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Bioavailability profiling shows differences in OA, DTX1 and DTX2 toxins that justify their toxicity

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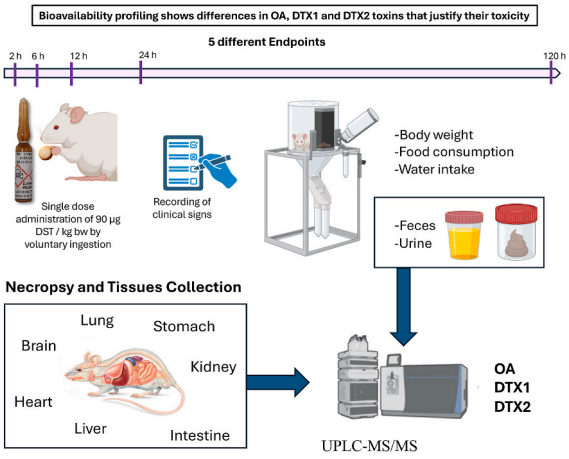
HIGHLIGHTS

- A single ingestion of DSTs through voluntary intake of bread is a non-invasive method suitable for animal welfare.
- 90 μg DSTs/kg bw is rapidly absorbed as indicated by a fast onset of diarrhea.
- Toxic effects of OA, DTX1 and DTX2 by voluntary feeding are related to their pharmacokinetic and should be considered for accurate risk assessments.

GRAPHICAL ABSTRACT



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ABSTRACT

The marine toxins of the Okadaic acid (OA) group are natural compounds produced by dinoflagellates that enters the food chain by accumulating in seafood. They are responsible for Diarrhetic Shellfish Poisoning (DSP) events in humans over the world and therefore are also jointly named as Diarrhetic Shellfish Toxins (DSTs). The main objective of this study was to evaluate symptoms, toxicity, absorption, distribution, and elimination of OA, Dinophysistoxin-1 (DTX1), and Dinophysistoxin-2 (DTX2) at the sublethal dose of 90 μg toxin/kg bw administered through voluntary feeding to mice. The toxin comparison highlighted that OA and DTX1 induced more severe and specific symptoms such as diarrhea. After oral ingestion toxins were distributed through the entire organism being detected in liver, kidney, stomach, small and large intestine. Predominant excretion of the toxins was observed in feces, with OA exhibiting fast elimination, while DTX2 was showing prolonged excretion. The

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passage and accumulation of toxins in gastrointestinal organs instigated macroscopic damage in the stomach, small and large intestine that could persist up to 120 h. These findings highlight the importance of pharmacokinetic of sublethal doses of DSTs administered by voluntary feeding in their toxicity and their implication for public health.

Abbreviations

CRM	Certified Reference Materials
DSP	Diarrhetic Shellfish Poisoning
DSTs	Diarrhetic Shellfish Toxins
DTX1	Dinophysistoxin-1
DTX2	Dinophysistoxin-2
OA	Okadaic acid
OECD	Organisation for Economic Co-operation and Development
TEF	Toxicity Equivalency Factor
UHPLC	Ultra-High Pressure Liquid Chromatograph

1. Introduction

The Okadaic acid group, that includes toxins such as OA, DTX1 and DTX2, are marine polyether compounds produced by dinoflagellates, specifically of the genera *Prorocentrum* and *Dinophysis* (Vilariño et al., 2018). These toxins, commonly named Diarrhetic Shellfish Toxins (DSTs), are associated with some Harmful Algal Blooms, particularly on European coasts (McNamee et al., 2016; Emery et al., 2021). Spain stands as the largest mussels' producer in Europe. They are bivalves, and as filter feeders, can accumulate DSTs in their tissues (Valdiglesias et al., 2013; Vilariño et al., 2018; Avdelas et al., 2021; Vaz et al., 2021). Accidental consumption of shellfish highly contaminated with these toxins can lead to Diarrhetic Shellfish Poisoning (DSP), characterized by severe gastrointestinal symptoms such as vomiting and diarrhea (Trainer et al., 2013; O'Mahony, 2018).

Toxins of the OA group are the most prevalent in edible bivalves, therefore the European Union implemented a mandatory biotoxin analysis in routine monitoring of shellfish in production areas (Comission, 2004; O'Mahony, 2018). Whenever the DSTs in molluscs exceeds the established regulatory limit of 160 μg OA equivalents/kg (Comission, 2004) bivalves harvesting activities are suspended and the product already collected is dumped (Fernández et al., 2014; Visciano et al., 2016). The EU legislation implemented the analytical control of marine toxins by means of liquid chromatography and mass spectrometric protocols (LC-MS), however the challenge is translating individual toxin concentrations into toxicity to determine whether regulatory limits have been exceeded (Union, 2011). LC-MS analyses provide accurate quantitation of the toxin analogues, but they have widely dissimilar potencies, this is why Toxicity Equivalency Factors (TEFs) are required for determining overall toxicity of the seafood (Abal et al., 2017a, 2017b; Louzao et al., 2021b). TEFs are the ratios between the toxicity of the analogues and that of a reference compound within the same toxin group. For accurate risk assessments of toxin mixtures containing different DSTs, TEF values must be accurately determined for each toxin (Botana et al., 2016). To properly reflect the poisoning of DSTs ingestion, specific symptoms such as diarrhea without death, the chronic toxicity of the OA group and pharmacokinetics at sublethal doses by oral administration should be considered.

Many DSTs toxicity studies are focused on OA and usually used high concentrations (Ito et al., 2002; Tubaro et al., 2003; Le Hégarat et al., 2006; Aune et al., 2012; Sosa et al., 2022). Some recent *in vivo* experiments also were performed at low concentrations in acute studies, administering the toxin in repeated doses or over a long term exposure (Liu et al., 2020; Louzao et al., 2021b; Costas et al., 2022; Park et al., 2023). Mice deaths from oral OA ingestion ranged from 400 to 2000 μg/kg, with associated liver, small intestine, and forestomach damage. Distribution of OA in Swiss mice following oral administration of various concentrations via oral gavage, revealed a correlation between the presence of this compound in the mouse intestine and the occurrence of diarrhea (Louzao et al., 2021a). Besides, OA and DTX1 were suggested to induce erosion of intestinal villi (Terao et al., 1986; Ito et al., 2002) which is inconsistent with recent reports (Park et al., 2023). OA was detected in liver, but no damage was observed in this organ in mice treated with doses from 75 to 400 μg/kg (Vieira et al., 2013). Previous studies have indicated that OA shows the longest residence time in liver (Ito et al., 2002) that could be in line with the enterohepatic circulation already reported (Matias and Creppy, 1999). OA cause acute diarrhea at the low dose of 100 μg/kg (Louzao et al., 2021b). Lower doses (80 μg/kg) over a long term exposure (30 days) could cause damage to the intestinal epithelium and increase the relative abundance of pathogenic bacteria leading to an easier pathogenicity (Liu et al., 2020). Besides toxicity of low doses of OA may be exacerbated by co-exposure to other toxins (Sosa et al., 2022) or marine pollutants such as microplastics (Huang et al., 2024).

