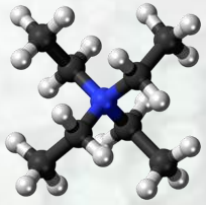
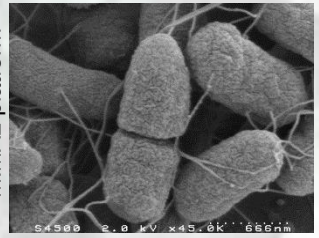
European FooD-STA

Evaluation and optimization of antimicrobial activity





MIMA2 platform

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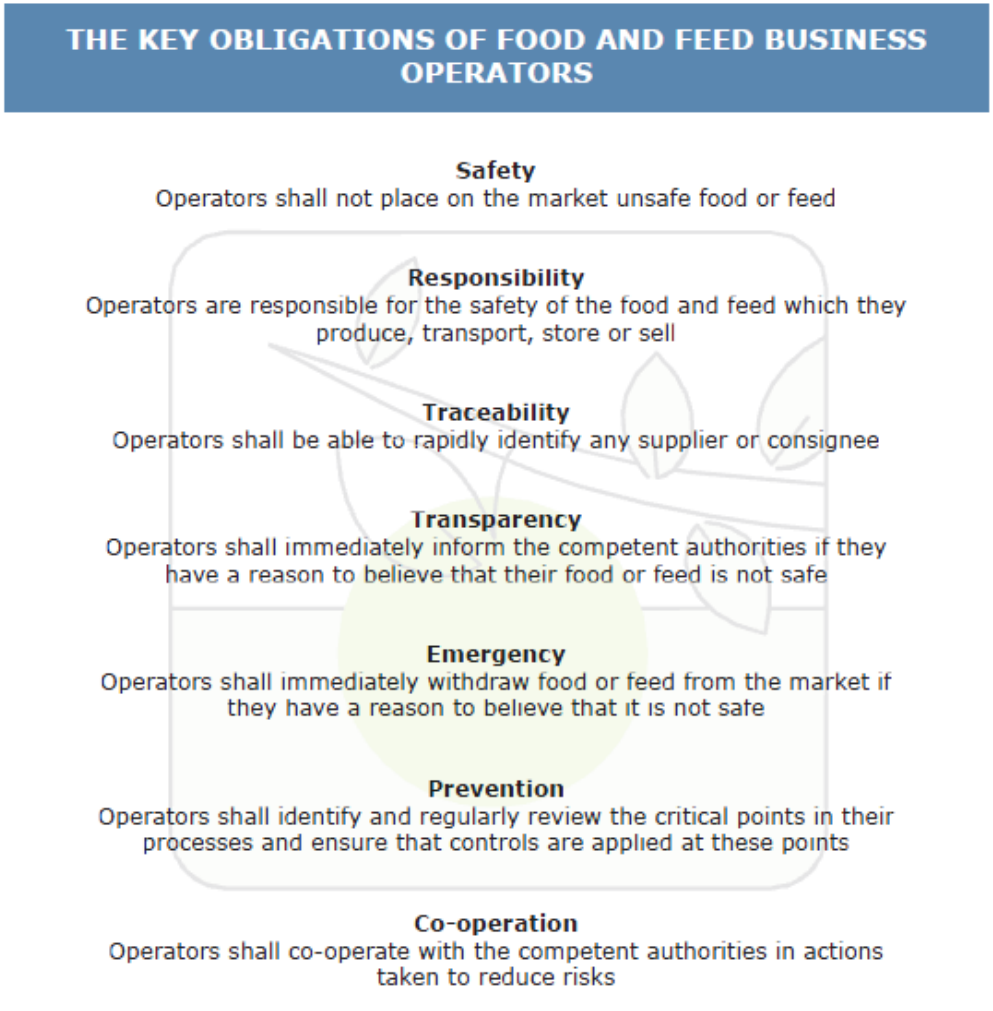
Regulation (EC) N° 178/2002 or « Food Law »

It lays down the definitions, principles and obligations covering <u>all stages</u> of food and feed production and distribution.

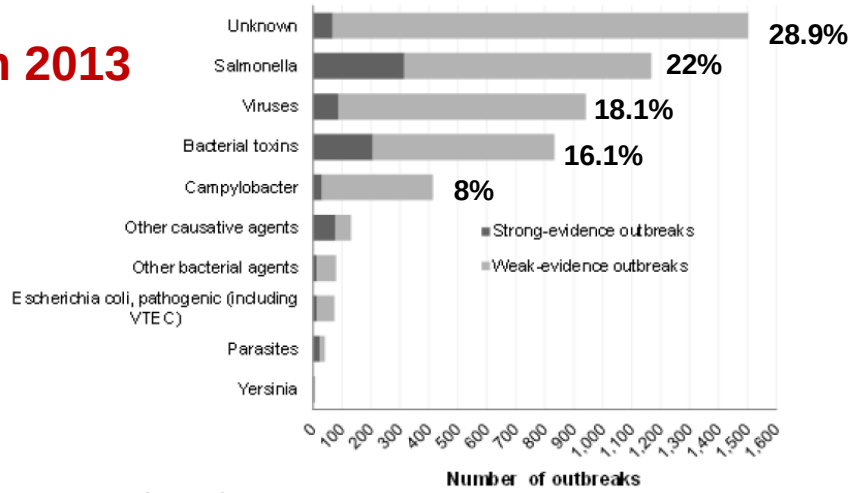
- ✓ **Establishing the** general principles of food **legislation**
- ✓ **Created the European Food Safety Authority (Efsa)**
- ✓ **Edicting procedures relative to food security** (rapid alert system)



Introduction

5,196 foodborne outbreaks in Europe in 2013

43,183 human cases, 11 deaths



(EFSA Journal 2015)

Bacterial toxins include toxins produced by *Bacillus*, *Clostridium* and *Staphylococcus*. Food-borne viruses include calicivirus, hepatitis A virus, flavivirus, rotavirus and other unspecified viruses. Other causative agents include mushroom toxins, marine biotoxins, histamine, mycotoxins and escolar fish (wax esters). Parasites include primarily *Trichinella*, but also *Cryptosporidium*, *Giardia* and other unspecified parasites. Other bacterial agents include *Listeria*, *Brucella*, *Shigella*, *Vibrio* and other unspecified bacterial agents. In this figure, the category 'Pathogenic *Escherichia coli* (including VTEC)' also includes one strong-evidence outbreak due to pathogenic *E. coli* other than VTEC.

Figure 2. Distribution of all food-borne outbreaks per causative agent in the EU, 2013

Causes that lead to foodborne outbreaks

Contaminated raw materials

Equipment

Contamination by staff

Mistakes during food preparation

Problem of preservation

In most of the cases, there are more than one cause!

(BEH, France 2013)

Introduction

Food preservation

Cleaning and disinfection of equipment

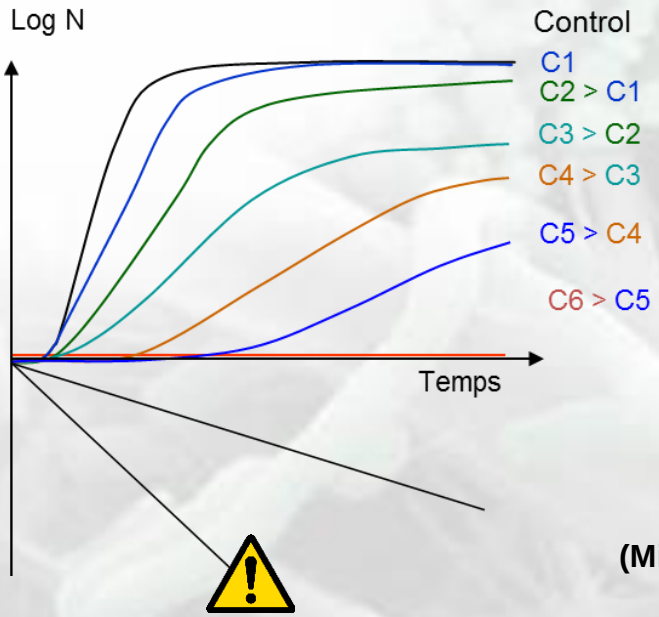
Antimicrobials can

Prevent microorganisms' growth

Inactivate microorganisms

Limit the increase of the initial level of contamination

Decrease the initial level of contamination



Inhibitory (bacteriostatic) activity

MIC determination

(Minimum inhibitory concentration)

bactericidal activity

MBC determination

(Minimum bactericidal concentration)

