Endotoxin testing of an exhaustive list of different autochthonous and common water contaminating Microorganisms – an LAL and rFC comparative study

USP Open Forum on Alternatives to Compendial Reagents used in the Bacterial Endotoxin Test

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PIONEERING DIAGNOSTICS

AGENDA

- **LAL and rFC Comparative Study 1**

  Typical water contaminating microorganisms

  - tested with two LAL assays and one rFC assay -

- **LAL and rFC Comparative Study 2**

  Autochthonous facility isolates

  - tested with three kinetic LAL, two turbidimetric LAL, and two rFC assays -

- **Potential impact of ß-glucans and other microbial sugars on LAL-rFC comparison studies**

LAL AND RFC COMPARATIVE STUDY 1
TYPICAL WATER CONTAMINATING MICROORGANISMS

METHOD

Goal:

Internal evaluation of comparability of rFC and LAL assays on non-purified LPS, including typical water contaminating bacterial strains.

Materials:

- Assays: 2 LAL (KCA 1, KCA 2), and 1 rFC
- Samples: 24 different strains of typical water contaminating microorganisms (Gram Negative Bacteria).
- Media: M9 and LB. Not all organisms grew in both media (→ 41 final samples)

Strain	Strain #	Strain	Strain #
Pseudomonas stutzeri	**S329**	*Enterobacter sakazakii*	**S2752**
Serratia marcescens	**S2947**	*Enterobacter sakazakii*	**S2753**
Yersinia enterocolitica	**S613**	*Klebsiella pneumoniae ssp. pneumoniae*	**S207**
Enterobacter aerogenes	**S205**	*Escherichia coli O113:H10*	**S3222**
Enterobacter cloacae	**S1582**	*Citrobacter sp.*	**S354**
Enterobacter sakazakii	**S2713**	*Proteus mirabilis*	**S1581**
Enterobacter sakazakii	**S2326**	*Escherichia coli*	**S396**
Klebsiella pneumoniae ssp. pneumoniae	**S206**	*Escherichia coli*	**S55**
Yersinia enterocolitica	**S2945**	*Escherichia coli*	**S162**
Serratia marcescens	**S2946**	*Escherichia coli*	**S78**
Salmonella enterica ssp. salamae	**S379**	*Escherichia coli O55:B5*	**S1268**
Vibrio natriegens	**S407**	*Escherichia coli*	**S498**

NOTES AND CONTROLS

Growth media used in the study:

LB: NaCl, yeast extract, tryptone, agar, WEF

M9: glucose 10%, $MgSO_4$, salt solution, WEF

Salt Solution: Na_2HPO_4 x 2 H_2O, KH_2PO_4, NaCl, NH_4Cl, WEF

Control of the media:

media	dilution	rFC			KCA 1			KCA 2		
		Measured value [EU/mL]	Endotoxin activity in the media [EU/mL] *	PPC	Measured value [EU/mL]	Endotoxin activity in the media [EU/mL] *	PPC	Measured value [EU/mL]	Endotoxin activity in the media [EU/mL] *	PPC
LB	1:10	< 0.005	< 0.05	87%	< 0.005	< 0.05	95%	0.022	0.22	155%
M9	1:10	< 0.005	< 0.05	108%	< 0.005	< 0.05	104%	< 0.005	< 0.05	136%

WEF: water endotoxin free; * Measured value multiplied with dilution factor

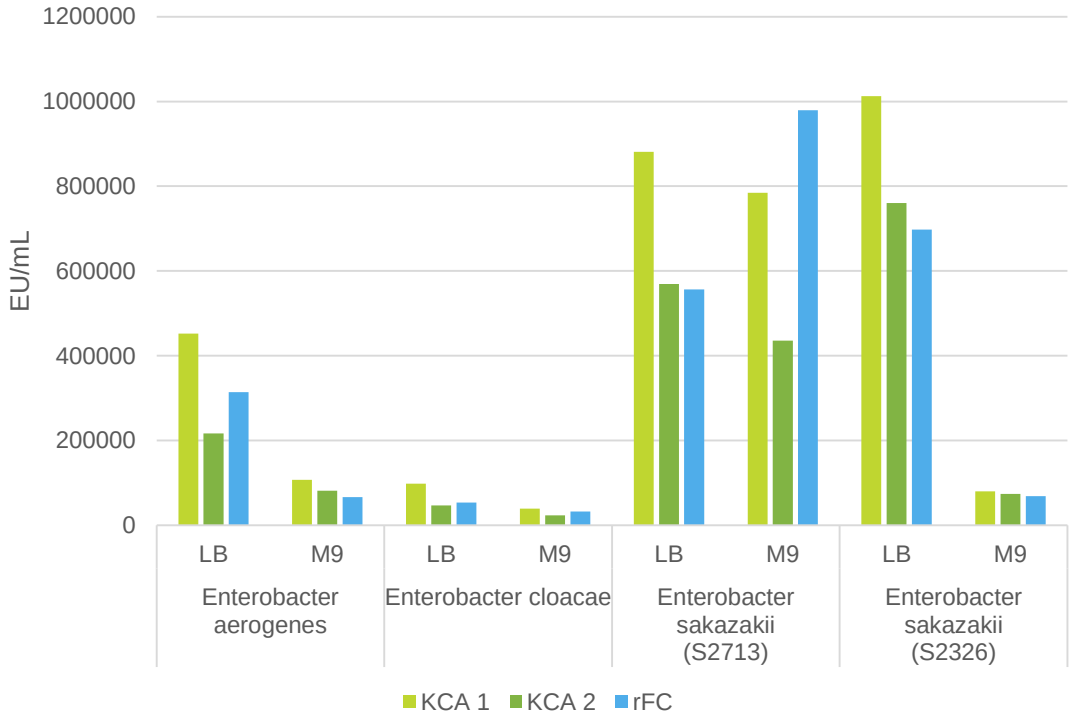
STUDY SETUP

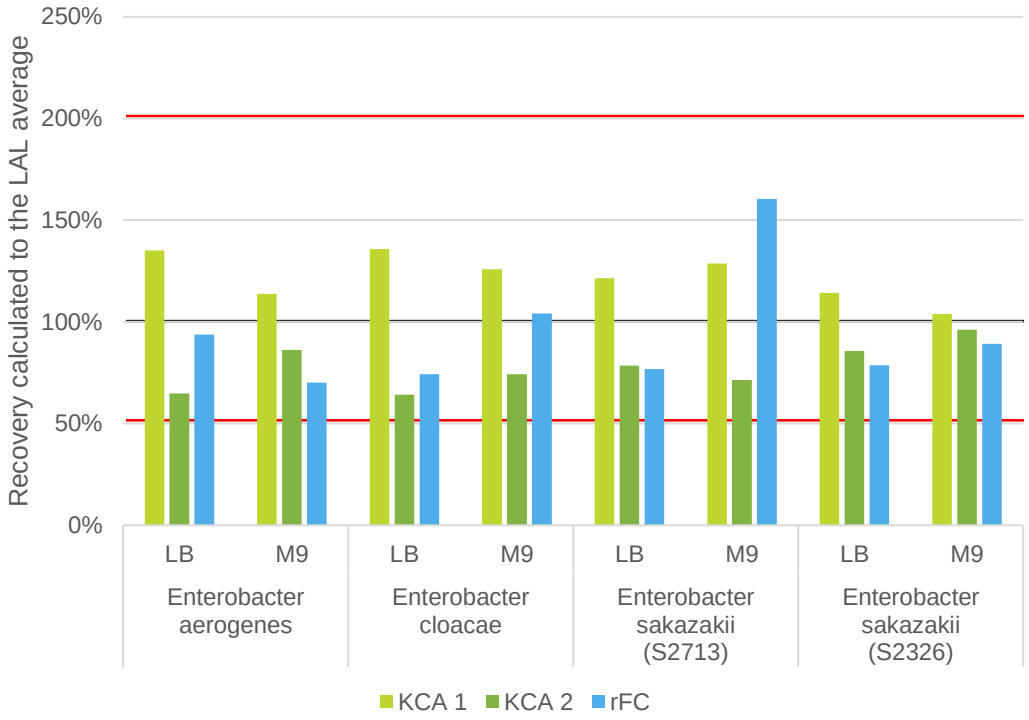
Sample measurements

- Serial dilution were prepared in endotoxin free water (1:1000 up to 1:1,000,000)
- Four different dilutions of each sample were analyzed
- All measurements were performed **at the same day using same dilutions for all assays**
- LAL and rFC assays were performed according to manufacturers instructions
- The experiments were performed at bioMérieux R&D laboratory:

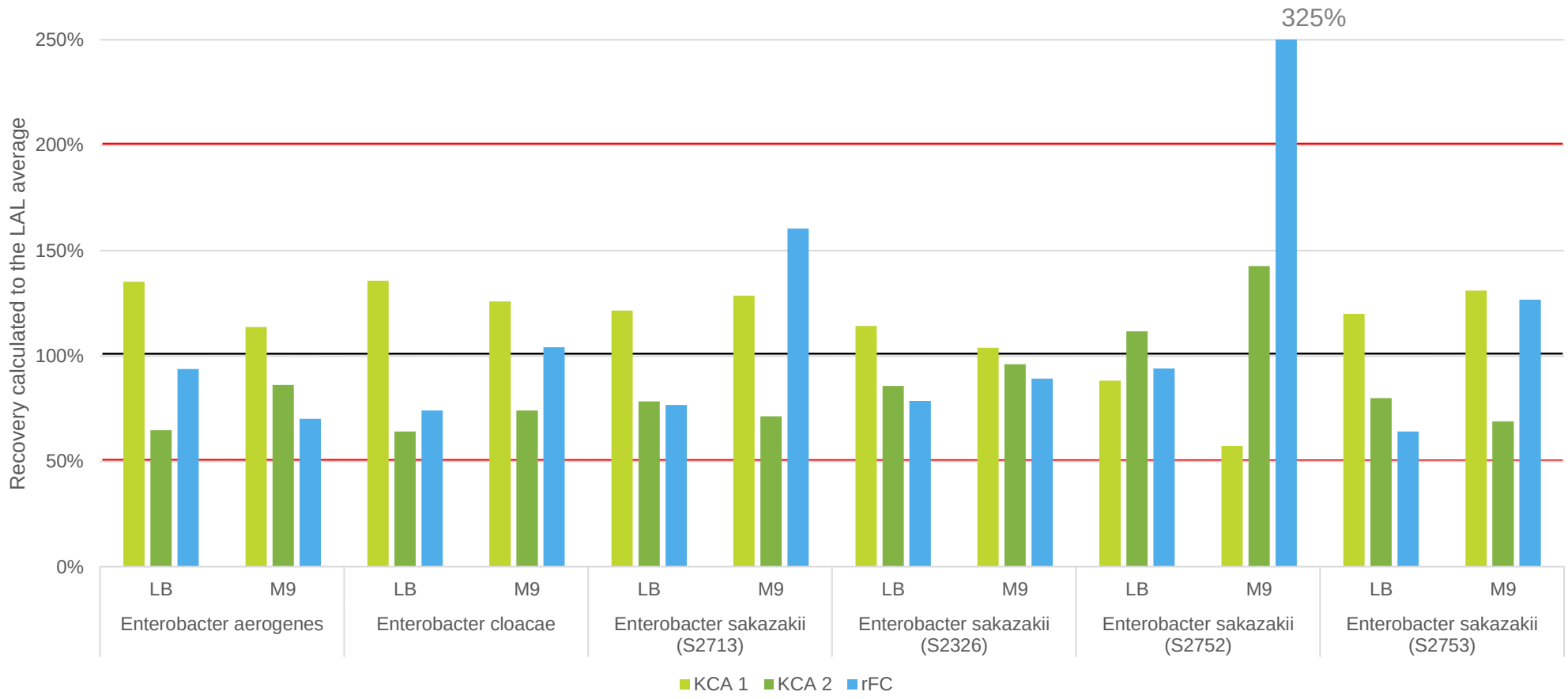
RESULT ANALYSIS

- To standardize the results, the average of the results of the 2 LAL assays was taken as 100% and the individual results compared to this average.

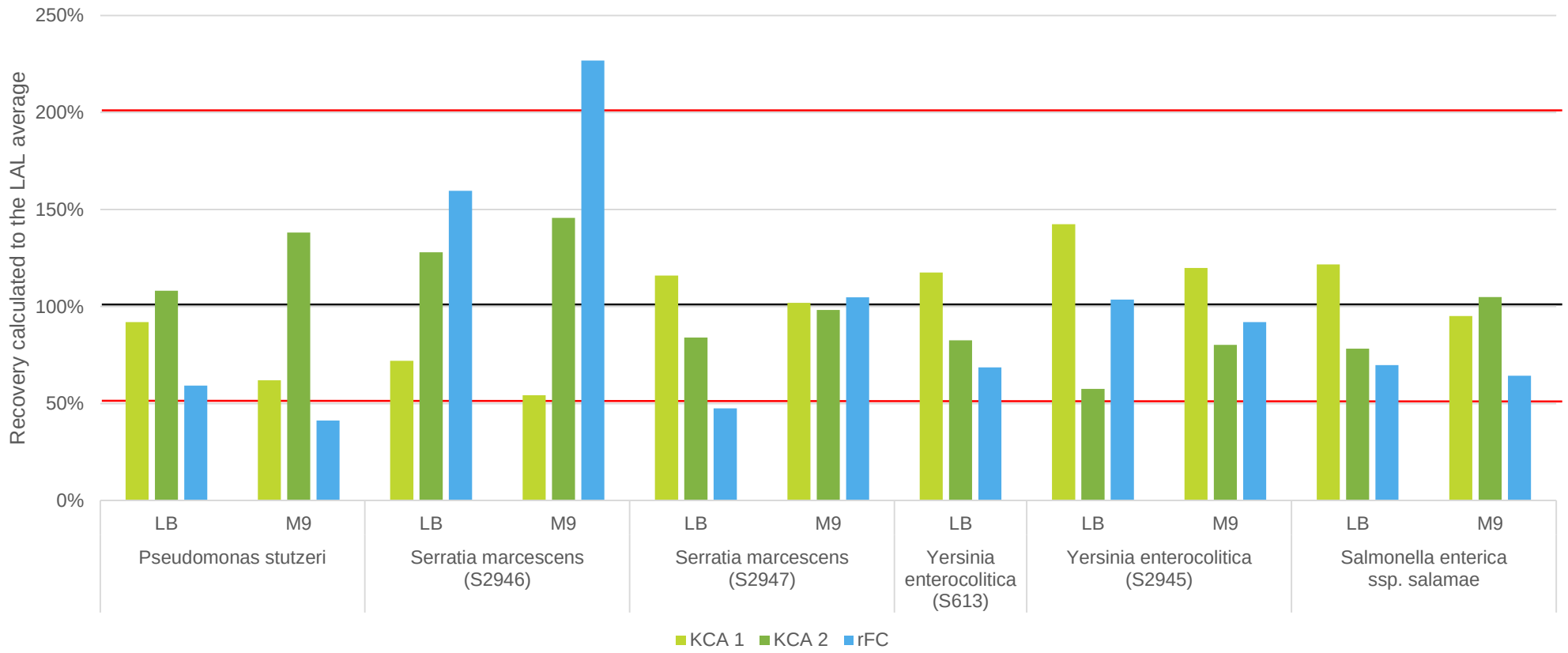




RESULTS 1


325%
250%
200%
150%
100%
50%
0%
Recovery calculated to the LAL average
LB
M9
Enterobacter aerogenes
Enterobacter cloacae
Enterobacter sakazakii (S2713)
Enterobacter sakazakii (S2326)
Enterobacter sakazakii (S2752)
Enterobacter sakazakii (S2753)
KCA 1
KCA 2
rFC

RESULTS 2


250%
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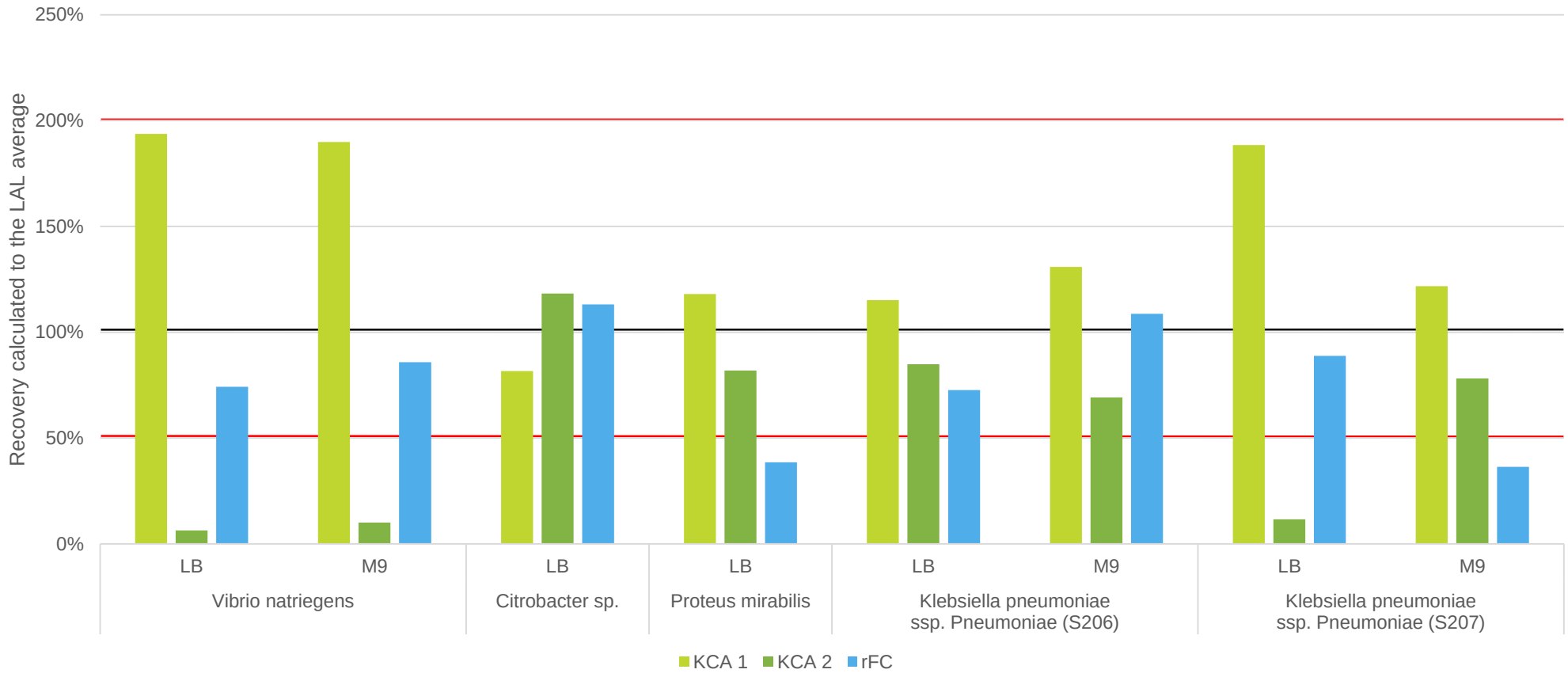
50%
0%
Recovery calculated to the LAL average
LB
M9
Pseudomonas stutzeri
Serratia marcescens (S2946)
Serratia marcescens (S2947)
Yersinia enterocolitica (S613)
Yersinia enterocolitica (S2945)
Salmonella enterica ssp. salamae
KCA 1
KCA 2
rFC

