Reduction of Acetylcholinesterase Inhibition Activity of Antiscolic Herbal Compounds by Bacilli Probiotics

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Abstract

Antiscolic herbal compounds (AHCs) are fast-growing biopesticides but must be removed afterwards from harvested crops due to their potential toxicities towards animal acetylcholine esterase (AChE). In this work, we investigated the inhibitory effect of several typical AHCs on AChE as well as their detoxification by bacilli probiotics, another group of green agricultural additives. Results showed the AChE inhibition activity of studied AHCs follows an order of berberine ($IC_{50} = 12.16 \mu\text{mol/L}$) > osthole ($233.21 \mu\text{mol/L}$) $\approx$ matrine ($262.41 \mu\text{mol/L}$) >> trigonelline (no inhibition). Molecular simulation predicted that berberine, matrine and osthole combine with AChE preferentially in the catalytic channel for acetylcholine (Trp84 and His440), but forming more stable complexes. At the IC_{50} dosage, the addition of *Bacillus subtilis* ($10^6 \sim 10^7$ CFU/mL) or several other bacilli species detoxified osthole from 50% AChE inhibition to around 30%. Slight detoxification of matrine was also observed but the toxicity of berberine was not influenced after incubated with bacilli probiotics. Conversely, the three AHCs could completely inhibited the growth of *B. subtilis* following an order of berberine (0.15 mmol/L) > Matrine (9 mmol/L) > osthole (1.5 mmol/L). *B. subtilis* Ruizhen[®] exhibited stronger tolerance to AHCs than other commercial strains. Among other tested species, *B. mucilaginosus* was less sensitive than *B. subtilis* while *B. laterosporus* and *P. polymyxa* were resistant to AHCs. The potential antagonistic actions between AHCs and bacilli probiotics maybe useful in removal of residual herbal toxicity with green bacterial sweeper.

Introduction

The harmful effects of synthetic pesticides on human health have caused worldwide attention in recent years. A promising solution is to develop natural compounds from antiscolic herbs as a substitute to meet the demand of sustainable agriculture (Chaudhary et al. 2017). Coptis root, sophora root, and fruit of *Fructus cnidii* are all traditional Chinese medicines for insect repellent (Zhang et al. 2018). Berberine extracted from coptis root is a rotenoid that strongly inhibits the mitochondrial respiratory chain of pests, resulting in respiratory disorders, paralysis and death (Li et al. 2018). Berberine also targets on water insects including aphids, thrips, beetles, spider mites, etc (Li et al. 2018). Matrine is an alkaloid isolated from saphoro root. It kills pests by paralyzing their nervous system after immediate contact and subsequently denaturalizing the albumen proteins in their bodies, which finally causes the insects to die of suffocation (Zanardi et al. 2015). Osthole is coumarin compound from *Fructus cnidii* which also acts on the nervous system of pests, causing non-functional contraction of the muscles and eventually dying (Yan et al. 2021). Some of these antiscolic herbal compounds (AHCs), i.e. berberine, has been commercially applied to replace synthetic organophosphorus pesticides for planting fruit trees and vegetables. However, many AHCs exhibit neurotoxicity not only towards insects but also to higher animals (Lu et al. 2014) especially when exceeding safe concentrations. Safe approaches to detoxify AHCs are currently in urgent need.

Bacilli probiotics have already applied in cultivation of herbs as well as in the composting of herbal medicine residue (Prakash et al. 2017; Chang et al. 2020). *Bacillus* is one of the most representative

families, among which, *B. subtilis* has been widely used as biofungicide for controlling pre- and post-harvest herbal plant pathogens through production of antibiotics, nutrient competition and induction of systemic resistance in the host plant (Fira et al. 2018). In addition to *B. subtilis*, *B. thuringiensis*, *B. pumilus*, *B. licheniformis* and several other species have also been approved in many countries for herbal plant protection and growth stimulation in agriculture (Jurat-Fuentes et al. 2021; Gutiérrez-Mañero et al. 2010). This raises us a question that would live bacteria reduce the residual toxicity of AHCs? The potential antagonistic role played by bacilli probiotics may arise a new detoxification approach for safe use of AHC biopesticides. On the other hand, previous authors have reported the antimicrobial activities of AHCs against bacterial strains including *Escherichia coli*, *Staphylococcus aureus* and *B. cereus* (Pierpaoli et al. 2021; Sun et al. 2021; More et al. 2017). Investigation of anti-bacilli activity of AHCs is also important for fully understanding the potential antagonistic effects between the two kinds of agricultural additives when used simultaneously or within a short service interval. However, to the best knowledge of us, the interactions between specific AHCs and live bacilli probiotics have not been well studied. This work focused on this topic and may help to optimize the application of AHCs and probiotics in for sustainable agriculture.

