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Review Article

Gossypol detoxification in the rumen and *Helicoverpa armigera* larvae: A review

Wei-Kang Wang [a], Hong-Jian Yang [a, b, *], Yan-Lu Wang [a], Kai-Lun Yang [c], Lin-Shu Jiang [d], Sheng-Li Li [a, b]

[a] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China
[b] Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China
[c] College of Animal Sciences, Xinjiang Agricultural University, Urumuqi, 830052, China
[d] College of Animal Science and Technology, Beijing University of Agriculture, Beijing, 102206, China

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ABSTRACT

Gossypol, a phenolic compound found in the cotton plant, is widely distributed in cottonseed by-products. Although ruminant animals are believed to be more tolerant of gossypol toxicity than monogastric animals due to rumen microbial fermentation, the actual mechanisms of detoxification remain unclear. In contrast, the metabolic detoxification of gossypol by *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae has achieved great advances. The present review discusses the clinical signs of gossypol in ruminant animals, as well as summarizing advances in the study of gossypol detoxification in the rumen. It also examines the regulatory roles of several key enzymes in gossypol detoxification and transformation known in *H. armigera*. With the rapid development of modern molecular biotechnology and -omics technology strategies, evidence increasingly indicates that research into the biological degradation of gossypol in *H. armigera* larvae and some microbes, in terms of these key enzymes, could provide scientific insights that would underpin future work on microbial gossypol detoxification in the rumen, with the ultimate aim of further alleviating gossypol toxicity in ruminant animals.

1. Introduction

Cottonseed by-products are used extensively as a source of energy and proteins in the diets of ruminant animals, although they contain gossypol, a polyphenolic compound found in cotton (*Gossypium* spp.) (Rogers et al., 2002; Santos et al., 2002). Excessive intake of gossypol can cause anaemia and impair animal reproductive functions, while consumption of animal products with excessive gossypol residues may affect human health (Brimer and Sørensen 2009). Due to the toxicity of excessive gossypol, many countries and regions have stipulated the dietary allowance limit of gossypol. For many years, three methods have been used in animal feed processing to reduce toxicity of gossypol in cottonseed by-products. These included mechanical processing, chemical treatment, and microbial fermentation. All of these methods play a role in gossypol detoxification, but microbial fermentation is the most promising method for gossypol detoxification compared with the adverse effects of other methods, because it not only has high detoxification efficiency but can also enhance the nutritive value of cottonseed powder (Weng and Sun 2006a, 2006b).

Owing to the existence of rumen microorganisms, ruminants are believed to be more tolerant of gossypol compared with monogastric animals (Reiser and Fu 1962). Chen et al. (2015) and Zhang et al. (2018) isolated *Bacillus* strains from the rumen with a high activity of gossypol degradation. Additionally, gossypol-degrading strains of bacteria and fungi isolated from cotton-planted soil,

* Corresponding author.
E-mail address: yang_hongjian@sina.com (H.-J. Yang).
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such as *Geotrichum candidum*, *Candida tropicalis*, *Torulopsis candida* and *Aspergillus* sp., applied in solid-state fermentation, have also been found to exhibit different degrading potentials to the free form of gossypol (FG) in cottonseed by-products (Zhang et al., 2007; Khalaf and Meleigy 2008; Sun et al., 2008; Lim and Lee 2011; Yang et al. 2011, 2012). However, these microbial detoxification studies were primarily based on the disappearance of FG, but to date the exact mechanism by which gossypol detoxification takes place in these microorganisms is not clear. In contrast with the rumen and other microorganisms, the mechanism of gossypol detoxification in *Helicoverpa armigera* larvae (Lepidoptera: Noctuidae) has been well studied and described. This is due to the extensive damage that *H. armigera* causes to cotton plants, which has attracted much attention in the cotton fibre industry (Krempl et al., 2016a). The objective of the present review is to determine whether any scientific insights can be gained by comparing what is known of gossypol degradation in the rumen and by *H. armigera* larvae, to better understand potential mechanisms of gossypol detoxification in ruminant animals.

