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Production and Degradation of 4-Ethylphenol in *LACTOBACILLUS SP.* pep8 Cultures and in Blended Swine Lagoon Enrichments

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PRODUCTION AND DEGRADATION OF 4-ETHYLPHENOL IN *LACTOBACILLUS SP*. pep8 CULTURES AND IN BLENDED SWINE LAGOON ENRICHMENTS

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Jerry Daday, Director of Thesis

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Dean, Graduate Studies and Research 8/20/12 Date

I dedicate this thesis to my parents, David and Annmary Hallen, who always inspire me to do everything in life to the best of my abilities. I also dedicate this work to my wife Sara, who helped edit and proofread this manuscript, as well as supported me throughout my time at WKU.

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Directed by: Kinchel Doerner, Rodney King, and Claire Rinehart

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4-Ethylphenol (4-EP) is a malodorant of swine waste and is derived from a component of lignin called p-coumaric acid (p-CA). The production of 4-EP from lignin in swine waste is untested. Additionally, the effect of Fe (III) on 4-EP levels is unknown. Four experiments were performed to determine if *Lactobacillus* sp. pep8 cultures, as well as enriched swine lagoon slurries, could liberate p-CA from lignin and convert p-CA to 4-EP. Furthermore, it was tested if the addition of Fe (III) influences the conversion of p-CA to 4-EP.

Experiment 1 tested *Lactobacillus* sp. pep8 cultures to determine if the addition of 10 mM Fe (III) and 0.2% sulphite lignin to *Lactobacillus* sp. pep8 cultures would stimulate production of 4-EP.

Experiment 2 tested the effect of 0.2% sulphite lignin and 10 mM Fe (III) on 4-EP production in the presence of enriched swine lagoon slurries. On day 0 there was no detectable 4-EP, for either 0.2% sulphite lignin addition or the 10 mmol l^{-1} Fe (III) additions.

Experiment 3 tested alternative forms of lignin, including 0.2% sulphite, indulin, or sigma lignin as potential source compounds for 4-EP production in enriched swine lagoon slurries. 4-EP produced in all three conditions are likely endogenous to the lagoon slurry additions.

Experiment 4 was designed to measure the degradation of exogenous 4-EP with varying final concentrations of 4-EP in enriched swine lagoon slurries. Data in Figure 7 indicate immediate degradation of 4-EP by day 5, however, by day 7 synthesis of 4-EP occurred until day 14 where 4-EP levels remained in a steady state.

Our results suggest that when both *Lactobacillus* sp. pep8 cultures and enriched swine lagoons are supplemented with p-CA, 4-EP is produced indicating that p-CA serves as a source of 4-EP. However, when supplemented with Fe (III) and/or sulphite, indulin, or sigma lignin, 4-EP production was not stimulated. This data indicates that, 4-EP production is not enhanced by the presence of Fe (III) in either *Lactobacillus* sp. pep8 cultures or in enriched swine lagoon slurries. Furthermore, lignin did not serve as a source of 4-EP.

INTRODUCTION

Animal feeding operations throughout the United States consistently produce an abundance of waste that is treated using waste lagoons. These lagoons produce volatile malodorous compounds that are becoming increasingly bothersome to the surrounding communities (Schiffman, 1998). These compounds have the potential to affect the health of the residents of the surrounding communities through toxicological effects, sensory irritation, neurochemical changes, and stored sensory perception (Schiffman, 1998). Also, as swine production facilities expand, there is an expressed concern over how to minimize fecal contamination of ground water, surface water, and the atmosphere (Merrill and Halverson, 2002). To process such large quantities of manure, animal feeding operations are building more manure lagoons closer to the farm buildings or even underneath the same buildings (Harkin, 1997). For the concerns outlined above, there is great interest in developing manure treatment systems that will reduce odor emissions (Williams, 1984).

Waste lagoons are primarily comprised of microorganisms found in the gastrointestinal tract of swine and cattle (Butine and Leedle, 1989). However, due to the open nature of these lagoons, a secondary inoculum of indigenous microorganisms originating from the clay liners and airborne particulates is possible. Animal age, health, bedding, and diet also affect the microbial population of the lagoons (Moore *et al*. 1987). In addition, environmental factors also impact the microbial diversity of the lagoons. For example, the microbial ecology of a lagoon can vary substantially with temperature, and with wet or dry seasons (Loughrin *et al*. 2006).

There are numerous malodourous compounds that are universally found in animal

waste lagoons such as 4-methlyphenol (4-MP), 3-methylindole (3-MI), and 4-ethylphenol (4-EP) (Wright *et al*. 2005). These compounds are produced through the anaerobic degradation of waste products. For 4-MP and 3-MI, the source compounds are L-tyrosine and L-tryptophan, respectively (Kridelbaugh and Doerner, 2009; Whitehead *et al*. 2008). However, in the case of 4-EP, the source compound in waste lagoons is currently unknown. 4-EP is naturally found in fermented foods and beverages, such as wine (Rodriguez *et al*. 2008a). In wine, 4-hydroxycinnamic acid (p-coumaric acid; p-CA) is released from lignin during processing and serves as the precursor to 4-EP (Rodriguez *et al*. 2008b). In fermented foods and beverages, *Lactobacillus sp*. typically carries out the conversion of p-CA to 4-EP (Rodriguez *et al*. 2008b). Similarly in waste lagoons, p-CA may be derived from the undigested plant lignin found in animal waste and in plant based bedding which is ultimately converted to 4-EP.

Doerner *et al* (2009) demonstrated that Fe (III) substantially increases the malodorants 4-MP and 3-MI in swine lagoon enrichments. The mechanism of this stimulation is unclear. Similar experiments using a culture of *Clostridium scatologenes* ATCC 25778 also showed increased 4-MP and 3-MI production in response to Fe (III). However, the effect of Fe (III) on 4-EP production has not been investigated.

Using reverse-phase high pressure liquid chromatography (RP-HPLC), *Lactobacillus* sp. pep8 (*Lacto*. sp. pep8) cultures, as well as enriched swine lagoon slurries, were tested for their ability to liberate p-CA from lignin and convert p-CA to 4-EP. Furthermore, any influence of the addition of Fe (III) on the conversion of p-CA to 4-EP was tested.

MATERIALS AND METHODS

Tryptone-Yeast Medium and Semi-defined medium

Tryptone-yeast medium (TYM): 5 g tryptone, 15 g yeast extract, 6 ml resazurin (200mg/100ml nanopure H_20), 1.5 ml hemin solution (25mg/100 ml nanopure H_20), 7.5 µl l^{-1} vitamin K1 (Product #: 103283, MP Biomedicals Co., Solon, OH, USA), 75 ml l^{-1} of each minerals #1 (6 g K_2HPO_4 in 1000 ml nanopure H_20) and # 2 (12 g NaCl, 12 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1.2 g $CaCl_2$, 2.5 g $MgSO_4$, in 1000 ml of nanopure H_20) (Bryant and Burkey, 1953), 341.5 ml diH_2O, (Final volume = 500 ml; Kridelbaugh and Doerner, 2009). The medium was boiled under N_2 gas until the solution was clear. While being flushed with N_2 gas the media was anaerobically dispensed to Balch bottles, capped, and autoclaved.

