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Article

# *Pediococcus pentosaceus* MZF16 Probiotic Strain Prevents *in vitro* Cytotoxic Effects of *Pseudomonas aeruginosa* H103 and Prolongs the Lifespan of *Caenorhabditis elegans*

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium, responsible of several life-threatening infection attributed to its multiple virulence factors and its problematic multi-drug resistance, hence the necessity to find out alternatives such as competitive probiotics. *Pediococcus pentosaceus* MZF16 is a LAB strain, isolated from traditional dried meat “Ossban”, with high probiotic potential. Our study investigated the capacity of *P. pentosaceus* MZF16 to counteract *P. aeruginosa* H103 using several tests on intestinal cells (analysis of cytotoxicity, inflammation, adhesion/invasion) and on the *in vivo* *Caenorhabditis elegans* model. The effect of MZF16 on the Quorum sensing of the pathogen was also examined. We found that *P. pentosaceus* MZF16 was able to reduce H103 cytotoxicity and inflammatory activity, prevented pathogen colonization and translocation across Caco-2/TC7 cells. MZF16 exerted also an anti-virulence effect by attenuating Quorum-sensing (QS) molecules and pyoverdine production and extended *C. elegans* lifespan. The obtained results highlight the potential of *P. pentosaceus* MZF16 probiotic strain as an anti-*Pseudomonas aeruginosa* alternative and establish a basis for elucidating the mechanisms of *P. pentosaceus* MZF16 involved in countering *Pseudomonas aeruginosa* virulence.

**Keywords:** *Pediococcus pentosaceus*; anti-virulence; *Pseudomonas aeruginosa*; Quorum sensing; probiotic; *Caenorhabditis elegans*

## 1. Introduction

Fermented foods are produced through controlled microbial growth, and enzymatic conversion of food components. Historically, food fermentation was a common preservation method, used to maintain food integrity and reduce the risk of contamination with pathogenic microorganisms, likely due to the production of antimicrobial metabolites such as organic acids, ethanol and bacteriocins [1]. In recent years, fermented foods have surged in popularity, because of their health-promoting properties especially for gastrointestinal health. These benefits are attributed to various mechanisms, including bioactive peptides and phenolic compounds conversion to biologically active compounds, as well as the beneficial effects of constituent microorganisms [2]. Fermented foods such as dairy products, fermented meats and vegetables, contain relatively resilient and stable microbial ecosystems, predominantly composed of lactic acid bacteria (LAB) and their primary metabolites including lactic acid [3]. Some LAB strains have long been recognized as probiotics due to their ancestral use and classification as beneficial bacteria with Generally Recognized As Safe (GRAS) status. Emerging probiotics are also being studied to better understand their potential health benefits. Among GRAS probiotics, the most well-known and studied bacteria belong to the *Lactobacillus* genus, recently reclassified into 23 genera [4]. Notably, *Lactobacillus* probiotic species including

*Lactocaseibacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, and *Lactiplantibacillus plantarum*. These species have demonstrated various health benefits, including anti-carcinogenic, anti-diabetic, antioxidant and anti-inflammatory and also anti-microbial effects against food-borne pathogens [5].

Despite their frequent presence in many fermented products (*i.e.* meat, fish, dairy, beer, wine, fruits, vegetables, and beverages)[6], probiotics from the genus *Pediococcus* have been less studied. This genus consists of several species, including *P. acidilactici*, *P. pentosaceus*, *P. damnosus*, *P. parvulus*, *P. inopinatus*, *P. halophilus*, *P. dextrinicus*, and *P. urinaeequi* [7]. Among these, *P. acidilactici* and *P. pentosaceus* probiotic strains are notable for their high production of bacteriocins (pediocins) and bacteriocin-like substances (BLS) [8]. Indeed, pediocins and other postbiotics metabolites (e.g. lactic acid, biosurfactants) from some *Pediococcus* species have exerted significant anti-bacterial and anti-virulence activities against several food spoilage pathogens, as *Listeria monocytogenes*, *Salmonella enterica* and also against the Multi-Drug Resistant (MDR) pathogen *Pseudomonas aeruginosa* [9,10,11,12,13,14].

*P. aeruginosa* is known to be a potential source of the spoilage of various foods, notably those with high-water content and nutrient-rich foods, such as milk, meats, and some fruits and vegetables [15]. It is also an opportunistic Gram-negative pathogen, widespread in the environment and responsible of health care-associated infections and foodborne diseases [16]. In some cases, this germ has been associated with diarrheal disease, systemic infections, enterocolitis and digestive cancers [17,18]. Its ability to cause severe acute and chronic life-threatening infections is attributed to its multiple virulence factors [19]. Moreover, the high versatility and adaptability of this pathogen are supported by its large genome (6 to 8 Mb), with over 8% dedicated to regulatory genes, enabling it to evade a broad spectrum of antimicrobial agents [20]. Thereby, alternative treatments, such as competitive probiotics, are urgently needed [21].

Some studies have explored the effects of postbiotic LAB metabolites, such as lactic acid, bacteriocins and bacteriocin-like substances produced by various *Pediococcus* species (*P. pentosaceus* and *P. acidilactici* ...) on *P. aeruginosa* [11,12,14]. However, interactions between these two species and also with the human host remain poorly characterized.

