Identification of specific probiotics capable of enhancing resistance to bacterial coldwater disease (CWD) in rainbow trout

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What are probiotics?

- “Microbial cells…with the aim of improving health” (Gatesoupe, 1999)
- Production of inhibitory compounds, competitive exclusion, improving host immunity, enhancing water quality, etc.
- Probiotics for humans and terrestrial animals are well established
  - Relatively new to aquaculture
    - Growing research – “green” approach to disease management

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Probiotics Background

Current selection methods:

- Probiotic candidates which inhibit specific pathogens

- Characteristics of probiotics
  - Enter/survive the gastrointestinal tract
  - Safe to animal and common in environment
  - Inhibit specific pathogenic bacteria

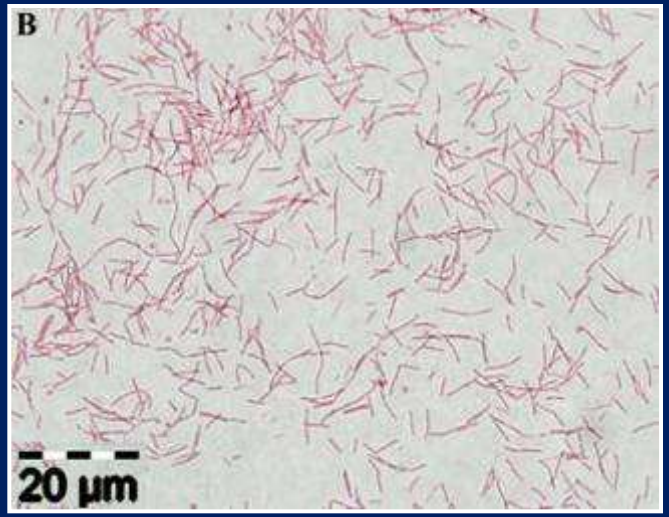
Coldwater Disease

- *Flavobacterium psychrophilum* is causative agent (Rainbow Trout Fry Syndrome in Europe)

- Significant economic losses affecting public and private salmonid aquaculture in the Pacific Northwest and worldwide

Management Options

- Licensed vaccine not yet available
  - Fish must be immunocompetent
- Antibiotics continue to be the main form of treatment
  - Risk of antibiotic resistance?
- Dramatic need for additional options
  - Probiotics as alternative?



Research Objectives

1. Identify candidate probiotics that fit standard definition with ability to inhibit *F. psychrophilum in vitro*

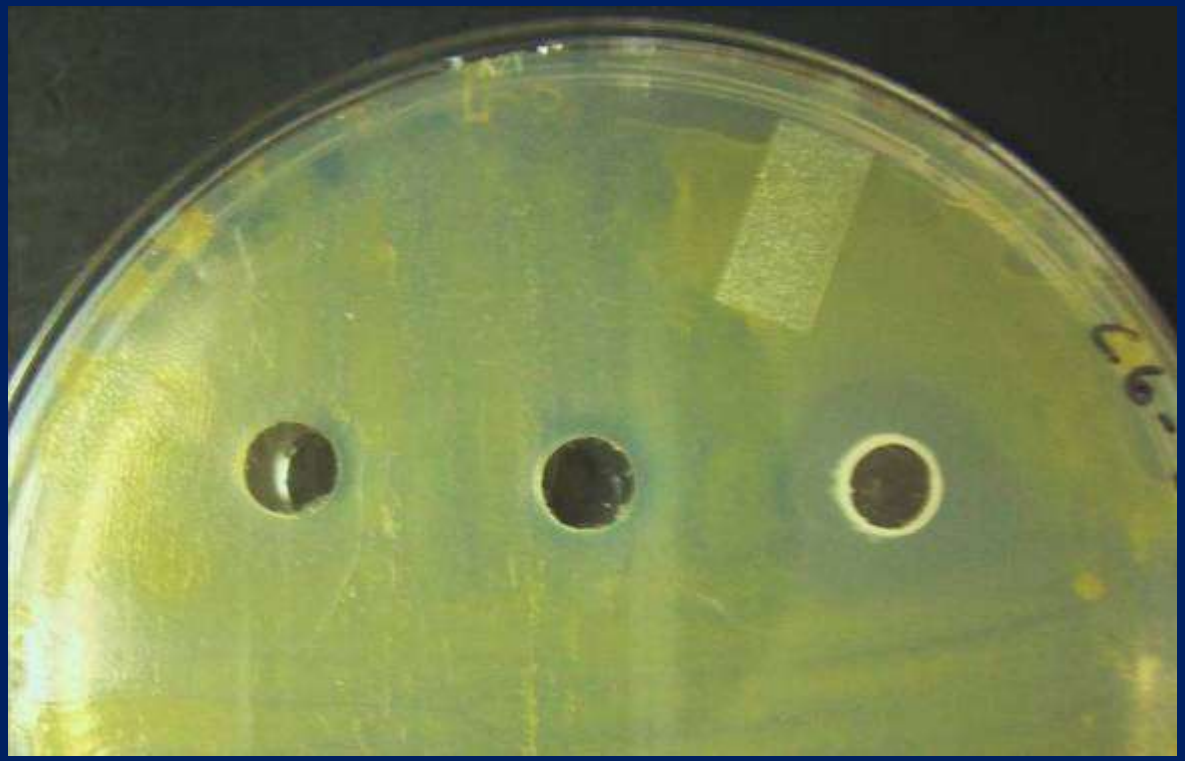
2. Test candidate probiotics through feed experiments and measure effects following challenge of fish with *F. psychrophilum*