Semi-defined media (SDM): 0.5 g glucose, 25 ml of each minerals #1 and #2 (Bryant and Burkey, 1953), 0.5 ml Pfenning trace mineral solution (0.1 g $ZnSO_4$, 0.03 g $MnCl_2$, 0.3 g H_3BO_3, 0.2 g $CoCl_2$, 0.01 g $CuCl_2$, 0.2 g $NiCl_2$, 0.03 g Na_2MoO_4, 1.5 g $FeCl_2$, 0.01 g Na_2SeO_3 in 1000 ml nanopure H_20) (Atlas, 2004; Genther et al. 1981) 1 g yeast extract, 2.5 g ammonium sulfate, 0.5 ml hemin solution (25mg/100ml nanopure H_20), 2 ml resazurin (200mg/100ml nanopure H_20), 447 ml diH_20 (Final volume = 500 ml; Kridelbaugh and Doerner, 2009). The medium was brought to a boil while being continuously flushed with nitrogen gas. After 15 minutes, the color of the medium changed from reddish-pink to clear. Once this change occurred, the flask was removed and cooled on ice. Once cooled to room temperature, 1.35 g sodium bicarbonate, 2.5 µl vitamin K1 (MP Biomedicals, Product #: 103283), 0.5 g cysteine-HCl, were added to the medium. The pH was adjusted to 7.1 using fresh 5 M sodium hydroxide. The medium

was then transferred to an anaerobic chamber (Coy Laboratory Products, Inc.; Grass Lake, MI, USA), which was maintained at a 95% CO_2 and 5% N_2 environment. The medium remained in the anaerobic chamber for 4 hours to equilibrate. It was then aliquoted into tubes (10 ml medium/tube) capped, and subsequently autoclaved.

p-Coumaric Acid Stock Solution

A stock solution of the phenolic compound p-CA was prepared by dissolving 0.164 g in 10 ml of 100% ethanol (final concentration of 100 mmol l^{-1}). The solution was filtered with a 0.2µm membrane filter (Fisher Scientific; Pittsburgh, PA, USA) to ensure there were no particulates in the solution.

4-Ethylphenol (4-EP) Stock Solution

A stock solution of 4-EP was prepared by dissolving 0.244 g into 10 mls of 100% ethanol to yield a 2 mM final concentration. Once dissolved, the solution was filtered through a 0.2µm membrane filter (Fisher Scientific; Pittsburgh, PA, USA) into an autoclave sterilized 10 ml glass tube.

Lignin Sources

Insoluble forms of lignin (indulin and sulphite) were provided by Dr. George Fahey, U. of Illinois-Urbana. A soluble form of lignin (Sigma lignin) was purchased from Sigma-Aldrich **(**Product # 471003**).**

Fe (III) stock solution

Fe (III) (100 mM) was prepared by dissolving 8.11g of $FeCl_3$ into 500 ml of diH_20. The pH was adjusted to 7.0 by adding fresh 5 M sodium hydroxide, and the solution was placed in an anaerobic chamber overnight to ensure solution was completely

anaerobic. The solution was then dispersed into 100 ml Balch bottles inside the anaerobic chamber, capped, and subsequently autoclaved.

***Lacto. sp.* pep8 Culture Conditions**

A 10 ml frozen stock of *Lacto.* sp. pep8 was thawed on ice followed by inoculating 0.1 ml of *Lacto.* sp. pep8 into 9.9 mls of anaerobic semi-defined medium (SDM) (Kridelbaugh *et al.* 2009). The initial inoculated culture was incubated at 37^{o}C for 24 hours. The initial inoculated culture was incubated at a higher temperature (37^{o}C) to ensure the *Lacto.* sp. pep8 culture was provided with optimal growth conditions. Every 24 hours, 0.1 ml of the *Lacto*. sp. pep8 culture was transferred anaerobically to 9.9 ml fresh SDM. By the third transfer there was discernible visual bacterial growth (based on visual turbidity). Once turbidity was seen 0.1 ml *Lacto.* sp. pep8 culture plus the appropriate supplements for each experimental condition were added to varying amounts of SDM. Each condition was individually replicated 3 times to ensure reproducibility. Additionally, 3 individual replicates of each condition were preformed to average the data of each condition at each time point. After the supplements were added, day 0 samples (1.5 ml) were taken and stored at -20^{o}C in 1.5 ml microfuge tubes. All experimental conditions were incubated at ambient temperature. In addition to day 0 samples, additional samples were then taken from each replicate at days 1, 2, 5, and 7. All samples were stored in microfuge tubes at -20^{o}C until HPLC analysis. Additionally, absorbance measurements were taken for conditions containing soluble materials at day 0, 1, 2, 5, and 7 (Figure 2) at 600 nm using a mini-1240 spectrophotometer (Shimadzu Corp., Kyoto, Japan) to ensure bacterial growth was not affected by the addition of ethanol, p-CA, or SDM.

Sample collection

Swine lagoon sediment was collected from the northeast corner of the primary lagoon at the WKU swine farm in two-1-liter containers, and immediately transported, on ice, to the laboratory. The sediment was blended, at maximum speed, for fifteen minutes using a Waring Blender (Waring Products; Torrington, CT, USA). Immediately after blending, 10 ml of slurry was added to anaerobic TYM containing specific treatments (as described below).

Enriched swine lagoons slurries

Ten ml of swine lagoon slurries were enriched with various amounts of anaerobic TYM augmented with the following treatments: 0.2% (wt/vol) sulphite lignin, 10 mls of Fe (III) (10 mmol l^{-1} final concentration), 0.2% (wt/vol) sulphite lignin and 10 mls of Fe (III) (10 mmol l^{-1} final concentration), 1 ml of p-CA (1 mmol l^{-1} final concentration), 10 mls of Fe (III) (10 mmol l^{-1} final concentration) and 1 ml of p-CA (1 mmol l^{-1} final concentration). Additionally, a control was run which contained 10 ml swine lagoon slurry and 90 ml anaerobic TYM. Samples were collected on days 0, 3, 7, 14, and 21 and stored in 1.5 ml microfuge tubes at -20°C until RP-HPLC analysis.

Enriched swine lagoon slurries augmented with varying lignin types

Control conditions contained 100 mls of anaerobic TYM augmented with 0.2% (wt/vol) sulphite lignin, indulin lignin, or sigma lignin. Experimental treatments contained 10 mls of swine lagoon slurry enriched with 90 ml of anaerobic TYM, and augmented with 0.2% (wt/vol) of varying lignin sources. The three lignin forms tested were 0.2% (wt/vol) sulphite lignin, indulin lignin, or sigma lignin. Sulphite and indulin are insoluble types of lignin (provided by Dr. George Fahey, U-of-I, Urbana), while

sigma lignin (Sigma-Aldrich, product #; 471003) is a synthetic water-soluble lignin. Samples were collected on days 0, 7, and 14 and stored in a 1.5 ml microfuge tube at -20^{o}C until RP-HPLC analysis.

Addition of exogenous 4-ethylphenol to enriched swine lagoon slurries

Ten mls of swine lagoon slurry were added to balch bottles containing varying amounts of anaerobic TYM followed by the addition of 1 ml of 2 mM 4-EP (2mM final concentration), 0.5 ml of 2 mM 4-EP (1 mM final concentration), or 0.25 ml of 2 mM 4-EP (0.5 mM final concentration) for a final volume of 100 ml. Three individual replicate samples were collected on days 0, 3, 5, 7, 10, 14, 18, and 21 and stored at -20^{o}C until analyzed by RP-HPLC**.** A vehicle control condition containing 89 ml TYM, 10 ml slurry, and 1 ml ethanol was tested to ensure ethanol did not inhibit bacterial growth or 4-EP production.

Extraction and RP-HPLC analysis

Hypersep C18 solid phase extraction columns (SPE) (Thermo scientific; Bellefonte, PA, USA) were used to isolate the desired analytes from samples. Initially each SPE column was equilibrated with two rinses of 1 ml HPLC grade methanol followed by two rinses of HPLC grade H_20. While equilibrating the SPE columns, samples were centrifuged to pellet any solid materials at 13k rpm for 10 minutes at 4^{o}C. The supernatant (500 µl) was added to the SPE column, followed by two rinses with 1 ml HPLC grade H_20. SPE columns were then placed in a 16x100mm borosilicate glass tubes with adapters which allowed for each SPE column to be suspended in a borosilicate glass tube. One ml of HPLC grade acetonitrile (Fisher Scientific; Pittsburgh, PA USA) was added to each suspended column and spun for 2 minutes at 1,000 rpm to elute the

analytes. Non-turbid samples were then transferred from each tube into 1 ml amber glass storage tubes (Fischer Scientific; Pittsburg, PA, USA). Turbid samples were filtered through a 0.45µm PTFE syringe filter (Fisher Scientific; Pittsburg, PA, USA) prior to being added into a 1 ml amber glass storage tubes for RP-HPLC analysis. The samples were labeled and stored at -20ºC for up to a week or analyzed immediately using RP-HPLC. Samples were standardized against pure 4-EP purchased from Sigma-Aldrich (Product # W31560). Standardized 4-EP (10^{-4} mmol l^{-1} final concentration) was used to create a standard curve for each HPLC run. Each standard curve was created by using 0.8, 1.6, 3.2, and 4.0 ng injection volumes of the standardized 4-EP, (10^{-4} mmol l^{-1} final concentration), creating a linear standard curve. Under these conditions, the lower detection limit for 4-EP was 0.5 ng.

