Science, Service, Stewardship

Discriminant analysis of oyster hemocyte immune functions as a predictor of potential probiotic candidates.

Diane Kapareiko*, Julie M. Rose, Jennifer H. Alix, Dorothy Jeffress and Gary H. Wikfors.

USDOC, NOAA, National Marine Fisheries Service, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

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The Milford Laboratory has isolated a naturally-occurring, safe and effective *Vibrio* sp. probiotic bacterium which improves survival during larviculture of the Eastern Oyster (*Crassostrea virginica)*:

Probiotic OY15

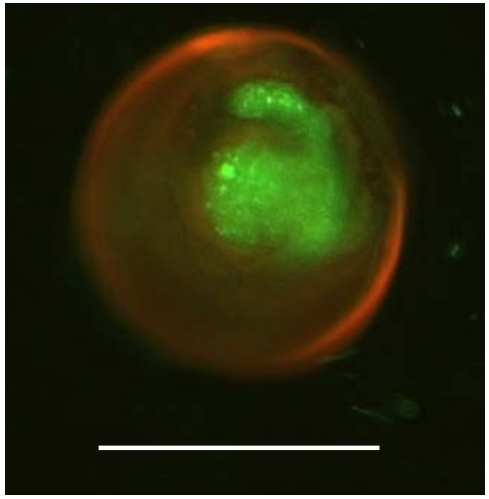
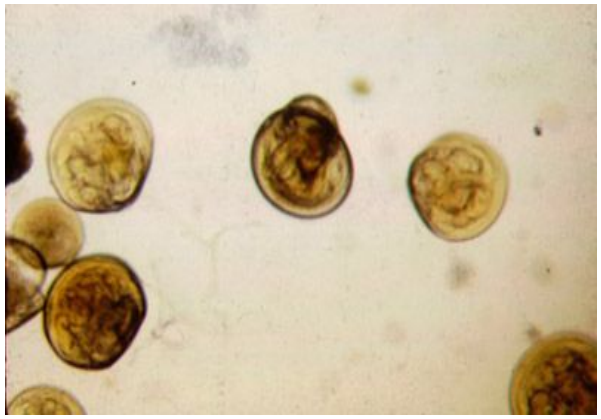


Fig. X. Eastern oyster larva 11 days post-fertilization exposed for 20 min to fluorescently-labeled (BacLite) probiotic bacterial strain OY-15 (scale bar = 100 μm). Fluorescent bacteria are concentrated within the esophagus and stomach of the larva; shell edge is seen as red, refracted light.

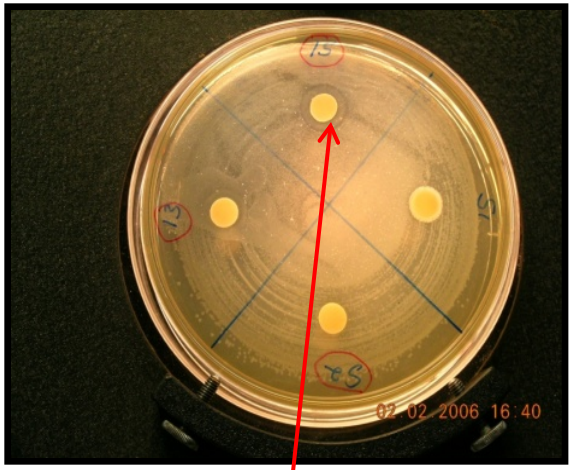


Lim et al. JSR, Vol. 30, No.3, 2011
Kapareiko et al. JSR, Vol 30, No. 3, 2011

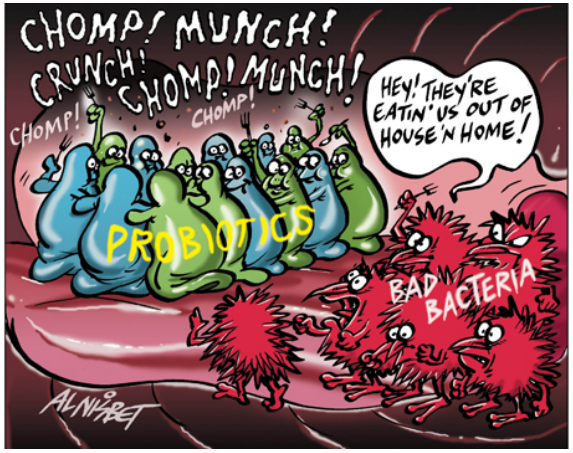
Initial selection process for probiotic candidates

- Selection process based on the assumption of Competitive Exclusion as a possible mechanism
- If OY15 inhibits pathogen B183 on solid agar, then it might do the same in culture buckets
- OY15 could out-compete pathogen B183 for nutrients
- OY15 could poison pathogen B183 by release of antimicrobial extracellular products.

Kirby-Bauer Disc Diffusion Method



OY15 inhibited shellfish-larval pathogen B183, (*Vibrio corallilyticus*)

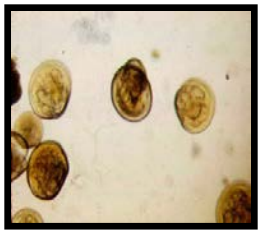


Findings:
No evidence of competitive exclusion as the mechanism of OY15's probiotic effects.