RESULTS 3


250%
200%
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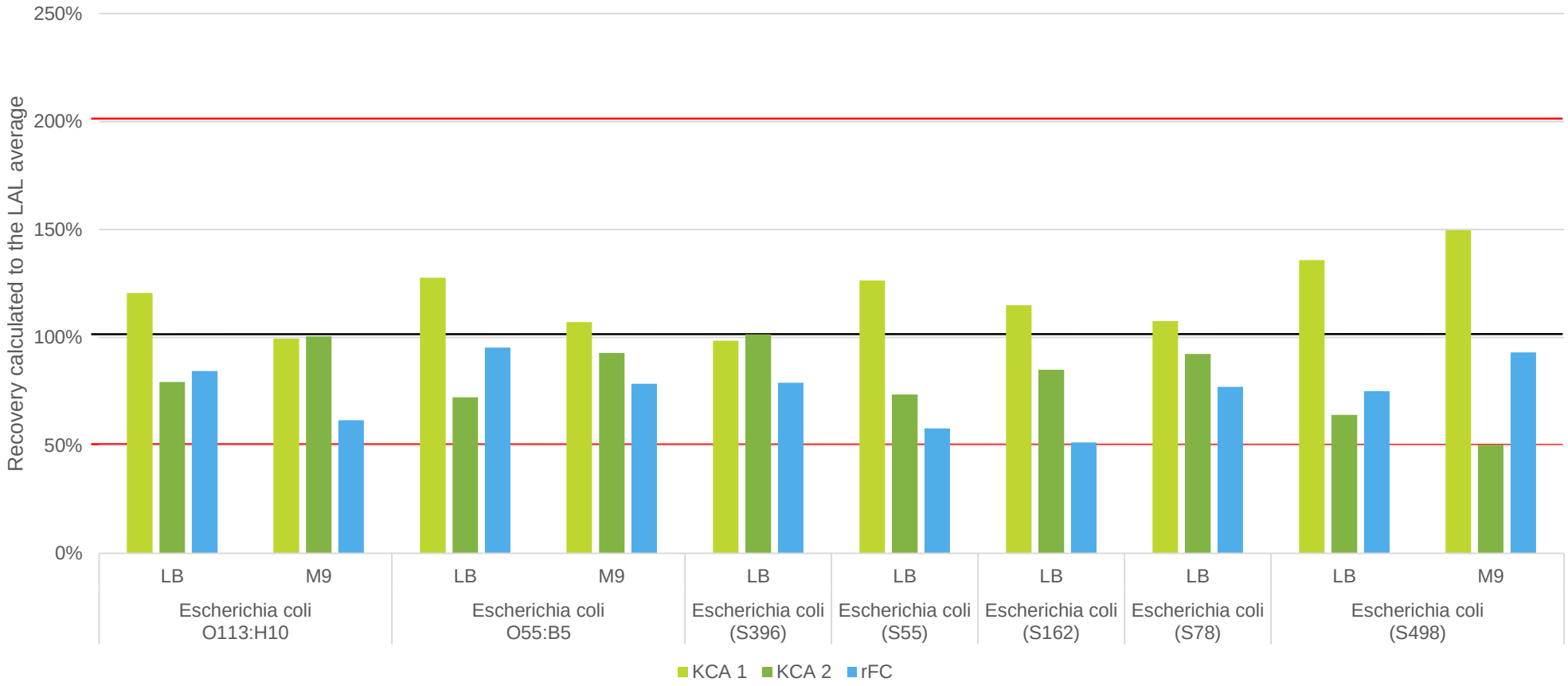
0%
Recovery calculated to the LAL average
LB
M9
Vibrio natriegens
LB
Citrobacter sp.
LB
Proteus mirabilis
LB
M9
Klebsiella pneumoniae ssp. Pneumoniae (S206)
LB
M9
Klebsiella pneumoniae ssp. Pneumoniae (S207)
KCA 1
KCA 2
rFC

RESULTS 4


250%
200%
150%
100%
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0%
Recovery calculated to the LAL average
LB
M9
LB
M9
LB
LB
LB
LB
LB
M9
Escherichia coli O113:H10
Escherichia coli O55:B5
Escherichia coli (S396)
Escherichia coli (S55)
Escherichia coli (S162)
Escherichia coli (S78)
Escherichia coli (S498)
KCA 1
KCA 2
rFC

SUMMARY

- rFC results are comparable with LAL when quantifying endotoxin from different gram negative bacteria growing under different environmental conditions.
- Comparison of the individual measured results with the average of the specific sample results measured with LAL assays:

Total sample number = 41	KCA 1	KCA 2	rFC
50% < n < 200%	41	38	36
n < 50%	0	3	4
n > 200%	0	0	1
concordant results regarding the average LAL results	100%	92.7%	87.8%

- For this data analysis the results of the LAL assays are defined as the “gold standard”. The results of the rFC assay matches in 87.8% with the results of the LAL assays.
- Even with this small test setup, the compendial methods show not always alignment in the results (92.7% for KCA 2).

LAL AND RFC COMPARATIVE STUDY 2
AUTOCHTHONOUS FACILITY ISOLATES

COMPARATIVE STUDY 2

Goal

To test if the variabilities of growth of autochthonous gram negative microorganisms and their endotoxin affects the ability of different BET methods to detect or quantitate them. It should be shown if rFC is comparable to LAL in quantification of endotoxin.

Materials

- Assays: 2 rFC, 5 LAL (3 Chromo, 2 Turbid), and 1 rLAL
- Samples: 7 different Autochthonous Gram Negative Organisms that were discovered during routine Bioburden testing in Pharmaceutical Facilities
- Media: up to four different growth media to simulate the GNB growing and adapting in low and high nutrients environments (→ 15 final samples).
  LB, M9, R2A, salt solution

STUDY SETUP

- The different facility isolates were added in BIOBALL format (550 CFU) to the different media.

- After ~ 5 days the samples were passed through 0.2 micron filters to remove any gram negative bacteria (GNB).
  The endotoxin was left in its natural state as it occurs in the environment (autochthonous).
- Samples were tested and diluted in endotoxin free water to approximately <1 EU/mL (using LAL)
- Samples were then tested using all different assays on two different days.
  Day 1: 4 LAL, 1 rLAL and 1 rFC assay
  Day 2: 1 LAL and 1 rFC assay
- LAL and rFC assays were performed according to manufacturers instructions and the results were all valid (PPC and CV).
- The experiments were performed at an independent 3rd party laboratory.