Statistical Analysis

The mean of three replicates was taken for all the experimental conditions. Mean data points were plotted for each condition and the standard deviation and standard error (S.E.) were determined for each data point. ($S.E.= s/\sqrt{n}$). An ANOVA was performed along with a Tukey's post-hoc test using the Mathematica 8 program (Wolfram research, Champaign, IL). The statistical significance used was $P \leq 0.05$.

RESULTS

Production of p-CA and conversion to 4-EP by *Lacto.* sp. pep8 cultures:

Lacto. sp. pep8 cultures were tested for the ability to liberate p-CA from lignin and to convert p-CA to 4-EP. Additionally, the affect of Fe (III) on 4-EP production was investigated. All conditions contained SDM, *Lacto.* sp. pep8 culture, and the indicated treatment. In conditions containing SDM and culture (the control), and SDM, *Lacto.* sp. pep8 culture, and Fe (III), only background levels (4-EP levels less than 50 μmol l^{-1}) of 4-EP were produced (Figure 1). Treatments augmented with Fe (III) and sulphite lignin or sulphite lignin alone did not produce detectable levels of 4-EP (data not shown). The highest level of 4-EP 2.47 μmol $l^{-1} \pm 2.47$ was detected on day 2 (Figure 1) whereas 4-EP production decreased to its lowest level on day 5 at 0.751 μmol l^{-1} (Figure 1). However, these data are not significantly different. Using ANOVA it was determined there was no significant difference between time points or treatments at a confidence level of 0.05. The levels of 4-EP on days 0, 1, 2, 5, and 7 stayed relatively constant, only varying slightly and never exceeding 3 μmol l^{-1}. 4-EP concentrations found in Figure 1 are considered background levels since they are below 50 μmol l^{-1}.

To determine if bacterial growth was inhibited in cultures augmented with ethanol, SDM, or p-CA the optical density (OD) was measured at days 0, 1, 2, 5, and 7 (Figure 2). Figure 2 depicts the three control conditions (culture control, ethanol vehicle control, or p-CA control) measured at 600nm. By day 5 the OD of all conditions reached their maximum OD between 0.45 -0.59 at 600 nm. Overall, the OD of the control conditions showed consistent bacterial growth indicating that control conditions did not inhibit bacterial growth. ANOVA analysis showed that there was no significant

difference between treatments at a confidence level of 0.05. However, day 0 was significantly different than all other days. Additionally, days 0, 1, 2, 5, and 7 were significantly different from one another at a confidence interval of 0.05. This indicates that bacterial growth was not affected by the addition of SDM, p-CA, or ethanol.

Figure 3 illustrates that when p-CA was added to the *Lacto.* sp. pep8 cultures, there was an increase in 4-EP levels. At time 0, 4-EP was not detected. With the addition of p-CA alone, the highest level of 4-EP produced was 176. 081 μmol l^{-1} ± 24.55 on day 1 (Figure 3), followed by a decrease on days 2 and 5 reaching the lowest concentration on day 7. Under the control condition augmented with p-CA and Fe (III), the concentration of 4-EP reached its maximum on day 1 of 240.96 μmol l^{-1} ± 29.02. However, with this condition there was a more rapid decrease in 4-EP concentration to almost background levels by day 7. Using ANOVA, it was determined that there were no significant differences between treatments. However, days 0 and 7 are significantly different than days 1, 2, and 5 but are not significantly different from one another at a confidence level of 0.05. This indicates that the addition of Fe (III) to conditions containing p-CA had little to no affect on the production of 4-EP. In addition, no detectable 4-EP levels were found when SDM supplemented with sulphite lignin, SDM supplemented with sulphite lignin and with Fe (III), or SDM supplemented with Ethanol was used to grow the *Lacto*. sp. pep8 cultures.

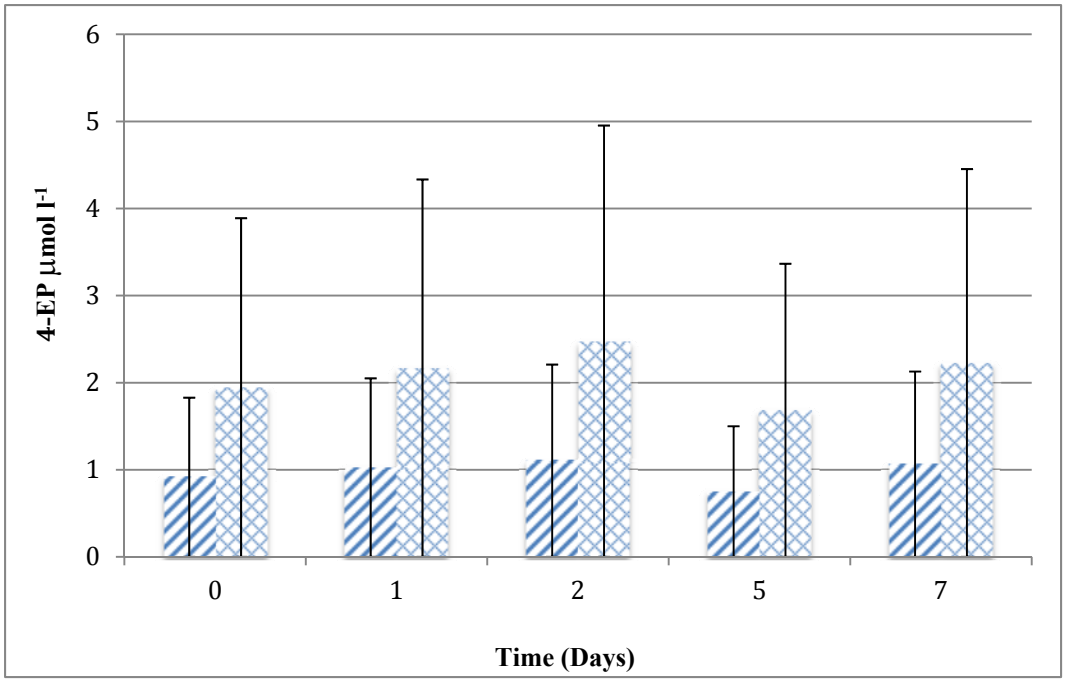

Figure 1: Levels of 4-ethylphenol in *Lactobacillus* sp. pep8 cultures grown in anaerobic semi-defined medium.

Treatment conditions: semi-defined medium + *Lacto.* sp. pep8 culture (diagonal lines), 10 mmol l^{-1} Fe (III) (final concentration) (crosshatched bars). Samples were incubated at ambient temperature for 7 days. All conditions were comprised of various amounts of semi-defined medium + 0.1 ml *Lacto.* sp. pep8 culture + treatment(s) for a total volume of 10 ml. Data are mean ± standard error (capped lines) of three replicates. Over all days the treatments were not statistically different from each other, Additionally, over the two treatments the days were not significantly different from each other.

