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SCOPE

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OBJECTIVE LENS

JOHN L. MCKILLIP, PH.D
MANAGING EDITOR, *FINE FOCUS*
ASSOCIATE PROFESSOR OF BIOLOGY,
BALL STATE UNIVERSITY

This issue you are reading now is the product of my Fall Semester spent in Iceland, where students at the University of Akureyri managed *Fine Focus* and successfully achieved several important goals for our journal's continued development. Among these goals was completing the application process for obtaining a Library of Congress International Standard Serial Number (ISSN) listing, which was granted in September. Secondly, the students were able to negotiate and secure a distribution contract with EBSCO Host, which will elevate our profile significantly, and allow published papers to be more readily accessible than ever before through a number of highly regarded databases and search engines. Thirdly, our Marketing Team revised our marketing plan to be more international in scope. Lastly, we successfully launched a crowdsource funding initiative on FirstGiving: (https://www.firstgiving.com/fundraiser/johnl-mckillip/FineFocus.)

This initiative will streamline donations important to augment our grant support. This financial need is ongoing, and goes 100% towards our production/print costs, student conference travel, and marketing materials. Since *Fine Focus* has no page charges, submission fees, or subscription price, our team this autumn decided to explore crowdsource funding as an exciting challenge to pursue, and we are very satisfied with this platform. Please consider visiting the site, viewing our posted video and other material, and donating to support undergraduate research in microbiology internationally. These four major goals were the framework surrounding our ongoing daily manuscript management, peer-review training, and other professional development that underscores every semester of *Fine Focus* student editors.

You can meet the team that raised the bar this past fall, by reading their perspectives at the end of this issue. Each delivered a unique and substantial contribution, and drew upon their distinct backgrounds and diverse expertise to produce an issue with more international flavor than ever before. Working with them in Iceland for a semester was rewarding and enriching.

I hope you enjoy reading this third issue of Fine Focus – the product of their dedication and commitment. We always welcome your input, suggestions, and general feedback on how we are doing. Let us know at finefocus@bsu.edu or look for us this Spring/Summer at ASMCUE, the ASM General Meeting in Boston, MA, the Indiana Branch ASM meeting in Fort Wayne, or the American Dairy Science Association (ADSA) July conference in Orlando, FL.

Best wishes for a productive spring and summer!

-JLM

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CHARACTERIZATION OF INDIGENOUS BACTERIAL COMMUNITIES IN CRUDE-OIL IMPACTED SITES AT OBAGI TOWN, ONELGA, RIVERS STATE, NIGERIA.

CHIOMA BLAISE CHIKERE*, CHINYERE AUGUSTA AJUZIEOGU, AND MICHAEL CHUKWUGOZIEM MILLER
DEPARTMENT OF MICROBIOLOGY, FACULTY OF SCIENCE, UNIVERSITY OF PORT HARCOURT, RIVERS STATE, NIGERIA

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CORRESPONDING AUTHOR

*Chioma Blaise Chikere
chioma.chikere@uniport.edu.ng

KEYWORDS

- Hydrocarbon-utilizers
- Indigenous
- Bacteria
- Crude-oil
- Bioremediation

ABSTRACT

Hydrocarbon utilizers are expected to be indigenous in crude-oil polluted environments. The isolation and characterization of hydrocarbon utilizers is often a key strategy in bioremediation of hydrocarbon-polluted environments. In this study, crude-oil polluted soil samples from Obagi town, Onelga, Rivers state were enumerated and characterized for putative hydrocarbon utilizing bacterial populations. Biochemical characterization identified five bacterial species representative of five genera: *Bacillus, Pseudomonas, Acinetobacter, Micrococcus* and *Staphylococcus*. Amongst the genera of bacteria isolated, *Bacillus* had the highest frequency of occurrence (40%). The mean count of total heterotrophic bacteria was 1.7 X 10^7 cfu/g, while hydrocarbon utilizing bacteria (HUB) count mean density was 1.0 X 10^7 cfu/g for the three soil samples. Statistical analyses revealed no significant difference at $p>0.05$ between Total Heterotrophic Bacterial (THB) and Hydrocarbon Utilizing Bacterial (HUB) counts, suggesting that most of the bacteria present in the sampled sites were hydrocarbon utilizers. Findings from this study suggest the presence of indigenous putative hydrocarbon utilizing bacteria in the crude-oil polluted soil of Obagi town. Hence, a promising potential exists for future bioremediation studies on the site.

INTRODUCTION

One of the major environmental problems today is hydrocarbon pollution by the petrochemical industry (1), and widespread release of aromatic hydrocarbons through spillages and leakage from underground tanks and steamers, causing extensive contamination of surface soils, ground water, seas and oceans (2). Mechanical and chemical methods for remediation of hydrocarbon polluted environments are often expensive, technologically complex and lack public acceptance (3).

Biodegradation by microorganisms is fundamental in the removal of hydrocarbons and xenobiotic substances (4). Irrespective of the wealth of research relating to microbial degradation of hydrocarbons, knowledge pertaining to which organisms are the key players in hydrocarbon degradation in the environment is limited (5).

In order to combat this challenge, it is pertinent to first assess the hydrocarbon degrading potential of the microorganisms before any bioremediation intervention rather than just focus on the removal of individual hydrocarbon compounds *via* mechanical and chemical methods for remediation (6). This approach will provide new insights for improving the management of such environments.

It has been observed that low molecular weight hydrocarbons like anthracene and naphthalene are usually readily degraded by bacteria in soil and under laboratory conditions (7). Other studies have also shown that petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast, and microalgae. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats (16). Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas*, *Marinobacter*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia* and *Gordonia* groups (8).

The aim of this study was to evaluate the microbial heterogeneity of crude-oil polluted soils at Obagi town, Onelga, Rivers state to be able to predict their inherent potential for hydrocarbon utilization.

MATERIALS AND METHODS

DESCRIPTION OF SAMPLE SITES

The study site, Obagi town, is a mangrove environment whose center lies at a latitude of 5.25114 and longitude of 6.61298. Surface soil from the three sample sites in Obagi town were collected to enumerate and characterize bacterial isolates that have the potential for utilizing hydrocarbons. The samples were placed in sterile polyethylene bags and transported to the laboratory for analysis.

ENUMERATION OF TOTAL HETEROTROPHS AND HYDROCARBON UTILIZING BACTERIA

One gram (1g) each of the soil samples were serially diluted (10^{-1} to 10^{-6}) in 9ml normal saline. Aliquots (0.1ml) from dilutions of 10^{-4}, 10^{-5} and 10^{-6} of soil samples were plated in duplicate on sterile Plate Count Agar (Merck, Germany) and incubated at 37°C for 24 hours for total culturable heterotrophic bacteria counts. For hydrocarbon utilizing bacterial counts, enumeration was performed as described by Hamamura *et al.* (15) where appropriate dilutions of soil sample suspensions were plated on Busnell-Haas Agar (Sigma-Aldrich, USA), and hydrocarbons were supplied through the vapour phase to putative hydrocarbon utilizers by placing sterile filter papers impregnated with 5ml Okono crude oil on the lids of the inverted plates and incubated for 7 days at 37°C.

PURIFICATION AND IDENTIFICATION OF PUTATIVE HYDROCARBON UTILIZING BACTERIAL ISOLATES

Discrete colonies of different putative hydrocarbon utilizing bacteria (HUB) were randomly picked using a sterile wire loop and subcultured for purification by streaking on nutrient agar plates and incubated at 37°C for 24 hours. Individual bacterial colonies were presumptively identified using morphological and biochemical tests as described in Bergy's

Manual for Determinative Bacteriology (Gram stain, motility test, catalase test, oxidase test, citrate utilization test, indole test, hydrogen sulphide test, urease test, triple sugar iron test, methyl red, and Voges–Proskauer test)(17).

STATISTICAL ANALYSIS

Data obtained from the study were subjected to statistical analysis using T-test and one way analysis of variance (ANOVA) at 0.05 confidence level ($p<0.05$).

RESULTS

Total heterotrophic bacterial counts for each soil sample were (1.9×10^7cfu/g, 2.0×10^6cfu/g, and 3.0×10^7cfu/g) (Fig. 1) and hydrocarbon utilizing bacterial counts were (2.1×10^6cfu/g, 1.4×10^6cfu/g and 2.7×10^7cfu/g) respectively (Fig. 2).

We observed that there was a significant difference between THB and HUB in soil sample A ($p=0.001$), which suggests that the hydrocarbon utilizers (HUB) present in soil sample A are not a majority proportion of the bacterial community (THB). However, in samples B and C (i.e, THB and HUB in sample B, and THB and HUB in sample C) there was no significant difference observed ($p=0.084$ and 0.441, respectively), which suggests that most of the culturable bacterial population (THB) have become putative hydrocarbon utilizers (HUBs).

A total of 28 bacterial species were isolated as THB, while 19 bacterial species were isolated on mineral salt medium (Bushnell Haas Agar) and identified morphologically and *via* biochemical tests.