*P. pentosaceus* MZF16 strain has been previously isolated in our laboratory from the artisanal Tunisian meat "Dried Ossban", and investigated for its probiotic potential [22,23]. The aim of the present study was to examine the interactions between *P. pentosaceus* MZF16 and the pathogen *P. aeruginosa* H103, with a particular focus on MZF16 strain ability to modulate the virulence of the pathogen and its interactions with the host.

## 2. Materials and Methods

### 2.1. Bacterial strains and culture conditions

The bacterial strains used in this study were the pathogen *P. aeruginosa* H103, a prototroph derivative of PAO1 strain [24], and the probiotic *P. pentosaceus* MZF16. They were respectively grown at 37 °C under rotary shaking (180 rpm) in Luria Bertani (LB) medium and in static conditions in Man Rogosa Sharpe (MRS) medium.

### 2.2. *P. aeruginosa* H103-gfp and *P. pentosaceus* MZF16-mCherry construction

*P. aeruginosa* H103 was grown in LB medium until reaching an OD<sub>600nm</sub> of 0.5, then centrifuged at 7000 rpm for 10 min at 4°C. The pellet was washed and resuspended in 300 mM sucrose buffer at a concentration of 10<sup>10</sup> CFU/mL. A 100 µL aliquot of electrocompetent *P. aeruginosa* was mixed with 50 ng of plasmid pHC60 (gfp, tetR)[25] and electroporated at 1.8 kV in a 0.2-cm cuvette. Immediately after electroporation, 1 mL of LB medium was added, and the mixture was incubated for 2 h at 37°C with shaking at 180 rpm. *P. aeruginosa* H103-gfp transformants were selected on LB agar supplemented with Tetracycline (100 µg/mL).

For *P. pentosaceus* MZF16-mCherry construction, electrotransformation was performed using plasmid pRCR12 (mCherry, CmR), provided by Pr Paloma Lopez (Madrid, Spain), and following the

method of Pérez-Ramos et al., (2018) [26]. Briefly, the bacterial pellet was washed and resuspended in 1 mL of lysozyme (2,000 U/ mL) and incubated for 20 min at 37 °C. Subsequently, 50 µL of electrocompetent cells were mixed with 5 µL of pRCR12 plasmid (0.5 µg) and electroporated at 2.5 kV in a 0.2-cm cuvette. After electroporation, 500 µL of MRS medium was immediately added, and the cells were incubated for 2 h at 37°C. *P. pentosaceus* MZF16-mCherry transformants were selected on MRS-agar supplemented with Chloramphenicol (10 µg/mL).

### 2.3. Caco-2/TC7 cell culture and infection

Human colon adenocarcinoma Caco-2/TC7 cells provided by Chantret (INSERM, Paris) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 20 % heat-inactivated fetal bovine serum (FBS, Sigma Aldrich) and penicillin/streptomycin (100 µg/mL). The cells were cultivated and incubated at 37 °C in 5% CO<sub>2</sub> atmosphere, and the medium was regularly changed. Cells were passed after reaching approximately 90% of confluence.

For adhesion, invasion, cytotoxicity assays and IL-8 quantification, cells were seeded in 24-well plates treated for tissue culture and grown to confluence. For bacterial translocation assay and transepithelial resistance (TER) measurements, cells were seeded on inserts (6.4 mm diameter, 3 µm pore size, Falcon) until full differentiation (19-21 days).

Bacterial suspensions of *P. aeruginosa* H103 and its mixture with *P. pentosaceus* MZF16 (1:100) were prepared in DMEM without FBS and antibiotics. These suspensions were added to confluent or differentiated Caco-2/TC7 cells and incubated at 37 °C in 5 % CO<sub>2</sub> for 3 h for adhesion and invasion assays, and overnight for the other experiments.

### 2.4. Cytotoxicity assay and Interleukin-8 quantification

Cytotoxicity and inflammation assays were performed on confluent Caco-2/TC7 cells grown in 24-well culture plates. Following overnight infection with bacterial suspensions, the supernatants were collected from Caco-2/TC7 monolayers. Then, cytotoxicity was assessed by measuring lactate dehydrogenase (LDH), a cytoplasmic enzyme released upon cell death, using an enzymatic assay (Cyquant Kit, Invitrogen). The concentration of IL-8 cytokine in the supernatants was quantified using the IL-8 Human ELISA Kit (Invitrogen).

### 2.5. In vitro adhesion and invasion assay

Bacterial adhesion and invasion into intestinal Caco-2/TC7 cells were assessed as follows. For the adhesion assay, Caco-2/TC7 cells were infected with suspensions of *P. aeruginosa* H103 and mixture with *P. pentosaceus* MZF16 (1:100 ratio) for 3 h. After incubation, cells were washed twice with Phosphate Buffer Saline (PBS) to remove non-associated bacteria and then lysed with 0.1% Triton X100. The lysates were serially diluted and plated on Cetrinide agar to quantify adherent pathogenic bacteria.