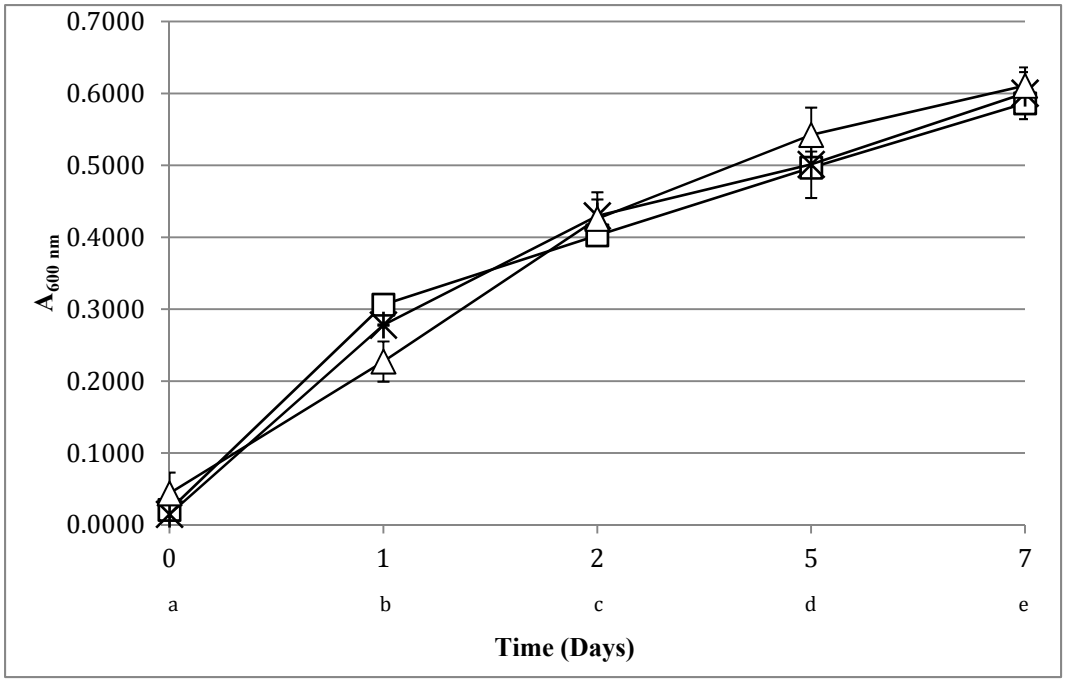

Figure 2: Growth of *Lactobacillus* sp. pep8 in anaerobic semi-defined medium monitored at 600 nm augmented with SDM, p-CA, or ethanol.

Treatment conditions: semi-defined medium + *Lacto.* sp. pep8 culture (□), 1 mmol l^{-1} p-coumaric acid (final concentration) (X), or ethanol vehicle control (Δ). An ethanol vehicle control was necessary as ethanol was used to dissolve soluble materials. All conditions were comprised of various amounts of semi-defined medium + 0.1 ml *Lacto.* sp. pep8 culture + treatment(s) for a total volume of 10 ml. Samples were not diluted prior to analysis. Data are mean ± standard error (capped lines) of three replicates. At any particular time point there was no significant difference between OD of the cultures indicating that there was no measurable affect of the different treatments on the growth of the cells. Over all days the treatments were not statistically different from each other at a confidence level of 0.05. Times that were coded with the same letter (a, b, c, d, or e) were not significantly different at a confidence level of 0.05.

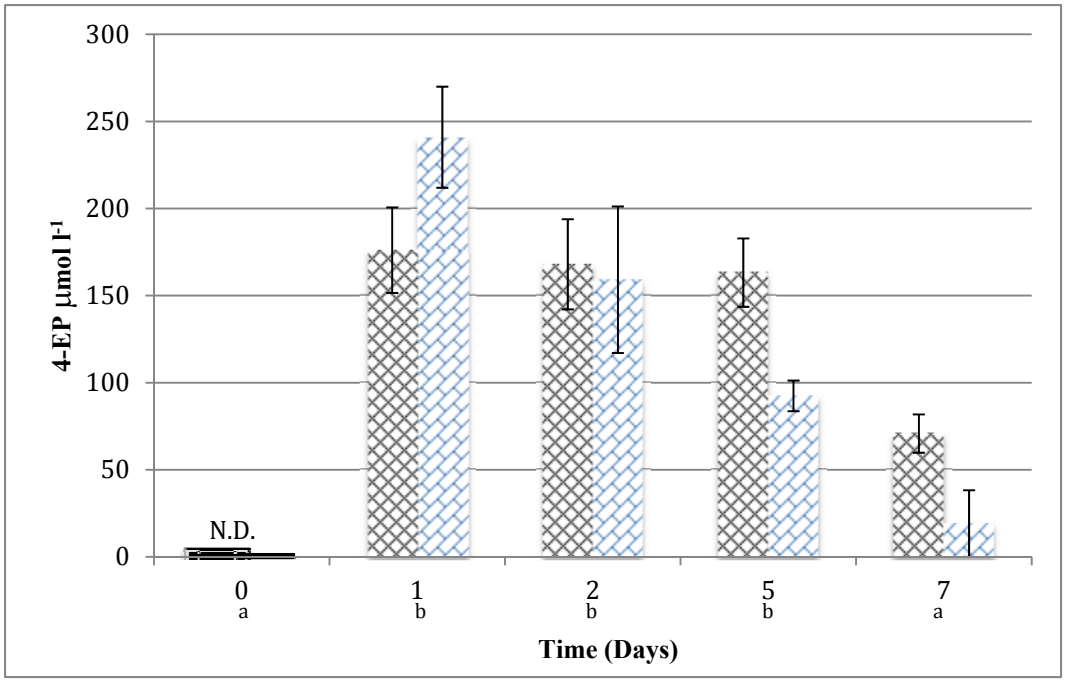

Figure 3: 4-Ehylphenol production by *Lactobacillus* sp. pep8 cultured in anaerobic semi-defined medium augmented with p-coumaric acid and Fe (III).

Treatment conditions: 0.1 ml p-coumaric acid (1 mmol l^{-1} final concentration) + 0.1 ml *Lacto.* sp. pep8 culture into 9.8 ml SDM (crosshatched bar). 10 mmol l^{-1} Fe (III) (final concentration) + 1 mmol l^{-1} p-coumaric acid (final concentration) (diagonal brick bars). Not detected (N.D.). Production of 4-EP lagged until day 1 when mean concentrations for both conditions reached their maximums. However, after day 1 levels of 4-EP decreased to near background levels by day 7. All conditions were comprised of various amounts of semi-defined medium + 0.1 ml *Lacto.* sp. pep8 culture + treatment(s) for a total volume of 10 ml. Samples were then augmented with the proper additions and incubated at ambient temperature for 7 days. Data are mean ± standard error (capped lines) of three replicates. The time points that were coded with different letters (a, or b) were not significantly different at a confidence level of 0.05. Over all days the treatments were not statistically different from each other at a confidence level of 0.05.

4-EP production in enriched swine lagoons slurries:

To further investigate the potential sources of 4-EP, 10 ml of blended swine lagoon sediments were enriched with TYM supplemented with either 0.2% sulphite lignin, or 10 mmol l^{-1} Fe (III), or both. In addition to investigating potential source compounds for 4-EP, the effect of Fe (III) on the production of 4-EP was also investigated (Figure 4). At day 0, there was no detectable 4-EP under any experimental condition tested. However, by day 7, 4-EP levels reached maximum levels for each condition, with the medium and slurry condition (Figure 4; diagonal bar) producing 626.202 μmol l^{-1} ± 45.33 of 4-EP on day 7. The experimental conditions produced approximately 125 μmol l^{-1} less 4-EP than the TYM and slurry condition. This suggests that 4-EP is likely endogenous to the swine lagoon slurries. Additionally, it may be possible that the addition of Fe (III) or sulphite lignin may hinder 4-EP production. Using ANOVA, it was determined that there was a significant difference between the control (TYM + Slurry = diagonal bars) and the condition containing 10 mmol l^{-1} and 0.2% sulphite lignin (horizontal bars) at a confidence interval of 0.05. Treatments containing more than one letter indicate that the condition is significantly different from certain conditions while not significantly different from all other conditions. Additionally, there was a significant difference between multiple time points. Days 0 and 21 are not significantly different while days 3, 7, and 14 are significantly different from one another as well as significantly different from days 0 and 21. This could indicate that the addition of both 0.2% sulphite lignin and 10 mmol l^{-1} hinders production of 4-EP based on the data in Figure 4.

Figure 5 illustrates the conditions containing TYM, 1 mmol l^{-1} p-CA, with or without the addition of 10 mmol l^{-1} Fe (III). 4-EP levels reached a maximum of 1026.39 μmol l^{-1} ± 18.13 on day 7 in in the absence of Fe (III) (Figure 5-horizontal bar). By day 14 there was a significant decrease in 4-EP concentration from 1026 μmol l^{-1} to below 200 μmol l^{-1} for both conditions. By day 21 the condition for the p-CA control without the addition of 10 mmol l^{-1}Fe (III) stayed around 200 μmol l^{-1} whereas the control condition containing both 10 mmol l^{-1} Fe (III) and 1 mM p-CA decreased to approximately 135 μmol l^{-1}. Using ANOVA, it was determined that there were no significant differences between treatments. Additionally, both day 0 and day 7 were significantly different than all other time points, while days 3, 14, and 21 were not significantly different at a confidence level of 0.05. This appears to indicate that there is little to no effect of Fe (III) on the production of 4-EP.