In culture water and in oyster larvae using 16S rRNA molecular analyses…

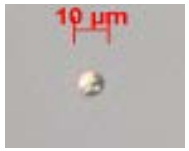
The signal for pathogen B183 gene sequences is gone one day post-challenge.

The signal for probiotic OY15 gene sequences was not found 2 days post-challenge with pathogen B183.



PCR reactions with 1055f/1392GCr Primers; 40-60% DGGE, 18 hours @60°C, 55 volts.

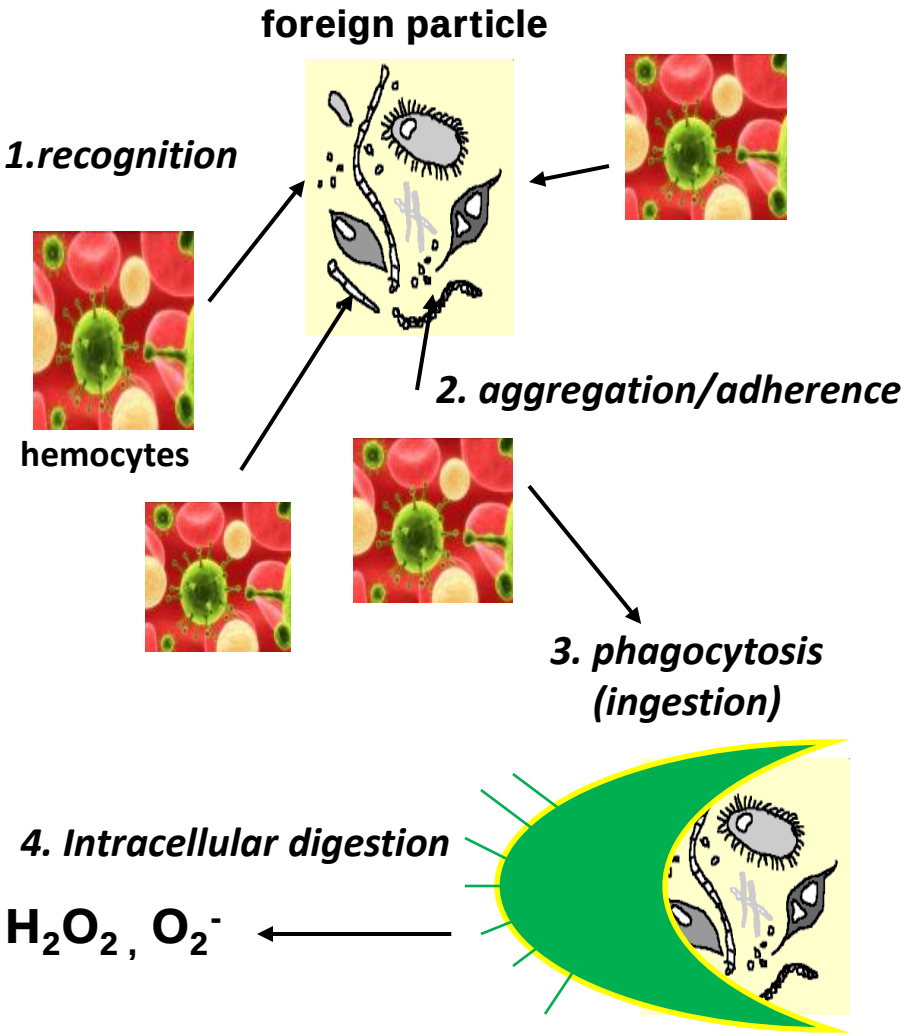
Hemocyte Immune Functions



- **<u>Oyster Cellular Immunity</u>**
  -open circulatory system
  -cell types (granular and agranular hemocytes)
  -functions (nutrition, waste disposal, defense)

- **<u>Defense functions</u>**
  Adhesion
  Phagocytosis
  ROS production

- **<u>Environmental Factors</u>**
  water quality (*temp, DO*)
  pollutant stressors
  food availability.



Strong evidence indicates mechanism responsible for improved survival of oyster larvae given probiotic OY15 is regulation of hemocyte immune functions

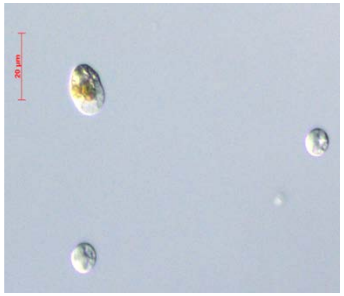
Previous Flow-cytometry work at the Milford Lab on oyster hemocytes demonstrated:

1. Probiotic OY15 Causes Up-regulation of Immune Response

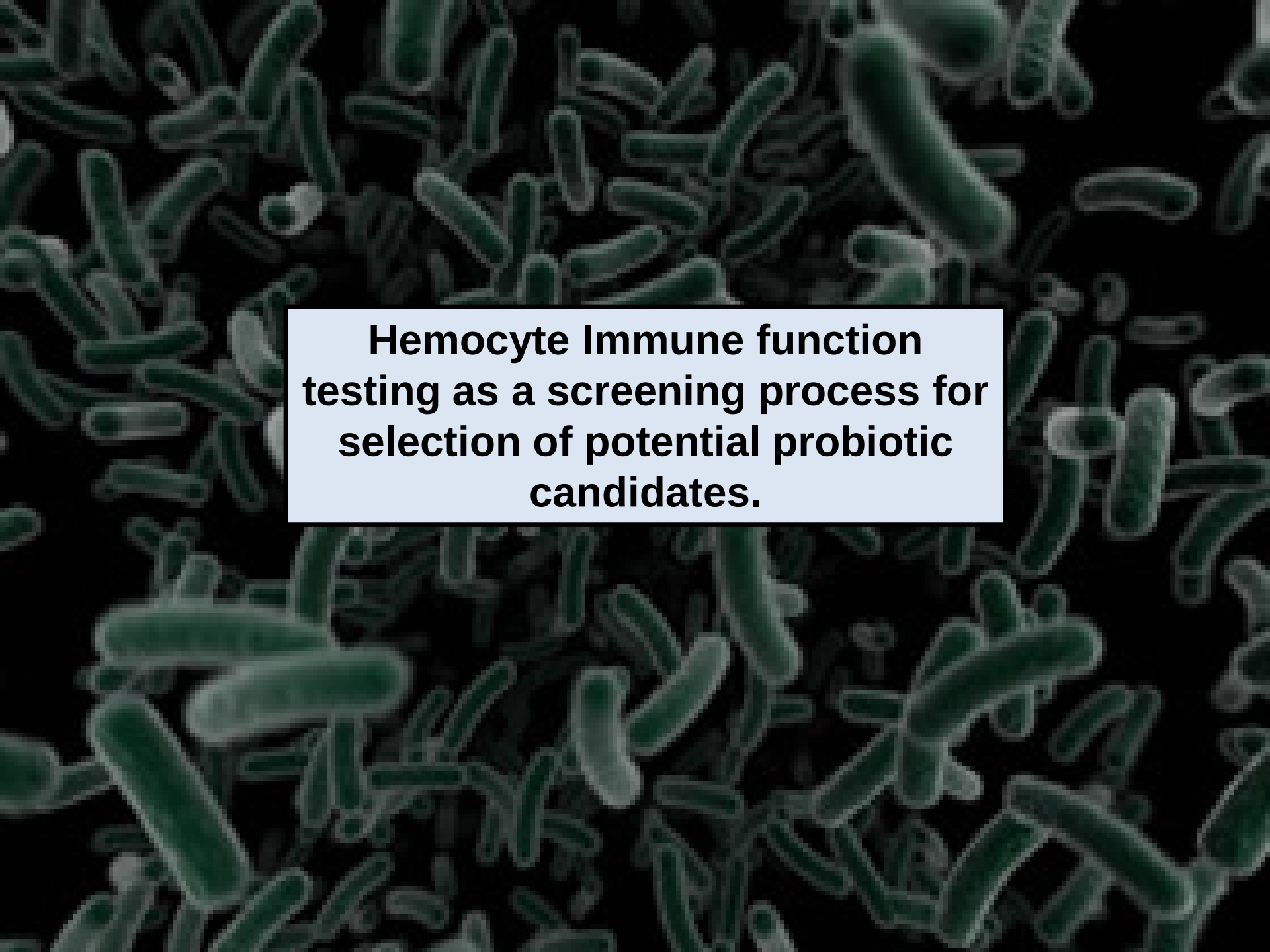
- **a: up-regulation of phagocytosis, adhesion, or ROS production.**
- **b: No effect on hemocyte mortality.**

2. Pathogen B183 had an immunosuppressive effect on hemocyte immune functions.

- **a. suppressed ROS production.**
- **b. significantly increased hemocyte mortality.**
- **c. no effect on phagocytosis or adhesion.**



The FACScan flow cytometer is a biomedical instrument designed for human blood cell analyses.

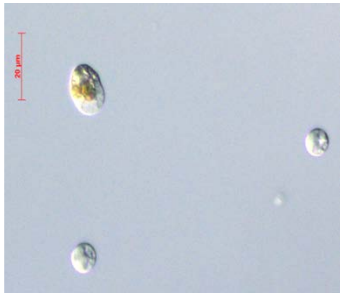


Hemocyte Immune function testing as a screening process for selection of potential probiotic candidates.

What patterns are we looking for?

- **Potential probiotic candidates: no hemocyte mortality, up-regulation of adhesion, phagocytosis and ROS production as previously seen with probiotic OY15.**

- **Potential Pathogens: hemocyte mortality, down-regulation of ROS production as previously seen with pathogen B183.**



Discriminant analysis of oyster hemocyte immune functions as a predictor of potential probiotic candidates- A “visual mapping process”