NOTES AND CONTROLS

- Control experiments of the pure media:

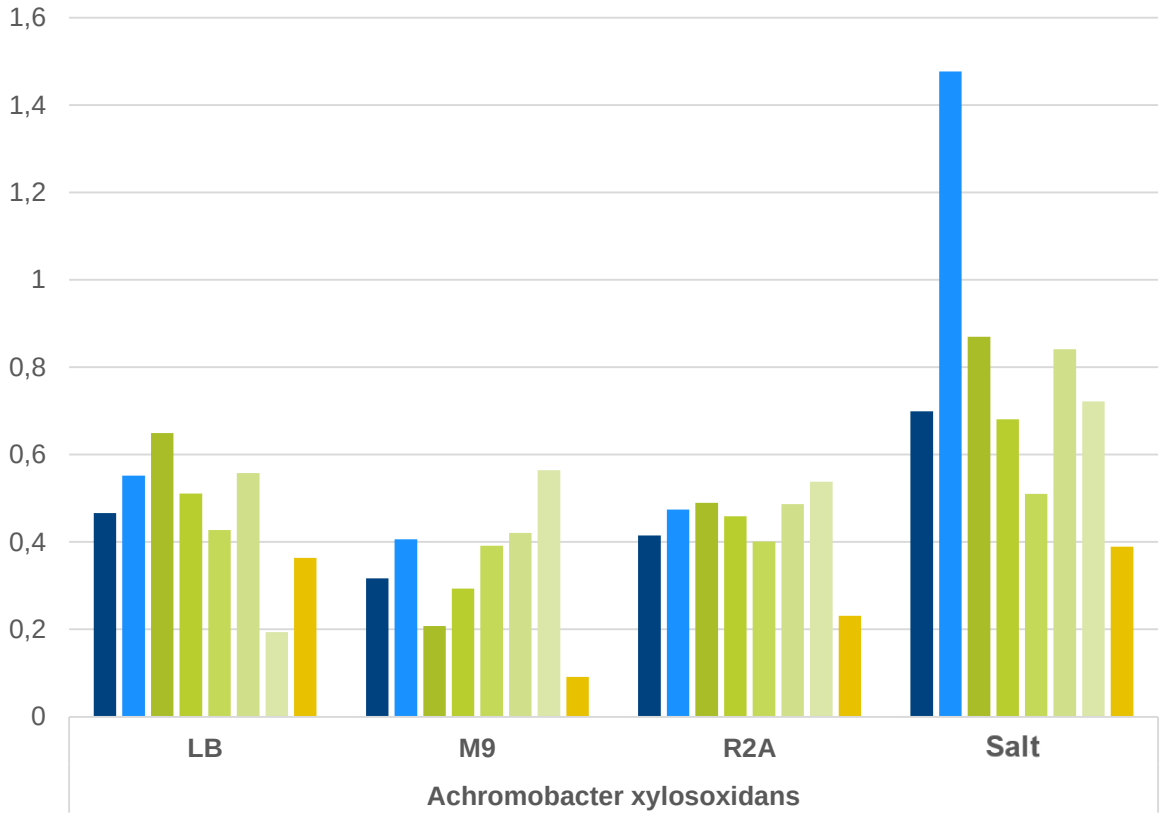
Media	dilution	rFC			KCA 1		
		Measured value [EU/mL]	Endotoxin activity in the media [EU/mL] *	PPC	Measured value [EU/mL]	Endotoxin activity in the media [EU/mL] *	PPC
LB	1:10	< 0.005	< 0.05	87%	0.022	0.22	155%
M9	1:10	< 0.005	< 0.05	108%	< 0.005	< 0.05	136%
R2A	1:100	< 0.005	< 0.5	98%	< 0.005	< 0.5	112%
salt	1:100	< 0.005	< 0.5	102%	< 0.005	< 0.5	148%

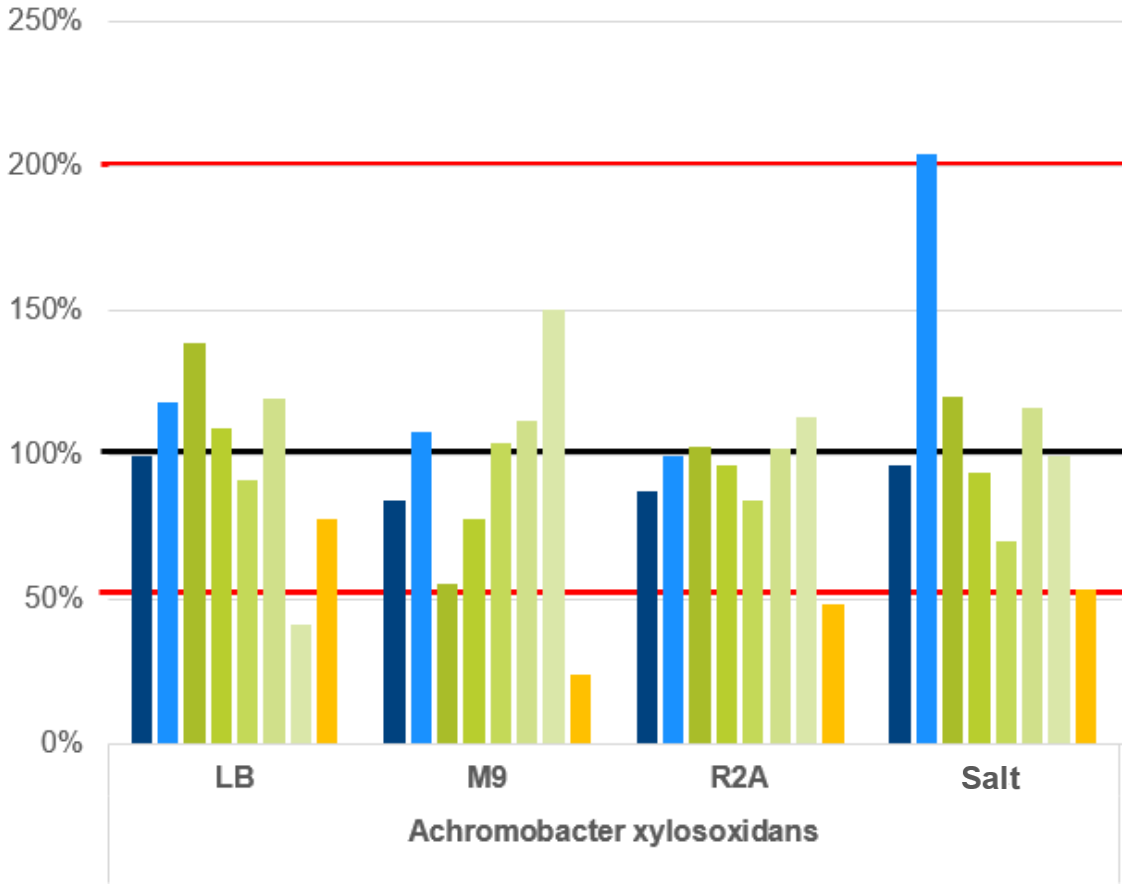
* Measured value multiplied with dilution factor

- All results had acceptable PPC and CV values. The samples were tested with the same dilution except for one KTA, for which some samples needed higher dilutions (1:2 to 1:10).
- Not all microorganisms grew in all media

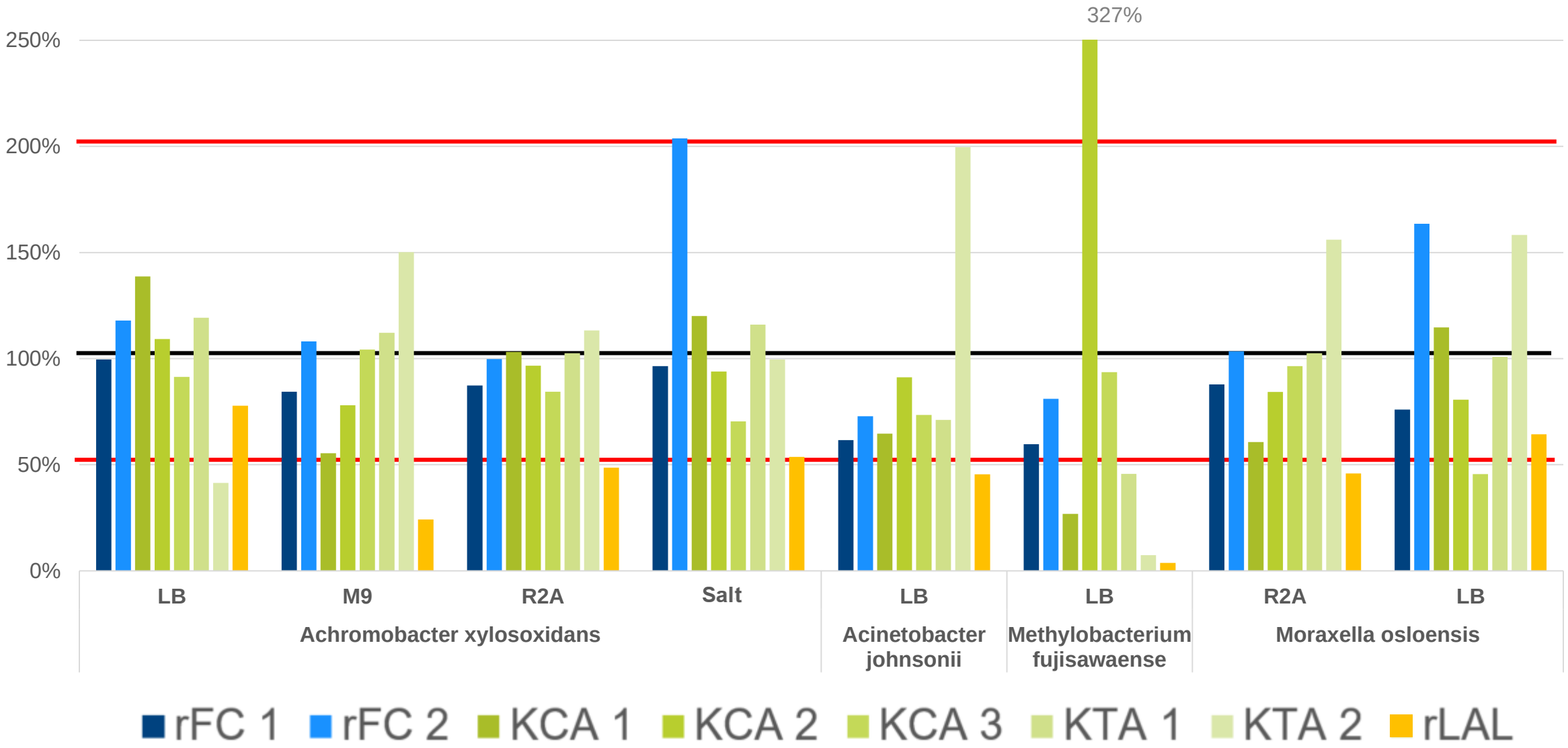
RESULT ANALYSIS

- To standardize the results, the average of the results of the 5 LAL assays was taken as 100% and the individual results compared to this average.