Objective 1: Isolation of candidate probiotics

- Collected rainbow trout from Idaho
  - Commercial production hatcheries
  - Aquaculture Research Institute (UI)
  - Spring Valley Reservoir (Troy, Idaho)
- Removed gastrointestinal tract, expelled contents, homogenized and plated (TSA, TYES agar)
  - Subcultured until isolation was achieved
- Collected 318 bacterial isolates from 29 rainbow trout

Objective 1: *F. psychrophilum* inhibition *in vitro*

- Out of 318 isolates, 24 were identified which inhibited *F. psychrophilum* growth *in vitro*



Objective 1: Discussion

- Further selection = 16 candidate probiotics
  - Capable of inhibiting *F. psychrophilum in vitro*
  - Evidence of survival in the GI tract of rainbow trout *in vitro*
  - Non-pathogenic to rainbow trout

Objectives

1. Identify candidate probiotics that fit standard definition with ability to inhibit *F. psychrophilum in vitro*

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Objective 2: Feed preparation

- Direct feeding of probiotics is the most practical and common method of introduction

- Probiotic feed preparation
  - Candidate probiotic grown to log phase
  - Centrifuge and re-suspend in menhaden oil to ~10^8 CFU ml^{-1}
  - Top-coated onto commercial trout feed

Objective 2: *In Vivo* Evaluation

- Evaluated the 10 most promising candidate probiotics through four separate trials

	Candidate probiotics
1	*Aeromonas caviae* [3]
2	*Aeromonas caviae* [4]
3	*Aeromonas caviae* [5]
4	*Aeromonas sobria* [3]
5	*Aeromonas sobria* [4]
6	*Aeromonas sobria* [5]
7	*Aeromonas sobria* [6]
8	*Aeromonas sobria* [7]

	Candidate probiotics
9	*Lysinibacillus fusiformis*
10	*Citrobacter freundii*
11	*Enterobacter* sp.[1] (C6-6)
12	*Enterobacter* sp.[2] (C6-8)
13	*Hafnia alvei*
14	*Plesiomonas shigelloides* [1]
15	*Plesiomonas shigelloides* [2]
16	*Staphylococcus equorum*

Discussion

- Two initial trials conducted: (tested 3 probiotics)
  - *Enterobacter* sp.[1] (C6-6) – appeared promising
  - Adjusted feeding regime for trials 3 and 4

- To observe maximum effect should feed probiotic prior to and during infection
  - Allow more time for colonization and establishment in GI tract