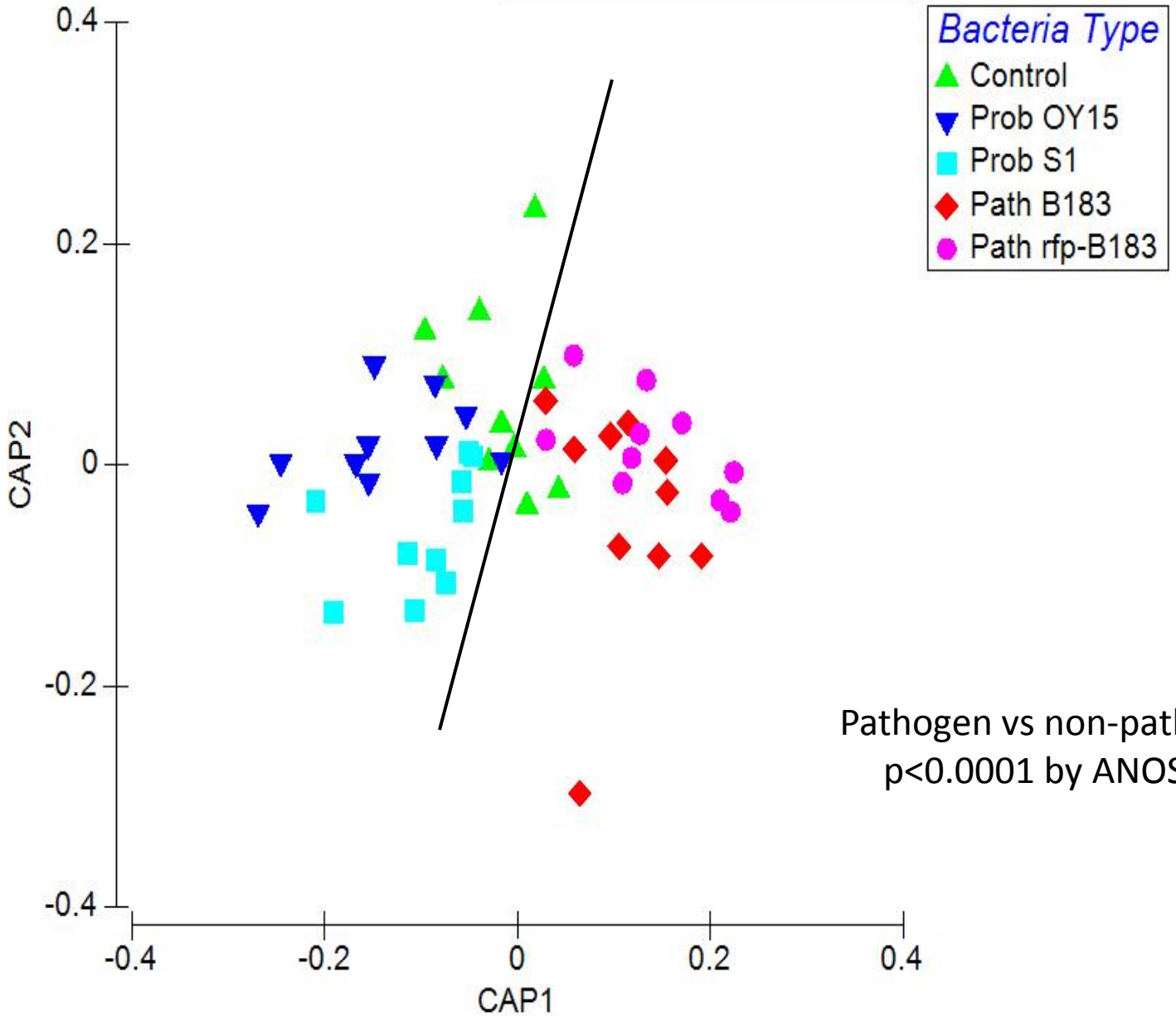
- Discriminant analysis (DA) is a multivariate statistical technique that can be used to classify data into categories based on a series of test variables.

- Simply put…the discriminant analysis classifies unknown bacterial isolates into groups or categories of the same type.

- Using B183 as a pathogen control, discriminant analysis separates and groups pathogens from non-pathogens.

