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# Effects of Live Yeast and Yeast Extracts with and without Pharmacological Levels of Zinc on Antimicrobial Susceptibilities of Fecal Escherichia coli in Nursery Pigs

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# Effects of Live Yeast and Yeast Extracts with and without Pharmacological Levels of Zinc on Antimicrobial Susceptibilities of Fecal Escherichia coli in Nursery Pigs


## Abstract

A total of 360 weanling barrows (Line 200 × 400, DNA Genetics; initial BW 12.4 ± 0.05 lb) were used in a 42-d study to evaluate yeast-based pre- and probiotics (Phileo by Lesaffre, Milwaukee, WI) in diets with or without pharmacological levels of Zn on antimicrobial resistance (AMR) patterns of fecal *Escherichia coli*. Pens were assigned to 1 of 4 dietary treatments with 5 pigs per pen and 18 pens per treatment. Dietary treatments were arranged in a 2 × 2 factorial with main effects of live yeast-based pre- and probiotics (none vs. 0.10% ActiSafSc 47 HR+, 0.05% SafMannan, and 0.05% NucleoSaf from d 0 to 7, then concentrations were lowered by 50% from day 7 to 21) and pharmacological levels of Zn (110 vs. 3,000 ppm from d 0 to 7, and 2,000 ppm from d 7 to 21 provided by ZnO). All pigs were fed a common diet from d 21 to 42 post-weaning without live yeast-based pre- and probiotics or pharmacological Zn. Fecal samples were collected on d 4, 21, and 42 from the same three pigs per pen for fecal *E. coli* isolation. The identification of *E. coli* was by PCR detection of *uidA* and *clpB* genes. The AMR patterns of E. coli were determined by microbroth dilution method using Sensititre CMV3AGNF panel containing 14 different antimicrobials. The addition of pharmacological levels of Zn had a marginally significant effect ($P$ = 0.051) to increase the minimum inhibitory concentration (MIC) values of ciprofloxacin; however, median MIC values were still under the Clinical and Laboratory Standards Institute (2018) classified resistant breakpoint for ciprofloxacin. There was no evidence for differences ($P$ > 0.05) for yeast additives or Zn for AMR of fecal *E. coli i*solates to any of the remaining antibiotics. In conclusion, pharmacological levels of Zn tended to increase the AMR of fecal *E. coli* to ciprofloxacin while the medians were below a resistant breakpoint. There was no influence of live yeast and yeast extracts on AMR patterns of fecal *E. coli* against tested antimicrobials.



## Keywords

nursery pigs, probiotics, yeast, yeast extracts, zinc





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# Effects of Live Yeast and Yeast Extracts with and without Pharmacological Levels of Zinc on Antimicrobial Susceptibilities of Fecal *Escherichia coli* in Nursery Pigs

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## Summary

A total of 360 weanling barrows (Line 200 × 400, DNA Genetics; initial BW 12.4 ± 0.05 lb) were used in a 42-d study to evaluate yeast-based pre- and probiotics (Phileo by Lesaffre, Milwaukee, WI) in diets with or without pharmacological levels of Zn on antimicrobial resistance (AMR) patterns of fecal *Escherichia coli*. Pens were assigned to 1 of 4 dietary treatments with 5 pigs per pen and 18 pens per treatment. Dietary treatments were arranged in a 2 × 2 factorial with main effects of live yeast-based pre- and probiotics (none vs. 0.10% ActiSafSc 47 HR+, 0.05% SafMannan, and 0.05% NucleoSaf from d 0 to 7, then concentrations were lowered by 50% from day 7 to 21) and pharmacological levels of Zn (110 vs. 3,000 ppm from d 0 to 7, and 2,000 ppm from d 7 to 21 provided by ZnO). All pigs were fed a common diet from d 21 to 42 post-weaning without live yeast-based pre- and probiotics or pharmacological Zn. Fecal samples were collected on d 4, 21, and 42 from the same three pigs per pen for fecal *E. coli* isolation. The identification of *E. coli* was by PCR detection of *uidA* and *clpB* genes. The AMR patterns of *E. coli* were determined by microbroth dilution method using Sensititre CMV3AGNF panel containing 14 different antimicrobials. The addition of pharmacological levels of Zn had a marginally significant effect ($P = 0.051$) to increase the minimum inhibitory concentration (MIC) values of ciprofloxacin; however, median MIC values were still under the Clinical and Laboratory Standards Institute (2018)[5] classified resistant breakpoint for ciprofloxacin. There was no evidence for differences ($P > 0.05$) for yeast additives or Zn for AMR of fecal *E. coli* isolates to any of the remaining antibiotics. In conclusion, pharmacological levels of Zn tended to increase the AMR of fecal *E. coli* to ciprofloxacin while the medians were below a resistant

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breakpoint. There was no influence of live yeast and yeast extracts on AMR patterns of fecal *E. coli* against tested antimicrobials.