Some studies have shed light on the distinct toxicological profiles for each DST toxin. Aune et al. (2007) provided the first comprehensive assessment of DTX2 toxicity in mice, revealing that the relative i.p. toxicity of DTX2 was about 0.6 fold that of OA (Aune et al., 2007). However, recent findings indicate that DTX2 exhibits even lower toxicity via oral ingestion compared to OA with a proposed TEF of 0.4 (Abal et al., 2017b). The assumption of a 1:1 TEF for DTX1 and OA could be in line with some recent results after repeated administration of sublethal doses (Park et al., 2023), However previous studies suggested that the toxicity of DTX1 surpasses that of OA (Fernández et al., 2014; Abal et al., 2018; Suzuki and Okada, 2018). Moreover, it was recently demonstrated the importance of considering the route of administration in assessing DSTs toxicity (Louzao et al., 2021a).

Despite being a regulated and well-known group of toxins in Europe with over 40 years of research (Tachibana et al., 1981; EFSA, 2008), there are scarce studies aimed at determining DSTs pharmacokinetic. Our study focuses on the pharmacokinetic assessment of DSTs in mice. In alignment with the European Union Regulatory Framework, we administered to mice doses in the legal limit considering the EFSA estimates on consumption data (EFSA, 2008, 2009, 2010). Furthermore, our approach fits the OECD's encouragement of voluntary feeding techniques over oral gavage, aiming to improve animal welfare and reduce stress during experimentation (OECD, 2010).

2. Material and methods

2.1. Toxins

The Toxins OA, DTX1 and DTX2, with purity >98%, were provided from Laboratorio CIFGA S.A. (Lugo, Spain). Stock solutions of toxins were in ethanol and stored at −80 °C. Certified Reference Materials (CRMs) from Laboratorio CIFGA S.A. were used for quantification.

2.2. Animals

Female Swiss (ICR background) 4-week-old mice, mean weight 20 g, with an individual weight variation not exceeding ± 20% were purchased from Centre for Experimental Biomedicine (CEBEGA) of the

University of Santiago de Compostela. They were maintained under standard conditions: light/dark cycles (12 h/12 h) in a temperature (23 ±2 °C) and humidity (60–70%) controlled room, with free access to water and pelleted diet. Animals were acclimatized for 2 weeks before the experiments, at standard conditions.

2.3. Procedure for in vivo experiment

Mice were randomly assigned to either the control group or the OA-treated, DTX1-treated or DTX2-treated group with 3 mice per group allocated to each specific timepoint (2, 6, 12, 24 or 120 h). 5 days before the experiment, animals were habituated to bread ingestion. Then, mice were weighed and individually placed in metabolic cages. After fasting overnight with free access to 5% glucose serum, mice were treated with DST by voluntary consumption. Mice had unlimited access to 5% glucose serum as previously described (Costas et al., 2023) to prevent hypoglycaemia, maintain adequate energy levels and reduce the stress associated with fasting, a measure carried out to promote increased appetite upon voluntary administration.

Toxins solutions for administration were freshly prepared in 0.9% saline solution to achieve the concentration of 90 μg DST/kg bw. 30 μL of toxin solution was added to a 100 mg piece of bread. The toxic bread was placed in the metabolic cages and the mice voluntary ingested it. Control mice received only vehicle. 90 μg DST/kg bw is a sublethal dose of the toxin and it is equivalent to the quantity of compound that could receive a consumer of seafood with DSTs in the regulatory limit (160 μg/kg mollusc). All toxin doses were mixed with bread and voluntary ingested by the mice. To facilitate this, the animals were habituated to bread consumption for 5 days.

Then, animals were provided with food and water *ad libitum* throughout the experiment. During the experimental period (2, 6, 12, 24 or 120 h), body, urine and feces weights were measured. Food and water ingestion was also quantified. Mice were also carefully observed for clinical signs. Considering the diarrhea as the main symptom of DSTs, a scoring system was employed to quantify its severity. This scoring system was defined as follows: normal feces were scored as 0, soft feces as 1, shapeless soft feces as 2, watery diarrhea as 3, and watery and repeated diarrhea over time as 4. Distinct episodes of diarrhea were considered if at least 20 min had passed since the last defecation.

All protocols were executed in accordance with European (EU directive 2010/63/EU) and Spanish legislation (Real Decreto 53/2013, Decreto 296/2008). Therefore, all procedures were accepted by the Institutional Animal Care Committee of the University of Santiago de Compostela (01/17/LU-002).

2.4. Necropsy and samples collection

At the end of the experimental period (2, 6, 12, 24 or 120 h), mice were euthanized by CO_2 inhalation, and subsequent necropsies were conducted. Organs macroscopic observation allows identifying signs of toxicity. The main organs (heart, lungs, liver, stomach, large and small intestine, kidney and brain) were also collected and weighed in each group of mice.

2.5. Toxin analysis in biological samples

Weighed organs were extracted with methanol as described in Louzao et al. (2021a). Briefly, 0.1 g of homogenized gastrointestinal organs were extracted with 400 μL methanol and after 1 min of vortex mixing and 30 s of sonication, the mixtures were centrifugated at 4000 rpm (2486×*g*) for 10 min. Supernatants were transferred to microtubes, and the pellets were extracted twice more. Combined supernatants were evaporated to dryness and reconstituted with 100 μL methanol. Next, 40 μL methanol and 10 μL of trichloroacetic acid 10% solution were added to boost protein precipitation. Finally, 50 μL acetonitrile was added and final extracts were filtered through 0.2 μm and centrifugated at 1400×*g* for 10 min to be analysed by UPLC-MS/MS. Feces, lungs, hearts, and brains samples were subjected to same protocol.

For the urine samples extractions, 40 μL methanol were added to 100 μL of homogenized samples. After vortexing for 1 min, 10 μL of trichloroacetic acid 10% solution were added and vortexed for protein precipitation. Finally, 50 μL acetonitrile were added and samples were filtered through 0.2 μm (14500×*g*; 10 min) before analysis by UPLC-MS/MS.

For the blood extraction, 1200 μL of 75% methanol were added to 300 μL of blood sample and vortexed for 1 min. Mixture was filtered through Amicon Ultra 0.5 mL centrifugal filter units (Ultracel 3K, regenerated cellulose membrane 3 KDa NMWL) at 13000 rpm (11334×*g*) for 30 min. Ultrafiltered solution was evaporated to dryness and reconstituted with 300 μL methanol. Final extracts were filtered (0.2 μm; 14500×*g*; 10 min) and analysed by UPLC-MS/MS.