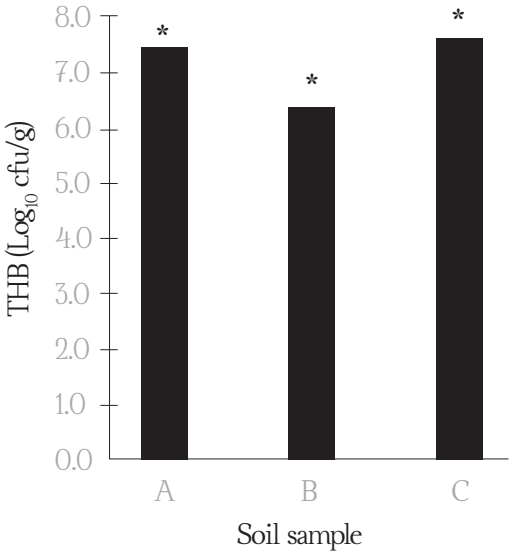

Figure 1. Log cfu/g of Total Heterotrophic Bacteria of the various oil impacted soil samples
*= significant difference exists at $p=0.05$

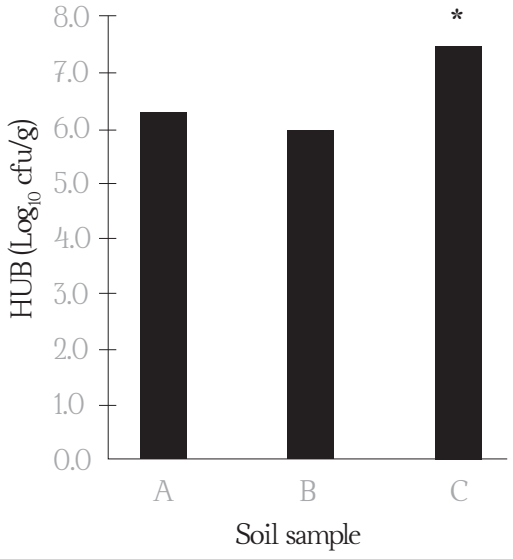

Figure 2. Log cfu/g of Hydrocarbon Utilizing Bacteria of the various oil impacted soil samples
*= significant difference exists at $p=0.05$

Table 1: Characterization of bacterial isolates.

Legends:
R: Rod; C: Cocci; +: Positive; –: Negative

Isolates	Gram reaction	Tentative identity
B2SA1	– R	*Klebsiella sp.*
B2SA2	+ R	*Corynebacterium sp.*
B2SA3	+ C	*Micrococcus sp.*
B2SA4	– R	*Enterobacter sp.*
B2SA5	– R	*Flavobacterium sp.*
B2SA6	– R	*Azotobacter sp.*
B4SA1	– R	*Escherichia coli*
B6SA1	+ C	*Proteus sp.*
B2SB1	+ C	*Staphylococcus sp.*
B2SB2	– R	*Proteus sp.*
B6SB1	– R	*Serratia sp.*
B8SB1	– R	*Pseudomonas sp.*
B8SC1	– R	*Pseudomonas sp.*
1A1	+ R	*Bacillus sp.*
1A2	+ R	*Bacillus sp.*
2A1	+ C	*Staphylococcus sp.*
2A2	– C	*Acinetobacter sp.*
2A3	+ C	*Micrococcus sp.*
1B1	– C	*Acinetobacter sp.*
1B2	– R	*Pseudomonas sp.*
2B1	+ C	*Micrococcus sp.*
2B2	– R	*Pseudomonas sp.*
2B3	+ R	*Bacillus sp.*
1C1	+ R	*Bacillus sp.*
1C2	+ C	*Staphylococcus sp.*
1C3	+ R	*Bacillus sp.*
2C1	+ R	*Bacillus sp.*
2C2	– R	*Pseudomonas sp.*

Table 2: Frequency of occurrence of hydrocarbon utilizing bacteria genera isolated from oil impacted soil samples.

Genus	Frequency of occurrence (%)
Bacillus spp.	31.6
Pseudomonas spp.	26.3
Acinetobacter spp.	10.5
Micrococcus spp.	15.8
Staphylococcus spp.	15.8

The characteristics of the Total Hydrocarbon Bacteria are presented in Table 1. The isolates were Gram positive and negative rods and cocci, and were tentatively identified to be representatives of the genera *Staphylococcus, Bacillus, Micrococcus, Pseudomonas Acinetobacter, Klebsiella,* and *Enterobacter*.

Nineteen putative HUB species were isolated with the dominance (frequency of occurrence) of representatives related to the genera *Bacillus and Pseudomonas* (Table 2). *Bacillus* spp. was observed to be the most isolated bacterial genera from the soil samples.

DISCUSSION

The study site, Obagi town, is a mangrove environment challenged by crude oil pollution. In this study, a culture dependent technique was used to isolate and characterize a putative hydrocarbon utilizing bacterial population indigenous in crude–oil impacted soil. Culture dependent techniques have been used by several researchers to isolate bacteria involved in petroleum hydrocarbon utilization (2,3,6,7,10,13,21,22–26).

Results showed that the mean values of total culturable heterotrophic and hydrocarbon utilizing bacterial counts from each soil sample (Soil A, B and C) were all moderate-to-high. These high counts may be attributed to the presence of organic matter content (nutrients) and favorable ecological factors that underpin the survival of these bacterial species. Consequently, it indicates a viable population with the potential to initiate and maintain hydrocarbon degradation. A similar finding was made by Abu and Dike (1) and Chikere and Ekwuabu (11).

Results revealed that most of the microorganisms present in the various sample sites were hydrocarbon degraders. A similar observation was reported by Chikere and Ekwuabu (11).

Furthermore, a one-way analysis of variance (ANOVA) showed that significant difference existed in the THB (Log10 cfu/g) counts in soil samples A, B and C with *p*-values of <0.0001 and HUB (Log10 cfu/g) counts in soil samples A, B and C with *p*-values of <0.0001.

It was also observed that HUB counts in Soil C were significantly higher compared to Soil A and B. This is likely due to the hydrocarbon concentration in Soil C. Soil C may have been chronically impacted by hydrocarbons compared to the other soils and the indigenous microbes as part of a survival strategy, and may have adapted to degrading/utilizing these hydrocarbons as carbon sources, thus increasing their counts in the area of concern. When environmental changes occur as a result of the presence of a pollutant, there will be the development of new metabolic pathways and acquisition of new functions by mutations. This increases diversity with one population by the formation of a new niche (hydrocarbon utilizers) such that under *in vitro* isolation processes, microorganisms with beneficial mutations have growth advantages in comparison to unadapted organisms (24).

A total of 28 bacterial isolates were obtained as THB while a total of 19 bacterial isolates were confirmed on mineral salt medium (Bushnell Haas Agar) with crude oil as the carbon source. These bacterial species were Gram positive and mostly Gram negative representative of the *Gammaproteobacteria* group. This correlates with earlier studies (11,20). The putative hydrocarbon utilizing bacterial isolates were observed to be representative of the genera *Bacillus*, *Staphylococcus*, *Acinetobacter*, *Micrococcus*, and *Pseudomonas*. These genera of bacteria have been reported as hydrocarbon utilizing bacteria by several researchers (9,12,18,27,28), and this suggests that these species are key players in biodegradation of hydrocarbons in these sites.

Bacillus spp. was recorded to be the dominant bacterial genus from the soil samples having 6 out of 15 isolates, followed closely by *Pseudomonas* spp. having 5 as shown in Table 1. This corroborates results observed by Chikere *et al.* and Kadali *et al.* (9,19). *Bacillus* spp. have been reported to be more tolerant to high concentrations of hydrocarbons in soils due to their resistant endospores (14,19,29). The capability of *Pseudomonas* species to grow and degrade different hydrocarbon content has also been reported. They have been isolated from worldwide polluted sites (5).

CONCLUSION

Mangroves are intertidal ecosystems along coastlines of tropical and subtropical regions, thus they are prone and subjected to urban and industrial effluent discharges and accidental oil spills. Results from this study revealed the utilization of hydrocarbons by the indigenous bacterial community in crude-oil polluted mangrove soil of Obagi town, evidenced by appreciable total heterotrophic bacterial and hydrocarbon utilizing bacterial counts from a mineral salt medium (Bushnell Haas) containing crude-oil as the only carbon source. The isolated bacterial species such as *Bacillus* and *Pseudomonas* amongst others have also been isolated and implicated as hydrocarbon utilizers in a number of studies. This study therefore provides a database of the indigenous hydrocarbon utilizing bacterial community present in Obagi town for application in bioremediation studies, although these data are based on culturable microbial populations present in the sampled locations. Hence, molecular methods that characterize functional genes would be a more powerful approach to study hydrocarbon utilizers in a mixed microbial community (8,30,31).

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FREQUENCY OF ANTIBIOTIC RESIDUES IN A CENTRAL WISCONSIN DAIRY

MORGAN L. BEILKE AND JEFFERY D. FRITZ*
DEPARTMENT OF BIOLOGICAL SCIENCES
UNIVERSITY OF WISCONSIN – MARSHFIELD/WOOD COUNTY
MARSHFIELD, WI USA

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ABSTRACT

Antibiotics, used to maintain healthy dairy animals, persist in milk produced by treated animals for several days after therapy. Antibiotic residues, if present in the milk supply, negatively impact suitability for processing and consumption. This is an area of concern in the dairy industry and for the general public. This study explores the incidence of antibiotic residues in milk provided to a Central Wisconsin dairy. Random, unidentified samples were obtained over a four month period from producers located within a 100–mile radius of a dairy located in Granton, Wisconsin. All samples were tested within one week of their receipt, and maintained at refrigerated temperatures at all times prior to testing. Following testing, the origin of the samples was revealed. Samples were derived from two milk populations: those intended for human consumption (bulk milk), and those from cows under surveillance following antibiotic treatment (milk removed from the commercial supply). All bulk milk samples tested negative for over 55 different antibiotic residues, while all samples from the surveillance population tested positive for antibiotic residues. Our results are consistent with those observed nationally, and suggest that dairy producers recognize the ongoing concern of antibiotic contamination in the milk supply and are taking steps to prevent antibiotic contamination in milk. Our findings also suggest the public should have minimal concern with respect to antibiotic residues in the commercial milk supply.