## Introduction

Feeding pharmacological levels of Zn (2,000 to 3,000 ppm) has become a concern for AMR to antimicrobials of importance to human and animal medicine. One potential replacement strategy for pharmacological levels of Zn in the early nursery is the use of pre- and probiotics. This report is a companion to our previous paper in which we evaluated the effects of pharmacological levels of Zn with or without the addition of the live yeast *Saccharomyces cerevisiae* strain NCYC Sc 47 and yeast-based prebiotics derived from *Saccharomyces cerevisiae* on weanling pig growth performance.[6] This paper reports the effects of live yeast and yeast-based prebiotics on AMR patterns for *E. coli* isolated from nursery pig feces.

## Materials and Methods

### *General*

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this experiment. The study was conducted at the Kansas State University Segregated Early Weaning Facility in Manhattan, KS. The facility has two identical barns that are completely enclosed, environmentally controlled, and mechanically ventilated. Treatments were equally represented in each barn. Each pen contained a 4-hole, dry self-feeder and a cup waterer to provide *ad libitum* access to feed and water. Pens (4 × 4 ft) had metal tri-bar floors and allowed approximately 2.7 $ft^2$/pig.

### *Animals and treatment structure*

A total of 360 barrows (Line 200 × 400, DNA Genetics; initially 12.4 ± 0.05 lb BW) were used in a 42-d study with 5 pigs per pen and 18 pens per treatment (9 pens per barn). Details as to pig allotment, experimental design, and diet preparation and analysis can be found in Chance et al. (2021).[6]

Briefly, dietary treatments were arranged in a 2 × 2 factorial with main effects of yeast-based pre- and probiotics (none vs. 0.10% ActiSafSc 47 HR+, 0.05% SafMannan, and 0.05% NucleoSaf from d 0 to 7, then concentrations were lowered by 50% from day 7 to 21) and pharmacological levels of Zn (110 vs. 3,000 ppm from d 0 to 7, and 2,000 ppm from d 7 to 21 provided by ZnO). All pigs were fed a common diet from d 21 to 42 post-weaning without added yeast products or pharmacological levels of Zn. The live yeast *Saccharomyces cerevisiae* strain NCYC Sc 47 (ActiSaf Sc 47 HR+; Phileo by Lesaffre, Milwaukee, WI) served as the yeast-based probiotic. The yeast-based prebiotics included a yeast cell wall fraction with concentrated mannan-oligosaccharides and β-glucans from *Saccharomyces cerevisiae* (SafMannan; Phileo by Lesaffre, Milwaukee, WI) and a yeast extract containing ≥6% unbound nucleotides from *Saccharomyces cerevisiae* (NucleoSaf; Phileo by Lesaffre, Milwaukee, WI).

[6] Chance, J. A., J. T. Gebhardt, J. M. DeRouchey, H. I. Calderón, M. D. Tokach, J. C. Woodworth, R. D. Goodband, and J. A. Loughmiller. 2021. Effects of Live Yeast and Yeast Extracts with and without Pharmacological Levels of Zinc on Nursery Pig Growth Performance and Fecal Consistency. *Kansas Experimental Station Research Reports: Vol. 7, Issue 11.*

### *Fecal collection*

Fecal samples were collected on d 4, 21, and 42 of the experiment to isolate *E. coli* and determine AMR. Fecal samples were collected directly from the rectum of the same three randomly selected pigs from each pen and pooled by pen to form one composite sample. Fecal samples were collected using a sterile, single-use cotton tipped applicator (Fisher Healthcare, Pittsburgh, PA), stored in a clean, single-use zipper storage bag placed on ice and delivered the same day to the laboratory of Dr. Raghavendra Amachawadi at the Kansas State University College of Veterinary Medicine for bacterial isolation and further characterization.