For the invasion assay, after 3 h of infection, adherent and non-internalized bacteria were eliminated by treating the cells with 300 µg/mL of gentamycin for 1 h. Following treatment, the cells were lysed, and internalized bacteria were quantified by plating.

For the visualization of bacterial adhesion, Caco-2/TC7 cells were seeded on glass-bottom 24-well plates (Sensoplate, Greiner) and infected with fluorescent strains *P. aeruginosa* H103-gfp and *P. pentosaceus* MZF16-mCherry for 3 h. Afterward, Caco-2/TC7 cells were stained with DAPI (4',6-diamidino-2-phenylindole) and visualized using Confocal Laser Scanning Microscope (CLSM).

### 2.6. Bacterial translocation and Transepithelial Electrical Resistance (TER) measurement

After an overnight infection of Caco-2/TC7 differentiated cells cultured on inserts, aliquots from the basolateral compartment were collected. The number of translocated *P. aeruginosa* that crossed the epithelial monolayers was determined by serial dilution and plating on Cetrinide agar.

To assess the integrity of the epithelial barrier, the transepithelial electrical resistance (TER) of infected and non-infected monolayers was measured using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA).

### 2.7. Antimicrobial activity test

The antagonistic activity of *P. pentosaceus* MZF16 against *P. aeruginosa* H103 was assessed using the disk diffusion method. Overnight cultures of both strains were centrifuged at 8500 rpm for 5 min. Supernatants of *P. pentosaceus* MZF16 were either pH-adjusted (pH=7) or not before being filtered (0.22  $\mu$ m membrane).

A suspension of *P. aeruginosa* H103 at a concentration of 10<sup>6</sup> CFU/mL was spread on Muller Hinton Agar plate. Disks impregnated with Cell Free Supernatants (CFS) of MZF16 were placed on the agar surface, and the plates were incubated at 37 °C for 24h.

### 2.8. Bacterial aggregation assay

Auto and coaggregation assays were performed as described previously [27] with minor modifications. Briefly, overnight cultures of H103 and MZF16 were centrifuged at 10 000 g for 10 min. The pellets were washed twice and resuspended in sterile phosphate buffer saline (PBS). The bacterial suspensions were adjusted to an OD<sub>600</sub> of approximately 0.3.

For the coaggregation assay, equal volumes of the standardized bacterial suspensions were mixed. Both individual and mixed suspensions were aliquoted into hemolysis tubes (4 mL) and incubated at room temperature without agitation.

The OD<sub>600</sub> of the suspensions was measured at the start of the experiment (A<sub>0</sub>) and after 3 h of incubation at 37 °C (A<sub>t=3h</sub>). The auto-aggregation percentage was calculated as:

$$\text{Autoaggregation (\%)} = \left[ 1 - \left( \frac{A_{t=3h}}{A_0} \right) \right] \times 100$$

The coaggregation percentage was determined using the formula:

$$\text{Coaggregation (\%)} = \left[ \left( \frac{(A_{H103+MZF16}) - (A_{mix})}{(A_{H103+MZF16})} \right) \right] \times 100$$

where A<sub>H103</sub> and A<sub>MZF16</sub> are the OD<sub>600</sub> values of the mixed bacterial suspension at time 0, and A<sub>mix</sub> is the OD<sub>600</sub> of the mixed suspension at t=3 h.

### 2.9. Pyoverdine production measurement

Pyoverdine, a key siderophore and virulence factor of *P. aeruginosa*. Its production was quantified by spectrophotometry at A<sub>405 nm</sub>. Bacterial suspensions of *P. aeruginosa* H103 and the mix with the probiotic *P. pentosaceus* MZF16 were grown in colorless DMEM medium supplemented with 20% Fetal bovine serum (FBS). After incubation, pyoverdine production was measured from collected supernatants. Results are expressed as the relative pyoverdine levels (mean  $\pm$  SEM) compared to the control, *P. aeruginosa* H103 culture.

### 2.10. Extraction and quantification of AHLs and HAQs molecules

N-acyl-homoserine lactones (AHLs) and 2-alkyl-4-quinolones (HAQ, PQS system) signaling molecules were extracted from *P. aeruginosa* H103 cultures and co-cultures with *P. pentosaceus* MZF16 (1:100), grown in DMEM medium, following the protocol described by Fletcher et al., (2007)[28]. For quantification, *Escherichia coli* carrying plasmid pSB401 (*luxRI*::*luxCDABE*) and *P. aeruginosa* PAO1  $\Delta$ *pqsA* CTX-*lux*::*pqsA* strain were used as biosensors for AHLs and HAQs, respectively. The assay was performed using a combined spectrophotometer/luminometer approach. Briefly, overnight

cultures of the biosensor strains were adjusted to an  $OD_{580\text{ nm}} = 1$  and then diluted to 1:50 and 1:100 ratios. Crude extracts of quorum-sensing (QS) molecules were diluted in LB medium and added to a 1:50 dilution of the biosensors. The 3-oxo-C12-HSL, C4-HSL, HHQ, and PQS synthetic standards (Sigma-Aldrich, Saint-Louis, MO) at final concentrations of 5  $\mu\text{M}$  were added to a 1:100 dilution of the biosensor strains as positive controls.

