Exploring the bioactive metabolites of *Raphidiopsis raciborskii* strains: beyond cylindrospermopsin

Pavel Babica[1,2*], Polina Shpet[1,3], Petra Labohá[1], Nada Tokodi[4,5], Dariusz Dziga[4]

[1] RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic

[2] Department of Experimental Phycology and Ecotoxicology, Czech Academy of Sciences, Brno, Czech Republic

[3] Department of Natural Drugs, Faculty of Pharmacy, Masaryk University, Brno, Czech Republic

[4] Laboratory of Metabolomics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

[5] Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia

** pavel.babica@recetox.muni.cz*

Introduction

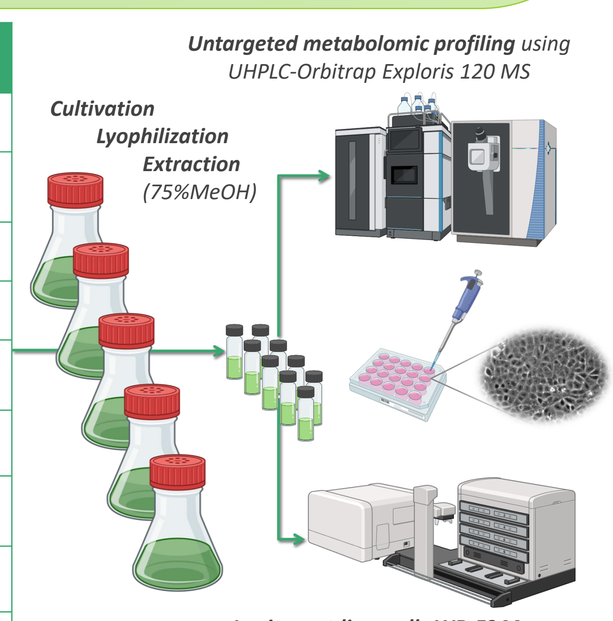
- ***Raphidiopsis raciborskii*** is a globally distributed, bloom-forming cyanobacterium. Its major recognized toxic metabolites include **cylindrospermopsins (CYNs)**, produced by strains in Oceania, South, and East Asia, while **saxitoxins (SXTs)** can be produced by South American strains.
- Although **European and Mediterranean** strains apparently do not synthesize CYNs or SXTs, they may produce other **bioactive and potentially toxic metabolites**.
- This warrants further exploration of their metabolic and toxicity profiles, including the strains recently isolated from the lakes in **Poland**.
- In this study, ***R. raciborskii*** isolates **from Polish lakes** were characterized by **untargeted metabolomics**, in comparison with a CYN-non-producing strain from Israel and two CYN-producing Australian strains.
- In parallel, **toxic effects** of these strains were evaluated *in vitro* in the rat liver stem-like cells WB-F344.
- Specifically, the effects on **gap junctional intercellular communication (GJIC)** were investigated along with markers of **cytotoxicity**, to evaluate the ability of *R. raciborskii* metabolites to impact one of the key processes responsible for the maintenance of tissue homeostasis, whose disruption is linked to **carcinogenesis**.

Major Findings

- Metabolomic analysis identified **the most distinct metabolite profiles** among the **CS-505, CS-506, KLL07, and UAM/DH-KmRr and -PnRr strains**.
- These strains elicited **different concentration- and time-dependent inhibitory effects** on **GJIC and cell viability** in rat liver cells *in vitro*.
- Toxic effects **did not correlate with the production of CYN**. In contrast, the **CYN-lacking strain UAM/DH-PnRr** from Pniewskie Lake induced the **most potent** GJIC-inhibiting and cytotoxic effects.
- Inhibition of GJIC was induced by **non-cytotoxic concentrations** of the extracts, indicating that bioactive metabolites from *R. raciborskii* strains affected **specific mechanisms controlling GJIC**, such as receptors or signal transduction pathways.
- Depending on the strain, inhibition of GJIC could be observed after **short and/or prolonged** exposures, suggesting that **different GJIC-controlling mechanisms were targeted by different extracts**.
- GJIC is known to be disrupted primarily via **nongenotoxic mechanisms** during cancer promotion and progression, thus its inhibition by *R. raciborskii* extracts suggests the presence of **bioactive metabolites with possible pro-tumorigenic activities**. *In vitro* GJIC assessment could be utilized for their **further identification**.

