

## SHORT COMMUNICATION

# Assessment of the vasoactive effects of the (S)-epimers of ergot alkaloids in vitro

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## Abstract

Ergot alkaloids are produced by the fungus *Claviceps purpurea* and their levels are carefully monitored in animal and human diets due to their harmful effects and widespread contamination of cereal crops. Ergot alkaloids exist in two forms known as the (R)- and (S)-epimers with only the former being monitored in diets in North America. The (S)-epimers of ergot alkaloids are thought to be biologically inactive and, therefore, harmless. A major mechanism by which the (R)-epimers of ergot alkaloids produce their toxic effect is through vasoconstriction. Therefore, the objective of this study was to examine the vasoactivity potential (contractile response) of four (S)-epimers, namely ergocryptinine, ergocristinine, ergocorninine, and ergotaminine utilizing an in vitro arterial tissue bath system. Bovine metatarsal arteries ( $n = 6$ , ergocryptinine and ergocorninine;  $n = 6$ , ergocristinine and ergotaminine;  $n = 6$  arteries/(S)-epimer, total  $n = 12$ ) were collected from healthy mixed-breed beef steers immediately after slaughter, cut into 3-mm arterial cross sections, and suspended in a tissue bath with continuously oxygenated Krebs–Henseleit buffer. To assess the contractile response of each (S)-epimer, a cumulative contractile dose–response curve was constructed by incubating arteries with increasing concentrations ( $1 \times 10^{-11}$  to  $1 \times 10^{-6}$  M) of that (S)-epimer. Contractile responses were recorded as grams of tension and were normalized to an initial contraction of phenylephrine. Contrary to the widespread belief, all tested (S)-epimers were found vasoactive and produced a concentration-dependent arterial contractile response similar to what has been reported for the (R)-epimers. The arterial contractile response to ergotaminine was strongest and was significantly greater than that of ergocryptinine and ergocristinine at the highest concentration used ( $P \leq 0.01$ ). Our results indicate that the (S)-epimers are biologically active and are likely harmful similar to the (R)-epimers. The levels of (S)-epimers should be carefully monitored in human and animal diets worldwide.

**Key words:** bovine, epimers, ergot alkaloids, metatarsal artery, vasoconstriction

## Introduction

The fungus *Claviceps purpurea* produces ergot alkaloids that invade cereal crops globally. In livestock feed, it is well established

that the presence of high concentrations of ergot alkaloids are associated with clinical manifestations, including gangrene, abortion, stillbirth, decreased birth weight, decreased immunity,

### Abbreviations

EC50	effective concentration giving half-maximal response
GEE	generalized estimating equations
PE	phenylephrine

and decreased fertility in multiple animal species (Klotz, 2015a). These effects are mainly related to the vasoconstrictive effects of ergot alkaloids, through structural similarity to biological amines binding to their receptor, on multiple arterial and venous vascular beds impacting the blood supply to multiple organs.

Over 90 ergot alkaloids have been isolated, which are separated into two main categories, including amide derivatives of lysergic acid and small peptides (ergopeptines), based on chemical structure (Liu and Jia, 2017). Small peptide ergot alkaloids exist in two conformational forms known as the (R)-epimers and the (S)-epimers. The two forms are in equilibrium with the transformation of (R)-epimers to their respective (S)-epimers being complex and dependent on several factors, including temperature, pH, and solvent for experimental use. The transformation passes through an intermediate conformation. The (R)-epimers contain the suffix “ine” whereas the (S)-epimers contain the suffix “inine” (Krska and Crews, 2008).

Both forms have been shown to have different chemical, physical, and biological properties (Smith and Shappell, 2002). It is currently thought, under normal physiological conditions, that the (R)-epimers exert biological effects, whereas the (S)-epimers are inactive (Smith and Shappell, 2002; Klotz, 2015b; Guo et al., 2016; Kudupoje et al., 2018). Original reports from the 1970s to 1980s are often cited in current publications stating inactiveness (Stadler and Stürmer, 1970; Pierrri et al., 1982).