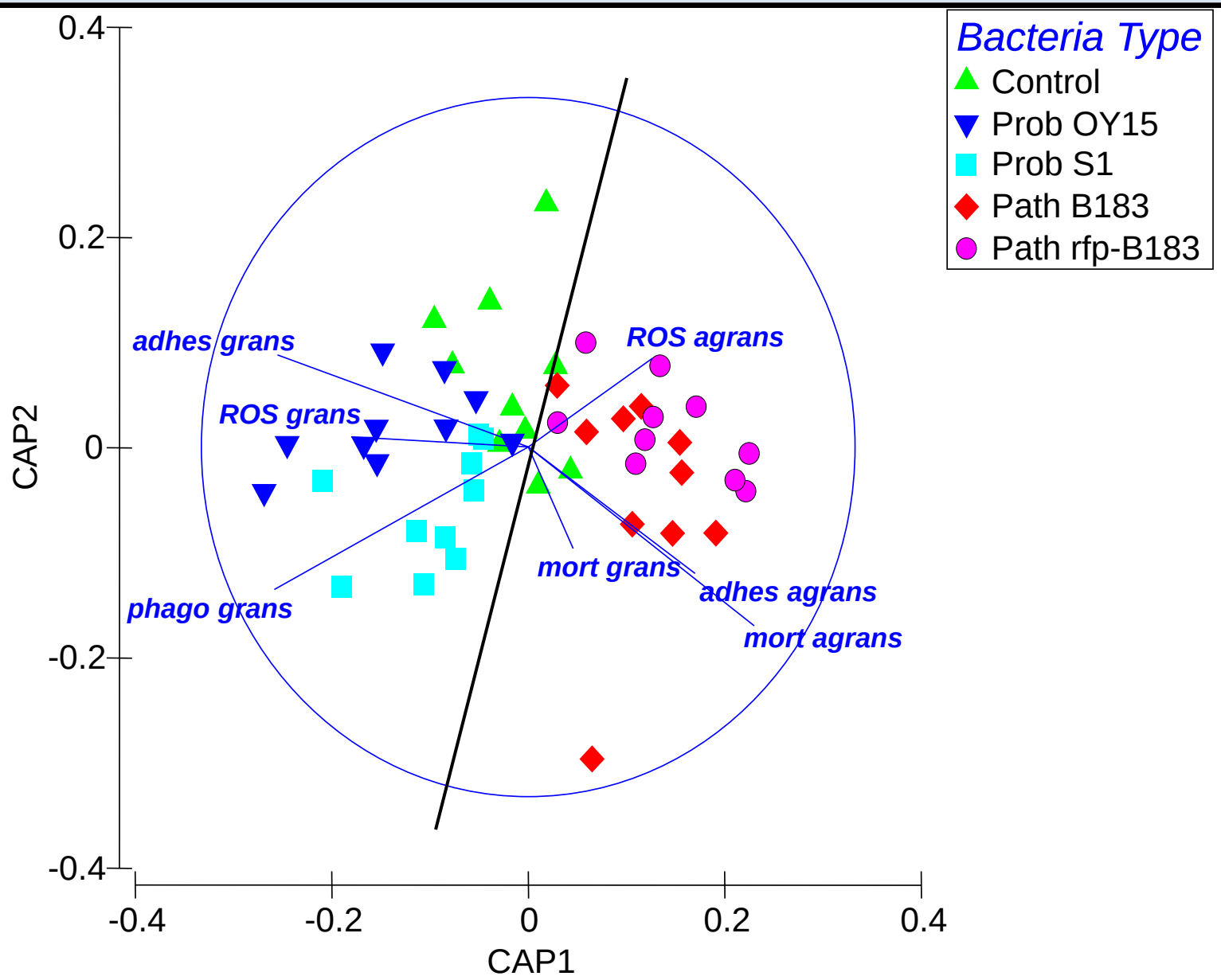
- Using OY15 as a probiotic control, probiotic candidates can be identified based on up-regulation of several immune functions.

DA plot for Probiotic OY15, Probiotic candidate S1 and Pathogens B183 and rfp-B183

