A novel oil produced by *Aureobasidium* has antibacterial activity with specificity for species of *Streptococcus*.

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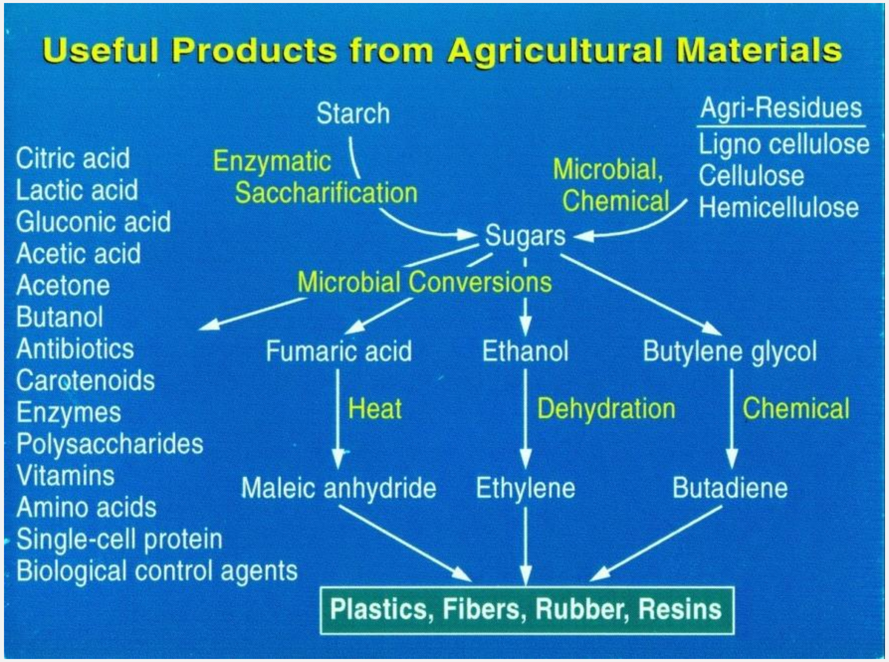
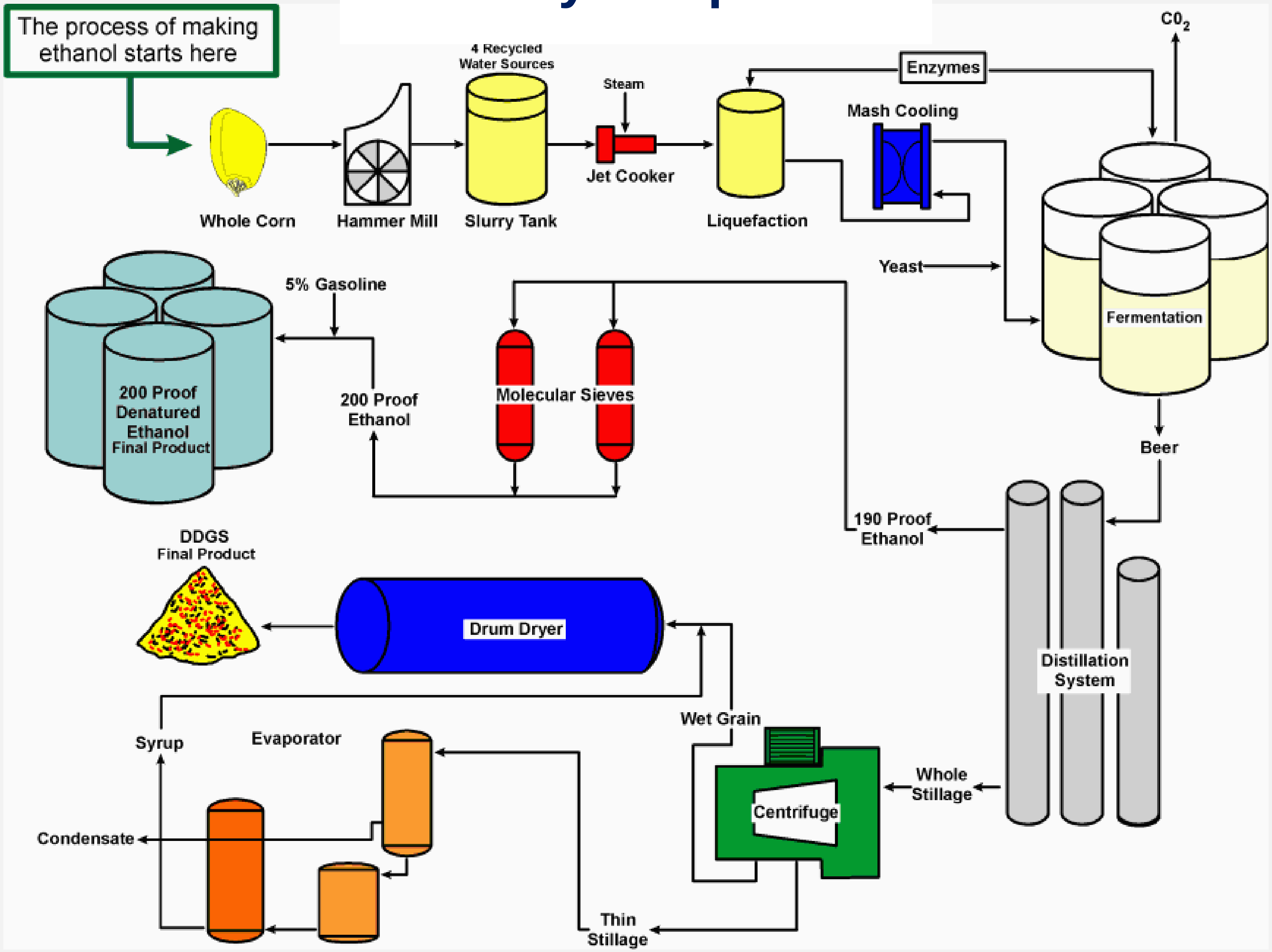
Renewable Product Technology Research



Mission: Develop new biobased products and improve the biochemical processes for the biorefining industry.

