statistically significant. Besides, the USEARCH software (version 10.0.240), the Silva database (http://www.arb-silva.de), and the Ribosomal Database Project (RDP) Classifier (version 2.14) were carried out for the analysis of gut microbial composition according to a previous study (Wu et al., 2021).

3. Results and discussion

3.1. Effects of the controllable de-esterification treatment (CDT) on physicochemical and structural characteristics of STP

3.1.1. Comparison of physicochemical properties of original and de-esterified STPs

Previous experimental results have clarified that pectic polysaccharides' physicochemical properties (*e.g.*, polysaccharide content, protein content, and uronic acid content) are almost stable after the de-esterified treatment under a low temperature and a low concentration of sodium hydroxide conditions (Li, Feng, et al., 2024; Li, Lei, et al., 2024; Zhang et al., 2021). Therefore, in order to confirm whether the CDT treatment can affect the chemical property of STP, the basically chemical compositions of original and de-esterified STPs were compared in this study. Obviously, as shown in Table 1, the levels of total polysaccharides and total proteins in STP-HDE, STP-MDE, and STP-LDE were almost stable, which were in the ranges of 88.90 mg/100 mg – 90.55 mg/100 mg and 1.51 mg/100 mg – 1.54 mg/100 mg, respectively. However, the levels of total uronic acids in de-esterified STPs slightly reduced from 29.49 mg/100 mg to 26.71 mg/100 mg, which is comparable to recent studies that the β-elimination reaction induced by the CDT treatment at a low concentration of sodium hydroxide could slightly break the backbone of HG domain in pectin molecules (Li, Wang, et al., 2024; Zhang et al., 2021). Besides, the levels of bound phenolics in de-esterified STPs decreased from 4.47 mg GAE/100 mg to 3.12 mg GAE/100 mg, which could be attributed to the reason that the mild alkaline condition of the CDT treatment could easily destroy the ester bonds to liberate bound phenolics (Li, Wang, et al., 2024). Furthermore, as displayed in Table 1, the esterified degree of the original STP notably declined from 47.01 % (STP-HDE) to 7.66 % (STP-LDE) after the CDT treatment at pH 12.0 and 4 °C for 30 min, which is comparable to recent studies (Li, Wang, et al., 2024; Liang et al., 2022; Zhang et al., 2021). Overall, these findings inferred that the CDT treatment could effectively reduce STP-HDE's esterified degree and its level of bound phenolics, while had minor effects on its contents of total polysaccharides, uronic acids, and proteins.

Table 1
Effects of the controllable de-esterification treatment on the physicochemical properties of pectic polysaccharides from sweet tea (STP).

	STP-HDE	STP-MDE	STP-LDE
Chemical compositions			
Total polysaccharides (mg/100 mg)	89.99 ± 0.96 ^{a}	88.90 ± 1.23 ^{a}	90.55 ± 1.26 ^{a}
Total uronic acids (mg/100 mg)	29.49 ± 0.73 ^{a}	28.36 ± 0.58 ^{a}	26.71 ± 0.55 ^{b}
Total proteins (mg/100 mg)	1.54 ± 0.03 ^{a}	1.51 ± 0.01 ^{b}	1.53 ± 0.02 ^{ab}
Total phenolics (mg GAE/100 mg)	4.47 ± 0.01 ^{a}	3.23 ± 0.05 ^{b}	3.15 ± 0.05 ^{b}
Degree of esterification (%)	47.01 ± 0.62 ^{a}	33.53 ± 0.94 ^{b}	7.66 ± 0.63 ^{c}
Molecular mass and its distribution			
$M_w \times 10^4$ (Da, error)	4.472 ± 0.037 ^{a}	4.376 ± 0.038 ^{a}	4.268 ± 0.055 ^{b}
M_w/M_n	1.749	1.712	1.659
Monosaccharides and molar percentage (mol%)			
Galacturonic acid (GalA)	31.67	30.75	29.24
Galactose (Gal)	29.16	30.51	30.96
Arabinose (Ara)	22.03	22.89	23.67
Rhamnose (Rha)	6.39	6.73	7.29
Xylose (Xyl)	4.67	4.44	4.50
Glucose (Glc)	3.20	1.77	1.72
Glucuronic acid (GlcA)	1.99	2.09	1.79
Mannose (Man)	0.89	0.82	0.83
Homogalacturonan (HG)	25.29	24.02	21.94
Rhamnogalacturonan I (RG-I)	63.96	66.85	69.22
Average length of RG-I sidechain	8.02	7.94	7.49

STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; Data were presented as mean ± standard deviation; Different letters (a-c) indicate statistical significances ($p < 0.05$) among STP-HDE, STP-MDE and STP-LDE. HG (mol%) = GalA (mol%) - Rha (mol%); RG-I (mol%) = GalA (mol%) - HG (mol%) + Rha (mol%) + Gal (mol%) + Ara (mol%); RG-I sidechain length = (Gal + Ara)/Rha.

3.1.2. Comparison of molecular mass distributions and rheological properties of original and de-esterified STPs

Recent studies have demonstrated that the mild alkaline treatment exhibit minor impacts on the molecular mass and its distribution of pectin molecules (Li, Feng, et al., 2024; Li, Lei, et al., 2024; Liang et al., 2022). Herein, to confirm the impact of the CDT treatment on the molecular mass distribution and rheological property of STP, the molecular mass distributions and apparent viscosities of original and de-esterified STPs were compared in this study. As displayed in Fig. 2A, there was a high overlap in size-exclusion elution curves of STP-HDE, STP-MDE, and STP-LDE, indicating that the CDT treatment possessed limited impacts on the molecular mass distribution of STP. In fact, their molecular masses and molecular mass distributions were in the range of 4.268×10^4 (STP-LDE) – 4.472×10^4 Da (STP-HDE) and 1.659 (STP-LDE) – 1.749 (STP-HDE), respectively. In addition, Fig. 2B showed the effects of the shear rate on apparent viscosities of original and de-esterified STPs. Obviously, the apparent viscosities of original and de-esterified STPs were very similar, implying that the rheological property of STP was overall stable after the CDT treatment, which is comparable to the change trend of STP's molecular mass. Furthermore, both original and de-esterified STPs showed non-Newtonian shear-thinning and near Newtonian fluid behaviors at different shear rates (Fig. 2B).