The assay was performed in 96-well microtiter plates (white-sided, clear-bottom). Bioluminescence (relative light units, RLU) and absorbance ( $A_{580}$ ) were measured every 15 min for 24 h at 37 °C using a Spark 20M multimode microplate reader (Tecan, Männedorf, Switzerland). The bioluminescence data were normalized to the  $A_{580}$  values.

### 2.11. *In vivo* *Caenorhabditis elegans* killing assay

The *C. elegans* slow killing assay was performed as previously described by Tan *et al* [29]. *C. elegans* wild-type Bristol strain N2 worms were grown at 22°C on nematode growth medium (NGM) agar plates using *Escherichia coli* OP50 strain as the nutrient. *P. aeruginosa* culture and its coculture with *P. pentosaceus* MZF16 were spread onto 35 mm NGM solidified agar plates before an overnight incubation at 37°. The plates were cooled to room temperature for 4 h. Then 20 to 30 L4-synchronized worms prepared as described by Blier *et al* [30] were plated and incubated at 22°C in a humid environment to prevent plate drying. Worm survival was scored daily for 16 days using an optical microscope (Leica CME, Switzerland). The worms were considered dead when they remained static without grinder movements for 20 s or did not respond to light flashes. Results are expressed as a percentage of living worms. The results are the average of at least three independent assays. The nematode survival was calculated using the Kaplan–Meier method, and survival differences were tested for significance using the log–rank test (GraphPad Prism version 4.0).

### 2.12. Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software version 10.2.2 with two-sample unpaired test. All tests were conducted at least three times, and statistical significance was considered at  $p < 0.05$ ,  $p < 0,01$  and  $p < 0,001$ .

## 3. Results

### 3.1. Autoaggregation and coaggregation

The autoaggregation and coaggregation abilities of *P. pentosaceus* MZF16 and *P. aeruginosa* H103 were measured in PBS after 3 h incubation at room temperature (Table 1). *P. pentosaceus* MZF16 demonstrated a high capacity for autoaggregation (44.7%) and coaggregation with *P. aeruginosa* H103 (45.7%).

**Table 1.** Aggregation activity of *P. pentosaceus* MZF16.

	<i>Pediococcus pentosaceus</i> MZF16
Autoaggregation	44.74% $\pm$ 2.58
Coaggregation with <i>P. aeruginosa</i> H103	45.77% $\pm$ 3.58

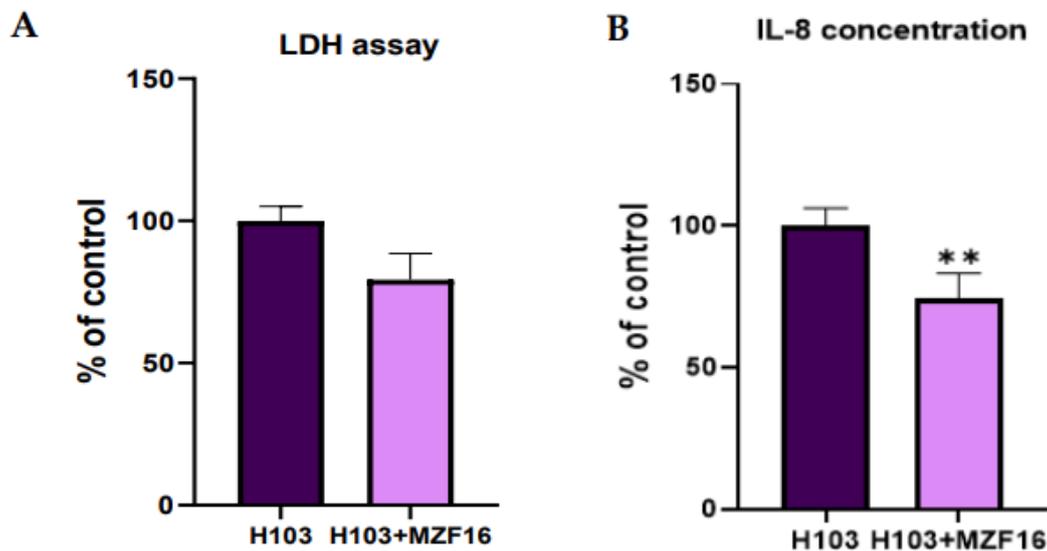
### 3.2. Antimicrobial activity test

The cell-free supernatant of *P. pentosaceus* MZF16 exhibited no antibacterial activity against *P. aeruginosa* H103 in our conditions, suggesting the absence of an anti-*Pseudomonas* bacteriocin or bacteriocin-like metabolite in our strain.