2. Gossypol in cottonseed by-products and its toxicity to ruminants

2.1. The presence of gossypol in cottonseed by-products

Gossypol is a yellow pigment compound, a polyphenolic binaphthyl dialdehyde (Fig. 1), found in cotton stems, leaves and flower buds, and it is especially rich in cottonseed. Gao et al. (2011) noted that gossypol content was positively correlated with crude fat content in different transgenic cottonseeds in China.

There are 2 forms of gossypol present in cottonseed by-products: the free form is gossypol having active hydroxyl and aldehyde groups. The bound form (BG) is gossypol bound to proteins, amino acids or other substances (Alexander et al., 2008). Phenolic and carbonyl groups of gossypol can covalently bind to free epsilon-amino groups from lysine and arginine through the browning or Maillard reaction (Bressani et al., 1964), and its dimeric structure facilitates cross-linking of proteins (Abou-Donia, 1976). Additionally, gossypol can chelate metal ions, presenting both pro-oxidant and antioxidant characteristics (De and Wang 1993).

Generally, the FG content in cottonseed ranges from 0.02% to 6.64% (Price et al., 1993), varying between different cotton varieties (Randel et al., 1992). In the seeds, almost all the gossypol is found in the free form. Heat and moisture processing can convert the free form into the less toxic, bound form (Alexander et al., 2008). Most of the BG cannot be absorbed in the digestive tract and is thus generally considered nontoxic to ruminants. However, some BG in cottonseed products may be released in the digestive tract as toxic FG, and the latter can then pass through the mucosa into the blood circulation of host animals (Noftsger et al., 2000). Additionally, the gossypol compound has 2 distinct stereoisomer forms, (−) and (+) gossypol; the (−) isomer presents greater toxicity to vertebrates than the (+) isomer because it is more slowly eliminated, with a longer residue time in body tissue (Noftsger et al., 2000; Rogers et al., 2002).

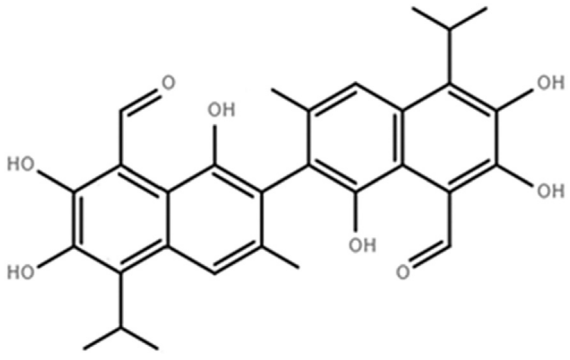

Fig. 1. The structure of free gossypol ($C_{30}H_{30}O_8$).

2.2. The toxicity of gossypol to ruminant animals

The main clinical signs of gossypol poisoning are weakness, apathy, impaired body weight gain, respiratory distress, and even death within a short period in various ruminant animals (Risco et al., 1992; Zelski et al., 1995; Alexander et al., 2008). A gossypol–iron complex formed by the active groups of gossypol can inhibit the absorption of iron, and iron deficiency may adversely affect erythropoiesis and cause anaemia in dairy cows after ingestion of gossypol for an extended time (Mena et al., 2004; Câmara et al., 2016). Gossypol can also enhance the activity of cytosolic Ca^{2+} which can initiate cell membrane contraction, and stimulate the eryptosis of erythrocytes (Zbidah et al., 2012).