Figures 4 and 5 both show a similar trend in that there is no detectable 4-EP on day 0, but by day 7, maximum 4-EP levels were reached for all conditions. Furthermore, in both graphs there was a subsequent decrease in 4-EP concentration to lowest levels by day 21.

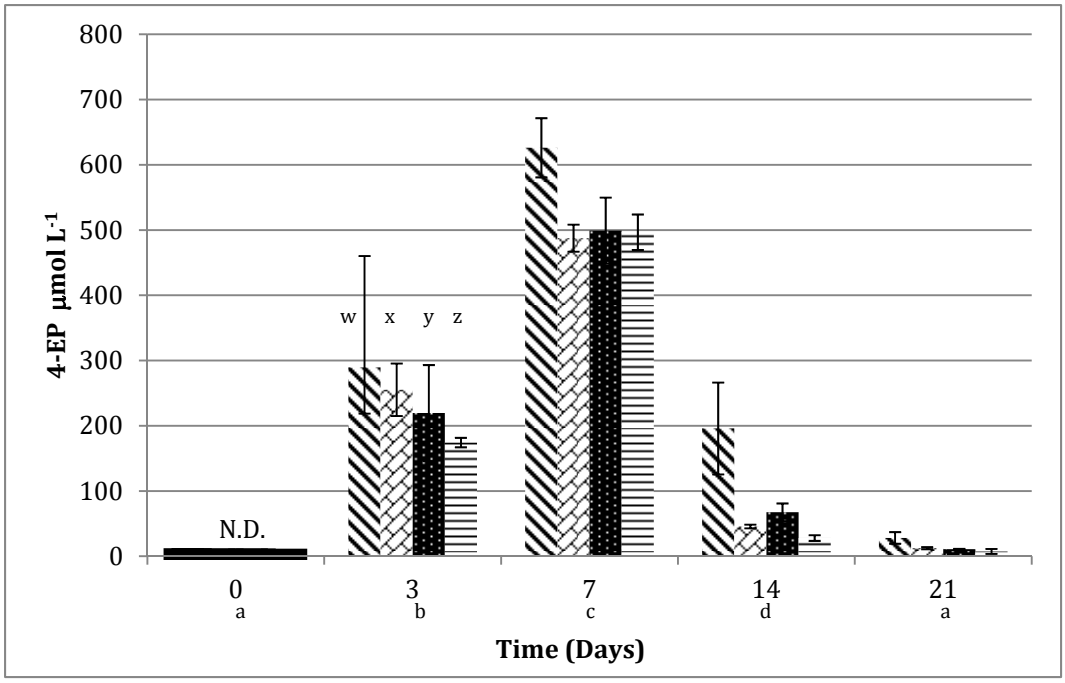

Figure 4: Production of 4-ethylphenol in enriched swine lagoon slurries cultured in anaerobic tryptone yeast medium containing sulphite lignin and Fe (III).

Treatment conditions: w = slurry and anerobic TYM (diagonal bar), x = slurry, anaerobic TYM and 0.2% (wt/vol) sulphite lignin (brick bar), y = slurry, anaerobic TYM and 10 mmol l^{-1} Fe (III) (final concentration) (black dotted bar), z = slurry, anaerobic TYM, 10 mmol l^{-1} Fe (III) (final concentration) and 0.2% (wt/vol) sulphite lignin (horizontal bar). All conditions contained various amounts of tryptone yeast medium + 10 ml lagoon slurry + treatment(s) for a total volume of 100 ml. Samples were collected on June 18th, 2010 with an outside temperature of 26^{o}C. Samples were then augmented with the proper additions and incubated at ambient temperature for 21 days. Not detected (N.D.). Data are mean ± standard error (capped lines) of three replicates. The time points that were coded with different letters (a, b, c, or d) were significantly different at a confidence level of 0.05. Treatments w, x, and y were not significantly different at a confidence interval of 0.05 over all days. Additionally, treatments x, y, and z were not

significantly different. Treatments W and Z are significantly different from one another over the course of all days at a confidence level of 0.05.

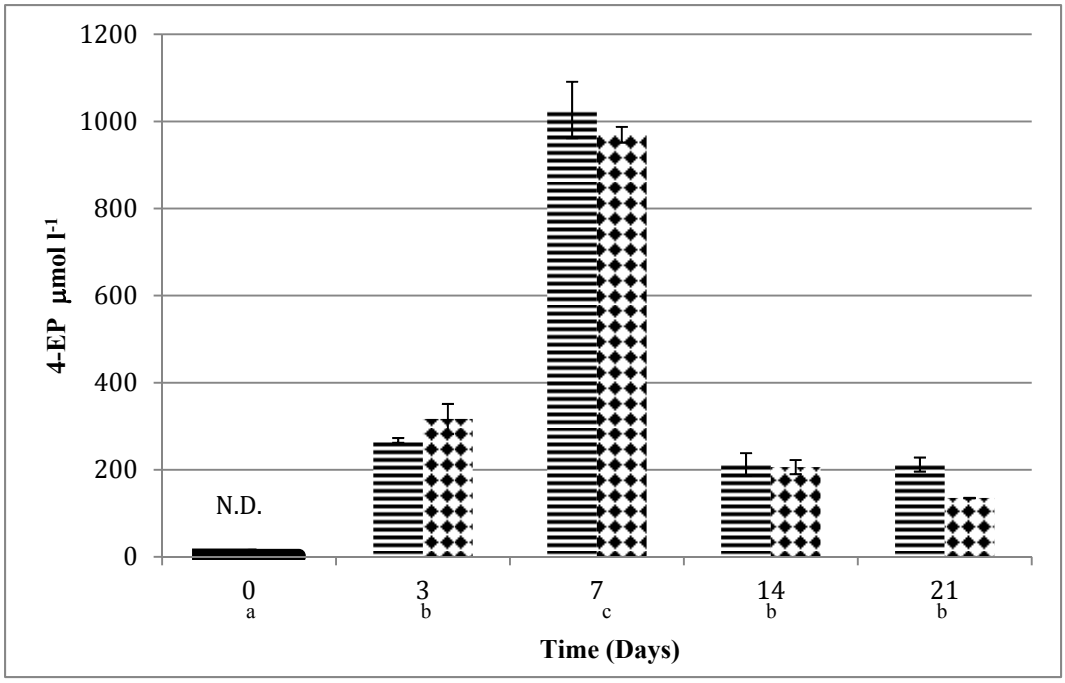

Figure 5: Growth conditions for enriched swine lagoon slurries containing p-coumaric acid and Fe (III).

Treatment conditions: 1 mmol l^{-1} p-coumaric acid (final concentration) (horizontal bars), 10 mmol l^{-1} Fe (III) (final concentration) and 1 mmol l^{-1} p-coumaric acid (final concentration) (diamond bars). Not detected (N.D.). All conditions contained various amounts of tryptone yeast medium + 10 ml lagoon slurry + treatment(s) for a total volume of 100 ml. Samples were collected on June 18th, 2010 with an outside temperature of 26^{o}C. Samples were then augmented with the proper additions and incubated at ambient temperature for 21 days. Data are mean ± standard error (capped lines) of three replicates. The time points that were coded with different letters (a, or b) were significantly different at a confidence level of 0.05. Over all days the treatments were not statistically different from each other at a confidence level of 0.05.

4-EP levels in enriched swine lagoon slurries with exogenous lignin additions:

Data suggest that the production of 4-EP was likely due to endogenous sources in the swine lagoon slurries (Figures 4 and 5). In this experiment, TYM enriched swine lagoon slurries were supplemented with exogenous lignin. Additionally, control conditions containing TYM augmented with either sulphite, sigma, or indulin lignin was tested. According to previous experiments, (Figure 4) data suggest that sulphite lignin is not the precursor to 4-EP. Therefore, in this experiment two additional forms of lignin were tested (sigma and indulin) in addition to the previously studied sulphite lignin.