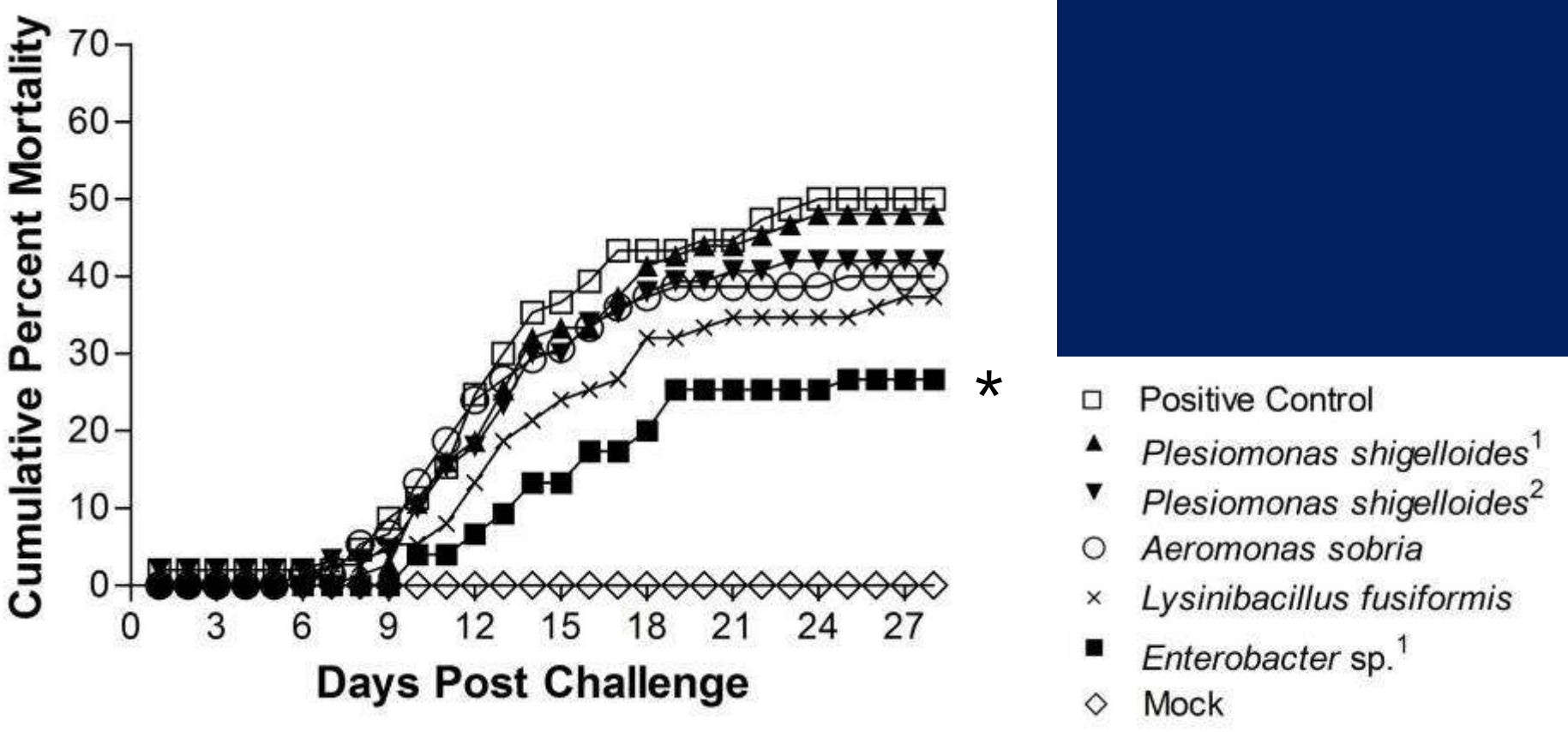
Objective 2: Trial 3 and 4

- Evaluated 7 remaining probiotic treatments in two challenges - also included *Enterobacter* sp.[1] (C6-6)
- Triplicate groups of 25 fish per probiotic treatment
- Fed candidate probiotic for 10 days prior to injection challenge with *F. psychrophilum*
- Continued to feed probiotics throughout 28 day challenge period