CORRESPONDING AUTHOR

*Jeffery D. Fritz
jeffery.fritz@uwc.edu

KEYWORDS

- Antibiotics
- Drug residues
- Dairy foods
- Food safety
- *Bacillus stearothermophilus* var. *calidolactis*

INTRODUCTION

Antibiotic use on dairy farms is imperative to treating ill cows. Use of antibiotics is most common in the treatment of mastitis, a painful inflammation of the cow's udder (8). There are approximately 40 drugs approved for use in lactating dairy cows (14). Once an ill cow is treated, the antibiotics may be retained in the cow body for several days. This makes it important for producers to test regularly for drug residues in treated cows. Generally, all antibiotics should be excreted from milk within 72 hours of treatment (7). However, if not used properly, antibiotics can be retained in the cow for longer periods. The Food and Drug Administration surveys indicate that improper use of drugs in the

control of mastitis is the major source of antibiotic residues found in milk supply (10).

If present, antibiotic drug residues in our milk supply pose a public health problem. Specifically, the consumption of milk contaminated with antibiotics can detrimentally influence our ability to treat human infections, because infectious agents adapt to the continual exposure of drugs (12). Further, for those individuals who are already sensitized to antibiotics, overuse of these antibiotics in dairy cows could result in allergies if these products make their way to our local grocery stores. For example, penicillin is the most frequent human drug allergy and is common – affecting approximately 10% of the population with anaphylaxis in 0.01% and fatal reactions in 0.0015% of cases (1).

Most concern with antibiotic persistence is placed on the development and spread of virulent and resistant bacteria within the dairy animal. These antimicrobial-resistant pathogens may emerge in the food production chain, and can be transmitted to humans, and cause infection. Salyers and Shoemaker outline the potential concern of the ability of bacteria to exchange resistance genes through their exposure to the intestinal tracts of consumers, as well as their potential to interact with any pathogenic bacteria in passage through the human colon (13).

Human exposure to resistant bacteria from non-human usage of antibiotics can create new, more severe, infections that would not have otherwise occurred, and cause an increased frequency of treatment failures (5).

Because the foodborne route is a major transmission pathway for bacteria and resistance genes (5), as well as antibiotic residues themselves, understanding farming practices relating to managing and treating ill cows becomes relevant to our everyday lives, especially for those purchasing commercial milk and milk products.

Consequently, educating the public on milk sanitation practices and the safety of our nation's milk supply is an important area of research. Our investigation was aimed at exploring the possible public health issue of antibiotic persistence in milk products through the collection and testing of raw milk samples collected from a dairy in Granton, WI. Current national findings place the frequency of antibiotic persistence in the commercial milk supply below 0.015% (14-16), and suggest that the public, at present, should have minimal concern regarding antibiotic residues when consuming commercial milk products. We hypothesize that the commercial milk supply contains very little (and likely undetectable levels) antibiotic residues.

MATERIALS AND METHODS

Milk samples were obtained from Lynn Dairy Inc. (located in Granton, Wisconsin), between March 09th, 2015 – June 07th, 2015. At the time of collection, samples were identified through a six-digit number with no indication of milk supplier or type of milk. Milk suppliers for these samples spanned a 100-mile radius from the dairy plant where milk samples were processed. Samples were received in batches of 25 and each batch was tested within a week of receipt. Prior to testing, samples were stored in a refrigerator below 6°C.

A total of 268 samples were tested for antibiotic residues using DSM's Delvotest SP NT, a simple and reliable test that detects over 55 different types of antibiotics including beta lactams, sulphonamides, aminoglycosides, quinolones, macrolides and tetracyclines (4). The Delvotest incubator allows for 10 ampoules to be tested at a time. In this study, 9 milk samples and 1 control sample of penicillin were tested each time. Separate pipettes were used to add 0.1 ml of each milk sample into the designated ampoules. Each ampoule was marked with the six-digit sample number provided at time of collection for sample identification. Individual pipettes were dipped 1 cm into the sample which was then transferred onto the agar medium in the designated ampoule. The incubation temperature was set at 64 degrees Celsius and testing took approximately 3 hours (2).

The test was read through analysis of the solid and buffered agar medium containing a pH indicator and a test organism, *Bacillus stearothermophilus* var. *calidolactis* (3). A purple/blue reading implies the reduced growth of the test organism, and therefore a positive test for antibiotics in the raw milk. A green/yellow test implies the growth of the test organism, and therefore a negative test result for antibiotics in the sample.

Table 1. Antibiotic residue analysis of milk samples in this study

Source of Sample	Total Samples	Number Positive	Percent Positive	Disposition per Pounds
Bulk Milk Pickup Tanker	3,147,302	429	0.014%	17,754,000
Pasteurized Fluid Milk and Milk Products	37,707	0	0.000%	0
Producer	445,223	266	0.060%	240,000
Other	49,953	8	0.016%	99,000

RESULTS

Following testing, milk sample numbers were identified as originating from bulk milk samples or milk from animals under surveillance for antibiotic residues. The bulk milk samples total 264 of the 268 samples tested negative for antibiotic residues. The remaining four samples were obtained from animals under surveillance for antibiotic residues and all four of these samples tested positive for antibiotic residues (Table 1).

DISCUSSION

BULK TANK SAMPLES

All 264 samples originating from bulk milk samples were those that were deemed fit for bulk tank inclusion by the dairy producer, and thus added to the truckload. However, prior to acceptance, dairy processors will test the truckload once it arrives at the processing establishment as a precautionary measure. If the bulk tank is negative for antibiotic residues, it is presumed that the tank is antibiotic free and safe to use. If a bulk tank is positive for antibiotic residues, the truckload will be dumped. Additionally, individual bulk, raw milk samples from the truckload will be tested in order to determine the cause of contamination. In our study, all samples deemed fit for bulk tank inclusion were completely antibiotic free.

NON-BULK TANK SAMPLES FROM ANIMALS UNDER SURVEILLANCE

Next, it is important to note that the four positive test results observed in our study were not considered "true positives" for antibiotics as these milk samples were not intended to be included into the bulk tank by the dairy producer. Specifically, milk producers may send individual milk samples from treated cows to milk processors for antibiotic testing. This precautionary routine is used to see if the drug still persists within the treated cow's system (6). As it is unlawful to place unfit milk on the market for human consumption, this primary screening method is integral in determining whether the milk is fit for inclusion in the bulk tank (6). Importantly, these samples are relevant to our data as it provides evidence that milk producers are exercising safe milk sanitation practices by monitoring treated cows and separating their milk from the central milk supply.

CONCLUSIONS

Our findings are consistent with surveys from the Food and Drug Administration's annual report on national milk drug residues. The national survey under the FDA has been conducted since 1994, and illustrated the findings of bulk milk antibiotic testing over the course of 20 years (16). These surveys show a peak in 1996 with 0.1% of bulk milk samples testing positive for antibiotic residues to its current low of 0.014% in 2014 (16).

The most recent FDA survey was conducted from October 1, 2013 to September 30, 2014 (16). A total of 4,008,662 tests were conducted, consisting of nine different groups of individual drugs or different drug families. A total of 3,680,185 samples were collected and analyzed for animal drug residues. Of those samples, only 703 were positive for a drug residue. The majority of samples (3,147,302) were samples from bulk milk pickup tankers of raw milk. Only 429 samples, or 0.014%, tested positive for a drug residue. Importantly, no antibiotic residues were found in samples that came from pasteurized fluid milk, or samples considered "retail-ready" (16). This would suggest that for our study, over 7000 bulk milk samples from our dairy would need to be surveyed before a single positive test would be likely.

The National Milk Drug Residue Database is a voluntary industry reporting program that identifies the following: the extent of national testing activities, the analytical methods used, the kind and extent of the animal drug residue identified, and the amount of contaminated milk (16). Mandatory reporting of drug residues in milk is required under the Pasteurized Milk Ordinance; a strict set of requirements

for milk production, transportation, pasteurization, equipment sanitation, and labeling (15). Dairy farmers and processors work closely with the Food and Drug Administration, the U.S. Department of Agriculture (USDA), and state regulatory agencies to promote the highest safety standards for milk (9). As such, it is not surprising that we found no bulk milk samples testing positive for antibiotic residues in our study given its sample size and these control measures.

Reducing the use of antibiotics minimizes the likelihood of antibiotic resistant bacteria to develop, as well as drug residues to exist in the milk supply. Maintaining antibiotic free milk begins with the efforts of dairy producers in implementing effective milk sanitation practices. Further, it is essential that producers and milk processors build trusting partnerships that allow for effective and economically sound business relationships. Preliminary screening methods for drug residues at milk processing establishments, previously discussed in this study, are critical in the continued prevention of contaminating truckloads of milk. In conclusion, our study is in agreement with national surveys, which have demonstrated that these efforts are being maintained, as our results, along with results on a national level, show extremely low incidences of antibiotic persistence in milk. Ultimately, these findings should provide reassurance that milk products will continue to be safe for consumption, and that efforts in reducing antibiotic contamination are ongoing and well implemented by the industry.

ACKNOWLEDGMENTS

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ASPERGILLUS SCLEROTIORUM FUNGUS IS LETHAL TO BOTH WESTERN DRYWOOD (*INCISITERMES MINOR*) AND WESTERN SUBTERRANEAN (*RETICULITERMES HESPERUS*) TERMITES.