According to Figure 6, at time 0, 4-EP was not be detected in any experimental conditions. However, by day 7, all three conditions produced approximately 1000 μmol l^{-1} of 4-EP (Figure 6). By day 14 there was a decrease in 4-EP levels (approximately half that found on day 7). When TYM was augmented with 0.2% sulphite, indulin, sigma lignin and lacking swine lagoon slurry additions, 4-EP was not produced.

Using ANOVA, statistical significance was determined at a confidence level of 0.05. The results showed no significant difference between treatments. Additionally, day 0 was significantly different from days 7 and 14, which were not significantly different from each other at a confidence level of 0.05**.**

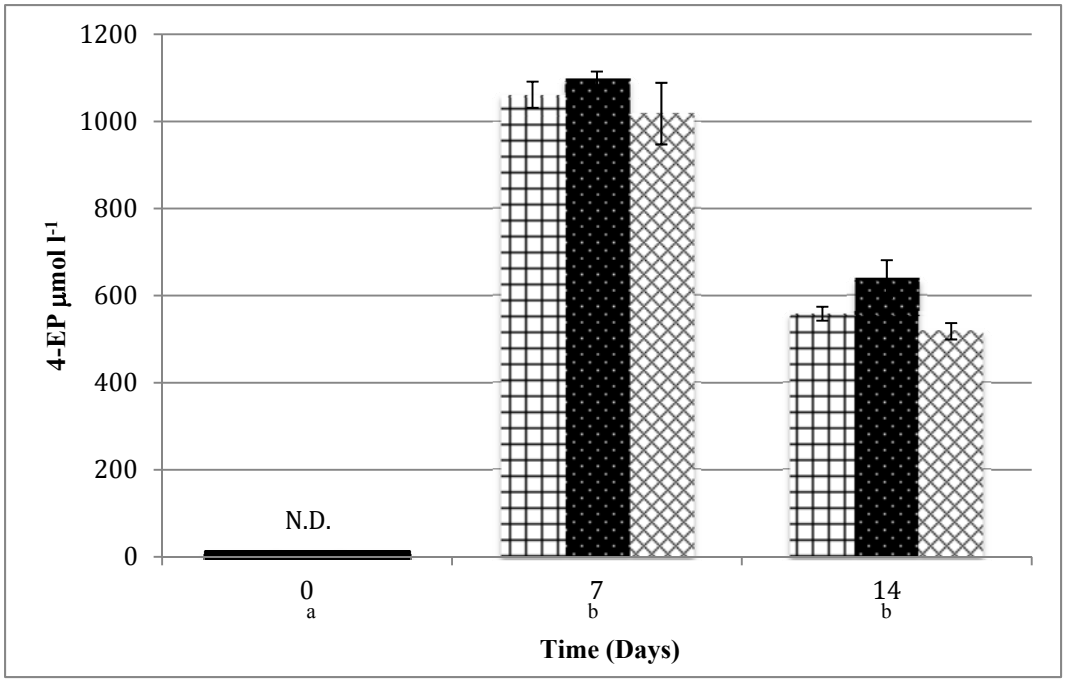

Figure 6: 4-Ethylphenol produced in blended swine lagoon slurries cultured in anaerobic tryptone yeast medium with varying lignin types.

Conditions: Each condition was inoculated with 10 mls of lagoon slurry into 90 mls (100 ml = total volume) of anaerobic tryptone yeast medium augmented with one of the following 0.2% (wt/vol) sulphite lignin (squares bar), 0.2% (wt/vol) indulin lignin (black dotted bar), 0.2% (wt/vol) sigma lignin (crosshatched bar). Not detected (N.D.). Samples were collected on February 2nd, 2011 with an outside temperature of 1.2°C approximately 60% of the swine lagoon was covered in ice (not including our sample area). Samples were then augmented with the proper additions and incubated at ambient temperature for 14 days. 4-ethylphenol concentrations are likely endogenous to swine lagoon slurries based on the comparison of control conditions that lacked slurry additions, which produced no 4-ethylphenol. Data are mean ± standard error (capped lines) of two replicates. Times coded with the same letter (a, or b) are not significantly

different at a confidence level of 0.05. Over all days the treatments were not statistically different at a confidence level of 0.05.

Degradation of exogenous 4-EP in enriched swine lagoon slurries:

Results from both previous swine lagoon enrichment experiments exhibited a similar trend in that the production of 4-EP was low initially, followed by a drastic increase in 4-EP levels and then a subsequent decrease by the end of the time course (Figures 4, 5, and 6). To determine if 4-EP was degraded in enriched swine lagoon sediments, exogenous 4-EP was supplemented into the TYM enriched slurry. In the control containing TYM, slurry, and ethanol (Figure 7: black dotted bars), the amount of 4-EP present on day 0 was 1140.63 μmol l^{-1} ± 65.30 but decreased drastically on day 3 to 475.97 μmol l^{-1} ± 47.18. After day 3, there was an increase in 4-EP on days 5, 7, and 10 (Figure 7: black dotted bars) from 787.14 μmol l^{-1} ± 34.07 to 1052.82 μmol l^{-1} ± 49.38, and to 1927.98 μmol l^{-1} ± 45.47, respectively. After day 10, there was only a slight decrease in 4-EP levels. In the control, production after day 10 never decreased below 1600 μmol l^{-1} (Figure 7). However, the experimental conditions gave conflicting results. In contrast to previous experiments, all conditions contained detectable levels of 4-EP at day 0 due to the addition of the exogenous 4-EP, whereas previous experiments contained no exogenous 4-EP. The experimental conditions each contained anaerobic TYM, slurry, and either 0.5, 1, or 2 mmol l^{-1} final concentrations of 4-EP. Each of these conditions, augmented with exogenous 4-EP, had levels between 1100 μmol l^{-1} and 1400 μmol l^{-1} on day 0. In each condition, there was a significant decrease in 4-EP levels by day 3 and a slight increase in production by day 5. 4-EP levels reached their maximum under all conditions by day 10. The only exception was for the condition containing 89.75 ml TYM, 10 ml slurry and [0.5 mmol l^{-1}] 4-EP (Figure 7: crosshatched bar), which reached maximum levels on day 18. Consequently, for most conditions, there was only a slight

decrease in levels of 4-EP after day 10 as opposed to a significant decrease to near background levels found in previous experiments.

Statistical significance was determined using ANOVA. The treatment containing 2 mmol l^{-1} and the control condition (TYM + ethanol + slurry) were significantly different from all other treatments. Additionally, the 4-EP levels detected at different time points were significantly different. Day 0 data were significantly different than data on days 3, and 5. However, day 7 data returned to the same significance level of day 0 data. Days 10, 14, 18, and 21 were significantly different than days 0, 3, 5, and 7. Additionally, days 0 and 7 were significantly different than days 3 and 5. This indicates that initial 4-EP levels were significantly degraded by days 3, and 5. Additionally, 4-EP levels significantly increased from day 5 to day 7 concentrations, by day 10 concentrations began to remain in a steady state and were not significantly different. Overall, the statistical data could support our theory of a varying bacteria community at different time points.