Corn Dry Mill process



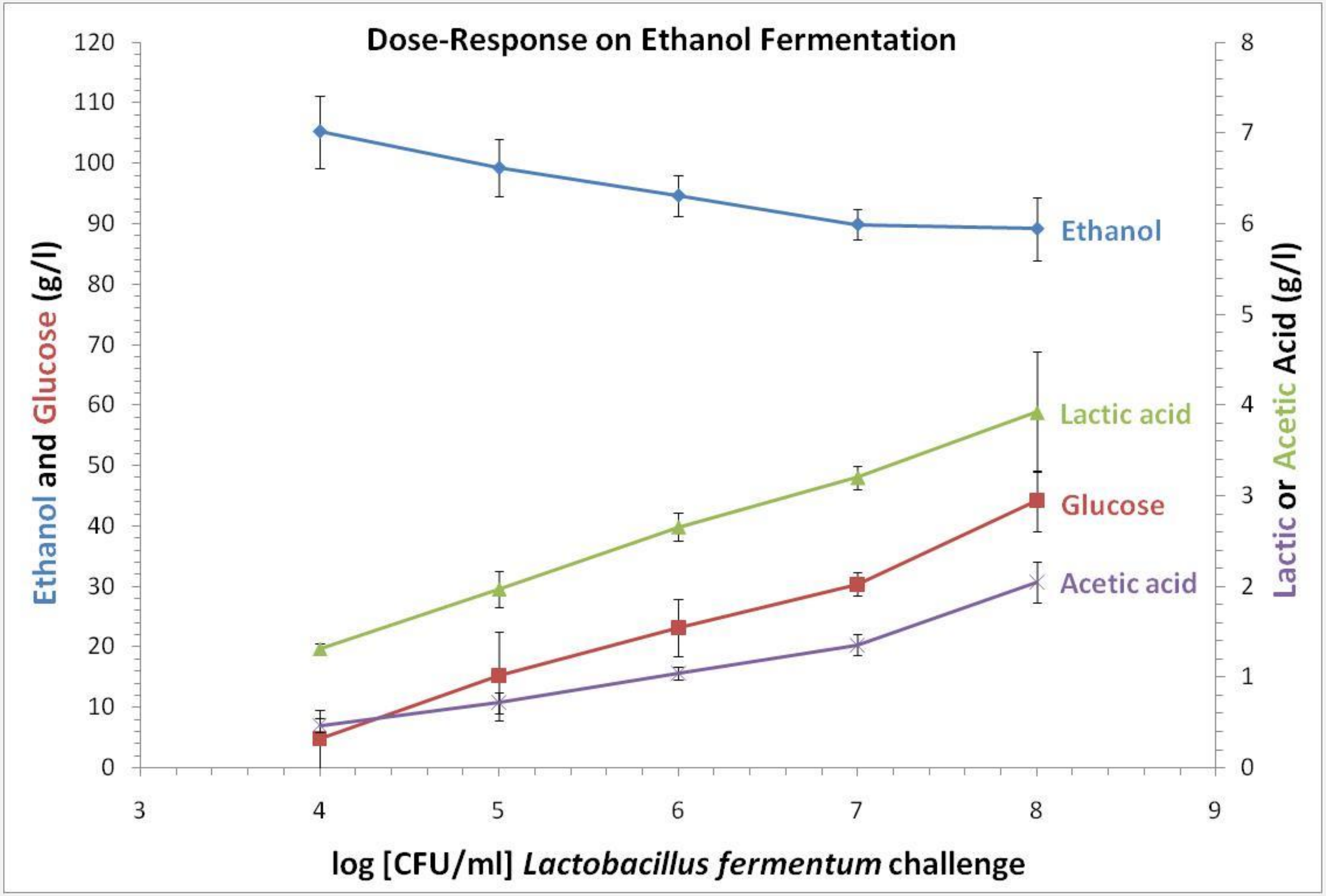
Source: http://www.distillersgrains.org/files/grains/CornDryMillProcess.html

Survey of bacterial contaminants in fuel ethanol plants

Genus	% of total[a]		
	Wet mill	Dry Grind #1	Dry-Grind #2
Bifidobacterium	0 – 20	1 – 2	0
Clostridium	0 – 9	0	0
Lactobacillus	**44 – 60**	**37 – 39**	**69 – 87**
Lactococcus	0 – 4	0 – 6	0
Leuconostoc	0 – 6	1 – 8	0 – 8
Pediococcus	0 – 6	19 – 24	0 – 4
Weisella	0 - 2	18 – 24	0 – 6

[a]Values represent a range over multiple samplings.

Skinner and Leathers, 2004



Bischoff et al., *Biotechnol. Bioeng.* 103:117-122 (2009)

Antibiotics in the ethanol industry

Table 2. List of some of the antibiotics used in the ethanol industry, their modes of action and activity spectrum.

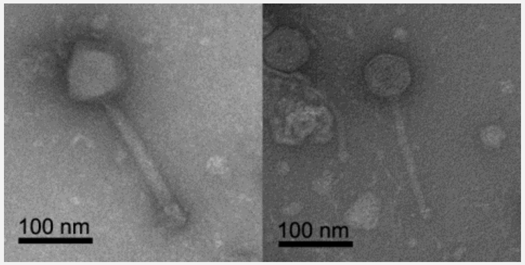
Antibiotic	*Mechanism*	*Bactericidal/static*	*Spectrum*
1a. Penicillin G 1b. Penicillin V*	Inhibits cell wall synthesis	Bactericidal	Gram(+) bacteria
2. Bacitracin	Affects cell wall	Bactericidal	Gram(+) bacteria
3. Tetracycline	Protein synthesis inhibitor	Bacteriostatic	Gram(+) & Gram(-) bacteria
4. Streptomycin	Protein synthesis inhibitor	Bactericidal	Gram(+) & aerobic Gram(-) bacteria
5. Erythromycin	Protein synthesis inhibitor	Bacteriostatic, cidal at high doses	Gram(+) & Gram(-) bacteria
6. Polymixin	Affects cell membrane	Bactericidal	Gram(-) bacteria
7. Virginiamycin	Protein synthesis inhibitor	Bactericidal	Gram(+) bacteria
8. Monensin	Affects cell membrane	Bactericidal	Gram(+) bacteria
9. Chloramphenicol	Protein synthesis inhibitor	Bactericidal (or) static	Gram(+) & Gram(-) bacteria. Good against anaerobes. Heat stable?

* Has the same properties as penicillin G but is more stable at acidic pH.

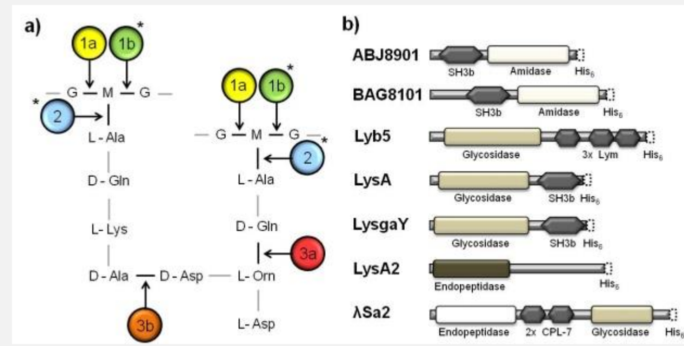
Source: Narendranath, (2003) *In*: The Alcohol Textbook, 4th Edition, pp 287-298.

