



# Stachyose Alleviates Corticosterone-Induced Long-Term Potentiation Impairment *via* the Gut–Brain Axis

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Stress can induce learning and memory impairment; corticosterone is often used to study the effects and mechanisms of stress in animal models. Long-term potentiation (LTP) has been widely used for tackling the mechanisms of memory. Liuwei Dihuang decoction-active fraction combination (LW-AFC) can improve stress-induced LTP and cognition impairment; stachyose is an oligosaccharide in LW-AFC. The effects and mechanisms of stachyose on stress are unknown. In this study, stachyose showed protective effects against LTP impairment by corticosterone *in vivo* only *via* intragastric administration for 7 consecutive days, but there was little effect even after direct intracerebroventricular injection; the protective effect of stachyose could be canceled by non-absorbable antibiotics (ATB) which disturbed gut flora. 16S rRNA sequencing, alpha diversity, and principal coordinate analysis (PCoA) revealed that the gut flora in corticosterone-treated mice was disturbed and stachyose could improve corticosterone-induced gut flora disturbance. Bacteroidetes were decreased and Deferribacteres were increased significantly in corticosterone-treated mice, and stachyose restored Bacteroidetes and Deferribacteres to the normal level. D-serine, a coactivator of NMDA receptors, plays an important role in synaptic plasticity and cognition. Here, corticosterone had little effect on the content of D-serine and L-serine (the precursor of D-serine), but it reduced the D-serine release-related proteins, Na<sup>+</sup>-independent alanine–serine–cysteine transporter-1 (ASC-1), and vesicle-associated membrane protein 2 (VAMP2) significantly in hippocampus; stachyose significantly increased ASC-1 and VAMP2 in corticosterone-treated mice, and ATB blocked stachyose's effects on ASC-1 and VAMP2. NMDA receptors co-agonists L-serine, D-serine, and glycine significantly improved LTP impairment by corticosterone. These results indicated that stachyose might indirectly increase D-serine release through the gut–brain axis to improve LTP impairment by corticosterone in the hippocampus *in vivo*.

**Keywords:** stachyose, corticosterone, long-term potentiation, D-serine, Bacteroidetes, Deferribacteres

**Abbreviations:** AD, Alzheimer's disease; APP/PS1, PrP-hAβPPswe/PS1ΔE9; Aβ, amyloid β-protein; CA-30, oligosaccharide fraction of LW-AFC; Cort, Corticosterone; HFS, high-frequency stimulation; HPA, hypothalamic–pituitary–adrenal; LTP, long-term potentiation; LW-AFC, LW-active fraction combination; LWB-B, polysaccharide fraction of LW-AFC; LWD-b, glycoside fraction of LW-AFC; NIM, neuroendocrine immunomodulation; SAMP8, senescence-accelerated mouse prone 8; TCM, traditional Chinese medicine.

## INTRODUCTION

Stress can activate the hypothalamic–pituitary–adrenal (HPA) axis and elevate glucocorticoids in the body (cortisol in humans and corticosterone in rodents) (de Kloet et al., 2005). Glucocorticoid receptors are abundant in the hippocampus and play an important role in stress-induced cognition alteration (De Kloet et al., 1998; Kim and Yoon, 1998; Rogalska, 2010). Long-term potentiation (LTP) is widely accepted as the cellular mechanism of learning and memory (Lüscher and Malenka, 2012). Severe stress could induce cognition impairment (Diamond and Rose, 1994; Baker and Kim, 2002; Diamond et al., 2006); corticosterone is often used to modeling cognitive impairment induced by stress (de Quervain et al., 2000; Khaksari et al., 2007). LTP deficit and cognitive impairment always coexist in stress models, and LTP impairment is often considered as one of the mechanisms for stress-induced cognitive deficits (Pavlidis et al., 1993; Kim et al., 2006; Howland and Wang, 2008; Aisa et al., 2009). N-methyl-D-aspartate (NMDA) receptors play critical roles both in normal synaptic functions and excitotoxicity in the central nervous system (CNS). D-serine, a coactivator of NMDA receptors, plays an important role in the brain function (Wolosker, 2018). Our recent research found that corticosterone could decrease D-serine release-related protein and Na<sup>+</sup>-independent alanine–serine–cysteine transporter-1 (ASC-1), causing hypofunction of NMDA receptors, which is an important reason for LTP impairment by corticosterone (Wang et al., 2021).

Prebiotic is a group of non-digestible food ingredients that “beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (Gibson and Roberfroid, 1995). Oligosaccharide is a type of prebiotics and is often served as a gut flora modulator (Gibson and Roberfroid, 1995; Davani-Davari et al., 2019). It has reported that oligosaccharides and gut flora could modulate the D-serine pathway (Savignac et al., 2013; Kawase et al., 2017) in the brain. So, oligosaccharides may affect brain functions *via* modulating gut flora.