Activity depends on concentration

Introduction

Preservatives in foods

Regulations CE/1331/2008 and CE/1333/2008

Substances which prolong the shelf-life of foods by protecting them against deterioration caused by microorganisms and/or which protect against growth of pathogenic microorganisms

Examples for canned or botttled fruit and vegetables

E-number	Name	Maximum level (mg/l or mg/kg as appropriate)
E 263	Calcium acetate	*quantum satis*
E 270	Lactic acid	*quantum satis*
E 296	Malic acid	*quantum satis*

Disinfectants on surfaces

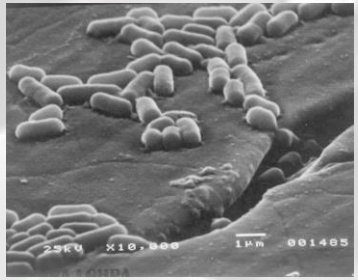
Regulation (EU) N°528/2012

Positive lists

Substance or mixture with the intention of destroying, rendering harmless or controlling effect on harmful organism

Product-type4 : food and feed area
Products used for disinfection of equipment, containers, consumption utensils, surfaces and pipework associated to the production, transport, storage or consumption for food and feed for humans and animals

MIMA2 platform



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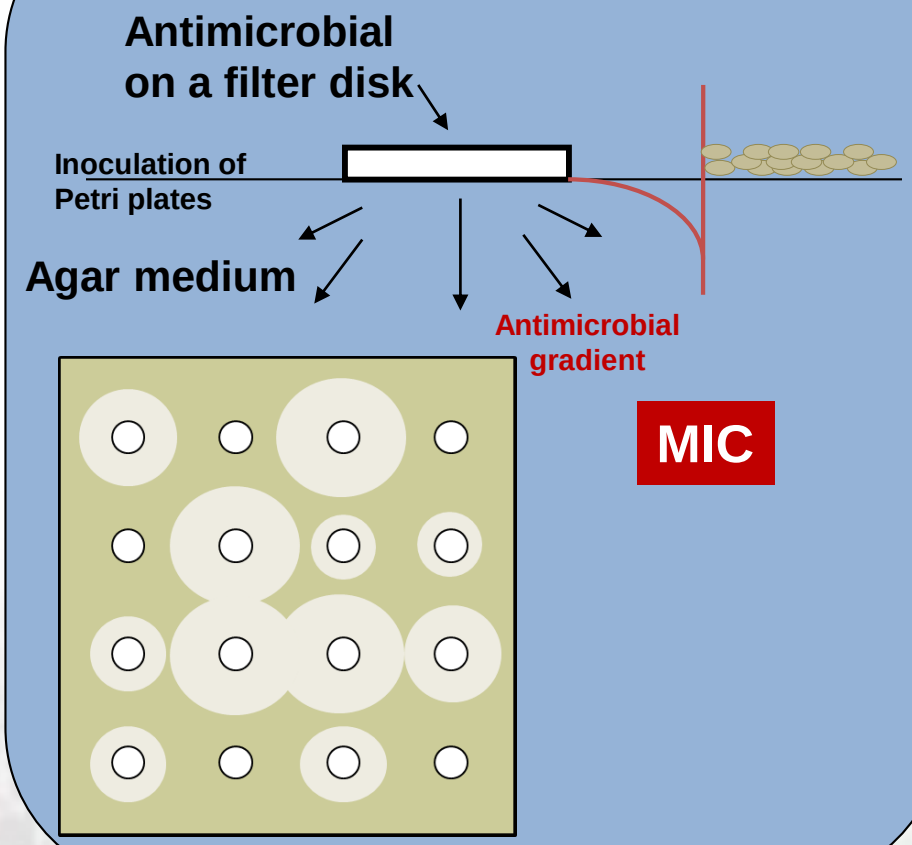
Part I

How can food operators better evaluate the efficiency of preservatives and optimize their use?

Better characterization of inhibitory activity (MIC)

Classical methods to determine bacteriostatic activity of an antimicrobial compound

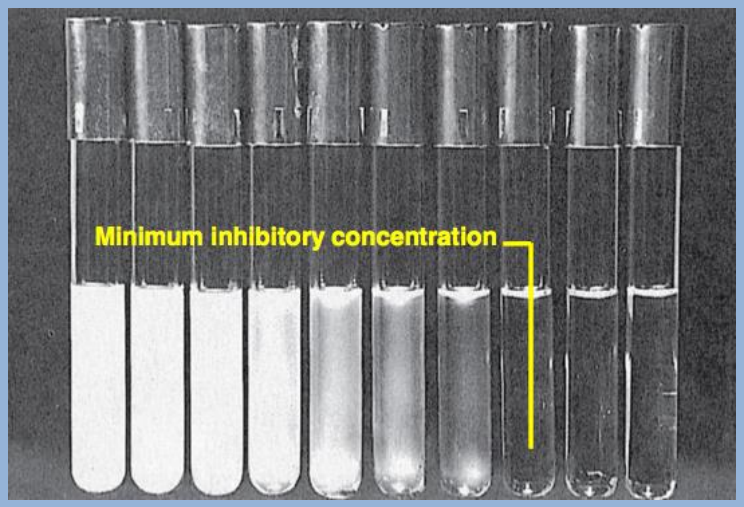
Diffusion method



MIC

Dilution method

Inoculation of nutrient broth containing different antimicrobial concentrations



Control without antimicrobial | C1 C2 C3 C4 C5 C6 C7 C8 C9

Growing concentrations of antimicrobials

C6<MIC<C7

Comparison of strain sensitivity or antimicrobial efficiency

Better characterization of inhibitory activity (MIC)

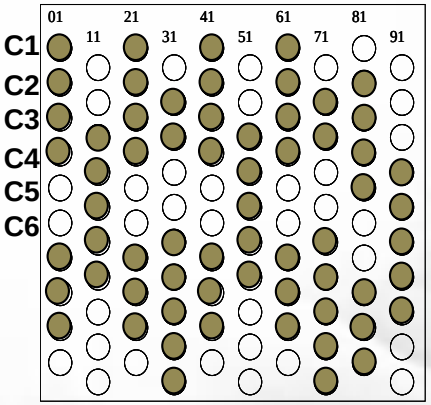
Method

Improvement of dilution method by modeling

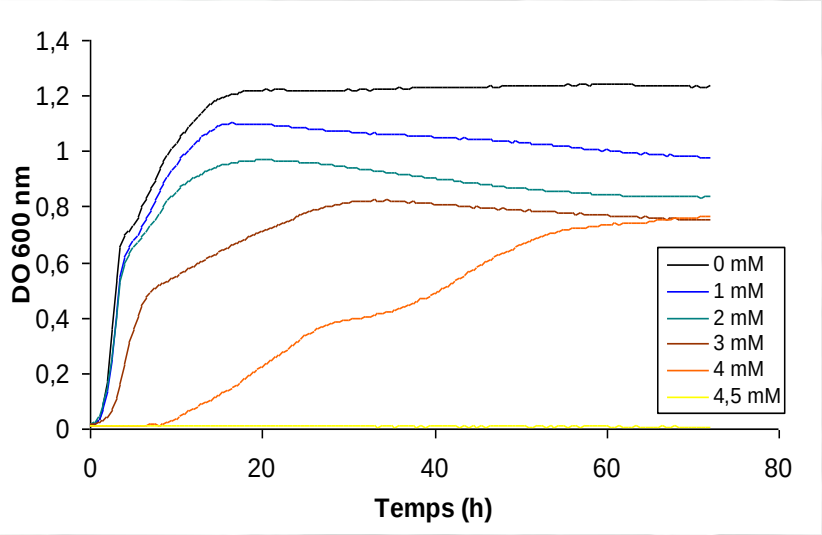
1rst step: continuous recording of absorbance during bacterial growth