Non-antibiotic intervention methods

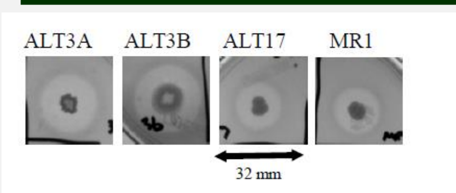
Bacteriophage



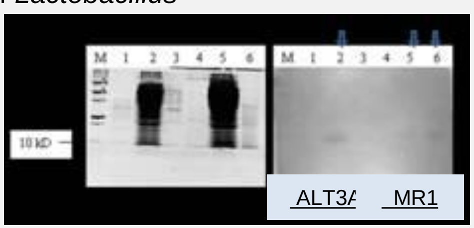
Lytic enzymes



Antibacterial peptides

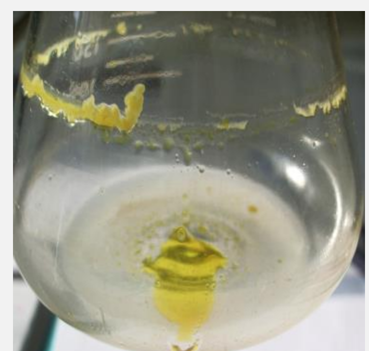


Inhibition of *Lactobacillus*

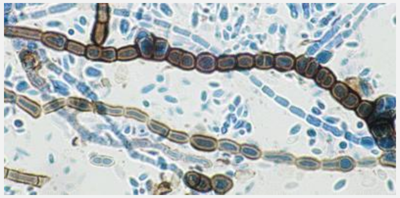


Zymogram

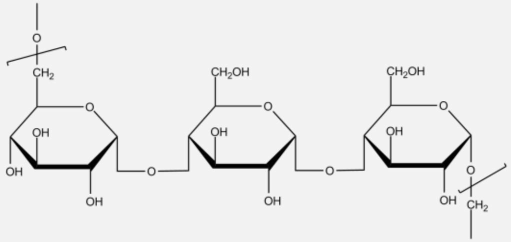
Natural products: Liamocins



Aureobasidium pullulans products have biotechnological applications:

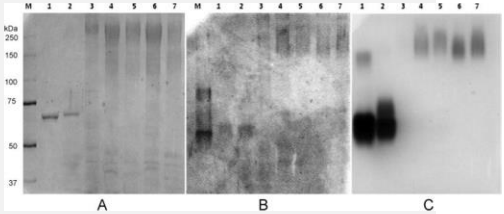


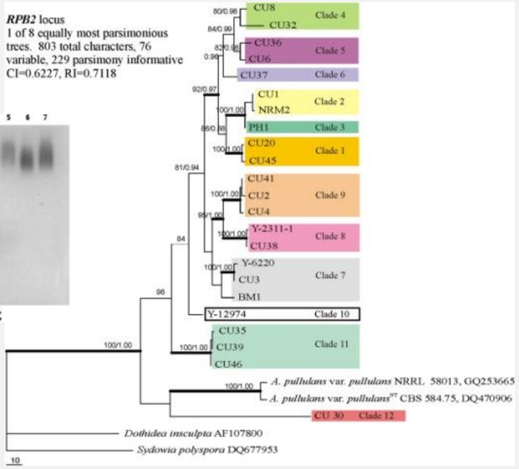
- **Exopolysaccharide (pullulan)**



α-1,4- ;α-1,6-glucan i.e. α-1,6-maltotriose units

- **Enzymes: Xylanase, lipase, laccase**



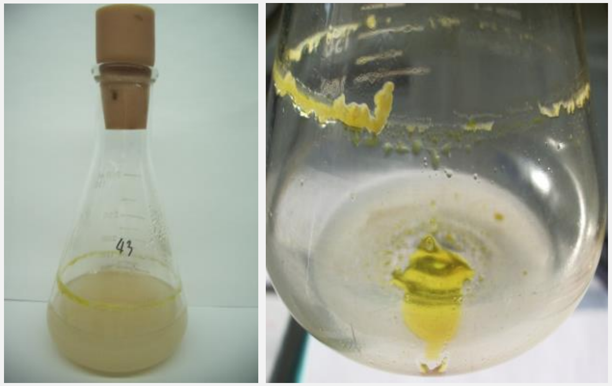


- **poly(β-L-malic acid) (PMA)**

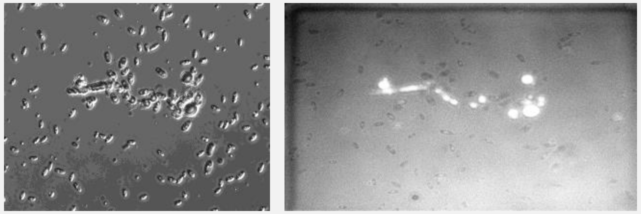
Heavy oils produced by *Aureobasidium pullulans*

Manitchotpisit et al., *Biotechnol. Lett*. **33:**1151-1157 (2011)

- 21/50 strains produced heavy oil.
- Yields: 0.5 - 6.0 g oil / L culture media
  0.01 – 0.12 g oil / g sucrose
- Extracellular polyol lipids from *Aureobasidium* was previously reported by Kurosawa et al., (1994).
- Surface active (biosurfactant).



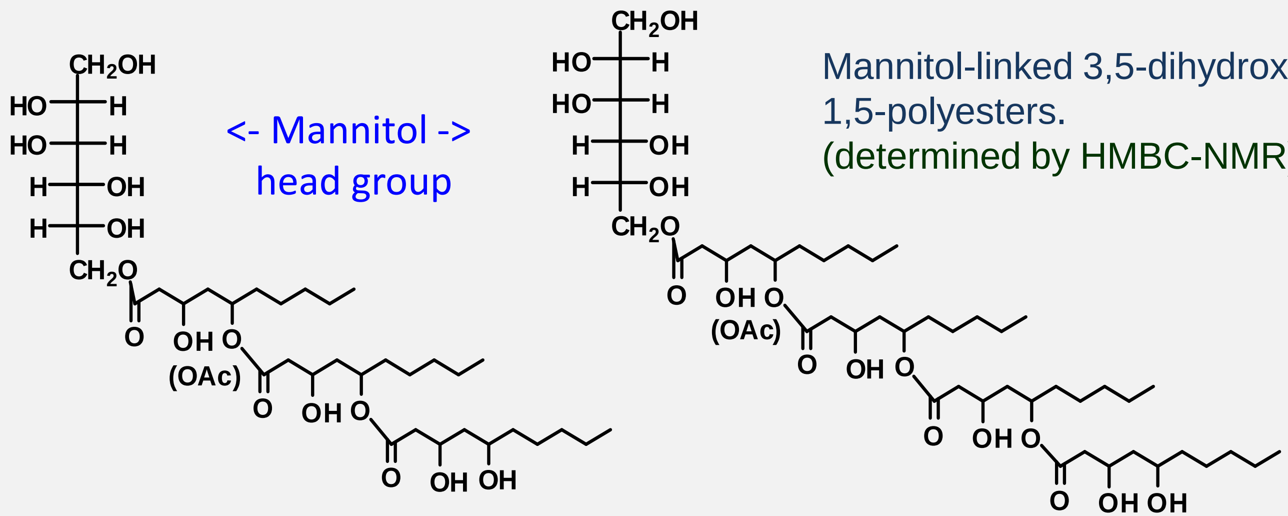
A. pullulans **in liquid culture**



Microscopy: *A. pullulans* on agar.
A. Visible **B. Fluorescence**

Structural characterization of extracellular liamocins (mannitol oils) produced by *A. pullulans* strain NRRL 50380