### *E. coli isolation*

Approximately 1 g of fecal sample was suspended in 9 mL of phosphate-buffered saline. Fifty microliters of the fecal suspension were then spread-plated onto a MacConkey agar (Becton Dickinson, Sparks, MD) for the isolation of *E. coli*. Two lactose-fermenting colonies were picked from each MacConkey agar; each colony was individually streaked onto a blood agar plate (Remel, Lenexa, KS) and incubated at 98.6°F for 24 h. An Indole test was done and indole-positive isolates were stored in cryo-protect beads (Cryocare, Key Scientific Products, Round Rock, TX) at -112°F. Species confirmation of *E. coli* was by polymerase chain reaction (PCR) detection of *uidA* and *clpB* genes.

### *Antimicrobial susceptibility testing of E. coli isolates*

Antimicrobial susceptibility testing was done on *E. coli* isolates recovered on d 4, 21, and 42. The microbroth dilution method as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2018)[5] was used to determine the minimum inhibitory concentrations (MIC) of several antibiotics (Table 1). Each isolate, stored in cryo-protect beads, was streaked onto a blood agar plate and incubated at 98.6°F for 24 h. Individual colonies were suspended in demineralized water (Trek Diagnostic Systems, Cleveland, OH) and turbidity was adjusted to 0.5 McFarland turbidity standards. Then, 10 µL of the bacterial inoculum was added to Mueller–Hinton broth and vortexed to mix. A Sensititre automated inoculation delivery system (Trek Diagnostics Systems) was used to dispense 100 µL of the culture into National Antimicrobial Resistance Monitoring System (NARMS) panel plates designed for Gram-negative (CMV3AGNF, Trek Diagnostic Systems) bacteria. *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) strain was included as quality control. Plates were incubated at 98.6°F for 18 h and bacterial growth was assessed using Sensititre ARIS and Vizion systems (Trek Diagnostic Systems). Clinical and Laboratory Standards Institute[5] guidelines were used to classify each isolate as susceptible, intermediate, or resistant according to the breakpoints established for each antimicrobial. The MIC values greater than the susceptible breakpoint but lower than the resistant breakpoint were considered intermediate. The antimicrobials evaluated included: amoxicillin/clavulanic acid 2:1 ratio, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole.

### *Statistical analysis*

For each of the 14 antimicrobials, minimum inhibitory concentration (MIC) data were summarized with appropriate descriptive statistics by treatment group at each sampling day. Due to the lack of variability, MICs of tetracycline were excluded from the statistical analysis since all isolates were resistant. The MIC data of the remaining

antimicrobials were analyzed using the linear mixed model. To better achieve model assumptions, data underwent natural log transformation before statistical modeling. Statistical analysis was performed using the MIXED procedure of SAS (v. 9.4, SAS Inst., Inc., Cary, NC) with option DDFM=KR in the MODEL statement. Fixed effects of the model included Zn, yeast, time, and their second- and third-order interactions. Random effects included block and pen. Treatment effect was assessed via back-transformed least squares means, i.e., geometric means of the MIC values. The variance-covariance structure of pen was taken as compound symmetry, first-order autoregressive, or unstructured according to the model fitting criteria. Differences between treatments were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$.

## Results and Discussion

From fecal samples collected and *E. coli* isolated, there were no two-way or three-way interactions observed for any of the antibiotics tested. Thus, MIC values of fecal *E. coli* isolates in response to the inclusion of yeast-based pre- and probiotics, pharmacological levels of Zn, and sampling day were further explored (Table2).

All fecal *E. coli* isolates were susceptible to azithromycin, ciprofloxacin, gentamicin, nalidixic acid, sulfisoxazole, and trimethoprim/sulfamethoxazole at all three sampling time points (d 4, 21, and 42) regardless of the inclusion of live yeast and yeast extracts or pharmacological levels of Zn. Regardless of diet or sampling day, fecal *E. coli* isolates were intermediate to amoxicillin:clavulanic acid (2:1 ratio), ampicillin, cefoxitin, ceftiofur, and chloramphenicol. Fecal *E. coli* isolates from all dietary treatments were resistant to streptomycin at all three sampling time points. On d 4 and 21, fecal *E. coli* isolates were considered intermediate to ceftriaxone but were resistant on d 42 based off CLSI[5] antimicrobial breakpoints for ceftriaxone.

There was evidence for increased ($P < 0.05$) MIC values over time for ampicillin, cefoxitin, ceftriaxone, ciprofloxacin, nalidixic acid, sulfisoxazole, and trimethoprim/sulfamethoxazole with a tendency ($P < 0.10$) for increased MIC values of chloramphenicol and gentamicin. Azithromycin was the only antibiotic that had evidence for decreased ($P < 0.001$) MIC values over time.