3.1.3. Comparison of structural properties of original and de-esterified STPs

In order to further confirm whether the CDT treatment can alter STP's basic chemical structure, the compositional monosaccharide, FT-IR, and NMR analysis were conducted to systematically compare structural properties of original and de-esterified STPs. Fig. 2C displayed that the HPLC profiles of original and de-esterified STPs were extremely similar, which indicated that STP's compositional monosaccharides were almost stable after the de-esterification. This phenomenon is comparable to previous studies that the alkaline treatment under a low concentration of sodium hydroxide condition has limited impacts on the compositional monosaccharides of pectin molecules (Li, Feng, et al., 2024; Li, Lei, et al., 2024). As shown in Table 1, the molar percentages of each monosaccharide in STP-HDE, STP-MDE, and STP-LDE were extremely similar. In detail, the molar percentages of galacturonic acid (GalA), galactose (Gal), arabinose (Ara), and rhamnose (Rha) in original and esterified STPs were in the ranges of 29.24 % – 31.67 %, 29.16 % – 30.96 %, 22.03 % – 23.67 %, and 6.39 % – 7.29 %, respectively. The molar percentage of GalA in STP slightly decreased from 31.67 % (STP-HDE) to 29.24 % (STP-LDE) after the CDT treatment. Moreover, according to the molar percentages of GalA, Gal, Ara, and Rha that commonly exist in pectin molecules, the ratios of HG and RG-I regions in original and de-esterified STPs can be assessed, and the average length of RG-I sidechain can be also estimated (Hou et al., 2021). As displayed in Table 1, the ratios of HG and RG-I domains in original and de-esterified STPs were in the ranges of 21.94 % (STP-LDE) – 25.29 % (STP-HDE) and 63.96 % (STP-HDE) – 69.22 % (STP-LDE), respectively, suggesting that the CDT treatment could slightly decrease the molar percentage of HG domain in STP, thereby slightly increasing its RG-I domain. This phenomenon may be owing to the reason that the β-elimination reaction induced by the CDT treatment could break the backbone of HG domain (Zhang et al., 2021). Besides, the average length of RG-I sidechain in STP also slightly decreased from 8.02 to 7.49, indicating that the CDT treatment possessed limited impacts on the RG-I sidechain.

In addition, the FI-TR spectra of STP-HDE, STP-MDE, and STP-LDE

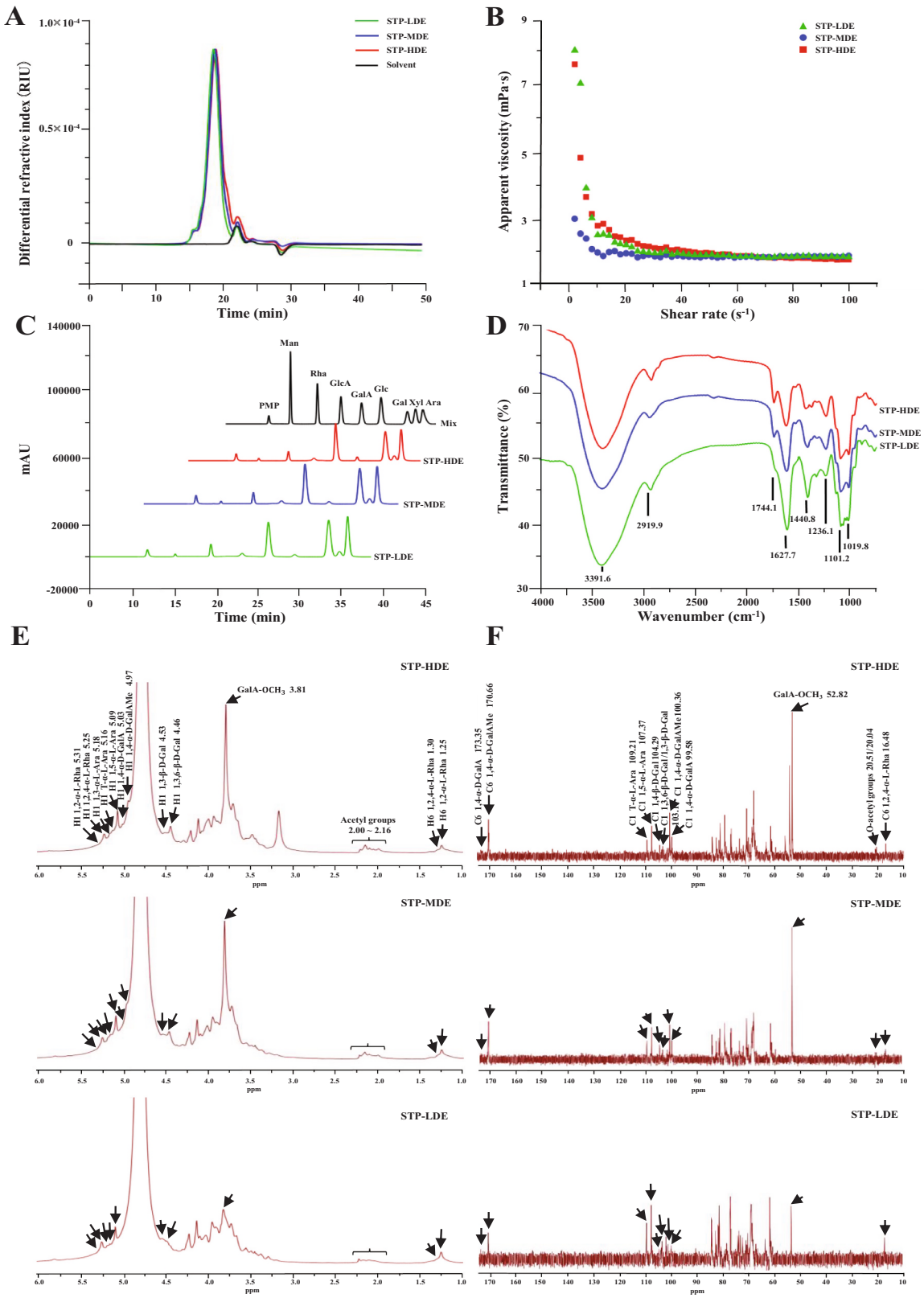

Fig. 2. Size exclusion chromatography profiles (A), apparent viscosities (B), HPLC profiles of constituent monosaccharides (C), FT-IR spectra (D), and NMR spectra (E and F) of original and de-esterified pectic polysaccharides from sweet tea (STP).
STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; PMP, 1-phenyl-3-methyl-5-pyrazolone; Man, mannose; Rha, rhamnose; GlcA, glucuronic acid; GalA, galacturonic acid; Glc, glucose; Gal, galactose; Xyl, xylose; Ara, arabinose.

were compared. As shown in Fig. 2D, both original and de-esterified STPs possessed similar chemical groups, and the similarly characteristic signals were observed at 3391.6, 2919.9, 1744.1, 1627.7, 1440.8, 1236.1, 1101.2, and 1019.8 cm^{-1} (Li, Feng, et al., 2024; Liang et al., 2022), respectively. Nevertheless, it could be obviously found that the intensity of signal at 1744.1 cm^{-1} in STP decreased after the CDT treatment, while the intensity of signal at 1627.7 cm^{-1} increased. In fact, based on the peak areas of 1744.1 and 1627.7 cm^{-1} (Hou et al., 2021), the esterified degrees of STP-HDE, STP-MDE, and STP-LDE were calculated to be 47.01 %, 33.53 %, and 7.66 %, respectively.