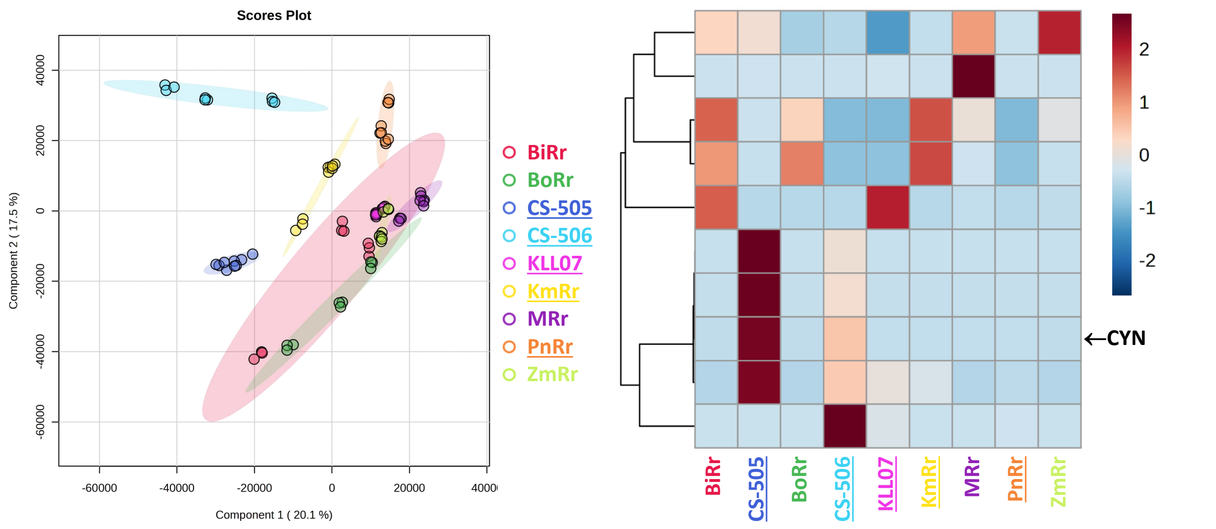
Strain code*	Origin	CYN
CS-505	Solomon Dam, Australia	✓
CS-506	Solomon Dam, Australia	✓
KLL07	Lake Kinneret, Israel	✗
UAM/DH-BiRr	Biskupinskie Lake, PL	✗
UAM/DH-BoRr	Boczowskie Lake, PL	✗
UAM/DH-KmRr	Kierskie Male Lake, PL	✗
UAM/DH-MRr	Miejskie Lake, PL	✗
UAM/DH-PnRr	Pniewskie Lake, PL	✗
UAM/DH-ZmRr	Zninskie Male Lake, PL	✗

**The underlined strains were selected for the in vitro experiments.*



***In vitro* rat liver cells WB-F344:**

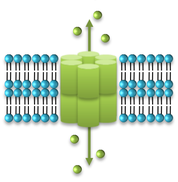
- *GJIC, Cell density, %Dead cells*
- *Rapid (30 min) and prolonged (24 h) effects*



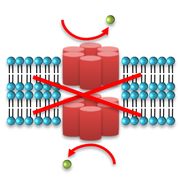
Untargeted metabolomic analysis identified over 880 unique compounds based on their mass-to-charge ratio (m/z) and enabled the annotation of more than 120 compounds. *Left:* **Partial Least Squares Discriminant Analysis** revealed that the CYN-producing strains CS-505 and CS-506, along with two Polish strains (UAM/DH-KmRr and UAM/DH-PnRr), displayed metabolic profiles most distinct from the other strains, including KLL07, which clustered together. *Right:* **Hierarchical clustering** revealed that different strains produced distinct dominant metabolites. Based on these results, the strains CS-505, CS-506, KLL07, UAM/DH-KmRr, and UAM/DH-PnRr were selected for the evaluation of effects in mammalian cells *in vitro*.