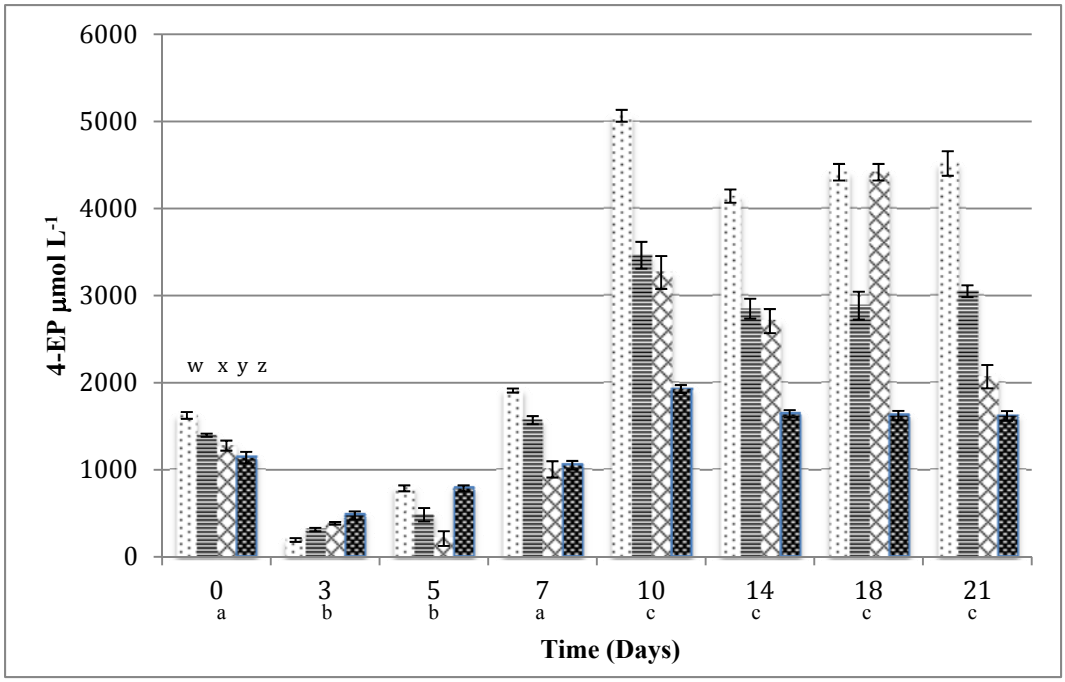

Figure 7: Effects of varying levels of exogenous 4-ethlyphenol on 4-ethylphenol levels in blended swine lagoon slurries cultured in anaerobic tryptone yeast medium.

Treatment conditions: w = 2 mmol l^{-1} 4-ethylphenol (final concentration) (white dotted), x = 1 mmol l^{-1} 4-ethylphenol (final concentration) (horizontal bars), y = 0.5 mmol l^{-1} 4-ethylphenol (final concentration) (crosshatched bars), z = ethanol vehicle control (black dotted bar). The ethanol vehicle control contained 10 ml swine lagoon slurry + 89 ml TYM + 1 ml ethanol. This control was performed to ensure 4-EP production was not hindered by ethanol, which was used to dissolve the exogenous 4-EP in the stock solution. All conditions were comprised of various amounts of tryptone yeast medium + 10 ml lagoon slurry + treatment(s) for a total volume of 100 ml. Swine lagoon samples were collected on June 13th, 2011 with an outside temperature of 26.6^{o} C. Lagoon samples were taken immediately to our laboratory where additions were made to specific treatments which were then incubated at ambient temperature for 21 days. Data are mean ± standard error (capped lines) of three replicates. Times that are coded with the

same letter (a, b, or c) are not significantly different at a confidence level of 0.05. Over all days, treatments x and y are not significantly different. Treatments w and z are significantly different than all other treatments across all days at a confidence level of 0.05.

DISCUSSION

Previous research identified 4-EP as a swine lagoon malodorant (Spoelstra, 1978), but the source compound of 4-EP in swine lagoon facilities is unknown. In fermented foods and wines, 4-EP is derived from p-CA (Rodriguez *et al*. 2008a) the conversion of p-CA to 4-EP is shown in Figure 8 (Van Beek and Priest, 2000). Due to the health concerns such as sensory irritation, neurochemical changes, and stored sensory experiences associated with swine lagoon systems (Schiffman, 1998), there is a need for further investigation to determine the source compound of p-CA in hopes of deterring the potential health concerns. p-CA is a cross-linker of lignin in plant cell walls (Ralph and Helm 1993), indicating plant material in feedstuffs may act as a source for 4-EP. Additionally, studies by this laboratory established that the inclusion of Fe (III) to enriched swine lagoon slurries increased production of common malodorants, 4-MP, and 3-MI. The inclusion of Fe (III) alone increased 3-MI levels by 1.85 fold when compared to the addition of tryptophan alone, and 4-MP levels increased 4.4 fold when compared to the addition of tyrosine alone (Doerner *et al*. 2009). Thus, in addition to the presence of p-CA, which may serve as a precursor for the production of 4-EP, the presence of Fe (III) may stimulate 4-EP synthesis. This increase occurred as a consequence of the reduction of Fe (III) to Fe (II). An electron is accepted by Fe (III), therefore converting Fe (III) to Fe (II). This reduction was coincidental with an increase in 3-MI and 4-MP production (Doerner *et al*. 2009). However, the inclusion of Fe (III) did not increase 4-EP levels (Figures 4 and 5). Figure 8, shows the metabolic pathway in which p-CA is converted to 4-EP. Overall, data from this study (Figures 3, 4, and 5) demonstrates Fe (III) does not influence production levels of 4-EP in swine lagoon slurries.

Results suggest that sulphite lignin, indulin lignin, and sigma lignin do not serve as precursors for 4-EP. There are many forms of lignin found in the environment (Whetton and Sederoff, 1995) thus multiple types of lignin preparations were tested. In 3 of the 4 experiments performed, there was no measurable 4-EP found on day 0, however, by day 3 there was slight production followed by maximum production of 4-EP by day 7 (Figures 4, 5, and 6). Additionally a similar trend can be seen in the data shown in Figures 4, 5, and 6; an increase from 0 μmol l^{-1} to maximum levels by day 7 and then a subsequent decrease to near background levels by day 14. The three control conditions which contained 100 mL TYM and 0.2% of sulphite lignin, indulin lignin, or sigma lignin did not produce any detectable 4-EP (data not shown).

Based upon the observed degradation of endogenous 4-EP (Figures 4, 5, and 6) we determined if exogenous 4-EP could also be degraded (Figure 7). Exogenous 4-EP with final concentrations of 2, 1, and 0.5 mmol l^{-1} were supplemented into experimental conditions containing TYM and swine lagoon slurry. We predicted that the exogenous 4-EP would be similarly degraded. Treatments were compared to the control containing media, slurry, and ethanol (Figure 7: black dotted bar). The data presented in Figure 7 conflicts with data shown in Figures 3, 4, 5, and 6. Figure 7 data show an immediate decrease in 4-EP concentrations from day 0 to day 5. 4-EP levels then increase on day 7 and 10 followed by 4-EP concentrations reaching steady state levels on days 14, 18, and 21. The concentration of 4-EP at day 0 in the control condition was 1140.66 μmol L^{-1}, which suggests the level of 4-EP in the control is endogenous to the slurry additions.

A confounding factor when interpreting the data in Figure 7 is that when adding exogenous 4-EP, there should be an additive effect on the already present endogenous 4-

EP (Figure 7: black dotted bar). However, this was not observed. Based on Figure 7 (black dotted bar), the control condition containing only media, slurry, and ethanol, there should be approximately 1140 μmol l^{-1} of 4-EP. Since exogenous 4-EP was added to endogenous 4-EP, the concentrations should be additive (Figure 7: black dotted bars), therefore the exogenous amounts of 4-EP in each condition (2 mM, 1 mM, and 0.5 mM) should be added to the 1140 μmol l^{-1} (Figure 7:black dotted bars, day 0) of endogenous 4-EP. The expectation was that the addition of 2mM of 4-EP to an experimental condition, the concentration of 4-EP would be additive to the control concentration (approximately 1140 μmol l^{-1}) resulting in approximately 3140 μmol l^{-1} of 4-EP. However, the 4-EP levels detected by HPLC analysis were significantly lower than expected. There are several possible reasons why the data in Figure 7 are inconsistent with the expected total 4-EP concentrations. It is possible there is variation in the swine lagoon samples based on the fact there is endogenous 4-EP on day 0 unlike the data shown in Figures 4, 5, and 6. This could be due to an over abundance of bacterial producers or lack of bacterial degraders (of 4-EP) in the swine lagoon at the time the sample was taken. It is possible when samples were taken from the swine lagoon a specific community of organisms (4-EP producers or degraders) was obtained at the location of sampling. Due to the high endogenous levels it is likely that there is a lack of 4-EP degraders. However, it is possible that by day 3, an expanded population of bacteria that degrade 4-EP had begun to flourish in the enriched medium and consumed much of the present 4-EP. This is then followed by an increase of in the number of bacteria that produce 4-EP at day 7. By day 10 concentrations of 4-EP reached their maximum concentrations for all conditions. Days 10 thru 21, the production of 4-EP appears to “level off” or remain in a steady state,

which would indicate that, the number of producers and degraders of 4-EP have reached an equilibrium.