Furthermore, the ^{1}H and ^{13}C NMR spectra of original and de-esterified STPs were compared. As displayed in Fig. 2E and Fig. 2F, both original and de-esterified STPs exhibited similar one-dimensional NMR spectra, implying that the basic structural feature of STP, except the esterified groups, was almost stable after the CDT treatment. In detail, as shown in Fig. 2E and F, the characteristic NMR signals of anomeric protons and anomeric carbons that corresponded to HG and RG-I pectic regions could be observed in original and de-esterified STPs. For instance, the anomeric protons of 1,3-β-D-Gal*p*, 1,3,6-β-D-Gal*p*, 1,4-α-D-GalAMe*p*, 1,4-α-D-GalA*p*, 1,5-α-L-Ara*f*, T-α-L-Ara*f*, 1,3-α-L-Ara*f*, 1,2,4-α-L-Rha*p*, and 1,2-α-L-Rha*p* were observed at 4.46 ppm, 4.53 ppm, 4.97 ppm, 5.03 ppm, 5.09 ppm, 5.16 ppm, 5.18 ppm, 5.25 ppm, and 5.31 ppm, respectively (Golovchenko et al., 2022; Huang et al., 2024; Li, Feng, et al., 2024; Yao et al., 2021). Besides, the anomeric carbons of 1,4-α-D-GalA*p*, 1,4-α-D-GalAMe*p*, 1,3-β-D-Gal*p*/1,3,6-β-D-Gal*p*, 1,4-β-D-Gal*p*, 1,5-α-L-Ara*f*, and T-α-L-Ara*f* were observed in the range of 99.58 ppm – 109.21 ppm (Golovchenko et al., 2022; Huang et al., 2024; Li, Feng, et al., 2024; Yao et al., 2021). However, compared with the original STP (STP-HDE), the characteristic NMR signals (3.81 ppm and 52.82 ppm) that corresponded to the GalA-OCH3 remarkedly weakened in the de-esterified STP-LDE (Li, Wang, et al., 2024; Yao et al., 2021), indicating that the methyl-esterified degree of STP-LDE was reduced after the CDT treatment. In addition, compared with the original STP (STP-HDE), the characteristic NMR signals (2.00 ppm, 2.16 ppm, 20.04 ppm, and 20.51 ppm) that corresponded to acetyl groups of GalA*p* residues also obviously weakened in the de-esterified STP-LDE (Li, Wang, et al., 2024; Yao et al., 2021), suggesting that the acetyl-esterified degree of STP-LDE was also reduced. Overall, these findings confirmed that the CDT treatment possessed minor effects on the basic chemical structure of STP, except the obvious reduction of esterified degree.

3.2. Impacts of esterified degrees of original and de-esterified STPs on their biological functions

3.2.1. Comparison of in vitro antioxidant functions of original and de-esterified STPs

Recent experimental results have revealed that sweet tea polysaccharides possess excellent antioxidant functions, which vary with their esterified degrees, uronic acids, molecular weights, and bound phenolics (Guo et al., 2021; Lei et al., 2022; Wu et al., 2022). However, the impact of STP's precise structural property on its biological functions is still limited and unclear. Herein, the impact of STP's esterified degree on its antioxidant functions was uncovered in this study. Figs. 3A-3D displayed the FRAP values, NO radical scavenging abilities, DPPH radical scavenging abilities, and ABTS radical scavenging abilities of original and de-esterified STPs. It could be seen that both original and de-esterified STPs possessed remarkable antioxidant functions. In detail, the values of FRAP for STP-HDE, STP-MDE and STP-LDE at the highest concentration were detected to be 1.033 ± 0.008 (absorbance at 700 nm), 0.335 ± 0.008 (absorbance at 700 nm), and 0.492 ± 0.006 (absorbance at 700 nm), respectively. Besides, the IC_{50} values for STP-HDE, STP-MDE, and STP-LDE against NO free radicals were detected to be 0.923 ± 0.008 mg/mL, 1.951 ± 0.018 mg/mL, and 1.171 ± 0.003 mg/mL, respectively. Additionally, the IC_{50} values for STP-HDE, STP-MDE, and STP-LDE against DPPH free radicals were detected to be 0.996 ± 0.006 mg/mL, 2.018 ± 0.033 mg/mL, and 1.652 ± 0.014 mg/mL, respectively. Finally, the IC_{50} values for STP-HDE, STP-MDE, and STP-LDE against ABTS free radicals were detected to be 0.616 ± 0.001 mg/mL, 1.375 ± 0.003 mg/mL, and 1.093 ± 0.012 mg/mL, respectively. These findings confirmed that the CDT treatment could notably regulate STP's antioxidant functions, in accordance with recent studies (Li, Feng, et al., 2024; Li, Lei, et al., 2024; Li, Wang, et al., 2024). In detail, compared with the original STP (STP-HDE), the antioxidant functions of both STP-MDE and STP-LDE significantly declined, mainly owing to the notable decrease of their bound phenolics after the CDT treatment (Table 1). This phenomenon is mainly attributed to the reason that the bound phenolics in dietary polysaccharides/fibers are regarded as the most vital contributors among all of their physicochemical structures to their antioxidant functions (Fernandes & Coimbra, 2023). Additionally, compared with the STP-MDE, the antioxidant function of STP-LDE significantly improved, which was mainly owing to the notable decrease of its esterified degree after the CDT treatment (Table 1). This phenomenon is mainly attributed to the reason that the methyl-esterified degree (unmethylated uronic acid) of pectic polysaccharides is considered as an important factor that can affect the antioxidant function (Fernandes & Coimbra, 2023). Recent experimental results also uncovered that the antioxidant function of high-esterified pectic polysaccharides was lower than that of low-esterified pectic polysaccharides (Li, Feng, et al., 2024). Overall, these findings inferred that STP's antioxidant function was positively linked to its bound phenolics, while negatively linked to its esterified degree.

