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Effects of patulin and ascladiol on porcine intestinal mucosa: an *ex vivo* approach

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Abstract

Patulin (PAT) is a secondary metabolite mainly produced by *Aspergillus* and *Penicillium* that is frequently found contaminating apples and rotten fruits. Patulin can be transformed in potentially less toxic compounds such as ascladiol (ASC). Toxic effects of patulin were described in rats and in *in vitro* models, however concerning ascladiol, data are restricted to metabolic pathways. The aim of the present study was to evaluate the effects of different concentrations of PAT (10 μ M, 30 μ M, 100 μ M) and ASC (30 μ M, 100 μ M) on intestinal tissue using the jejunal explant model. Explants from pigs were exposed for 4 hours to PAT and ASC and after this period were processed for histological, morphometrical and immunohistochemical analysis. Mild histological changes were observed in jejunal explants exposed to PAT and ASC, however no significant difference in the lesional score or villi height was observed between the PAT/ASC-groups and the control. Also, explants exposed to 100 μ M of PAT showed a significant decrease in goblet cells density and a significant increase in cell apoptosis. These results indicate that high levels of patulin can induce mild toxic effects on intestinal mucosa whereas ascladiol apparently is non-toxic to intestinal tissue.

Keywords: intestinal explants, mycotoxins, goblet cells, histopathology, toxicity.

1. Introduction

Mycotoxins are secondary fungal metabolites and are found as food contaminants with harmful impact on human and animal health. Their occurrence in commercialized food and feed is a consequence of fungal attacks on crops in the field and/or on stored products (Ferrer et al., 2015; Ianiri et al., 2016).

Patulin (PAT) is a polyketide mycotoxin mainly produced by *Aspergillus*, *Penicillium* and *Byssochlamys* (Mohan et al., 2012). It is a frequently found contaminant in spoiled fruits, especially apples and related products (Beretta et al., 2000; Puel et al., 2010; Yang et al., 2014), but also described in vegetables products (Lugauskas et al., 2005), stored cheese and cereal products (Lopez-Diaz et al., 1996; Lopez-Diaz and Flannigan, 1997). This mycotoxin is easily transferred into the products during the processing owing to its solubility in water and stability to heat in acidic medium (Raiola et al., 2012).

The World Health Organization (WHO) established a safety level of 50 µg/L (0.32 µM) for apple juice (Food and Agriculture Organization of the United Nations/WHO, 2003) which was taken over by many European Union countries and the United States (Commission of the European Communities, 2006; Glaser and Stopper, 2012). The maximum permitted levels of PAT have been set at 50 µg/kg for apple based products, 25 µg/kg for solid apple products and maximum level of 10 µg/kg for all products intended for infants and young children (Appell et al., 2009; Hawar et al., 2013). Patulin levels in apple juices range from 0.7 to 845 µg/L worldwide (Oroian et al., 2014; Rahimi and Rezapoor Jeiran, 2015; Saxena et al., 2008), however levels until 16,402 and 44,572 µg/kg were reported in conventional and organic fresh apples, respectively (Piemontese et al., 2005).

In vivo studies have indicated that acute ingestion of PAT (48 mg/kg) induces toxic effects to the gastrointestinal tract of rats, including mucosal ulceration and inflammation (McKinley and Carlton, 1980). Mice submitted to an ingestion of 152.5 µg/kg of PAT for six weeks showed liver necrosis, glomerular degeneration and neurotoxicological effects (Al-Hazmi, 2014). Additionally, intestinal cell lines (HT-29-D4 and Caco-2-14) exposed to PAT (15 to 100 µM) showed a significant decrease of transepithelial resistance (TER) without major signs of toxicity (Mahfoud et al., 2002). On the other hand, toxic effects were reported in kidney cell lines (Riley and Showker, 1991; Pillay et al., 2015), chinese hamster ovary cell line (CHO-K1) (Ferrer et al., 2009) and human promyelocytic leukaemia (HL-60) cells (Liu et al., 2007) exposed to 0.1 to 100 µM of PAT.