The analysis of DSTs was carried out using an Acquity UPLC BEH C18 100 mm × 2.1 mm × 1.7 μm column (Waters, Milford, CT, USA). Mobile phases were as follows: (A) water and (B) acetonitrile (95% v/v) both containing 5 mM of ammonium acetate. Gradient: 0–1.0 min 30% B; 1.0–4.0 min 90% B; 4.0–4.5 min 100% B; 4.5–9.0 min 100% B; 9.0–9.1 min 30% B; 9.1–11.0 min 30% B. Automatic sampler temperature was maintained at 8 °C, and the column temperature was set at 30 °C. Flow was settled at 0.45 mL/min, and the injection volume was 5 μL. Retention times for OA, DTX2 and DTX1 were 2.90, 3.10 and 3.50 min respectively.

Mass analysis was performed using a Xevo TQ MS mass spectrometer from Waters (Manchester, UK) operated with the following parameters (Multiple Reaction Monitoring, MRM, electrospray ionization ESI-): cone gas flow 50 L/h N_2, desolvation gas flow 900 L/h, desolvation temperature 350 °C, source temperature 120 °C, capillary potential 3 kV. Argon was used as the collision gas at 0.12 mL/min. DSTs were quantified against the certified reference standards CRM-00-OA batch 22-001 (18.9 μmol/kg, purity ≥99%), CRM-00-DTX1 batch 21-001 (10.52 μmol/kg, purity ≥96%) and CRM-02-DTX2 batch 19-001 (5.2 μmol/kg, purity ≥98%) sourced from Laboratorio CIFGA S.A. (Lugo, Spain) by using the following transitions: OA and DTX2 (*m/z* 803.5 > 113.1/255.2 (Q)) and DTX1 (817.1 > 113.1/255.2 (Q)). Calibration standards were prepared from standard solutions and a 7-points calibration curve was generated with variable concentrations ranging from 2 to 100 ng/g solution.

The matrix effect (ME), extraction efficiency (EE), recovery (R), limits of detection (LoD) and quantification (LoQ) were calculated for each organ (including fluids) by pre- and post-spiking samples at 3 different DSTs concentration levels (10, 50 and 100 ng/g organ). LoD ranged 1.12–9.08 ng/g while LoQ ranged 3.71–30.25 ng/g depending on organ type. (see Tables S1 and S2).

2.6. Statistical analysis

The results were analysed using the Kruskal-Wallis test in R (v.4.2.1). Statistical significance was set at $p \leq 0.05$. Graphical analysis was performed with GraphPad Prism 5.0.

3. Results

3.1. Clinical signs

Mice administered with 90 μg/kg bw of DSTs (OA, DTX1 or DTX2) were carefully monitored throughout the entire experiment and any signs of toxicity were documented. Non-specific symptoms onset, like piloerection or lordosis, became promptly noticeable as early as 30 min, with diarrhea episodes in the first 2 h. The symptoms are defined below. A scale between 0 and 2 was used for quantification, with "0″ not present, "1″ moderately or intermediate present and "2″ obviously present.

Clinical signs included:

Orbital tightening: The mouse closes its eyelids, leading to a

narrowing of the orbital area.

Apathy: Lack of motivation or reduction of goal-directed behaviours. (Tanaka and Hamaguchi, 2019).

Lordosis/Kyphosis: Body position with increased anterior curvature of the thoracic spine.

Ear position back: Rotation of ears outwards, away from the face, with the possibility of folding to create a pointed shape and an increased space between the ears.

Piloerection: Raised or standing hair on the body, typically induced by pain or stress.

The clinical signs of DSTs revealed different patterns in their impact on mice (Table 1). While mice that received OA showed a fast score 3 diarrhea and a broad spectrum of long-lasting symptoms, including also score 2 diarrhea, orbital tightening, apathy, lordosis and piloerection, mice treated with DTX1 have notable effects on score 2 diarrhea, orbital tightening, lordosis, ear position back and piloerection. DTX2 primarily induces the score 2 diarrhea, lordosis and piloerection. The comparison highlighted that OA and DTX1 induced the most severe symptoms, followed by DTX2.

3.2. Impact of DSTs on physiological parameters

In our study of mice that ingested 90 μg/kg bw of DSTs by voluntary feeding, as expected, none died. Mice were kept individually in metabolic cages, allowing us to quantify food and water intake.

Fig. 1 illustrates variations in different physiological parameters for both the control group and mice administered with 90 μg/kg of OA, DTX1, or DTX2 at the different endpoints. Body weight loss is a common physiological parameter that reflects overall health status. After the voluntary feeding of DSTs the effect on body weight over time was only significant between mice treated with OA and DTX1 after 12 h of toxin administration. A significant increase in relative water consumption was observed in the DTX1 group at 2 h compared to OA and DTX2. Conversely, the quantity of urine in the OA treated group is significantly higher than control and DTX2 treated group 2 h after toxin ingestion. DTX1 group showed increased water consumption at 12 h relative to controls and OA. However, it is the DTX2 treated group that excreted more urine than controls and DTX1 treated mice 12 h after toxin administration. At 24 h, although no significant differences in water intake were observed, the urine weight in DTX2 treated mice was higher than controls and OA treated mice.

At 2 h, no significant differences were observed in either relative food consumption or fecal weight. Between 2 and 12 h, food consumption did not vary significantly between treatments. However, at 6 h the average fecal weight in the OA treated group standed higher than controls. At 24 h, OA had higher food consumption than DTX2 and also higher fecal weight than DTX1 and DTX2. At 120 h, significant differences were detected in food consumption between OA and DTX1 and between DTX1 and DTX2, with DTX1 showing lower intake.

The whole heart, lung, liver, stomach, kidney, brain, and small and large intestine were isolated at each endpoint of the experiment period (2, 6, 12, 24 and 120 h). Time-course changes of the weight ratio between each organ and body (%) are illustrated in Fig. 2. For the heart weight ratio, only significant differences were detected between OA treated groups and DTX2 group at 6 h and DTX1 at 12 h. For the lungs DTX1 treated groups showed significant differences with DTX2 and OA treated groups after 120 h of toxin administration. The values of brain weight were lower in DTX2 treated mice in comparison to mice administered with DTX1 and OA 2 h after administration and lower than OA treated mice at 12 h. DTX1 decreased the kidney weight in mice in comparison to DTX2 after 12 h. OA treated mice decreased their kidney weight versus DTX2 12 and 24 h after toxin administration. In the liver weight ratio, significant differences were found between DTX2 treated mice and mice that received OA and DTX1 24 h after toxin administration and to DTX1 at 120 h.

After checking the stomach results, the following significant differences were observed: at the 2, 6 and 24 h times, the OA group showed higher relative stomach weight compared to the control group. In addition, at the 6 and 120 h, the OA group had a higher relative stomach weight than DTX2.

2 h after DSTs ingestion the small intestine weight was higher than that of controls, at this stage small intestine showed important swelling. Nevertheless, 120 h after the OA administration the mice small intestine weight was significantly lower than in controls and DTX1 group. In the large intestine, there was a significant increase in the weight of the DTX2 group versus controls and OA group 2 h after ingestion.