3.2.2. Comparison of in vitro anti-diabetic functions of original and de-esterified STPs

Recent experimental results have clarified that sweet tea polysaccharides exhibit potential *in vitro* anti-diabetic function through inhibiting the enzymatic activity of α-glucosidase, and their inhibitory effects on α-glucosidase vary with their esterified degrees (unmethylated uronic acids), molecular weights, and bound phenolics (Guo et al., 2021; Lei et al., 2022; Wu et al., 2022). Herein, the impact of STP's esterified degree on its inhibitory effects on α-glucosidase and α-amylase was uncovered. Fig. 3E and Fig. 3F showed that the inhibitory effects of original and de-esterified STPs on α-glucosidase and α-amylase were obviously different, indicating that the CDT treatment could regulate STP's anti-diabetic function. In detail, the IC_{50} values for STP-HDE, STP-MDE, and STP-LDE against α-amylase were detected to be 1.034 ± 0.006 mg/mL, 2.712 ± 0.019 mg/mL, and 1.303 ± 0.004 mg/mL, respectively. The IC_{50} values for STP-HDE, STP-MDE and STP-LDE against α-glucosidase were detected to be 0.069 ± 0.002 mg/mL, 1.315 ± 0.023 mg/mL, and 0.762 ± 0.023 mg/mL, respectively. Compared with the original STP (STP-HDE), the inhibitory effects of both STP-MDE and STP-LDE against α-glucosidase and α-amylase significantly declined, which might be owing to the obvious decrease of bound phenolics after the CDT treatment (Table 1). Because recent experimental results have uncovered that the anti-diabetic function of dietary polysaccharides/fibers is positively linked to their bound phenolics (Huang et al., 2022). Besides, compared with STP-MDE, the anti-diabetic function of STP-LDE notably improved, which might be owing to the decrease of esterified degree to expose more free carboxyl groups after the CDT treatment (Table 1). That's because pectic polysaccharides can restrain the enzymatic activity of digestive enzymes by binding with their amino acids to change their structures (Wu et al., 2017), and more free carboxyl and hydroxyl groups in pectic polysaccharides can increase their binding capacity to digestive enzymes, thereby exhibiting stronger inhibitory effects on digestive enzymes. Therefore, these results uncovered that STP's anti-diabetic function was positively linked to the bound phenolics, but negatively associated with the esterified degree.

3.2.3. Comparison of in vitro immunostimulatory functions of original and de-esterified STPs

Recent experimental results have clarified that sweet tea polysaccharides possess notable *in vitro* immunostimulatory functions, and

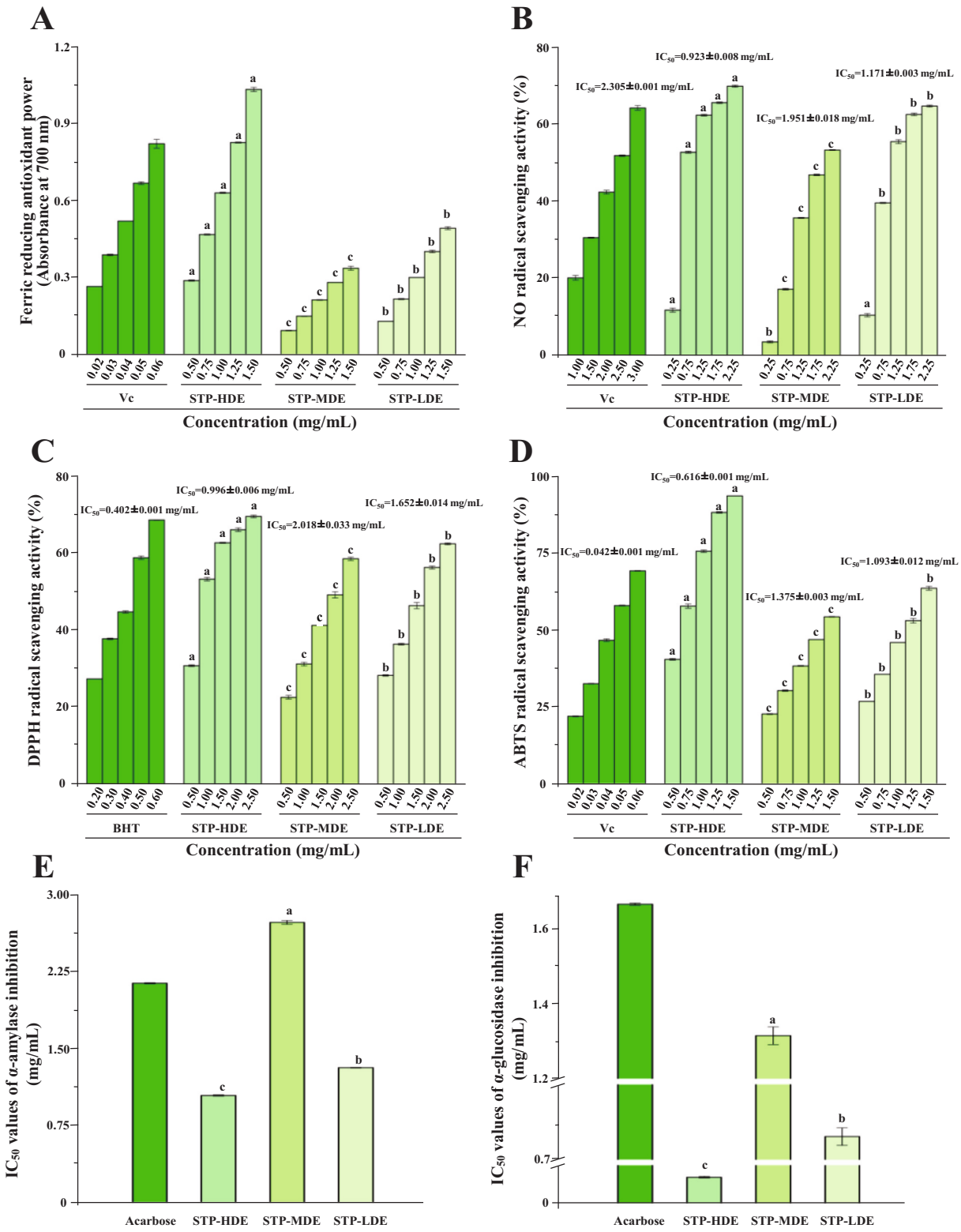

Fig. 3. *In vitro* antioxidant (A, B, C, and D) and anti-diabetic functions (E and F) of original and de-esterified pectic polysaccharides from sweet tea (STP). A, Ferric reducing antioxidant power; B, NO radical scavenging activity; C, DPPH radical scavenging activity; D, ABTS radical scavenging activity; E, inhibitory effect on α-amylase; F, inhibitory effect on α-glucosidase; STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; The error bars are standard deviations; Significant ($p < 0.05$) differences among STP-HDE, STP-MDE, and STP-LDE are shown by data bearing different letters (a-c);

their immunostimulatory functions are associated with the combined effect of their esterified degrees, molecular weights, and contents of uronic acids (Lei et al., 2022; Wu et al., 2022). Herein, the impact of STP's esterified degree on its immunostimulatory function was uncovered in this study. As displayed in Fig. S1, both original and de-esterified STPs showed no toxic effects on RAW 264.7 macrophages. Additionally, as displayed in Figs. 4A-4C, both original and de-esterified STPs could improve the generation of pro-inflammatory mediator (nitric oxide, NO) and cytokines (interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)) from RAW 264.7 macrophages, thereby exhibiting remarkable immunostimulatory functions. Indeed, compared with the original STP (STP-HDE), the immunostimulatory functions of both STP-MDE and STP-LDE gradually promoted, thereby indicating that their immunostimulatory functions were negatively associated with the esterified degree. Similar