The toxic effects that PAT exerts on cells may be due to its electrophilic nature, reacting with sulphhydryl moieties, and cross linking proteins and peptides (Ciegler et al., 1976; Mohan et al., 2012) including covalent inactivation of anti-oxidants (Fliege and Metzler, 2000). This way, PAT induces oxidative stress by lowering the concentration of the antioxidant peptide glutathione, and through generation of reactive oxygen species (ROS) (Barhoumi and Burghardt, 1996). ROS generation plays a role in the molecular events leading to apoptotic processes particularly by inducing peroxidation of membrane lipids and oxidative DNA damage (Ferrer et al., 2009; Zhou et al., 2009; De Melo et al., 2012). There is increasing evidence supporting a role for apoptosis in the toxicity of PAT (Saxena et al., 2009; De Melo et al., 2012; Kwon et al., 2012).

Patulin can be transformed in potentially less toxic compounds such as ascladiol (ASC) and desoxypatulinic acid. Previous studies using *Saccharomyces cerevisiae* (Suzuki and Iwahashi, 2011; Shao et al., 2012), *Rhodosporidium kratochvilovae* (Castoria et al., 2011)

and *Gluconobacter oxydans* (Ricelli et al., 2007) showed that these microorganisms are able to reduce PAT levels by a biotransformation process. There is an increasing interest in the use of yeasts as a biocontrol alternative to reduce or avoid patulin production in fruits, especially apples. Nevertheless, toxicological information about the resulting metabolites is scarce (Suzuki et al. 1971; Tannous et al. 2016).

Over the years, *in vivo* models were conducted to evaluate systemic effects of patulin (Speijers et al., 1988; Gc et al., 1998; Saxena et al., 2009; De Melo et al., 2012) and *in vitro* models reported individual cellular response to this mycotoxin (Schumacher et al., 2005; Zhou et al., 2009; Glaser and Stopper, 2012), but no *ex vivo* model was performed to achieve toxicological effects of PAT or ASC on intestinal mucosa. Moreover, histological aspects were rarely focused in these previous reports. In the context of further studies in intestinal toxicity induced by PAT and ASC, this report aimed to evaluate the effects of different concentrations of patulin and its metabolite ascladiol on porcine jejunal explants. To assay this, we performed histological, immunohistochemical and morphometrical analysis on intestinal tissue using an *ex vivo* model. Intestinal explants from pigs allow preservation of the normal histological structure (Basso et al., 2013) and represent a less costly model.

2. Materials and Methods

2.1 Animals

For explants sampling, six male 4-5 week-old crossbred piglets were used, housed in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France). The experimental procedures were conducted in accordance with European Guidelines for the Care and Use

of Animals for Research Purposes and were approved by the INRA local ethical committees for animal experimentation (C3155513).

2.2 Patulin and Ascladiol

Patulin standard was acquired from Sigma-Aldrich (Saint Quentin Fallavier, France). The E-ascladiol standard used in this study was obtained by chemical synthesis as previously described (Shao et al., 2012; Tannous et al., 2016). Mycotoxins were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20°C until use. Patulin was dissolved to a final concentration of $10\ \mu\text{M}$, $30\ \mu\text{M}$ and $100\ \mu\text{M}$ and ascladiol to $30\ \mu\text{M}$ and $100\ \mu\text{M}$.

2.3 Jejenum explants culture

The animals were used to obtain explants of jejunal tissue and the procedures for the culture of explants were performed as previously described (Kolf-Clauw et al., 2009). Fragments of 5 cm of medial jejunum were sampled immediately after euthanasia, and washed with buffered saline solution (PBS) and opened longitudinally. Explants were incubated for 4 h in Dulbecco's modified Eagle medium enriched with glutamine (Gibco, Cergy-Pontoise, France), supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids (Sigma-Aldrich) and 0.5% gentamicin (Eurobio, Courtaboeuf, France) at 37°C under a CO_2 -controlled atmosphere with orbital shaking.

2.4 Explants exposure to patulin and ascladiol

The explants were deposited with the mucosa facing upwards (2 explants/well) and incubated at 37°C for 4 hours in the presence of PAT at $10\ \mu\text{M}$, $30\ \mu\text{M}$ and $100\ \mu\text{M}$ and ASC at $30\ \mu\text{M}$ and $100\ \mu\text{M}$.