Although dietary gossypol had been found to have no direct harmful effect on postpartum oestrus nor on the artificial insemination of cows, long-term intake of high gossypol diets does decrease the conception rate, and has been associated with increased incidence of abortions in dairy cows (Santos et al., 2003), as well as decreased quantity of viable ovarian follicles released in ruminants (Câmara et al., 2015). The interference of gossypol in male reproduction is considerably better understood than in the female. Dietary intake of gossypol was reported to have a detrimental effect on the cauda epididymal sperm through its damage to cellular membranes, and caused degeneration of seminiferous tubules in the parenchyma of the testicles in bulls (Chenoweth et al., 2000; Hassan et al., 2004; Yuan and Shi, 2005).

High gossypol diets also decreased milk production and milk protein and increased the somatic cell score in lactating cows (Higginbotham et al., 2004). Fortunately, gossypol residue in milk was far below the maximum residue limits of FG specified by the Food and Agriculture Organization and the US Food and Drug Administration, which are set at 600 and 450 mg/kg, respectively (Wang and Plhak 2004; Zhong 2007; Wang et al., 2012).

Toxicity often occurs to ruminants when the dietary intake level of FG exceeds the detoxifying capacity of rumen microbes, or when it is overfed to young ruminants with a functionally underdeveloped rumen (Randel et al., 1992). The European Union has stipulated that the use of FG should be less than 500 mg/kg in adult ruminant diets and 100 mg/kg in calves or lambs (Knutsen et al., 2017).

3. The detoxification of gossypol in the rumen: current knowledge based on ruminant studies

The high tolerance of gossypol by ruminants has been attributed to its detoxification in the rumen, which involves its binding to soluble proteins and degradation by rumen microbes (Fig. 2) (Reiser and Fu 1962; Wang 1995). As most differences in metabolism between ruminant and non-ruminant animals can be traced to the activity of rumen microorganisms, most studies on the detoxification mechanisms of gossypol in ruminant animals have focused mainly on the determination and explanation of the transformation of FG into BG. For instance, Smith (1957) speculated that ruminants may detoxify gossypol in the rumen by binding it to soluble proteins or by dilution and slowed absorption. Later, Reiser and Fu (1962), in a series of experiments, concluded convincingly that the mechanism of ruminant detoxification of