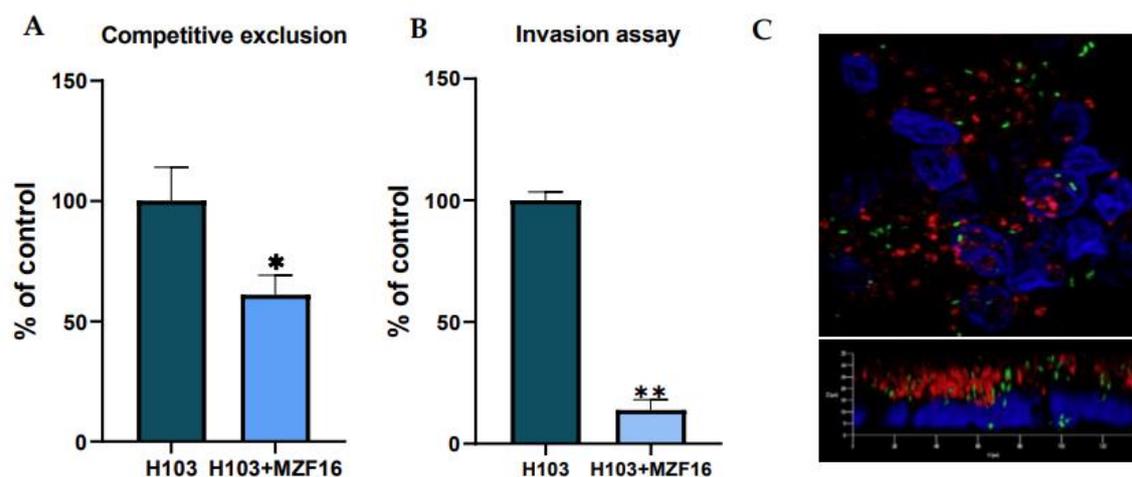
### 3.3. Cytotoxicity assay and Interleukin-8 quantification

The cytotoxicity and pro-inflammatory activity of *P. aeruginosa* H103 towards the intestinal Caco-2/TC7 cells were evaluated after overnight incubation in the presence or absence of *P.*

*pentosaceus* MZF16. The results demonstrated that MZF16 slightly decreased the level of lactate dehydrogenase (LDH) produced by the eukaryotic cells in response to the cytotoxic effect of the pathogen, with a reduction of about 20% (Figure 1A). In addition, MZF16 significantly reduced the level of IL-8 by more than 25% (Figure 1B).



**Figure 1.** Effect of *P. pentosaceus* MZF16 on the cytotoxic activity of *P. aeruginosa* H103 toward Caco-2/TC7 cells (A) and on its pro-inflammatory potential and interleukin-8 (IL-8) secretion (B). Cytotoxicity was evaluated by measurement of Lactate Dehydrogenase (LDH) released from damaged cells after an overnight infection (\*\* p < 0.01).



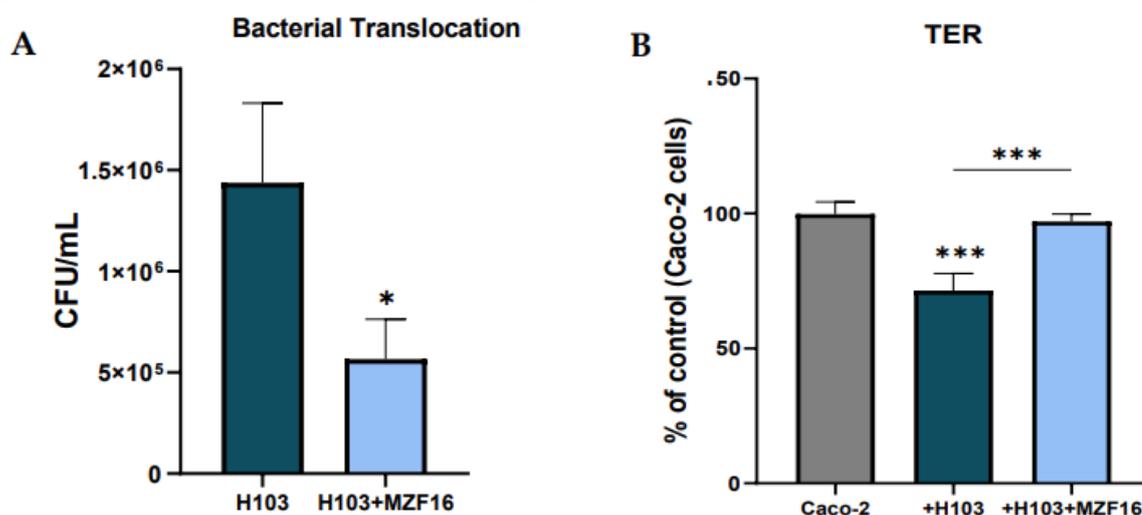
**Figure 2.** Inhibition of *P. aeruginosa* adhesion (A) and invasion (B) to Caco-2/TC7 cells by *P. pentosaceus* MZF16. The adhesion of the pathogen into Caco-2/TC7 cells in presence of *P. pentosaceus* MZF16 (1 :100) was examined by plating cells lysate, after 3 h of contact, on Cetrimide agar, while invasive bacteria was selected by gentamycin (300 µg/mL) treatment for 1 h before plating the lysate. Each experiment was assayed at least four times (\* p<0,05, \*\* p<0,01). (C) CLSM visualization of H103-gfp and MZF16-mCherry to DAPI-colored Caco-2/TC7 cells.