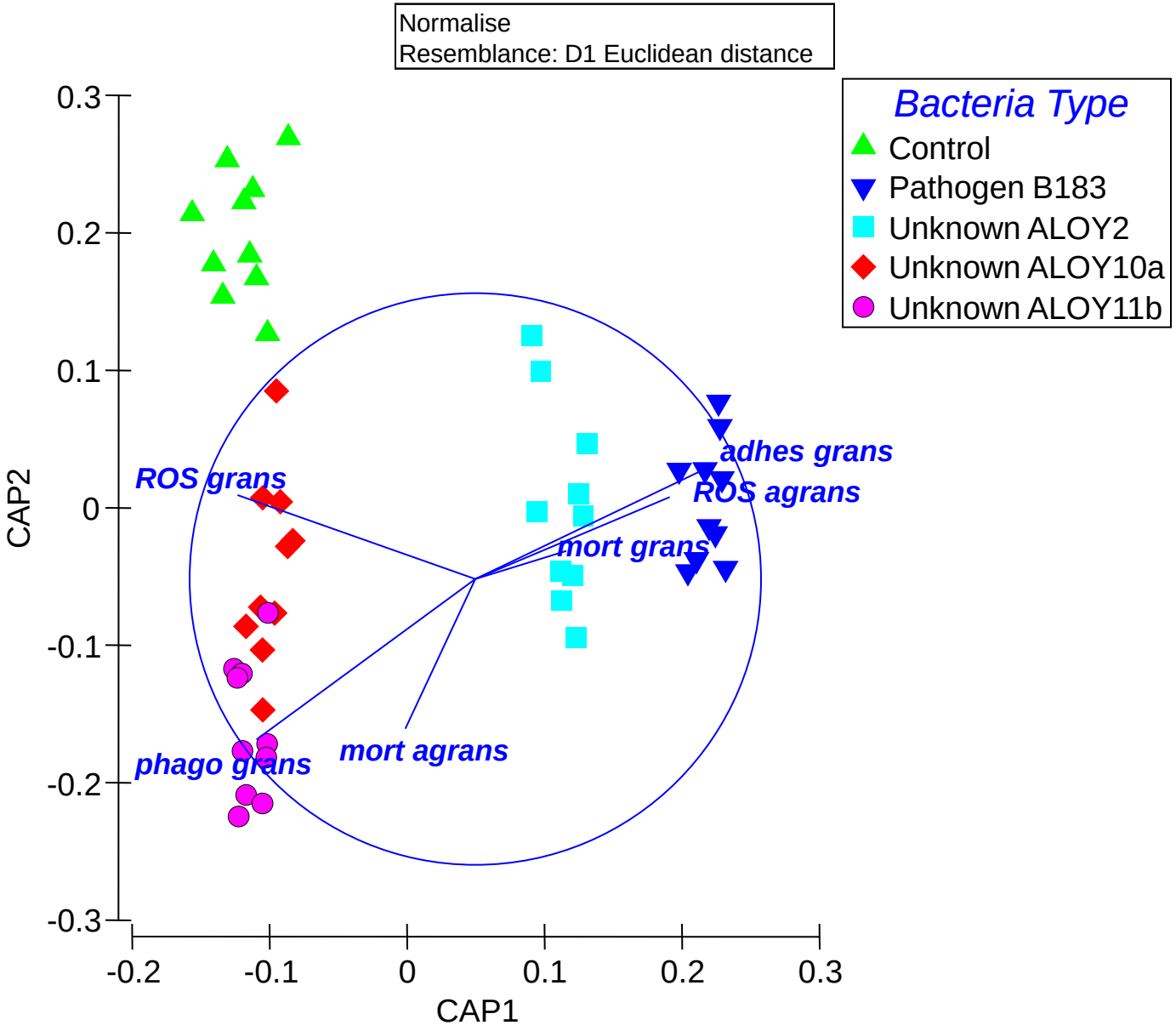


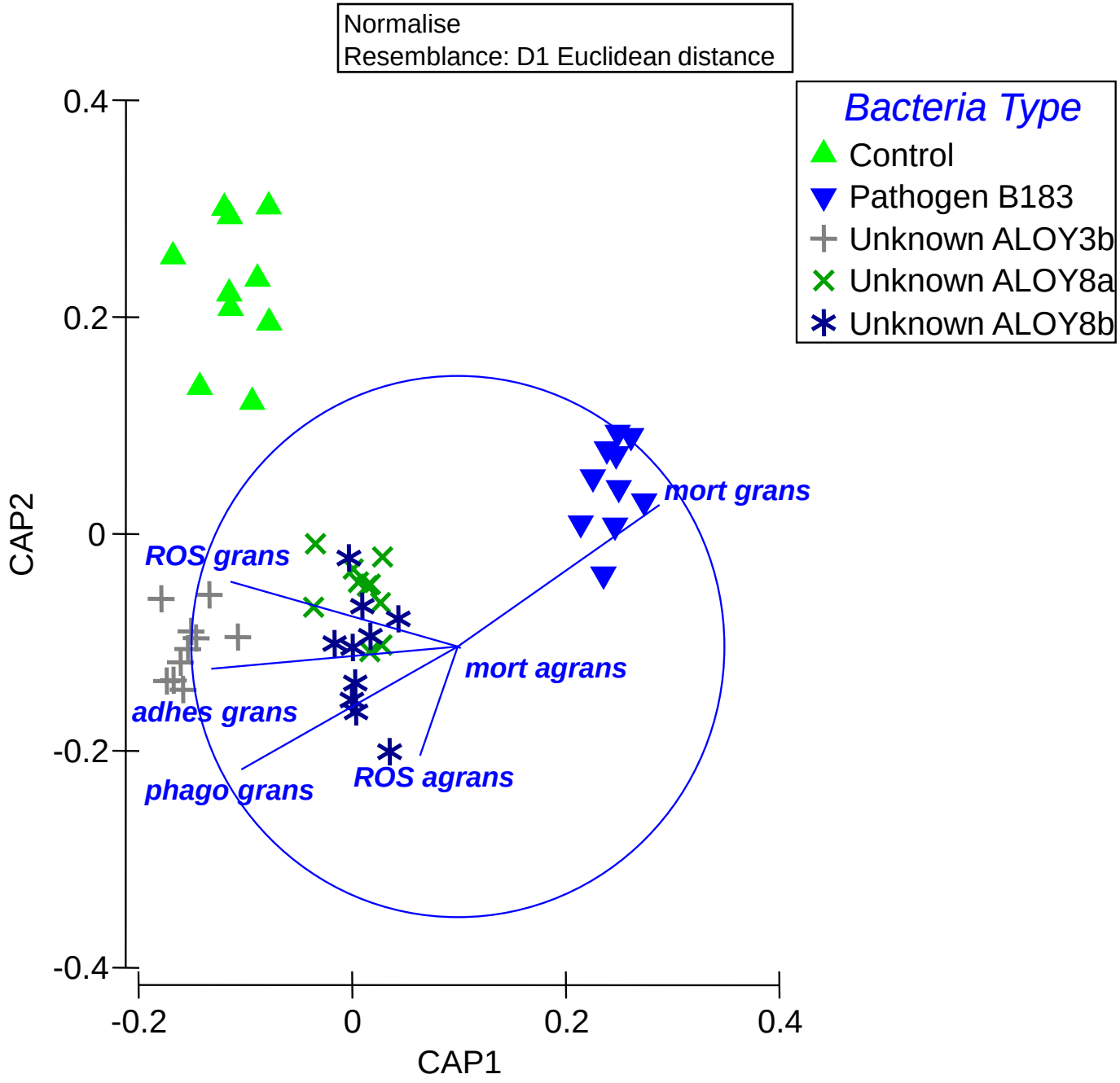
Pathogen vs non-pathogen
$p<0.0001$ by ANOSIM