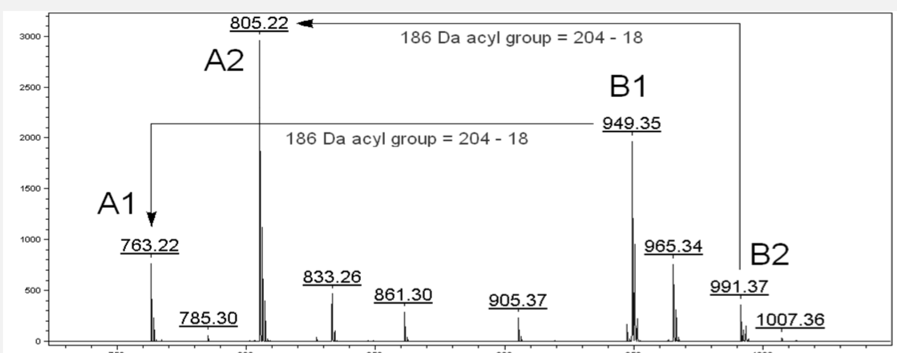
Price et al., *Carbohydrate Res* **370**:24-32 (2013)



Liamocins A1 and A2

Liamocins B1 and B2

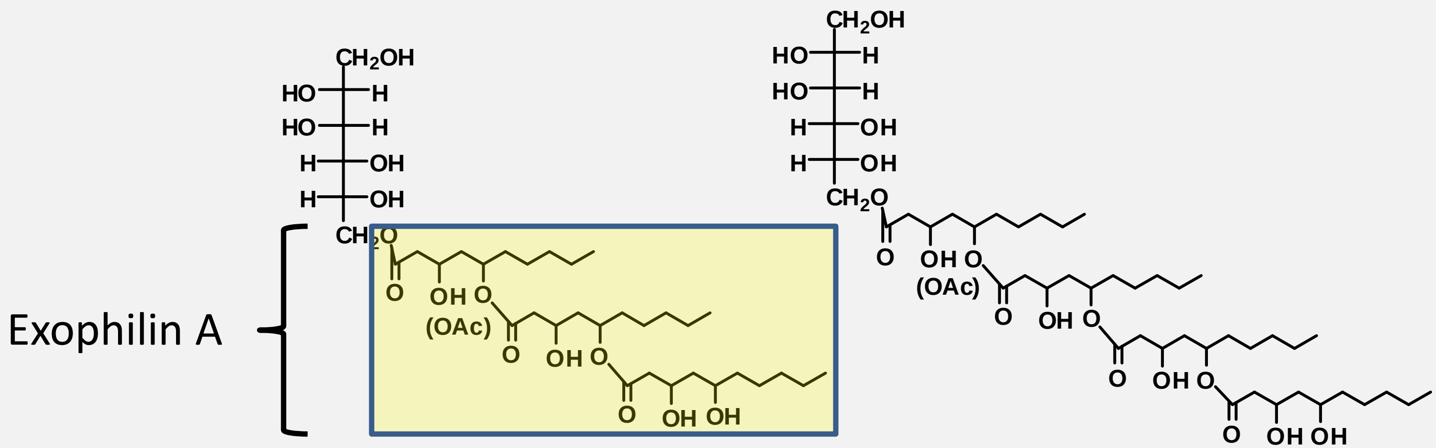
Mannitol-linked 3,5-dihydroxydecanoate 1,5-polyesters.
(determined by HMBC-NMR)



MALDI-TOF mass spec of types of liamocins:

A. Trimers
B. Acetylated trimers
C. Tetramers
D. Acetylated tetramers

Structural similarity to Exophilin A



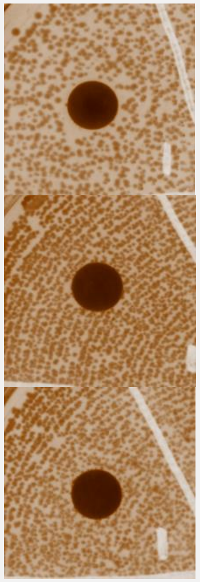
Liamocins A1 and A2

Liamocins B1 and B2

- Exophilin A reported by Doshida et al., (1996).
- Product of *Exophiala pisciphila* (now *Aureobasidium pullulans*).
- Antibacterial activity against Gram positives.

Do liamocins inhibit bacterial contaminants of bioethanol fermentation?

Qualitative antibacterial assays



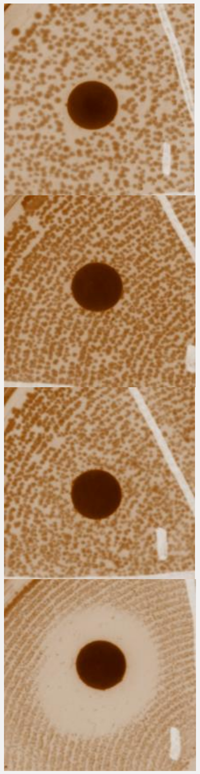
Lactobacillus fermentum
BR0315-1

Lactobacillus brevis
5-37

Lactobacillus plantarum
5-38

Agar diffusion assay. Bacteria (20 μl) at a density of 0.5 McFarland units were evenly spread on agar media, and paper discs (6 mm diameter) were placed on the surface. Oil from *A. pullulans* NRRL 50380 was dissolved to a concentration of 50 mg/ml in solvent (1:1 dimethylsulfoxide:2-butanone), and 10 μl applied to each disc. Plates were incubated at 37°C overnight.

Qualitative antibacterial assays

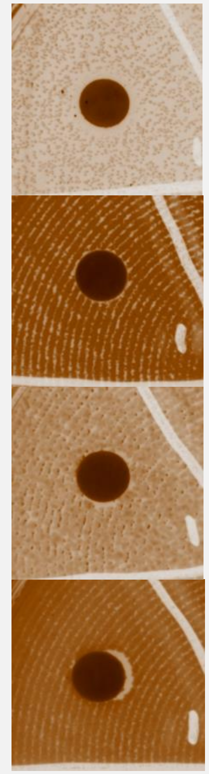


Lactobacillus fermentum
BR0315-1

Lactobacillus brevis
5-37

Lactobacillus plantarum
5-38

Streptococcus agalactiae
KU-MU-3B



Enterococcus faecalis
ATCC 29212

Staphylococcus aureus
ATCC 29213

Psuedomonas aeruginosa
ATCC 27853

Escherichia coli
ATCC 25922

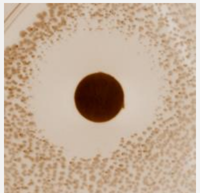
Agar diffusion assay. Bacteria (20 µl) at a density of 0.5 McFarland units were evenly spread on agar media, and paper discs (6 mm diameter) were placed on the surface. Oil from *A. pullulans* NRRL 50380 was dissolved to a concentration of 50 mg/ml in solvent (1:1 dimethylsulfoxide:2-butanone), and 10 µl applied to each disc. Plates were incubated at 37°C overnight.

