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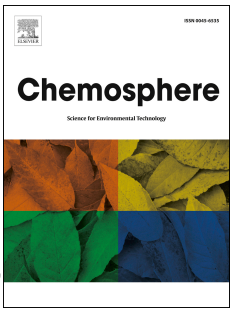

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Biological evaluation of microbial toxin degradation by microinjected zebrafish (*Danio rerio*) embryos

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Abstract

The use of microinjection of newly fertilized zebrafish eggs as an appropriate tool for qualifying the biodetoxification properties of toxin-degrading microbes was investigated. Ochratoxin A (OTA), bacterial degradation products of OTA and bacterial metabolites of the *Cupriavidus basilensis* ŐR16 strain were microinjected. Results showed that variations in the injected droplet size, and thus treatment concentrations, stayed within ±20%, moreover embryo mortality did not exceed 10% in controls, that is in accordance with the recommendations of the OECD 236 guideline. The highest lethality was caused by OTA with a significantly higher toxicity than that of bacterial metabolites or OTA degradation products. However, toxicity of the latter two did not differ statistically from each other showing that the observed mortality was due to the intrinsic toxicity of bacterial metabolites (and not OTA degradation products), thus, the strain effectively degrades OTA to nontoxic products. Sublethal symptoms also confirmed this finding.

Results confirmed that microinjection of zebrafish embryos could be a reliable tool for testing the toxin-degrading properties of microbes. The method also allows comparisons among microbial strains able to degrade the same toxin, helping the selection of effective and environmentally safe microbial strains for the biodetoxification of mycotoxins in large scale.

Keywords: *Cupriavidus basilensis*, mycotoxin, ochratoxin, biodegradation, biodetoxification

1. Introduction

Ochratoxin A (OTA) is a hazardous mycotoxin produced during the secondary metabolism of filamentous fungi belonging to the genera *Aspergillus* and *Penicillium* (Bui-Klimke and Wu, 2015). OTA is a potent nephrotoxic mycotoxin that has several harmful effects in Vertebrates, including fish, such as hepatotoxicity (Gagliano et al., 2006), teratogenicity (Haq et al., 2016; O'Brien et al., 2005) and immunosuppression (Marin and Taranu, 2015). OTA has been reported to play a role in the development of different types of tumors in Rodent models and humans (Pfohl-Leszkowicz and Manderville, 2007). Chronic OTA exposure proved to be a leading factor in mycotoxin-induced porcine nephropathy and Balkan endemic nephropathy (BEN) in humans (Stoev and Denev, 2013; Vrabcheva et al., 2000).

The toxin is present in various agricultural products (e.g., fruits, cereals, meats, coffee beans, spices) (Bui-Klimke and Wu, 2015) and survives many common food-processing procedures, such as roasting, brewing and baking, thus, it can be found in bread (Scudamore et al., 2004), juicy fruits (Fernández-Cruz et al., 2010), beer (Odhav and Naicker, 2002) and wine (Otteneder and Majerus, 2000). Because of its potential health risks, many countries and international organizations have introduced a limit value for the OTA content of cereals and cereal products (ECR, 2006; FAO, 2003).

Global occurrence of mycotoxins in the food chain is a problem worldwide, so several strategies have been developed to decrease mycotoxin levels in animal feeds and human food e.g. prevention, physical and chemical methods and biodegradation (Binder, 2007; EFSA, 2010). Among these, toxin biodegradation by microorganisms or their enzymes is the most promising approach which could be an important postharvest strategy to reduce or eliminate mycotoxin contamination.

There is growing need for the selection of microbial strains for efficient mycotoxin biodegradation in large scale use, which are able to eliminate the hazardous effects of a toxin

and its breakdown products in addition to the degradation of their chemical structure (Ferenczi et al., 2014; Sheikh-Zeinoddin and Khalesi, 2018; Vanhoutte et al., 2016). Traditional analytical and immunological methods are sufficient to test biodegradation of the parent compound, but they are unable to detect the toxic effects of potential degradation products and bacterial metabolites. In addition, biodegradation does not always mean biodetoxification. According to the statements described above and the scientific advice of EFSA (EFSA, 2010), it is important to develop and use new *in vivo* toxicological approaches for investigating biodegradation and detoxification efficiency directly.

Various microorganisms have been reported to be suitable for degrading and detoxifying OTA, some of them are highly efficient (Abrunhosa et al., 2014; Hathout and Aly, 2014). Two pathways may be involved in OTA microbiological degradation. The primary is the hydrolytic cleavage of the amide bond in OTA, resulting in the production of phenylalanine and ochratoxin α (OTα), which - in most cases – is the major degradation product. Since OTα and phenylalanine are presumably non-toxic, this mechanism can be considered as a detoxification pathway. The second is a hypothetical process where OTA is degraded via the hydrolysis of the lactone ring (Karlovsky Petr, 1999). In this case, the final degradation product is an opened lactones form of OTA, which has similar toxicity to the parent compound (Li et al., 1997; Xiao et al., 1996).

In the present report, *Cupriavidus basilensis* (ŐR16 strain), the first *Cupriavidus* species with proven OTA degradation potency has been selected. The strain ŐR16 can degrade almost 100% of OTA in solutions with concentrations below 20 mg/L in laboratory conditions during 5 days of incubation, and the major metabolite of OTA is OTα. The degradation efficiency of the strain was tested in mice, where neither the metabolites produced in a modified LB medium, nor the degraded OTA residuals evoked pathological disorders, or disturbed the expression of the examined genes (Ferenczi et al., 2014). Based on these

phenomena, the strain ŐR16 seems to be suitable for developing new *in vivo* test methods for Vertebrate models to examine and evaluate the detoxification ability of mycotoxin degrading microorganisms.

Zebrafish embryo tests are widely used bioassays in toxicological and ecotoxicological testing, and are often used to analyze organic-matter rich samples (e.g. waste water and sediment samples) (Braunbeck et al., 2005; Nagel, 2002). Since these assays should be carried out at temperatures above 25°C, many factors may interfere with toxicity evaluation, of which low oxygen supply in the embryo test vessel is one of the most important (Küster and Altenburger, 2008; Strecker et al., 2011). Deviations from oxygen saturation increase the frequency of malformations or suspension of embryo development, and distinction between effects of hypoxia and the toxicity of a sample is not always possible. For organic-matter rich samples, the microinjection of fish embryos could be an alternative method to eliminate the secondary effects of hypoxia.

Microinjection is a simple way to introduce substances into newly fertilized fish eggs. It has previously been used for testing polar and nonpolar substances in many fish species (Colman et al., 2004; Mizell and Romig, 1997; Walker et al., 1992). Effects on embryonic development are visible shortly after microinjection, and even minor toxic effects can be distinguished from background mortality and other sublethal symptoms. Although, microinjection of substances into the yolk of zebrafish eggs is feasible, introduction of accurate volumes (e.g. constant volumes) through a series of injections seems to be problematic so nominal and real injected volumes may be different (Schubert et al., 2014).