Table 1
Clinical signs observed in Controls (C) and mice administered with 90 μg/kg Okadaic acid (OA), Dinophysistoxin-1 (DTX1) or Dinophysistoxin-2 (DTX2). The data is the percentage of mice with the clinical sign and time after toxin administration (from 2 to 120 h), a score to each clinical sign was also included as superscript.

Clinical signs	Time (h)											
	2				6				12			
	C	OA	DTX1	DTX2	C	OA	DTX1	DTX2	C	OA	DTX1	DTX2
Orbital tightening	0	33.3^{1}	66.7^{1}	33.3^{1}	0	66.7^{1}	66.7^{1}	33.3^{1}	0	66.7^{1}	33.3^{1}	0
Apathy	0	0	33.3^{1}	0	0	0	66.7^{1}	$67.7^{1,2}$	0	0	0	33.3^{1}
Lordosis	0	$66.7^{1,2}$	66.7^{2}	66.7^{2}	0	100^{2}	66.7^{2}	100^{2}	0	100^{2}	100^{2}	100^{2}
Ear position	0	33.3^{1}	33.3^{2}	33.3^{1}	0	33.3^{1}	$100^{1,2,2}$	33.3^{1}	0	33.3^{1}	33.3^{1}	66.7^{1}
Piloerection	66.7^{1}	100^{2}	33.3^{2}	33.3^{2}	0	100^{2}	66.7^{2}	$100^{1,2,2}$	0	100^{2}	100^{2}	$100^{1,2,2}$
Diarrhea	0	66.7	33.3	33.3	0	0	33.3	0	0	0		0
Onset (h)		1h16' 1h52′	1 h	1 h 16′			4 h 40′					
Diarrhea Sc.	–	3	2	2	–	–	2	–	–	–	2	–

Clinical signs	Time (h)							
	24				120			
	C	OA	DTX1	DTX2	C	OA	DTX1	DTX2
Orbital tightening	33.3^{1}	$100^{1,1,2}$	33.3^{1}	0	0	66.7^{1}	33.3^{1}	0
Apathy	0	0	0	33.3^{1}	0	66.7^{1}	0	0
Lordosis	0	100^{2}	66.7^{2}	$66.7^{1,2}$	0	66.7^{2}	100^{2}	100^{2}
Ear position	0	66.7^{1}	$66.7^{1,2}$	33.3^{1}	0	33.3^{1}	33.3^{1}	33.3^{1}
Piloerection	0	100^{2}	100^{2}	100^{2}	100^{2}	100^{2}	100^{2}	100^{2}
Diarrhea	0	66.7	33.3	0	0	0	0	0
Onset (h)		10–24 h	10–24 h					
Diarrhea Sc.	–	2	2	–	–	–	–	–

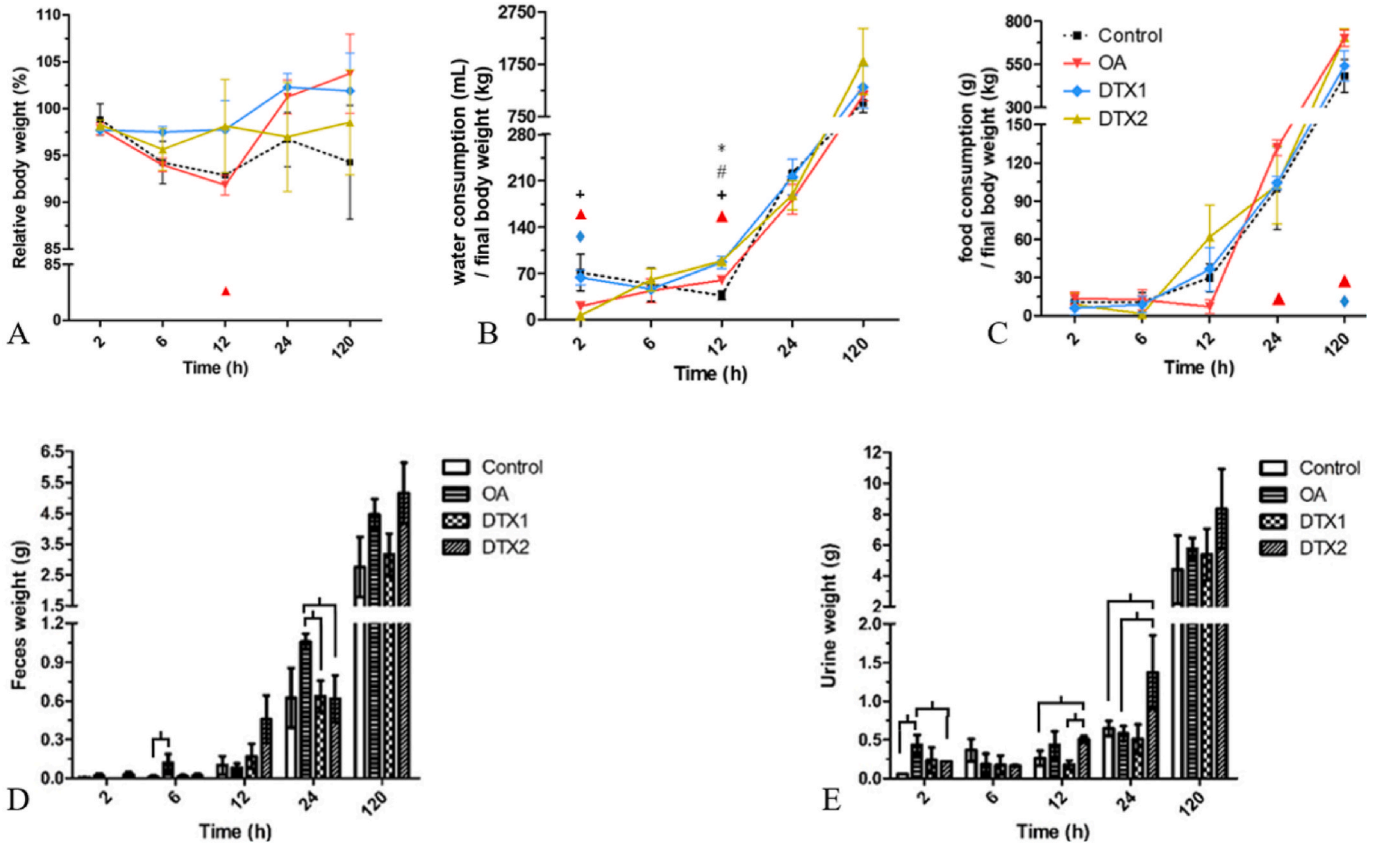

Fig. 1. Variation in the final body weight in control and mice administered with 90 μg/kg OA, DTX1 or DTX2 over the time (A). Time course of the relative water intake (B) and food consumption (C) represented as the ratio of water intake (mL) or food consumption (g) to final body weight (kg). Time course of feces (D) and urine weight (E) in controls and mice administered with 90 μg/kg OA, DTX1 or DTX2. Mean ± SEM. Significant differences in Fig. 1A-C are represented with the following signs ($p < 0.05$): Ctrl vs. OA: *; Ctrl vs. DTX1: #; Ctrl vs. DTX2: +; OA vs. DTX1: ▲; OA vs. DTX2: •; DTX1 vs. DTX2: ◆. Significant differences in Fig. 1D-E are represented with brackets ($p < 0.05$).

