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Drugs for Superbugs: Antibiotic Discovery, Modes of Action And Mechanisms of Resistance

Session: Repurposing & Antimicrobial Adjuvants (S4)

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UNIVERSITÀ DEL SALENTO

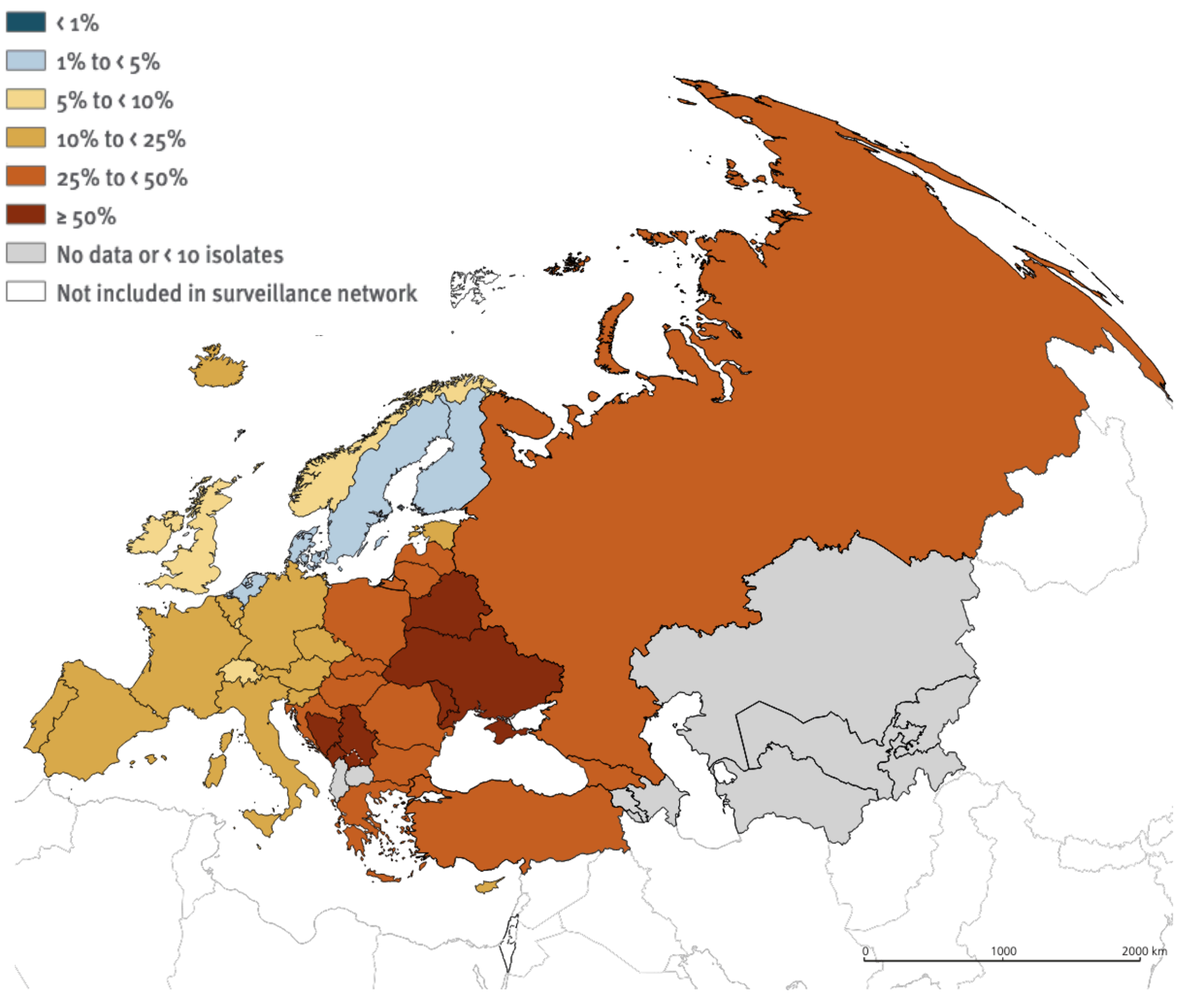
L'Ateneo tra i due mari

Off-target activity of spiramycin disarms *Pseudomonas aeruginosa* by inhibition of biofilm formation, pigment production and phenotypic differentiation

MATTEO CALCAGNILE & PIETRO ALIFANO

Identikit of *Pseudomonas aeruginosa*

- Gram-negative bacterium
- one of the most common pathogens in chronic lung infection
- the third most common pathogen associated with nosocomial urinary tract infections (UTIs)
- multi-drug resistance (acquired resistance)