2.5 Histological and morphometric analysis

After the incubation period, explants were fixed in 10% buffered formalin solution, dehydrated in increasing alcohols and embedded in paraffin for histological analysis. Explants were sectioned of 5 μm thicknesses parallel to the villi axis, stained with hematoxylin and eosin (HE) or periodic acid of Schiff (PAS), and mounted with coverslips. The histological changes were evaluated using an adapted tissue score based on the intensity and severity of lesions as previously described (Cheat et al., 2015). The maximum score (39) indicates the overall integrity of the intestine. The criteria included in tissue score were number of villi, villi atrophy, villi fusion, cellular necrosis, presence of cellular debris, interstitial edema and morphology of enterocytes. The lesional score was calculated by taking into account the degree of severity (severity factor) and the extent of each lesion (according to intensity or observed frequency (scored from 0 to 3) (Table 1). Villi height was measured as the distance between the crypt mouth and the top of the villi randomly on 10 villi using the Motic Image Plus 2.0 software (Motic Instruments, Richmond, Canada).

2.6 Goblet cells assessment

Goblet cells density was performed separately throughout villus and crypt axis in histological sections of jejunal explants stained with PAS. Positively stained goblet cells were counted randomly in five fields per slide at 40x magnification, and the means were subjected to statistical analysis.

Table 1. Criteria used to establish the lesional score – endpoints used and severity factors.

	Criteria (severity factor)	End-point	Score
Lesional score	Enterocyte morphology (2)	Columnar epithelium	3
		<50% cuboidal epithelium	2
		>50% cuboidal epithelium	1
	Apical denudation of villi (3)	Flattened epithelium	0
		0-11%	3
		12-40%	2
		41-70%	1
		71-100%	0
	Lesions of lamina propria (1)	Absent	3
		Localized <30%	2
Multifocal >31%		1	
Diffuse		0	
Morphological score	Villi fusion (2)	0-11%	3
		12-40%	2
		41-70%	1
		71-100%	0
		>30	3
	Number of villi (2)	20-29	2
		10-19	1
		<10	0
		0-11%	3
	Villi atrophy (2)	12-40%	2
		41-70%	1
		71-100%	0
		Absent	3
Debris		Localized <30%	2
	Multifocal >31%	1	
	Diffuse	0	

2.7 Apoptosis assessment

Evaluation of apoptosis was performed on jejunal explants using antibody anti-cleaved caspase-3 (CCasp3) (clone Asp 175, 1:200 dilution, Cell Signaling Technology, Beverly,

MA). The immunohistochemical procedures were performed as described previously (Silva et al., 2014). The immunoexpression of CCasp3 in jejunal explants was estimated by counting strongly positive immunostaining of cell cytoplasm in 500 epithelial cells at 400x magnification. The results were expressed as the mean number (\pm standard deviation) of immunostained cells. The immunohistochemical assay was performed in explants exposed to 100 μ M of patulin and in control explants considering the results obtained in goblet cells assessment.

2.8 Statistical analysis

The experimental design used in the present study was entirely randomized with 24 repetitions for each treatment (each explant representing one repetition). Data were statistically analysed by the free software Action 2.3 (Campinas, SP, Brazil) using normality (Shapiro-Wilk's test) and homogeneity (Bartlett) tests. When these two assumptions were met the lesional score, the intestinal morphometry and the number of goblet cells were analysed by ANOVA followed by Tukey's test. Student's t-test was used to analyse the mean number of positive immunostained caspase-3 cells. The p value of ≤ 0.05 was considered significant.

3. Results

3.1 Effects of patulin and ascladiol on histological and morphometric analysis

After four hours of incubation, untreated explants (control group) presented mild villi atrophy and a mean histological score of 29.32. Explants exposed to PAT and ASC showed similar histological aspects in all concentrations. Additionally, some explants treated with PAT (100 μ M) also exhibited apical villi necrosis and lateral intercellular

disruption (Fig.1). The mean histological score for the explants exposed to PAT (10, 30 and 100 μM) was 28.92, 29.45 and 29.50 and to ASC (30 and 100 μM) was 29.95 and 28.88, respectively. No statistical difference was observed in the lesional score between explants exposed to PAT and ASC compared to the control group.