Liuwei Dihuang decoction (LW) is a classic formula of traditional Chinese medicine (TCM) and has been used for nearly one thousand years for various diseases with characteristic features of kidney-yin deficiency, which is related to disturbance of the neuroendocrine immunomodulation (NIM) network (Zhou et al., 2016). LW consists of six botanical drugs including Dihuang [*Rehmannia glutinosa* (Gaertn.) DC.], Shanyao (rhizome of *Dioscorea polystachya* Turcz.), Shanzhuyu (fruit of *Cornus officinalis* Siebold & Zucc.), Mudanpi (root bark of *Paeonia × suffruticosa* Andrews), Zexie (rhizome of *Alisma plantago-aquatica* L.), and Fuling [sclerotia of *Wolfiporia extensa* (Peck) Ginns] in the weight ratio 8:4:4:3:3:3. The preparation of LW has been described in our previous study in detail (Cheng et al., 2007). LW-active fraction combination (LW-AFC) is obtained from LW, which contain three kinds of main active fractions: polysaccharide fraction (LWB-B, 20.3%), glycosides fraction (LWD-b, 15.1%), and oligosaccharide fraction (CA-30,

64.6%) (Wang et al., 2016a; Wang et al., 2017a), and the preparation of LW-AFC has been described in our previous study in detail (Wang et al., 2016a). Previous researches showed that rebalancing NIM network was involved in the cognitive improving effects of LW-AFC on Alzheimer’s disease (AD) animal models, including the PrP-hA $\beta$ PPswe/PS1 <sup>$\Delta$ E9</sup> (APP/PS1) mouse model and senescence-accelerated mouse prone 8 (SAMP8) strain (Wang et al., 2016a; Wang et al., 2017a). As a main form of synaptic plasticity, long-term potentiation (LTP) is believed to represent the cellular correlates of learning and memory (Lüscher and Malenka, 2012), and stress or corticosterone-induced cognitive impairment are closely related to LTP deficiency (Pavlidis et al., 1993; Kim et al., 2006; Howland and Wang, 2008; Aisa et al., 2009). Modulating gut flora contributes to the neuroprotective effects of LW-AFC and CA-30 (Huang et al., 2019; Wang et al., 2019; Cheng et al., 2020a). CA-30 contains many oligosaccharides; if there is an oligosaccharide that can replace CA-30 and play its effects fully that will make the quality of LW-AFC more controllable. However, the active ingredients and mechanisms of CA-30 are not well understood yet.

Stachyose, an oligosaccharide, is an ingredient of CA-30 (Cheng et al., 2020a). It remains unknown whether stachyose has neuroprotective effects. For further understanding the active ingredients and mechanisms of CA-30, in this study, we focused on the effects of stachyose, on LTP impairment by corticosterone, gut flora, and the D-serine pathway.

## MATERIALS AND METHODS

### Animals

Male BALB/c mice (18–22 g) were procured from the animal center of Academy of Military Medical Sciences (AMMS). The animals were housed in plastic cages on a 12-h light/dark cycle with controlled room temperature (24–26°C) and humidity (50–60%). Mice were acclimatized to the laboratory environment for 7 days prior to experiments with free access to a standard diet of pellets and water. Institute Animal Care and Use Committee (IACUC) of National Beijing Center for Drug Safety Evaluation and Research (NBCDSER) approved all the experimental protocols.

### Drug Administration

Corticosterone (Cort, TCI chemicals, dissolved in saline with 5% ethanol) was injected subcutaneously (50 mg/kg) 60 min before LTP was induced by high-frequency stimulation (HFS), and control animals were subcutaneously injected with a vehicle (saline with 5% ethanol). For single administration, stachyose (STA, provided by Chengdu Biopurify Phytochemicals Ltd., and the purity is 99%) was intracerebroventricularly (i.c.v., 500  $\mu$ g), intraperitoneally (i.p., 20 mg/kg), and intragastrically (i.g., 450 mg/kg) administered 30 min before corticosterone. For multiple administration, stachyose was administered (i.g., i.p.) for 7 days. For disturbance of gut flora, a combination of non-absorbable antibiotics (ATB, 2.5 mg/kg pimaricin, 50 mg/kg bacitracin, and 50 mg/kg neomycin dissolved in one solution)

were used in this study (Verdú et al., 2006; Huang et al., 2019), and animals received ATB (i.g.) every day 30 min before stachyose administration for 7 days. D-serine (Sigma-Aldrich), L-serine (Sigma-Aldrich), and glycine (Sigma-Aldrich) were dissolved in artificial cerebrospinal fluid (ACSF) for stocking and then diluted in ACSF before use. D-serine (300 nmol per mouse in 5  $\mu$ L), L-serine (300 nmol per mouse in 5  $\mu$ L), and glycine (100 nmol per mouse in 5  $\mu$ L) was administered (i.c.v.) 30 min before corticosterone.

## LTP Recording

The method of LTP recording was described previously (Huang et al., 2012; Huang et al., 2013; Li et al., 2016). In brief, mice were anesthetized with urethane (1.2 g/kg, i.p.) and then fitted with ear cuffs to place in a stereotaxic frame. An incision was made along the midline of the head that made the sutures visible; a stimulating electrode was placed into the perforant path, and the recording electrode was placed into the dentate gyrus. The stimulus was delivered by a stimulator (A-M Systems Model 2100), and the responses were received by an amplifier (A-M Systems Model 1800). The WinLTP program (<http://www.winltp.com>) was used to record and analyze data. After a 30 min baseline recording, LTP was induced by HFS (three trains 10 s apart, 250 Hz, eight 0.1 m pulses in each train) and recorded for another 60 min. The mean population spikes (PS) amplitudes during the baseline period (0–30 min) were normalized to 100%; after HFS, the relative PS amplitudes (31–90 min) were normalized relative to the baseline period at every point.

## Gut Flora Analysis

The method has been described previously (Wang et al., 2016b). In brief, about 180–220 mg fresh stool was collected from proximal colon 1 h after corticosterone administration for each mouse. After the isolation (QIAamp DNA Stool Mini Kit, 51504, Qiagen) and validation of total genomic DNA (gDNA), two universal primers 356F (5'CCTACGGGNGGCWGCAG3') and 803R (5'GACTACHVGGGTATCTAATCC3') were employed to amplify V3-V4 regions of bacterial 16S rRNA gene from gDNA. The libraries were sequenced on the Illumina Miseq platform (Illumina, San Diego, CA, United States) to generate 2  $\times$  300 bp pair end sequencing reads using the standard protocol under standard conditions. The data quality control, chimera removal, operational taxonomic unit (OTU) clustering, taxonomic assignment, and principal coordinate analyses (PCoA) were performed in QIIME.