The objective of this *in vivo* toxicological study was to investigate whether microinjection of newly fertilized zebrafish eggs could be an appropriate tool for qualifying the biodetoxification efficiency of toxin-degrading microbes. Therefore OTA, breakdown products of OTA and bacterial metabolites of *Cupriavidus basilensis* ŐR16 strain were

injected into zebrafish eggs at different volumes and mortality and sublethal effects were compared. Additionally, we investigated the injected volume fluctuations during a series of microinjections, to see if desired treatment concentrations are reached and to ensure that the results are reliable.

2. Material and methods

2.1. Animal protection

The Animal Protocol was approved under the Hungarian Animal Welfare Law (XIV-I-001/2303-4/2012).

2.2. Zebrafish maintenance and egg collection

Laboratory-bred AB strain zebrafish were held in breeding groups of 30 females and 30 males at the Department of Aquaculture, Szent István University, Hungary, in a Tecniplast ZebTEC recirculation system (Tecniplast S.p.A., Italy) at 25.5°C ± 0.5°C, pH 7.0±0.2, conductivity 550±50 µS (system water) and light:dark period of 14 h:10 h. Fish were fed twice a day with dry granulate food (Zebrafeed 400-600 µm, Sparos Lda., Portugal) supplemented with freshly hatched live *Artemia salina* twice a week. Fish were placed in breeding tanks (Tecniplast S.p.a.) late in the afternoon the day before the experiment and allowed to spawn by removing the dividing walls next morning. Spawning of individual pairs was delayed through time to allow a continuous supply of 1-cell embryos.

2.3. Bacterial strain cultivation and metabolite preparation

The bacterial *Cupriavidus basilensis* ŐR16 strain (stored at −80 °C) was thawed on ice, streaked on Luria-Bertani (LB) agar plates (10 g tryptone, 5 g yeast extract, 9 g sodium-chloride and 18 g bacteriological agar (Biolab Ltd., Hungary) in 1L (pH 7.0) ion-exchanged water) and incubated at 28 °C for 72 hours. Then single colonies were inoculated into 50 mL 100% LB medium (10 g tryptone, 5 g yeast extract and 9 g sodium-chloride in 1L (pH 7.0) ion-exchanged water) in 250 mL flasks and cultures were grown for 120 h at 28 °C, 170 rpm in a shaking incubator (Sartorius Certomat BS-1, Germany). Liquid cultures were centrifuged at 3220 g, 4 °C for 20 min (Eppendorf 5810R, Germany), the pellet was resuspended in 50

mL 20% LB medium (100% LB medium diluted with ion-exchanged water), then was centrifuged again at the same conditions. The procedure was repeated twice. After resuspension, the optical density of the culture was measured at 600 nm (OD_{600}) (GENESIS 10S UV-VIS, Thermo Fischer Scientific) and adjusted to 0.6±0.05 to prepare bacterial inoculum. 5 mL bacterial suspensions were inoculated into 45 mL sterile 20% LB medium in triplicates and incubated on a laboratory shaker at 28 °C, 170 rpm for 120 h. Cultures were then centrifuged at 3220 g, 4 °C, for 15 min. Supernatants were filtered through 0.2 µm syringe filters (VWR International Ltd., Hungary) to gain bacteriologically sterile samples containing bacterial metabolites only. Samples were stored at −20 °C until microinjection.

2.4. Ochratoxin A biodegradation and OTA concentration measurement

Bacterial inocula (5 mL) were prepared as above, and added to 45 mL 20% LB medium containing OTA (7 mg/L final concentration). Similar inocula were prepared in parallel without OTA to test the effects of bacterial metabolites. Uninoculated LB medium (20%) contaminated by OTA (7 mg/L) was used as negative control. Both of the cultures and control were incubated at 28 °C, 170 rpm for 120 h in triplicates. After the incubation, cultures were centrifuged at 3220 g, 4 °C, for 20 min. Supernatants were filtered with 0.2 µm syringe filters, and samples were stored at −20 °C until microinjection.

For the measurement of OTA concentration, high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) was applied. Prior to measuring toxin concentration, 100 µL sample were mixed with 25 µL isotope-labelled internal standard ($^{13}C_{20}$-OTA), the mixture was evaporated under nitrogen gas, thereafter it was reconsituted in 50-50 V/V% A-B eluent (A : water, 5mM ammonium-acetate, 0.1% acetic acid ; B : methanol, 5mM ammonium-acetate, 0.1% acetic acid). For the separation Agilent 1100 HPLC (Agilent Technologies, USA) equipped with Agilent Zorbax C18 column (3.5µm, XDB-C18, 2.1 x

50mm) was used. 10 µL prepared samples were injected into the mobile phase containing A-B eluent. 400 µL/min flow rate and 40°C column temperature was set. 3200 QTRAP LC/MS/MS system (Applied Biosystems, USA) in positive ion mode was used for the determination of OTA concentration in samples. During the measurement, LOD was 2 µg/L and LOQ was 6 µg/L.

2.5. Microinjection

A Narishige (Japan Model PN-31) micropipette puller (heater level: 89.1, magnet sub level: 15.7, magnet main level: 84.3) was used to pull microinjection pipette tips (injection needle) (Narishige Japan G-1 borosilicate glass capillary, 1 mm o.d. x 0.6 mm i.d., 90 mm length). Injection needles were backfilled with 20 µL substance without air bubbles by a Microloader pipette tip (Eppendorf, Germany).

The needle was placed in the microinjection manipulator (microINJECTOR MINJ-2, TriTech Research Inc. Los Angeles, USA) connected to a nitrogen gas bottle. Injections were carried out under a stereomicroscope at 15× magnification (Leica LED2500, Leica Microsystems GmbH, Germany). Injection volumes were determined in immersion oil (Merck Ltd., Hungary, An affiliate of Merck KGaA, Darmstadt, Germany) on the basis of droplet diameters by a calibrated software (Leica M205 FA, Leica DFC 7000T camera, Leica Application Suite 3.4.2.18368, Leica Microsystems GmbH, Germany). Injection volumes were administered five times into the oil droplet until appropriate volume was achieved (pressure or capillary orifice size change). According to the sphere volume formula ($V=1/6\pi d^3$), a sphere diameter of 50 µm corresponded to an injection volume of 0.22 nL, 100 µm to 0.52 nL, 150 µm to 1.77 nL, and 200 µm to 4.17 nL. Injection volume needed to be measured and adjusted for each solution, concentration and control.