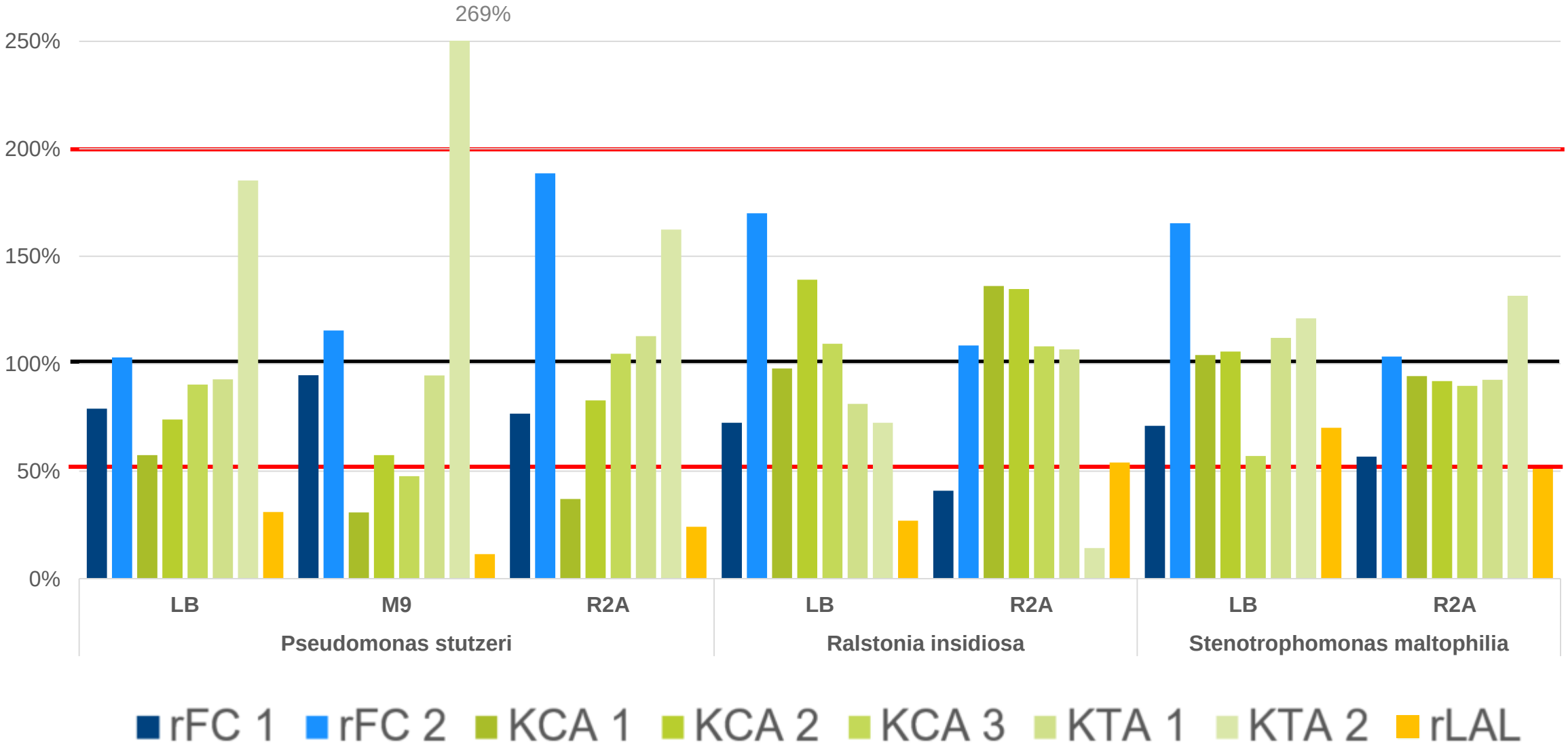




RESULTS 1


327%
250%
200%
150%
100%
50%
0%
LB
M9
R2A
Salt
LB
LB
R2A
LB
Achromobacter xylosoxidans
Acinetobacter johnsonii
Methylobacterium fujisawaense
Moraxella osloensis
rFC 1
rFC 2
KCA 1
KCA 2
KCA 3
KTA 1
KTA 2
rLAL

RESULTS 2


269%
250%
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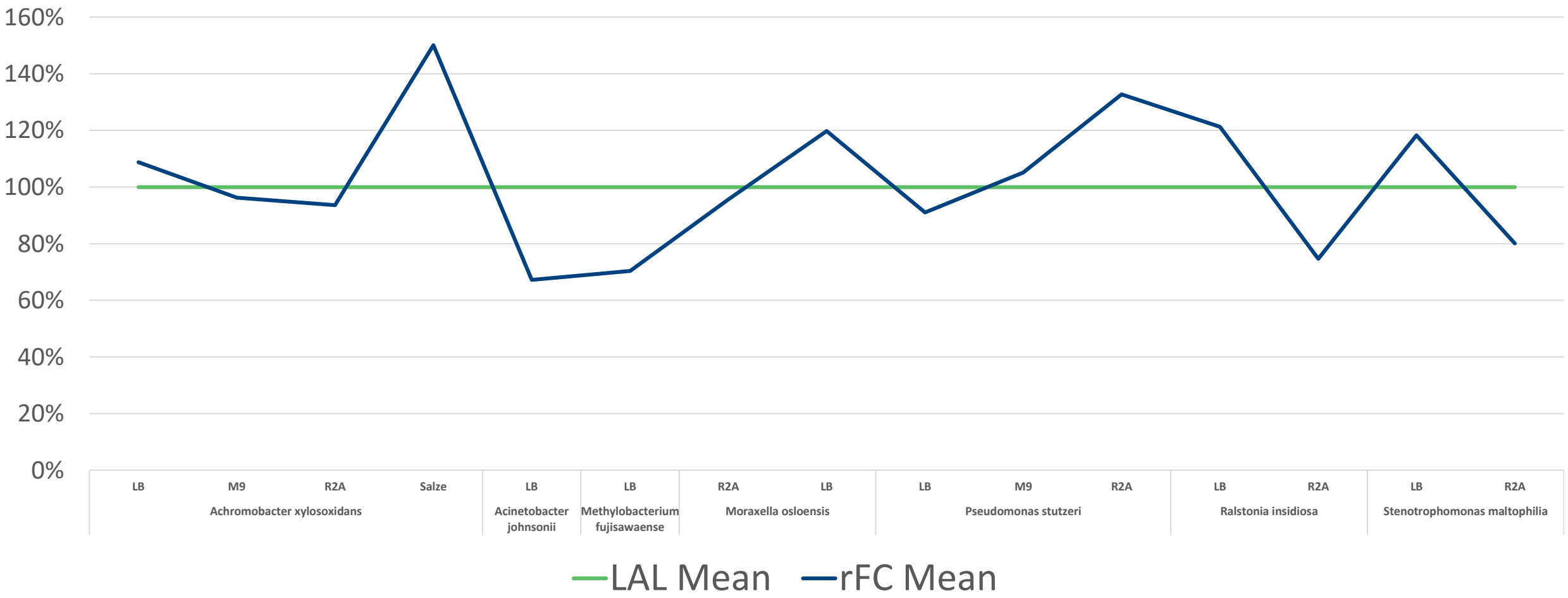
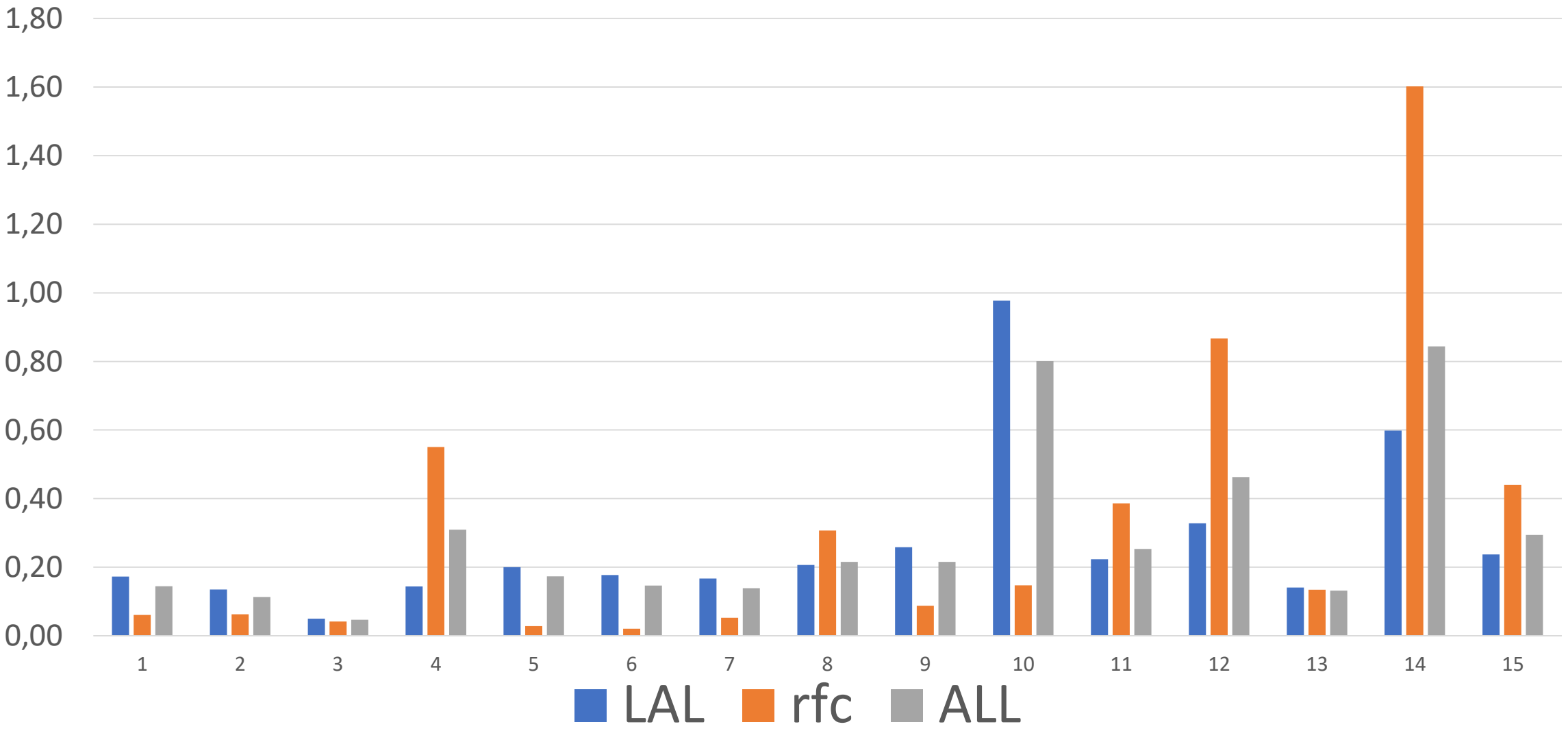
LB
M9
R2A
LB
R2A
LB
R2A
Pseudomonas stutzeri
Ralstonia insidiosa
Stenotrophomonas maltophilia
rFC 1
rFC 2
KCA 1
KCA 2
KCA 3
KTA 1
KTA 2
rLAL