Materials And Methods

Chemicals

All chemicals were purchased from Macklin Chemical Reagent Co. Ltd. (Shanghai, China). The basic information of 4 AHCs, matrine, berberine, osthole and trigonelline were obtained from the supplier as summarized in Table 1. Trigonelline is a major soluble alkaloid extracted from *Trigonella foenum graecum* seed, a Chinese traditional antiscolic herbal medicine.

Acetylcholinesterase (AChE) Inhibition Test

The AChE inhibition by AHCs and bacillus were quantitatively determined using a HPER-40T pesticide residue detector and the matching assay kit (HPER Scientific Instrument Co., Ltd., Guangzhou, China). The following reagents were pipetted in a 4-mL cuvette: 0.14 mL of phosphate buffer (20 mM, pH 7.6), 0.05 mL of acetylthiocholine iodide (5 mM), and 0.01 mL of AHC solution (water as reference). After incubation at 38°C for 10 min, the reaction was stopped with 1.8 mL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)-phosphate ethanol reagent supplied by HPER. The color change at 412 nm was recorded immediately from which the concentration to inhibit 50 % of AChE (IC₅₀) was reported.

Molecular Simulation

An AChE model, 1UT6, was obtained from the Protein Data Bank database (<http://www.rcsb.org>) for simulating the binding of AHCs. All the 3D structural files of AHCs were obtained from ChemSpider. In the software Autodock 4.2, the PDB files of AChE and AHCs were rewritten to be PDBQT format, following with docking procedures based on Lamarckian Genetic Algorithm. The gridbox parameters were set as below: number of points in x, y and z dimension was 126, 126 and 126 respectively; spacing = 0.5 Å;

center $x = 4.85$, $y = 64.409$, $z = 56.065$. The number of GA runs was 200 and the population size was 400. The output data were redrawn in UCSF Chimera software. The amino acids interacted with AHC ligands were analyzed using the LigPlus tools.

Bacterial Strains and Culture Conditions

B. subtilis Ruizhen® biofungicide was purchased from Beijing Coway BioWorks Biotech Co., Ltd. (Beijing, China) in the form of dried powder. The active endospores were 1×10^{10} CFU/g as denoted by the supplier and the filler in powder products was soluble and non-nutrient. Colonies of live cells were obtained after spreading 10 μ L diluent (10^8 dilution) of RuiZhen® biofungicide on plates of Chromogenic *Bacillus Cereus* Agar (CBCA, Huankai, Guangdong, China) and incubating at 30°C for 24 h. A single colony was inoculated in nutrient broth (peptone 10 g/L, beef extract 3 g/L, NaCl 5 g/L, pH 7.3 $\pm$ 0.2) and cultivated at 30°C, 120 rpm for 2 h. Then the broth was centrifuged at 4°C, 10000 rpm for 5 min. The precipitate was washed with sterile saline for 3 times and finally diluted to a suspension containing living cells of 1×10^7 CFU/g. All the plates with isolated colonies were preserved only for experiment and were heat sterilized after this research.

Other two commercial *B. subtilis* products were purchased from BION Tech Inc. (BTI, Taiwan, China) and Beihai Qiangxing Biotech Co., Ltd. (Guangxi, China), respectively, denoted as Y1336 and BHQX according to the suppliers. *Bacillus mucilaginosus*, *Brevibacillus laterosporus* and *Paenibacillus polymyxa* were obtained from Guangdong Microbial Culture Collection Center (GDMCC).

Bacterial activity test

All cell suspensions of bacteria were properly diluted, inoculated (20 μ L) and spread onto CBCA plates. The plates were incubated at 30°C for 24 h. The number of *Bacillus* colonies with blue-green color was counted and reported into CFU/mL.

Results And Discussion

AchE Inhibition by AHCs

The AchE inhibitory effect of the tested 4 AHCs was determined as shown in Figure 1 from which the respective IC₅₀ values were obtained as summarized in Table 2. Berberine hemisulfate exhibited the strongest inhibition with an IC₅₀ value (12.16 μ mol/L) an order of magnitude lower than that of matrine (262.41 μ mol/L) or osthole (233.21 μ mol/L). No toxicity for trigonelline chloride was detected in the tested range.