One-cell stage zebrafish embryos were lined up against the side of a microscope slide placed in a 10 cm diameter Petri dish. Excess water was removed with a plastic pipette. Treatment groups of 20 eggs were injected in a minimum of three replicates per treatment. Following microinjection, eggs were incubated in system water with methylene blue (2 mL 0.1% methylene blue in 1 L system water) (25°C ± 2°C) in 10 cm diameter Petri dishes. After 2 hours, coagulated and/or non-fertilized eggs were discarded and developing embryos were transferred in groups of twenty into 6 cm diameter Petri dishes. Embryos were then incubated in system water at 26°C ± 1°C and a 14 h:10 h-light:dark period and checked for lethal and sublethal effects under a microscope. System water was replaced in every 24 hours until 120 hpf (hours post-fertilization). Digital images of embryos (72 hpf) and larvae (120 hpf) in lateral orientation were taken under a stereomicroscope at 30× magnification (Leica M205 FA, Leica DFC 7000T camera, Leica Application Suite 3.4.2.18368, Leica Microsystems GmbH, Germany).

2.6. Determination of the variations in the injection volume

Zebrafish Ringer's solution (ZFR) (116 mM sodium-chloride, 2.9 mM potassium-chloride, 1.8 mM calcium-chloride and 5 mM HEPES (pH 7.2) (Sigma-Aldrich, Hungary) in system water, filtered with 0.2 µm syringe filters) was injected into the yolk of zebrafish eggs. Prior to treatments, microinjection parameters (pressure and capillary orifice size) were set according to the volumes calculated on the basis of injected droplet sizes in immersion oil. When the desired volume was reached, five eggs were injected, and the diameter of five droplets was measured again in immersion oil. This egg injection - droplet measurement cycle was repeated five times to test the accuracy of injection.

2.7. Effect of the highest used injection volume and the LB media on the viability of embryos

The effect of the largest injection volume (4.2 nL) on egg viability was tested with Zebrafish Ringer's solution, the negative control of the experiments. The effect of the bacterial growth medium and the effects of the solvent were tested following the injection of 4.2 nL of 20% LB medium and 20% LB medium with acetone (250 µL acetone in 50 mL 20% LB medium).

2.8. Determination of the initial OTA concentration of the reference curve

OTA (99.5% Fermentek, Israel) was dissolved in acetone (98.8% Sigma-Aldrich, Hungary) at 1000 mg/L concentration, of which 1; 7; 10 mg/L concentrations were prepared in 20% LB medium. OTA contaminated medium was injected in 0.22 nL, 0.52 nL, 1.77 nL and 4.17 nL volumes into the embryos to find the optimal concentration for the reference curve.

2.9. Examining the toxicity of samples derived from OTA degradation experiment

Samples containing ŐR16 metabolic products as well as OTA degradation products were injected in 0.22 nL, 0.52 nL, 1.77 nL and 4.17 nL volumes into the zebrafish embryos.

2.10. Examination of injected embryos

Embryo mortality was determined at 72 and 120 hpf on the basis of egg coagulation, the lack of somite formation and the lack of heart function. Sublethal effects were examined at 72 and 120 hpf, the endpoints were pericardial edema, yolk edema, tail deformation, craniofacial deformation and disintegrated abnormal embryo shape. Abnormalities were recorded separately, irrespective of the number of deformities per individual.

239 *2.11. Statistics*

240 Results were analysed and graphs were plotted by GraphPad Prism 6.01 (GraphPad Software,
241 San Diego, USA). Data were checked for normality with Shapiro-Wilk normality test and
242 non-compliance with the requirements of parametric methods was established. Significant
243 differences were verified by Kruskal-Wallis analysis with Dunn's multiple comparisons test.

244

3. Results and discussion

3.1 Examination of variations in the microinjection volume

In toxicology including ecotoxicology, the concentrations used should remain as stable as possible to obtain reliable results. The microinjection method may cause volume fluctuations, the rate of which depends on the injection time, the applied pressure, the diameter of the needle tip and the viscosity of the cytoplasm of the injected cell (Minaschek G. et al., 1989; Schubert et al., 2014). These volume variations cause concentration shifts, and so nominal and real concentrations may differ from each other.

The best method for volume determination is the measurement of droplet diameters in the yolk after each injection (Schubert et al., 2014). However, with diffuse substances – such as those used in these experiments – this is not possible, therefore droplet size was measured in immersion oil, prior to microinjection to the yolk. To examine alterations in the injection volume during the microinjection procedure, a microinjection series was carried out with zebrafish Ringer's solution and the diameter of injected droplets were measured after the injection of every 5 embryos. In general, no significant difference was observed between replicates compared to the desired diameter (Fig 1 A). Minimal and maximal droplet volumes calculated from the measured diameters are shown in Fig 1 B. The largest decrease in volume was detected in case of the 1.77 nL droplet size (17.51% (1.46 nL)), while the largest volume increase was seen in case of the 0.22 nL droplet size (18.18% (0.26 nL)).

According to the OECD 236 guideline for the Fish Embryo Toxicity Test, nominal and real concentrations should not differ from each other by more than ±20% (OECD236, 2013). In this experiment deviations from the nominal volume stayed within this range for all volumes tested, thus presumably our experiments would meet this basic requirement.

Results show that with the above described experimental settings, the method is dimensionally stable for all used droplet sizes, if the capillary is not clogged during injection.

3.2 Effect of the largest injection volume and the media on the viability of embryos

Prior to testing bacterial products, potential toxic effects of three basic media, the Zebrafish Ringer's solution, the LB medium, the medium supplemented with the solvent acetone (which served as a bacterial propagation medium and carrier for OTA) and the largest used injection volume (selected according to the work of Schubert and co-workers (2014)) was examined. The injection volume is a critical factor in postinjection embryo survival, but potentially does not cause egg trauma if the administered volume is bellow 10% of the total volume of the yolk (Walker et al., 1992). For the same substance and same concentration, smaller injection volumes cause less mortality and malformations in injected embryos (Zabel et al., 1995). According to these, LB media were administered in the largest droplet volume too.

As the conditions did not have significant toxic effects, only results for 5 days of exposure are shown in Figure 1 C. In the non-injected control no dead embryos were found, and the average mortality rate was also very low in case of the LB medium (5%), the solvent supplemented LB (5%) and the ZFR (5.83%) and there was no significant difference between treatments. Malformations were not detected either in injected or non-injected (control) embryos (Fig. 1 D).

The OECD guideline for fish embryo test allows a maximum of 10% lethality in the control during an experiment (OECD236, 2013). This criterion was fulfilled in this study, since LB media and ZFR caused lower lethality. Based on the mortality and morphology results, the injection settings and droplet sizes used here seemed to be suitable for further work, the examined conditions are not toxic to zebrafish embryos and so do not affect the outcome of subsequent tests.