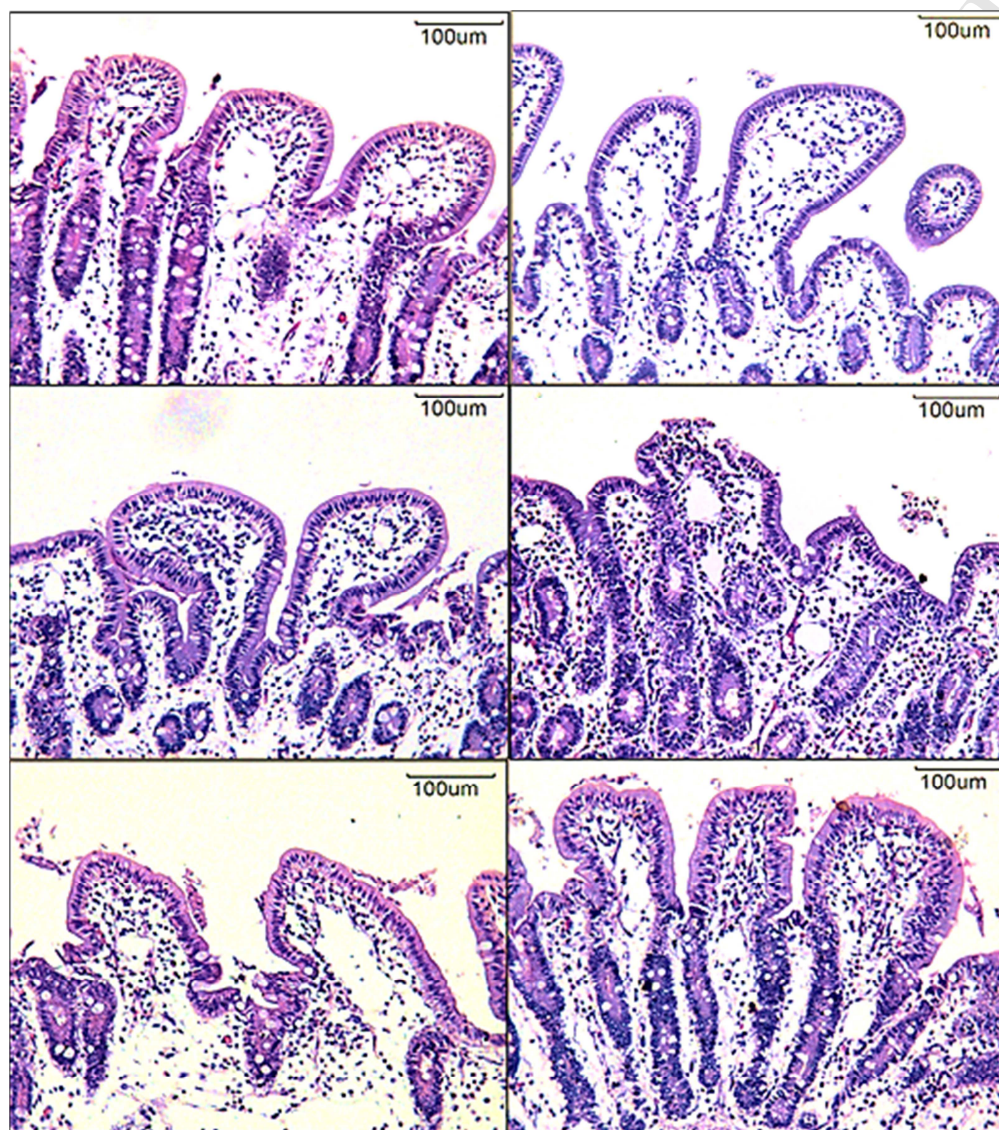


Figure 1. Histological aspects of jejunal explants exposed to patulin and ascladiol. Epithelial cells remained columnar in all treatments. Control group (A), Patulin 10 μM (B), Patulin 30 μM (C), Patulin 100 μM with cellular disruption (D), Ascladiol 30 μM (E), Ascladiol 100 μM (F). Haematoxylin-eosin, Bar 100 μm .