The bioactivity, in terms of vasoactivity, of (R)-epimers is commonly assessed using an in vitro tissue bath system where the contractile response of dissected arteries is monitored after incubation with increasing concentrations of a particular (R)-epimer (Klotz et al., 2010; Foote et al., 2011; Klotz and McDowell, 2017). Specifically, cattle arteries (Oliver et al., 1993; Klotz et al., 2010; Foote et al., 2011), such as the dorsal metatarsal artery (Oliver et al., 1993; Yonipam, 2018), have been utilized. It is hypothesized, based on a similar chemical structure, that the (S)-epimers of ergot alkaloids will cause arterial contraction similar to (R)-epimers. Therefore, the objective of this study was to examine the vasoactivity potential (contractile response) of bovine metatarsal arteries after incubation with one of four (S)-epimers, including ergocryptinine, ergocristinine, ergocorninine, and ergotaminine using an in vitro tissue bath system.

## Materials and Methods

No ethics approval was required. The primary investigator (A.N.A) was required to submit a form to the University Ethics Committee indicating only the number of animals to be slaughtered. Animal slaughter was performed according to slaughterhouse guidelines by slaughterhouse employees.

### Animals and arteries

Dorsal metatarsal arteries were collected from healthy mixed-breed beef steers ( $n = 12$ ) aged 15 to 20 mo immediately after the slaughter at a local abattoir. One foot limb, with the hide removed, was given to the researcher located in a separated space within the abattoir approximately 7 min after animal was euthanized (one foot limb collected per day). From each foot limb, muscle, nerves, and tissues were removed to access a

15-cm segment of the dorsal metatarsal artery that was located directly next to the bone. Once the segment was carefully removed using surgical scissors and a scalpel, it was placed in a small container with a lid containing modified Krebs–Henseleit oxygenated buffer, (95% oxygen/5% carbon dioxide mixture; pH = 7.4; composition in g/2 liters deionized water: 13.68 NaCl, 0.7 KCl, 0.28 MgSO<sub>4</sub>, 0.32 KH<sub>2</sub>PO<sub>4</sub>, 3.6 NaHCO<sub>3</sub>, 1.8 glucose, and CaCl<sub>2</sub>, made in house) and on ice for transport to the laboratory (Klotz and McDowell, 2017). Upon arrival to the laboratory, excess fat and connective tissue were removed from each artery segment (Klotz and Barnes, 2014), which were sliced into 3-mm arterial cross sections (Klotz and McDowell, 2017). Four arterial cross sections were suspended horizontally in a four-chamber (one arterial cross section per chamber) tissue bath (Radnoti [159906], Monrovia, CA, USA) containing 15 mL of continuously oxygenated (95% oxygen/5% carbon dioxide) modified Krebs–Henseleit buffer, same as above, at 37 °C. Arterial cross sections were then allowed to equilibrate for 1 h with a resting tension of 2 g, with buffer solution replacement every 15 min (Foote et al., 2011; Yonipam, 2018). Arterial cross sections were then exposed to an alpha-adrenergic agonist, phenylephrine (PE) ( $1 \times 10^{-4}$  M, Fisher Scientific, Fair Lawn, NJ), to verify viability and to provide contraction information to normalize experimental contractions. Arterial cross sections were then washed every 15 min with incubation buffer to restore resting tension with 4 to 6 replacements made.

### Ergocristinine, ergocorninine, ergocryptinine, and ergotaminine cumulative concentration–response

For each experimental run, two of the four arterial cross sections, from a 15-cm artery segment, would receive one (S)-epimer and the other two would receive a different (S)-epimer. Values for the contraction of each artery segment were based on the average of duplicated arterial cross sections (two (S)-epimers and two arterial cross sections/(S)-epimer). Twelve ( $n = 12$ ) arteries (artery segments) in total were used with six ( $n = 6$ ) exposed to ergocryptinine and ergocorninine, and the other six ( $n = 6$ ) exposed to ergocristinine and ergotaminine ( $n = 6$  arteries/(S)-epimer). After resting tension was restored, (S)-epimers were added separately to each chamber in the tissue bath from lowest concentration ( $1 \times 10^{-11}$  M) to highest ( $1 \times 10^{-6}$  M) for an incubation period of 29 min, with one buffer replacement, followed by a 2.5-min washout period and 1 min recovery before the next (S)-epimer addition (Klotz and McDowell, 2017, with slight modification). Preliminary investigation indicated that the arteries do not relax after the (S)-epimers were removed from the buffer within the tissue bath. The arteries continued to increase in contractility several minutes after the (S)-epimers were washed out. This is consistent with a previous study with (R)-epimers (Pesqueira et al., 2014). In comparison, when PE was washed out from the buffer solution, the contraction abated almost immediately. A maximal response was not achieved in a 9- or 14-min incubation period, which had been used in previous validated assays (Klotz et al., 2006; Klotz and McDowell, 2017); therefore, a longer incubation period for each (S)-epimer was employed to ensure contraction was not overlooked. After the last concentration, PE ( $1 \times 10^{-4}$  M) was added again to ensure viability of all arterial cross sections. Arterial cross sections exposed to PE at the conclusion of an experimental run had similar contractile responses to the first PE additions. Stock solutions of ergocristinine (Romer Labs, Vancouver, BC), ergocorninine (Romer Labs, Vancouver, BC), ergotaminine (U.S. Pharmacopeia, Rockville, MD) were prepared in 100% methanol (Fisher Scientific,