## Determination of Neurotransmitters

D-serine and L-serine were analyzed by high-performance liquid chromatography (HPLC) in this study according to a published protocol (Wang et al., 2021). In brief, hippocampus was homogenized in 20-volume methanol, centrifuged for 15 min at 15,000 g (4°C). The supernatant was stored at –20°C until use. In total, 20  $\mu$ L sample solution was added into 20  $\mu$ L o-phthaldialdehyde (OPA)/N-acetyl-L-cysteine (NAC) derivatization solution (30 mg OPA and 30 mg NAC dissolved in 1 ml ethanol and then diluted in 0.2 mol/ml borate buffer to a final volume of 5 ml, pH = 9.8) for 3 min on ice before injection.

An Agilent reversed-phase chromatographic column (C-18, 4.6 mm  $\times$  250 mm, 5  $\mu$ m) was used. The mobile phase consisted of ammonium acetate:methanol (96:4, v/v); the flow rate was set at 1.0 ml/min; an electrochemical detector was used to detect the signals of D-serine and L-serine; and the detector potential was + 0.75 V, and the sensitivity was set at 50 nA full-scale detection. The retention times for L-serine and D-serine are 33.92 and 38.11 min, respectively. The area under curve (AUC) was used to calculate the concentration.

## Western Blotting

The method has been described previously (Huang et al., 2017). In brief, 1 h after corticosterone or vehicle injection, animals were sacrificed with CO<sub>2</sub> and their hippocampal samples were obtained. The hippocampal samples were homogenized in a lysis buffer (100 mg tissue in 1 ml lysis buffer), extracted by supersonic quassation, and then centrifuged at 15,000 g for 15 min (4°C). A Bradford protein assay kit (Galen Biopharm International Co., Ltd.) determined the protein concentrations. Equal amounts of protein (30  $\mu$ g) were boiled in a loading buffer for 5 min before loading on an 8% SDS-polyacrylamide gel. Electrophoresis was performed at 60 V for 30 min and then 100 V for 90 min, followed by wet transfer onto a nitrocellulose membrane at 100 V for 60 min. The membrane was blocked for 60 min in blocking solution (5% non-fat dry milk) and then incubated at 4°C overnight with anti- $\beta$ -actin (1:1000, Abcam), anti-ASC-1 (1:100, Abcam), and anti-VAMP2 (1:1000, Abcam). After three washes with phosphate-buffered saline (contain 0.05% Tween 20) for 30 min, the primary antibodies were detected with the horseradish peroxidase-conjugated secondary antibodies and chemiluminescent HRP substrate (Thermo Fisher Scientific). Band density values were normalized to  $\beta$ -actin.

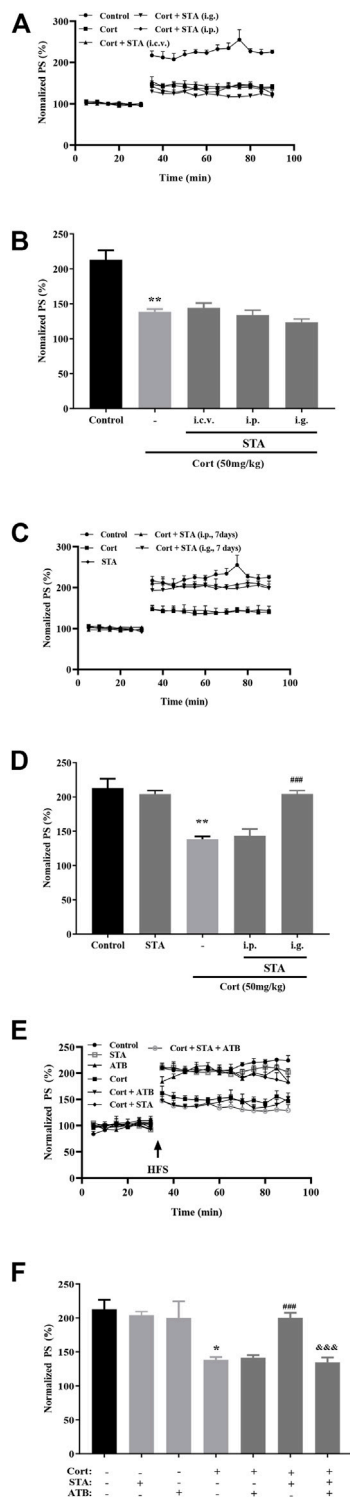
## Data Analysis

The results are presented as the mean  $\pm$  SEM. GraphPad Prism 8 (GraphPad Software, Inc., United States) was used to plot and analyze the data. Student's unpaired *t*-test was used to compare two groups. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to compare more than two groups. *p* < 0.05 were considered statistically significant.