Automatic absorbance microplate reader

200 simoultaneous growth curves



C4 < CMI < C5

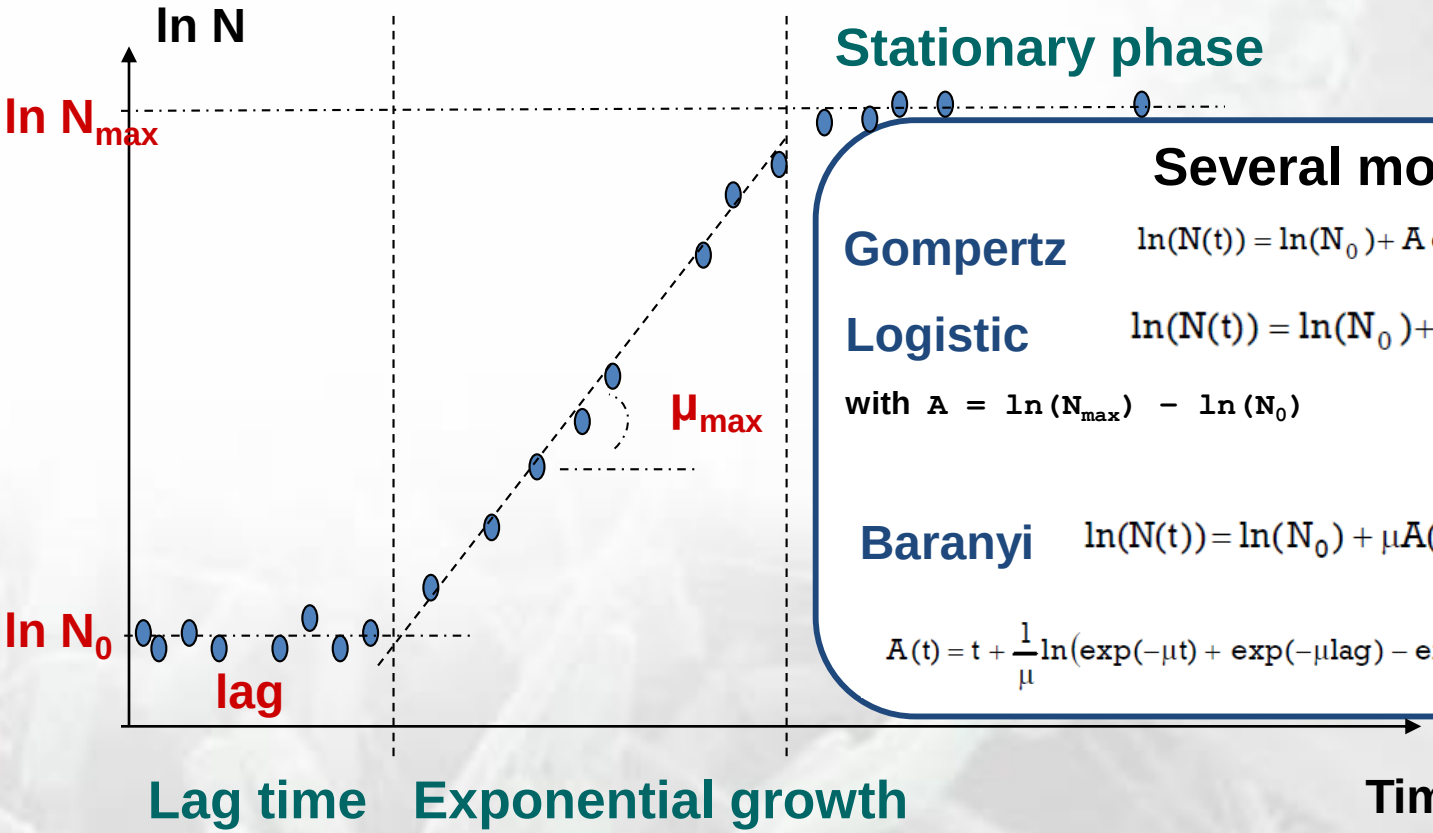


- 200-400 µl of broth with growing concentrations of antimicrobials
- inoculation with the bacterial culture (1%)
- setting of temperature, agitation
- setting of duration of recording (ex: every 30 min during 72h)

Method

Improvement of dilution method by modeling

2nd step: modeling growth as a function of time to determine μmax for each antimicrobial concentration



Several models

Gompertz

$$\ln(N(t)) = \ln(N_0) + A\exp\left(-\exp\left(\frac{\mu e}{A}(lag - t) + 1\right)\right)$$

Logistic

$$\ln(N(t)) = \ln(N_0) + \frac{A}{1 + \exp\left(\frac{4\mu}{A}(lag - t) + 2\right)}$$

with $A = \ln(N_{max}) - \ln(N_0)$

Baranyi

$$\ln(N(t)) = \ln(N_0) + \mu A(t) - \ln\left(1 + \frac{\exp(\mu A(t)) - 1}{\frac{N_{max}}{N_0}}\right)$$

$$A(t) = t + \frac{1}{\mu}\ln\left(\exp(-\mu t) + \exp(-\mu lag) - \exp(-\mu t - \mu lag)\right)$$

Method

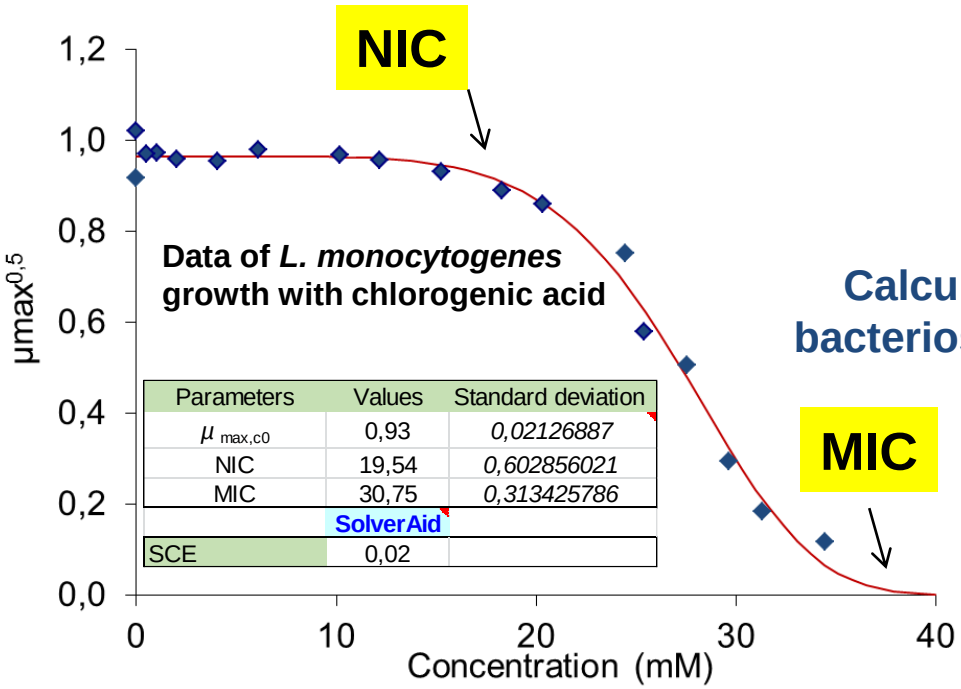
Improvement of dilution method by modeling

3rd step: modeling the impact of antimicrobial concentration on µmax

Lambert-Pearson model (2000)

$$\sqrt{\mu_{max}(c)} = \sqrt{\mu_{max}(c=0)} \cdot g(c)$$

with $$g(c) = \exp\left[-\left(\frac{c}{MIC / \exp\left(\frac{\ln(NIC/MIC)}{-e}\right)}\right)^{\left(\frac{-e}{\ln(NIC/MIC)}\right)}\right]$$



Parameters	Values	Standard deviation
$\mu_{max,c0}$	0,93	*0,02126887*
NIC	19,54	*0,602856021*
MIC	30,75	*0,313425786*
	SolverAid	
SCE	0,02	

Calculation of two parameters that characterize bacteriostatic activity of an antimicrobial compound