RFC & LAL COMPARABILITY WITH AUTOCHTHONOUS ENDOTOXIN



STANDARD DEVIATION



CONCLUSIONS

- rFC results are comparable with LAL when quantifying autochthonous endotoxin from GNB that have been discovered in pharmaceutical environments
- Stressing the microorganisms and endotoxin in nutrient rich and nutrient poor environments has no effect on the detection or quantification of endotoxin.
- rFC and LAL use the same endotoxin detection enzyme (Factor C) and are comparable when detecting and quantifying endotoxin

	LAL	rFC
Overall test number	75	30
50% < n < 200%	63	28
n < 50%	9	1
n > 200%	3	1
concordant results regarding the average LAL results	84.0%	93.3%

The rLAL is not included in this analysis.
The results of the LAL measurements are defined as the “gold standard”.

Potential impact of β-glucans and other microbial sugars on LAL-rFC comparison studies

Kevin Williams, Senior Scientist, bioMerieux November 15th, 2021

GLUCANS: ARE UBIQUITOUS IN NATURE (FUNGI/PLANTS/ETC.)

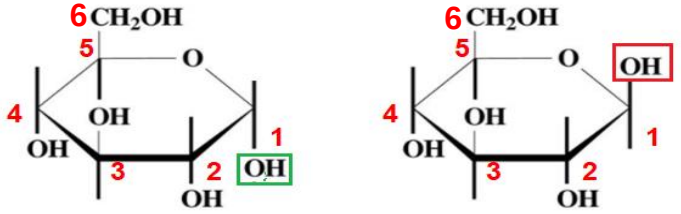
And Consist of wildly diverse structures!

Background: Look at structures and some membrane types they occur in

Data generated: Try and answer **3 basic questions** around chrome vs turb β-glucan reactivity and utility of the beta glucan blocking buffer.

- Plants /grass
- Fungi
- Yeast
- Algae
- Lichen
- Cellulosic (wood)
- Some bacteria (*Agrobacterium*)
- Seaweed

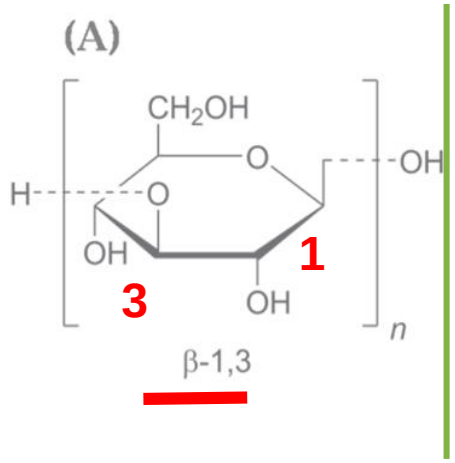
BETA GLUCANS – MONOMERS > POLYMERS



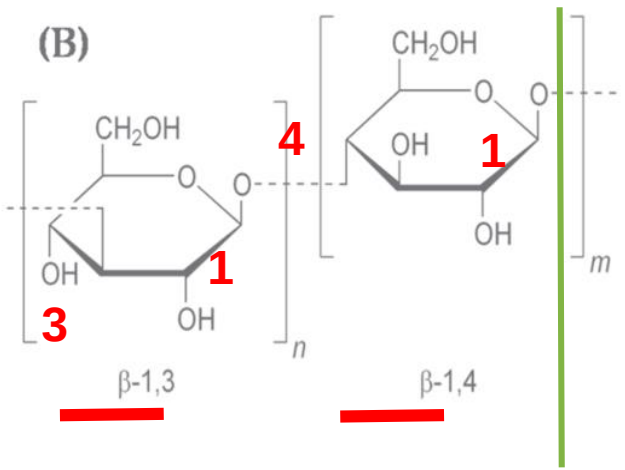
α-Glucose β-Glucose

D-Glucose

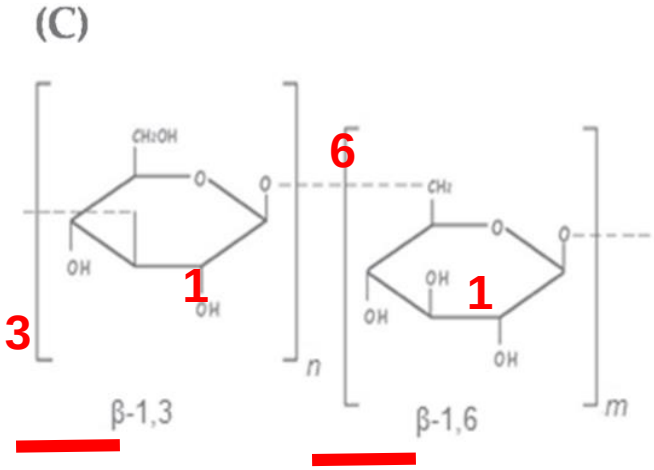
Alpha glucose and beta glucose ring structure