It was worth to mention that osthole was dispersed in the form of emulsion due to it is slightly soluble in water. When testing a suspension of osthole in water instead of emulsion, at most 24% AchE activity loss was obtained at a content of 30 μ mol/L. The activity loss was maintained at about 20% within a wide range of osthole content in aqueous suspension up to 244 μ mol/L (Figure S1). This inhibition limit

probably corresponds to the dissolution ratio of osthole in water, and only those dissolved part non-productively binds with AchE.

The binding of AHCs in AchE was simulated using Autodock and the results were shown in Figure 2. Berberine, matrine and osthole preferentially bound with AchE at the catalytic channel which is also anchored by the regular substrate, acetylcholine. The binding energies were -9.11, -9.41 and -7.99 kcal/mol, respectively (Table 2). The absolute values were much higher than that of acetylcholine (-4.75 kcal/mol), suggesting that the three AHCs formed more stable complex conformations with AchE than acetylcholine. Trigonelline was significantly different from them. It took up a favorite binding site away from the catalytic channel, suggesting there is no AchE inhibition effect of trigonelline. This was in accordance with the data as illustrated in Figure 1.

The amino acids interacted with AHC ligands were summarized in Table 2. Trp84, Phe330 and His440 were identical binding sites for both substrate (acetylcholine) and inhibitor (matrine, berberine or osthole). In previous papers, amino acids Trp84, Tyr130, Phe330 and Phe331 are responsible for the molecular recognition of quaternary ammonium ligands and also the catalytic anionic sites (Lee et al. 2015; Takomthong et al. 2021). Therefore, the three AHCs probably inhibit AchE through competitively occupation of the active catalytic channel. Berberine and matrine were stronger inhibitors than osthole probably due to that their higher binding energies (Table 2). Trigonelline preferably anchored the hydrophilic valley at the N-terminal of AchE, showing no competition with acetylcholine and thus no inhibition. It can be further deduced that trigonelline is probably not the active component in *Trigonella foenum graecum* seed which has been considered as a kind of antiscolic herb.

Detoxification of AHCs by *Bacillus*

B. subtilis Ruizhen[®] was added into the AchE inhibition test system together with berberine, matrine and osthole at their respective IC₅₀ concentration. The total live cell number of Ruizhen[®] was 1×10⁶ CFU/mL. These bacteria at this content level did not show any impact on the AchE activity (Figure 3a). After incubation at 30°C for 2 h, both berberine and matrine caused half loss of the AchE activity in presence of Ruizhen[®] (Figure 3a). Interestingly, in the test of osthole incubated with Ruizhen[®] at 10⁶ CFU/mL level, the AchE activity loss was decreased to 66.57%. The results clearly indicate a detoxification of osthole with the addition of Ruizhen[®].

At a higher content of live cells, i.e. 10⁷ and 10⁸ CFU/mL, *B. subtilis* Ruizhen[®] independently depressed AchE activity by about 15% and 22%, respectively. These inhibition data were much lower than the alarming line (50% loss) and were considered to be safe in general (Printes et al. 2004). After incubating Ruizhen[®] and the three AHCs at 30°C for 2 h, only berberine kept the same toxicity level to AchE. The measured AchE loss by combination of Ruizhen[®] and berberine was almost exact half of the value of reference. In another word, there was no detoxification effect on berberine by incubation with bacillus Ruizhen[®].

However, the toxicities of matrine and osthole as represented by AchE inhibition were obviously weakened after incubated with Ruizhen[®] (10^7 or 10^8 CFU/mL). Their retained AchE activities were all above the expected half-loss line as shown in Figure 3a. The antagonistic effect suggests that Ruizhen[®] is not suitable for mixed use together with biopesticides containing matrine and osthole and can somehow detoxify these two biopesticides residues.

Detoxification effects were also found when incubating AHCs with other bacilli species (Figure 3b). Such effect on osthole was more obvious than on matrine. At the IC_{50} dosages, the addition of *Bacillus subtilis* (10^7 CFU/mL) or several other bacilli species detoxified osthole from 50% AchE inhibition to around 30%. There was still no obvious change found in the case of berberine. On the other hand, *P. polymyxa* gave stronger AchE toxicity than *B. subtilis*. Mixing these two probiotics (10^7 CFU/mL) with berberine (IC_{50} concentration) resulted in remarkable AchE inhibition degree up to 67%.