294

295 *3.3 The effect of metabolites produced by the Cupriavidus basilensis ŐR16 strain on the*
296 *survival of microinjected embryos*

297 Bacterial metabolites produced during the primary metabolism of the strain (ŐR16) might
298 also have toxic effects on embryos, therefore the effect of the LB medium following 3 and 5
299 days of bacterial incubation was tested in 4 injection volumes (Fig. 2 A and B). The solution
300 decreased the survival rate of embryos at 72 and 120 hpf too and dose-response relationship
301 was found between injection volumes and lethality. After 72 hours of exposure, significant
302 increase was detected in mortality in the groups injected with 1.77 and 4.17 nL ($p < 0.05$)
303 compared to the control, and the group injected with the largest volume (4.17 nL) and the
304 group injected with 0.22 nL ($p < 0.01$). Mortality in the groups injected with the two largest
305 volumes was 22.92% (1.77 nL) and 26.15% (4.17 nL), with no significant difference between
306 the groups. Mortality increased in all injected groups after 120 hours of exposure, but
307 compared to the control, significant difference was only detected in the groups injected with
308 the two largest volumes where mortality was 32.92% (1.77 nL, $p < 0.01$) and 50.13% (4.17
309 nL, $p < 0.01$). Results clearly show that the strain ŐR16 produces toxic metabolites that –
310 following administration by microinjection – decrease the survival of zebrafish embryos.
311 Ferenczi et al. (2014) examined OTA biodegradation efficiency of the strain ŐR16 and the
312 toxicity of breakdown products derived from degradation in feeding experiments with mice.
313 Animals were exposed to ŐR16 metabolites via intragastric gavage once a day through 21
314 days. Toxic effects were examined via the expression of several marker genes and
315 histolopathological examination of the kidney and spleen. In mice, metabolic products of the
316 strain ŐR16 did not seem to be toxic compared to the control. According to the results
317 described above, zebrafish embryos seem to be more sensitive to the bacterial metabolites
318 than mice, however, difference may be due to different exposition pathways.

3.4 Determination of the initial OTA concentration for further experiments

In order to determine the initial OTA concentration for further degradation experiments, OTA was injected into the yolk of embryos in 1, 7 and 10 mg/L concentration, in different volumes. All concentrations fell within the degradable concentration range of strain ŐR16. Mortality was checked at 3 and 5 dpf and results were plotted on a dose response curve for mortality. The graph of the potentially optimal initial concentration should serve as a reference for further experiments even if toxicity is higher following degradation, so should meet the following requirements: the maximum mortality should not exceed that of the bacterial metabolic products and the curve should not reach its maximum early.

Mortality increased along with the injection volume in case of all three OTA concentrations, and reached the maximum after 72 hours of exposure in all cases. Mortality did not change significantly for 120 hours following exposure (Fig. 3 A and B).

The slope of the dose-response curve for 1 mg/L OTA was lower than the others and mortality maximum was reached only with the largest injection volume following 72 and 120 hours of exposure. In case of lower injection volumes, mortality was below 10%.

Dose-response relationship was detected between injection volumes and mortality in case of 7 mg/L OTA as well. Mortality increased gradually with injection volumes at 72 and 120 hours of exposure too, and the maximum (100%) was reached with the largest injection volume. From 0.52 nL, significant difference ($p < 0.05$) was detected in mortality compared to the control. Differences between mortality values of the groups injected with volumes ≤0.52 nL compared to the 1.77 ($p < 0.001$) and 4.17 nL injection volumes were also significant ($p < 0.05$), however, no significant difference was found in case of the two largest volumes (Fig. 3C and D). Mortality reached its maximum (75%) early with 10 mg/L OTA with the lowest injection volume (0.52 nL) and did not show to be higher with larger volumes.

On the basis of our results, 7 mg/L was selected to be an initial concentration in further experiments. The mortality curve of this concentration shown here served as reference for subsequent tests.

The present study was the first to examine acute toxic effects of OTA following microinjection, and high mortality was detected even after short exposures to low concentrations. However, these results are difficult to compare to the results of classical tests where embryos are exposed via waterborne exposure. It is still unclear how substances are distributed in the yolk following injection but it is inhomogenous in most cases, so presumably embryos are not exposed uniformly. Moreover, zebrafish embryos consume their yolk sac completely to 165 ±12 hpf (Litvak and Jardine, 2003), thus, some of the substance may remain unabsorbed during the exposition period presented here, however, with longer exposure the experiment would fall under animal testing regulations. The microinjection technique enables the administration of exact amounts, so theoretically it would be possible to determine doses per bodyweight as seen in feeding experiments with vertebrates.

3.5 Toxicity of samples derived from ochratoxin degradation experiment

In order to clarify the toxicity of OTA-metabolites produced during microbial toxin degradation with strain ŐR16, degradation products were microinjected in four concentrations into zebrafish embryos. Mortality was examined on the 3rd and 5th day of exposure.

Mortality increased with the injected volume as seen previously. At 72 hours of exposure, mortality in the non-injected control, and in the 0.22 nL and 0.52 nL injection volumes was bellow 10%, and did not reach 30% even with the highest volumes. No significant difference was observed between treated groups (Fig. 4 B). At 120 hours of exposure, dose-response relationship was found between the injected volume and embryo mortality, as mortality increased gradually along with the injection volume and reached 38.5% in the largest volume.

Statistically significant decrease was detected in the number of survivals in the groups injected with 0.52 nL, 1.77 nL and 4.17 nL compared to the non-injected control ($p < 0.05$), and the two largest injection volumes compared to 0.22 nL ($p < 0.05$) (Fig. 4 C).

OTA degrading efficiency of strain ŐR16 was tested prior to exposure and it was found to be 95.6% (Fig. 4 A). OTA degradation of the strain ŐR16 is possibly mediated by a peptidase enzyme. Ferenczi et al. (2014) showed that the major metabolite of OTA degraded by strain ŐR16 is ochratoxin alpha (OTα). They found that OTA content in the supernatants decreased gradually, OTα content increased in parallel during the 5-day incubation period and OTA was completely degraded (94% decrease was measured by ELISA and 100% by HPLC), that is in accordance with the results of the above described experiments. OTα is not potentially toxic, according to the results of previous Vertebrate studies (Bruinink, 1998; Ferenczi et al., 2014). Haq and co-workers (2016) tested the toxicity of OTα with ZETA test on zebrafish embryos in concentrations ≤2.5 μM. In contrast to OTA, no significant difference was detected between the mortality of embryos exposed to OTα and the untreated negative controls during the 5 days exposure. On the basis of these, mortality in our experiments is probably due to other metabolites of strain ŐR16.

Ferenczi et al. (2014) also studied OTA degradation products of strain ŐR16 in mouse feeding experiments. Subchronic exposure did not cause mortality in mice and physiological or gene expression alterations in the examined organs, compared to controls. However degradation products were lethal to injected zebrafish embryos, so the zebrafish embryo is probably a more sensitive model, than the mouse.

3.6 Comparison of mortality values of 7 mg/L OTA, and the bacterial and degradation products of the strain ŐR16

Mortality caused by 7 mg/L OTA, and the bacterial and degradation products of the strain ŐR16 were plotted on joint graphs. In order to investigate the degradation characteristics of the bacterial strain, mortality values of equal volumes were compared to each other (Fig. 5 A and B).