A second possibility, though unlikely, is that there may have been an error with HPLC detection of 4-EP. When analyzing large numbers of samples, there is a risk of variation in the retention time of the compound. However, by performing a standard curve at the beginning and the end of the analysis, it would be very obvious that something was wrong if the standards were incorrect. For this experiment, (Figure 7) both front and back end standards were run, and both were normal, giving a linear best-fit line as well as an "r^2" value of .99. The exogenous 4-EP detected in Figure 7 should have been additive to the endogenous 4-EP of the control condition, but this was not observed; for unknown reasons there was no additive effect.

Another difference between the data shown in Figure 7 and the data shown in Figures 4, 5, and 6, in that after day 7, there was synthesis of 4-EP. The 4-EP concentration in all previous experiments increased to maximum levels by day 7 and decreased to near background levels by day 14. However, the data shown in figure 7, 4-EP concentrations starts high on day 0 although not as high as expected, decreases to their lowest levels by day 5 but then increases to maximum concentration levels on day 10. After reaching maximum levels by day 10, the 4-EP concentration remains constant. If possible, the experiment would have been repeated, which was not feasible.

Swine lagoon slurries as well as the swine gastrointestinal tract are dynamic environments. A consortium of organisms work in conjunction with each other to achieve numerous things such as carbohydrate fermentation, facilitating nutrient absorption by the host, and mediation of metabolic functions like vitamin synthesis, and absorption of

ions (Mackie *et al*.1998; Sears, 2005). Microorganisms are often associated with one another on the surfaces of swine lagoon sediments forming biofilms. It is possible that the conversion of p-CA to 4-EP in swine lagoon slurries requires multiple organisms**.** These aggregations are likely separated from one another during blending. When blending, communities of organisms found in the lagoon slurry may be separated from each other due to the force of the blender creating a homogenous mixture. However, if the disassociation of these communities occurs, the production of 4-EP would likely decrease because disassociated communities can no longer function together to produce 4-EP. Conversely, the data in Figures 4, 5, and 6 indicate that production of 4-EP reached its highest concentration after 7 days, suggesting that reestablishment of the consortium requires a certain amount of time (5-7 days). Our data is consistent with the idea that the consortium of organisms may need time to re-associate before production of 4-EP can resume.

The time of year each sample was taken can also contribute to variation in the endogenous production of 4-EP (Loughrin *et al*. 2006). Our samples were taken at various times throughout the year. Merrill and Halverson (2002) have concluded that the concentrations of volatile compound found in swine waste lagoons vary based on seasonal changes including temperature and pH of the lagoon. Samples for our first enriched swine lagoon experiment were taken during the summer of 2010 (June 23rd). This was a particularly dry summer (http://water.weather.gov/precip/index.php) with temperatures regularly above 26^{o}C (http://www.crh.noaa.gov/images/lmk/pdf/2010). These extreme temperatures may have had an effect on the microbial flora of the primary lagoon. Enriched swine lagoon samples for experiment 3 (Figure 6) were taken during the

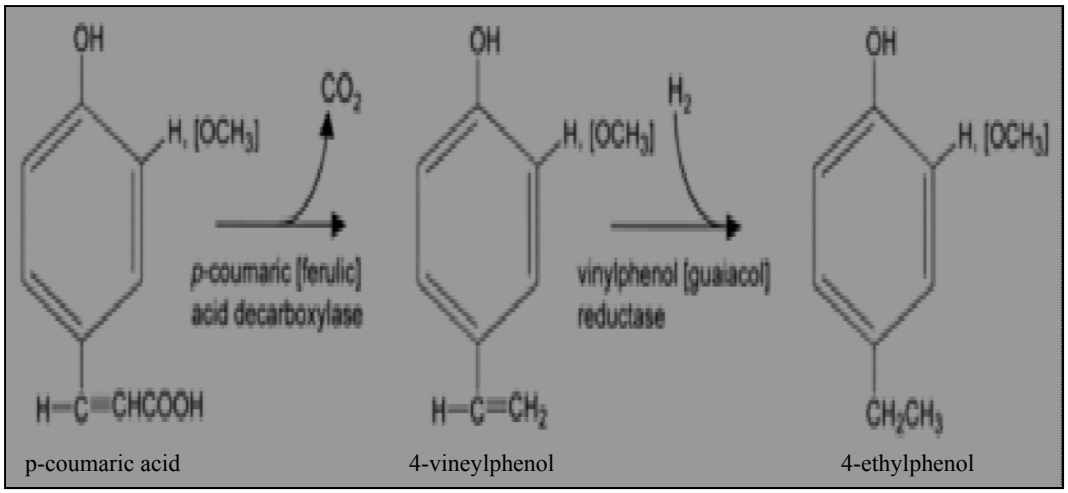

Figure 8: Conversion of p-coumaric acid to 4-ethylphenol.

Conversion of p-coumaric acid to 4-ethylphenol in fermented foods and beverages such as wines and beer. p-Coumaric acid is first decarboxylated by cinnamate decarboxylase giving off a by-product of carbon dioxide and yielding an intermediate of 4-vinylphenol. The intermediate 4-vinylphenol has the 4-carbon side chain double bond reduced by vinylphenol reductase. Next, two electrons are added yielding a final compound of 4-ethylphenol (Van Beek, S. and Priest. F.G., 2000).

late winter season (February 2nd, 2011). The primary lagoon was not frozen over completely but it did have some areas where ice was present (approximately 60% coverage). This could have potentially changed the microbial flora found in the primary lagoon, as many organisms do not grow or have significantly reduced metabolic activities at such low temperatures.

Lastly, enriched swine lagoon samples supplemented with exogenous 4-EP were collected during the summer (June 12th, 2011) with temperatures above 27°C (http://www.crh.noaa.gov/images/lmk/pdf/2011). Additionally, it was a particularly wet summer (http://water.weather.gov/precip/index.php). This may have caused the primary swine lagoon and the secondary lagoon to flow freely between one another mixing the sediment found in the primary lagoon where our samples were taken. If the primary and the secondary lagoons were allowed to mix, it could lead to significant changes in the microbial communities.

Environmental conditions are impossible to control. Samples were collected as needed regardless of time of year and current environmental conditions. However, our data suggests that time of year, temperature, or rainfall did not influence 4-EP concentrations. Throughout all swine lagoon enrichment experiments, 4-EP concentrations stayed relatively constant (Figures 4, 5, and 6). This suggests that there is a somewhat stable "community" of bacteria throughout the entire year.

In conclusion, data indicate that *Lacto. sp*. pep8 and enriched swine lagoon slurries fail to liberate p-CA from lignin. Though the pathway in which p-CA is converted to 4-EP is well known in fermented foods, it is possible that another pathway mediates the conversion of p-CA to 4-EP in swine waste lagoons. To test this would

require detection of p-CA in swine lagoon enrichments. However, to-date, an assay for the detection of p-CA using RP-HPLC has not been developed.

The inclusion of different forms of lignin as well as the addition of Fe (III) failed to stimulate production of 4-EP in both enriched swine lagoon slurries as well as *Lacto.* sp. pep8 cultures. Furthermore, 4-EP levels are likely endogenous to the swine lagoon slurries which does not exclude other potential lignin sources as the source compound of 4-EP. Based on our data it has been established that neither sulphite, indulin, nor sigma lignin are precursors to 4-EP. To further understand 4-EP production, it is necessary to explore other potential source compounds, such as different forms of lignin produced through different extraction techniques. Different forms of lignin are achieved based on the method in which the lignin is extracted from plant material.

Additionally, by testing non-enriched (without supplementation with culture medium) swine lagoon slurries there is a possibility of determining 4-EP concentrations endogenous to the swine lagoon over a longer period of time. By doing so, future research could possibly determine steady state levels of 4-EP in a swine waste lagoon.

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