## RESULTS

### The Effects of Stachyose on LTP Impairment by Corticosterone

Results of electrophysiological experiments showed that the average values of the relative PS amplitudes after HFS in the 50 mg/kg corticosterone group were significantly lower than that in the Control group, indicating that corticosterone significantly impaired hippocampal LTP. After single administration (i.c.v., i.p., and i.g.), stachyose had no protective effect on LTP impairment by corticosterone (**Figures 1A,B**; Cort vs. Control: *p* = 0.0011; STA (i.c.v.) vs. Cort: *p* = 0.8226, STA (i.p.) vs. Cort: *p* = 0.8997, STA (i.g.) vs. Cort: *p* = 0.2286). After intraperitoneal administration for 7 days, stachyose had no protective effect,



**FIGURE 1 |** Effects of stachyose (STA) on LTP impairment by corticosterone (Cort). **(A)** Time course of average relative PS amplitudes. **(B)** Average relative PS amplitudes after HFS. Cort/vehicle was injected 60 min before HFS, and STA were administrated (i.v., i.p., i.g.) 30 min before Cort. **(C)** Time course of average relative PS amplitudes. **(D)** Average relative PS amplitudes after HFS. STA were administrated (i.p., i.g.) for 7 days, and the last administration was 30 min before Cort. **(E)** Time course of average relative

(Continued)

while stachyose showed protective effect after intragastric administration for 7 days (**Figures 1C,D**, Cort vs. Control:  $p = 0.0011$ ; STA vs. Control:  $p = 0.8698$ ; STA (i.p.) vs. Cort:  $p = 0.8259$ ; STA (i.g.) vs. Cort:  $p < 0.001$ ).

ATB was used to disturb gut flora, results showed that administration of ATB had no effect on hippocampal LTP in control and corticosterone-treated mice. Administration (i.g.) of stachyose for 7 days significantly improved LTP impairment by corticosterone, while ATB canceled the beneficial effect of stachyose on LTP impairment by corticosterone (**Figures 1E,F**, Cort vs. Control:  $p = 0.0191$ , ATB vs. Control:  $p = 0.8174$ , Cort + ATB vs. Cort:  $p = 0.8816$ , Cort + STA vs. Cort:  $p < 0.001$ , Cort + STA + ATB vs. Cort + STA:  $p = 0.0004$ ), suggesting that stachyose may display its protective effects *via* gut flora.

## The Effects of Stachyose on Gut Flora

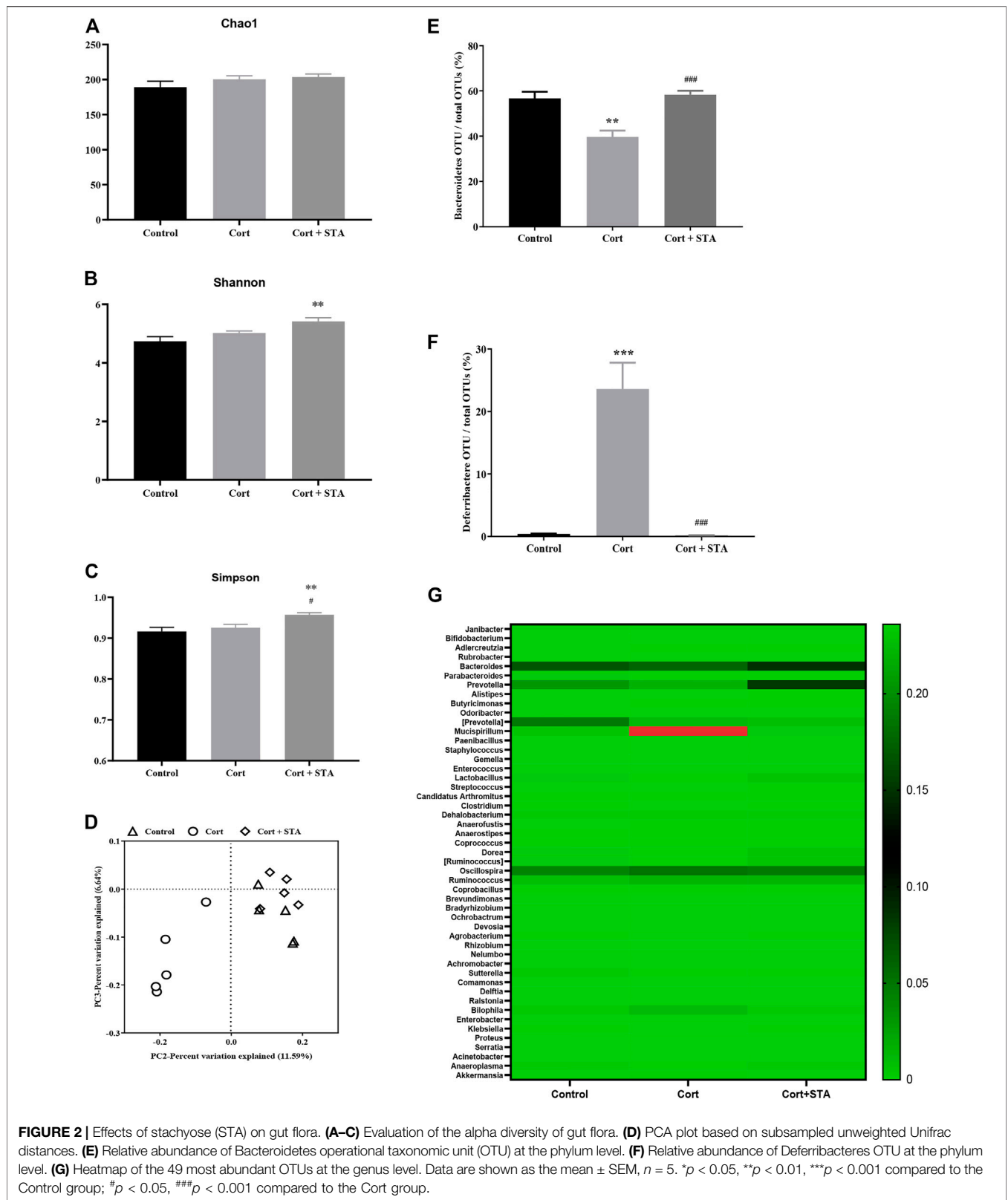
The Chao1, Shannon, and Simpson indices were evaluated to estimate the alpha diversity of gut flora. Corticosterone had little effects on these indices, and stachyose increased the Shannon and Simpson indices significantly (**Figures 2A–C**, Shannon, Cort vs. Control:  $p = 0.2679$ , Cort + STA vs. Control:  $p = 0.0049$ , Cort + STA vs. Cort:  $p = 0.0894$ ; Simpson, Cort vs. Control:  $p = 0.6952$ , Cort + STA vs. Control:  $p = 0.0078$ , Cort + STA vs. Cort:  $p = 0.0341$ ), indicating that stachyose increased the diversity of gut flora. The principal coordinate analysis (PCoA) showed that the gut flora in corticosterone-treated mice was distinct from that in the control group, and the gut flora in stachyose-treated mice clustered together with that in the control group (**Figure 2D**).