***P. aeruginosa*: percentage of invasive isolates with **resistance to carbapenems,** by country/area, WHO European Region, 2020 (Antimicrobial resistance surveillance in Europe 2022 - 2020 data- ECDC)**

Pseudomonas aeruginosa

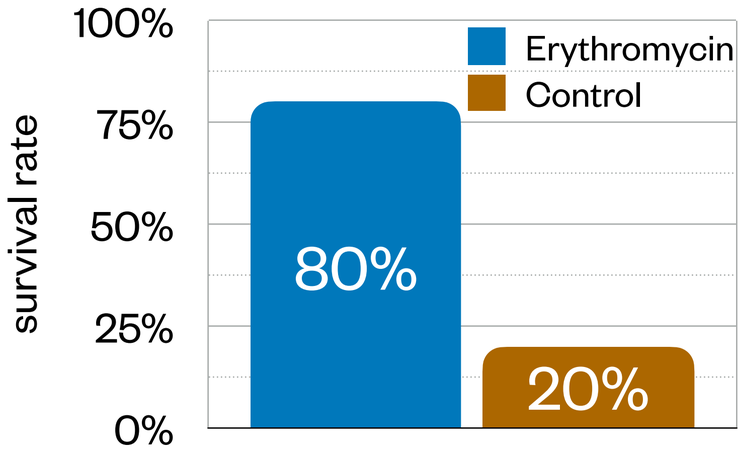
in vitro evidence:

spontaneously resistance to macrolides

Treatment with Macrolides of *P. aeruginosa* infection

1 mouse model of *P. aeruginosa* bacteraemia, treatment with erythromycin

INCREASE SURVIVAL RATE (HIRAKATA ET AL., 1992).



2 "A 4–6-month trial of azithromycin is justified in children with cystic fibrosis who do not respond to conventional treatment. The mechanism of action remains unknown." (Equi et al., 2002)

Speculation on macrolides:

- down-modulation of the inflammatory response (host)
- down-modulation of the *P. aeruginosa* virulence.

