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The role of methylparaben as an environmental contaminant in enhancing the pathogenicity of drinking water bacteria

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Abstract Parabens, commonly used as preservatives in personal care products, can affect drinking water (DW) environmental microbial communities, posing an emerging global concern for public health. This study examines the impact of MP at 15 µg/L on the virulence of *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* both isolated from a drinking water distribution system (DWDS). MP was able to change the production and properties of bacterial released outer membrane vesicles (OMVs) by affecting the OMVs hydrodynamic size, lipid content and OMVs concentration. Furthermore, MP exposure increased the ability of *A. calcoaceticus* cells to invade human gingival fibroblasts (HGF). These findings highlight how MP can amplify interconnected virulence mechanisms - including OMV production, and host cell invasion - posing potential health risks in DWDS.

Keywords: bacterial virulence, biofilms, internalization, invasion, methylparaben, outer membrane vesicles

1. Introduction

Parabens are increasingly detected in drinking water distribution systems (DWDS) (A. R. Pereira et al., 2023b). Methylparaben (MP) is the most prevalent due to its widespread use in personal care products. Although MP poses ecological and human health risks due to its endocrine-disrupting properties, its impact on microbial communities in DWDS, critical for ensuring water quality and safety, remains poorly understood. Previous studies have shown that MP at trace levels significantly enhances biofilm thickness, bacterial culturability, and total cell density while increasing the production of virulence factors and tolerance to disinfection (A. R. Pereira et al., 2023a; Pereira and Gomes, 2024). This highlights its potential role in promoting bacterial virulence and antimicrobial resistance. However, the bacterial virulence mechanisms promoted by parabens exposure are not well understood.

This study explores the effects of MP (15 µg/L) on the interconnected mechanisms of outer membrane vesicles (OMVs) production - key mediators of bacterial interactions and virulence - and host cell invasion in *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia*, providing insights into public health risks associated with environmental contamination.

2. Methods

2.1. Bacteria conditions and MP exposure

A. calcoaceticus and *S. maltophilia* were used as model bacteria of DW bacteria. Bacteria cultures were prepared in R2A broth medium overnight at 25 °C with 120 rpm of agitation. Two bacterial growth conditions were evaluated: (1) isolated bacteria from 7-days old dual-species biofilms and continuously exposed to MP at 15 µg/L (Pereira and Gomes, 2024); and (2) planktonic bacteria continuously exposed to 15 µg/L of MP for 7 days.

2.2. OMVs bacterial extraction and quantification

OMVs from MP-exposed and non-exposed bacteria were isolated and purified as described by Chutkan et al. (2013). Bacteria were grown in R2A medium (25 ºC, 120 rpm) until reach the early stationary phase, and then were centrifuged (3772 ×g, 10 min, 4 ºC). The supernatants containing OMVs were filtered through 0.45 and 0.22 µm cellulose nitrate filters for *A. calcoaceticus* and *S. maltophilia*, respectively. Vesicle-containing pellets were achieved through another centrifugation (38400 ×g, 1 h, 4 ºC), resuspended in Dulbecco's phosphate-buffered saline supplemented with salts (DPBSS), and further ultracentrifuged at 100000 ×g for 1 h at 4 ºC. OMVs were then ressupended in DPBSS for analysis. OMV concentration and size were assessed using nanoparticle tracking analysis (NTA), and lipid content quantified with Nile red fluorescence (Bertozzini et al., 2011). Protein content was quantified using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma, USA).

2.3. Bacterial ability to invade human cells

HGF were used to assess the effect of MP exposure on the invasive potential of both DW bacteria. HGF were cultured in α-MEM + 10% FBS at 37 ºC in a humidified 5% CO_2 atmosphere. Then, bacterial suspensions were

added to HGF monolayers (2.0×10^4 cells/well) to achieve a final concentration of 10^6 bacteria/mL. Plates were incubated for 90 min at 37 ºC in a 5% CO2 atmosphere to allow bacteria-cell interactions. After washing co-cultures, HGF were lysed with 0.2% Triton X-100 for 30 min, followed by plating the lysates on tryptic soy agar (TSA).