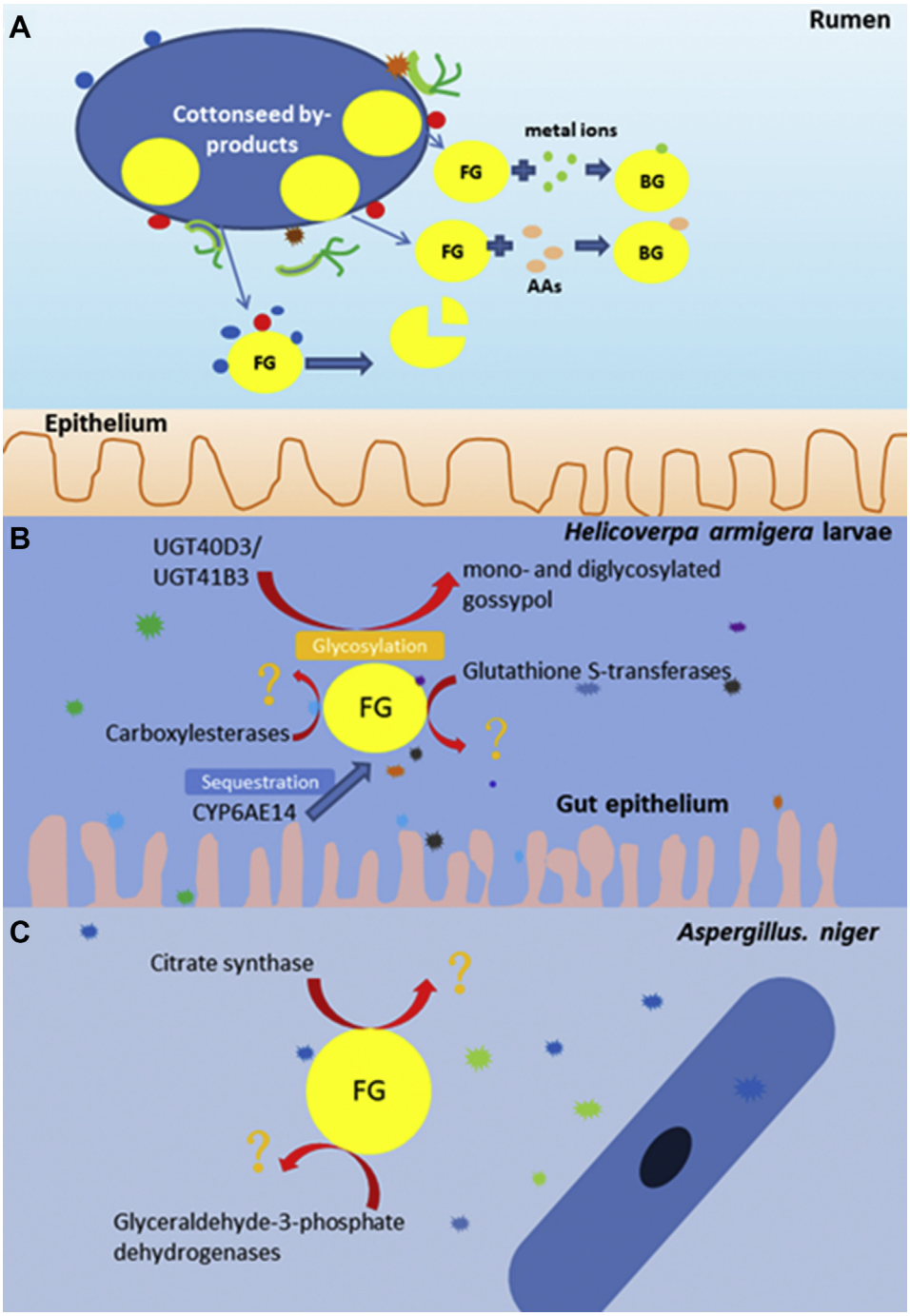

Fig. 2. Current knowledge of gossypol detoxification mechanism. (A) Rumen; (B) *Helicoverpa armigera* larvae; (C) *Aspergillus niger*. AA = amino acids; BG = bound gossypol; FG = free gossypol.

gossypol was by binding to soluble proteins and that the bond was permanent during protein digestion. A few reports directly considered microbial detoxification of FG in cottonseed by-products. For instance, it was reported that 95.2% of the gossypol in cottonseed was degraded by rumen microbes during fermentation in vitro for 24 h, but that the degradation rate of FG decreased significantly after the addition of antibiotics. This demonstrated the degradability of gossypol by rumen microbes (Wang 1995). Chen et al. (2015) isolated a *Bacillus* strain from the rumen with high activity of gossypol degradation, and the liquid state fermentative gossypol degradation rate reached 94% after the *Bacillus* strain was applied, leading to the disappearance of up to 80% of FG in the fermented cottonseed meal. Zhang et al. (2018) isolated a bacterial strain from rumen fluid that used gossypol as its sole carbon source, and the strain was then identified by 16S rDNA sequencing to be 98% homologous to the sequence of *Bacillus subtilis* strain GH38. According to the results of Zhang et al. (2018), in optimum fermentation conditions, the FG and total gossypol (TG) content in fermented cottonseed meal decreased 78% and 49%, respectively, relative to the control. The FG and TG content in fermented cottonseed meal was significantly lower than in the unfermentated cottonseed meal, demonstrating not only that FG

could be converted into BG by being bound to proteins, lipids and nucleic acids, but also that FG can be degraded by rumen microorganisms efficiently. In addition, an in vitro study showed that gossypol was degraded rapidly by rumen microbes and this degradation was not enantioselective. At 6 h, 67.4% and 85.7% of (−)-gossypol were degraded for the 500 and 1000 μg/g ramic gossypol added groups, respectively, which increased to 83.6% and 92.5% disappearance, respectively, at 12 h. From 12 to 48 h, the degradation rates varied slightly. These results demonstrated the strong degradation of gossypol by rumen microbes which partly explains the high tolerance of gossypol among ruminants (Tang et al., 2018). However, it is not clear exactly what the microbial detoxification activities were for gossypol in this instance.