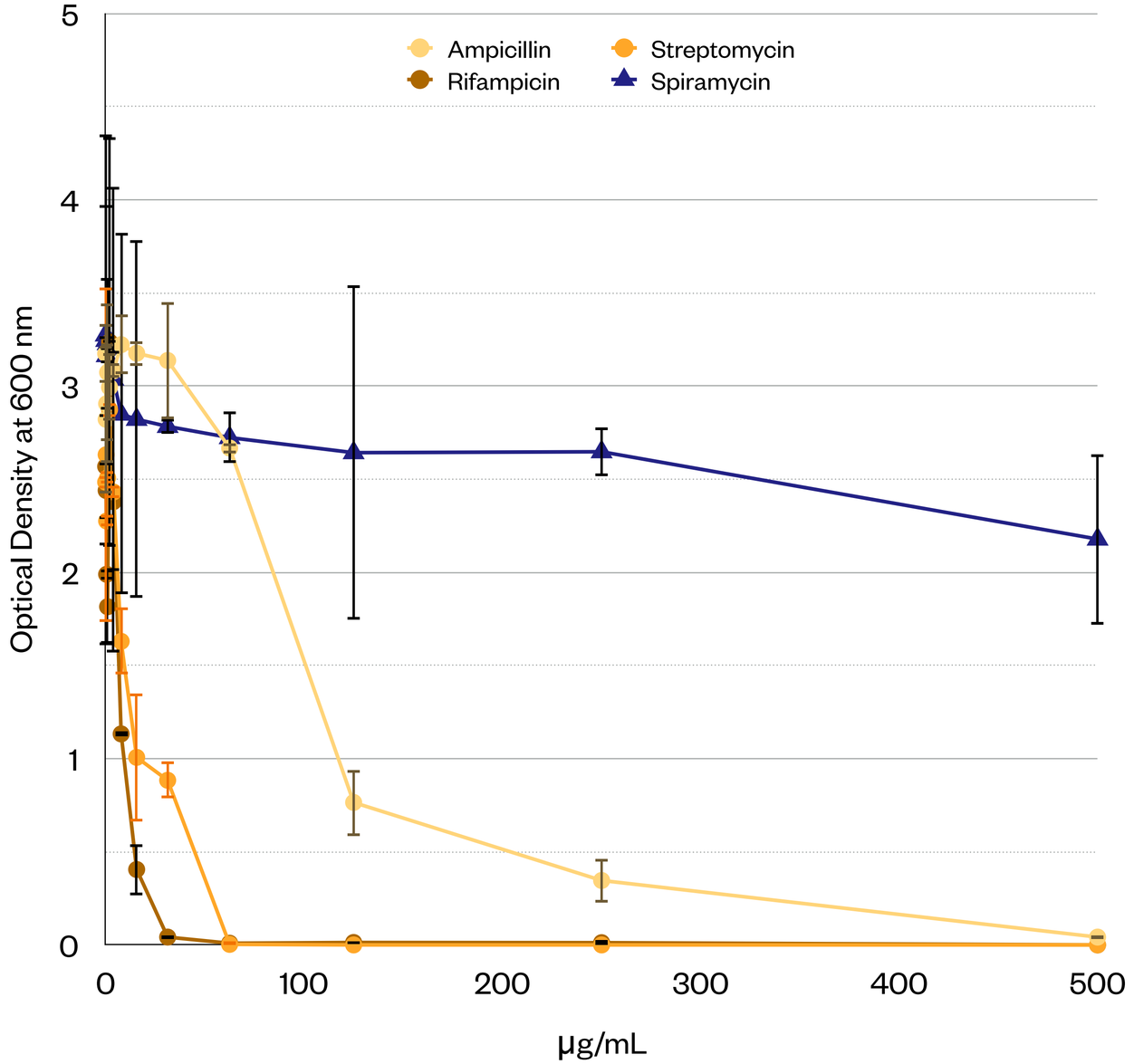
Serendipity in drug repurposing

We studied the sensitivity of *P. aeruginosa* to antibiotics. We observed that this bacterium was resistant to spiramycin.

Usually, the bacterium is green due to the pigments (e.g., pyocianin). With spiramycin (from 15 µg/mL), the strain was white but continued to grow.

µg/mL	Ampicillin	Streptomycin	Rifampicin	Spiramycin
500	-	-	-	+
250	+	-	-	+
125	+	-	-	+
62.5	+	-	-	+
31.25	+	+	-	+
15.625	+	+	+	+

-	Inhibited Bacteria
+	Active growing Bacteria



Spiramycin

- 16-membered macrolide
- produced by *Streptomyces ambofaciens*
- Effective against Gram-positive pathogens (*Staphylococcus aureus*, streptococci of groups A, B, C and D, and pneumococcus)
- It is also effective against Gram-negative pathogens (*Neisseria, Legionella*)
- It is an antiparasitic agent (active against *Toxoplasma spp*.)
- The antibacterial activity was associated with its ability to bind the 50S ribosomal subunit and to block the translation

Effect of spiramycin on Pyocianin production

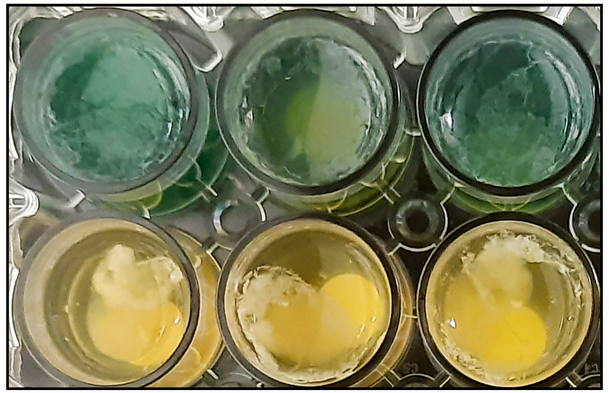
Spiramycin (from 500 µg/mL to 1 µg/mL) does not inhibit the growth of *P. aeruginosa* in LB medium in both liquid and solid (Fig. A and Fig. B) cultures

MIC (minimal inhibitory concentration)

Estimation of biomass using optical density (O.D.) show as *P. aeruginosa* is sensitive to ampicillin, Rifampicin and Streptomycin (Fig. B).

Spiramycin (from 500 µg/mL to 30 µg/mL) affects the production of pigments in *P. aeruginosa* cultures (Fig. C) or solid (Fig. A).