Mean villi height was assessed in explants exposed to different concentrations of PAT and ASC, however no significant difference was observed when compared to control group. The control group showed mean villi height of $155.62 \pm 8.43 \mu\text{m}$, while explants exposed to PAT (10, 30 and $100 \mu\text{M}$) and ASC (30 and $100 \mu\text{M}$) showed mean villi height of $154.16 \pm 11.63 \mu\text{m}$, $147.43 \pm 13.18 \mu\text{m}$, $148.02 \pm 10.66 \mu\text{m}$, $148.55 \pm 6.48 \mu\text{m}$ and $149.75 \pm 6.16 \mu\text{m}$, respectively.

3.2 Effects of patulin and ascladiol on goblets cells density

The number of goblet cells in explants treated with PAT $100 \mu\text{M}$ were significantly lower in villus ($p=0.05$) and crypts ($p=0.03$) comparing to the control group. Explants treated with $10 \mu\text{M}$ and $30 \mu\text{M}$ PAT and ASC remained statistically similar to control group (Fig.2)

3.3 Effects of patulin on apoptosis index

In order to evaluate a potential role of apoptosis in the decreased number of goblet cells an immunohistochemical assay was performed in jejunal explants. Considering that only the explants exposed to $100 \mu\text{M}$ of PAT showed a significant difference in this parameter compared to the control group, the immunohistochemical expression of CCasp3 was achieved in these two groups. A significant increase in the number of immunostained cells to CCasp3 was observed in the villi (6.47 ± 1.77) and the crypts (109.75 ± 14.30) of explants exposed to $100 \mu\text{M}$ of PAT when compared to the control group (2.56 ± 0.53 for villi, $p=0.05$; 46.26 ± 11.02 for crypts, $p=0.0089$).

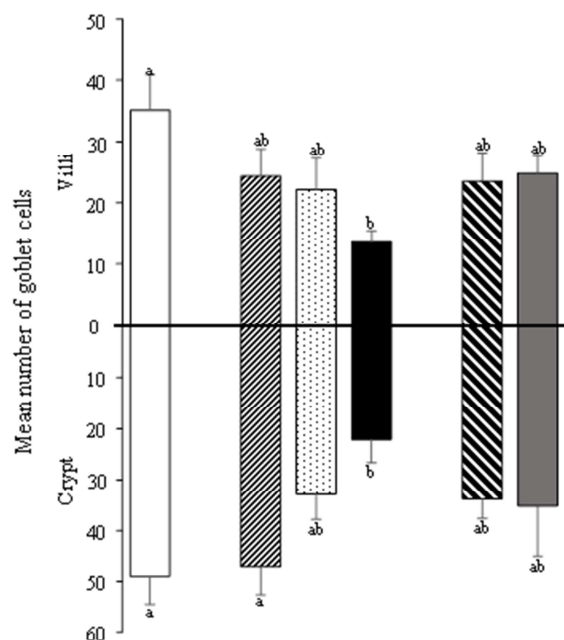


Figure 2. Mean goblet cell density on villi and crypts of explants exposed to control treatment (□), PAT to 10 μM (▨), PAT to 30 μM (▩), PAT to 100 μM (■), ASC to 30 μM (▧) and ASC to 100 μM (■). Values are mean \pm SD represented by vertical bars. a,b Mean values with unlike letters were significantly different ($p \leq 0.05$). Tukey's test. ($p \leq 0.05$).