At the phylum level, compared with the control group, relative abundance of Bacteroidetes decreased and relative abundance of Deferribacteres increased significantly in corticosterone-treated mice, and stachyose restored Bacteroidetes and Deferribacteres to the normal level (**Figure 2E**, Bacteroidetes, Cort vs. Control:  $p = 0.0030$ , Cort + STA vs. Cort:  $p = 0.0005$ . **Figure 2F**, Deferribacteres, Cort vs. Control:  $p = 0.0006$ , Cort + STA vs. Cort:  $p = 0.0005$ ). The heatmap of the 49 most abundant OTUs at the genus level (**Figure 2G**) showed that Bacteroides and Prevotella (belonging to Bacteroidetes at phylum level) decreased in corticosterone-treated mice, and stachyose restored them; Mucispirillum (belonging to Deferribacteres at phylum level) increased in corticosterone-treated mice, and stachyose restored them, further demonstrating that stachyose may exert moderating effect on gut flora.

## The Effects of Stachyose on D-serine Metabolic Pathway

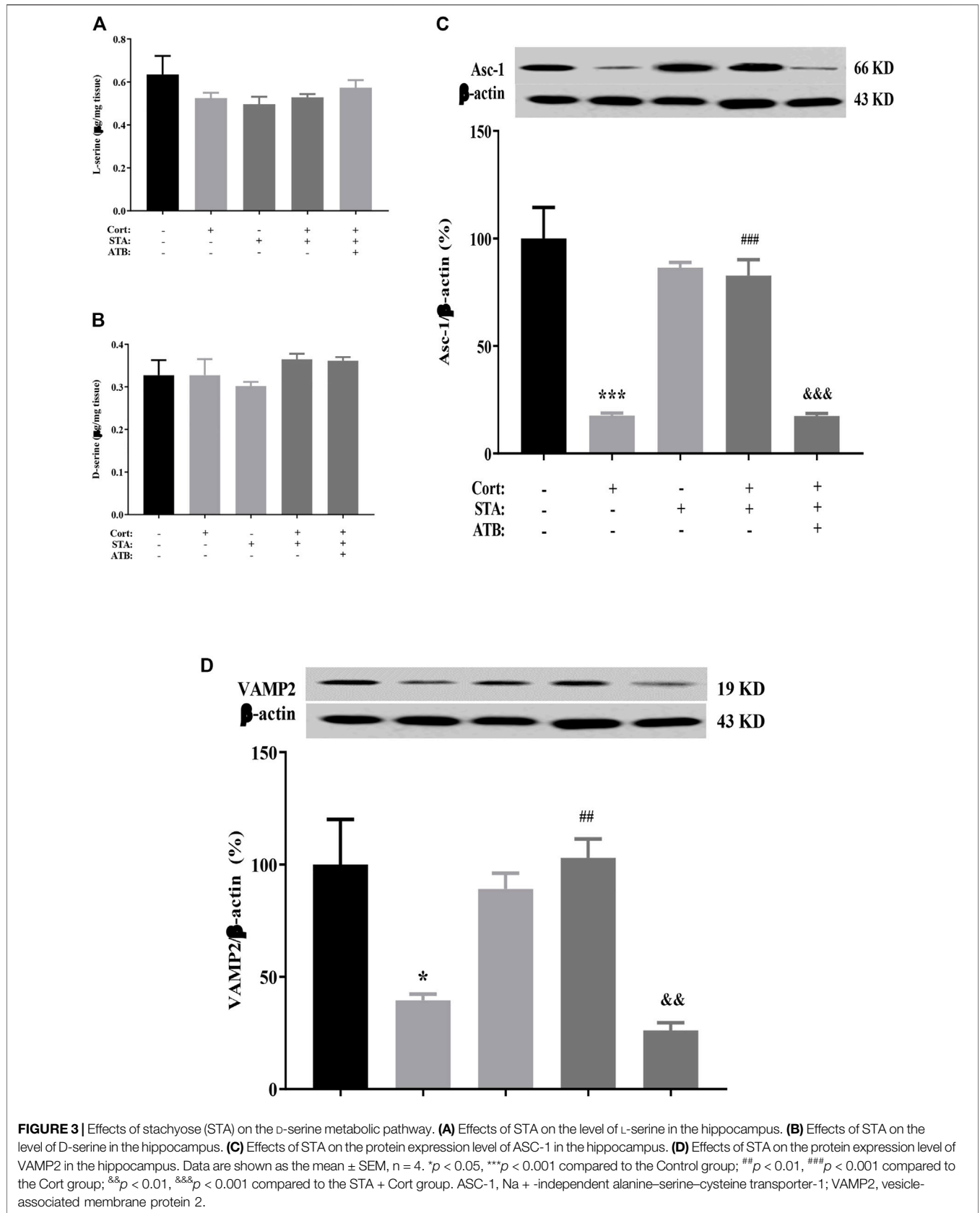
HPLC results showed that the content of D-serine and its precursor L-serine in the hippocampus did not change in

**FIGURE 1 |** PS amplitudes. **(F)** Average relative PS amplitudes after HFS. ATB was administrated 30 min before STA (i.g.). Data are shown as the mean  $\pm$  SEM,  $n = 4–6$ . \* $p < 0.05$ , \*\* $p < 0.01$  compared to the Control group; ### $p < 0.001$  compared to the Cort group; &&& $p < 0.001$  compared to the STA + Cort group.