Fair Lawn, NJ), and ergocorninine prepared in 100% ethyl acetate (Fisher Scientific, Fair Lawn, NJ) (Klotz et al., 2010) within the manufacturer amber vials, all stored at  $-20^{\circ}\text{C}$  (Smith and Shappell, 2002; Hafner et al., 2008). From the stock solutions, dilutions were made to obtain the desired concentrations, the day of the experimental run, while arterial cross sections were equilibrating. Each concentration of the working standards was made within a 1.5-mL microcentrifuge tube with a snap cap (Labcon North America, Petaluma, CA) and stored in dark and on ice within a coved styrofoam container until use. The volume of solvent used for each concentration of (S)-epimer did not exceed 0.5% of the 15 mL buffer within the tissue bath.

### Data collection and statistical analysis

Contractile responses of each arterial cross section exposed to PE or tested (S)-epimers were recorded as grams of tension using Biopac Student Lab PRO (professional version 3.7.1, Goleta, CA), which was connected to the four-chamber tissue bath. At the end of the 29-min incubation period of each (S)-epimer, grams of tension were recorded and corrected to the baseline tension. The baseline tension was calculated from average tension prior to the demonstration of contraction (increased tension) to ensure that the natural resting baseline tension of each arterial cross section was not missed. These values were then normalized as a percent of the first addition of PE ( $1 \times 10^{-4}\text{ M}$ ) to compensate for the variability of animals as well as artery size. These data were used as a source for data analysis. All data were reported as mean  $\pm$  SD, with contractile response expressed as a percent of PE contraction for each (S)-epimer. Data were plotted with nonlinear regression using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA).

All analyses were performed using a commercial software package (IBM SPSS Statistics for Windows, version 23, IBM Corp., Armonk, NY). Statistical tests evaluated if mean % contractile response of PE for bovine arterial segments was affected by the two variables, epimer ((S)-epimers of four alkaloids) and concentration, when considered together. The (S)-epimers were ergotaminine, ergocristinine, ergocorninine, and ergocryptinine;  $n = 6$ , ergocryptinine and ergocorninine;  $n = 6$ , ergocristinine and ergotaminine ( $n = 6$  arteries/(S)-epimer, total of  $n = 12$  arteries). Data were included for each concentration where a contraction was observed ( $1 \times 10^{-7}$  and  $1 \times 10^{-6}\text{ M}$ ). Statistical analysis was undertaken using generalized estimating equations (GEE) with an identity link function, robust errors, and an unstructured correlation matrix to account for repeated measures (segments of the same artery measured at different concentrations and for different (S)-epimers). GEE is able to correctly analyze results from an unbalanced design with incomplete repeated measures (each steer artery did not receive all (S)-epimers). Differences were considered to be significant at  $P < 0.05$ . In the presence of a significant interaction effect between (S)-epimer type and concentration on the percent contractility, GEE analysis was performed separately at each individual concentration to test whether a difference in effect existed between (S)-epimers. This was followed by multiple pairwise comparisons with a sequential Sidak correction. To test if arterial contraction from each (S)-epimer/concentration combination was different from zero, an individual one-sample t-test was utilized.