4. Discussion

In the present study an alternative model to evaluate intestinal mucosal toxicity of PAT and ASC was used, based in previous studies (Pierron et al., 2015) where jejunal explants from pigs proved to be a reliable model to investigate the effects of food and feed contaminants, especially regarding the relevance of pig model-species compared to humans (Helke and Swindle, 2013). The doses of patulin used in this study were chosen to include the lowest observed adverse effect level (LOAEL) for chronic study in rats and a three and ten-fold higher dose (Becci et al., 1981). Levels of patulin in natural contaminations most frequently

are below the maximum permitted level (50 µg/kg) (Harris et al., 2009), however occasionally high levels of PAT (until 44,572 µg/kg) were reported (Piemontese et al., 2005). Alternatively, considering that no data about LOAEL was available for ascladiol similar doses were used.

In this study, the histological analysis revealed similar histological aspects in all explants exposed to PAT and ASC. Also, no significant difference in the lesional score or villi height was observed between explants exposed to different concentrations of these mycotoxins and the control group. Controversial results are reported in *in vitro* models. Caco-2 cell exposed to PAT (100 µM) (Mahfoud et al., 2002; Mclaughlin et al., 2009) showed no change in apical, basolateral or basal sides, after 12 and 5 hours of incubation; moreover, epithelial monolayers remained confluent and expressed cell viability. On the other hand, exposure of Caco-2 cells to concentration above 12 µM of PAT induced a significant reduction in cell viability, mainly by changes in zonula occludens-1 levels (Assuncao et al., 2016). In addition, changes in transepithelial electric resistance (TEER) were also reported in intestinal cell lines Caco 2-14 and HT-29-D4 exposed to PAT (50 to 90 µM). The value of TEER is considered a good parameter for the protein cell junction organization and for the paracellular permeability (Mahfoud et al., 2002; Mohan et al., 2012; Assuncao et al., 2016). Altogether, literature data provide evidence of differences in toxicity according to cell type and patulin concentration, however, there is no previous study assessing the effects of ascladiol on intestinal tissue.

It is interesting to note that although no significant difference was observed in the lesional score between the mycotoxin-exposed groups and control explants, histological analysis revealed a mild enterocyte cell-cell contact disruption in jejunal explants treated with 100 µM of PAT. This change suggests that high levels of PAT induce a loss of tissue

integrity and may be related to alterations in the expression of TJs proteins (Katsuyama et al., 2014; Assuncao et al., 2016). The toxicity of PAT is thought to be due to the impairment of the intestinal barrier caused by the destruction of TJs in the epithelial cell layer (Assuncao et al., 2016) induced by ROS generation (Ferrer et al., 2009; Kawauchiya et al., 2011; Boussabbeh et al., 2015) and activation of apoptosis signaling (Wu et al., 2008; Boussabbeh et al., 2015; Zhang et al., 2015). These two proposed mechanisms are associated with the electrophilic properties of the conjugated double bond system, and include covalent inactivation of antioxidants and proteins (Fliege e Metzler, 2000).

Mucin synthesis and secretion play an important role as a physical barrier (highly glycosylated proteins) on intestinal mucosa (Pelaseyed et al., 2014). Changes in this dynamic may facilitate pathogen invasion and loss of tissue homeostasis. In this study a significant decrease (two fold) in goblet cells density was observed in explants exposed to high levels of PAT (100 μ M), both on villus and crypt region. Furthermore, a significant increase in cell apoptosis (~2.5 fold) was observed in explants exposed to 100 μ M of PAT. Taken together, these results suggest that high levels of PAT induce a reduction in the number of goblet cells by an apoptotic pathway. Apoptosis induced by a ROS-dependent mechanism has been previously described in human intestinal and kidney cells exposed to PAT. Specifically, PAT induces both an endoplasmic reticulum stress and a drop in mitochondrial membrane potential leading to activation of the caspase pathway (Boussabbeh et al., 2015; Zhang et al., 2015). In addition, a decrease in the expression of mucin-related genes was reported in explants exposed to deoxynivalenol resulting in a reduction in the synthesis of mucin (Pinton et al., 2015). To the best of author's knowledge it is the first report to evaluate goblet cell density and apoptosis induced by patulin using

the explant model; however the effects of PAT and ASC in mucin-related genes remain to be investigated.