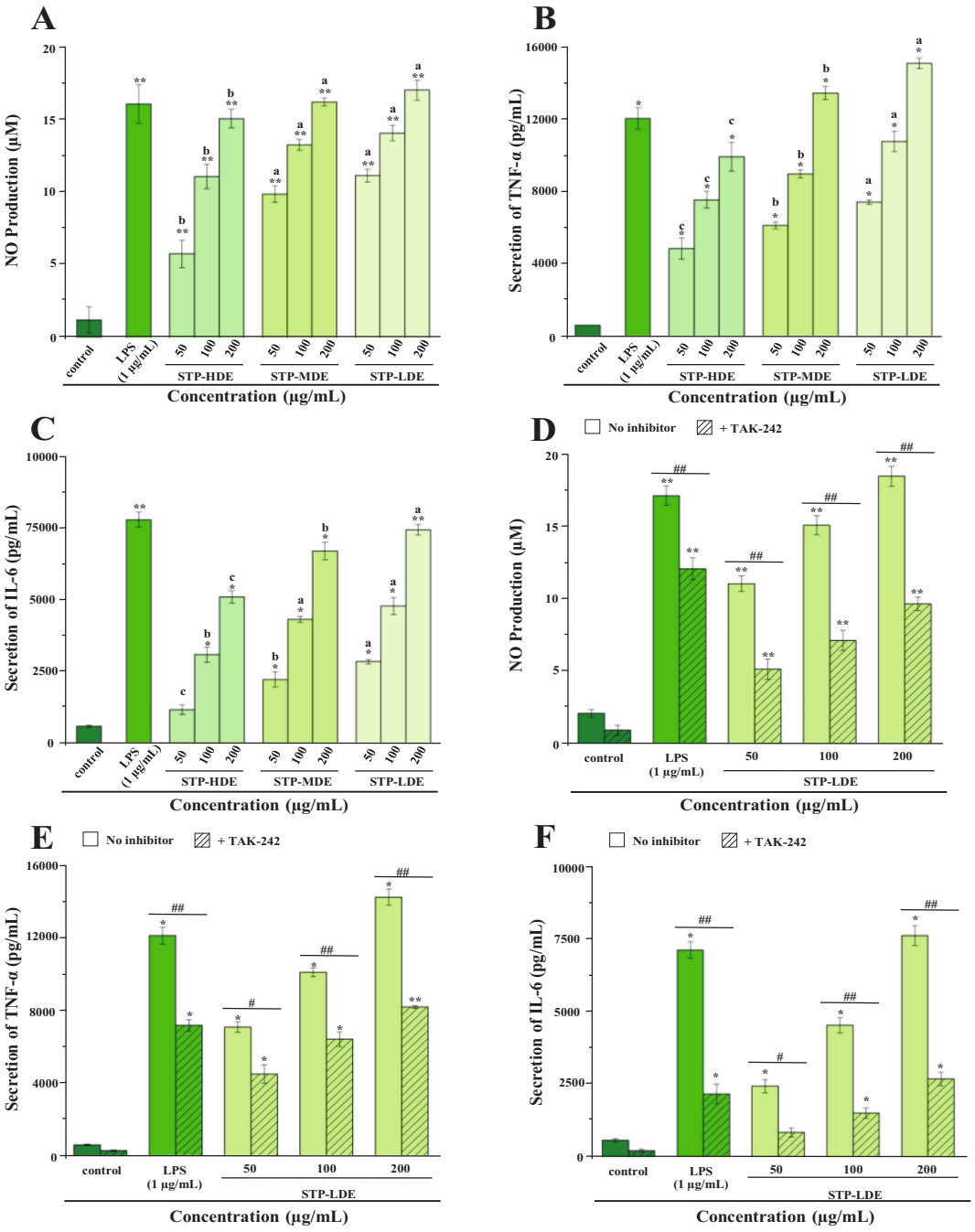

Fig. 4. Immunostimulatory functions (A, B, and C) of original and de-esterified pectic polysaccharides from sweet tea (STP) and their potential mechanisms of action (D, E, and F) related to TLR4-mediated signaling pathway.
A, B, and C indicate the production of pro-inflammatory mediator (NO) and cytokines (TNF-α and IL-6) from RAW 264.7 macrophages induced by original and de-esterified STPs, respectively; D, E, and F indicate the impacts of the TAK-242 inhibitor on the generation of pro-inflammatory mediator (NO) and cytokines (TNF-α and IL-6) from RAW 264.7 macrophages induced by lipopolysaccharide (LPS) and STP-LDE, respectively; STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; The error bars are standard deviations; Significant differences ($p < 0.05$) among STP-HDE, STP-MDE, and STP-LDE are shown by data bearing different letters (a-c); Significant differences in the generation of NO, IL-6, and TNF-α in LPS and sample groups *vs.* control are shown by $^{*}p < 0.05$ and $^{**}p < 0.01$. Significant differences between samples treated with the TAK-242 inhibitor and samples treated without the TAK-242 inhibitor are shown by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

experimental results clarified that the low-esterified pectic polysaccharides exerted stronger immunostimulatory functions than the high-esterified pectic polysaccharides (Ferreira et al., 2023; Li, Feng, et al., 2024; Li, Wang, et al., 2024). Besides, previous experimental studies demonstrated that pectic polysaccharide's immunostimulatory function was positively linked to the ratio of RG-I to HG domains (Ho et al., 2015). Thereby, compared with the original STP (STP-HDE), the increase of immunostimulatory function of both STP-MDE and STP-LDE might be also owing to the slight enhancement of their ratios of RG-I to HG domains (Table 1).

Generally, pectin molecules can exert immunostimulatory function through their recognition by specific receptors (*e.g.*, Toll-like receptors) (Hyun et al., 2023). Herein, to uncover whether the STP can interact with the TLR4, the impact of the TAK-242 inhibitor on the generation of pro-inflammatory mediator and cytokines from RAW 264.7 macrophages induced by STP-LDE were detected. As displayed in Figs. 5D-5F, the TAK-242 inhibitor could significantly ($p < 0.05$) suppress the generation of pro-inflammatory mediator and cytokines from RAW 264.7 macrophages induced by STP-LDE, thereby suggesting that STP-LDE could recognize the TLR4 receptor to exert strong immunostimulatory function, which is comparable to a recent study that pectic-like polysaccharides could enhance immune responses on macrophages *via* TLR4/nuclear factor-κB signaling activation (Liao et al., 2024).