MIC = minimal inhibitory concentration

NIC = non-inhibitory concentration

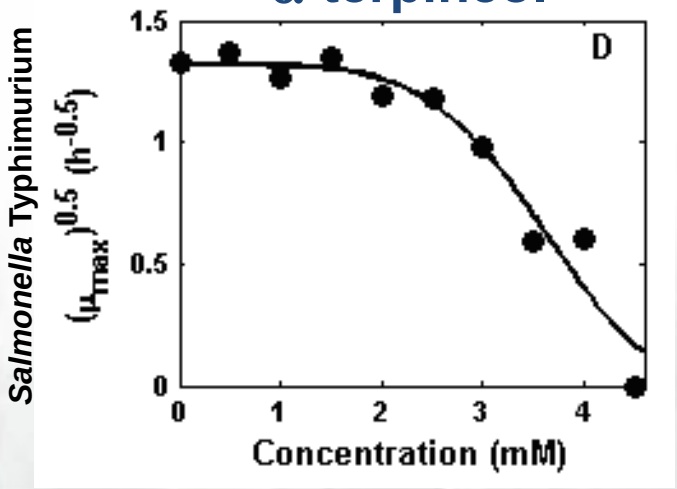
Method

Improvement of dilution method by modeling

3rd step: modeling the impact of antimicrobial concentration on µmax

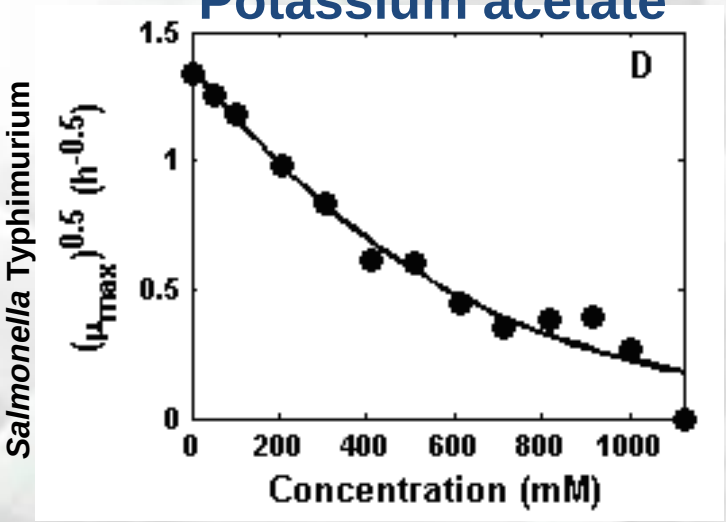
Several profiles that can guide the choice of the best antimicrobial according to the context

α-terpineol



MIC (72h)= 4,10 mM [3,75-4,46]
NIC (72h)= 2,31 mM [1,67-2,96]

Potassium acetate



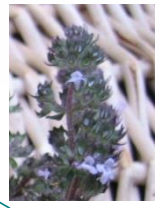
MIC (72h)= 801,40 mM [556,78-1028,30]
NIC (72h)= 34,91 mM [0-128,94]

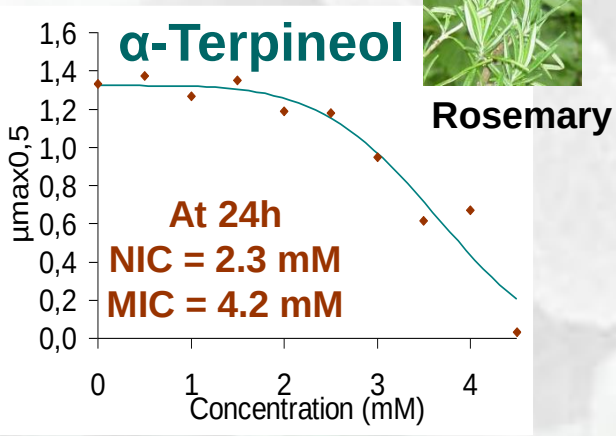
Guillier, L., *et al.* (2007). *Journal of Food Protection 70(10): 2243-2250*

Better characterization of inhibitory activity (MIC)

Application

Phyto-phenols as growth inhibitors for *Salmonella* Typhimurium





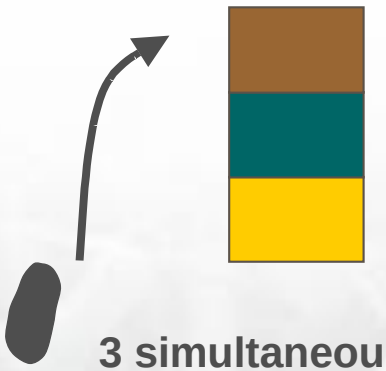
Guillier, L., *et al.* (2007). *Journal of Food Protection 70(10): 2243-2250*

Optimization of combinations

Application

Multifactorial preservation with combinations of several antimicrobial compounds

To maintain food safety while lowering the doses of each compound



3 simultaneous barriers

Roller (2000)

Benefits :

- **To remain easily below regulation limits (if there are some)**
- **To remain below the organoleptic threshold (for compound such as essential oils)**
- **To optimize costs (balance between expensive/efficient and cheaper/less efficient compounds)**

How can food operators optimize antimicrobial combinations?

Optimization of combinations

Method

Two-by-two combinations

Checkerboard method

NIC →

	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
0										X	X
0.1									X	X	X
0.2								X	X	X	
0.3							X	X	X		
0.4						X	X	X			
0.5					X	X	X				
0.6				X	X	X					
0.7			X	X	X						
0.8		X	X	X							
0.9	X	X	X								
1	X	X									

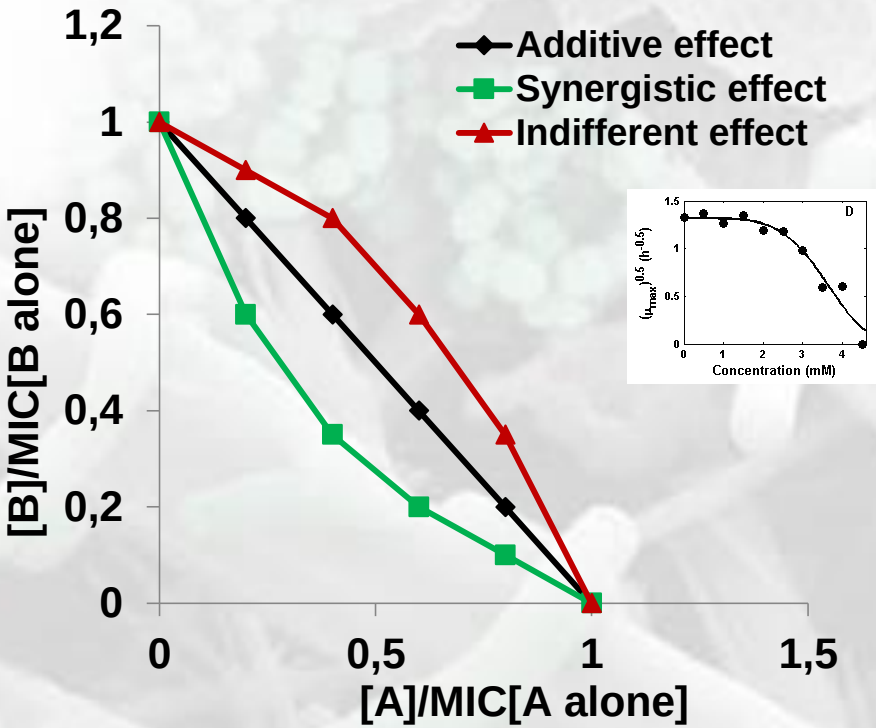
MIC →

Antimicrobial B

Antimicrobial A $\frac{C_A}{MIC_A\ alone}$

Isobole

curve connecting equivalent doses of both compounds that lead to total bacterial inhibition



$$FIC_{index} = \frac{MIC_A\ combination}{MIC_A\ alone} + \frac{MIC_B\ combination}{MIC_B\ alone}$$