Scientific data suggest that there is a wide variation in the results obtained among cell lines, time of exposure and concentration of toxins. Given the potentially high concentration of PAT in spoiled food products (Oroian et al., 2014; Van De Perre et al., 2014) and the high intestinal bioaccessibility of patulin (González-Arias et al., 2013) it is important to develop a database with results that will enable an understanding of the effects of this mycotoxin on the structure and function of the intestinal mucosa. Also, considering the potential use of microorganisms as biocontrol agents to reduce patulin contamination it is necessary to evaluate the toxicological effects of metabolites as ascladiol. Our results also demonstrated, for the first time, that ascladiol induced no toxic effects on the intestinal tissue.

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Conflict of interest

The authors declare no conflict of interest.

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Table legends

Table 1 - Criteria used to establish the lesional score – endpoints used and severity factors.

Figure legends

Figure 1 - Histological aspects of jejunal explants exposed to patulin and ascladiol. Epithelial cells remained columnar in all treatments. Control group (A), Patulin 10 μ M (B), Patulin 30 μ M (C), Patulin 100 μ M with cellular disruption (D), Ascladiol 30 μ M (E), Ascladiol 100 μ M (F). Haematoxylin-eosin (HE), Bar 100 μ m.

Figure 2. Mean goblet cell density on villi and cripts of explants exposed to control treatment (\square), PAT to 10 μ M (▨), PAT to 30 μ M (▩), PAT to 100 μ M (\blacksquare), ASC to 30 μ M (⊞) and ASC to 100 μ M (\blacksquare). Values are mean \pm SD represented by vertical bars. a,b Mean values with unlike letters were significantly different ($p \leq 0.05$). Tukey's test. ($p \leq 0.05$).

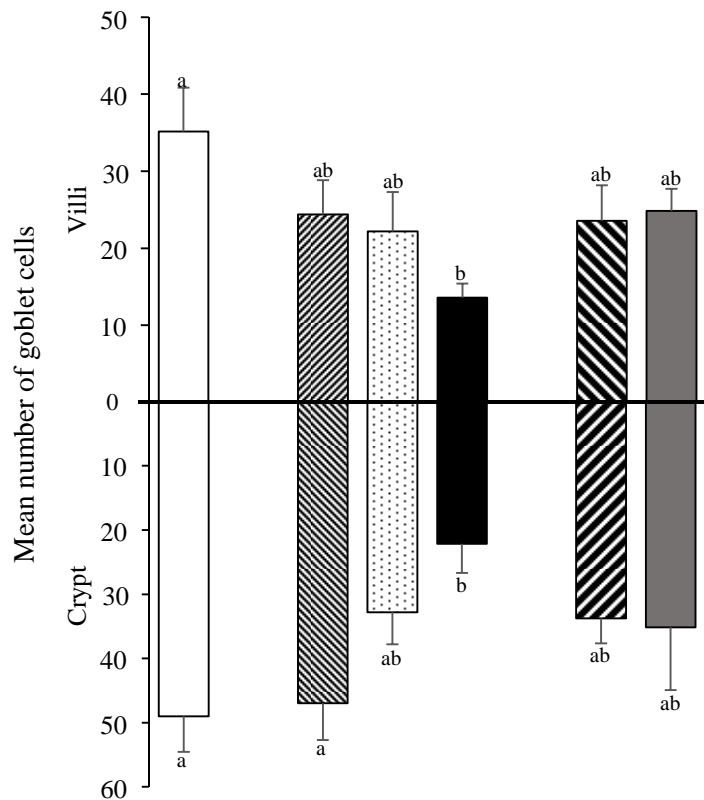


Figure 2. Mean goblet cell density on villi and cripts of explants exposed to control treatment (□), PAT to 10 μM (▨), PAT to 30 μM (▩), PAT to 100 μM (■), ASC to 30 μM (▧) and ASC to 100 μM (■). Values are mean \pm SD represented by vertical bars. a,b Mean values with unlike letters were significantly different ($p \leq 0.05$). Tukey's test. ($p \leq 0.05$).

Highlights

- Patulin induces mild toxic effects on intestinal tissue.
- Ascladiol induces no toxic effects on intestinal tissue.
- Biotransformation of patulin seems to be a reliable process of detoxification .

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