3.3. Necroscopic analysis

During the necropsies, the following organs of the digestive system were carefully analysed: liver, stomach, small intestine, large intestine, and kidney. To compare the results, a categorization system was established based on the damage to each organ between 0 and 2, with 0 for normal and 2 for a large accumulation of fluid (Table S3). Macroscopic alterations were observed in the stomach, small intestine, and large intestine of mice that received 90 μg DSTs/kg (Table 2).

Stomach of some mice that received DTX1 contained liquid and gas up to 12 h whereas it was only detectable in the first 2 h in mice treated with DTX2. Mice that ingested OA presented moderate pale fluid in the lumen or swollen content in the small intestine the first 24 h. This effect was only appreciable the first 6 h in mice treated with DTX2. On the large intestine, none of the control mice showed any soft feces or diarrhea. Mice treated with OA showed a soft content in the large intestine that persisted up to 120 h, while DTX1 group exhibited this effect in only at 6 h. DTX2-treated mice displayed diarrhetic content, but only until 6 h.

3.4. Distribution and excretion of DSTs

DSTs were distributed in most mice organs. Gastrointestinal organs including the stomach, small intestine, and large intestine, had the highest toxin level. Also, it was observed a greater quantity of toxins excreted in feces (Fig. 3).

The stomach seemed to show different patterns in relation to DSTs. Mice that received DTX1, had the highest toxin concentration in the stomach with its maximum value at 6 h being significantly different than OA and DTX2 and then decreased over time. In contrast, mice with OA exhibited a moderate level of toxin in stomach during the initial 2 h, followed by a gradual decrease to eventual undetectability at 120 h. 12 h after toxin administration OA was significantly different than DTX1 and DTX2. In a similar way, DTX2 had the highest concentration at 2 h, with a quick and more pronounced reduction until disappearing at 24 h.

Examining the transit of the toxins from the stomach to the small intestine, DTX1 stayed longer in the stomach, while DTX2 was eliminated faster. At 2 h, DTX2 showed significantly higher levels in the small intestine compared to OA and DTX1, indicating more efficient transit. This trend changed at 24 h, when DTX2 levels in the small intestine were lower compared to OA and DTX1, suggesting faster elimination of DTX2 from the body. OA had a low peak in small intestine at 12 h, with concentrations significantly different than DTX1, followed by a gradual decrease. Levels of DTX1 were significantly lower than OA and DTX2 6 h after toxin administration and with similar values along the time until being reduced at 120 h.

In the large intestine, the three DSTs exhibited a different trend. The concentration of OA was higher than the other toxins with significant differences with DTX1 at 2, 6, 12 and 24 h and also with DTX1 and DTX2 at 6 h and 12 h. The highest concentration of OA was found in the large intestine, with important values higher than 1000 ng/g up to 12 h, then there was a fast decrease in concentration which indicates a fast elimination of OA over time.

The highest amount of DTX2 was also detected in the large intestine with a lower than OA peak concentration at 6 h and significant differences with DTX1 at 2 h and 6 h. From 6 h onwards, DTX2 levels decreased, becoming undetectable at 120 h. This trend indicated a relatively rapid elimination of DTX2. Opposite, DTX1 was lower in the