3.3. Impacts of esterified degrees of original and de-esterified STPs on their microbial fermentation characteristics.

3.3.1. Changes in total carbohydrates and reducing sugars of original and de-esterified STPs during the fecal fermentation.

Generally, pectic polysaccharides are resistant to be digested in the upper gastrointestinal tract due to the lack of carbohydrate active enzymes in human body. Therefore, pectic polysaccharides' biological functions are closely linked to their microbial metabolisms in the large colon (Lee et al., 2022; Tan & Nie, 2020). Recent investigations have demonstrated that sweet tea-derived pectic polysaccharides can be utilized by gut microbiota, which can positively regulate the gut microbial composition to release beneficial microbial metabolites, *e.g.*, propionic acid and *n*-butyric acid (Lei et al., 2022). Nevertheless, the impact of STP's esterified degree on its microbial fermentation characteristics is still unclear. Thus, the microbial fermentation characteristics of original and de-esterified STPs were systematically compared in this study. Fig. 5A, B, and C exhibited the alterations of total carbohydrates, reducing sugars, and fermentation rates of original and de-esterified STPs during *in vitro* fecal fermentation. It could be clearly seen that the contents of total carbohydrates of STP-HDE, STP-MDE, and STP-LDE sharply decreased from 9.05 mg/mL to 5.12 mg/mL (STP-HDE), from 8.92 mg/mL to 4.19 mg/mL (STP-MDE), and from 8.86 mg/mL to 3.63 mg/mL (STP-LDE), respectively, after the *in vitro* fermentation for 48 h (Fig. 5A), while the contents of reducing sugars significantly increased at the initial fermentation period (0 h – 6 h) and then gradually decreased from 6 h to 48 h (Fig. 5B). These results confirmed that both original and de-esterified STPs could be degraded and utilized by intestinal microorganisms, similar to previous studies that pectic polysaccharides can be degraded by carbohydrate active enzymes that encoded by human gut microbiota (Tan & Nie, 2020). Interestingly, it could be obviously found that the fermentation rates of original and de-esterified STPs varied with different esterified degrees (Fig. 5C). In detail, compared with the original STP (STP-HDE) (46.82 %), the de-esterified STP (STP-LDE) exhibited a higher fermentation rate (60.81 %), indicating that the STP with a lower-esterified degree could be more easily consumed by gut microbiota, which is comparable to previous studies (Dongowski et al., 2000; Xu et al., 2024). This result might be due to the reason that the degradation of pectic polysaccharides (HG domain) with a high-esterified degree requires different types of carbohydrate active enzymes (*e.g.*, carbohydrate esterases, polysaccharide lyases, and glycoside hydrolases) that encoded by intestinal microorganisms.

3.3.2. Changes in pH values and SCFAs production of original and de-esterified STPs during the fecal fermentation

Pectic polysaccharides can be degraded by intestinal microorganisms to generate various SCFAs (*e.g.*, acetic acid and propionic acid), thereby resulting in the decrease of the pH value of the fermentation mixture (Lee et al., 2022; Tan & Nie, 2020). Thus, the alterations of pH values and short chain fatty acids in the fermented mixtures of original and de-esterified STPs were compared. Fig. 5D showed that the pH values of the fermentation broth of STP-HDE, STP-MDE, and STP-LDE decreased sharply from 0 h to 12 h, and continued to decrease to 5.59, 5.49, and 5.35 at 48 h, respectively. This phenomenon is consistent with previous studies showing that the pH of fermentation broths containing pectic polysaccharides significantly declines after *in vitro* fecal fermentation (Lei et al., 2022; Wu et al., 2021), suggesting that original and de-esterified STPs could be metabolized by intestinal microorganisms. In fact, short chain fatty acids are one of the importantly end products of pectic polysaccharides that metabolized by intestinal microorganisms (Lee et al., 2022). Fig. 5E, F, G, and H displayed the alterations in the contents of SCFAs in the blank (BLK, negative control group), STP-HDE, STP-MDE and STP-LDE groups after *in vitro* fecal fermentation. The findings displayed that the levels of total SCFAs in the STP-HDE (38.07 mmoL/L), STP-MDE (45.37 mmoL/L), and STP-LDE (52.47 mmoL/L) groups were obviously higher than the BLK group (17.55 mmoL/L), further demonstrating that original and de-esterified STPs could be utilized and metabolized by intestinal microorganisms (Lei et al., 2022; Wu et al., 2021). Interestingly, the production of acetic acid, propionic acid, and *n*-butyric acid in the fermented broth of STP-LDE was notably higher than that of STP-HDE (Fig. 5E, F, and G), implying that the STP with a lower-esterified degree could be easily metabolized by gut microbiota to generate more SCFAs. The phenomenon is consistent with a previous study that a higher content of total SCFAs can be generated from pectin molecules with a lower-esterified degree after the *in vitro* fecal fermentation for 48 h (Dongowski et al., 2000).

3.3.3. Effects of original and de-esterified STPs on gut microbial compositions

Gut microbiota acts a critical role in the host health, and the gut dysbiosis is considered as a major hallmark of various metabolic disorders (Fan & Pedersen, 2021). A recent study has revealed that sweet tea pectic polysaccharides can positively regulate the microbial composition in an *in vitro* fermentation model through improving the relative abundances of several beneficial bacteria (Lei et al., 2022). Nevertheless, the impact of esterified degrees of original and de-esterified STPs on their microbial regulation effects is still unclear. Herein, the regulation effects of original and de-esterified STPs on the gut microbial composition were investigated, and the 16S rRNA sequencing technique was carried out to evaluate the impacts of original and de-esterified STPs on the gut microbial compositions. As displayed in Fig. 6A, the BLK group was mainly consisted of *Proteobacteria* (64.33 %), *Fusobacteria* (18.16 %), *Bacteroidetes* (10.85 %), and *Firmicutes* (5.23 %). Compared with the BLK group, the STP-HDE, STP-MDE, and STP-LDE groups could completely inhibit the growth of *Fusobacteria*, which is generally considered as a conditional pathogen (Xu et al., 2020). Besides, compared with the BLK group, the de-esterified STP-MDE and STP-LDE could also significantly inhibit the growth of *Proteobacteria*. *Proteobacteria* is associated with diabetes, inflammation, and cancer, which is a potential indicator of gut microbial dysbiosis (Shin et al., 2015). Furthermore, compared with the BLK group, the supplement of original and de-esterified STPs could significantly promote the relative abundance of *Bacteroidetes*. This phenomenon may be owing to the reason that most *Bacteroidetes* species encode various carbohydrate active enzymes, *e.g.*, polysaccharide lyases, carbohydrate esterases, and glycoside hydrolases, which can degrade HG and RG-I pectic domains to support their growth (Tan & Nie, 2020). Interestingly, it could be clearly seen that the relative abundance of *Bacteroidetes* in the de-esterified STP-LDE group (47.43 %) was notably higher than the STP-HDE group (28.93 %)