Of the fecal samples collected and *E. coli* isolated, MICs were not influenced by the presence of dietary addition of live yeast and yeast extracts. Only fecal *E. coli* isolated from pigs fed pharmacological levels of Zn from d 0 to 21 had a marginally significant effect ($P = 0.051$) where the AMR to ciprofloxacin was higher compared to those that were not fed added Zn. However, all median MICs were still well under the CLSI[5] antimicrobial breakpoint for ciprofloxacin.

In conclusion, there was minimal impact on the AMR of fecal *E. coli* of pigs fed diets with pharmacological levels of Zn and/or yeast-based pre- and probiotics. However, feeding high levels of Zn tended to increase the possibility of fecal *E. coli*'s AMR to ciprofloxacin, but based on the breakpoint the isolates were considered susceptible.

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**Table 1. Resistance breakpoints and evaluated concentrations for antimicrobials of National Antimicrobial Resistance Monitoring System Gram-negative bacteria panel (CMV3AGNF; WHO, 2018)[1]**

| Antimicrobial | WHO classification[2] | Susceptible breakpoints, µg/mL | Intermediate breakpoints, µg/mL | Resistant breakpoint, µg/mL |
|---|---|---|---|---|
| Amoxicillin:clavulanic acid 2:1 ratio | Critically important | ≤ 8/4 | 16/8 | ≥ 32/16 |
| Ampicillin | Critically important | ≤ 8 | 16 | ≥ 32 |
| Azithromycin | Critically important | ≤ 16 | N/A[3] | ≥ 32 |
| Cefoxitin | Highly important | ≤ 8 | 16 | ≥ 32 |
| Ceftiofur | Critically important | ≤ 2 | 4 | ≥ 8 |
| Ceftriaxone | Critically important | ≤ 1 | 2 | ≥ 4 |
| Chloramphenicol | Highly important | ≤ 8 | 16 | ≥ 32 |
| Ciprofloxacin | Critically important | ≤ 0.06 | ≥ 0.12 | ≥ 0.12 |
| Gentamicin | Critically important | ≤ 4 | 8 | ≥ 16 |
| Nalidixic acid | Critically important | ≤ 16 | N/A | ≥ 32 |
| Streptomycin | Critically important | ≤ 16 | N/A | ≥ 32 |
| Sulfisoxazole | Highly important | ≤ 256 | N/A | ≥ 512 |
| Tetracycline | Highly important | ≤ 4 | 8 | ≥ 16 |
| Trimethoprim/sulfamethoxazole 1:19 ratio | Highly important | ≤ 2/38 | N/A | ≥ 4/76 |

[1] Breakpoints established by Clinical and Laboratory Standards Institute (CLSI, 2018) which are categorized as susceptible (treatable), intermediate (possibly treatable with higher doses), and resistant (not treatable). The MIC values greater than the susceptible breakpoint but lower than the resistant breakpoint were considered intermediate. (Clinical and Laboratory Standards Institute (CLSI). 2018. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard, 5th ed. CLSI supplement VET08. CLSI, Wayne, PA.)
[2] World Health Organization (WHO) categorization of antimicrobials according to importance for human medicine (WHO, 2018).
[3]N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

**Table 2. Main effects of yeast probiotics and pharmacological levels of Zn over time on antimicrobial susceptibilities of fecal *Escherichia coli* according to National Antimicrobial Resistance Monitoring System (CLSI, 2018) established breakpoints[1,2]**