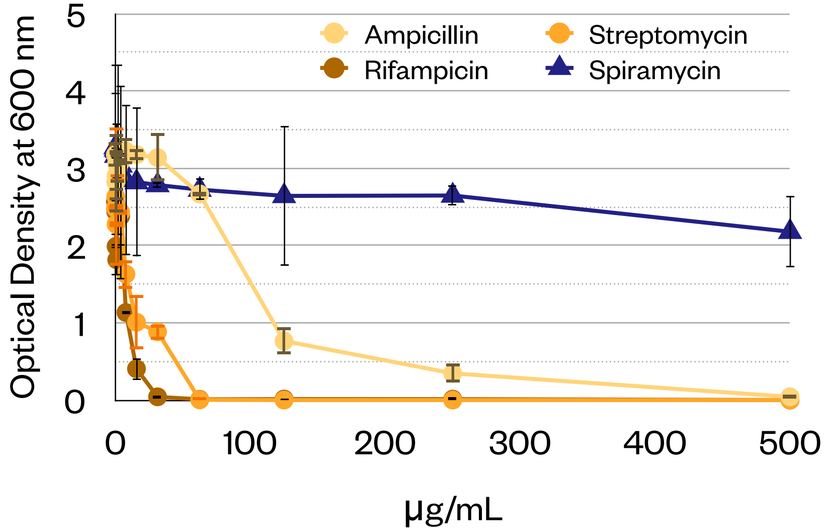
A



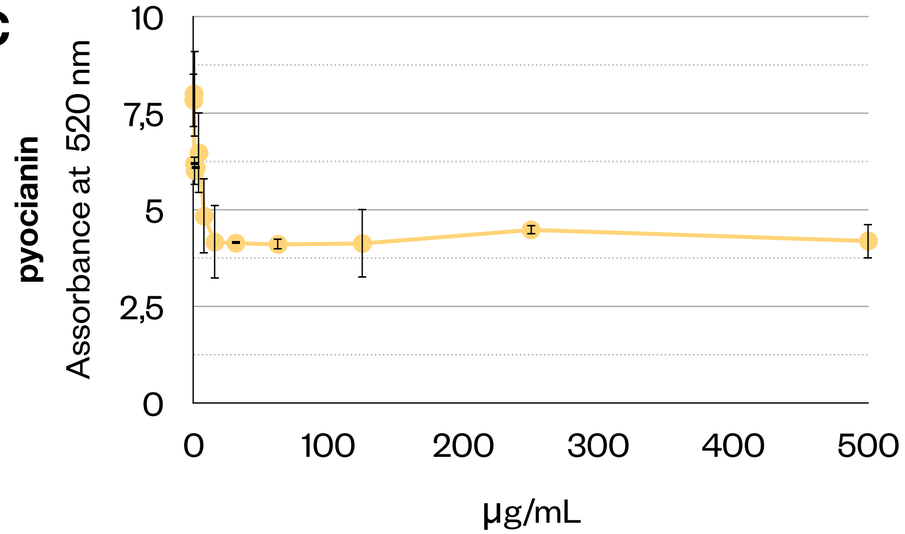
Control

Spiramycin 30 µg/mL

B



C



Effect of spiramycin on biofilm formation

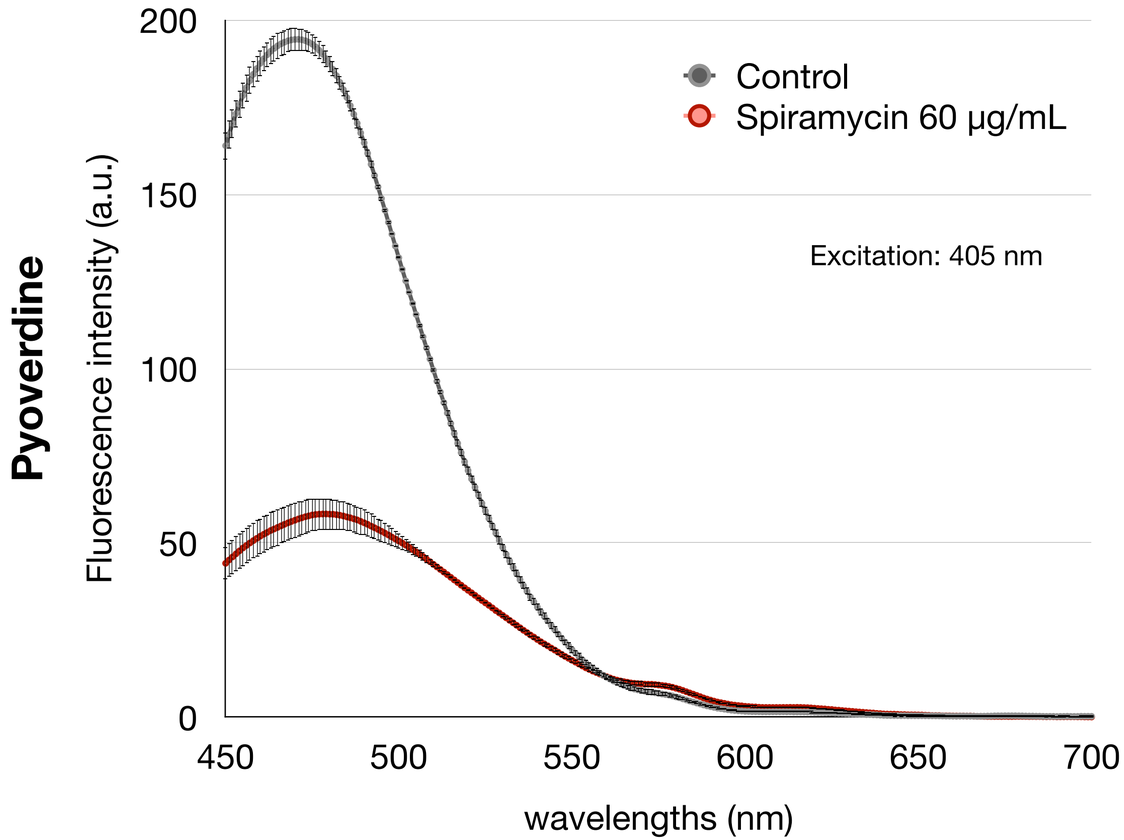
We cultured *P. aeruginosa* using hydroxyapatite dishes as supports to form a biofilm.