GREGORY M. HANSEN, TYLER S. LAIRD, ERICA WOERTZ, DANIEL OJALA, DARALYNN GLANZER, KELLY RING, AND SARAH M. RICHART*

DEPARTMENT OF BIOLOGY AND CHEMISTRY, AZUSA PACIFIC UNIVERSITY, AZUSA CA

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CORRESPONDING AUTHOR

*Sarah M. Richart
srichart@apu.edu

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- Entomopathogenic
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- Biological control
- Pest management
- *Aspergillus sclerotiorum*
- *Reticulitermes hesperus*
- *Incisitermes minor*
- Termites

ABSTRACT

Termite control costs $1.5 billion per year in the United States alone, and methods for termite control usually consist of chemical pesticides. However, these methods have their drawbacks, which include the development of resistance, environmental pollution, and toxicity to other organisms. Biological termite control, which employs the use of living organisms to combat pests, offers an alternative to chemical pesticides. This study highlights the discovery of a fungus, termed "APU strain," that was hypothesized to be pathogenic to termites. Phylogenetic and morphological analysis showed that the fungus is a strain of *Aspergillus sclerotiorum*, and experiments showed that both western drywood (*Incisitermes minor*) and western subterranean (*Reticulitermes hesperus*) termites die in a dose-dependent manner exposed to fungal spores of *A. sclerotiorum* APU strain. In addition, exposure to the *A. sclerotiorum* Huber strain elicited death in a similar manner as the APU strain. The mechanism by which the fungus caused termite death is still unknown and warrants further investigation. While these results support that *A. sclerotiorum* is a termite pathogen, further studies are needed to determine whether the fungal species has potential as a biological control agent.

INTRODUCTION

Termites are a common structural and economic pest worldwide. While estimates vary considerably, termites are responsible for between $500 million and $1 billion of wood damage and cost $1.5 billion in control measures per year in the United States alone (7,32). An array of problems have been associated with the use of chemical pesticides to control pests like termites, such as the development of resistance in target organisms, environmental pollution, and toxicity to non-target organisms, including humans (25). Avoidance behavior to such chemicals is also a significant challenge in controlling termite populations (33). Because of such complications, biological termite control agents that are natural insect pathogens have been proposed, including parasitoids, viruses, bacteria, protozoa, nematodes, and fungi (7).

Fungi, especially those that occur naturally in termite habitats, are promising candidates for use as biological control agents because they are well-adapted to survive in such environments (33). Entomopathogenic fungal species such as *Beauveria bassiana*,

Paecilomyces fumosoroseus, and *Metarhizium anisopliae* have been suggested as potential termite control agents, with the latter being the best studied and most effective of the three (17,19,28). There are limitations, however, to the effectiveness of fungi as termite control agents in their native habitats, due to specific termite behaviors such as allogrooming (6), spore avoidance (22), and vibrational alarm signals in response to fungal spores (22,29). In addition, naturally occurring antimicrobial compounds in termite feces and alimentary canals have been described (6, 23).

In this current study, we hypothesize that a novel isolated fungus is entomopathogenic to termites. The research objectives are to genetically and morphologically identify the APU strain, experimentally infect two different species of termites, *Incisitermes minor* and *Reticulitermes hesperus*, with APU strain, and determine if dose has any effect on termite survival.

MATERIALS AND METHODS

TERMITE COLLECTION

Incisitermes minor and *Reticulitermes hesperus* termites were obtained either from decomposing firewood in Arcadia, CA, or from branches found in the foothills of the San Gabriel Mountains in Glendora, CA. Termite-containing wood was stored in plastic bins in a cool, dark room until used in experiments. To collect termites, wood was broken apart and termites were picked up with lightweight forceps or paintbrushes, and placed in Petri dishes with a brown paper towel as food, with additional moisture for *R. hesperus*. Termites were left two or more days in Petri dishes after collection and before the start of an experiment to ensure that healthy, uninjured insects were used.

FUNGAL CULTURES, DRY SPORE COLLECTION, AND QUANTIFICATION.

A strain of fungus believed to have eliminated an *I. minor* colony at Azusa Pacific University in 2009 (called "APU strain") was isolated from a deceased termite and cultured on Difco Sabouraud Dextrose Agar (SDA) (BD Diagnostics, Franklin Lakes, NJ). Fungus was repeatedly subcultured on SDA plates to ensure a pure culture. *Aspergillus sclerotiorum* strain Huber (16892, American Type Culture Collection, Rockville, MD) was used as a comparison strain. For spores that were used to infect termites, cultures of both strains were grown in the dark on SDA plates with penicillin-streptomycin (100 U/ml penicillin and 100 mg/ml streptomycin, diluted from 100X stock solution, HyClone Laboratories, Logan, UT) at 25°C for approximately 3 weeks, or until a confluent layer of yellow spores was present. Each plate was then either used at 3 weeks or later to infect termites directly.

To collect spores, confluent plates were inverted and placed atop a 0.45 mm sterile vacuum filter, then gently and repeatedly tapped to dislodge spores. Spores were then left in the filter apparatus under vacuum to dry for approximately 3 hours. The resulting dry spore powder was quantified by resuspending in a solution of 0.1% (v/v) Tween-20 and counting in a hemocytometer. Live spore quantification was determined by standard plate count on SDA in duplicate or triplicate for each dilution, and fungal colonies were counted on days 2–4, depending on the growth.

ITS REGION AND β-TUBULIN GENE POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

The species identification of the strain of fungus that was presumed to kill a colony of *I. minor* termites in 2009 (called "APU strain") was initially performed by PCR amplification of the internal transcribed spacer region (ITS) of fungal ribosomal DNA isolated using ZR Fungal/Bacterial DNA miniprep kit (Zymo, Irvine, CA) according to the manufacturer's protocol, with the following additional step: prior to DNA extraction, fungal samples suspended in water were frozen at –20°C. The primer pair made for amplification of the fungal ITS region was ITS1 and ITS4 (sequences as previously described) which amplifies a region that includes the entire 5.8S rRNA gene (31). PCR was also performed using primers (Bt2a and Bt2b), designed to amplify *Aspergillus* β–tubulin, as previously described (10). PCR products were purified using the Wizard SV Gel and PCR Clean–up kit (Promega, Madison, WI), and sequenced at the DNA Sequencing Core Lab (City of Hope, Duarte, CA).

PHYLOGENETIC ANALYSIS OF ITS AND β-TUBULIN GENE REGIONS OF APU STRAIN

The APU strain ITS sequence was subjected to a megablast search using the fungi RefSeq ITS database in BLAST (National Center for Biotechnology Information). The top eight sequences ranked by maximum score were used. *Aspergillus tanneri*, the 8th sequence, was used as an outgroup, similar to a previous phylogenetic study (27). For ITS, sequences were aligned along with the APU strain sequence using ClustalW in MEGA6 software. Default settings were used for the alignment. Under the pairwise alignment and multiple alignment sections, the gap opening penalties were set to 15, and the gap extension penalties were set to 6.66. Under the multiple alignment section, the gap opening penalty was set to 15 and the gap extension penalty to 6.66. The DNA weight matrix was set to IUB. The transition weight was 0.5. The use of a negative matrix was turned off. The delay of divergent cutoff was set to 30%. After alignment, the sequences were trimmed to 562bp, and used to construct maximum likelihood phylogenetic trees using MEGA6. The Tamura 3–parameter model was selected, as well as gamma distributed with invariant sites (G + I), as done previously (27). The number of discrete gamma categories was 5. Gaps and missing data were subject to complete deletion. The ML Heuristic method was set to Nearest–Neighbor–Interchange, and the initial tree was set to Default – NJ/BioNJ. The branch swap filter was set to very strong. The number of threads selected was 1. Bootstrap analysis of 1000 replicates was performed.

The sequence of a region of the β–tubulin gene, a commonly sequenced gene in fungi, was also subjected to a megablast using BLAST. The first 14 strains of unique species similar to APU strain were chosen for phylogenetic tree construction. The *Aspergillus tanneri* β–tubulin sequence was acquired from NCBI as an outgroup, as done previously (27). A total of 15 sequences of β–tubulin regions were acquired and aligned along with the APU strain sequence in MEGA6 using the same parameters as mentioned above for the ITS region. After alignment, the sequences were trimmed to 579bp. A maximum likelihood phylogenetic tree was constructed in MEGA6. All selected options were the same as done for the ITS region as described earlier except that the Kimura 2–parameter was used as done

previously (27). In addition, the first, second, third, and noncoding sites were selected. Bootstrap analysis was performed with 1000 replicates.

METHODS FOR MORPHOLOGICAL STUDIES AND MICROSCOPY

For light microscopy analysis, fungal microcultures were set up by inoculating each of the 4 edges of a 1 cm x 1 cm square of Sabouraud dextrose agar (SDA) on top of a microscope slide, and covering the inoculated medium with a sterile glass microscope cover slip. The slide culture was set on a small platform in a Petri dish, and extra water was put in the bottom of the dish below the level of the slide to allow proper moisture. Fungi were grown in the dark at 25°C for 3–4 days. To view, cover slips were carefully removed and placed on top of clean microscope slides with lactophenol cotton blue stain (Medical Chemical Corp., Torrance, CA) and initial photographs were taken using a Leica DM70 microscope. For measuring APU strain features, fungus was inoculated onto a SDA square but grown directly on a micrometer, and viewed using a Nikon TMS microscope. To view macroscopic fungal colony growth, fungi were inoculated onto Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA) plates and grown in the dark at 25°C for 7 days, as previously described (14).