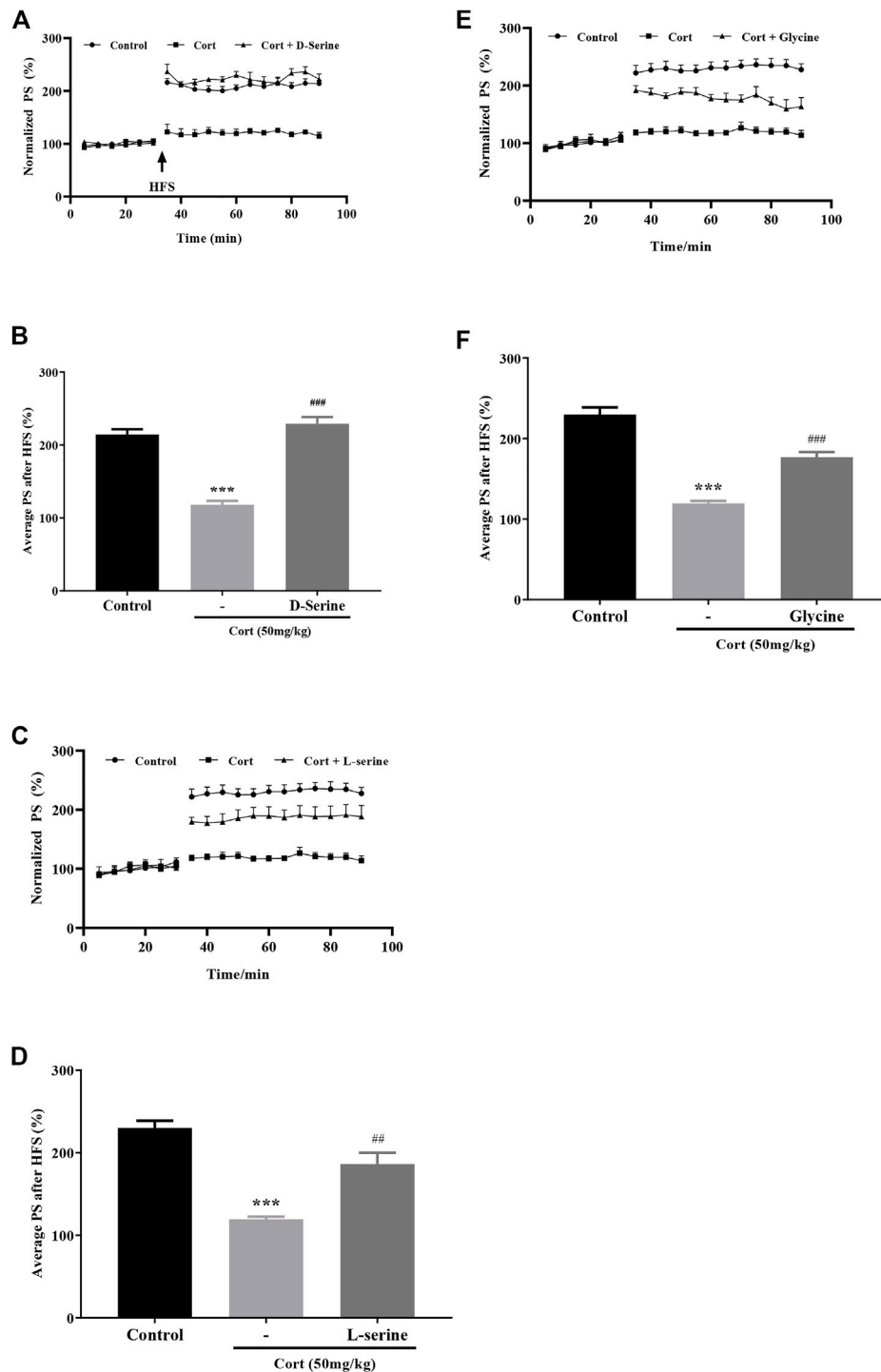


corticosterone-treated mice. Stachyose had no effect on the level of hippocampal D-serine and L-serine in control and corticosterone-treated mice. ATB had no effect on the level of

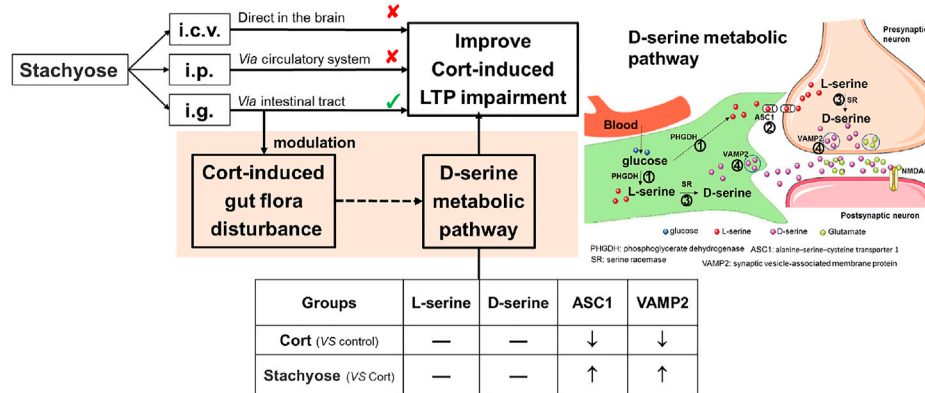
hippocampal D-serine and L-serine in stachyose and corticosterone-treated mice (**Figure 3A**, L-serine, Cort vs. Control:  $p = 0.4774$ , STA vs. Control:  $p = 0.2651$ , Cort + STA