Objective 2: Trial 3 and 4

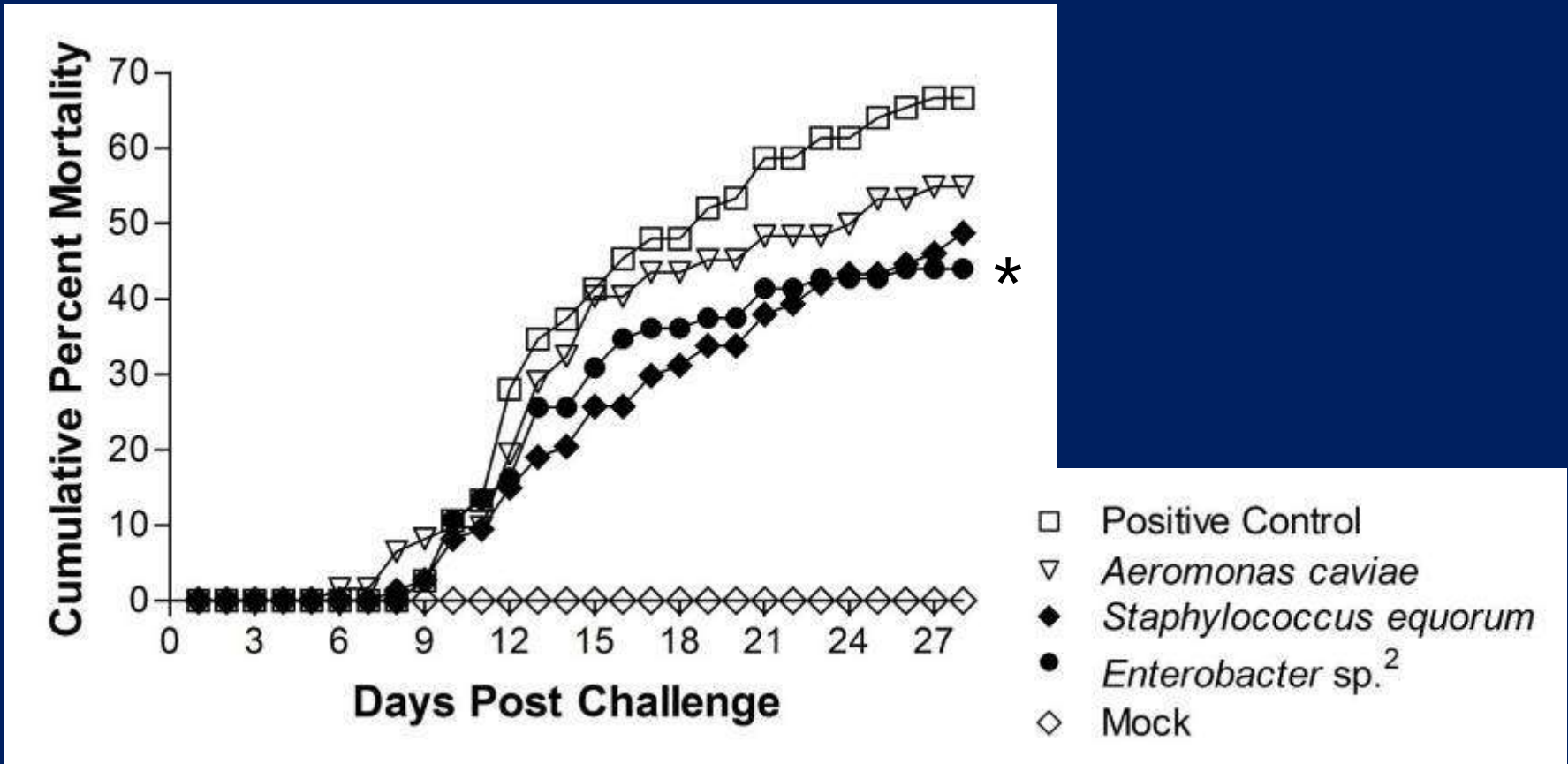
- Positive control comprised of triplicate groups of 25 fish
  - *F. psychrophilum* injection and feed with oil only
- Mock infected group comprised of triplicate groups of 25 fish - PBS injection and fed standard trout diet
- Monitor mortalities for 28 days
- Attempted to reisolate *F. psychrophilum* and candidate probiotics from at least 20% of daily mortalities