Growth Inhibition of Bacilli by AHCs

The antimicrobial activity of AHCs were tested with *B. subtilis* Ruizhen[®] and the results were demonstrated in Figures 4 and 5. Separate green round colonies occurred after 24-h incubation of diluted cell suspensions of Ruizhen[®] spread on CBCA plates. Numbers of colonies were decreased due to the addition of AHCs. There was a narrow tolerance range for Ruizhen[®] to berberine (0~0.012 mmol/L), matrine (0~4.2 mmol/L) and osthole (0~0.53 mmol/L). Within the respective tolerant range, the loss of living cells was less than 15%. Berberine, matrine and osthole completely inhibited the growth of *B. subtilis* (4.2×10^7 CFU/mL) at a concentration of 0.43, 3.37 and 9.22 mmol/L, respectively.

Compared with Ruizhen[®], the other two *B. subtilis* strains, BHQX and Y1336, showed much lower resistance to berberine, matrine and osthole. They were completely inhibited by berberine at 0.1 mmol/L and by matrine at 2.25 mmol/L. Osthole was also very toxic to them, inhibited 94.4% and 82.2% of live cells at a content of 0.5 mmol/L. As a comparison, no significant inhibition occurred in the test of Ruizhen[®]. This indicates the importance of training bacterium strains to be tolerant to certain AHCs before applying them to herbal plants.

Unlike *B. subtilis*, the other three bacilli species generally showed higher tolerance when exposed to berberine, matrine and osthole, following the order: *B. mucilaginosus* > *B. laterosporus* ~ *P. polymyxa* (Table 3). The only exception is that *B. mucilaginosus* was more sensitive to matrine and osthole than *B. subtilis* Ruizhen[®]. Among all the tested strains, *B. laterosporus* had the highest tolerance to AHCs. Little inhibitory effect was detected in the presence of herbal compounds within the tested concentration ranges.

Results also suggests the importance of training probiotic microbes that tolerate the anti-microbial activity of AHCs if they are used for detoxification purpose. The studied three AHCs could completely inhibited the growth of *B. subtilis* following an order of berberine (0.15 mmol/L) > Matrine (9 mmol/L) > osthole (1.5 mmol/L). In literature, the potential of berberine as an antimicrobial agent against mixed

microorganisms containing *E. coli* and *B. subtilis* has been reported by Kong et al. (2012). They found a low concentration (20 µg/mL) of berberine began to inhibit the growth of *E. coli* and mixed microorganisms, while promoting the growth of *B. subtilis* (Kong et al. 2012). Deep mechanism study revealed that berberine can inhibit not only *B. subtilis* cell growth, but also spore outgrowth, including inhibiting protein synthesis during this period (Wang et al. 2015). The presence of L-valine prevented the effect of berberine (200 µg/mL) on *B. subtilis* spore germination (Wang et al. 2015). Impressively, our data in Table 3 showed berberine blocked the growth of *B. subtilis* strains but more compatible with *B. mucilaginosus*, *B. laterosporus* and *P. polymyxa*. In another word, *B. mucilaginosus* and *B. laterosporus* were less sensitive to berberine (0.1 mmol/L) than *B. subtilis* strains. There might be metabolites by these two species that play a role in detoxification of berberine. The combination of *P. polymyxa* and berberine could be further explored through insecticide for developing conjugated bio-insecticides.

Antimicrobial properties of matrine are associated with glycerophospholipid metabolism and the sphingolipid metabolic signaling pathways (Huang et al. 2016). Previous authors reported matrine enhanced the pathogenicity of *Beauveria brongniartii* against agricultural insect pests (Wu et al. 2019). Osthole kill both Gram positive and negative bacteria by inhibiting the absorption of Ca^{2+} , which affects the growth and spore germination of the bacteria and inhibits chitin deposition of the bacterial cell wall (Sun et al. 2021). Supplemental of soluble calcium salts in decomposition of osthole-containing herbal residues by *Bacillus* may help preserve the living cells.