Following 72 hours of incubation, mortality did not show significant difference between groups injected with the smallest volumes. In case of larger volumes, there was no difference between results of the bacterial metabolites and the degradation products of the strain, however, mortality values of 7 mg/L OTA differed significantly from these ($p < 0.05$). Highest mortality was caused by 7 mg/L OTA injected in 0.52 nL and above.

120 hours after microinjection, no significant difference was seen between the mortality values of groups injected with 0.22 nL. In case of the groups injected with 0.52 nL, significant difference was detected between 7 mg/L OTA and the metabolites of the strain ŐR16 ($p < 0.05$). Mortality values of the degradation products of strain ŐR16 did not differ nor from that of the bacterial metabolites neither from the OTA solution. In larger volumes, only mortality values of 7 mg/L OTA differed significantly from other groups ($p < 0.001$ - degradation products, $p < 0.01$ – ŐR16 bacterial metabolites), however, bacterial metabolites of the strain and degradation products of OTA did not show significant difference. The highest mortality was detected in 7 mg/L OTA injected in 0.52 nL and above.

As no statistical difference was found between the mortality values of the bacterial and degradation products, it can be concluded that OTA breakdown products are not toxic, and mortality is probably caused by metabolites of the strain ŐR16. Results also show that exposure via microinjection is a potential, functional, alternative way to test the detoxification efficiency of toxin degrading microbes on zebrafish embryos *in vivo*. Mortality in itself may provide a sufficient endpoint when testing the differences between the toxicity of the bacterial metabolites of a strain and the degradation products of the toxin following microinjection, and

toxicity of toxin degradation products can be predicted. There was no detectable difference between the mortality curves of 3 and 5 days of exposure, so it seems that a 3 days exposure period is sufficient for studying the degradation characteristics of bacterial strains.

3.7 Sublethal effects in injected embryos

Beyond mortality, sublethal endpoints were also analyzed in treated embryos following 72 and 120 hours of exposure. Generally, compared to the non-injected controls all treatments with all injected volumes increased the frequency and severity of developmental deformities (Fig. 6 A and B). Following 72 hours of exposure, the highest frequency of morphological disorders was detected in the 7 mg/L OTA group, and in some replicates of treatments with the highest volumes of this concentration, all surviving embryos showed abnormalities. A statistically significant difference was only observed between the 1.77 nL OTA (7 mg/L), and 1.77 nL samples containing bacterial metabolites or degradation products ($p < 0.01$). Following 120 hours of exposure, it was also evident that compared to other treatment groups the ratio of deformed embryos was the highest in the groups treated with OTA from 0.52 nL and above. Statistically significant differences were observed in ŐR16 bacterial metabolites ($p < 0.01$) and breakdown products of OTA ($p < 0.05$) compared to 7 mg/L OTA, injected in 1.77 nL. Significant differences were also found between OTA 7 mg/L and metabolites of strain ŐR16 ($p < 0.01$) or degradation products ($p < 0.05$), injected in 4.17nL. However no significant difference was detected between the deformation frequencies in the groups injected with the bacterial metabolites of the strain and OTA degradation products during the whole exposure period with any injection volumes.

It can be concluded that notwithstanding the significant differences detected in morphology, OTA degradation products seem to be nontoxic on the basis of deformation frequencies, however the metabolites of the strain were proved to be toxic.

Figure 7. shows representative development dysfunctions in embryos from treatment groups with statistically significant differences. Following 3 days of OTA injection (1.77 nL) embryos displayed craniofacial deformities, small eyes, curvature of the body axis, yolk deformities, reduced growth rates and edemas in some cases. Most of them have previously been described in OTA treated zebrafish embryos (Haq et al., 2016), and teratogenic effect was observed at sub-micromolar concentrations with an EC50 of 20 nM OTA.

Similarly to zebrafish OTA proved to be teratogenic in the amphibian *Xenopus laevis* model (FETAX) too, causing mainly craniofacial deformities (O'Brien et al., 2005) like in the experiments described above. These developmental abnormalities (craniofacial deformities) were also detected in a wide range of Vertebrates, including rats (Brown and Purmalis, 1976), mice (Arora, 1983), hamsters (Hood et al., 1976) and chicken (Wiger and Starrmer, 1990). Decreased hatching rate described by Haq et al. (2016) was not seen in our experiments.

Embryos injected with the same volume (1.7 nL) of bacterial metabolites and OTA degradation products displayed shorter body, yolk sac deformations, grey coloration in the yolk, pericardial edema, small eyes and deformities of lower facial structures in embryos following 3 days of injection. Curvation of the body as a common sign of OTA exposure has not been detected.

Five days after microinjection, sympthoms got more pronounced in OTA treated embryos and severe deformations appeared all through the body. Embryos injected with bacterial metabolites of the strain ŐR16 and OTA degradation products displayed shorter body, yolk sac deformations, pericardial edema, edema around the abdomen, small eyes, small and not well defined olfactory region and deformities of lower facial structures on the 5th day of exposure. As in 3 dpf exposed embryos, curvation of the body axis was not seen here either.

In contrast, OTα did not seem to be toxic in Vertebrates. Haq and co-workers (2016) examined the effects of OTα (along with OTA) on zebrafish embryos and neither

468 teratogenicity nor mortality differed significantly from that of the negative control embryos
469 during 5 days of exposure. Ferenczi *et al.* (2014) demonstrated apparent hydrolysis of OTA to
470 OTα, and consequent detoxification by using a bacterial species *Cupriavidus basilensis*, as
471 evidenced by comparative toxicological studies in a mouse model of nephrotoxicity.
472 In the present study, morphological examination showed that phenotype of OTA treated
473 embryos differed significantly from the morphology of embryos exposed to bacterial
474 metabolites or OTA degradation products in both experimental time points, however embryos
475 in the latter groups showed similar phenotypes. In conclusion, it seems that strain ŐR16
476 degrades OTA to nontoxic metabolites, the strain is able to degrade OTA even in 7 mg/L
477 concentration, and deformations resulted from the injection of OTA degradation products are
478 probably due to the metabolites of the bacteria. In addition, zebrafish exposed via
479 microinjection appeared to be more sensitive to the metabolites of strain ŐR16 than mice.
480 All injected solutions contained high levels of organic matter. No deformation implied
481 oxygen deprivation in morphological examinations of exposed zebrafish embryos (Küster and
482 Altenburger, 2008; Strecker et al., 2011). Results suggest that microinjection can be an
483 alternative way to test samples with high organic matter content.
484 High organic matter content of samples often causes hypoxia during zebrafish embryo tests,
485 and its effects (developmental disorders, suspension of embryo development) can hardly be
486 differentiated from those of the sample itself (Küster and Altenburger, 2008; Strecker et al.,
487 2011). With microinjection hypoxic effect of such samples can be avoided and results can
488 easily be evaluated.
489

4. Conclusions

Microinjection is a simple way to introduce organic matter-rich test substances into newly fertilized fish eggs and helps to eliminate hypoxia that cause a wide range of secondary effects. If the method is well optimized, injection volume variations can be kept within ±20%, according to the OECD 236 test guideline's recommendations and so result reliability can be ensured.