### Results

All four (S)-epimers caused a measurable contraction of bovine dorsal metatarsal arteries at a concentration of  $1 \times 10^{-7}\text{ M}$ . The arterial contractile response increased at the next highest concentration of  $1 \times 10^{-6}\text{ M}$  (Figure 1). Of the four (S)-epimers, at

the highest concentration, ergotaminine generated the strongest contraction of followed by ergocorninine, ergocristinine, and ergocryptinine. This study demonstrated a statistically significant interaction effect between (S)-epimer type and concentration on the percent contractility ( $P < 0.001$ ). Due to a significant interaction, GEE analysis was performed separately at each individual concentration to test whether a difference in effect existed between (S)-epimers ( $P < 0.001$  for each concentration). Specifically, at  $1 \times 10^{-7}\text{ M}$ , ergocryptinine resulted in a lower percentile contraction to ergotaminine ( $P = 0.011$ ). At  $1 \times 10^{-6}\text{ M}$ , ergocryptinine and ergocristinine each resulted in a significantly lower percentile contraction than ergotaminine ( $P = 0.011$  and  $P = 0.001$ , respectively) (Table 1). The arterial contraction from each (S)-epimer/concentration combination was different from zero ( $P < 0.05$ ), with the exception of both ergocryptinine and ergocorninine at  $1 \times 10^{-7}\text{ M}$ . The addition of PE at the conclusion of the experiment resulted in a large increase in contraction similar to the first PE addition.

### Discussion

The bovine metatarsal artery was selected due to its peripheral location within the limb and potential for clinical disease (lameness). The presence of a contractile response of bovine metatarsal arteries exposed to increasing concentrations of (S)-epimers, namely ergotaminine, ergocorninine, ergocristinine, and ergocryptinine, illustrates vasoactivity and the potential for vasoconstriction leading to toxic effects. The findings of the present study demonstrate the biological activity of (S)-epimers, which have been previously stated as inactive (Smith and Shappell, 2002; Klotz, 2015b; Guo et al., 2016; Kudupoe et al., 2018). Evidence from previous studies have implied that the (S)-epimers may be toxic due to accumulation within cell lysate (Mulac and Humpf, 2011; Mulac et al., 2013), although stating that more research need to be completed, especially on single epimers. It has been reported that (S)-epimers negatively affected the blood-brain barrier (Mulac et al., 2012), which also supports (S)-epimers having biological activity, due to not only

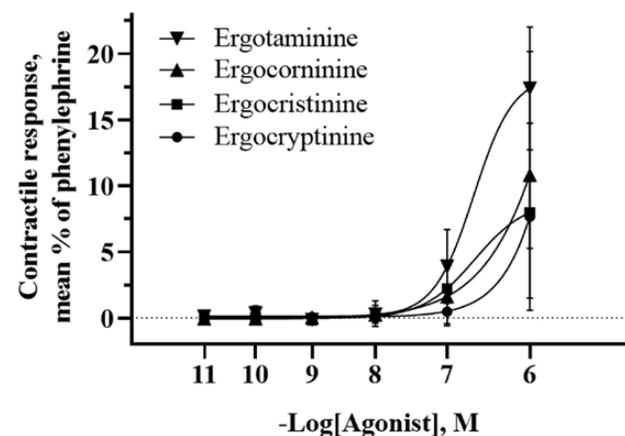


Figure 1. Contraction of bovine arteries from (S)-epimers. Mean contractile response, % of first addition of PE, of bovine metatarsal arteries to increasing concentrations of (S)-epimers, namely ergocryptinine, ergocristinine, ergocorninine, and ergotaminine ( $n = 6$ , ergocryptinine and ergocorninine;  $n = 6$ , ergocristinine and ergotaminine;  $n = 6$  arteries/(S)-epimer, total  $n = 12$ ). Error bars are SD.