| | Yeast probiotics[3] | | | Zn[4] | | | Day | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | No yeast | Yeast | *P* = | Low Zn | High Zn | *P* = | 4 | 21 | 42 | *P* = |
| Amoxicillin:clavulanic acid 2:1 ratio[5] | 13.5 ± 1.0 | 13.3 ± 1.0 | 0.905 | 13.1 ± 1.0 | 13.6 ± 1.0 | 0.721 | 11.3 ± 1.1[a] | 14.4 ± 1.4[a] | 14.7 ± 1.4[a] | 0.134 |
| Ampicillin | 23.1 ± 1.7 | 24.3 ± 1.8 | 0.625 | 22.9 ± 1.7 | 24.4 ± 1.8 | 0.541 | 18.3 ± 2.1[a] | 23.1 ± 1.9[a] | 31.4 ± 0.6[b] | <0.001 |
| Azithromycin | 9.2 ± 0.64 | 9.1 ± 0.63 | 0.876 | 8.8 ± 0.60 | 9.6 ± 0.66 | 0.272 | 11.7 ± 0.96[b] | 6.8 ± 0.56[a] | 9.7 ± 0.80[b] | <0.001 |
| Cefoxitin | 13.5 ± 1.0 | 14.6 ± 1.1 | 0.367 | 13.0 ± 1.0 | 15.1 ± 1.1 | 0.112 | 11.9 ± 1.1[a] | 14.1 ± 1.3[ab] | 16.5 ± 1.6[b] | 0.030 |
| Ceftiofur | 2.5 ± 0.30 | 2.7 ± 0.32 | 0.606 | 2.7 ± 0.32 | 2.4 ± 0.29 | 0.505 | 2.1 ± 0.33[a] | 2.5 ± 0.39[a] | 3.2 ± 0.49[a] | 0.189 |
| Ceftriaxone | 2.6 ± 0.49 | 3.1 ± 0.58 | 0.443 | 3.1 ± 0.59 | 2.6 ± 0.48 | 0.336 | 1.9 ± 0.46[a] | 2.4 ± 0.60[a] | 4.9 ± 1.20[b] | 0.011 |
| Chloramphenicol | 20.2 ± 1.3 | 20.7 ± 1.3 | 0.774 | 20.4 ± 1.3 | 20.4 ± 1.3 | 0.999 | 19.8 ± 1.6[ab] | 18.3 ± 1.7[a] | 23.5 ± 1.5[b] | 0.087 |
| Ciprofloxacin | 0.031 ± 0.004 | 0.031 ± 0.004 | 0.996 | 0.026 ± 0.004 | 0.038 ± 0.005 | 0.051 | 0.024 ± 0.0031[a] | 0.028 ± 0.0036[a] | 0.046 ± 0.0084[b] | 0.007 |
| Gentamicin | 2.9 ± 0.43 | 3.7 ± 0.54 | 0.232 | 3.6 ± 0.52 | 3.0 ± 0.44 | 0.386 | 2.5 ± 0.41[a] | 4.0 ± 0.65[b] | 3.4 ± 0.55[ab] | 0.072 |
| Nalidixic acid | 3.2 ± 0.23 | 3.2 ± 0.23 | 1.000 | 3.0 ± 0.21 | 3.4 ± 0.24 | 0.253 | 2.9 ± 0.25[a] | 2.9 ± 0.21[a] | 3.9 ± 0.40[b] | 0.029 |
| Streptomycin | 50.1 ± 2.8 | 53.8 ± 3.0 | 0.288 | 51.1 ± 2.9 | 52.7 ± 3.0 | 0.633 | 51.7 ± 3.9[a] | 51.3 ± 3.8[a] | 52.8 ± 4.0[a] | 0.958 |
| Sulfisoxazole | 217 ± 13 | 203 ± 12 | 0.447 | 208 ± 12 | 211 ± 13 | 0.879 | 176 ± 17[a] | 219 ± 15[ab] | 239 ± 10[b] | 0.010 |
| Trimethoprim/Sulfamethoxazole 1:19 ratio[5] | 0.25 ± 0.025 | 0.24 ± 0.025 | 0.848 | 0.23 ± 0.023 | 0.27 ± 0.027 | 0.210 | 0.20 ± 0.020[b] | 0.15 ± 0.013[a] | 0.51 ± 0.089[c] | <0.001 |

[1]A total of 360 barrows (initially12.4 ± 0.05lb) were used in a 42-d study with 5 pigs per pen and 18 pens per treatment. Data reported as geometric mean of minimal inhibitory concentration (MIC) ± standard error of the mean. (Clinical and Laboratory Standards Institute (CLSI). 2018. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard, 5th ed. CLSI supplement VET08. CLSI, Wayne, PA.)

[2]Fecal samples from the same 3 pigs/pen were collected on d 4, 21, and 42 for *E. coli* isolation and further characterization.

[3]Yeast pre- and probiotics included ActiSaf Sc 47 HR+ at 0.1%, SafMannan at 0.05% and NucleoSaf at 0.05% in phase 1 diets, and then concentrations were lowered by 50% in phase 2 diets (Phileo by Lesaffre, Milwaukee, WI).

[4]Zinc oxide was added to supply 3,000 ppm of Zn for the duration of phase 1, and 2,000 ppm of Zn for the duration of phase 2.

[5]The MIC numerator of the ratio was reported for the antimicrobial's amoxicillin:clavulanic acid 2:1 ratio and trimethoprim/sulfamethoxazole 1:19 ratio.

[a,b,c] Superscripts signify statistical difference among sampling days at $P < 0.05$.