Results clearly showed that investigation of zebrafish embryos microinjected with toxin solutions, metabolites of bacterial strains and OTA degradation products could provide an alternative way for studying the toxin detoxification-properties of microbial strains. The zebrafish embryo – thanks to their sensitivity – proved to be a good model for the studies. Toxicity differences between substances may be detected even after 3 days of exposure on the basis of mortality, that can be completed and further refined by the evaluation of sublethal data.

Microinjection enables the selection of microbial strains that are able to degrade the toxin and the identification of the most effective and environmentally safe microbes from the selected strains.

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520 **5. References**

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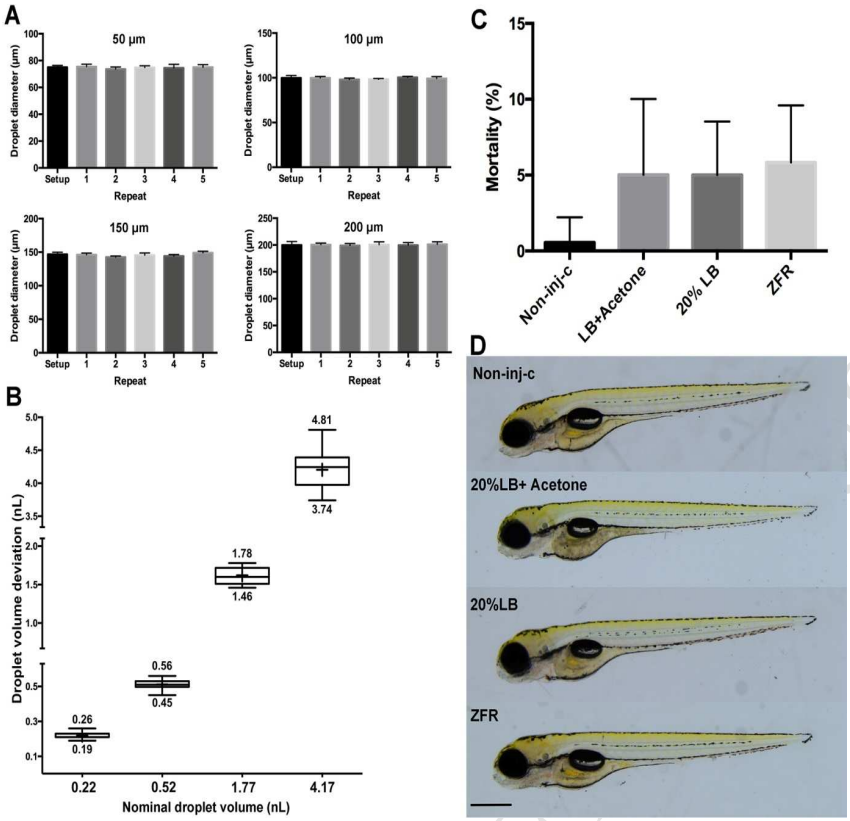
643 **Tables and figures**

644



645 **Fig1 Variations in the diameter (A) and volume (B) of the injected droplet and mortality**
646 **(C) and morphology (D) effects of control solutions.** The largest decrease in volume was
647 detected in case of the 1.77 nL droplet size (17.51% (1.46 nL)), while the largest volume
648 increase was seen in case of the 0.22 nL droplet size (18.18% (0.26 nL)). Droplet diameter
649 and volume stayed within ±20%, and no significant difference was detected between
650 measurements. Average mortality rate of 120 hpf embryos injected with 4.17 nL was very low
651 in all cases ((Non-inj-c (non-injected control): 0%, 20% LB: 5%, 20% LB + Acetone: 5%,
652 ZFR (Zebrafish Ringers's solution): 5.83%). There was no significant difference between
653 treatment groups and no malformations were detected in any case. Scale bar: 500µm.

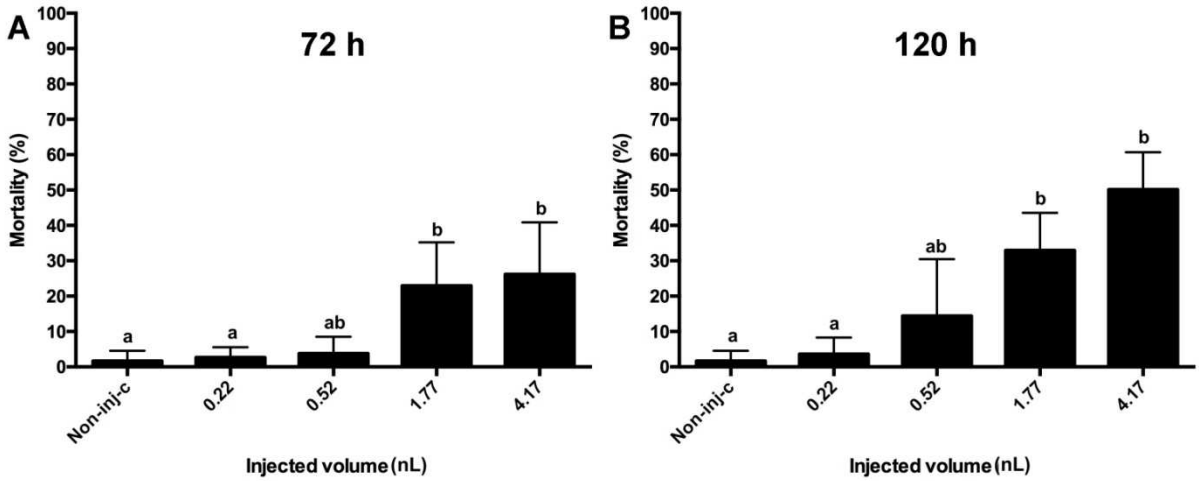


Fig 2 Effects of the metabolites of *Cupriavidus basilensis* ŐR16 strain, injected in different volumes, on the mortality of zebrafish embryos at 72 (A) and 120 hpf (B). After 72 hours of exposure, statistical significant differences were observed between the non-injected control and 1.77 nL ($p < 0.05$), non-injected control and 4.17 nL ($p < 0.05$), 0.22 nL and 4.17 nL ($p < 0.01$). Lethality was below 10% in the non-injected control, 0.22 nL and 0.52 nL. After 120 hours of exposure statistical significant differences were observed between the non-injected control and 1.77 nL ($p < 0.01$), non-injected control and 4.17 nL ($p < 0.01$), 0.22 nL and 4.17 nL ($p < 0.001$). Mortality was below 10% in the non-injected control and 0.22 nL.