Gap junctional intercellular communication (GJIC)

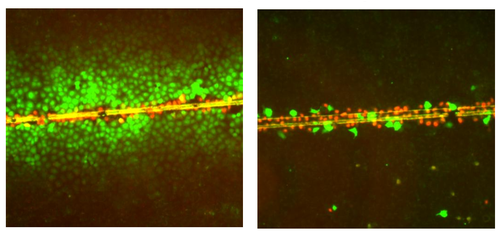
Adjacent cells in healthy mammalian tissues communicate via **gap junctions**, which are membrane channels formed by **connexin** proteins.

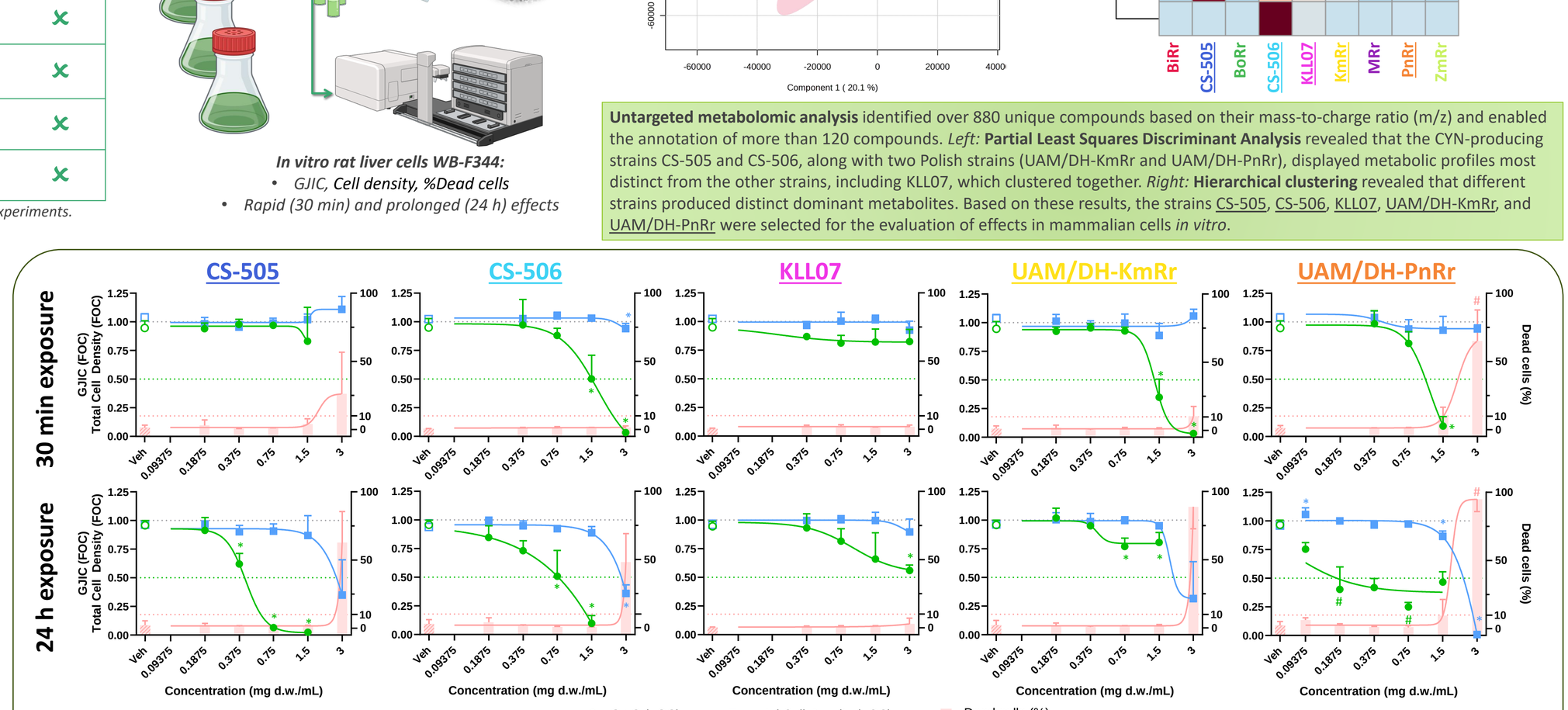


Disruption of GJIC is associated with various **diseases**, including **cancer**, often in response to **chemical exposures**.