### 3.4. Adhesion and invasion

The competitive exclusion of pathogen adhesion and invasion to Caco-2 cells by *P. pentosaceus* MZF16 was evaluated over 3 h by spreading cell lysates on Cetrimide agar. The simultaneous addition of *P. pentosaceus* MZF16 with *P. aeruginosa* H103 to intestinal Caco-2/TC7 cells resulted in a decrease of  $39\pm 8\%$  in the number of adhering *P. aeruginosa* H103 (Figure 2A), and a reduction of  $85\pm 4\%$  in invasive bacteria number (Figure 2B). Visualization of bacterial adhesion to Caco-2 cells using confocal laser scanning microscopy (CLSM) showed that MZF16-mCherry formed a layer on the cells. In contrast, H103-gfp appeared more scattered in the different stacks, likely due to its mobility and invasive ability (Figure 2C).

### 3.5. Transepithelial electric resistance (TER) and translocation

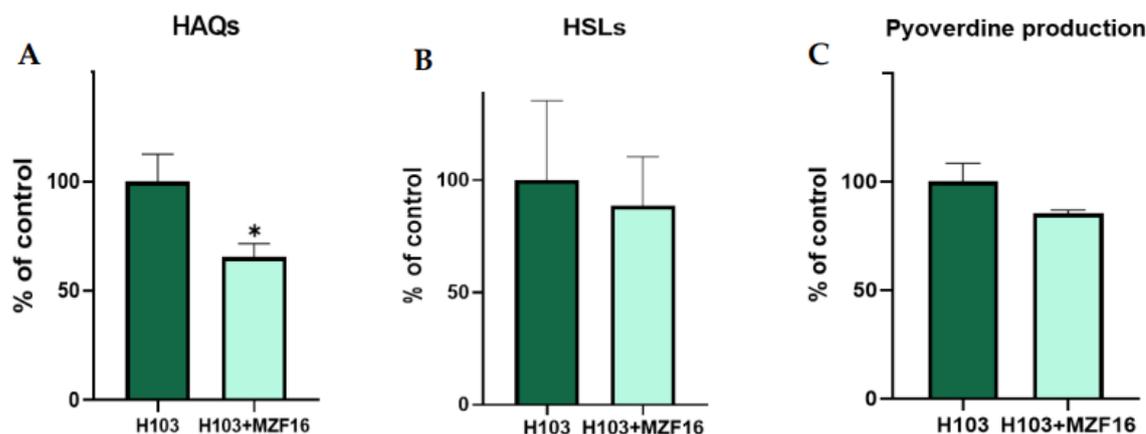
The transepithelial resistance (TER) and the bacterial translocation of *P. aeruginosa* H103 were evaluated on differentiated Caco-2/TC7 cells after an overnight infection. Results showed that *P. pentosaceus* MZF16 prevented the reduction of TER provoked by *P. aeruginosa* H103, which was about 30% (Figure 3A). Additionally, *P. pentosaceus* MZF16 significantly reduced the translocation of the pathogen across the Caco-2 cells by three times (Figure 3B).



**Figure 3.** *P. pentosaceus* MZF16 effect on bacterial translocation of *P. aeruginosa* (A) and on Caco-2/TC7 differentiated cells transepithelial resistance (B). *P. aeruginosa* translocation was assessed by plating the bottom of the well on cetrimide medium after an overnight infection of Caco-2/TC7 with the pathogen H103 strain and its coculture with *P. pentosaceus* MZF16 (1 :100) (\* p<0,05, \*\*\* p<0,001).

### 3.6. Quorum-sensing and pyoverdine production

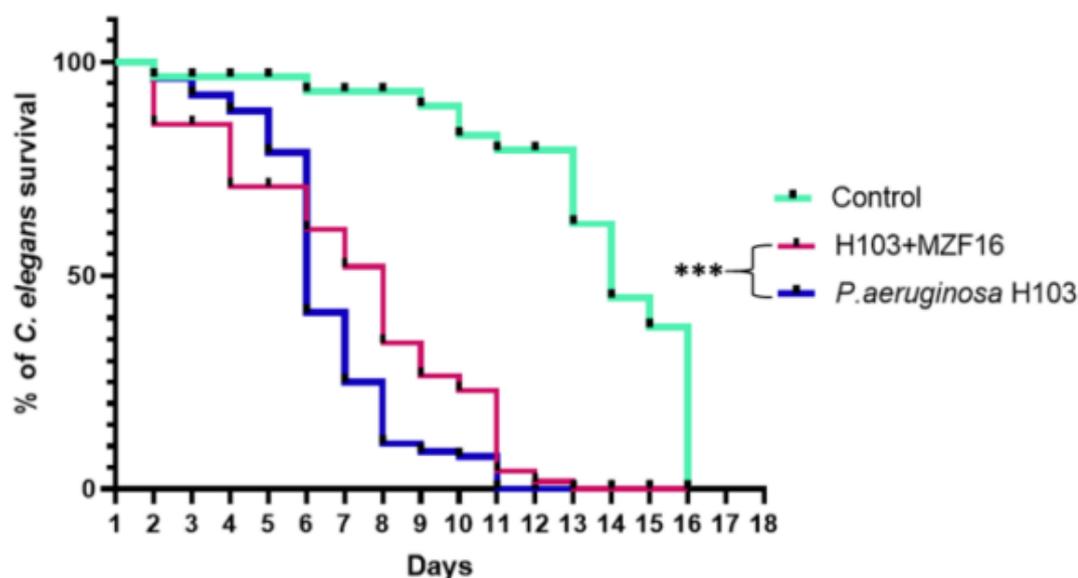
*N*-acyl-homoserine lactones (AHL) production was assessed using the biosensor strain *E. coli* harboring the plasmid pSB401 (*luxRI*::*luxCDABE*), able to detect short (C4) and long (C12) HSL chains produced by *P. aeruginosa*. The production of 2-alkyl-4-quinolones (HAQ) signaling molecules (HHQ and PQS) was assessed using the *P. aeruginosa* PAO1  $\Delta$ *pqsA* CTX-*lux*::*pqsA* biosensor strain, which detects HAQ derivatives produced by *P. aeruginosa*. Both molecules production was examined in presence and absence of MZF16 strain. Results showed that *P. pentosaceus* MZF16 significantly reduced (of about 35%) the production of HAQs molecules by *P. aeruginosa* H103 (Figure 4A), but had no effect on HSLs (Figure 4B). Additionally, the effect of MZF16 on *P. aeruginosa* H103 pyoverdine production was studied, showing a slight decrease in the presence of the probiotic (Figure 4C).