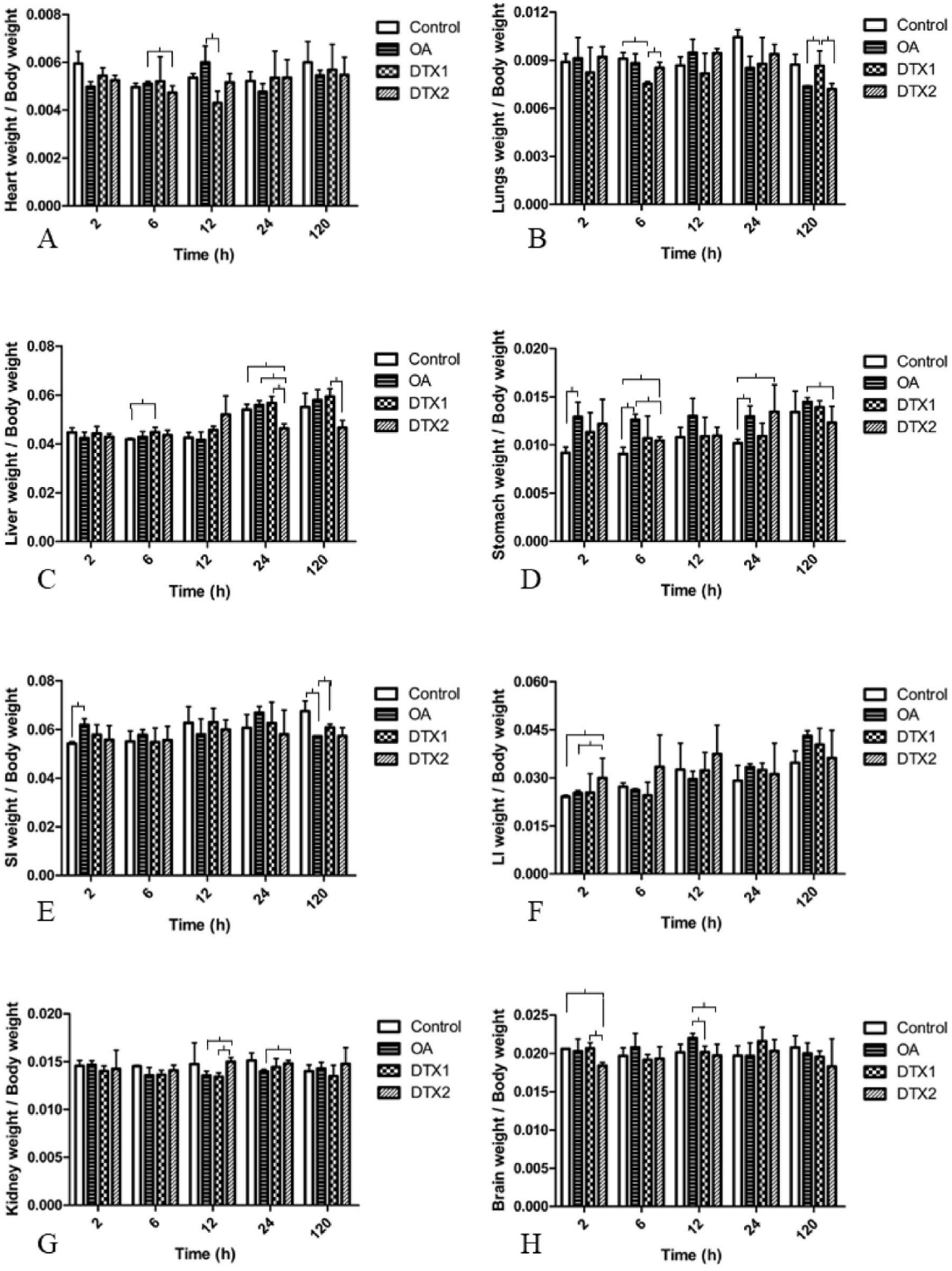

Fig. 2. Ratios between heart (A), lungs (B), liver (C), stomach (D), small intestine (SI), large intestine (LI), kidney (G), brain (H) and body weight in control mice and mice administered with OA, DTX1 and DTX2 at the five different endpoints. Mean ± SEM. Significant differences between treatments or treatments and control are marked with brackets ($p < 0.05$).

large intestine than in stomach, reaching its peak concentration at 12 h with an approximated value of 300 ng/g. Then the concentration decreases which suggests a progressive elimination in the large intestine over time. It should be highlighted that concentration of DTX1 was lower in intestines that may be due to the accumulation in stomach showing a different toxin behaviour.

Subsequently, our focus was directed towards the organs responsible for detoxification, kidney and liver were the amount of toxin detected was low. In the kidney, OA levels reached a very small peak at 6 h, before declining that were significantly different than DTX2. Regarding DTX2, the higher concentration was identified 2 h after toxin ingestion, followed with a fast decrease, being detectable at 12 h. DTX1 was

Table 2
Macroscopic evaluation of digestive organs in response to treatment. Each number corresponds to one animal.

Treatment	Control					OA				
Time (h)	2	6	12	24	120	2	6	12	24	120
Stomach	0,0,1	0,0,0	0,0,0	0,0,0	0,0,0	0,2,0	0,0,0	1,1,0	0,0,0	1,0,0
Small intestine	0,1,0	0,0,0	0,1,0	0,0,0	0,0,0	2,1,1	0,1,1	0,0,2	0,0,2	1,0,0
Large intestine	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,1	1,1,0	1,1,0	0,0,0	1,0,0
Treatment	DTX1					DTX2				
Time (h)	2	6	12	24	120	2	6	12	24	120
Stomach	1,0,2	2,0,2	0,0,2	0,0,0	0,0,0	2,0,2	0,0,0	0,0,1	0,0,0	0,0,0
Small intestine	1,2,2	1,0,0	0,2,1	0,0,0	0,0,0	2,0,1	1,0,1	0,0,0	0,0,0	0,0,0
Large intestine	0,0,0	0,0,1	0,0,0	0,0,0	0,0,0	2,0,0	2,2,0	0,0,0	0,0,0	0,0,0

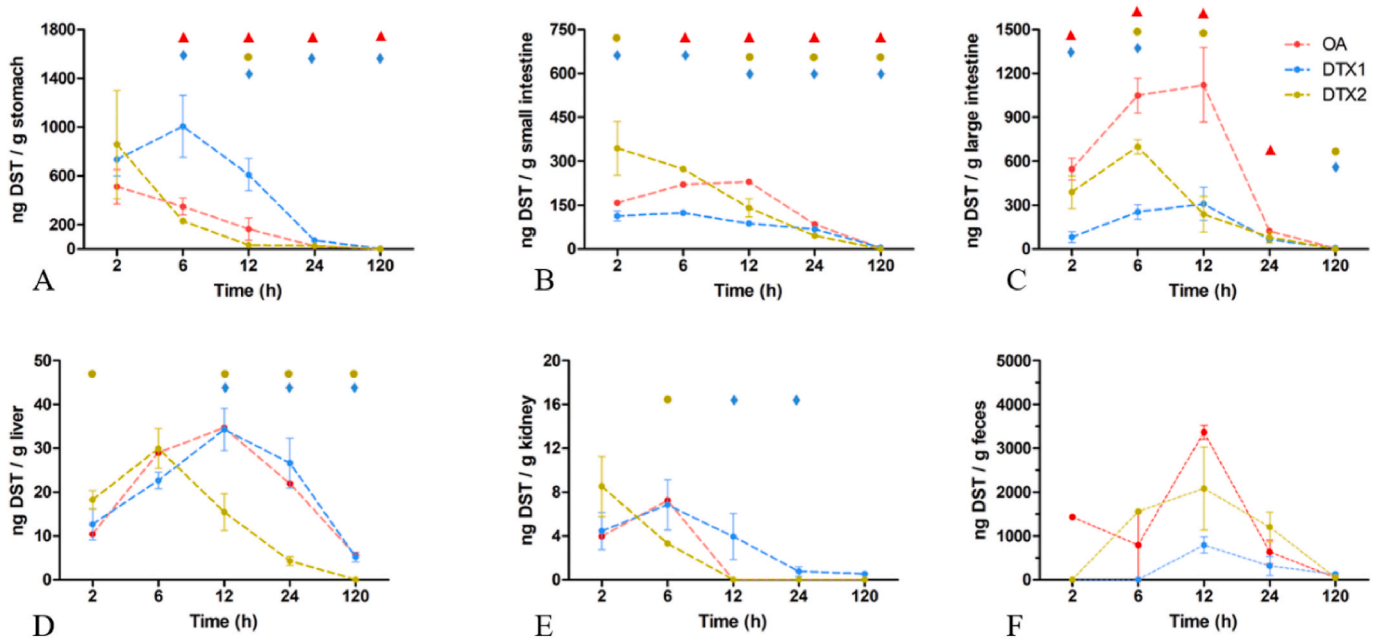

Fig. 3. Concentration of toxin (ng/g) in stomach (A), small intestine (B), large intestine (C), liver (D), kidney (E) and feces (F), represented as Mean ± SEM. In the case of feces, n = 0 in 2 h DTX1; n = 1 in 2 h OA, 6 h DTX1, 2 h DTX2, 6 h DTX2; n = 2 in 6 h OA, 12 h OA, 12 h DTX1. n = 3 in the rest of cases. Significant differences between treatments are represented with the following signs ($p < 0.05$): OA vs. DTX1: ▲; OA vs. DTX2: •; DTX1 vs. DTX2: ◆.

significantly higher than DTX2 at 12 h, and 24 h after toxin administration, DTX1 was still detected although a very low concentration.

In the initial 2 h, the hepatic levels of both OA and DTX1 remained low being OA significantly different from DTX2. However, OA and DTX1 showed an increase in liver, reaching a peak at 12 h followed by a gradual decrease in toxin levels up to 120 h. Relating to DTX2, its concentration in the liver peaks at 6 h, and subsequently experienced a rapid fall indicating a faster elimination of DTX2 by liver being significant compared to OA and DTX1 at 12, 24 and 120 h.