**Table 1.** Comparison of mean arterial contractile responses to increasing concentration of four (S)-epimers of ergot alkaloids, namely ergotaminine, ergocorninine, ergocristinine, and ergocryptinine after normalization to PE at two concentrations where contraction was observed

	Concentration ( $1 \times 10^x$ ), M	Contraction, g as % PE contraction,		
		Mean $\pm$ SD	Min. <sup>1</sup>	Max. <sup>2</sup>
Ergotaminine	-7	3.90 $\pm$ 2.80 <sup>a</sup>	1.04	8.78
	-6	17.39 $\pm$ 4.64 <sup>c</sup>	10.06	22.44
Ergocorninine	-7	1.64 $\pm$ 2.21 <sup>a,b</sup>	-0.55	5.57
	-6	10.85 $\pm$ 9.35 <sup>c,d</sup>	0.00	27.87
Ergocristinine	-7	2.24 $\pm$ 1.94 <sup>a,b</sup>	0.44	5.63
	-6	7.98 $\pm$ 2.71 <sup>d</sup>	4.01	12.13
Ergocryptinine	-7	0.49 $\pm$ 0.91 <sup>b</sup>	-0.02	2.06
	-6	7.69 $\pm$ 7.10 <sup>d</sup>	-1.71	18.62

<sup>1</sup>Minimum arterial contractile response, % contractile response of PE.

<sup>2</sup>Maximum arterial contractile response, % contractile response of PE.

<sup>a-d</sup>(S)-epimers demonstrating significant differences between contraction of bovine arteries are designated by different letters at  $10^{-7}$  M (a,b) and  $10^{-6}$  M (c,d) (n = 6 arteries/(S)-epimer, total n = 12, GEE, multiple pairwise comparison with Sidak correction, P < 0.05).

accumulation but also perhaps receptor activation as seen in the present study.

Few studies examined the contractile response of (R)-epimers in bovine metatarsal arteries to provide published comparators for the present study. In a previous study examining the contractile response of (R)-epimers using a bovine ruminal artery, the contractile response of ergotamine was first observed at  $10^{-6}$  M, while ergocornine and ergocryptine did not cause contraction until the  $10^{-5}$  M addition and only the highest dose of ergocristine ( $10^{-4}$  M) elicited a contractile response (Foote et al., 2011). Two other in vitro studies reported an arterial contractile response for lysergamide and ergovaline using bovine metatarsal and uterine arteries, respectively. Lysergamide first elicited a contractile response at  $10^{-7}$  M, while ergovaline resulted in slow but sustained contractions over 2 h suggesting a high affinity to the receptors (Dyer, 1993; Oliver et al., 1993). Comparing results in the present study to previous investigations indicate that the (S)-epimers may be more potent than their corresponding (R)-epimers. The contractile response seen in the present study at the two concentrations,  $10^{-7}$  and  $10^{-6}$  M, was visually similar to graphed results provided for similar-sized arteries exposed to (R)-epimers (Klotz et al., 2010; Klotz and McDowell, 2017). The difference/similarity, however, could be related to the use of various arterial segments and experimental differences in the studies mentioned. It is anticipated that incubation with higher concentrations of the (S)-epimers would generate similar higher comparable contractile responses as demonstrated in other studies using the corresponding (R)-epimers.

It is important to note that the current study was conducted using only the lower end of the concentration range than what is commonly used for the (R)-epimers studies ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M; Klotz and McDowell, 2017). The (S)-epimers are available commercially from few sources and only in very limited quantities (0.125 mg/vial). To achieve concentrations exceeding  $1 \times 10^{-6}$  M of an (S)-epimer within a single tissue bath was practically unrealistic. Also, a measurement of potency such as the effective concentration giving half-maximal response (EC50) is commonly used in in vitro arterial tissue bath studies (Oliver et al., 1993; Klotz et al., 2010). However, it was not appropriate to include EC50 values in the present study because arterial contraction was observed at only two concentrations. A reliable EC50 value requires multiple data points to be useful. Kinetic modeling would not have generated reliable results for this descriptive study.