After 72 h we detected:

- Pigments production (Fig. A, B e C)
- Biofilm formation (Fig. D and E)
- Planktonic cell (Fig. F)

Spiramycin (60 µg/mL) leads to the inhibition of both biofilm and pigments (Pyocyanin & Pyoverdine)

	Units	Control	Spiramycin (60 µg/ml)
Biofilm	CFU/support	$1x10^6$	$5x10^3$
Planktonic cells	Total protein (µg/µL)	1.5	2
Pyocyanin	Absorbance at 520 nm	4.2	9.22



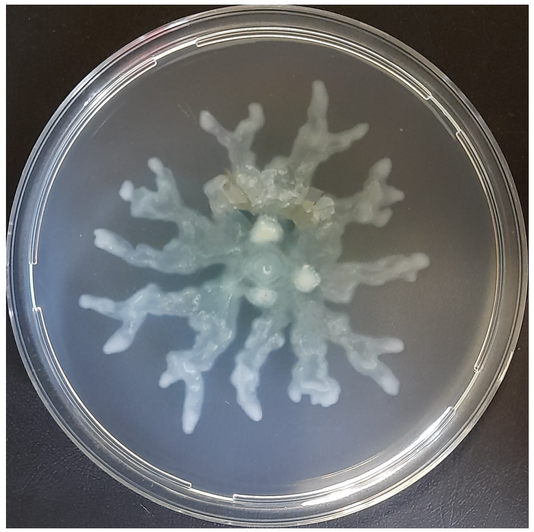
Effect of spiramycin on motility and rhamnolipids production

We tested the ability of spiramycin to inhibit the motility of the bacterium using an appropriate minimal medium (Fig. A, control; Fig. B, spiramycin 60 µg/mL).

We cultured *P. aeruginosa* in 250 ml flasks filled with 50 ml of LB (72 h, 37 ° C, 180 rpm). We measured rhamnolipids using orcinol.

Spiramycin inhibits motility and reduces the amount of rhamnolipids (biosurfactants) produced

A



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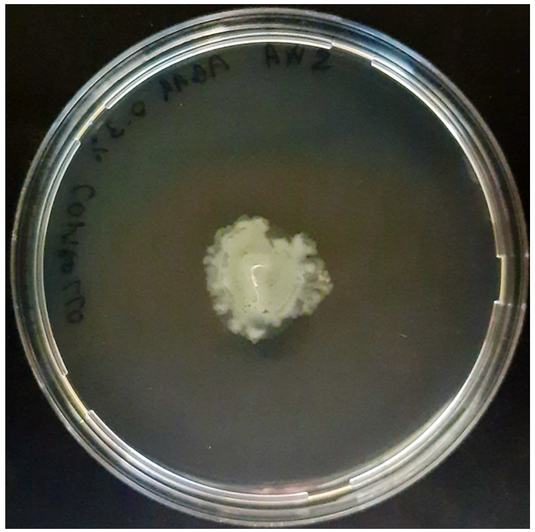