Concerning blood, DTX2 was not detected in any sample, OA was just detected in one sample at 12 h, while DTX1 was found in 1 sample at 6 h, 3 samples at 12 h and 2 samples at 24 h, all of them below LOQ. In the case of urine, toxin presence was only detected in 5 out of 45 samples, exhibiting values below LOQ. Finally, attention was given to the feces, revealing the predominant excretion of DSTs.

OA showed an early elimination in feces at 2 h, while DTX2 was not detected in the same period. It is worth noting that only one mouse treated with DTX2 had feces during this period, while none of the DTX1-treated mice had. At 6 h feces showed levels of OA significantly higher than DTX1, where not toxin was found. 12 h after toxin administration, all three toxins recorded their peak concentrations in feces, although DTX2 and OA exhibited concentrations approximately double and triple that of DTX1, respectively. At 24 h, OA and DTX1 showed important decreases, while DTX2 maintained its presence to a greater extent. After 120 h, OA and DTX2 were almost no detected in feces.

It should be noted that OA displayed progressive increase up to 12 h in liver and large intestine also detected in feces followed with a fast drop, DTX1 exhibited a fast increase in the stomach in the first 6 h followed by a rapid decrease in toxin up to 24 h. In small and large intestine as well as in liver the increase of DTX1 was lower up to 12 h followed by a prolonged toxin reduction. DTX2 reached the concentration peak at 6 h in large intestine and liver while in feces was at 12 h and then showed a progressive decrease in concentration.

4. Discussion

Many routes for toxin administration in mice can be invasive and stressful confounding experimental outcomes. In animal research the oral route is one of the most desirable to explore and is typically achieved by oral gavage which allows the delivery of precise doses. Oral administration by voluntary ingestion is an alternative non-invasive method suitable both for animal welfare and for accomplishing robust experiments (Teixeira-Santos et al., 2021). We herein describe for the first time a method for oral administration of DSTs through voluntary intake of bread developed for mice. Animals ingested it in less than 5 min controlling the amount and timing of toxin ingestion. This

procedure is suitable to observe the toxicity, absorption, distribution and elimination of toxins over time after ingestion of DSTs just once. Our results evidence that OA, DTX1 and DTX2 has effect in mice at the low dose of 90 μg/kg bw. This dose in mice is equivalent to the toxin that could be ingested by consumption of bivalves with DSTs concentration in the threshold limit for shellfish harvesting (160 μg OA eq/kg) as defined in the European Union by the Regulation (EC) No 853/2004 (Comission, 2004). Mice that ingested 90 μg OA/kg bw through voluntary intake of bread exhibited a broad spectrum of long-lasting symptoms, including orbital tightening, apathy, lordosis, and piloerection. Although there is also evidence that OA may have long-term toxicity acting as a potent tumour promotor in animals (Messner et al., 2006; Jimenez-Carcamo et al., 2020) and it might be the cause of cancer cases reported in epidemiological studies (Cordier et al., 2000; Lopez-Rodas et al., 2006; Manerio et al., 2008). Due to the frequency of appearance, the health concerns about DSP in humans are gastrointestinal symptoms including diarrhea. The low dose of OA and DTX1 used in this study have caused diarrhea with high score shortly after ingestion between 30 min and 2 h and within the first 24 h, whereas animals with DTX2 had only diarrhea in the first 2 h. The fast onset of diarrhea is according to effects reported after DSTs administration by oral gavage (Matias and Creppy, 1999; Abal et al., 2017b; Louzao et al., 2021a) showing a rapid poisoning in mice and manifesting suitability of the method for oral administration. Compared with OA and DTX1, DTX2 seems less potent in triggering and maintaining specific symptoms. The unspecific symptom piloerection could be related to hypothermia described after OA administration (Levy and Dubois, 2006; Suzuki, 2021).

It was previously reported that DSTs did not induce body weight loss (Tubaro et al., 2004), even after repeated administration for 8 days (Park et al., 2023), so water and food consumption were checked in this study to establish potential correlations. After voluntary feeding, only mice treated with OA had significantly lower weight than DTX1 treated mice at 12 h time point coincident with lower water ingestion. However, mice treated with DTX1 showed a decrease in food consumption compared to OA treated mice after 24 and 120 h of toxin administration.

Even though DSTs have mostly been recognized for their acute effects in the gut, they are able to pass the intestinal barrier and reach most of the organs (Wuerger et al., 2023). At each endpoint of the experiment, (2, 6, 12 24 and 120 h after ingestion of DSTs) macroscopically liver, kidney, brain, lung and heart were indistinguishable between control and treated mice. However, some histological lung or liver injuries were previously detected but with higher doses of OA (Ito et al., 2002; Vieira et al., 2013; Abal et al., 2018; Louzao et al., 2021a). The relative liver weight of mice administered with DTX2 was significantly lower than that of mice with OA and DTX1 at 24 h. However, no changes were detected in the relative liver weight in mice with OA versus controls, as was reported by Sosa et al. (2022). DTXs exhibit relatively low hepatic levels in comparison to intestines or stomach. However, OA and DTX1 reach peak concentration at 12 h while DTX2 has the peak 6 h after toxin ingestion followed by a rapid decline significantly lower than DTX1 and OA up to 120 h showing a faster elimination. Concentration of DTX1 in kidney was significantly higher than OA and DTX2 after 12 h of toxin administration. DSTs values in the liver were higher than in the kidney, indicating that liver distribution of DTXs plays a major role, which is interesting since it suggests that liver may be an important organ in the metabolism of toxins. Nevertheless, no macroscopic damage was found in the liver according to previous findings at similar doses (Aune et al., 2012; Park et al., 2023). Interestingly, this might influence with previous studies showing that OA can interfere with glucose metabolism in hepatocytes by increasing gluconeogenesis and glucose output (Louzao et al., 2005; Abal et al., 2018). Decrease in kidney weight was detected in mice treated with OA and DTX1 in comparison to mice with DTX2, but the kidney has no macroscopic lesions as was previously reported for OA and DTX1 (Louzao et al., 2021a; Park et al., 2023).