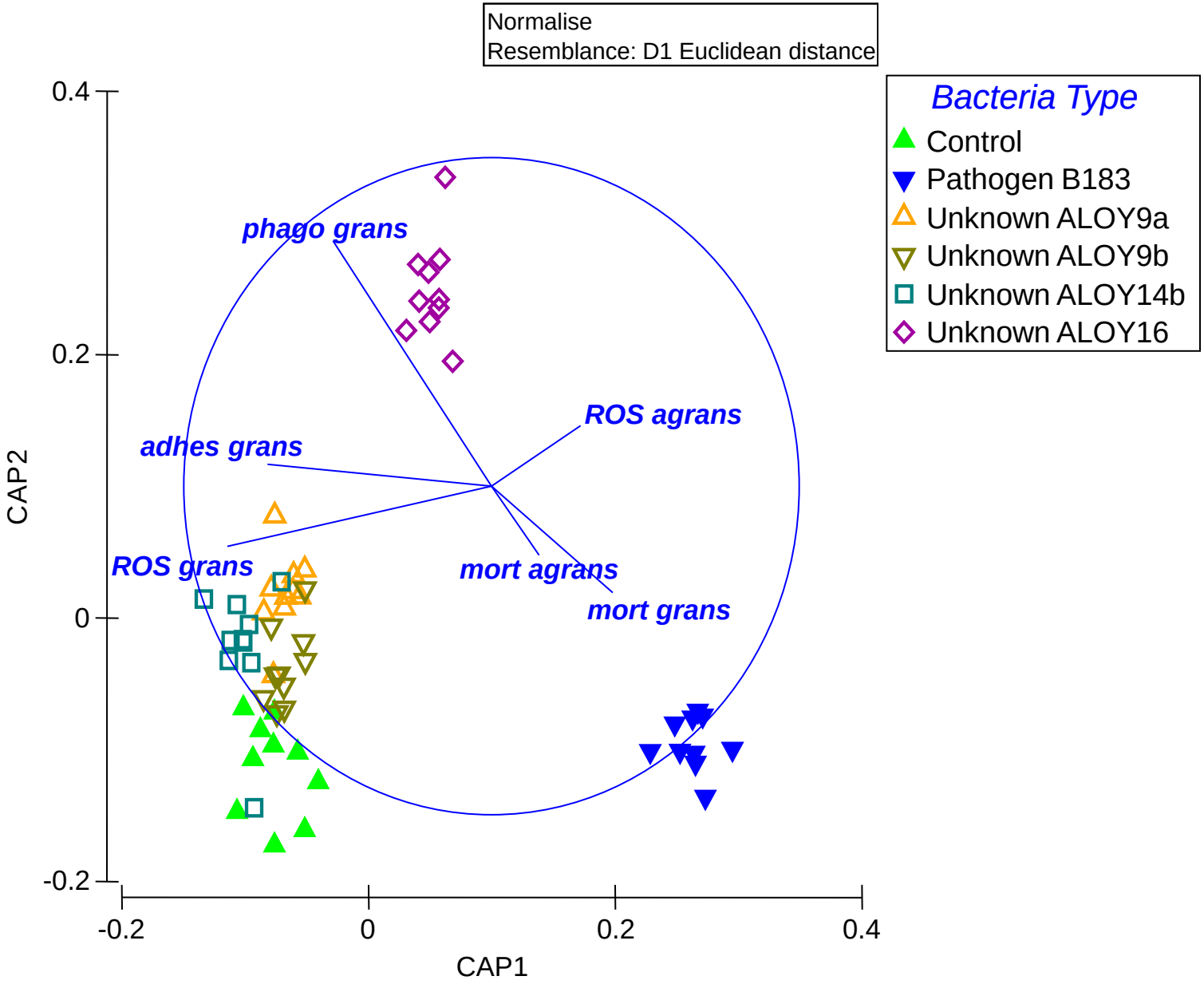
DA plot for Probiotic OY15, Probiotic candidate S1 and Pathogens B183 and rfp-B183



Discriminant Analysis plots for unknown isolates


Normalise
Resemblance: D1 Euclidean distance
Bacteria Type
Control
Pathogen B183
Unknown ALOY2
Unknown ALOY10a
Unknown ALOY11b
ROS grans
adhes grans
ROS agrans
mort grans
mort agrans
phago grans
CAP2
CAP1
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.2
-0.1
0
0.1
0.2
0.3


Normalise
Resemblance: D1 Euclidean distance
Bacteria Type
Control
Pathogen B183
Unknown ALOY3b
Unknown ALOY8a
Unknown ALOY8b
mort grans
ROS grans
mort agrans
adhes grans
phago grans
ROS agrans
0.4
0.2
0
-0.2
-0.4
CAP2
-0.2
0
0.2
0.4
CAP1


Normalise
Resemblance: D1 Euclidean distance
Bacteria Type
Control
Pathogen B183
Unknown ALOY9a
Unknown ALOY9b
Unknown ALOY14b
Unknown ALOY16
phago grans
ROS agrans
adhes grans
ROS grans
mort agrans
mort grans
0.4
0.2
0
-0.2
CAP2
-0.2
0
0.2
0.4
CAP1

Summary of Results

- Discriminant analysis can be utilized as a screening tool to separate unknown bacterial isolates into groups of the same type. Using hemocyte immune functions, DA can separate potential probiotic candidates with the same mechanism of immune regulation from pathogenic organisms.