**FIGURE 4 |** Effects of D-serine, L-serine, and glycine on LTP impairment by corticosterone (Cort). **(A)** Time course of average relative PS amplitudes. **(B)** Average relative PS amplitudes after HFS; Cort/vehicle was injected 60 min before HFS, and D-serine was administered 30 min before Cort. **(C)** Time course of average relative PS amplitudes. **(D)** Average relative PS amplitudes after HFS; Cort/vehicle was injected 60 min before HFS, and L-serine was administered 30 min before Cort. **(E)** Time course of average relative PS amplitudes. **(F)** Average relative PS amplitudes after HFS; Cort/vehicle was injected 60 min before HFS, and glycine was administered 30 min before Cort. Data are shown as the mean  $\pm$  SEM,  $n = 4-7$ . \*\*\* $p < 0.001$  compared to the Control group; ## $p < 0.01$ , ### $p < 0.001$  compared to the Cort group.



**FIGURE 5 |** Summary of effects and mechanisms of stachyose. Stachyose can protect LTP impairment against corticosterone via intragastric administration, but there is little effect when administration via intraperitoneal injection or intracerebroventricular injection. Modulating the gut flora is involved in the beneficial effects of stachyose. Stachyose can restore the D-serine metabolic pathway disturbed by corticosterone.

vs. Cort:  $p = 0.9949$ , Cort + STA + ATB vs. Cort + STA:  $p = 0.9454$ ; **Figure 3B**, D-serine, Cort vs. Control:  $p = 0.9998$ , STA vs. Control:  $p = 0.9450$ , Cort + STA vs. Cort:  $p = 0.8185$ , Cort + STA + ATB vs. Cort + STA:  $p = 0.8652$ ). However, the protein expression level of hippocampal ASC-1 and VAMP2 were significantly decreased in corticosterone-treated mice. Stachyose significantly increased the expression level of hippocampal ASC-1 and VAMP2 in corticosterone-treated mice and had no effect on the level of hippocampal ASC-1 in control mice. ATB significantly canceled the increased effect of stachyose on the protein expression level of hippocampal ASC-1 and VAMP2 in corticosterone-treated mice (**Figure 3C**, ASC-1, Cort vs. Control:  $p = 0.0009$ , STA vs. Control:  $p = 0.4631$ , Cort + STA vs. Cort:  $p = 0.0010$ , Cort + STA + ATB vs. Cort + STA:  $p = 0.0009$ . **Figure 3D**, VAMP2, Cort vs. Control:  $p = 0.0237$ , STA vs. Control:  $p = 0.7700$ , Cort + STA vs. Cort:  $p = 0.0020$ , Cort + STA + ATB vs. Cort + STA:  $p = 0.0011$ ), suggesting that stachyose may restore D-serine release to improve LTP impairment by corticosterone.

## The Effects of D-Serine, L-Serine, and Glycine on LTP Impairment by Corticosterone

Results showed that administration (i.c.v.) of endogenous NMDA receptor glycine site full agonist D-serine (300 nmol) significantly improved corticosterone-induced hippocampal LTP impairment (**Figures 4A,B**, Cort vs. Control:  $p < 0.001$ , Cort + D-serine vs. Cort:  $p < 0.001$ ). Administration (i.c.v.) of L-serine (300 nmol), the precursor of D-serine, also significantly improved corticosterone-induced hippocampal LTP impairment (**Figures 4C,D**, Cort vs. Control:  $p < 0.001$ , Cort + L-serine vs. Cort:  $p = 0.0037$ ). Results also showed that administration (i.c.v.) of endogenous NMDA receptor glycine site partial agonist glycine (100 nmol) significantly improved corticosterone-induced hippocampal LTP impairment (**Figures 4E,F**, Cort vs. Control:  $p < 0.001$ , Cort + glycine vs. Cort:  $p = 0.0002$ ), demonstrating that stachyose may enhance the function

NMDA receptors by restoring D-serine release to improve LTP impairment by corticosterone.

## DISCUSSION

The dosage of stachyose is based on the dosage of LW-AFC. According to our previous study, 0.8 g/kg to 3.2 g/kg of LW-AFC (intragastric administration) could protect LTP impairment against corticosterone (Huang et al., 2019); LW-AFC contains 64.6% oligosaccharide fraction, that is, the theoretical effective dosage of oligosaccharide fraction is ranged from 520 mg/kg to 2080 mg/kg. As one of the main ingredients in oligosaccharide fraction, the effective dosage of stachyose might equal or less than that of oligosaccharide fraction. So, 450 mg/kg of stachyose (intragastric administration) will not exceed a pharmacologically meaningful level. Furthermore, the toxicity of stachyose was very low, whereas intracerebroventricularly (500  $\mu$ g) and intraperitoneally (20 mg/kg) administered relative high dosage showed little effect on normal animals. Because the effects of LW-AFC and oligosaccharide fraction has been fully demonstrated (Wang et al., 2016a; Wang et al., 2016b; Wang et al., 2016c; Wang et al., 2017a; Wang et al., 2017b; Wang et al., 2017c; Huang et al., 2019; Zeng et al., 2019; Cheng et al., 2020b; Wei et al., 2021) and stachyose is one of the main ingredients in oligosaccharide fraction, we used a single effective dosage of stachyose to study the mechanisms.