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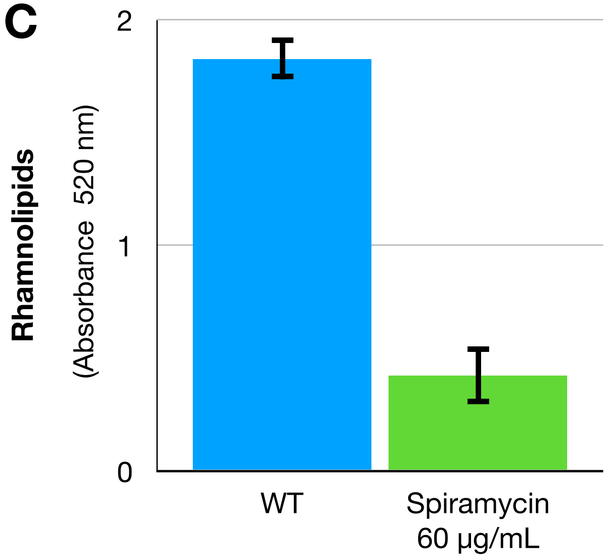


Infection of *G. mellonella* larvae - Protocol

We used *Galleria mellonella* larvae as an in vivo infection model.

1. **Inoculum:** a bacterial suspension formed by an injection solution (10 mM $MgSO_4$) and bacteria (O.D. at 600 nm of 0.1)

2. **Dilution:** we diluted the solution obtained 7 times (dilution 1:10).

3. **Injection:** we injected 5 µl aliquots into the larvae through the last left leg using a 10 µl Hamilton syringe.

We prepared two different samples:

1) control (without spiramycin);

2) treated. In the second case, both LB (medium of growth) and injection solution contained spiramycin (60 mg/mL).

We incubated the larvae at 37°C for 24 hours.

Infection of *G. mellonella* larvae - Results

CONTROL

P. AERUGINOSA INFECTION

P. AERUGINOSA INFECTION AND SPIRAMYCIN (60 mg/mL)

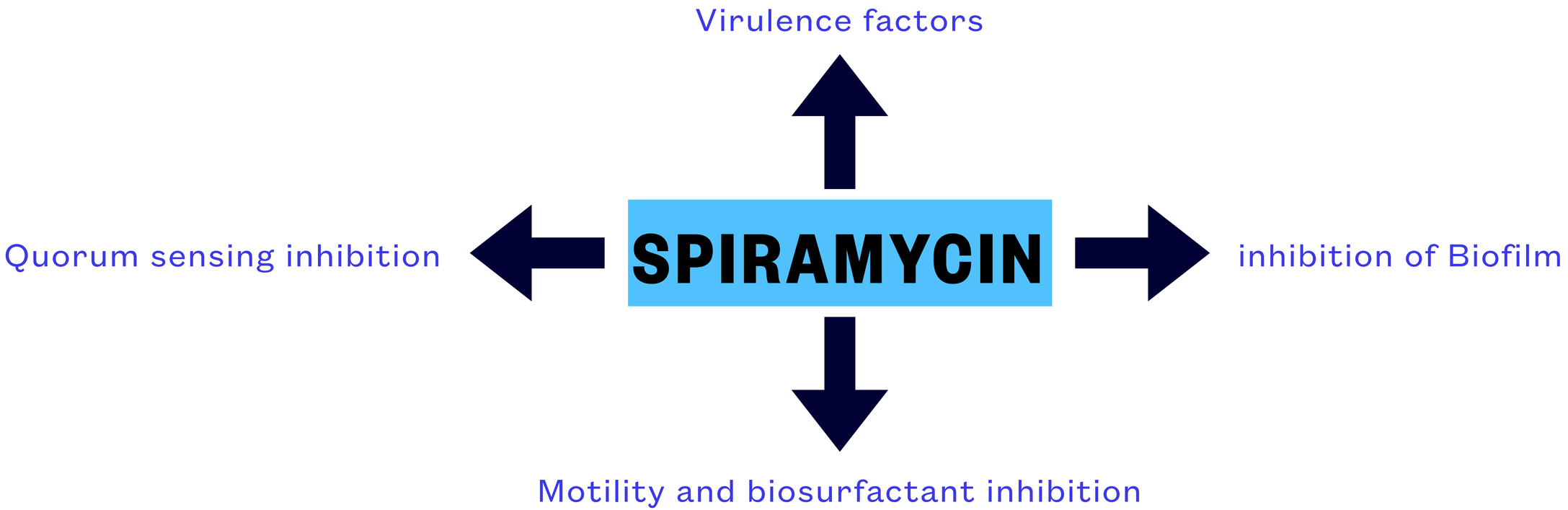
The results show that infecting the larvae with only *P. aeruginosa* mortality is about 92%.

Mortality drops to 30% when the bacterium is inoculated with spiramycin.

These results are preliminary and will be replicated in the future.

One Hypotesis

Spiramycin binds the quorum-sensing regulators. This phenomenon leads to an inhibition of biofilm formation, motility and more.