An electron micrograph was taken by directly putting a sample on a conductive double-sided adhesive (PELCO Image Tab™, Ted Pella, Inc, Redding, CA) and viewed using a Hitachi TM-1000 tabletop scanning electron microscope.

I. MINOR TERMITE INFECTION WITH APU STRAIN OR HUBER STRAIN

An initial study was conducted to assess the lethality of APU strain to *I. minor*. Thirty *I. minor* termites of differing developmental stages were individually inoculated on the back by inoculation loop with APU strain from an overgrown SDA culture plate. Thirty control termites were untreated. Each group was placed in a Petri dish with 90 mm Whatman No. 3 paper for food. Live termites were counted each day for 7 days.

To compare the effect of APU strain to *A. sclerotiorum* Huber strain on *I. minor* termites, 40 termites per group were placed on a SDA plate overgrown with either the APU strain or Huber strain and allowed to walk around for 2 minutes, during which they became covered in fungal spores. Forty control termites were removed from the same colony as the experimental group and were left untreated. Each group of 40 was then placed on a piece of 90 mm Whatman No. 3 paper in a Petri dish, and live termites were counted daily except on day 5 for the duration of the experiment.

DOSE RESPONSE OF *I. MINOR* TERMITES TO APU STRAIN

The response of *I. minor* termites to increasing doses of *A. sclerotiorum* APU strain was determined for 4 different doses (150 mg, 70 mg, 30 mg, and 3 mg) by inoculating n=3 groups containing 25 termites each of differing developmental stages with each dose. The 3 mg doses were prepared by adding 9 parts powdered sugar to 1 part fungal spores, and using 30 mg of the sugar/fungal mixture per Petri dish. Each dose of fungal spores were uniformly spread on a 90 mm disk of Whatman No. 3 filter paper in a Petri dish,

where termites were kept throughout the experiment and thus exposed to spores for the duration of the experiment. The 0 mg control groups (*n*=2) were prepared in the same manner, except spread with 75 mg of powdered sugar instead of fungal spores. The termite groups were kept in the dark, and the number of live individuals was determined daily (approximately every 24 hours) over 9 days. Each mg of spores corresponded to 1.07 x 10^8 spores by hemocytometer estimation. Viable counts were not performed. A one way ANOVA followed by a TukeyHSD test (RStudio Version 0.99.441) was performed in order to determine the significance of the doses on the number of living termites on days 5 and 9 of the experiment.

DOSE RESPONSE OF *R. HESPERUS* TERMITES

Ten or 25 mL of a 1.5% (w/v) agar solution (Bacto–agar, BD Difco Franklin Lakes, New Jersey) was first pipetted into glass Petri dishes and allowed to solidify to provide enough moisture for subterranean termites and inhibit further fungal growth (33). Whatman No. 3 filter paper disks (90 mm) were placed on top of the solidified agar as a food source. Various quantities of dry APU strain *A. sclerotiorum* spores were evenly spread on top of the paper. The following quantities of spores were used: 25, 50, 75, 100 mg. Each mg corresponded to 1.05 x 10^8 total spores as counted by hemocytometer, and 4.86 x 10^5 viable spores by plate count determination. For the control groups, 75 or 100 mg of powdered sugar was spread on the filter paper so as to mimic the powdery nature of the spores. Termites were separated into groups of 35 termites of differing developmental stages. The groups were then introduced into dishes containing either fungal spores or powdered sugar (*n*=2 for 100 mg spores; *n*=3 for all other dosages, where data were pooled from two separate experiments). The termites lived in the dishes for the duration of the experiment, and their survival was recorded daily for 9 days. All of the dishes were stored in the dark and only exposed to light when survivability was recorded. A one way ANOVA followed by a TukeyHSD test (RStudio Version 0.99.441) was performed in order to determine the significance of the doses on the number of living termites on day 9 of the experiment.

RESULTS

FUNGUS ISOLATE EXHIBITS *I. MINOR* TERMITE PATHOGENICITY

Initially, *I. minor* termites were exposed to APU strain spores and the results of this experiment showed that the number of live termites inoculated with the APU strain declined throughout one week of observation, beginning with the first inoculated termite death on day 3 (Fig. 1A). By day 7, all 30 of the inoculated termites were dead. By contrast, all 30 uninoculated control termites were alive until day 6, and on day 7, there were still 27 live control termites. Inoculated termites that died were examined using light microscopy for signs of fungal infection, such as shown in Fig. 1B, and upon transfer to fungal media (Sabouraud Dextrose Agar), grew fungus that appeared very morphologically similar (data not shown). Scanning electron microscopy of dead inoculated termites indicated fungal growth, seemingly of one type that, due to its unenclosed spores, belonged to the phylum Ascomycota (Fig. 1C).

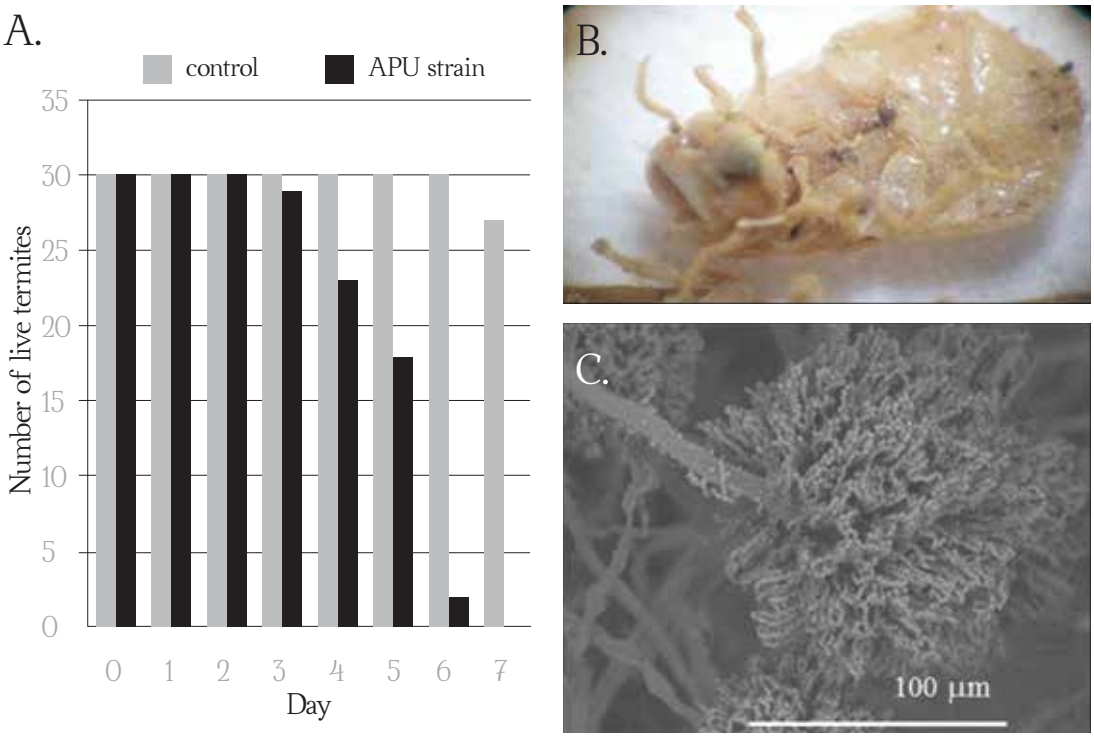


Figure 1: Healthy *Incisitermes minor* termites inoculated with APU strain show decreased viability over time. A. Survivability of infected termites over time. Control termites were unaffected. B. Light micrograph of a representative deceased *I. minor* termite after inoculation with APU strain with characteristic yellow spores visible. C. Scanning electron micrograph of fungal fruiting body found on deceased termite (800x magnification).

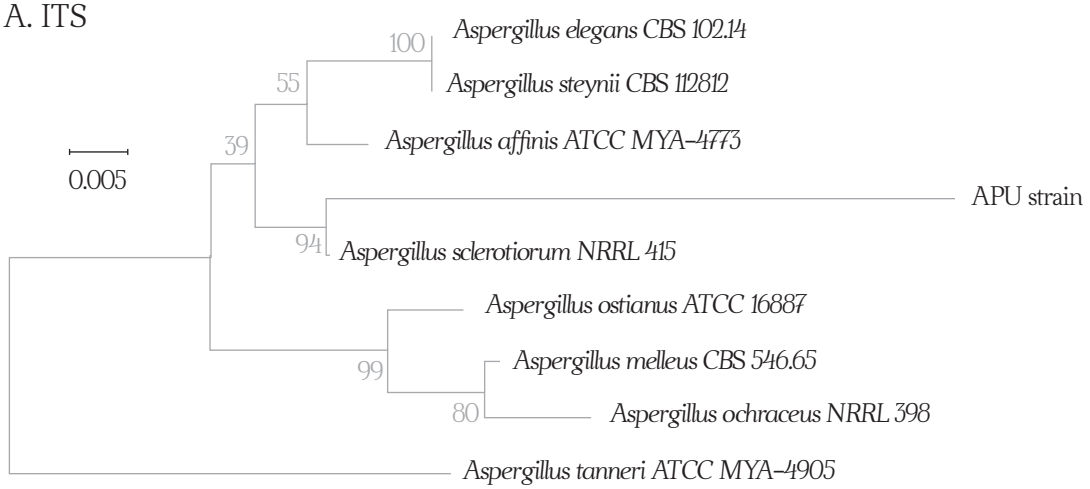
IDENTIFICATION OF APU STRAIN AS *ASPERGILLUS SCLEROTIORUM*

In order to determine if the APU strain was a known insect or termite pathogen, it was necessary to identify the species of fungus. Once the ITS sequence of the APU strain was determined, and a BLAST comparison using validated reference sequences (RefSeq, National Centers for Biotechnology Information) was performed, a phylogenetic tree was constructed consisting of 8 species with the most similar ITS sequences (Fig 2A). *A. sclerotiorum* had the closest sequence to the APU strain ITS region, making it the probable identity of the APU strain. Identification of APU strain was confirmed to be *A. sclerotiorum* by an independent lab (Accugenix, Newark, DE) that analyzed a smaller region of ITS (ITS2).