Przybylski et al. (2009) found that when the content of gossypol reached 12.5, 25, 50 or 100 μg/mL, there was decreased growth of *B. cereus* ATCC 11778, *S. aureus* ATCC 25923, *S. aureus* NCTC 4163 and *M. luteus* ATCC 9341, respectively, and it was suggested that heterocyclic derivatives of gossypol in particular, should be considered as candidates for new and effective antibacterial agents based on gossypol substrate. Although this study demonstrated the antimicrobial activity of gossypol, this minimal inhibitory concentration was higher than the maximum gossypol concentration allowed in the diet of adult ruminants, and the result showed that gossypol only has an inhibitory effect on the tested microbes, not necessarily on all microbes. Additionally, Schneider et al. (2002) found that TG concentration did not change during in vitro fermentation, thereby confirming the results of Reiser and Fu (1962). They speculated that FG must be complexed by rumen components during fermentation, reducing their ability to reach the bloodstream, and that the complexes must be broken down during their derivatization with D-alaninol. The rumen environment is much more complex than an in vitro fermentation system, and many rumen microorganisms cannot be cultured in vitro. Although there is no evidence of mechanisms by which rumen microbes participate in gossypol detoxification, many researchers have isolated gossypol degradation microbes from the rumen, which use gossypol as their only carbon source (Chen et al., 2015; Zhang et al., 2018). Thus, further study of the potential detoxification mechanisms of gossypol by rumen microbes is necessary.

4. Several enzymes associated with gossypol detoxification

Generally, the metabolic detoxification of a toxin goes through three stages as follows (Krempl et al., 2016a). First, the activity and hydrophilicity of toxic molecules are enhanced by introducing or releasing of functional groups with the direct action of cytochrome P450 monooxygenases or carboxylesterases (Janocha et al., 2015). Second, the water-solubility of toxins are promoted by the action of glutathione S-transferases (GSTs), UDP-glycosyltransferases (Robertson et al., 1999; Chrysostomou et al., 2015), which could prevent toxins from penetrating the cell membrane. Third, enzymes, such as ATP-binding cassette transporters, enable the excretion of toxins by facilitating the transfer activity of toxins across the membranes (Rowland et al., 2013).

4.1. Advances in understanding of gossypol detoxification mechanisms by microbes

Yang et al. (2011) noted that gossypol was detoxified by *Aspergillus niger* through its protease or other protein products (Fig. 2). Using 2-dimensional electrophoresis, they identified 51 differentially expressed proteins secreted by *A. niger* between 2 carbon sources, that could be involved in gossypol degradation. Of these, there were 13 small molecular proteins whose weights (less than 18.4 kDa) were considered to play key roles in the biodegradation of gossypol. According to further analysis by MALDI-TOF MS, proteins identified as kinesin family protein, citrate synthase and glyceraldehyde-3-phosphate dehydrogenases were higher expressed in the carbon source of gossypol, and these proteins were considered to be involved in energy metabolism.

Gossypol is a polyphenolic hydroxyl binaphthalene compound, so the metabolic pathway of naphthalene is an essential process of the biodegradation of gossypol. The degradation process of naphthalene needs a greater consumption of energy because of its aromatic ring, which would explain the higher expression of these energy-related protein enzymes in gossypol, and these results of Yang et al. (2011) demonstrated the essential role of energy metabolism in gossypol degradation. Additionally, the functions of 15 other unnamed proteins were identified by extrapolating, e.g. laccase is the one of the most prominent oxidases of polyphenols, and may be involved in the biodegradation of gossypol. Further study of the function of these hypothetical protein enzymes is required to better understand the biodegradation mechanisms of gossypol in the rumen.