3. Results and discussion

3.1. OMVs quantification and characterization

OMVs production by *A. calcoaceticus* remained consistent across all conditions. However, MP exposure reduced OMVs production in *S. maltophilia*, with a more pronounced effect in planktonic cells compared to biofilm-derived ones ($P < 0.05$). For *A. calcoaceticus*, lipids content per OMVs was similar for all conditions assessed, irrespective of MP exposure. In contrast, MP-exposed biofilm-derived *S. maltophilia* showed a 15-fold increase in lipids content per OMVs compared to non-exposed counterparts. Conversely, MP exposure led to a two-fold decrease in lipids content per OMVs in planktonic *S. maltophilia* ($P < 0.05$). Regarding protein content per OMVs, the exposure to MP did not alter OMVs proteins content of both bacterial strains. In terms of OMVs size, MP-exposed biofilm-derived *A. calcoaceticus* produced larger OMVs (181 ± 5 nm) compared to the non-exposed control (103 ± 14 nm), whereas MP-exposed planktonic *A. calcoaceticus* produced smaller OVMs (143 ± 9 nm) relative to the control (207 ± 0.3 nm). For *S. maltophilia*, the size of OMVs remained consistent, regardless of MP exposure.

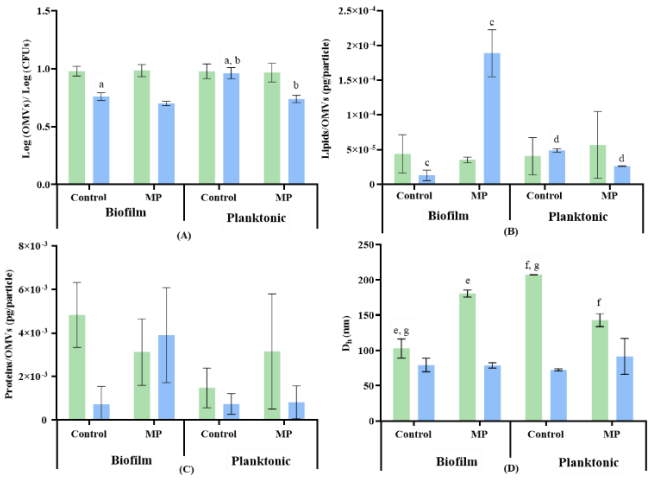

Figure 1 - Concentration of OMVs per CFUs (A), lipids per OMVs (B), proteins per OMVs (C) (pg/particle) and size (nm) (D) of OMVs produced by non-exposed and MP-exposed biofilm-derived and planktonic cells of *A. calcoaceticus* (■) and *S. maltophilia* (■). [a to f] – samples were statistically different between them. (t-test, $P < 0.05$).

3.2. Bacterial invasion into HGF

Biofilm-derived *A. calcoaceticus* showed similar invasion levels regardless of MP exposure ($P > 0.05$). In contrast, planktonic MP-exposed *A. calcoaceticus* exhibited significantly higher invasion ability than the control resulting in an increase of 126% in the number of culturable bacterial cells able to invade fibroblasts ($P < 0.05$). MP exposure did not impact *S. maltophilia* invasion capacity ($P > 0.05$).

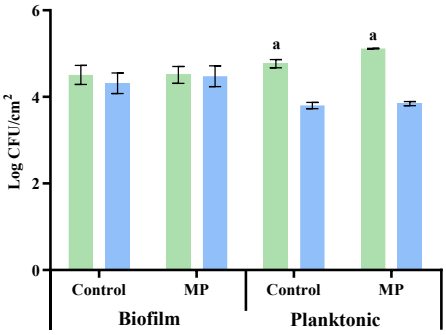

Figure 1 - Effects of MP exposure on *A. calcoaceticus* (■) and *S. maltophilia* (■) biofilm-derived and planktonic cells invasion ability of HGF. [a] - Conditions were statistically different between them (t-test, $P < 0.05$).

4. Conclusion

This study highlights the significant impact of MP exposure on the virulence of both biofilm-derived and planktonic cells of *A. calcoaceticus* and *S. maltophilia*. Exposure to environmentally relevant concentrations (15 µg/L) of MP alter the production, size, and lipid content of OMVs, which may have impactful modifications on OMVs functions, modulating virulence mechanisms.

5. Aknowledgements

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