Broth dilution susceptibility testing

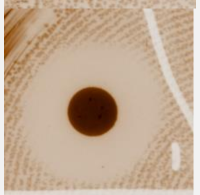
Species	Minimum Inhibitory Concentration (μg liamocins / ml)
Streptococcus agalactiae	40
Enterococcus faecalis	312
Bacillus subtilis	640
Staphylococcus aureus	> 1250
Lactobacillus fermentum	> 1250
Escherichia coli	> 1250
Pseudomonas aeruginosa	> 1250

MICs of oil from *A. pullulans* NRRL 50380 were determined by broth dilution method, with serial two-fold dilutions of oil ranging from 1250 μg/ml to 20 μg/ml.

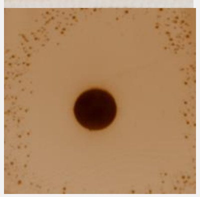
Qualitative antibacterial assays



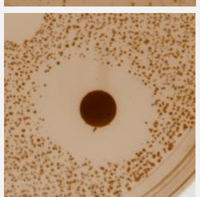
S. agalactiae NRRL B-1815



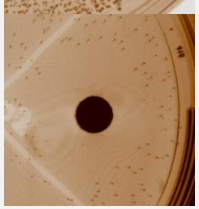
S. uberis



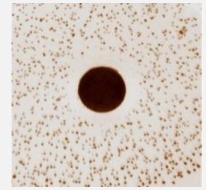
S. suis ATCC 43765



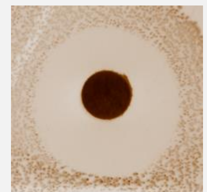
S. pyogenes ATCC 12344



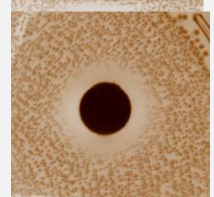
S. pneumoniae ATCC 55143



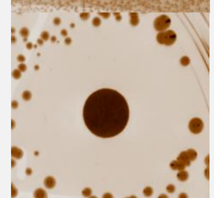
S. mutans ATTC 25175



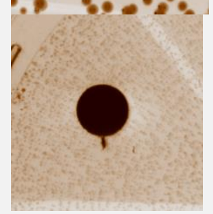
S. mitis NRRL B-14574



S. infantarius NRRL B-41208



S. salivarius NRRL B-3714



S. sobrinus NRRL B-4468

Broth dilution susceptibility testing

Strain	Minimum Inhibitory Concentration (μg liamocins / ml)
Streptococcus species	
S. agalactiae KU-MU-3B	40
S. agalactiae NRRL B-1815	20
S. uberis	80
S. suis ATCC 43765	≤ 10
S. pneumoniae ATCC 55143	≤ 10
S. pyogenes ATCC 12344	16
S. mutans ATCC 25175	80
S. mitis NRRL B-14574	20
S. infantarius NRRL B-41208	80
S. salivarius NRRL B 3714	≤ 10
S. sobrinus NRRL B-4468	> 1250

MICs of oil from *A. pullulans* NRRL 50380 were determined by broth dilution method, with serial two-fold dilutions of oil ranging from 1250 μg/ml to 10 μg/ml.

Feedstocks for production of liamocins

Carbon Source	Minimum Inhibitory Concentration (μg/ml)
Arabinose	≤ 20
Glucose	39
Sucrose	39
Xylose	39
Wheat straw	156
AHP-treated corn fiber	625
Oat spelt xylan	312

A. pullulans was grown in media containing the indicated carbon source, and oil extracted from the culture was tested for antibacterial activity. MICs for *Streptococcus agalactiae* were determined by broth dilution susceptibility testing.

Polyols, not sugars, determine the structure of the liamocin headgroup.

Table 2. Liamocins from *A. pullulans* NRRL 50380 grown on different sugars or polyols as the sole carbon source.

Carbon	**% Polyol Headgroups from Acid-Hydrolyzed Liamocins[2]**								
Source[1]	**Gro**	**Thr**	**Ery**	**Rib**	**Ara**	**Xyl**	**Man**	**Glc**	**Gal**
Sucrose	-	-	-	-	-	-	100	-	-
Lactose	-	-	-	-	-	-	100	-	-
D-fructose	-	-		-	-	-	100	-	-
D-glucose	-	-	-	-	-	-	100	-	-
D-mannose	-	-	-	-	-	-	100	-	-
D-galactose	-	-	-	-	-	-	100	-	-
D-arabinose	-	-	-	-	-	-	100	-	-
L-arabinose	-	-	-	-	-	-	100	-	-
D-xylose	-	-	-	-	-	-	100	-	-
D-mannitol	-	-	-	-	-	-	100	-	-
D-glucitol	-	-	-	-	-	-	65	35	-
D-galactitol	-	-	-	-	-	-	73	8	19
D-arabitol	-	-	-	-	98	-	2	-	-
L-arabitol	-	-	-	-	62	-	38	-	-
D-xylitol	-	-	-	-	27	45	28	-	-
D-ribitol	-	-	-	18	63	-	19	-	-
D/L-threitol	-	78	-	-	-	-	22	-	-
erythritol	-	-	5	-	-	-	95	-	-
D-glycerol	75		-	-	8	-	17	-	-

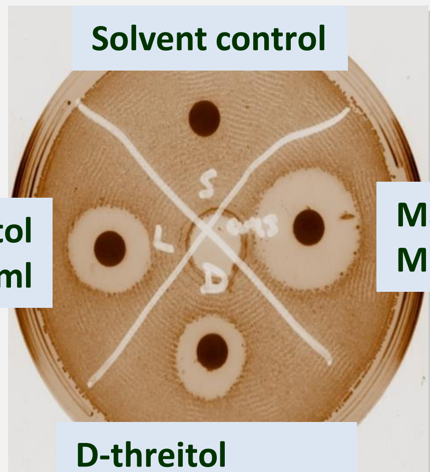
[1]Sole carbon source at 5% in PM medium (above the line the carbon sources are sugars, and below are polyols).

[2]Analyzed by GC/MS. The dry weight yields of the total liamocins were 1.2 – 1.5 g/L.

Susceptibility of *S. agalactiae* to structural analogs of liamocins.