In this study, single administration of stachyose via i.p. and i.g., even direct in the brain via i.c.v., has little effect on LTP impairment by corticosterone. Many studies have confirmed that stachyose can regulate the gut flora balance and act as prebiotic (Li et al., 2013; Li et al., 2017; Liu et al., 2018), so stachyose would have protective effects against LTP impairment by corticosterone via intragastric administration for a few days. The data in this study showed that 7-consecutive-day administration of stachyose via i.g. had protective effect, and there was little effect via i.p. To disturb gut flora, a combination of non-absorbable antibiotics (ATB) (Huang et al., 2019) were



applied, the results showed that ATB canceled the protective effect of stachyose without affecting LTP in control and corticosterone-treated mice, suggesting that stachyose may display its protective effects against LTP impairment by corticosterone *via* gut flora.

The alpha diversity showed that corticosterone did not change the diversity and richness of gut flora, and stachyose could increase the diversity, indicating that higher gut flora diversity and richness might contribute to the protective effect of stachyose against corticosterone. The PCoA analysis of gut flora showed that corticosterone-treated mice were distinct from mice in the control group, and the gut flora in stachyose-treated mice clustered together with that in the control group. Also, Stachyose could also restore Bacteroidetes and Deferribacteres to normal level. It has been reported that Bacteroidetes is related to cognitive development in human infants and dementia (Carlson et al., 2018; Saji et al., 2019). We found that Bacteroidetes decreased in corticosterone treated mice, which is similar to previous report (Qiu et al., 2019). Shi's study (Shi et al., 2020) showed that restored Bacteroidetes level was related to cognitive improving effect of  $\beta$ -glucan. Results in this study showed that stachyose could increase the relative abundance of Bacteroidetes and restore it to normal level. Increased Deferribacteres was associated to inflammation (Berry et al., 2015), and decreased Deferribacteres level was reported to relate to cognitive improving effect of methylene blue (Gureev et al., 2020). Here we found that Deferribacteres increased significantly in corticosterone-treated mice, and stachyose could restore it to normal level. These results indicated that modulating gut flora such as Bacteroidetes and Deferribacteres might be an important factor for stachyose's neuroprotective effect.

D-serine is synthesized by the enzyme serine racemase (SR) which is mainly localized in neurons (Benneyworth et al., 2012; Balu et al., 2014), playing an important role in brain function.  $\text{Na}^+$ -independent alanine-serine-cysteine transporter-1 (ASC-1) and vesicle-associated membrane protein 2 (VAMP2) are responsible for the transport and release of D-serine (Mothet et al., 2005; Rosenberg et al., 2013; Neame et al., 2019). Previous studies indicated that D-serine could improve memory deficit by stress through activation of NMDA receptors (Choi et al., 1987; Guercio et al., 2014; Wang et al., 2017d; Gonçalves-Ribeiro et al., 2019), suggesting that hypofunction of NMDA receptors might be an important factor for stress-induced cognitive impairment. And we recently found that corticosterone decreased ASC-1 and VAMP2, which are important for D-serine release, leading to hypofunction of the NMDA receptors and LTP impairment (Wang et al., 2021). In this study, the results showed that stachyose had no effect on the content of L-serine or D-serine in hippocampal tissue, but increased D-serine release-related

proteins ASC-1 and VAMP2. Along with a combination of non-absorbable antibiotics, the effect of stachyose on ASC-1 and VAMP2 was blocked. Modulating gut flora might contribute to the effect of stachyose on the D-serine pathway. It has reported that D-serine and L-serine were lower in the brain of specific pathogen-free (SPF) mice than in those of germ-free (GF) mice, indicating that gut flora might be involved in the D-serine metabolic pathway in the brain (Kawase et al., 2017). Oligosaccharides, such as galacto-oligosaccharides (GOS), administrated *via* i.g. could increase hippocampal D-serine level (Savignac et al., 2013). D-serine is a coactivator of NMDA receptor, binding to the glycine site of NMDA receptor (Wolosker, 2018). These results indicated that increase D-serine release might contribute to neuroprotective effect of stachyose against corticosterone *via* modulating gut flora. It is a limitation that how the gut flora modulates the D-serine pathway in the brain also remains elusive.

Altogether, stachyose have a very similar effect on LTP impairment by corticosterone, which makes it a candidate for replacing CA-30 in LW-AFC. Stachyose could increase the D-serine release-related proteins ASC-1 and VAMP2 *via* gut-brain axis, protecting LTP from deteriorative effect of corticosterone (Figure 5). This study contributes to the understanding of active ingredients and mechanisms of LW-AFC. Further study is needed to uncover the relation between gut flora and the D-serine metabolic pathway.

## DATA AVAILABILITY STATEMENT

The sequence data have been deposited in the National Center for Biotechnology Information (NCBI) BioProject database with project number PRJNA773852. All other data are available upon request from the authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Care and Use Committee (IACUC) of National Beijing Center for Drug Safety Evaluation and Research (NBCDSER).

## AUTHOR CONTRIBUTIONS

YH and W-XZ conceived the study and participated in its design. DL conducted all experiments. YH, CW, and DL wrote the manuscript. YH and W-XZ revised the manuscript. NS participated in the analysis of the results and manuscript preparation.

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