$FIC_{index} < 0.5$ ➡ Synergistic effect

$0.5 < FIC_{index} < 1$ ➡ Additive effect

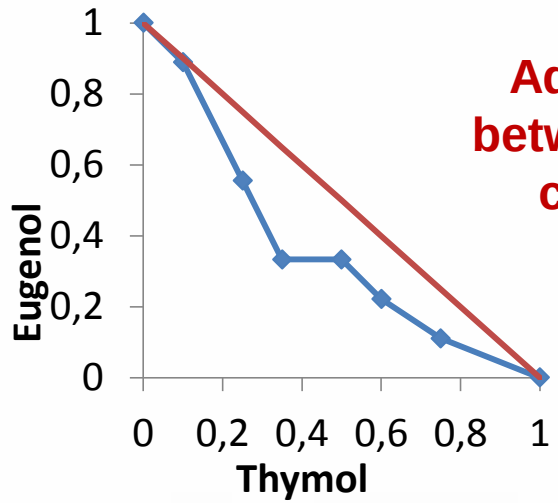
$1 < FIC_{index} < 4$ ➡ Indifferent effect

$FIC_{index} > 4$ ➡ Antagonistic effect

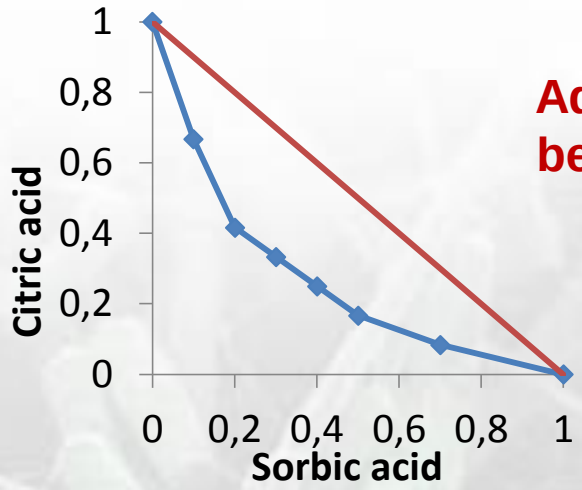
Optimization of combinations

Application

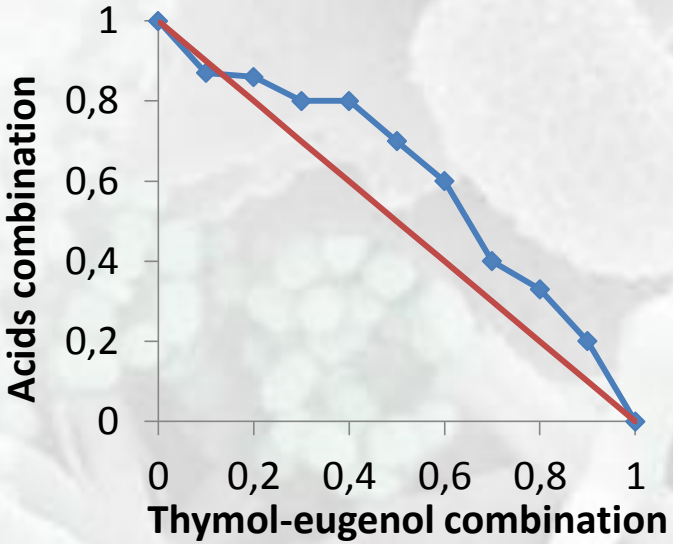
Real case of improvement of an antimicrobial mixture for feed products



Additive effect between aromatic compounds



Additive effect between acids



Indifferent effect between both groups

Part I conclusion

Better evaluate and optimize the efficiency of preservatives in food

Conclusion

The dilution methodology associated to modeling

Highly efficient for fast and accurate screening of numerous compounds

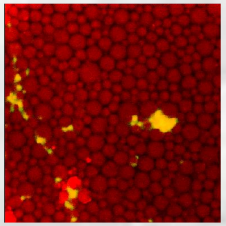
- **MIC : more accurate data with confidence intervals**
- **NIC : minimum concentration to use**

Limits

Not applicable in food matrices

Plate counts or

Confocal Laser Scanning Microscopy



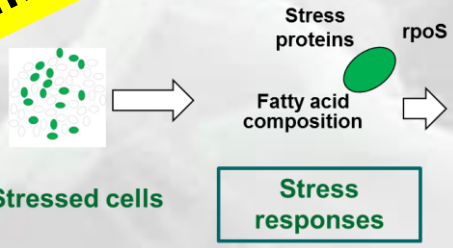
Conclusion

The use of combinations

Highly efficient FIC method for accurate evaluation of synergistic / antagonistic / indifferent antimicrobial effect between compounds

Limits

Contact with low doses of antimicrobials



Risk of adaptation or cross-resistance

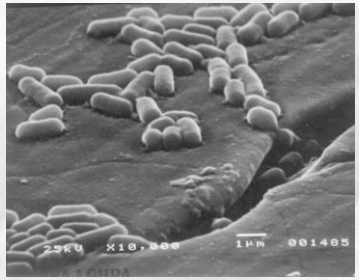
Better characterize the lethal activity

Disinfectants on surfaces

Regulation (EU) N°528/2012

Substance or mixture with the intention of destroying, rendering harmless or controlling effect on harmful organism

Product-type4 : food and feed area
Products used for disinfection of equipment, containers, consumption utensils, surfaces and pipework associated to the production, transport, storage or consumption for food and feed for humans and animals



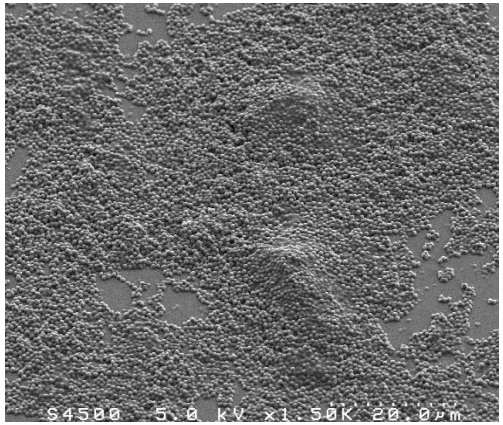
MIMA2 platform

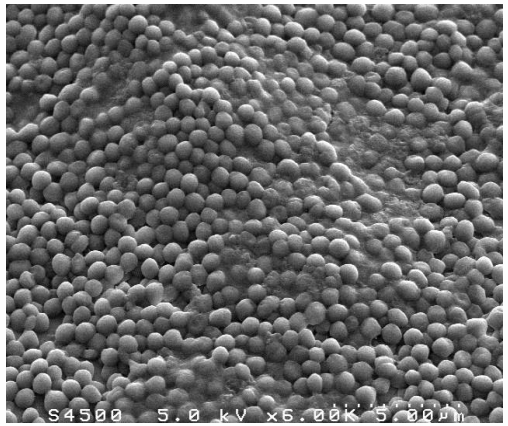
Part II

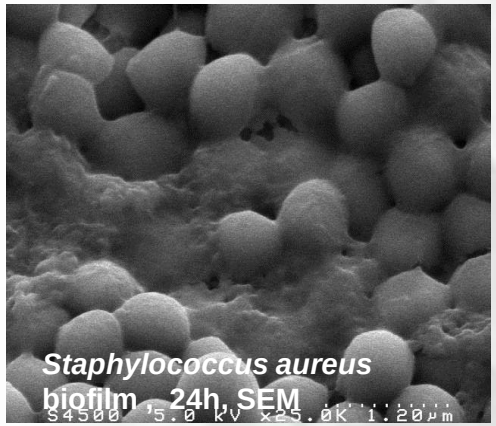
How can food operators better evaluate the efficiency of biocides on food contact surfaces?