Solvent control

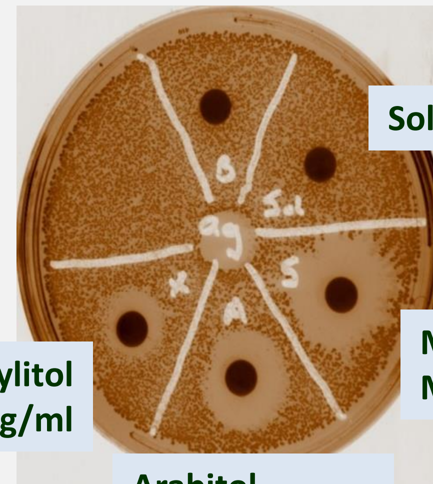
L-threitol
MIC = 156 µg/ml



Mannitol
MIC = 16 µg/ml

D-threitol
MIC = 156 µg/ml

Solvent control

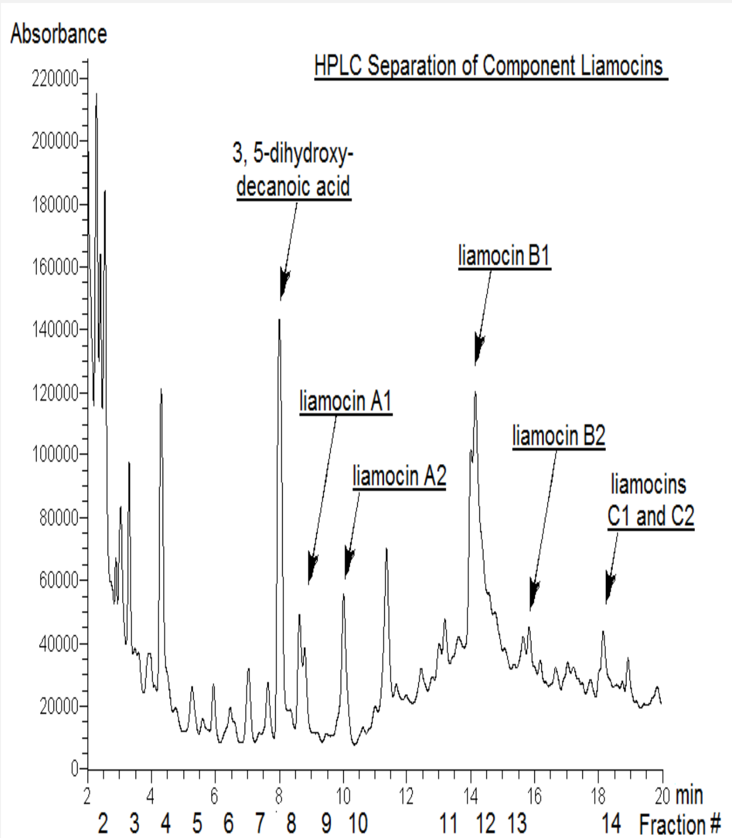


Xylitol
MIC = 128 µg/ml

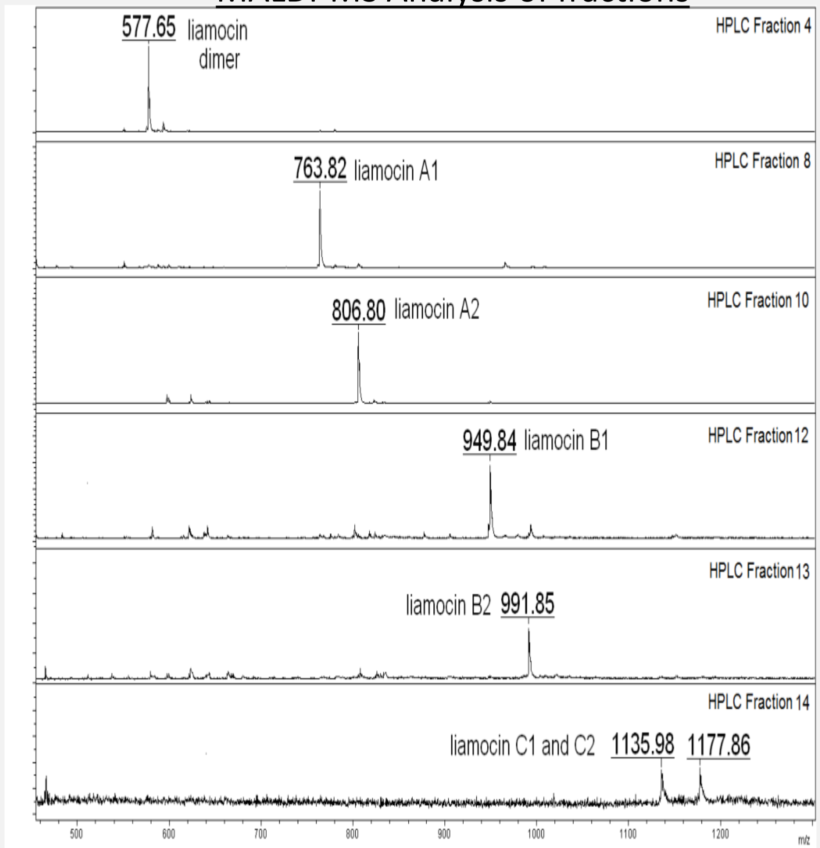
Mannitol
MIC = 16 µg/ml

Arabitol
MIC = 64 µg/ml

Reverse phase HPLC purification of component liamocins.