In vitro GJIC assessment by a **multiparametric Scrape Loading Dye Transfer (mSLDT) assay**





Effects of the *R. raciborskii* extracts on GJIC, Total Cell Density, and % Dead cells in rat liver cells WB-F344. GJIC and Total Cell Density are presented as a Fraction of the Control (FOC) relative to the untreated control (FOC=1) and plotted on the left y-axis. % Dead cells are expressed as the percentage of dead cells from the total cell count in the culture and plotted on the right y-axis. Data represent means ± S.D. from independently prepared and tested extracts. Treatments significantly different from the vehicle control (Veh) based on ANOVA/Dunnett's test are marked with *, while treatments with non-normal distribution and/or unequal variances were tested by Kruskal-Wallis ANOVA on ranks/Dunn's test with significant differences marked with #. The summary of IC_{50} and EC_{10} values (mg d.w./mL) estimated using non-linear regression is provided in the Table.

Strain	GJIC IC_{50} (mg d.w./mL) 30 min	GJIC IC_{50} (mg d.w./mL) 24 h	Total Cell Density IC_{50} (mg d.w./mL) 30 min	Total Cell Density IC_{50} (mg d.w./mL) 24 h	%Dead cells EC_{10} (mg d.w./mL) 30 min	%Dead cells EC_{10} (mg d.w./mL) 24 h
CS-505	> 1.5	0.42	> 3	2.67	1.76	2.59
CS-506	1.51	0.76	> 3	2.73	> 3	2.71
KLL07	> 3	> 3	> 3	> 3	> 3	> 3
UAM/DH-KmRr	1.36	> 3	> 3	2.02	2.99	2.55
UAM/DH-PnRr	1.05	0.18	> 3	2.28	1.51	1.51

Cultivation and extraction of *R. raciborskii* strains

Most strains were cultivated in WC medium (pH 7.4-7.8) with full nitrogen content (WC100N) or with $NaNO_3$ content reduced to 25% (WC25N), depending on their ability to fix nitrogen. The KLL07 strain was cultivated in CB medium (pH 8.0). The cultures were maintained under a 14/10-h light-dark cycle with a light intensity of 40 µmol m^{-2} s^{-1} at 20°C. Cyanobacterial biomass was harvested after 5 weeks of cultivation via centrifugation (15,000 ×g, 10 min). The biomass was lyophilized and then extracted with 75% (v/v) methanol (10 min ultrasonic bath, then 24 h extraction at 4°C). Extracts were clarified by centrifugation (15,000 ×g, 10 min) and supernatants air-dried.

Metabolic profiling

Untargeted metabolomics was conducted using Ultra High Pressure Liquid Chromatography (UHPLC)-Orbitrap Exploris 120 Mass Spectrometer (Thermo) with an ACQUITY UPLC BEH C18 column. Data analysis was performed with Compound Discoverer 3.3 Software. Metabolites were annotated through ChemSpider and CyanoMetDB. Variable Importance in Projection (VIP) score was utilized to determine each metabolite's contribution to group differentiation. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) and cluster analysis were performed using MetaboAnalyst 6.0. The extracts from the strains showing the most distinct metabolite profiles (CS-505, CS-506, KLL07, UAM/DH-KmRrr and UAM/DH-PnRr) were selected for the *in vitro* assay.

In vitro assessment of the effects on GJIC, cytotoxicity and cell injury

Gap junctional intercellular communication (GJIC) was evaluated using the rat liver cell line WB-F344 (Cytion #305201), a normal, non-tumorigenic line that exhibits properties of hepatic progenitor or oval cells. GJIC was assessed using a multiparametric Scrape Loading Dye Transfer (mSLDT) assay, simultaneously with cytotoxicity endpoints (cell density and the percentage of dead cells). Dried cyanobacterial extracts were dissolved in PBS and added to the cells at final concentrations equivalent to 0.1–3 mg dry weight (d.w.)/mL. The cells were exposed for short-term (30 min) and prolonged (24 h) exposure to detect both rapid—potentially transient—inhibition of GJIC as well as GJIC inhibition caused by slower mechanisms. From the dose–response curves, IC_{50} values (for GJIC inhibition and cell density reduction) and EC_{10} values (for increased cell death) were derived using non-linear 3- or 4-parameter regression with GraphPad Prism. Statistical analysis was done in SigmaPlot.

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