Objective 2: Trial 3



* significant difference ($p<0.05$) from positive control

Objective 2: Trial 4



* significant difference ($p < 0.05$) from positive control

Objective 2: Discussion

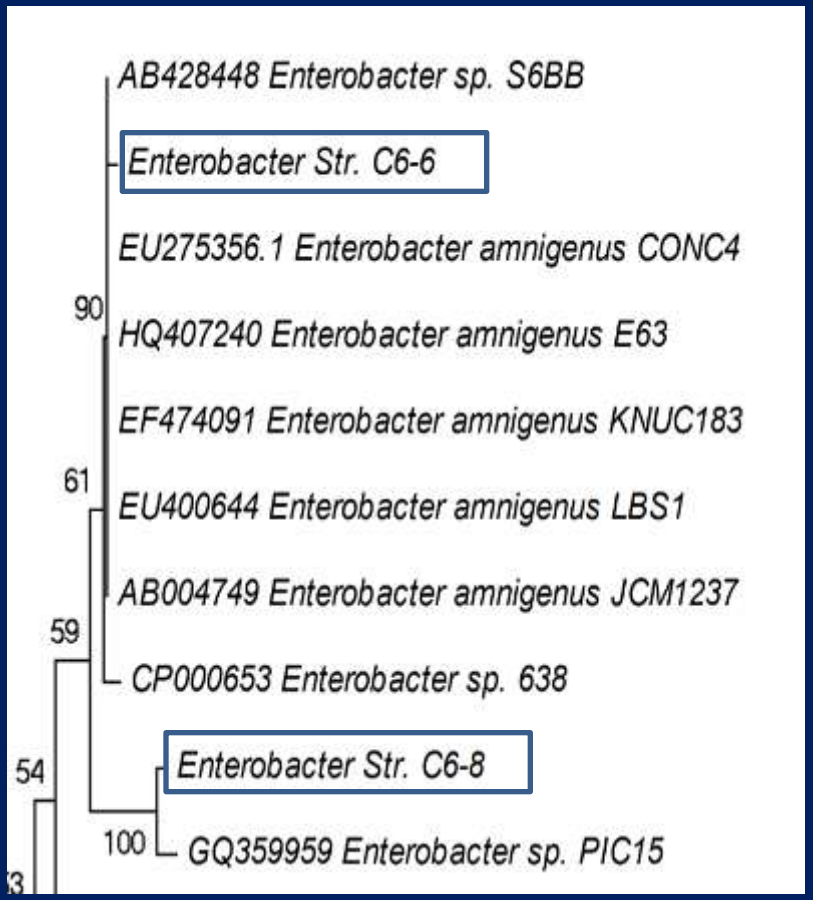
- Both *Enterobacter* sp. (C6-6 and C6-8) are promising probiotics
  - Significant reductions in mortality
  - Consistent reisolation from GI tract compared to other candidates

- All other treatments eliminated from further testing
  - Did not show “significant” decrease in mortality

Enterobacter sp.

- C6-6 and C6-8 believed to be different species based on lab culture characteristics
- 16S sequencing confirmed that these are separate *Enterobacter* species
  - Most closely related with *Enterobacter amnigenus* and *Enterobacter* sp. PIC15

Enterobacter sp.