To corroborate these results, a region of the β-tubulin gene from APU strain was chosen to be sequenced as a means of species identification. Similar to the ITS region, the sequence of the β-tubulin gene showed closest identity to *A. sclerotiorum* (Fig. 2B).

Since sequence results of both ITS and β-tubulin regions suggested *A. sclerotiorum* was the APU strain species, the APU strain was compared microscopically to a known reference strain of *A. sclerotiorum* (Huber strain). Microscopic examination of APU

A. ITS



B. β-tublin

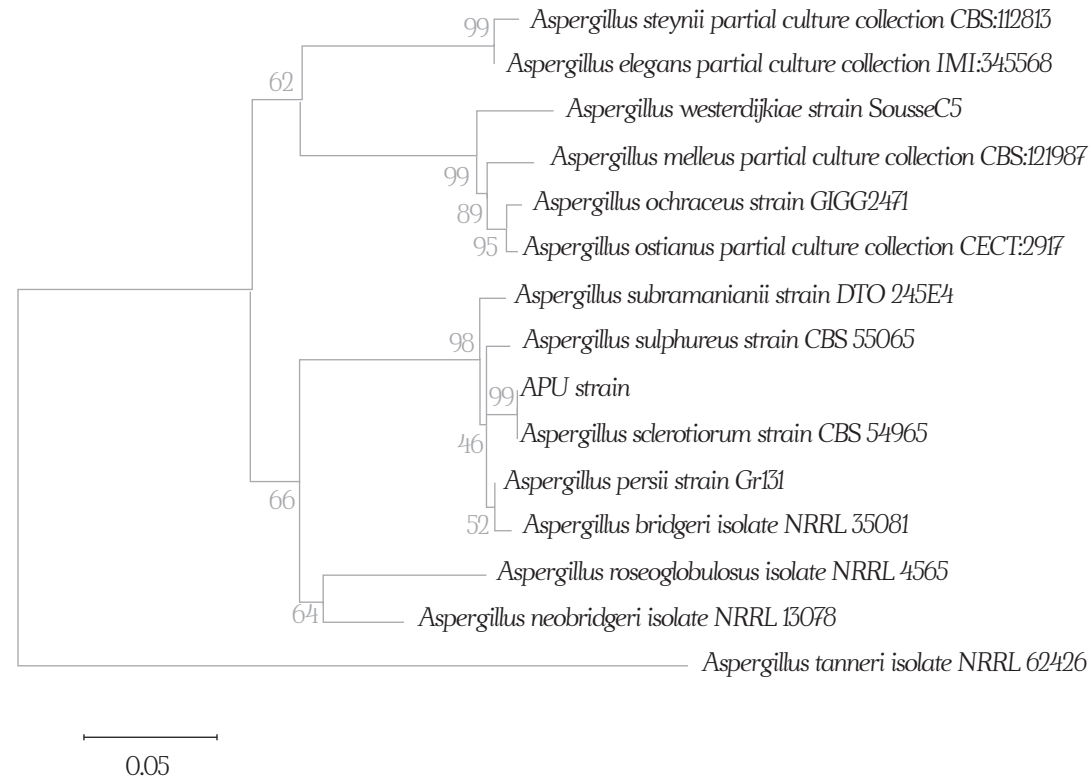
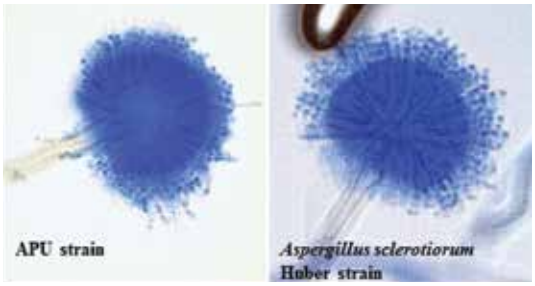


Figure 2. Phylogenetic trees based on ITS sequences and β-tubulin gene sequences of APU strain indicate identity is *Aspergillus sclerotiorum*. The phylogenetic tree for A. ITS and for B. β-tubulin genetic regions of APU strain were each constructed using the Maximum Likelihood method in MEGA6. Bootstrapping percentages are shown near the branch they refer to. A strain of *Aspergillus tanneri* was used as an outgroup for each tree.

A.



B.

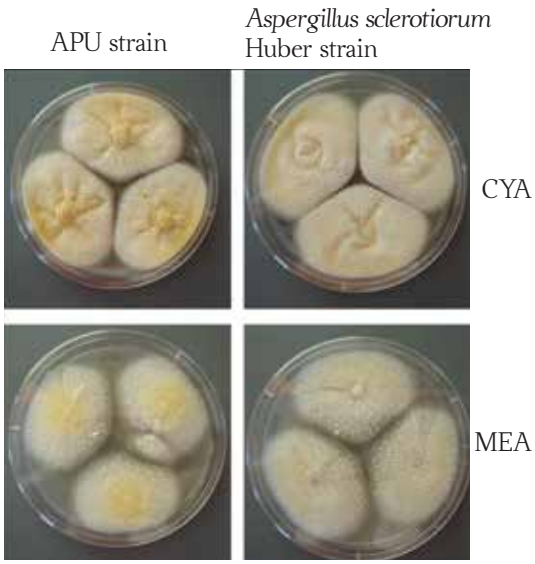


Figure 3. Microscopic and macroscopic comparison of APU strain and *A. sclerotiorum* Huber strain show similarities. A. Microscopic examination of fruiting body structures using white light microscopy. B. Fungal colony growth at day 7 on either Czapek Yeast Agar (CYA) or Malt Extract Agar (MEA) medium, as indicated.

Table 1. APU strain morphological features compared to those of *Aspergillus sclerotiorum*

Fungus	Metulae	Phialides	Vesicle		Spores	
			Shape	Diameter	Texture	Diameter
A. sclerotiorum[a]	7–12 µm	6–8 µm	pyriform/ spherical	17–35 µm	Smooth/finely roughened	2.5–3.0 µm
APU strain	8 µm	8 µm	spherical	23 µm	smooth	3.0 µm

[a]*all values for A. sclerotiorum are as previously described (14)*

and Huber strains showed very similar morphological features, such as the shape of the conidial head structures, which displayed similar overall spherical shapes with spherically shaped vesicles, as shown in Fig. 3A. Additionally, the vesicles of both fungi have biseriate appendages (made up of metulae and phialides) leading to the spherical conidia (spores). Various other fungal structures of the APU strain were observed or measured by light microscopy and compared to those of *A. sclerotiorum* from Klitch's key to identifying *Aspergillus* species (14). All structures observed or measured on the APU strain were found to fall within the range of structures found in *A. sclerotiorum* (Table 1), although they were not unique to *A. sclerotiorum.*

APU and Huber strains of fungus were also grown on media to compare colony morphologies. On both Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA), colonies of APU and Huber strains looked nearly identical in size, indicating a similar growth rate, as well as colony shape and texture (Fig. 3B). Both species displayed white mycelia and had liquid colony exudate. The major difference in colony appearance between the two fungal strains

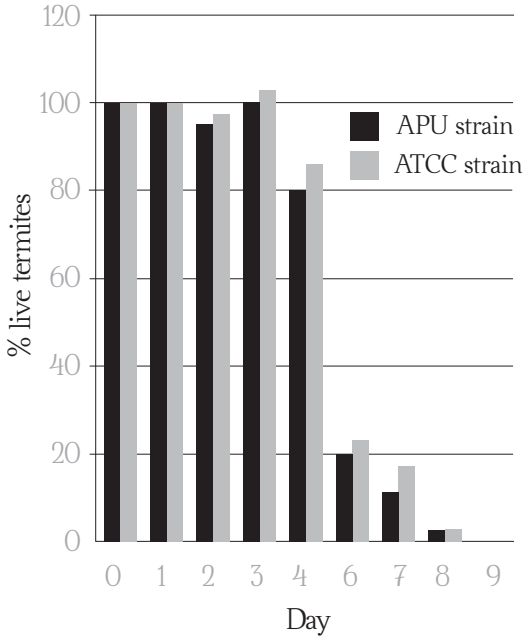


Figure 4. Comparison of survival of *Incisitermes minor* drywood termites over time infected with two different strains of *Aspergillus sclerotiorum*. *I. minor* termites were exposed to APU strain ("APU strain," black bars) or Huber strain ("ATCC strain," grey bars). Control termites were unexposed. Each group had 40 individuals from the same colony. Data are expressed as mean percent of the uninfected control live termites on each day.