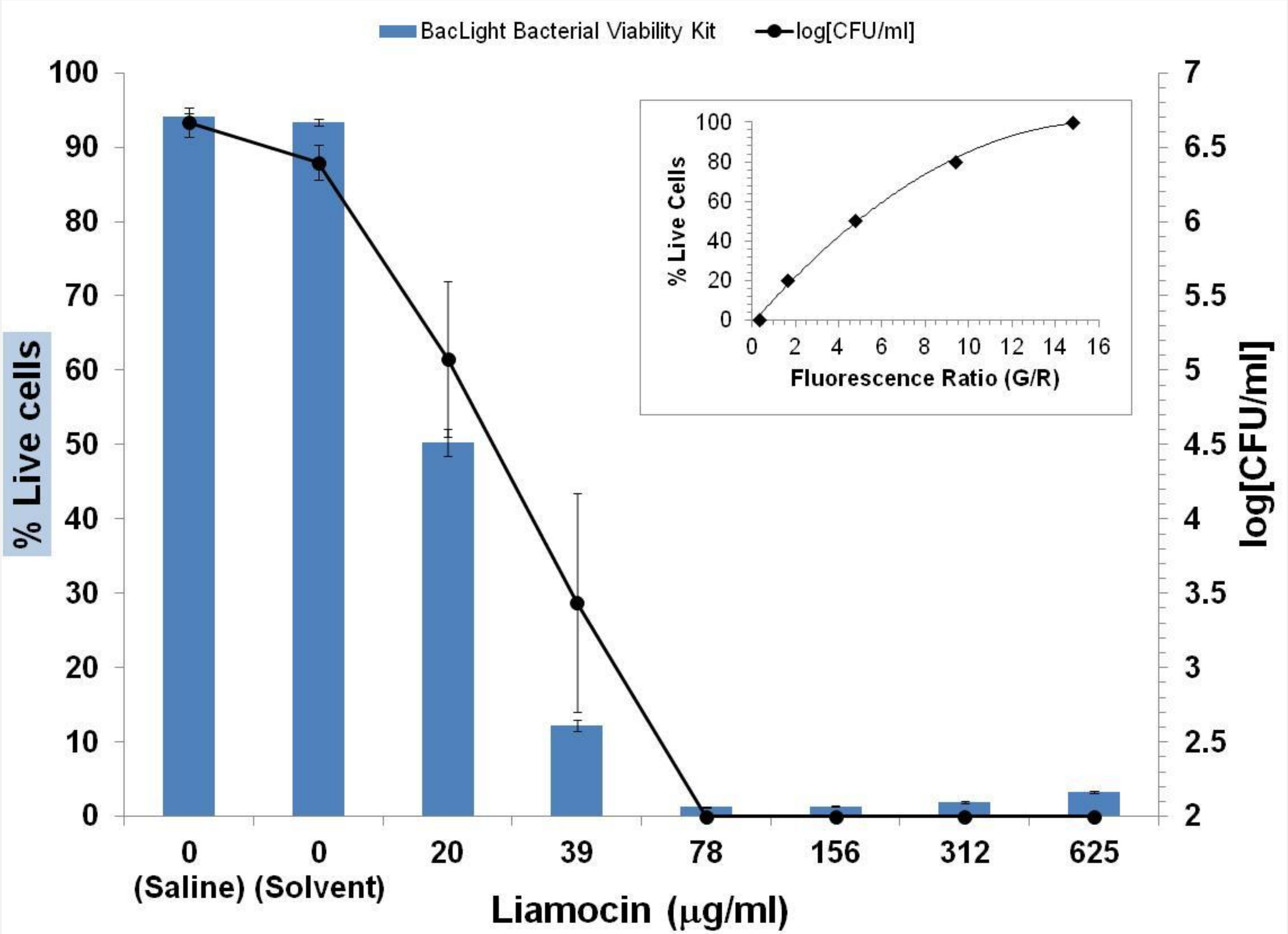
MALDI-MS Analysis of fractions



MIC (μg/ml)
>128
64
64
16
128
32

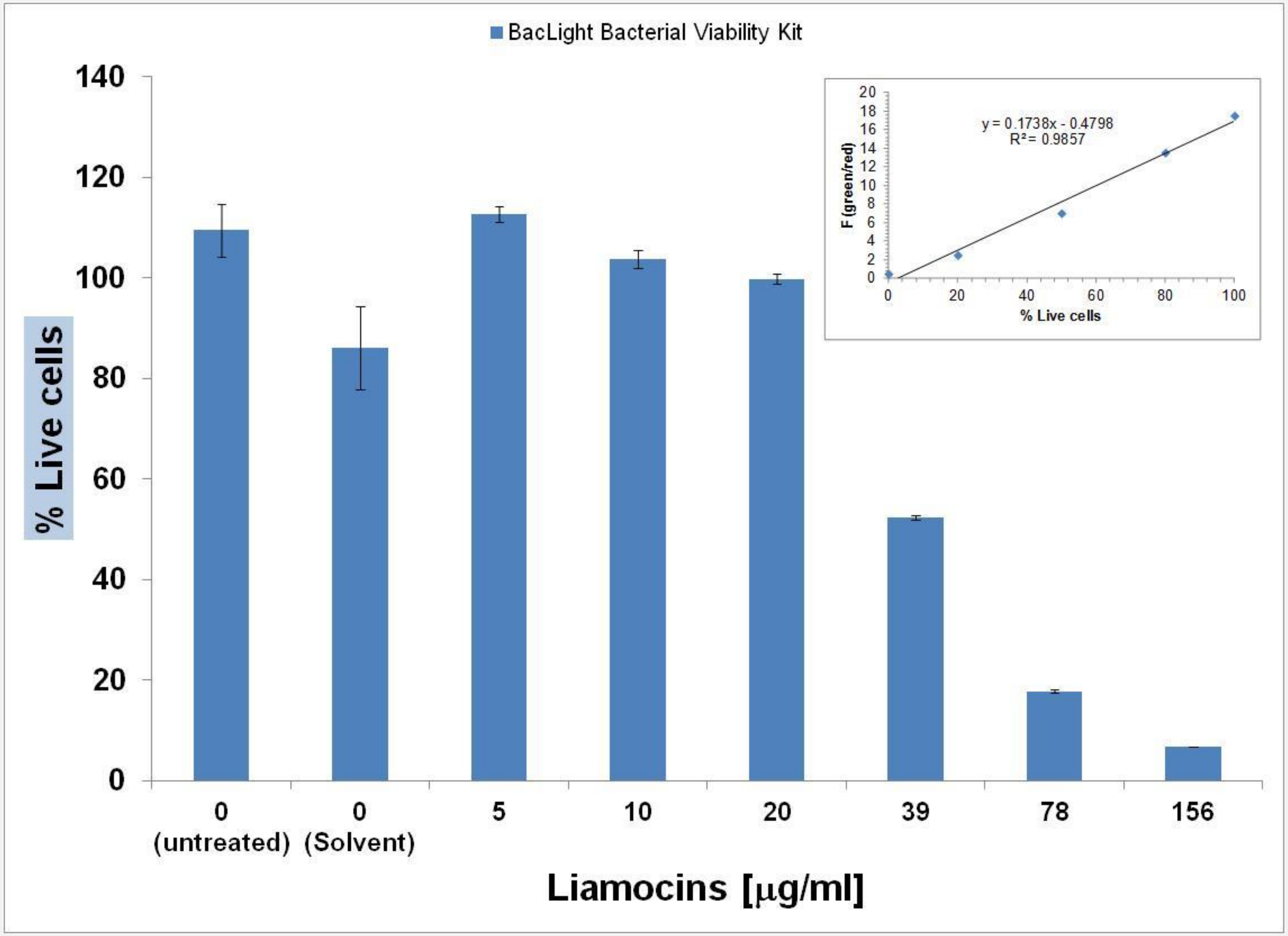
Oil from *A.pullulans* NRRL 50380 was fractionated on an RP18 HPLC column using a linear gradient of 50 – 100 % acetonitrile in water. The collected fractions were assayed by MALDI-MS, and tested for antibacterial activity by broth dilution.

Viability assays: *S. agalactiae*



Determined using LIVE/DEAD BacLight Bacterial Viability Kit (bars) and by enumeration of surviving cells (line).