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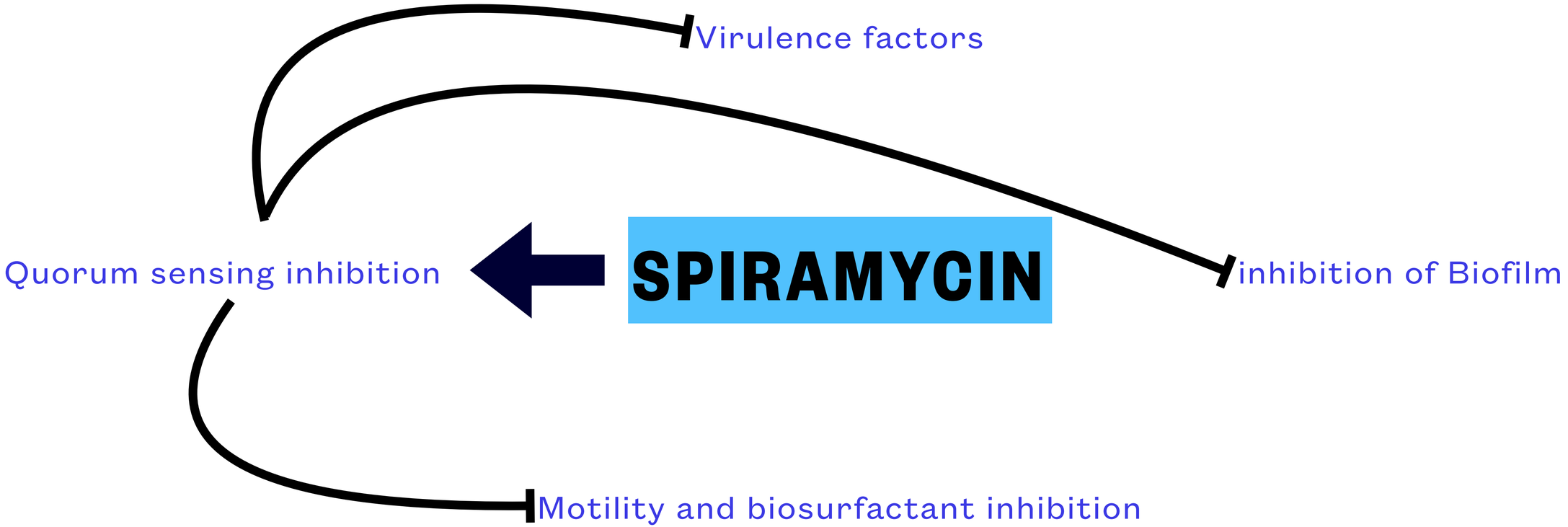
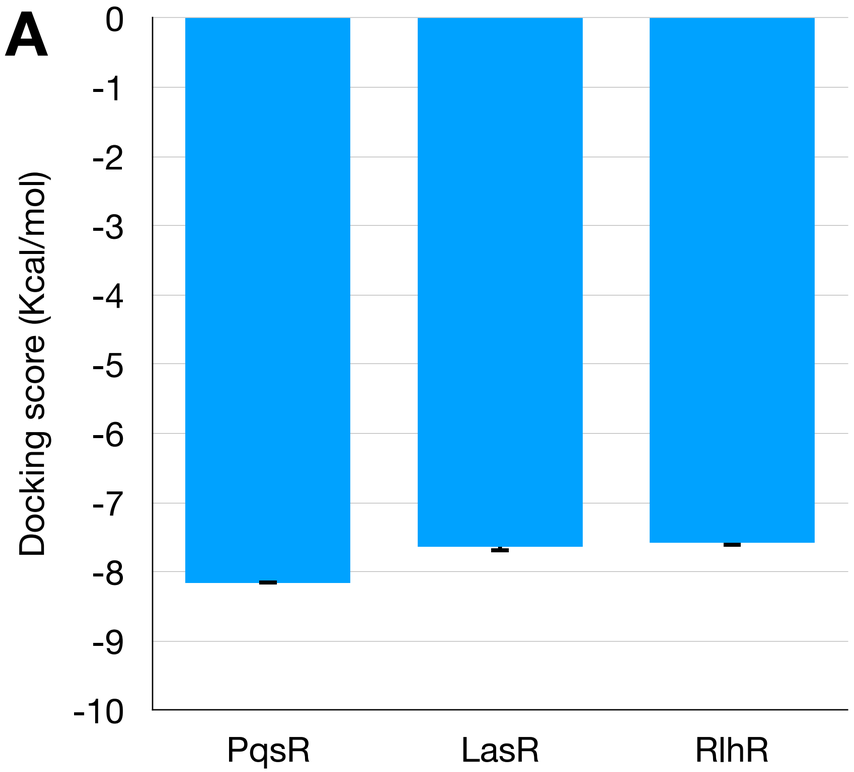
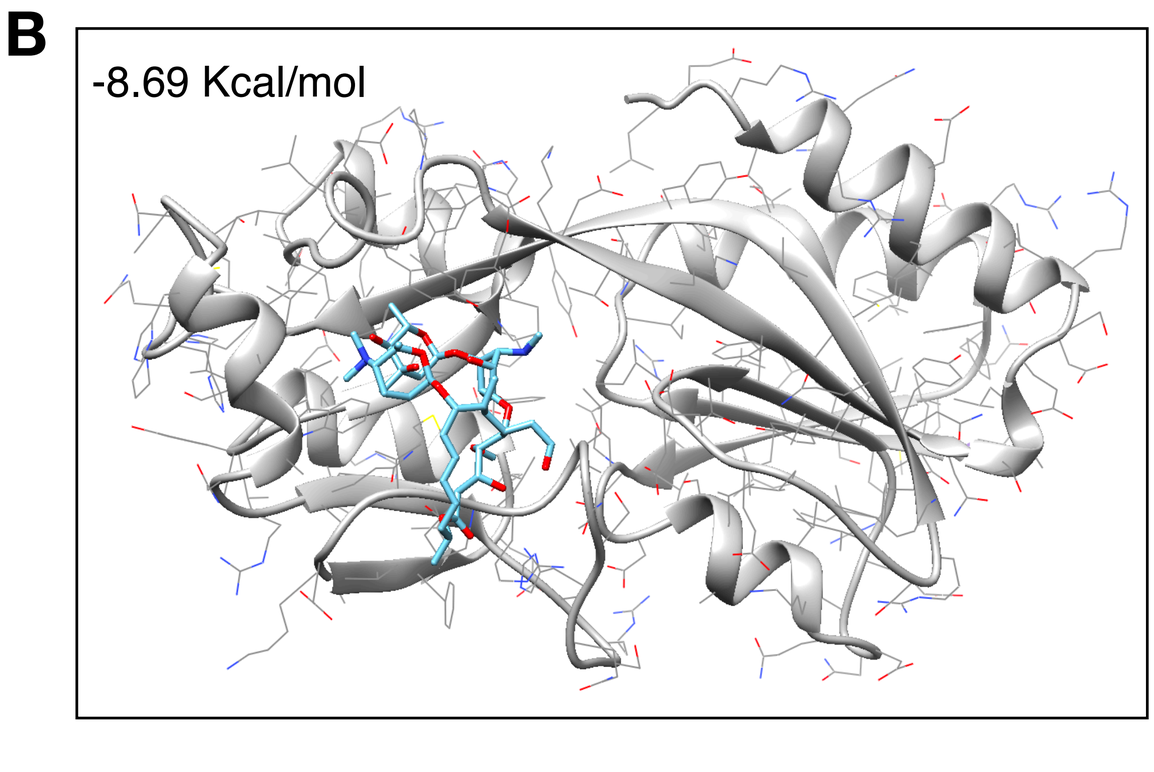