Conclusion

The three AHCs, berberine ($\text{IC}_{50} = 12.16 \mu\text{mol/L}$), matrine (262.41 µmol/L) and osthole (233.21 µmol/L) are all AchE inhibitors that could enter the catalytic channel of the enzyme and occupy key amino acids competing with the regular substrate. They could also completely inhibit the growth of *B. subtilis* following an order of berberine (0.15 mmol/L) > Matrine (9 mmol/L) > osthole (1.5 mmol/L). Addition of *Bacillus subtilis* ($10^6 \sim 10^7$ CFU/mL) or several other *Bacilli* species detoxified matrine and osthole to some extent suggesting obvious antagonistic action between *Bacilli* and some AHCs. The toxicity of berberine was not influenced by the incubation with *Bacilli* probiotics. Berberine also blocked the growth of *B. subtilis* strains but more compatible with *B. mucilaginosus*, *B. laterosporus* and *P. polymyxa*. Our results may be helpful for the further combined utilization of AHCs biopesticides and microbial probiotics.

Declarations

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Tables

Table 1 AHCs used in this work

Chemical name & CAS No.	Formula, Molecular weight & Purity	Molecular structure*
Matrine (CAS 519-02-8)	$C_{15}H_{24}N_2O$ 248.36 g/mol 98%
Berberine Hemisulfate (CAS 633-66-9)	$C_{20}H_{18}NO_4 \cdot 1/2SO_4$ 384.4 g/mol 95%
Osthole (CAS 484-12-8)	$C_{15}H_{16}O_3$ 540.6 g/mol 99%
Trigonelline chloride (CAS 6138-41-6)	$C_7H_8ClNO_2$ 173.6 g/mol 99%

Table 2 Inhibitory binding of AHCs to AchE simulated with Autodock

Ligand	IC ₅₀ (μmol/L)*	Binding energy (kcal/mol)	Inhibition constant (μM)	Amino acids residues at binding sites**
Acetylcholine	-	-4.75	331.81	Trp84, Gly117, Gly118, Gly119 , Tyr130, Glu199, Ser200 , Phe288, Phe290, Phe331, His440 , Gly441
Matrine	262.41	-9.41	0.13	Trp84, Glu199, Phe330, Tyr334, Trp432, Ile439, His440, Gly441, Tyr442
Berberine	12.16	-9.11	0.21	Gln69, Tyr70, Asp72, Trp84, Asn85, Pro86, Gly118, Ser122 , Gly123, Phe330, Tyr334, Trp432, Met436, Ile439, His440, Tyr442,
Osthole	233.21	-7.99	1.38	Asp72, Ser81, Trp84, Gly117, Ser122, Gly123, Leu127, Tyr130, Glu199, Phe330, Tyr334 , His440,
Trigonelline	ND	-4.48	520.33	Arg468, His471, Tyr472, Pro482, Asn483, Glu484, Lys491

* All IC₅₀ data were tested in triplicate;

**The amino acids participating the hydrogen bonding were marked in bold. .

Table 3 Growth inhibition of other bacilli species

Strain	Control	Matrine (mmol/L)		Berberine (mmol/L)			Osthole (mmol/L)	
		2.25	5.8	0.025	0.05	0.1	0.5	1.0
<i>B.s. Ruzen@</i>	42.0	38.0	15.5	35.0	13.5	2.4	40.3	0.5
<i>B.s.BHQX</i>	44.1	0.0	0.0	14.4	0.0	0.0	2.5	0.0
<i>B.s.Y1336</i>	24.6	0.0	0.0	7.5	2.0	0.0	4.4	2.1
<i>B.mucilaginosus</i>	36.2	17.3	17.3	31.6	33.6	27.8	18.4	11.6
<i>B.laterosporus</i>	43.4	43.1	40.5	44.5	35.1	37.7	42.0	39.0
<i>P. polymyxa</i>	39.5	32.8	30.6	42.6	38.2	28.2	33.3	37.9

Note: The unit of living cell content is million CFU/mL.

Figures

Figure 1

Inhibition of AchE activity by different AHCs at varied concentrations

Note: The test without addition of any AHC was set as reference (100%)

Figure 2

Binding of acetylcholine and AHCs in AchE

Note: green - acetylcholine, red - berberine, purple - matrine, cyan - osthole, blue - trigonelline

(a) *B. subtilis* Ruizhen®.

Note: IC₅₀ concentrations of matrine, berberine and osthole were used.

(b) Other Bacilli species

Note: 1×10^7 CFU/ml bacteria and IC₅₀ concentrations of matrine, berberine and osthole were used

Figure 3

Effects of *Bacillis* on the AchE toxicity of AHCs

Figure 4

Inhibition of *B. subtilis* Ruizhen[®] growth by AHCs

Note: Starting cell content 4.2×10^7 CFU/g

Figure 5

Effects of the AHCs on the growth of *B. subtilis* Ruizhen

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