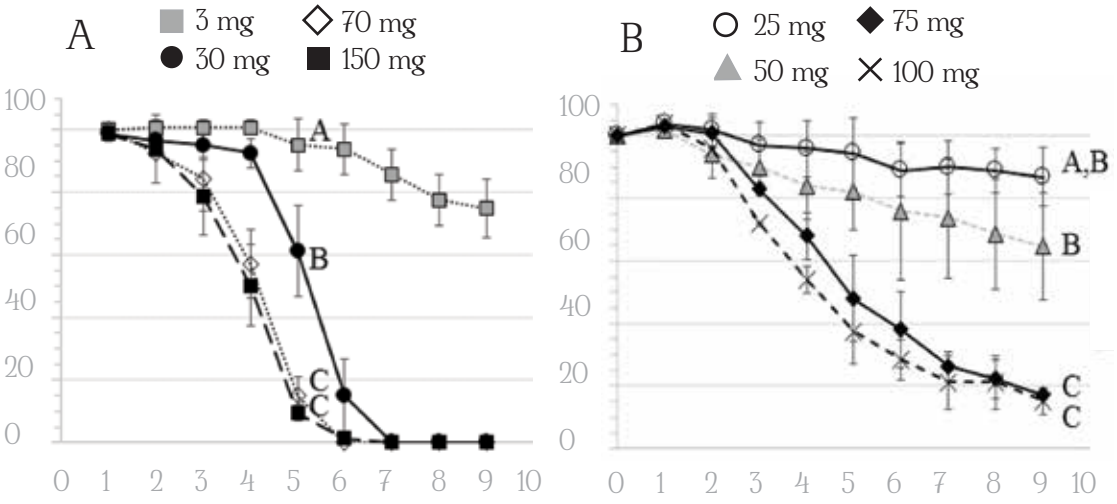


Figure 5. APU strain *Aspergillus sclerotiorum* affects *I. minor* and *R. hesperus* termites in a dose-dependent manner.
Termites were exposed to various total mg quantities of dried fungal spores as indicated. Data are expressed as mean percent ± S.D of the uninfected control live termites on each day. Control termites were exposed to powdered sugar. A. *I. minor* termites were placed into groups of 25 and exposed to each mg quantity of fungus (n=3) or 75 mg powdered sugar (n=2). Each mg contained 1.07 x 10^8 total spores by hemacytometer count. Viable counts were not performed. Different letters on day 5 indicate statistically significant differences ($p<0.05$) with the control belonging to group A. B. *R. hesperus* termites were placed into groups of 35 and exposed to each mg quantity of fungus (n=3 or n=2) or 100 or 75 mg powdered sugar (n=3) . Each mg contained 1.05 x 10^8 total spores by hemocytometer count, and 4.86 x 10^5 viable spores. Different letters on day 9 indicate statistically significant differences ($p<0.05$) with the control belonging to group A.

was the spore color: APU strain spores were bright yellow–orange in color, while the spores of Huber strain were beige (visible in Fig. 3B).

INCISITERMES MINOR TERMITES DIE AFTER EXPOSURE TO BOTH APU AND HUBER STRAINS OF *A. SCLEROTIORUM*

I. minor termites exposed to either APU or Huber strains showed very similar survivability kinetics over time, and all exposed termites died by day 9 (Fig. 4). Initially, for the first several days after fungal exposure, there was little termite death seen. A large decrease in live termites then occurred from day 4 to day 6 in both experimental groups.

BOTH *I. MINOR* AND *RETICULITERMES HESPERUS* TERMITES DIED AFTER EXPOSURE TO APU STRAIN IN A DOSE-DEPENDENT FASHION.

Since initial experiments involving *I. minor* termites walking on overgrown plates of APU strain (Fig 1A and Fig 4) or Huber strain (Fig 4) resulted in fewer live termites over time compared to controls, larger experiments were conducted with termites exposed to varying doses of fungal spores. The results shown in Fig. 5 clearly demonstrate that the numbers of live *I. minor* and *R. hesperus* termites decreased over time as the dose of dry fungal spores they were exposed to increased, in the range of 3 to 150 mg for *I. minor* (Fig. 5A), and 25 to 100 mg for *R. hesperus* (Fig. 5B). For *I. minor* termites, all individuals per group died by day 7 when exposed to 30 mg or higher doses (Fig. 5A), but on day 5, a statistical difference in the average percent of live termites ($p<0.05$) was seen for each dose except for 70 and 150 mg doses. Statistical analysis confirmed that there was a significant effect of dose in relation to *I. minor* termite survival on day 5 (ANOVA, $F = 66.64$, $Pr(>F) = 1.1e{-}06$), as well as on day 9 (ANOVA, $F= 289.4$, $Pr(>F) = 1.7e{-}09$). For *R. hesperus* termites infected with varying mg dose quantities of APU strain, there was a more gradual decline in the numbers of live termites over the 9 day course of the experiment, with no dose resulting in the death of all members in the groups. At day 9, however, 18% of termites treated with the highest dose of the spores survived compared to the controls. 86% of those treated with the lowest dose survived. Significantly different groups are shown in Fig. 5B ($p<0.05$), and statistical analysis confirmed that there was a significant effect of dose in relation to *R. hesperus* termite survival on day 9 (ANOVA, $F =36.2$, $Pr=1.49e{-}05$).

DISCUSSION

In 2009, a colony of *Incisitermes minor* western drywood termites housed at Azusa Pacific University (APU) died mysteriously, and dead termites were inspected and found to be covered in a fungus with yellow spores, which was subsequently isolated. It was possible that either the fungus was feeding off of termites that had died of other causes or it contributed to the termites' demise. Therefore, *I. minor* termites were initially infected with the purified APU strain, all of which died by day 7. This indicated that the fungus contributed to termite death.

To see if APU strain was a novel termite pathogen, it was identified by genetic analysis. APU strain was found to be most genetically similar to *Aspergillus sclerotiorum* fungus based on both its internal transcribed spacer (ITS) region sequence within the ribosomal RNA gene loci and its β-tubulin gene sequence (Fig. 2). This ITS region has been shown to be the most accurate "DNA bar coding" region of fungal genomes for species identification to date (24) and has the most numerous fungal sequence submissions to Genbank (approximately 800,000). External independent corroboration of the APU strain as *A. sclerotiorum* was also determined by sequence examination of a smaller ITS region, ITS2 (Accugenix). A second region of the APU strain genome, a portion of the β-tubulin gene, was also sequenced, since this region has been shown to be polymorphic and useful in identifying filamentous fungi within phylum Ascomycota (10). The species used to construct the β-tubulin tree (Fig. 2B) that were genetically similar to *A. sclerotiorum* were different from those used in the ITS region tree (Fig. 2A) because the β-tubulin gene sequences were used from the entire Genbank database, rather than selected ITS reference strain sequences in the RefSeq database. However, this β-tubulin phylogenetic tree shows similar species relationships to a phylogenetic tree previously published based on three DNA regions: β-tubulin gene, calmodulin gene, and ITS region (27). These three genetic analyses all confirm that APU strain belongs to *A. sclerotiorum*.

APU strain also had similar morphological features to a reference strain of *A. sclerotiorum* Huber when examined microscopically (Fig. 3A). More extensive observation of its microscopic morphology and measurement of several of its features (Table 1) fit within published parameters for *A. sclerotiorum* (14), although they did not rule out many related *Aspergillus* species that have similar features. This was helpful because it did not contradict the genetic analyses performed. The colony morphologies of APU and Huber strains when grown on solid media also appeared similar (Fig. 3B). The major difference in appearance between the two strains was the color of the spores, however, it is not unusual for strains of the same *Aspergillus* species to have a range of spore pigmentation (14). Taken together, these genetic and morphological approaches to species identification indicated that the identity of APU strain was *A. sclerotiorum*. This was especially interesting since *A. sclerotiorum* has not generally been studied for its entomopathogenicity.

Infection of western drywood termites with two different strains of *A. sclerotiorum* (APU and Huber) resulted in decreased viability of termites over time, and both strains displayed similar kinetics (Fig. 4). These similar kinetics further suggest that the two belong to the same species. This was not necessarily an expected result, as different strains of the same species of entomopathogenic fungi may show selective pathogenicity, depending on the source of their isolation (13,30). Since the APU strain of *A. sclerotiorum* was isolated from dead termites, and the Huber strain was originally isolated from a decaying apple in 1933, they could have had very different effects on drywood termites (12). Interestingly, they were both lethal to termites when termites were exposed to each fungus in the same manner, which suggests an inherent pathogenicity for termites. In addition, to our knowledge, it is the first demonstration of *A. sclerotiorum* as an *I. minor* termite pathogen.