Docking: to test this hypothesis we performed *in-silico* analysis

We included the three known QS regulators in *P. aeruginosa* in this analysis: LasR, PqsR and RhlR.

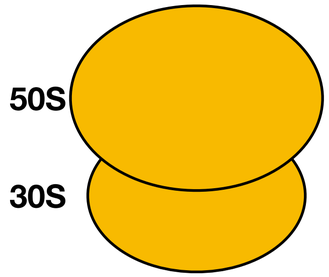




According to the results obtained with SwissDock, PqsR could be the target of spiramycin.

Another Hypothesis ...

Another hypothesis of the mechanism of spiramycin against *P. aeruginosa* could concern ribosomes:



Direct effect

The bond between ribosome and spiramycin changes the translation kinetics

indirect effect

Effect on proteins involved with the processing of secondary RNA structures.

Decrease of proteins involved in virulence (effect only on some mRNAs).

Conclusions & Perspective

- Our in vitro results show as spiramycin inhibits motility, biofilm formation, rhamnolipids production and pigment secretion by *P. aeruginosa*.
- Our in vivo model show as spiramycin inhibit the virulence of *P. aeruginosa* injected in *G. mellonella* larvae.
- We have elaborated two hypotheses on the mechanism including:
    1. QS inhibition (supported by docking analysis)
    2. Direct or indirect interaction with the ribosome

We expect to obtain data on the mechanism of action in the future.

The data presented could be the basis for the design of clinical trials.

Thanks !

References

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