Better characterize the lethal activity

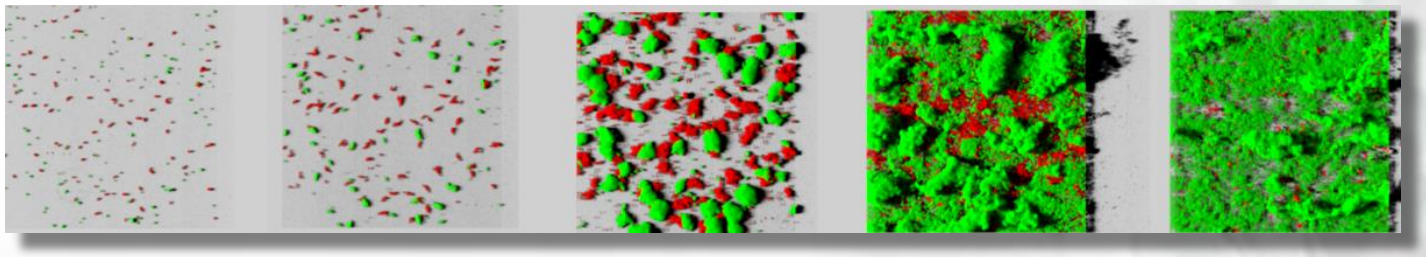
Biofilm settles on food-contact surfaces





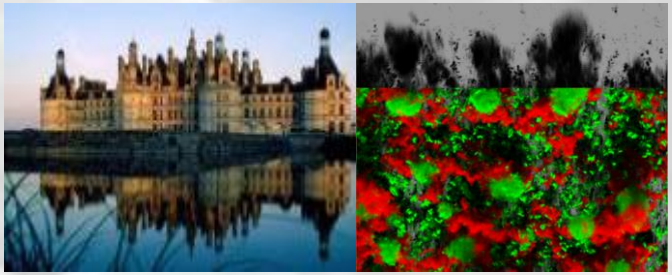


MIMA2 platform



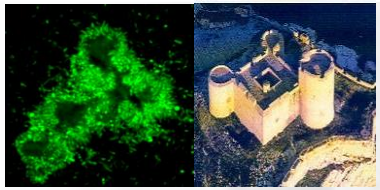
Biofilm: consortium of micro-organisms trapped in a matrix of organic polymers and adhering to a surface

Biofilms are bacterial fortresses



Better characterize the lethal activity

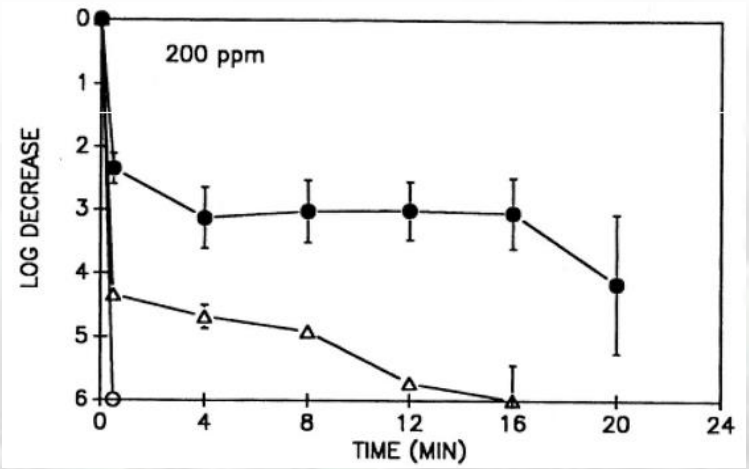
Biofilm: a structure of resistance

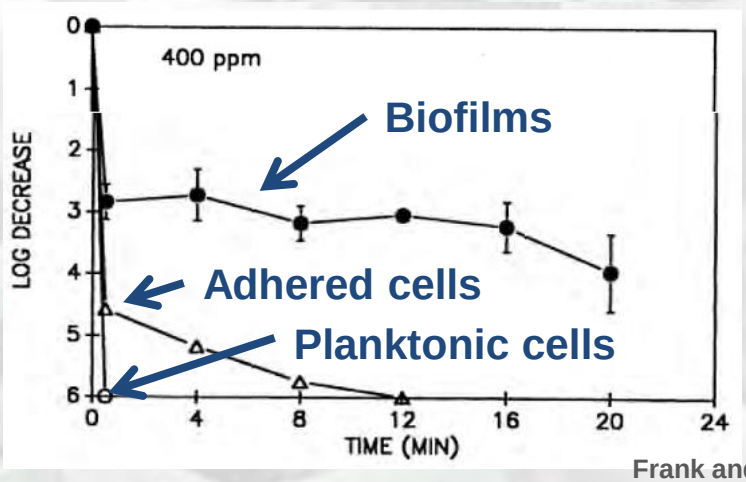


Disinfectants	Resistance coefficient (Rc) = Ratio of concentrations needed to obtain the same level of log reduction between biofilm and planktonic cells
oxidizing agents	5 - 600
QAC	10 - 1000

(Dubois-Brissonnet et al, 1995; Ntsama-Essomba et al, 1997; Campanac et al 2002, Luppens et al, 2002; Bridier et al 2011)

Activity of benzalkonium chloride on *Listeria monocytogenes* in the planktonic state (○), sessile (Δ) or biofilm state (●).





Frank and Koffi ,1990

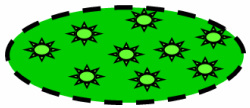
Inputs in advanced microscopic techniques

Method

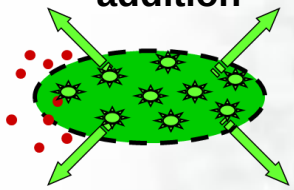
Real time visualization of antibiofilm action