In addition to the research noted above, some other microorganisms isolated from rumen (i.e. *Bacillus subtilis*) (Chen et al. 2015; Zhang et al., 2018) and cotton planted soil (i.e. *Candida utilis*, *Baclicus Lincheniformis*, *Lactobacillus plantarum*) (Hou et al., 2016) have been shown to be capable of gossypol degradation based on gossypol disappearance. However, the corresponding mechanisms by which this occurs, are still unclear.

4.2. Scientific insights into gossypol detoxification mechanisms of *H. armigera* larvae

The generalist moth *H. armigera* is an important pest species of cotton and causes considerable damage to plant tissue in many parts of the world. With the rapid development of modern molecular biotechnology and -omics technology strategies, gossypol detoxification by *H. armigera* and *Heliothis virescens* larvae has been well studied. Researchers have found some key enzymes which may be involved in the metabolism and transformation of FG as shown Fig. 2 (Mao et al. 2007, 2011; Celorio-Mancera et al., 2011; Krempl et al., 2016a), thus indicating some potential scientific insights into microbial gossypol detoxification in ruminant animals.

A study was conducted where a gossypol-containing diet was fed to *H. armigera* and *H. virescens* larvae, with the purpose to study the metabolic transformation mechanisms of gossypol (Krempl et al., 2016a). Using a microarray method, several mono- and diglycosylated gossypol isomers were found in the faeces of both larvae and confirmed that UGT41B3 and UGT40D1 as UDP-glycosyltransferases, were capable of glycosylating gossypol. In addition, other researchers found that an increase of gossypol intake upregulated the gene expression level of some UDP-glycosyltransferases, cytochrome P450s, carboxylesterases and a few GSTs in the gut of *H. armigera* larvae (Celorio-Mancera et al., 2011). *H. armigera* larvae fed on transgenic dsCYP6AE14 plants showed reduced growth on a gossypol-containing diet and a suppressed CYP6AE14 expression (Mao et al. 2007, 2011). Krempl et al. (2016b) in a subsequent in vitro study, demonstrated that CYP6AE14 could play an important role in the reduction of the general stress response of *H. armigera* larvae toward plant toxins by sequestrating gossypol within the gut wall.

Glycosylation of toxins is an important detoxification process, in which a lipophilic aglycone is converted into a more hydrophilic and readily excretable compound. The basic mechanism is a second order nucleophilic substitution catalyzed by UDP-glycosyltransferases (Radominska-Pandya et al., 2010). As one molecule of gossypol possesses 6 hydroxyl groups, there are several positions possible for the binding of the hexose moiety, and

a total of 9 isomeric diglycosides is theoretically possible. In addition to the direct effects of glycosylation on gossypol, such as reduced reactivity and enhanced excretion, another important effect may be a sterical hindrance of the reactive aldehyde groups, thus preventing the formation of Schiff bases with proteins. Cytochrome P450s belong to a kind of mixed-function oxidase system. One function of this enzyme is to catalyze the synthesis of active substances in the body, such as hormones and enzymes (Kramlinger et al., 2015); another function is to catalyze the metabolism of exogenous substances, such as plant toxins (Mizutani 2012). Additionally, it is also the most important drug-metabolizing enzyme in animals (Kulcsár et al., 2017). In order to metabolize gossypol to gossic acid, several oxidation steps are required (Abou-Donia, 1976), for which the cytochrome P450s are candidates. GSTs play an important role in detoxification through binding and sequestering a variety of toxic compounds and peptides, and also exhibit antioxidant activities, thanks to their selenium-independent glutathione peroxidase activities (Hamed et al., 2014). They are involved in the detoxification of xenobiotic compounds bearing sufficient active electrophilic centres by the addition of nucleophilic sulphhydryl groups (thiols) of the reduced glutathione (Jakoby 1978). Carboxylesterase belongs to the serine hydrolase family, which can effectively catalyze the hydrolysis of endogenous and exogenous substances with ester bonds, amide bonds and thiol bonds. Given that gossypol is a fat soluble substance (Zhang et al., 2015), we speculate that carboxylesterase may participate in the release of gossypol from lipids and indirectly promote the detoxification of gossypol.