The intestinal tract is the primary target organ of DSTs (Ito et al., 2002). To compare gastrointestinal toxicities after administration of a single dose of 90 μg/kg by voluntary ingestion we evaluated macroscopic changes and quantity of toxin. A single intake of DSTs caused stomach inflammation, a previously recorded effect but at higher toxin doses (Tubaro et al., 2003). The stomach weight of mice treated with OA and DTX2 initially was significantly higher than controls with similar values to those previously reported (Matias and Creppy, 1999). Macroscopic alterations as fluid and gas accumulation in the stomach were greater and more persistent in DTX1 treated mice coinciding with a significant high concentration of toxin. All treated mice showed an increase in small intestinal weight that could be related to the accumulation of fluid observed during necropsies. This effect was previously described after a single dose administration of high OA concentrations by oral gavage (Ito et al., 2002; Tubaro et al., 2003). Swollen intestine was found in OA-treated mice up to 24 h that still had diarrhea. In mice that ingested DTX1 the macroscopic damage disappears completely at 24 h, but during this first day mice had diarrhea at all endpoints with a score of 2. In the case of mice with DTX2, the macroscopic effect was more evident at 2 h, although changes were no longer visible at 12 h, in agreement with less toxin detected in small and large intestine being significantly different than OA and no diarrhea observed. This swollen intestine has been reported in previous studies after administration of DSTs by oral gavage and with higher doses (Abal et al., 2018; Louzao et al., 2021a). Since OA can stimulate sodium secretion by intestinal cells, leading to fluid accumulation in the intestinal lumen, and this could be the an important mechanism of OA-induced diarrhea (Fu et al., 2019; Costas et al., 2022; Park et al., 2023). Numerous data set *in vitro* are available demonstrating the effects of OA on proinflammatory signalling cascades (Wuerger et al., 2023; Würger et al., 2024). At the intestinal level, colon is essential for fluid absorption. The relative weight of the large intestine did not differ significantly over time between mice that ingested OA or DTX1 and controls. But after 2 h of toxin administration the large intestine of DTX2 treated mice was heavier than OA and DTX1 groups. However, in the necropsy we found that mice that ingested OA had large intestine with an intermediate swelling throughout the first 12 h coincident with the peak of toxin. This increase of intestinal fluid during the first hours and high OA amounts in the intestine was already reported with OA treatments at high concentrations (Matias and Creppy, 1999; Ito et al., 2002) and may be associated with colon dysfunction (Liu et al., 2023). It should be noted that DTX2 ingestion was accompanied by diarrheal large intestine during the first 6 h, but the effect disappeared thereafter. Coincident with the macroscopic manifestations DTX2 was found in the large intestine with the higher peak 6 h after ingestion, but then toxin decreased. This result is in accordance with the diarrhea which occurs fast but was not observed after 6 h. Interestingly, the peak of DTX2 in liver is observed at 6 h, while the peaks of OA and DTX1 are detected at 12 h, as the highest concentrations found in large intestine which agrees with a biliary excretion and enterohepatic circulation of DTXs.

Toxins distributing in intestines might not be intended for excretion, but compounds in feces are for sure eliminated out of the body. Kidney and intestines were traditionally considered to be excretory organs. The groups treated with OA excreted higher amounts of urine 2 h after toxin administration. After 12 h DTX2 treated group excreted more urine than controls and DTX1 treated mice and after 24 h onwards, the DTX2-treated group excreted significantly more urine than OA and the control group besides has higher kidney weight, but no toxin was detected. Regarding feces it should be noted that they were diarrheal in mice that ingested DTX1 and OA up to 24 h. There was a significant increase of feces weight in OA group at 6 h and at 24 versus DTX1 and DTX2 and this correlated with higher food consumption, although not statistically significant. It was previously reported that mice treated by oral gavage with DTX2 excreted less urine and feces than healthy individuals according to a reduction in water intake, and most animals treated with higher doses did not have urine excretion but experienced diarrhea (Abal et al., 2017b). Also, in our results feces of mice treated with DTX2 were normal after 6 h of toxin administration since no diarrhea was

observed after that time. At 24 h, OA treated mice had higher food consumption than DTX1 treated ones and also a higher fecal weight. Sosa et al. also observed slightly higher food consumption in OA-treated mice compared to the control group at 5 days, although in their study, this was preceded by a lower intake during the first 24 h (Sosa et al., 2022).

Therefore in the present study results show that toxins were eliminated out of the body mainly via feces after exposure, being detected in the feces but not in urine opposite to previous data (Matias and Creppy, 1999). The maximum elimination of DSTs was registered 12 h after toxin ingestion. However, OA showed an early elimination in feces at 2 h coincident with high concentration of toxin in large intestine and high score of diarrhea, while DTX1 and DTX2 were not detected at that time. 12 h after administration DSTs showed gradual decreases, although excretion was higher in mice that received OA or DTX2, while elimination of DTX1 was considerably lower, which is in correlation with the lower content of DTX1 found in intestines. This elimination process aligns with previously published (Wang et al., 2012). The DSTs in feces and elimination time supports reported results where the amount of DTX1 excreted after oral gavage administration was always lower than that of OA (Abal et al., 2018; Louzao et al., 2021a). The late appearance of DTX1 in feces 6 h after voluntary ingestion of bread with toxin suggest a possible inhibition of intestinal motility by this toxin supported also by the high levels found in stomach. (Abal et al., 2018; Suzuki and Okada, 2018; Ferron et al., 2014; Louzao et al., 2015; Solino et al., 2015; Huguet et al., 2020). Even though toxicity potential of DTX1 has remained underestimated as compared to that of OA, our results indicated that DTX1 might be more toxic than OA by voluntary ingestion in line with previous published *in vivo* (Abal et al., 2018; Suzuki and Okada, 2018) and *in vitro* experiments (Ferron et al., 2014; Louzao et al., 2015; Solino et al., 2015; Huguet et al., 2020)}.

5. Conclusions

Considering all the above, here we report for the first time that a single ingestion of 90 μg DSTs/kg bw through voluntary intake of bread is a non-invasive method suitable for animal welfare and for simulating human consumption of DSTs in the EU regulatory limit. At this dose OA, DTX1 and DTX2 were rapidly absorbed, showing all of them diarrhea in mice in the first 2 h. Pharmacokinetic characteristics of OA contributes to its toxicity with high absorption and well distribution in gastrointestinal organs causing significant changes in the stomach weight, macroscopic characteristics, and diarrhea that could be detected until the toxin is finally excreted. DTX1 is well distributed in gastrointestinal organs with the highest concentration peak in stomach 6 h after ingestion where macroscopic changes were observed. Diarrhea was detected in DTX1 treated mice in all endpoints as shapeless soft feces up to 24 h. This toxic effect last longer with DTX1 than with the other DSTs due to the lower excretion. The previously described low toxicity of DTX2 is also due to the scarce absorption and important excretion in feces, that could explain the fast disappearance of diarrhea. In conclusion, our data indicate that toxic effects of 90 μg DSTs/kg bw by voluntary intake are related to their pharmacokinetics and should be considered for accurate risk assessments.

CRediT authorship contribution statement

Luis Rodríguez-Santos: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Celia Costas:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **M. Carmen Louzao:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Eva Cagide:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Mercedes Alvarez:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Inés Rodríguez-Cañás:** Methodology, Formal analysis. **Sandra Raposo-García:** Visualization, Methodology, Investigation, Data curation. **Carmen Vale:** Writing – original draft, Methodology, Formal analysis, Data curation. **Mercedes R. Vieytes:** Writing – review & editing, Supervision, Methodology. **Manuel Lolo:** Supervision, Resources, Funding acquisition. **Luis M. Botana:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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