Bacterial Fungi / yeast
(*agrobacterium*)
Linear no branches

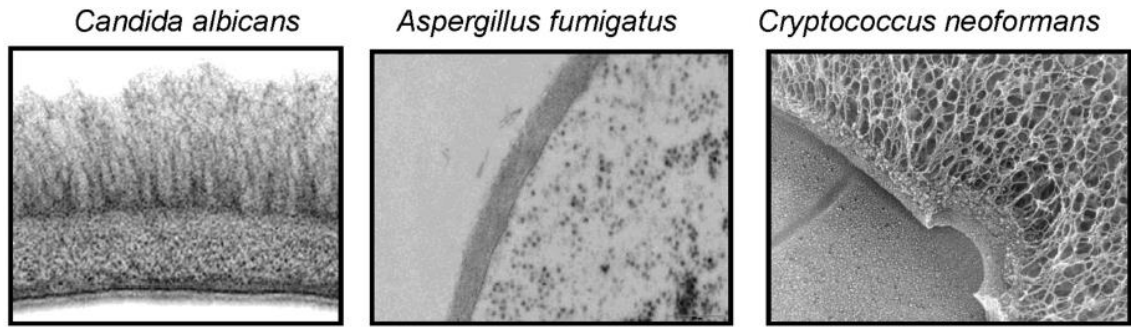


Cereals / lichens
Linear 1>3 or 1>4
No branches



Seaweed (Laminaria)
Fungi / yeast
1>3 backbone
with 1>6 branches

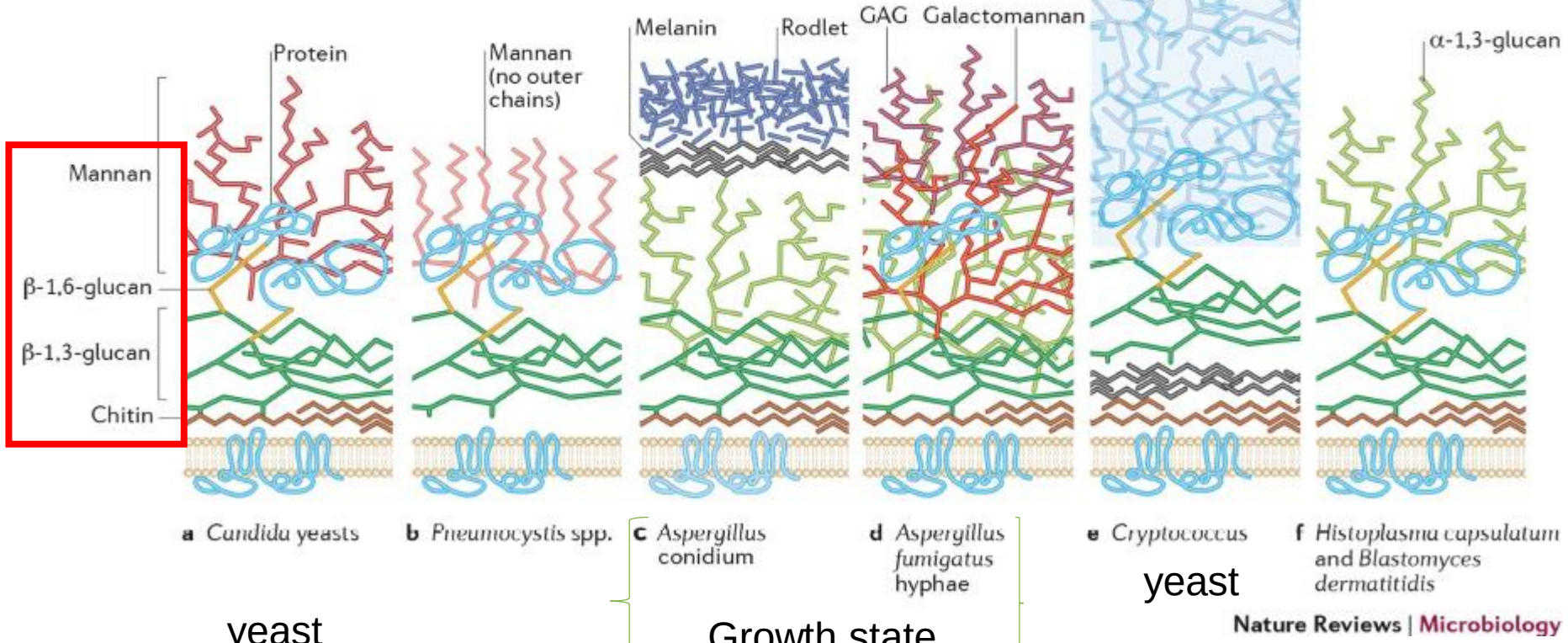
MORE COMPLEXITY/UBIQUITY THAN COMMONLY ACKNOWLEDGED



common in soil

GENERIC

- Mannans
- β glucans
- Chitin
- Phospholipid bilayer



SO NATURE IS NOT SO SIMPLE

Things to “worry about” if you are using non-filtered water for LAL and rfc comparisons.

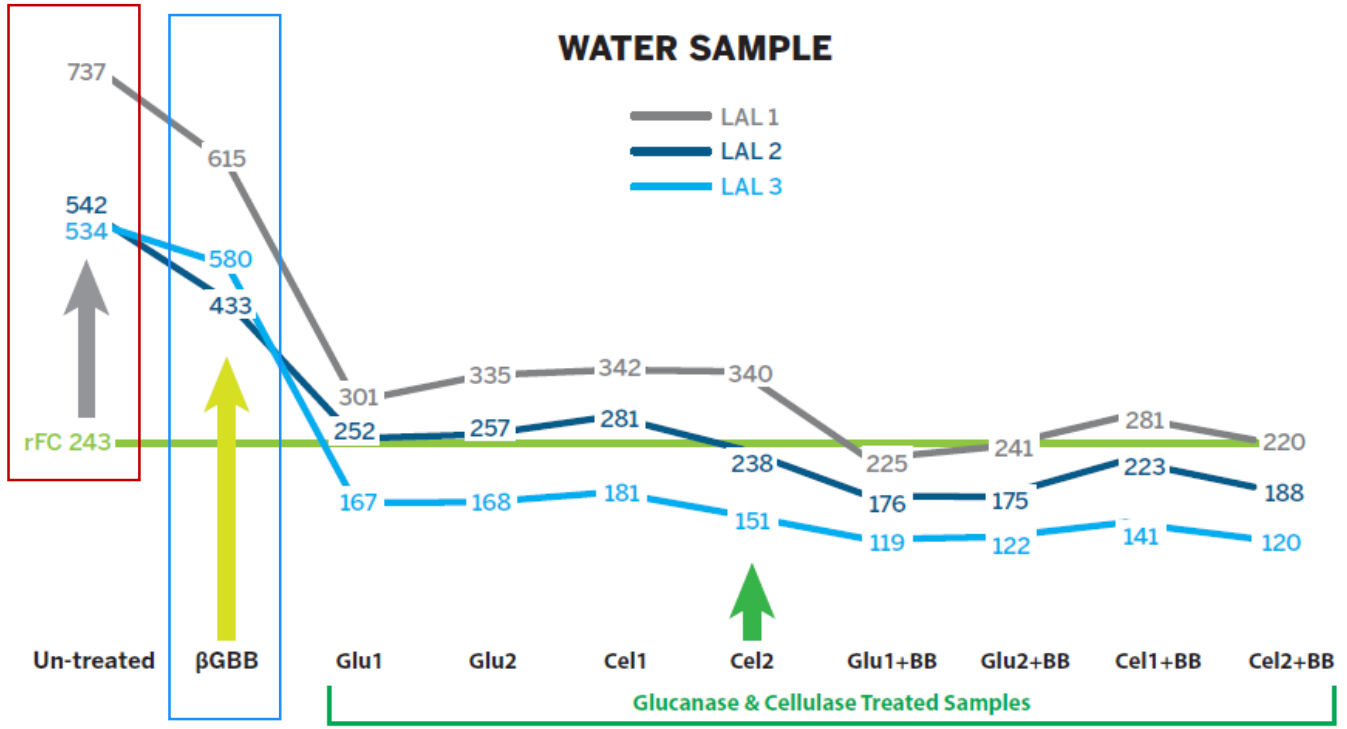
1. Do chrome and turb LAL methods give the same result in the presence of β-glucans?

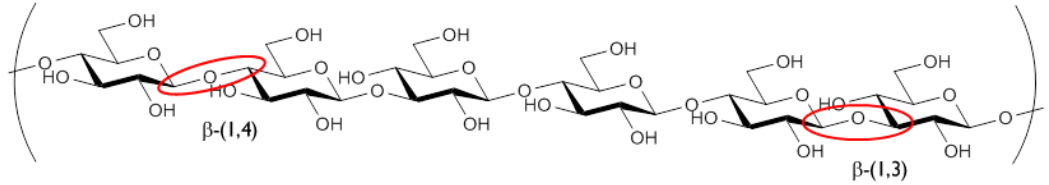
2. Can we really block all β-glucans by just using a blocking buffer?

3. Are there other microbial sugars that react with LAL?

ARE ALL GLUCANS BLOCKED WITH GB BUFFER?

- Initial test makes rFC look low
- Treatment with BGBB brings LAL results down a little
- But enzymatic treatment brings LAL values down more
- Repeated tests on 2 different natural water sources produced near identical results.





***Study from last year**

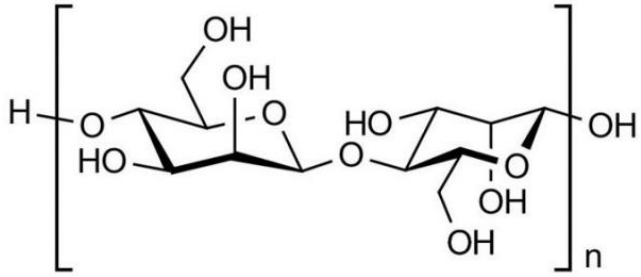
STUDIES FROM THIS YEAR

- Many fungi and yeast have prototypical β (1>3) D glucans, many do not
- LAL-RM from cellulosic filters are (1>4) β glucans – not included here

α vs β

- Plant mannans have β(1-4) linkages. They are a form of storage polysaccharide.
- Yeast mannans have α(1-6) linked backbones and α(1-2) and α(1-3) linked branches. It is serologically similar to structures found on mammalian glycoproteins.