**Figure 4.** QS signaling molecules and virulence factors production. PQS molecules (HAQs) (A) and HSLs (B) production, were quantified, in presence of *P. pentosaceus* MZF16 cells, comparing to the basic *P. aeruginosa* H103 production in DMEM medium. Bioluminescence measurements ( $\pm$  the SEM) normalized with  $A_{600\text{ nm}}$  along the bacterial growth of the AHL bioreporter strain, *E. coli* pSB401, and of the HAQ bioreporter strain, *P. aeruginosa* PAO1  $\Delta pqsA pqsA::lux$ . Histograms are a representation of both conditions at the peak of bioluminescence. The relative quantifications ( $\pm$  the SEM) of pyoverdine production (C) were determined by supernatants absorbance measurement at 405 nm. Each experiment was assayed at least four times independently. Statistics were determined by t-tests (\*  $p < 0,05$ ).

### 3.7. In vivo virulence test on *C. elegans*

A slow killing assay was monitored over 16 days (Figure 5). The results showed that in the presence of *P. pentosaceus* MZF16, the lifespan of *C. elegans* was significantly prolonged compared to when the worms were exposed only to the pathogen *P. aeruginosa* H103. Especially, on day 8 of the test, only 10% of the worms infected by H103 survived, while in the presence of *P. pentosaceus* MZF16, the survival rate of *C. elegans* infected by H103 was about 40%.



**Figure 5.** *C. elegans* nematodes lifespan in contact with *P. aeruginosa* H103 (blue curve), and cocultures with *P. pentosaceus* MZF16 (inoculated in 1:100) (pink curve) ratio. Experiment was assayed at least three times independently.

## 4. Discussion

*Pseudomonas aeruginosa* is a major opportunistic pathogen responsible for both acute and chronic infections, especially in immunocompromised individuals [31]. The rise of multidrug resistant *P. aeruginosa* strains has led to an urgent need for alternative treatments to antibiotics, such as probiotics.

*Pediococcus pentosaceus* MZF16 strain, isolated from Tunisian dried meat (Ossban), exhibits probiotic properties, including resistance to the harsh gastrointestinal conditions and the high ability of adhesion to intestinal cells, without any risks for human health [22]. These traits underscore its potential in fighting pathogens.

In the present work, we evaluated the capacity of *P. pentosaceus* MZF16 to compete with *P. aeruginosa* H103 and reduce its virulence. For this, undifferentiated intestinal Caco-2/TC7 cells were treated simultaneously with both bacteria and the cytotoxicity, inflammatory potential, and adhesion/invasion of H103 have been investigated. Our study demonstrated that *P. pentosaceus* MZF16 can mitigate *P. aeruginosa* H103-induced cytotoxicity and inflammation in intestinal Caco-2/TC7 cells. Specifically, MZF16 reduced both LDH release (necrosis marker) and IL-8 secretion. These findings align with previous research showing similar protective effects of probiotics against various pathogens. For example, *P. acidilactici* SPM138 increased the human intestinal epithelial cells HT-29 cells survival rate from 20% to 98% and reduced by more than 60% IL-8 levels released upon *Clostridium difficile* infection [32]. Similarly, *P. acidilactici* TMAB26 demonstrated immunomodulatory effects by alleviating *Klebsiella pneumoniae* endotoxin-induced gut inflammation [12].

*P. pentosaceus* MZF16 also reduced the adhesion, invasion and translocation of *P. aeruginosa* H103 across undifferentiated/differentiated Caco-2/TC7 cells, preserving epithelial barrier integrity as evidenced by TEER measurements. Similar anti-adherence effects have been reported for *P. acidilactici* IAH-5 against *S. Typhimurium* [33]. In another study, *P. pentosaceus* L1 isolated from pickled radish enable also the decrease of adhesion of an *E. coli* enterotoxigenic strain to porcine intestinal epithelial cells [34].