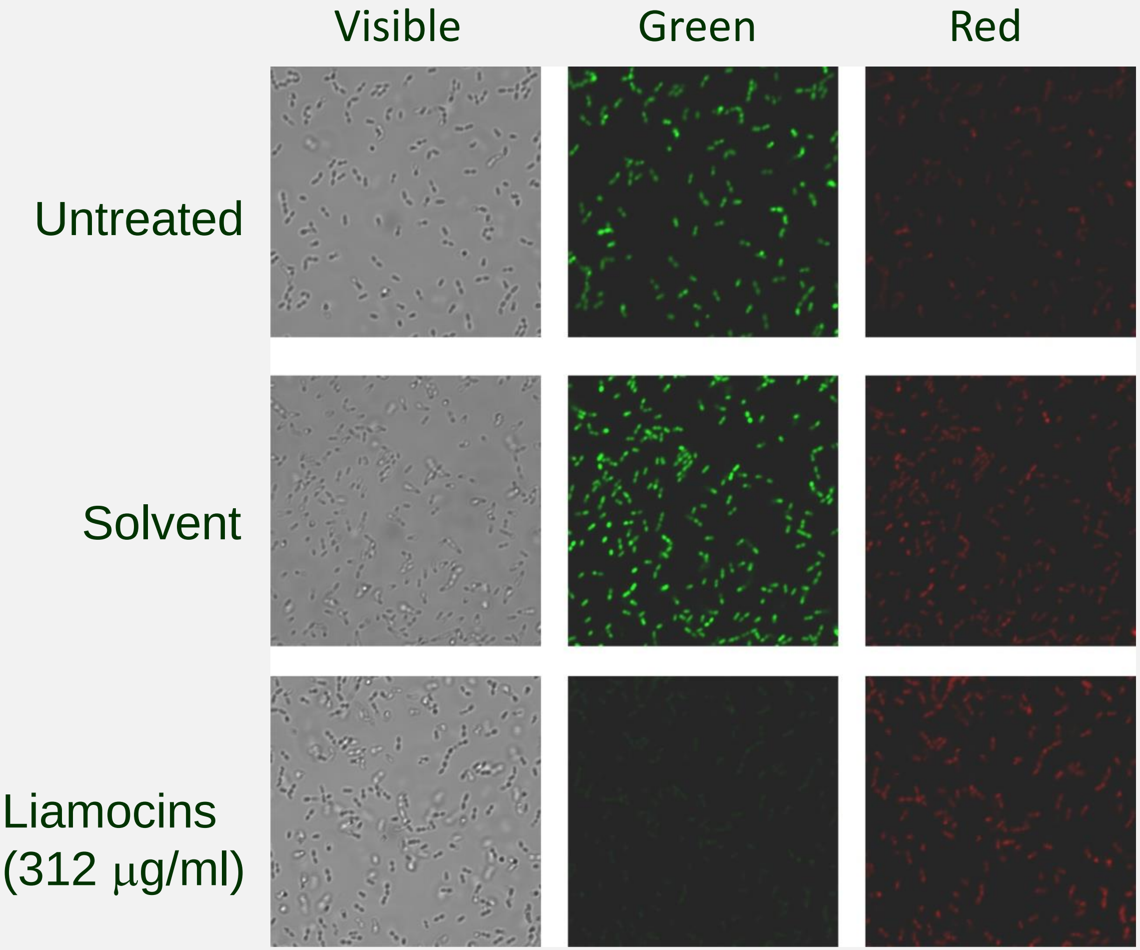
Viability assays: *S. suis* ATCC 43765



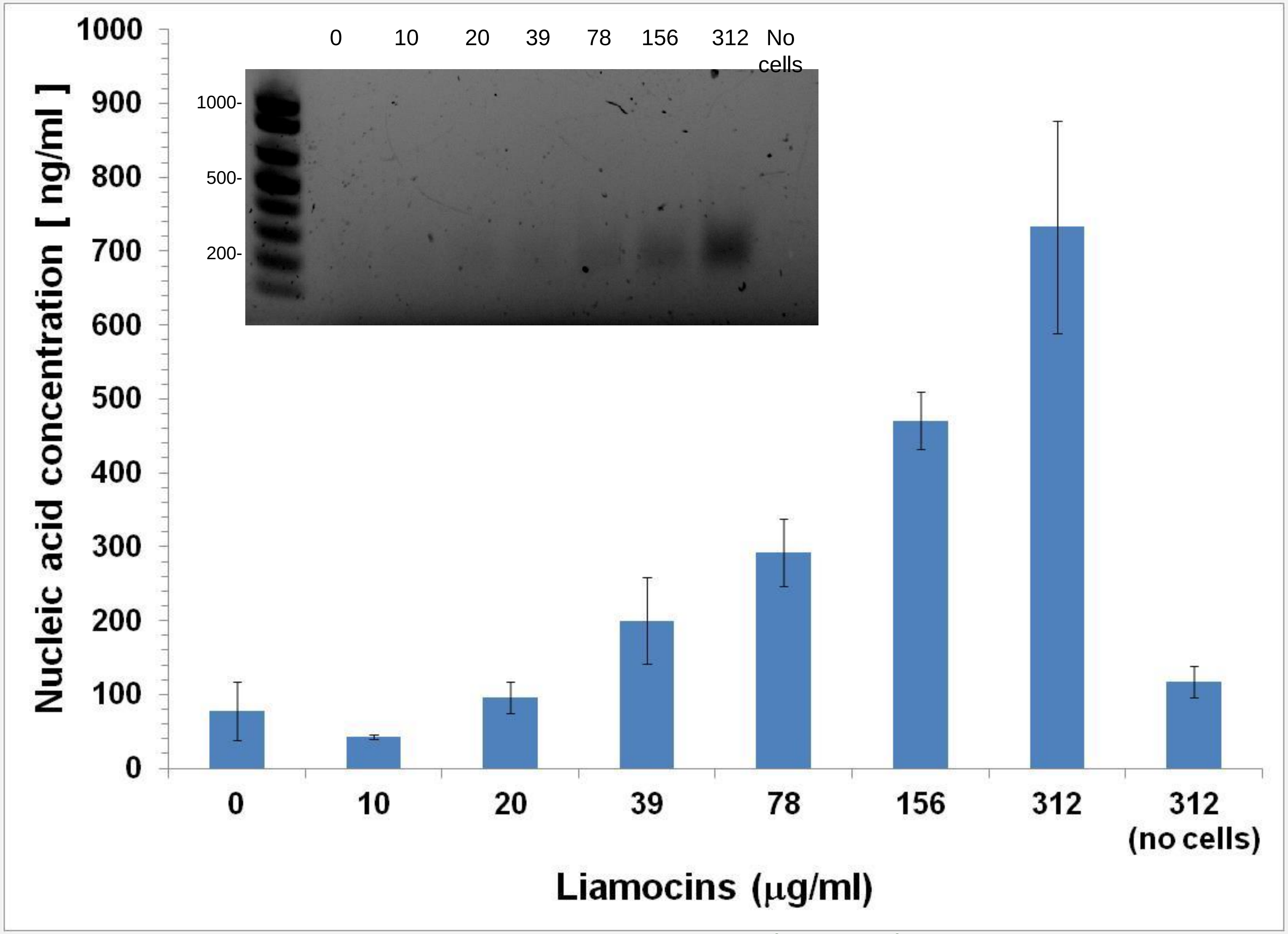
Determined using LIVE/DEAD BacLight Bacterial Viability Kit.

Fluorescence microscopy

S. suis ATCC 43765



Cell leakage assays: *S. suis* ATCC 43765



DNA concentration determined using Quant-iT PicoGreen dsDNA kit (Invitrogen).

Summary

- **Nine species of *Streptococcus* were susceptible to growth inhibition by *A. pullulans* liamocins.**
- ***Lactobacillus spp*., *S. aureus*, *E. faecalis*, *B. subtilis*, *E. coli*, and *P. aeruginosa* were not susceptible.**
- **Growth on polyols can alter the head group.**
- **Mannitol liamocin B1 (the non-acetylated tetramer) appears to be the most active type.**
- **Liamocins treatment results in loss of membrane integrity.**

Conclusions

- Liamocins have antibacterial activity with specificity for *Streptococcus*.
- Future directions:
  - Test against clinical/field isolates.
  - Determine mode of action.
  - Purify/synthesize liamocins sub-types.
  - Apply in veterinary/clinical models.
- Liamocins may be developed as a narrow spectrum antimicrobial agent that targets streptococcal pathogens but avoids disruption of the beneficial normal flora.

USDA

United States Department of Agriculture

Agricultural Research Service

Thank you.

Renewable Product Technology Research Unit

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