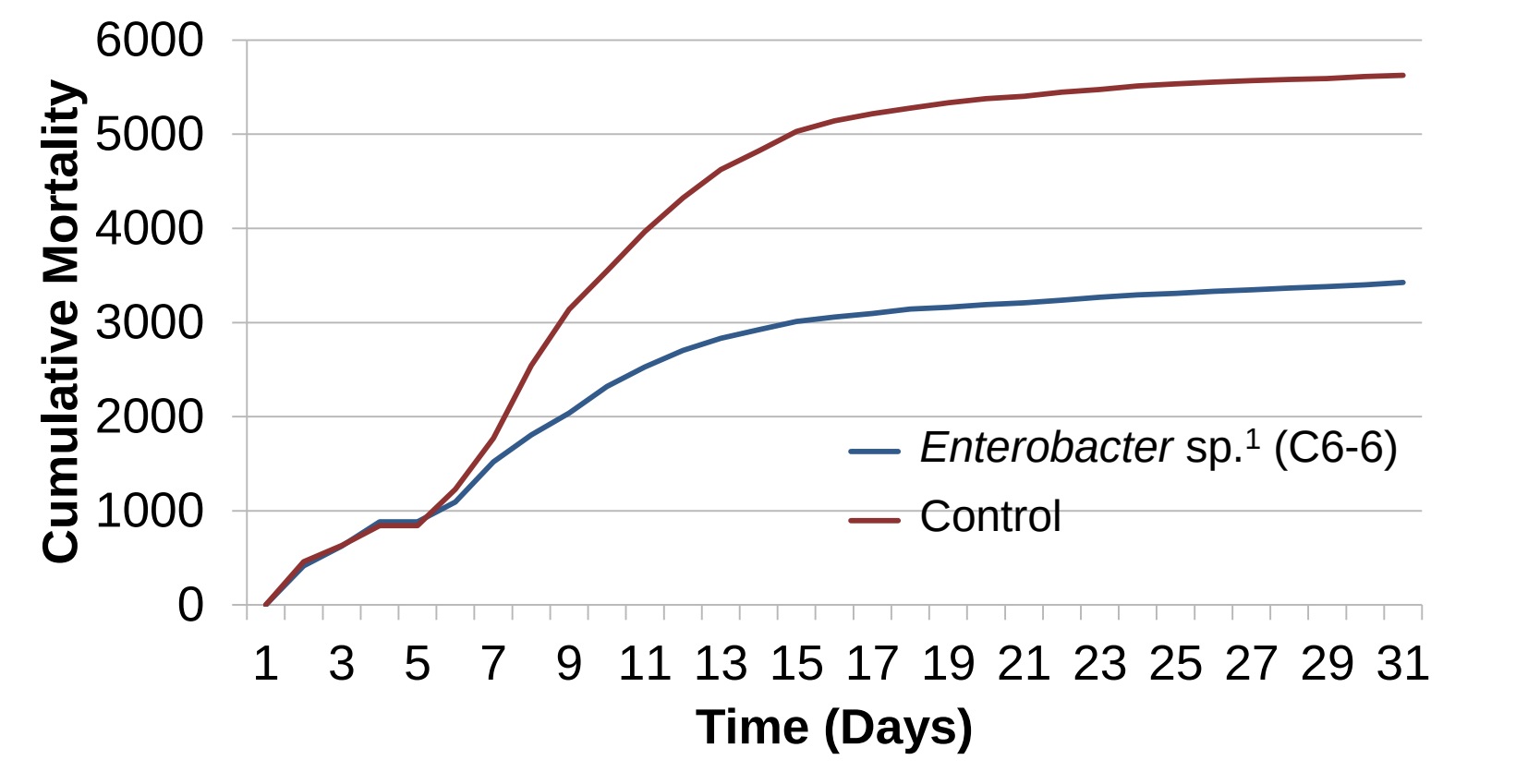


- *Enterobacter amnigenus* is an ubiquitous bacteria commonly found in the soil and water
- *Enterobacter* sp. PIC15 originally isolated from yellow catfish (*Pelteobagrus fulvidraco*)