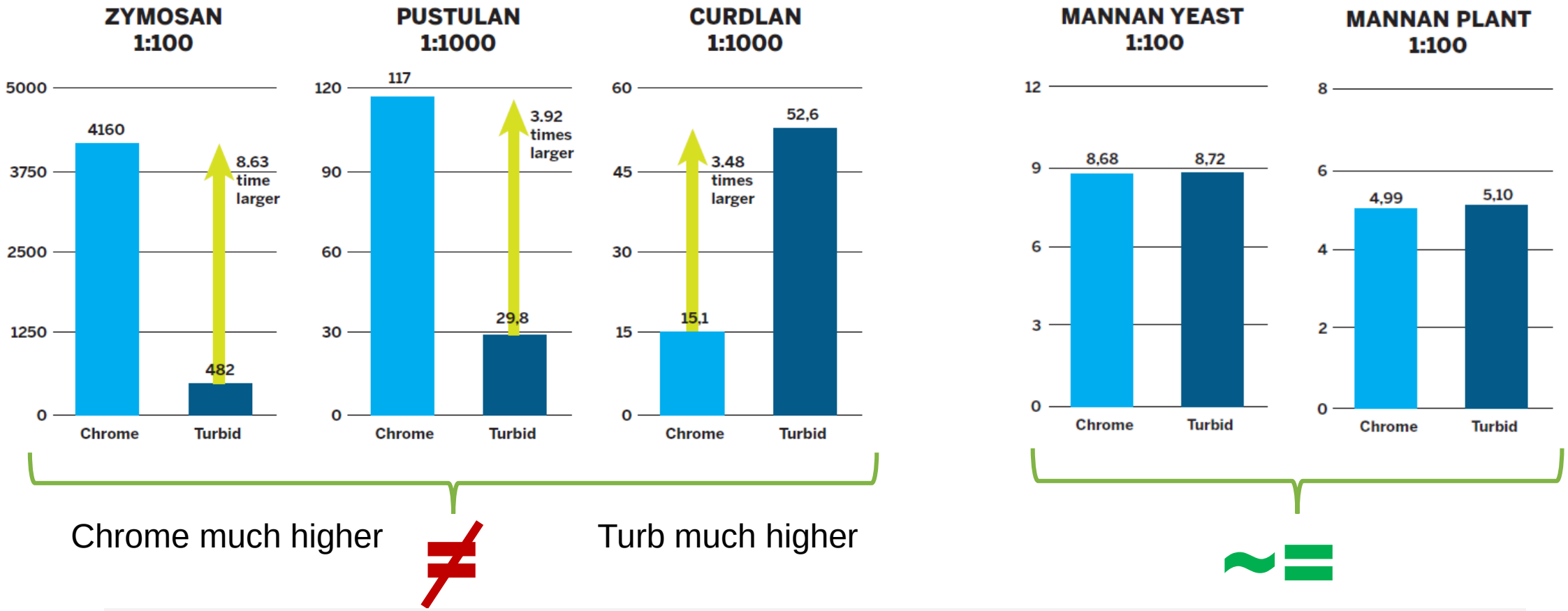
- Looked at mannan because some old LAL references mention its LAL reactivity. Not a beta glucan.



Mannan oligosaccharides

Q1. WHAT ABOUT CHROME VS TURB REACTIVITY?

If “gold standard” result then they should be equal

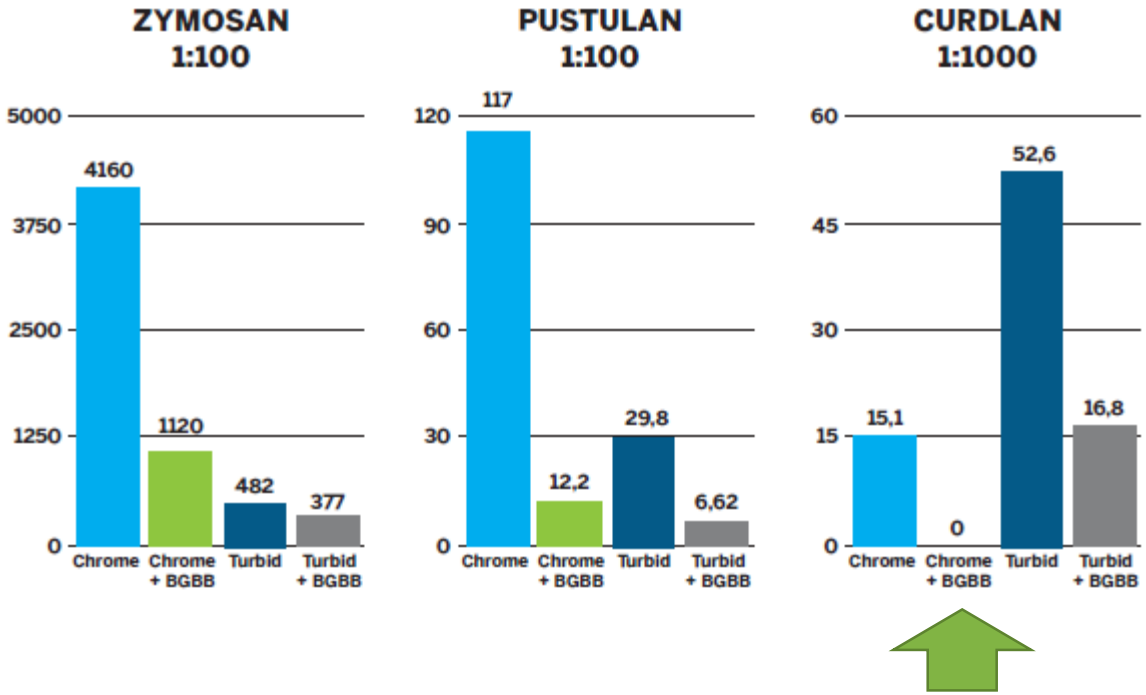


Chrome much higher ≠ Turb much higher

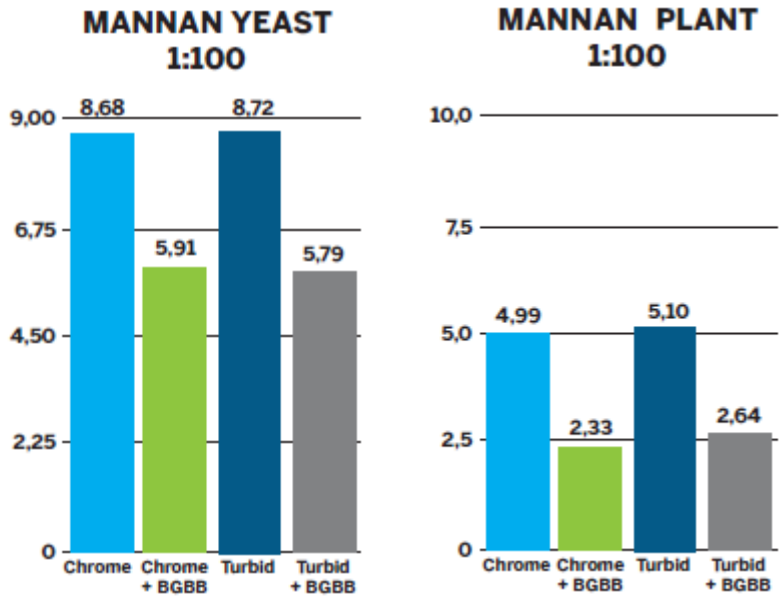
≈ =

1 mg/mL of each sugar dissolved in purified water, heated 70C (oven) for 1 hr. and vortexed for 30 min. Diluted 1:100 or 1:1000. Sample sugars were of the highest purity available and labeled <1 EU/mL as tested by human TLR4-expressing HEK 293 cells and as tested negative for endotoxin with rFC.

Q2. ARE ALL GLUCANS BLOCKED WITH GB BUFFER?
Q3. WHAT ABOUT NON CONVENTIONAL MICROBIAL SUGARS?



Curdlan - as advertised but only with chrome.
Other sugars have significant residual activity.



Blocking buffer does very little to counter mannan LAL activity

THE HISTORICAL SUCCESS OF LAL IS BUILT UPON…

- The presumption that purified water and other manufacturing process constituents are clean of non-endotoxin pyrogens and other reactants (β -glucans)
- β -glucans and other contaminants are excluded *during process validation*
- Only Gram negative bacteria can “spring up” quickly in purified water
- All LAL tests give basically the same answer (presumption challenged by this data when β -glucans are present)
- There is no β-glucan standard in the LAL test and therefore it can only “interfere” with a true endotoxin result
- **rFC continues the basic pharma LAL paradigm**

ANY QUESTIONS?

Thank you for your attention!

For questions and/or support, please contact

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PIONEERING DIAGNOSTICS