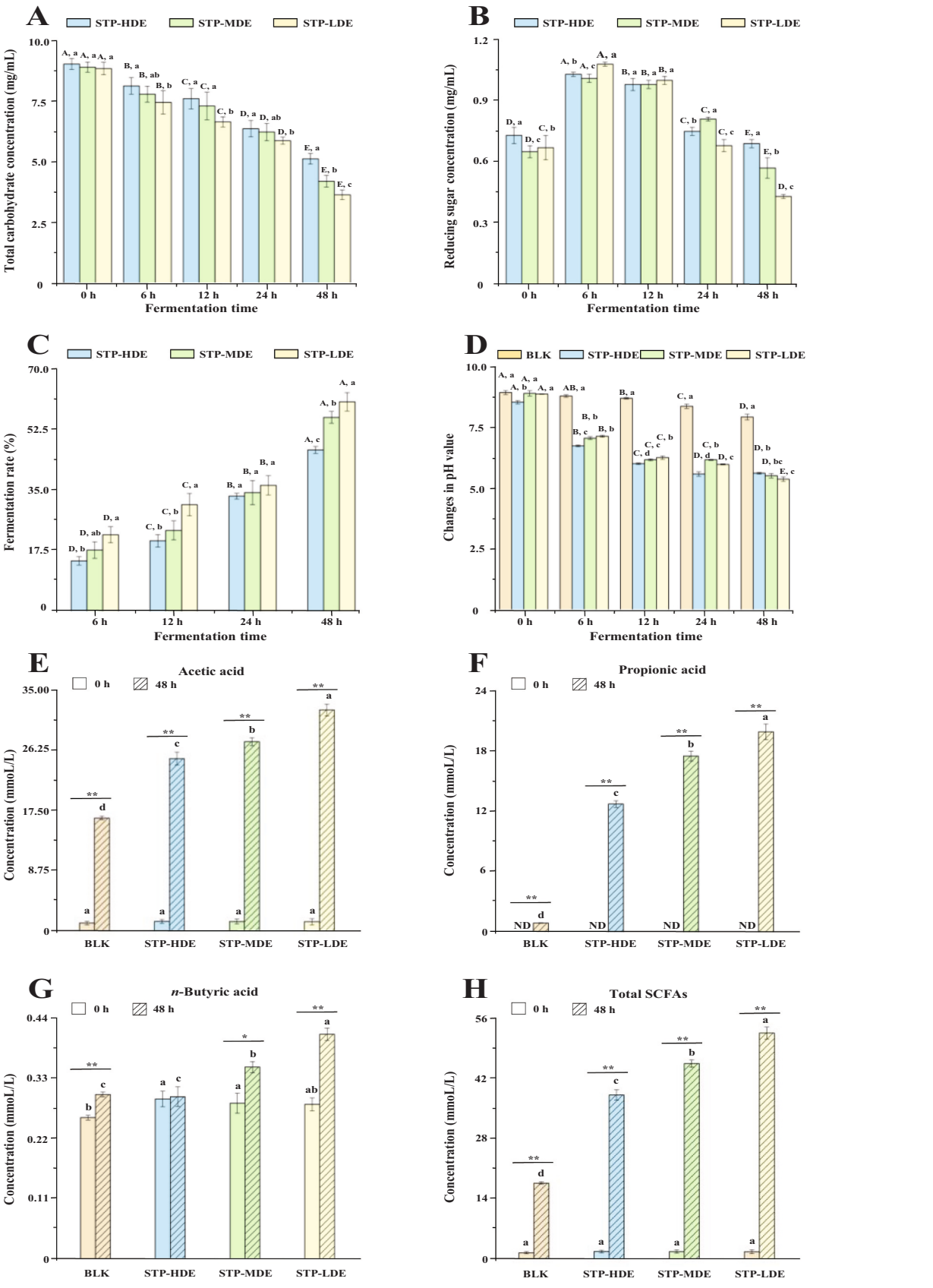

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Fig. 5. Changes in total carbohydrates (A), reducing sugars (B), fermentation rates (C), and pH values (D) as well as individual (E, F, and G) and total short chain fatty acids (H) of original and de-esterified pectic polysaccharides from sweet tea (STP) during *in vitro* microbial fermentation.
STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; BLK, blank group (negative control group); Significant differences ($p < 0.05$) in total carbohydrates, reducing sugars, fermentation rates, and pH values for the same sample at different fermentation stages are shown by different capital letters (A-E); Significant differences ($p < 0.05$) in total carbohydrates, reducing sugars, fermentation rates, and pH values for different samples at the same fermentation stage are shown by different lowercase letters (a-d); Significant differences ($p < 0.05$) in contents of short chain fatty acids among different samples at the same fermentation stage are shown by different lowercase letters (a-d); Significant differences in contents of short chain fatty acids between the same sample that fermented at 0 h and 48 h are shown by $*p < 0.05$ and $**p < 0.01$.