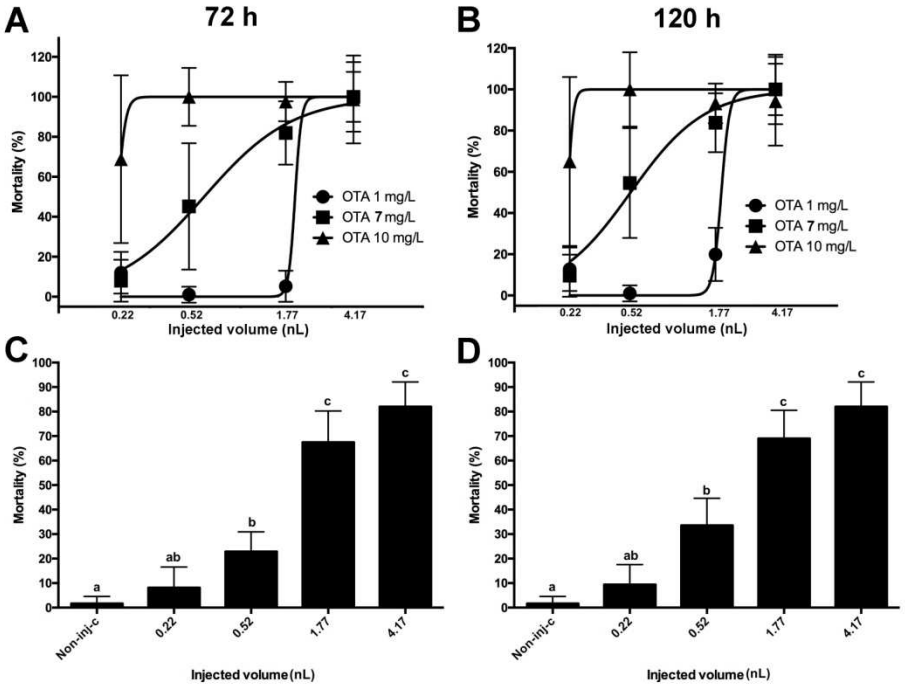
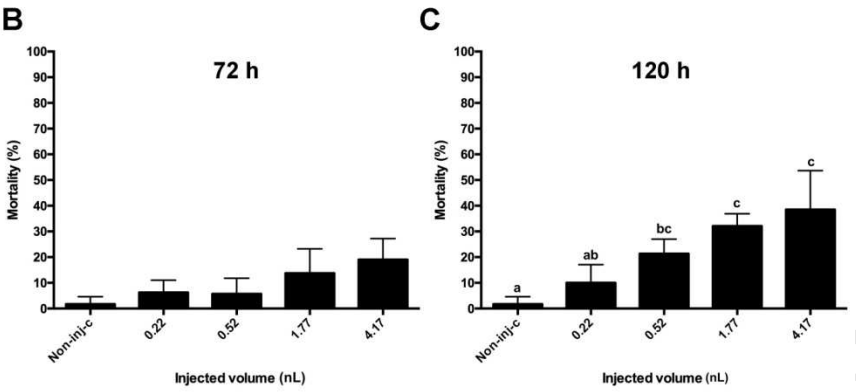


Fig 3 Effects of ochratoxin A (OTA) injected in different concentrations and volumes on the mortality of 72 (A) and 120 (B) hpf zebrafish embryos and the effects of 7 mg/L OTA injected in different volumes on the mortality of 72 (C) and 120 (D) hpf zebrafish embryos. At 72 hpf, lethality results in the non-injected control were below 10%. Statistical significant differences were observed in the 0.52 nL ($p < 0.05$), 1.77 nL ($p < 0.05$) and 4.17 nL ($p < 0.01$) groups compared to the non-injected control. Significant differences were detected between 0.22 nL and 1.77 nL ($p < 0.01$), 0.22 nL and 4.17 nL ($p < 0.0001$), 0.52 nL and 1.77 nL ($p < 0.0001$), 0.52 nL and 4.17 nL ($p < 0.05$) (C). At 120 hpf lethality results were below 10% in the non-injected control. Statistically significant differences were observed between the non-injected control and 0.52 nL ($p < 0.05$), 1.77 nL ($p < 0.05$) and 4.17 nL ($p < 0.01$) groups. Significant differences were detected between 0.22 nL and 1.77 nL ($p < 0.01$), 0.22 nL and 4.17 nL ($p < 0.0001$), 0.52 nL and 1.77 nL ($p < 0.001$), 0.52 nL and 4.17 nL ($p < 0.05$) (D).

A

OTA degradation efficiency			
	Replicate 1.	Replicate 2.	Replicate 3.
Before degradation	6.6 mg/L	6.7 mg/L	6.7 mg/L
After degradation	0.39 mg/L	0.28 mg/L	0.2 mg/L
Degradation efficiency (%)	94.1%	95.8%	97%



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683 **Fig 4 Ochratoxin A (OTA) degradation efficiency of *Cupravidus basiliensis* ŐR16 strain**
684 **following 120 hours of incubation with 7 mg/L OTA (A) and effects of OTA degradation**
685 **products injected in different volumes on the survival of zebrafish embryos at 72 (B)**
686 **and 120 (right) hpf (C).** At 72 hpf, no significant difference was observed between treatment
687 groups and mortality was less than 10% in the non-injected control (Non-inj-c), 0.22 nL and
688 0.52 nL groups. At 120 hpf, mortality was below 10 % in the non-injected control. Statistical
689 significant differences were observed between the non-injected control and 0.52 nL ($p < 0.05$),
690 non-injected control and 1.77 nL ($p < 0.05$), non-injected and 4.17 nL ($p < 0.05$).
691 Significant differences were detected between 0.22 nL and 1.77 nL ($p < 0.05$), 0.22 nL and
692 4.17 nL ($p < 0.05$).

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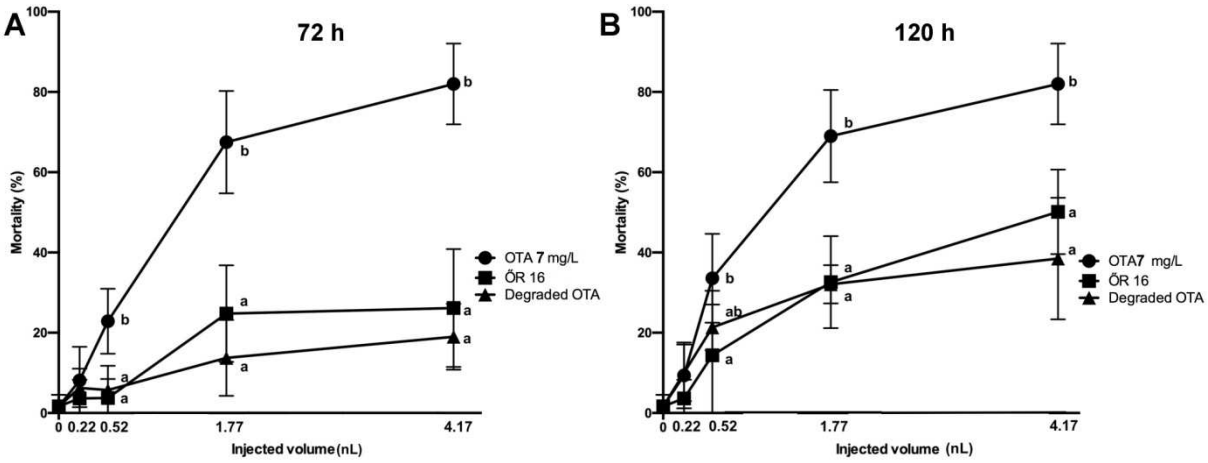