To the authors' knowledge, no studies have been performed to assess the contractile activity of the (S)-epimers or the contractile response resulting from the transformation of (R)-epimers to (S)-epimers. To assess the contractile response in vascular beds, dissected arteries or veins must be maintained alive under normal physiological conditions of pH (7.4), temperature (37 °C), nutrients, and gas. Under these experimental conditions, it is possible for the transformation between the (R) and (S)-epimers to occur until an equilibrium is reached (Komarova and Tolkachev, 2001; Smith and Shappell, 2002). We, therefore, cannot rule out the possibility that the contractile response seen in the present study was, in part, due to the transformation of the (S)-epimer to the (R)-epimer. However, this transformation was unlikely. The reason being, the transformation of the (S) to (R)-epimer is not favored and the (S)-epimer slightly dominates the equilibrium (Andrae et al., 2014). The (S)-epimer would have to undergo a more difficult rearrangement to switch to the intermediate and, therefore, the (R)-epimer. This is also supported by examining the transformation of a (R)- to (S)-epimer under physiological conditions (Smith and Shappell, 2002). When an aqueous solution was used to assess the epimerization of pure ergovaline (R) at 37 °C (pH 7.5), equilibrium was reached after 11 h and only 59.4% of ergovaline (R) remained. The transformation between epimers has been observed at 75 min (Smith and Shappell, 2002), which is longer than the time period used in the present study. At the highest concentration used, for specific (S)-epimers, there was observation of slight artery contraction within 14 min of incubation and an increase in contraction at the end of the 29-min incubation period. Analytical detection of each form of epimer in the buffer solution may provide evidence for which form is present and/or dominant. However, the time and methods needed for testing will likely result in an increase in epimer transformation. This will not accurately reflect the conformational form of an epimer at the time of artery contraction. Therefore, the time frame within the present experiment, along with the likelihood of the transformation of ergot alkaloids favoring the (S)-epimers within the conditions used, pH 7.5 and 37 °C, it is strongly suggested that the (S)-epimer administered to the buffer within the tissue bath did not transform into the (R)-epimer. This supports that the S-epimers were responsible for the arterial contraction observed.

The (R) and (S)-epimers of ergot alkaloids are found in almost all food products derived from *Claviceps*-infested crops. Along with animal feed, food products intended for human

consumption (including, but not limited to, baby formula, bread, and pasta) may contain ergot (S)-epimers (Debegnach et al., 2019). Studies often report that the “harmful” (R)-epimers convert to the “inactive” (S)-epimers after food processing intended for human consumption (Merkel et al., 2012). One study examined changes in concentrations of six (R)- and (S)-epimers during the production of rye bread (Bryła et al., 2019). The study reported that thermal processing (or baking) caused the concentration of the (R)-epimers to decrease but resulted in an increase of (S)-epimers. The study concluded that baking contributes to the reduction of toxicity of the final bread product. Similarly, a study examining the milling of durum and the production of pasta reported comparable results (Tittlemier et al., 2019). These studies emphasize the importance of understanding specifically the biological effect of (S)-epimers and the significance of the findings in the present study.

The (S)-epimers used in the present study are routinely encountered in cereal crops in Western Canada (Tittlemier et al., 2015). The mere presence of a contractile response after incubating arteries with four different (S)-epimers indicates that their presence in human diets and animal feed may be significant and should be considered in analytical and food safety assessments. Concentrations of (R)-epimers are monitored in human and animal feed worldwide by regulatory agencies in many countries, whereas (S)-epimers are not measured or evaluated in human and animal feed standards in North America (Coufal-Majewski et al., 2016). Since the (S)-epimers are thought to be biologically inactive, their concentrations in the feed are considered less important. Previously, studies report that the quantification of (R)-epimers should consider their epimerization toward (S)-epimers and, therefore, utilize extraction solvents and conditions that decrease the chance of epimerization (Smith and Shappell, 2002). However, because these conditions are not easy to maintain in an experimental setting and naturally contaminated feed contain (R)- and (S)-epimers, it is important to quantify both (Guo et al., 2016). The adverse manifestations such as ergotism seen in livestock, as a result of contaminated feed consumption, are likely produced by the combined action of the (R)- and (S)-epimers and not exclusively the (R)-epimers.

In conclusion, this study reports the demonstration of biological activity for four (S)-epimers of ergot alkaloids, namely ergotaminine, ergocorninine, ergocristinine, and ergocryptinine, through potential vasoconstriction as demonstrated by arterial contraction, which were thought to be previously inactive. Ergotaminine had the greatest contractile response compared with the other (S)-epimers with each having different degrees of contractility. This may be similar to that reported for (R)-epimers (Klotz et al., 2010). Future work would include the comparison of contractility between (R) vs. (S)-epimers. This will be beneficial for understanding the potencies of each form. The mere presence of a contractile response in bovine arteries stimulates future discussion concerning the biological activity of (S)-epimers in multiple settings, which may influence the direction of, and development of, regulatory standards for ergot concentrations relating to human and animal food safety worldwide.

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## Conflict of interest statement

We declare no competing interests.

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