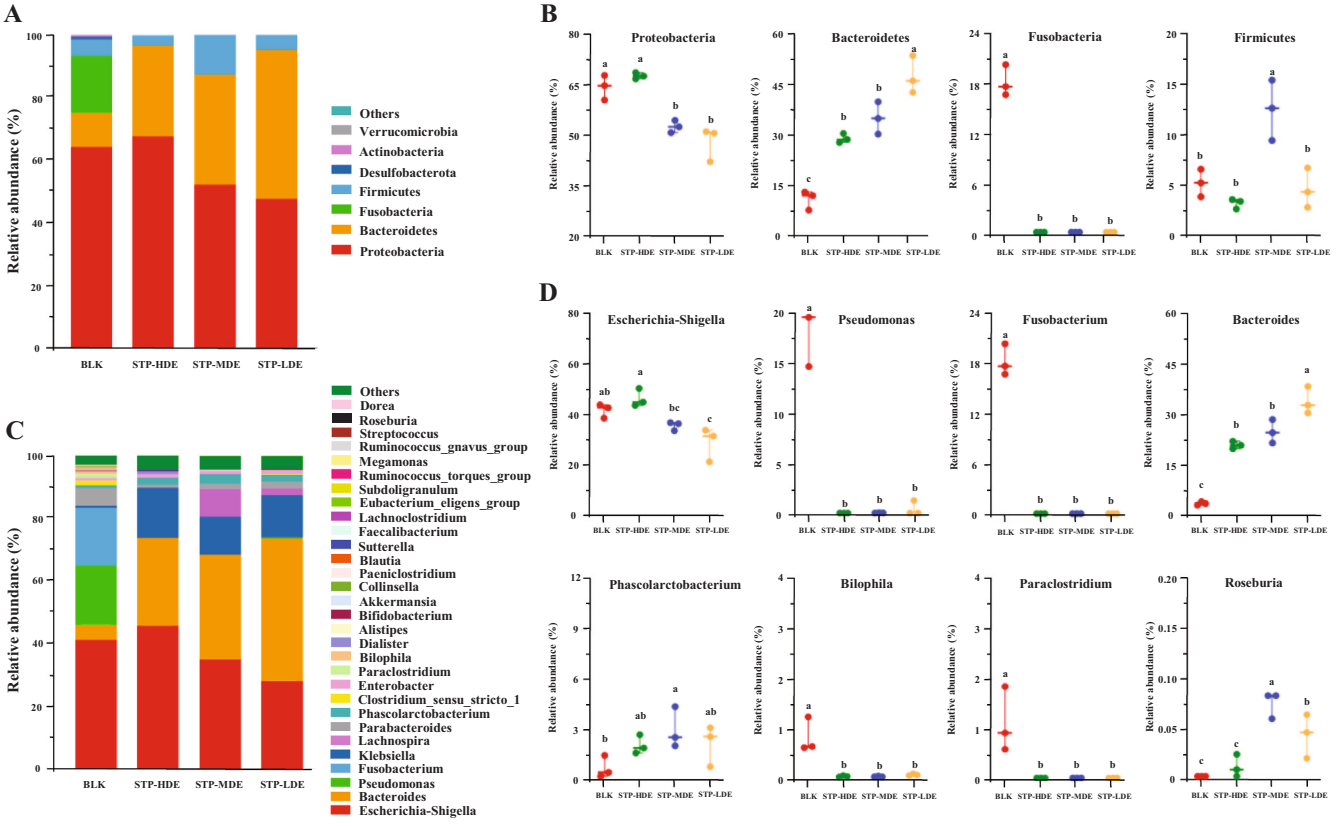

Fig. 6. Gut microbial composition at the phylum level (A and B) and the genus level (C and D).
STP-HDE, STP-MDE, and STP-LDE indicate the original STP with a high-esterified degree, the de-esterified STP with a middle-esterified degree, and the de-esterified STP with a low-esterified degree, respectively; BLK, blank group (negative control group); Different lowercase letters (a-c) indicate significant differences ($p < 0.05$) among different groups.

(Fig. 6B), indicating that the STP with a lower-esterified degree could be more easily degraded by *Bacteroidetes* to support its growth. Besides, the decrease in the ratio of *Bacteroidetes* to *Firmicutes* (B/F) is associated with the pathogenesis of obesity (Fan & Pedersen, 2021). Compared with the BLK group (2.07), both original and de-esterified STPs, especially the STP-LDE (10.22), could significantly increase the B/F ratio, thereby exhibiting potential anti-obesity function. Furthermore, at the genus level, the BLK group was dominantly consisted of *Escherichia-Shigella*, *Pseudomonas*, *Fusobacterium*, *Bacteroides*, and *Parabacteroides* (Fig. 6C). *Escherichia-Shigella*, *Pseudomonas,* and *Fusobacterium* are usually considered as pathogens or conditional pathogens (Castaño-Rodríguez et al., 2018; Ma et al., 2022). Obviously, the supplement of STP-LDE with a low-esterified degree could significantly inhibit the growth of several pathogens (*e.g.*, *Escherichia-Shigella*, *Pseudomonas,* and *Fusobacterium*) when compared with the BLK group (Fig. 6D). In addition, *Bilophila* and *Paraclostridium* may cause inflammatory bowel disease and colorectal cancer (Aziz et al., 2023; Cai et al., 2023), which were obviously inhibited by the supplement of both original and de-esterified STPs. In contrast, the relative abundances of some beneficial bacteria, *e.g.*, *Bacteroides*, *Lachnospira*, *Phascolarctobacterium*, and *Roseburia,* were significantly increased by the supplement of de-esterified STPs when compared with the BLK group. *Roseburia* is a butyric acid-producing bacterium (Venegas et al., 2019), and *Phascolarctobacterium* can promote the production of propionate (Zhang et al., 2015), thereby exhibiting beneficial effects on intestinal health. Collectively, the supplement of original and de-esterified STPs, especially the STP-LDE, could positively modulate gut microbial composition by inhibiting the growth of several pathogens and promoting the proliferation of several beneficial bacteria.

4. Conclusion

In this study, the precisely chemical modification of STP was achieved by the controllable de-esterification treatment (CDT). Compared with the original STP (STP-HDE, 47.01 %), the de-esterified STPs with a middle-esterified degree (33.53 %) and a low-esterified degree (7.66 %) were obtained, and their physicochemical structures were overall stable after the CDT treatment. Furthermore, the findings inferred that the anti-diabetic and antioxidant functions of STP were positively linked to the bound phenolics, while negatively linked to the esterified-degree.

Besides, the immunostimulatory function of STP was negatively linked to the esterified degree, which might be owing to the reason that the de-esterified STP could expose more free charge groups to interact with the TLR4 receptor to activate immune responses. Moreover, the microbial fermentation characteristic of STP was also affected by the esterified degree, and the STP-LDE with a lower-esterified degree was more easily to be metabolized by intestinal microorganisms in human feces to promote the production of SCFAs. Overall, these findings are conducive to unlocking the precise SFR of STP, and are also helpful for promoting STP's potential applications. Nevertheless, owing to STP's structural complexity, future studies regarding its other key structural features (*e.g.*, ratio of HG to RG-I and RG-I sidechain length) on the bioactivity should be carefully evaluated. Animal studies are also needed to confirm its health benefits beyond *in vitro* limitations.

CRediT authorship contribution statement

Yu-Jing Huang: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Jing Feng:** Validation, Investigation, Formal analysis. **Yong Deng:** Writing – review & editing, Funding acquisition, Formal analysis. **Jie Li:** Validation, Resources. **Hong-Yan Liu:** Writing – review & editing, Funding acquisition. **Qing Liang:** Resources. **Yi-Chen Hu:** Writing – review & editing, Formal analysis. **Jian-Yong Zhang:** Writing – review & editing. **Liang Zou:** Writing – review & editing, Formal analysis. **Ding-Tao Wu:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102462.

Data availability

Data will be made available on request.

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