Fig 5 Effects of 7 mg/L Ochratoxin A (OTA 7 mg/L), bacterial metabolites (ŐR16) and OTA degradation products (degraded OTA) derived from the biodegradation experiment with *Cupriavidus basilensis* ŐR16 strain on the survival of 72 (A) and 120 (B) hpf zebrafish embryos. At 72 hpf mortality in the non-injected control was below 10%. Statistical significant differences were observed between OTA and degraded OTA ($p < 0.05$), OTA and ŐR16 ($p < 0.05$) in case of 0.52 nL, OTA and degraded OTA ($p < 0.01$), OTA and ŐR16 ($p < 0.01$) in case of 1.77 nL, and OTA and degraded OTA ($p < 0.01$), OTA and ŐR16 ($p < 0.01$) in case of 4.17 nL. At 120 hpf mortality in the non-injected control was below 10%. Statistical significant differences were observed between OTA and ŐR16 ($p < 0.05$) in case of 0.52 nL, OTA and degraded OTA ($p < 0.05$), OTA and ŐR16 ($p < 0.01$) in case of 1.77 nL, and OTA and degraded OTA ($p < 0.001$), OTA and ŐR16 ($p < 0.01$) in case of 4.17 nL.

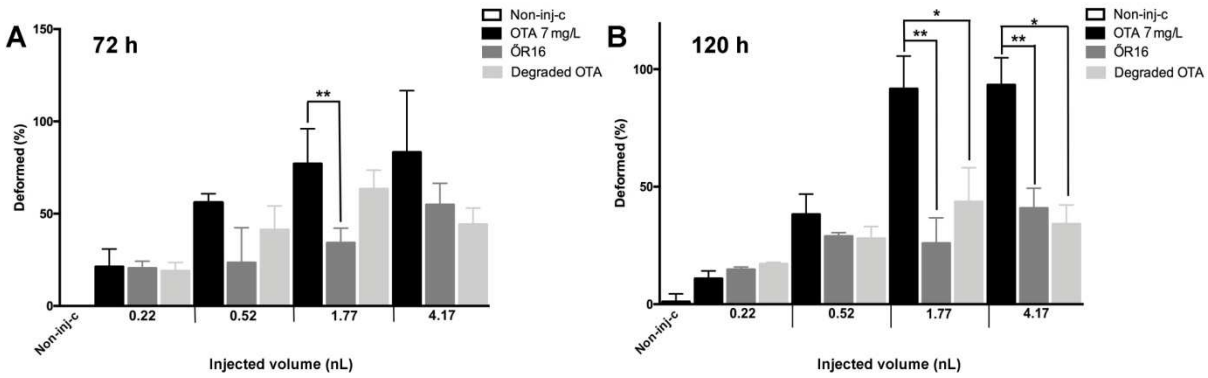

Fig 6 Effects of 7 mg/L Ochratoxin A (OTA 7 mg/L), bacterial metabolites (ŐR 16) and OTA degradation products (Degraded OTA) derived from the biodegradation experiment with *Cupriavidus basilensis* ŐR16 strain on the frequency of developmental deformities in 72 (A) and 120 (B) hpf zebrafish embryos. At 72 hpf, the highest frequency of morphological disorders was detected in the 7 mg/L OTA group. Statistically significant difference was only observed between the 1.77 nl OTA 7 mg/L and 1.77 nL ŐR16 ($p < 0.01$) groups. In 120 hpf embryos, the ratio of deformed embryos was the highest in the groups treated with OTA from 0.52 nL and above. Statistically significant difference was observed between OTA 7 mg/L and ŐR16 ($p < 0.01$) and OTA 7 mg/L and degraded OTA injected in 1.77 nL ($p < 0.05$), OTA 7 mg/L and degraded OTA 7 mg/L and ŐR16 ($p < 0.01$) and OTA 7 mg/L and degraded OTA ($p < 0.05$) injected in 4.17 nL. No significant difference was detected between the deformation frequencies in the groups injected with the bacterial metabolites and OTA degradation products.

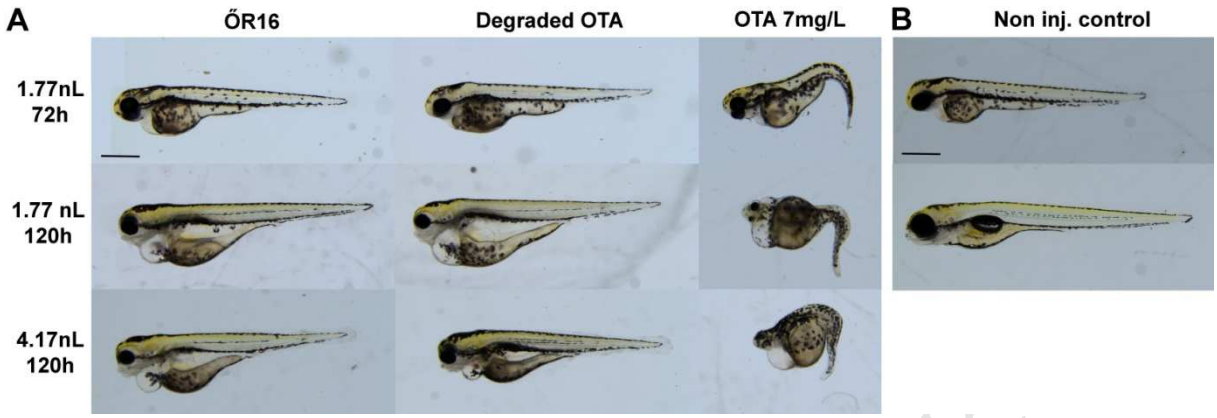

Fig 7 Representative development dysfunctions in zebrafish embryos following injection. Ochratoxin A (OTA 7 mg/L), bacterial metabolites (ŐR16) and OTA degradation products (Degraded OTA) derived from biodegradation experiment with *Cupriavidus basilensis* ŐR16 strain were injected in 1.7, 1.77 and 4.17 nL volumes and disorders were examined following 72 and 120 hours of injection (A). Non-injected control embryos (Non inj. control) are shown on Figure 8B. Scale bar: 500µm.

HIGHLIGHTS:

- ŐR16 degrades OTA to nontoxic products, however bacteria have intrinsic toxicity
- Toxicity differences between test solutions are detectable after 3 days of exposure
- Injection volume variations and control mortality correspond with OECD TG 236
- Microinjection is proper for qualifying the toxin-degrading properties of microbes
- The method helps in selecting the most effective, safe strains for detoxification