Biofilm formation on 96-well microtiter plates

Fluorescent labelling with a viability marker



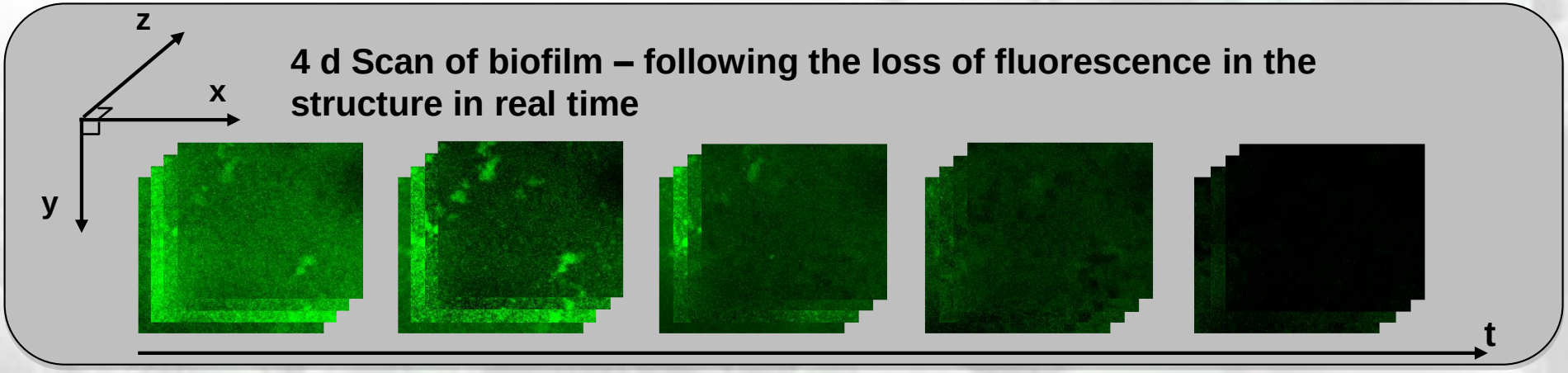
Biocide addition



Confocal Laser Scanning Microscope

MIMA2 microscopy platform

Inactivation dynamics are monitored by time lapse CLSM



Inputs in advanced microscopic techniques

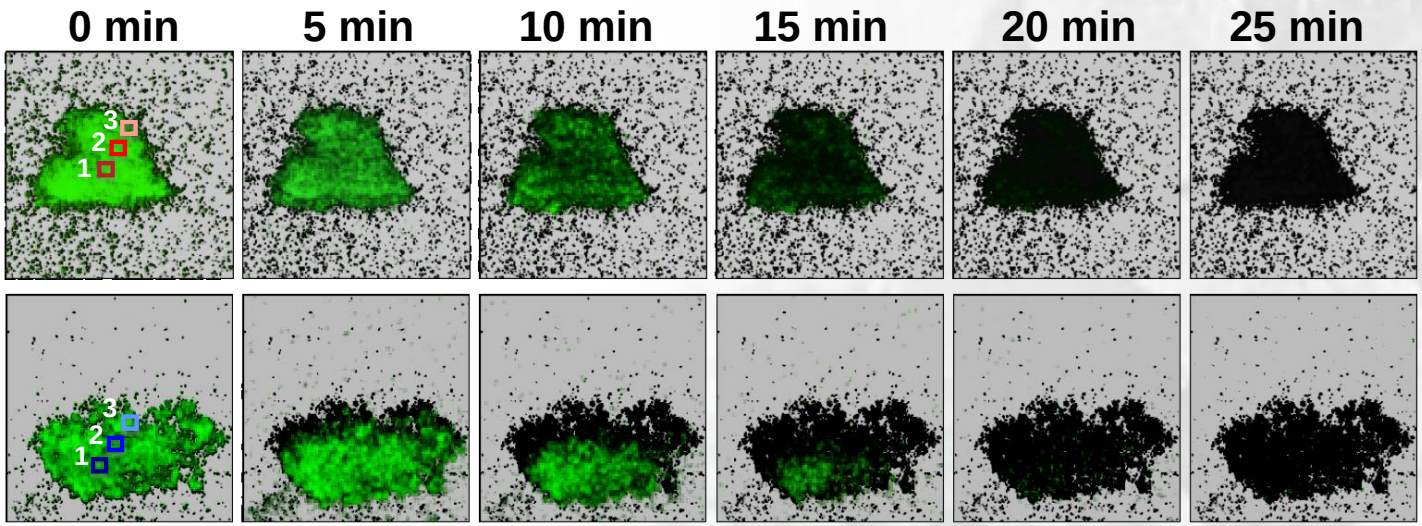
Spatio-temporal dynamics of disinfectant action with two different biocides

P. aeruginosa ATCC 15442

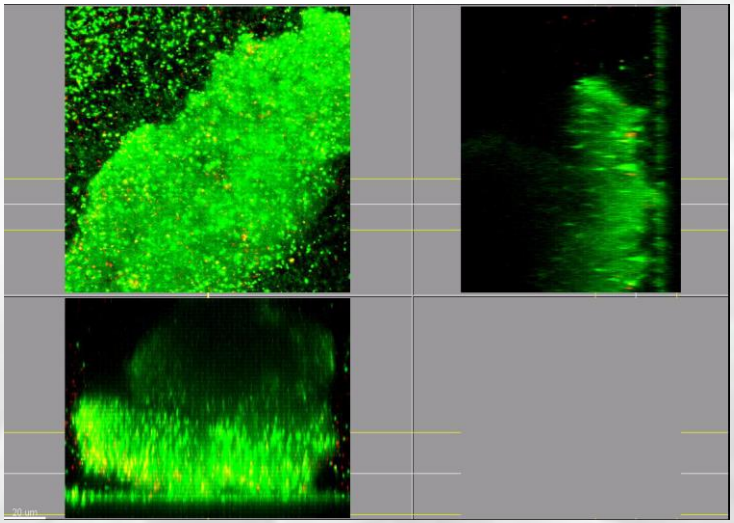
PAA (0,05%)

Fluorescence is recorded in three spots

BAC (0,5%)



BAC (0,5%)



Green = cells viable;
Red = permeabilized cells

(4D confocal laser scanning microscopy)

1 image/min

Bridier, A et al, 2011. Antimicrobial Agents and Chemotherapy 55, 2648-2654.

Inputs in advanced microscopic techniques

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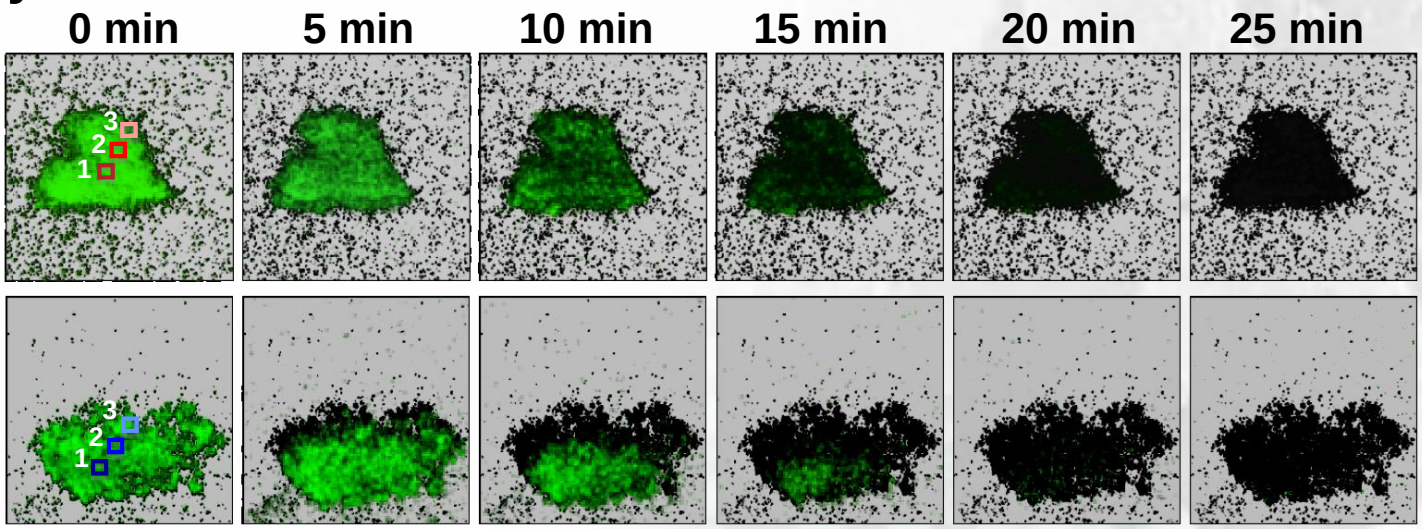
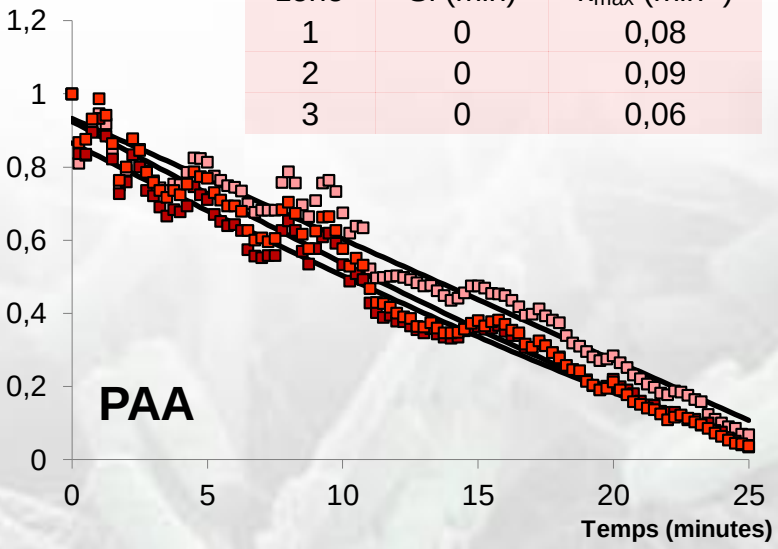
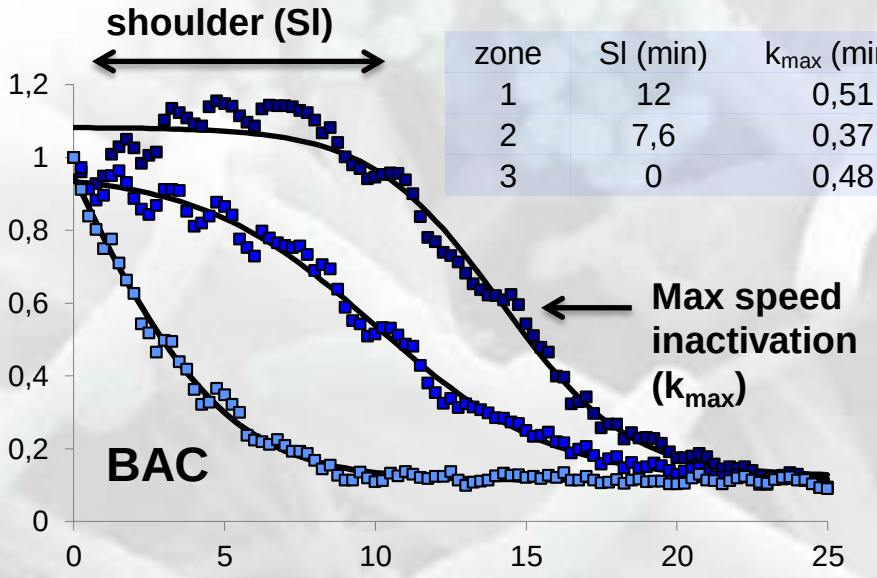


zone	Sl (min)	k_{max} (min^{-1})
1	0	0,08
2	0	0,09
3	0	0,06





zone	Sl (min)	k_{max} (min^{-1})
1	12	0,51
2	7,6	0,37
3	0	0,48

Bridier, A et al, 2011. Antimicrobial Agents and Chemotherapy 55, 2648-2654.