- A streamlined process of "mapping" hemocyte immune functions to predict potential probiotic candidates.

- Overlays of hemocyte immune function variables over plots from the discriminant analysis aids in "visualizing" the data.

- Ten unknown bacteria isolated from the digestive glands of adult Eastern oysters were analyzed.

- No obvious potential probiotics were found in this batch. However, one unknown, ALOY2 appears to be a pathogen based on discriminant analysis.

- In addition, ALOY16 is a non-pathogen but is separated from the other unknown non-pathogens. Does this mean it could be a potential probiotic?

Stay tuned!

Future work…

Discriminant analysis incorporating probiotic OY15 and pathogen B183 as controls to screen unknown isolates as potential probiotic candidates for oyster larviculture.

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Thank you!

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And
NOAA's Aquaculture Program
for funding

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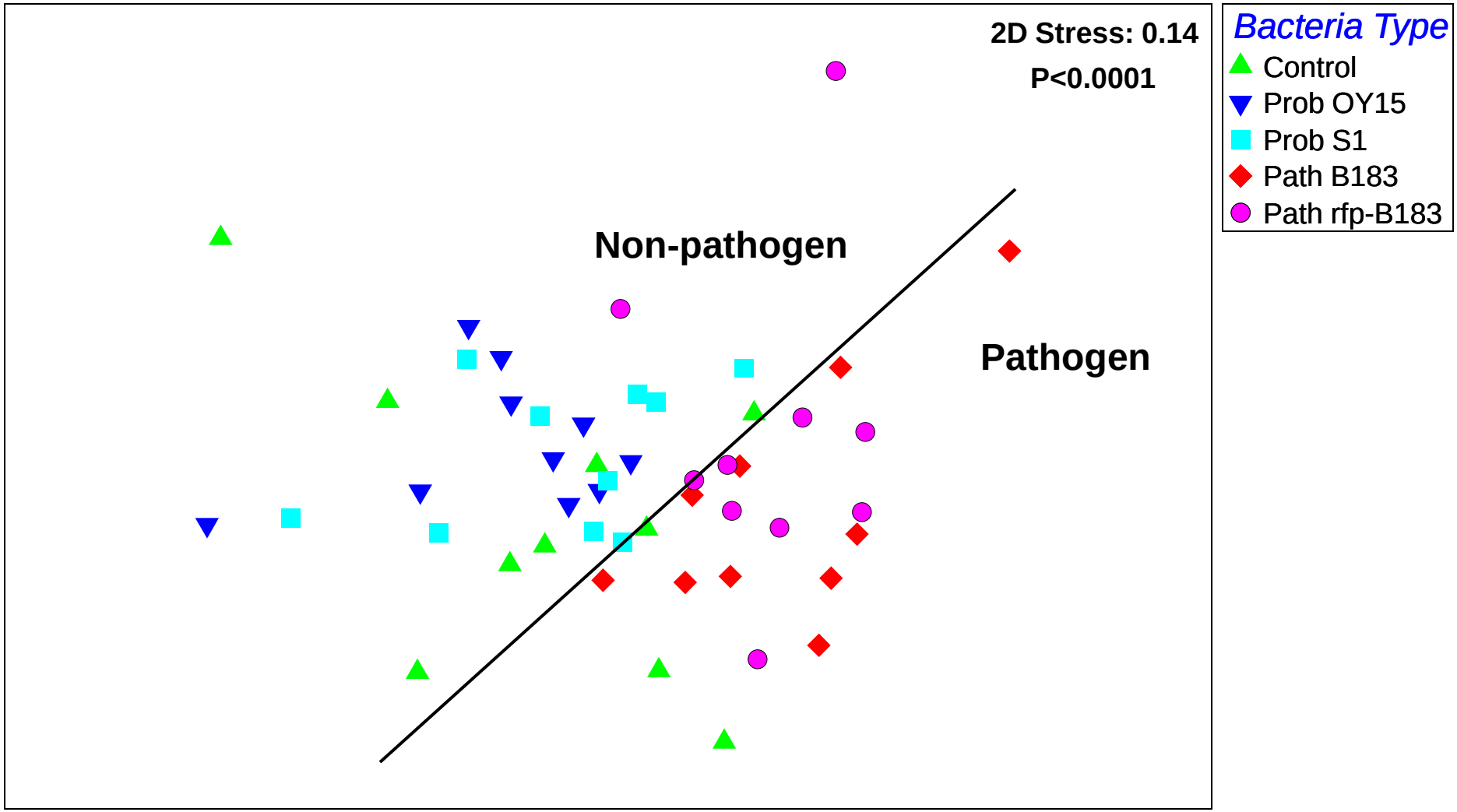
Multi-dimensional Scaling Plot

Normalise
Resemblance: D1 Euclidean distance