The observed reduction in adhesion/invasion and cytotoxicity may result from direct probiotic-pathogen interaction or a postbiotic effect mediated by secreted factors. In fact, autoaggregation is stated to be a prerequisite for probiotic bacteria adhesion to intestinal epithelium, whereas their coaggregation abilities with pathogens enable forming an effective barrier, preventing epithelium colonization by harmful bacteria. Our strain *P. pentosaceus* MZF16 exhibited high autoaggregation (44.7%) and coaggregation abilities with H103 (45.7%), supporting its role in forming a protective barrier. This coaggregation capacity aligns with previous findings for *P. pentosaceus* DHR005 which showed lower autoaggregation (28,6%) and coaggregation (22%) against *Salmonella* Typhimurium PTCC1609 [33].

*P. pentosaceus* MZF16 is able to produce lactic acid and coagulin bacteriocin [22], we investigated if the protective effect of the probiotic was related to one of these aspects. Interestingly, despite the ability of *P. pentosaceus* MZF16 to produce antibacterial metabolites, no direct antimicrobial activity was detected against *P. aeruginosa* H103 under our experimental conditions. Whereas, in other studies, bacteriocins from some *P. acidilactici* and *P. inopinatus* strains exerted antimicrobial activities against *P. aeruginosa* [10,14]. This lack of activity may be due to culture conditions, as bacteriocin production can vary based on time of exposition and media composition. Nonetheless, the ability of MZF16 to co-exist with H103 cocultured in DMEM medium (data not shown) suggests that in our experimental conditions, the protective effect of MZF16 was not directly linked to the production of lactic acid and/or the bacteriocin, but it may be related to a competitive mechanism for nutrients or epithelial binding sites [35].

Furthermore, other studies have shown that some *Pediococcus* species are able to produce exopolysaccharides and biosurfactants with a high ability to disrupt *P. aeruginosa* biofilms and display anti-microbial, anti-adhesive and anti-virulence properties [11,26].

The pathogenicity of *P. aeruginosa* involves the production of several virulence factors [36] such as pyoverdine and pyocyanin, regulated by the cell density-dependent signaling mechanism « quorum sensing (QS) » [37]. In *P. aeruginosa*, two main QS systems are defined, the system mediated

by *N*-acylhomoserine lactones (AHLs) known as Las and Rhl, which are responsible of the production of homoserine lactone signal molecules, and the Pqs system depending on 2-alkyl-4-quinolones (HAQ) and which is interlinked with the Las and Rhl systems. These systems are involved in the pathogenesis of *P. aeruginosa* and the synthesis of some virulence factors such as rhamnolipid, pyocyanin, pyoverdine and the production of enzymes like proteases and lipases and biofilm formation [38,39].

The inhibition of this signalization can attenuate QS-related bacterial virulence [40,41]. Thus, anti-QS may be a promising strategy to fight against some pathogens including *P. aeruginosa*. In this context; we aimed to evaluate the effect of MZF16 on *P. aeruginosa* H103 QS. Interestingly, we found that the production of HSL molecules and PQS level were notably reduced in presence of MZF16. Pyoverdine is a siderophore and an important virulence factor in *P. aeruginosa*, enabling iron sequestration from host to improve the bacterial establishment during the infection. Our results showed that *P. pentosaceus* MZF16 decreased the pyoverdine level released in the coculture media, suggesting that *P. pentosaceus* MZF16 may be able to modulate the QS of *P. aeruginosa* H103. In agreement with our findings, some strains of *Pediococcus*, i.e *P. acidilactici* M7 have shown a quorum quenching activity, inhibiting short-chain HSL production, motility, and virulence factors production (elastase, protease, pyocyanin, and biofilm production) of *Pseudomonas aeruginosa* isolates [42]. Another study on *P. acidilactici* HW01 bacteriocin showed a decrease of the production of virulence factors such as QS-regulated pyocyanin, protease, and rhamnolipid [10].

Finally, the slow killing assay on *C. elegans in vivo* model confirmed the anti-virulence effect of *P. pentosaceus* MZF16 against *P. aeruginosa* H103. Indeed, the presence of MZF16 has significantly enhanced the survival of *C. elegans* infected with *P. aeruginosa* H103. This result is consistent with studies showing that probiotics like *P. acidilactici* DM9 and *P. pentosaceus* SDL1409 extended remarkably *C. elegans* lifespan during *P. aeruginosa* PA14 infection [9].

## 5. Conclusions

This study highlights the potential of *Pediococcus pentosaceus* MZF16 as an effective probiotic against *Pseudomonas aeruginosa* H103. MZF16 is able to mitigate *in vivo* and *in vitro* cytotoxicity, decreases inflammation levels, and pathogen adhesion and invasion in intestinal models, preserves epithelial cells integrity, and modulates quorum-sensing pathways, reducing virulence factor production. These findings underscore the potential of using probiotics as adjunct therapies to combat multidrug-resistant pathogens. Further research is needed to decipher molecular mechanisms involved in these bacterial interactions.

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