Inputs in advanced microscopic techniques

Application

Towards the use of natural compounds as disinfectants?

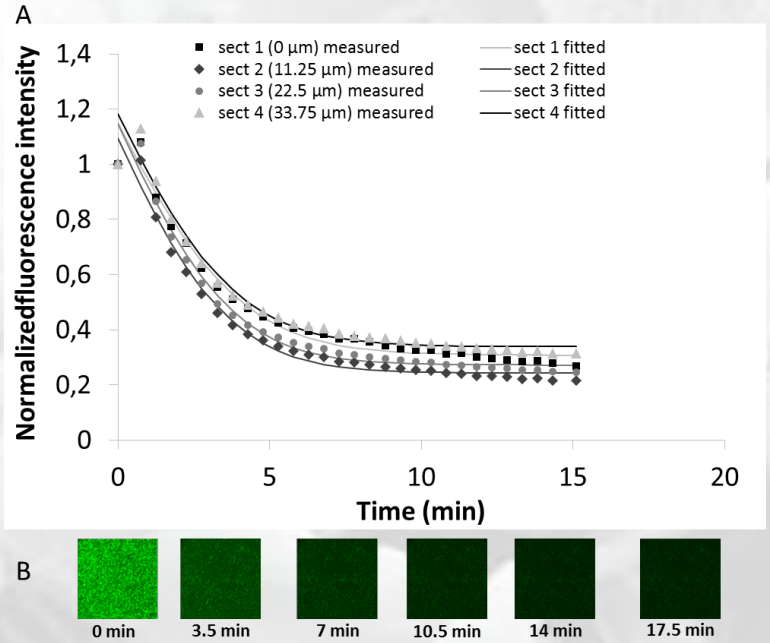
Thymbra capitata hydrosols as disinfectants

compared to BAC (benzalkonium ammonium chloride)

Resistance coefficient (Rc) =
$C_{biofilm}/C_{planktonic}$

Resistance coefficient (Rc)	
Benzalkonium chloride	208.3
Hydrosol	1.6

Hydrosol is nearly as efficient on biofilm than on planktonic cells contrarily to benzalkonium chloride



Hypothesis to explain the high antimicrobial activity

- ❖ **It contains a large amount of carvacrol associated with other antimicrobials**
- ❖ **It has an high water solubility**

Karampoula, F. et al, 2016. Applied and Environmental Microbiology (accepted)

Part II Conclusion

Conclusion

- Microscopic techniques are an interesting tool to evaluate heterogeneity of bacterial resistance within the biofilm structure

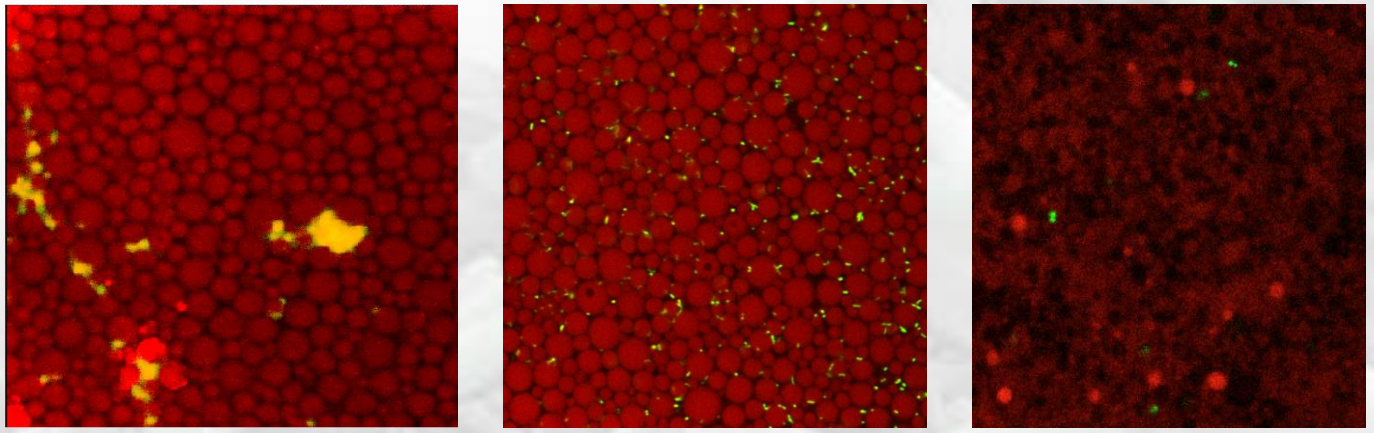
Limits

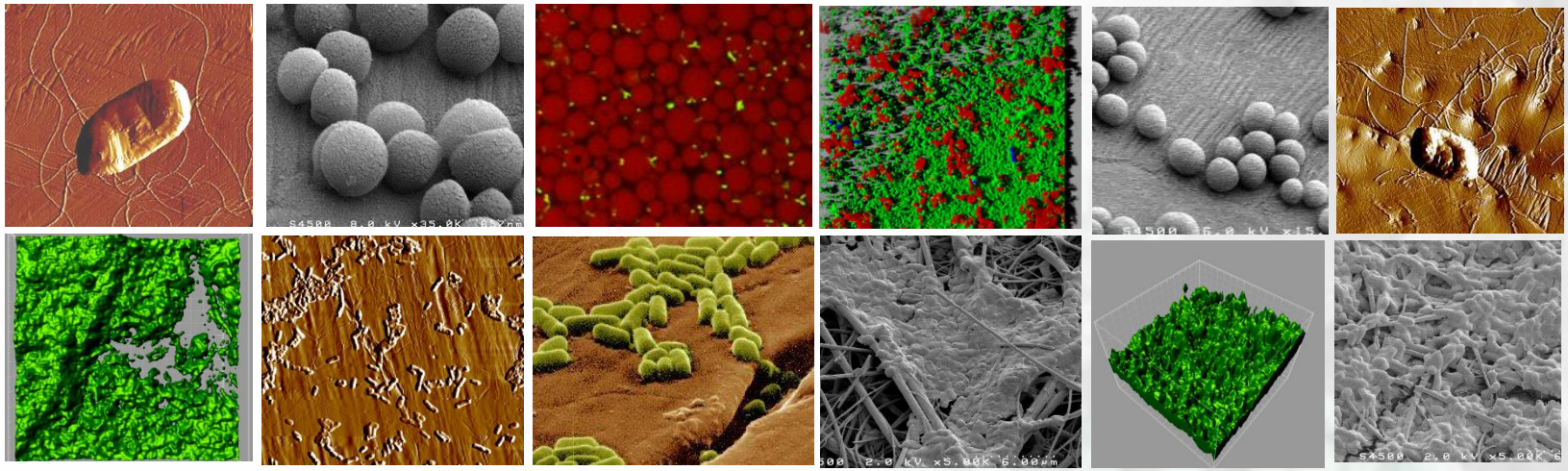
- Adequate labelling is needed (no interaction with the biocide)
- CLSM availability (microscopic platforms – possible to rent slot allocations)

Next challenges

Next challenge is to apply these microscopic techniques for evaluation of antimicrobial activities within food matrices

MIMA2 platform





Images from MIMA2 platform – UMR Micalis AgroParisTech INRA

Thank you for your attention !

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