Field Trials

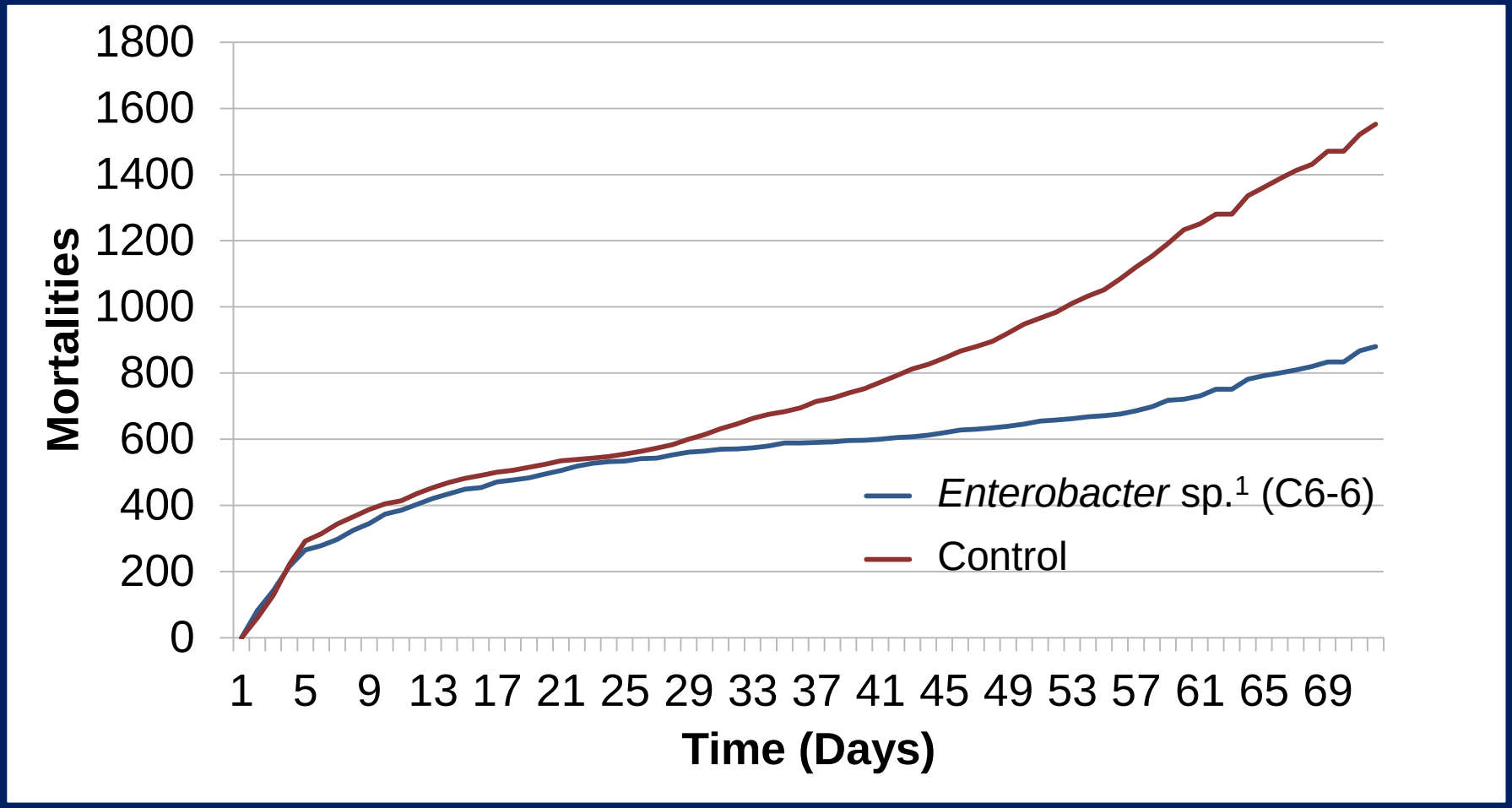
- *Enterobacter* sp.[1] (C6-6) provided to Utah Division of Wildlife Resources for field evaluations
  - Used at facilities experiencing *F. psychrophilum* outbreaks
  - Evaluated *Enterobacter* sp.[1] (C6-6) through several field trials

Field Trial 1



- ~ 38% decrease in total mortality from control group
- *F. psychrophilum* confirmed in both groups of fish

Field Trial 2



- ~ 43% decrease in total mortality from control group
- *F. psychrophilum* confirmed in both groups of fish

Discussion

- *Enterobacter* sp.[1] (C6-6) effective on a larger scale
  - Offers a relatively simple means of improving fish health (early life stages)

Summary (*Enterobacter* sp.)

- Two separate *Enterobacter* species acting similarly
  - Colonize the GI tract
  - Inhibit *F. psychrophilum* growth *in vitro*
  - Significantly reduce mortality due to *F. psychrophilum*
  - Non-pathogenic to rainbow trout and common in environment

- Other observations
  - Short doubling time ~ 30 min
  - Wide range of growth temperatures, 4 – 40 °C
  - Viability under storage conditions, -20, 4, 15 °C

Conclusions

Both *Enterobacter* species appear to represent a viable tool that can improve survival of fish infected with *F. psychrophilum*

- High potential (and need) for commercialization and application of these probiotics

It is possible that a combined approach using Probiotics at first feeding followed by Vaccination when fish are immunocompetent may dramatically reduce CWD problems

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