The results obtained in the above studies have implied that these key enzymes play an important role in gossypol detoxification, although the exact action mode remains unclear. Unlike the larvae, most rumen microorganisms are obligate anaerobes, but with the rapid development of gene sequencing technology, a sequence-based screening approach was successfully applied to identify genes from a rumen sample, which confirmed the existence of these key enzymes in the rumen. A total of 373 contigs encoding glycosyltransferase were identified from a buffalo rumen metagenome (Patel et al., 2014). Li et al. (2015) reported the gene expression level of glutathione in the ruminal wall by a ruminal transcriptomic analysis method. In addition, some carboxylesterase genes have been cloned and characterized from metagenomic libraries of cow rumina, based on solid-attached bacteria and liquid-associated bacteria in the rumen, and the protein-coding sequences of these carboxylesterases were reported to be more closely related to *Butyrivibrio fibrisolvens*, *Ruminococcus* sp., *Bacteroides* sp. and *Prevotella* sp. in the rumen (Liu et al., 2009; Islam et al., 2010; Privé et al., 2015). The above studies confirmed the existence of these key enzymes in rumen microbes. However, no direct confirmation exists to date that pure or mixed cultures of the rumen microbes which are capable of gossypol detoxification exhibit activity of these enzymes, and it is also not clear how these enzymes in rumen microbes are involved in the metabolic transformation of gossypol.

5. Recommendations for future work

Previous studies of gossypol detoxification in *H. armigera* larvae have achieved great advances in terms of elucidating the roles of UDP-glycosyltransferases, cytochrome P450s, GSTs and carboxylesterases. However, most of the work on these detoxification enzyme classes is based on transcriptional data, suggesting upregulation/induction in the organism after toxin ingestion. Upregulation may suggest that these enzymes are somehow involved in gossypol detoxification. Genes discovered through this transcriptional approach may be additional agents in gossypol detoxification and candidates for coping with gossypol-induced stress. With the rapid development of modern molecular biotechnology and -omics technology strategies, evidence has accumulated that the advances in understanding the mechanism of gossypol detoxification in *H. armigera* larvae could provide scientific insights that allow further study of the mechanism of gossypol detoxification in ruminant animals. The rumen is a complex fermentation system, and all rumen microbes play an important role in nutrient digestion and toxin removal. On the one hand, gossypol may be degraded by enzymes secreted by microbes directly; on the other hand, rumen microorganisms may catalyze the reaction of gossypol with other metabolites, thereby achieving detoxification. In future studies, it is essential to further investigate the specific detoxification mode of rumen microbes and the role of these enzymes in gossypol detoxification. Before starting such a study, numerous studies using pure cultures of the relevant rumen microorganism should first be conducted, and then combined with modern -omics technology to clarify the specific roles of these key enzymes which have been related to gossypol detoxification and transformation. Systematic elucidation of the microbial gossypol detoxification mechanism will have scientific and practical significance for the extensive utilization of cottonseed by-products in ruminant animals, and also in monogastric animals, and could contribute to reducing the treatment costs, and improving the nutritional value of cottonseed feed in the future.

Author contributions

Wei-kang Wang: Conceptualization, Methodology, Investigation, Writing-Original Draft, Visualization. **Hong-jian Yang:** Writing-Review & Editing, Project administration. **Yan-lu Wang:** Resources, Supervision. **Kai-Lun Yang:** Funding acquisition. **Lin-Shu Jiang:** Funding acquisition. **Sheng-Li Li:** Funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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