The APU strain of *A. sclerotiorum* was not only lethal to western drywood termites (family Kalotermitidae), but also to western subterranean termites (family Rhinotermitidae). Groups of termites of both species infected with *A. sclerotiorum* had fewer live termites than control termites over time, and this decrease in viability was dose–dependent (Fig 5). In this set of experiments, each group of termites as a whole was exposed to a particular fungal dose; this was preferable to individually exposing termites because it has been shown that the drywood termite *Incisitermes schwarti* is more susceptible to death by fungal infections when in isolation, rather than in groups of 10 or 25 (3). Additionally, in these experiments, control termites were exposed to powdered sugar, an inert but particulate substance, in order to establish that the fungus was entomopathogenic and not causing or contributing to termite death in some non–specific way, for instance, by physically blocking respiratory structures. The apparent greater lethal effect that the 30 and 70 mg doses of spores had on *I. minor* compared with the 25 and 75 mg doses with *R. hesperus* may either be due to a greater susceptibility that *I. minor* has for the fungus, or that there were more viable spores per mg in the *I. minor* experiments than in the *R. hesperus* experiments, although the total amounts of spores per mg were very similar. This second possibility is likely, because the experiments with *R. hesperus* were done much later than the *I. minor* experiments with the same preparation of spores, and it very likely lost viability in that time, although it is not possible at this point to rule out the first possibility. Low doses of fungal spores resulted in greater termite viability than higher doses, which suggests that both termite species have antifungal defenses to protect them from the lower doses of fungus, although their immune systems are largely uncharacterized. One complicating factor, however, is that the 3 mg dose of spores used to infect *I. minor* contained powdered sugar. This could possibly have given an immune advantage to the *I. minor* as has previously been reported in *Odontotermes formosanus* (8), and may explain why this dose was not significantly different from the control uninoculated termites on either day 5 or 9. In any case, this is the first study indicating that *A. sclerotiorum* is pathogenic to both of these species of termite pests, which are among the five important termite pest species out of 45 total termite species in the United States (26).

The mechanism for *A. sclerotiorum*'s infection of and entomopathogenicity to termites is unknown. Spores could be ingested and germinate in the alimentary tract, as seen in termites infected with *Beauveria bassiana*, or they could invade the termite by secreting cuticle–degrading enzymes, as seen in termites infected with *Metarhizium anisopliae* (4,15). *A. sclerotiorum* may then cause disease by one or more of its known excreted metabolites, such as the insecticidal aspochracin molecules (27). One aspochracin molecule previously isolated from *Aspergillus ochraceus* has been shown to be toxic to silkworm larvae (21). Control termites in the dose–dependent experiments in our study (Fig. 5) were treated with powdered sugar to try to control for the possibility that spores might be blocking termite respiratory spiracles or interfering with other aspects of their physiology as particles, and not by some fungus–specific mechanism. However, the powdered sugar is not uniformly sized compared to the spores, and so using inert particulates of the same size as the fungal spores, perhaps pollen, would make a better control. Finally, it is also possible that *A. sclerotiorum* pathogenesis is related to the termite's immune response to the fungus. Both the route of infection and the mechanism for pathogenesis of *A. sclerotiorum* in termites are currently being explored.

POTENTIAL USE OF *A. SCLEROTIORUM* AS A TERMITE BIOLOGICAL CONTROL AGENT

The use of microbiological agents as biological controls for pests like termites includes the use of the fungi *M. anisopliae* and *B. bassiana*. Any fungus introduced into an environment to control termites must be horizontally transmissible to other nest mates, since in many termite species, direct treatment of the nest may not be possible (22). Whether *A. sclerotiorum* is transmissible from infected to uninfected termites is currently under investigation, but seems likely since it was found on many individuals from the same colony upon initial isolation. If it proves to be transmissible, then infecting individuals and introducing them into nests or other approximations of termite nests, such as termite planar arenas, would be necessary to determine the feasibility of *A. sclerotiorum* as a termite control agent (5,6).

Biological control agents must also be benign to humans and other inhabitants of the area in which they are applied. As a candidate species for termite biological control, *A. sclerotiorum* shows minimal pathogenicity to humans. There have been only a few documented human cases of *A. sclerotiorum* disease, including nail infection (onychomycosis) and ear canal infection (otomycosis) (1,9,11). However, like most fungi, it could potentially pose a more serious threat to a person with underlying immunodeficiency. Less well-known are the effects of *A. sclerotiorum* on other insect species that may occupy similar niches as termites. *M. anisopliae*, the most extensively studied entomopathogenic fungus, has been shown to be lethal to all species of termites tested which is part of what makes it such a promising control for termites (20). However, *M. anisopliae* is also lethal to a number of other non-related insects, including locusts and grasshoppers (18), ticks (2), and mosquitoes (25), just to name a few. Whether *A. sclerotiorum* is less harmful to other insects than it is to termites is not well-established; to date, it has only reportedly been tested on mosquitoes. In that study, two species of mosquito larvae (*Culex quinquefasciatus* and *Aedes fluviatilis*) infected with low doses of *A. sclerotiorum* (4.5×10^5 or 1.75×10^5, respectively) displayed 0% or 18% mortality rates, respectively, by day 10 post-infection, which was lower than many other different species of *Aspergillus* tested in the same study (16). It will be interesting to see if *A. sclerotiorum* is less pathogenic to other insect types, as well. If it is less pathogenic, it may prove to be a better choice of fungal species for use in termite biological control.

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CHARACTERIZATION OF WATER-TYPES AND THEIR INFLUENCE ON THE ANTIMICROBIAL PROPERTIES OF KOMBUCHA FERMENTS AGAINST BACTERIA AND YEAST

JAMES K. LAWTON II [1,2] AND RENU B. KUMAR [1*]
[1] MINNESOTA STATE COLLEGE AND UNIVERSITY (MINNEAPOLIS COMMUNITY AND TECHNICAL COLLEGE)
[2] COLLEGE OF BIOLOGICAL SCIENCES, UNIVERSITY OF MINNESOTA – TWIN CITIES, MN

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ABSTRACT

Understanding the relationships between diet, gut microflora, and health is an increasingly important area of research. Recent studies have demonstrated that Kombucha tea provides variable antimicrobial activity against pathogenic microbes. In this study, we tested Kombucha tea for antimicrobial activity against various Gram-positive and Gram-negative bacteria, as well as yeast, using an agar diffusion method. Standard zone of inhibition assays were used to test the hypothesis that variance in antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* may be due to the varying levels of cations, like Ca^{2+}, found in different water-types (well water, artesian water, city water, type-II water and distilled water). Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) results indicated that high cationic (Ca^{2+}, Mg^{2+}, and Na^{+}) content water (well water) resulted in the largest zone of inhibition against *S. aureus*, with a 12.3% difference when compared to low cationic content water (type-II water). *E. coli* maintained a constant zone of inhibition regardless of water-type or batch-type, while *C. albicans* showed no zones of inhibition. Inhibition is either through a synergistic relationship with the pH conditions, the other cations present (Mg^{2+}, Na^{+}, Si, etc.) or a mix of both, as pH in the range of 4.5 – 3 is not enough to inhibit the growth of *S. aureus*. These results indicate that a direct relationship exists between cationic concentrations of water used to prepare Kombucha, and antibacterial activity against *S. aureus*, due to the improved fermentation of the tea with high concentrations of cations. Strong antimicrobial potential exists, particularly against *S. aureus*, which may be useful in determining novel approaches to synthesize antimicrobial drugs. Further study is needed to assess other *S. aureus* strains, as well as to determine how this relationship translates to human microbiota interactions and their microbial metabolic profiles.

CORRESPONDING AUTHOR

*Renu B. Kumar
renu.kumar@minneapolis.edu

KEYWORDS

- Fermented tea
- Kombucha
- Antimicrobial
- Cation

INTRODUCTION

Kombucha is a fermented tea produced with the help of a symbiotic culture of bacteria and yeast (SCOBY). The genus *Acetobacter* is an aerobic, nitrogen-fixing bacteria which produces acetic acid, gluconic acid, and cellulose. *Saccharomyces* is an aerobic to facultatively anaerobic, single celled yeast that produces ethanol and carbon dioxide. These two microorganisms have synergistic roles and are the most common components of SCOBY. The microbial composition of the SCOBY can vary and is dependent upon the origin of the culture (3). *Acetobacter* produces cellulose, which is seen as a thin white film originating on the top of the tea as early as the first day of fermentation (1). This is when the bacteria and yeast cell mass accumulates to begin the synergetic fermentation process. The synergy of the yeasts cleaving the sugar and bacteria producing the acidic components is what establishes the unique cider-like carbonated characteristics of Kombucha. *Saccharomyces* strains cleave sucrose into glucose and fructose, with the former used to produce ethanol and carbon dioxide. *Acetobacter* strains then oxidize ethanol to acetaldehyde and then into acetic acid (5). Glucose also leads to the production of cellulose and gluconic acid. The acidic levels attained during fermentation (pH 3.0 – 2.5) result in unsuitable growing conditions for most microorganisms, reducing contamination (14). Kombucha has been used as a beneficial health drink for several millennia. It has been suggested that Kombucha aids in digestion, prevents microbial infections, can vitalize the physical body, increase the efficacy of the gastrointestinal tract, and is believed to enhance immunity (8). The popularity of Kombucha in the United States has increased within the last 15 years, with annual reports showing Kombucha sales expected to surpass $500M by the end of 2015 (10). This increase in popularity highlights the importance of the overall health implications of Kombucha. Kombucha has been identified as having antimicrobial properties against *Helicobacter pylori*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Bacillus cereus*, *Shigella sonnei*, *Salmonella enteritidis* and *Escherichia coli* (8,12,15). No previous study has characterized water-types and identified its relationships with Kombucha ferments relative to its antimicrobial activity.

MATERIALS AND METHODS

WATER-TYPES

Five water types were selected on the basis of cationic concentration: 1.) Well water taken from a private well located in Prescott, WI USA; 2.) Artesian water purchased from a local Minnesota based company – Artesian Fresh (LeRoy, MN USA); 3.) City water taken from a tap located in Hennepin County, Minneapolis, MN USA; 4.) Type-II water; 5.) Distilled water from Millipore water purification systems. Water-types were analyzed for the major cations on a Thermo Scientific iCAP 6500 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) at the University of Minnesota's Analytical Geochemistry Laboratory. Triplicate measures were taken on each water sample, with the mean of each cation determined. High levels of Ca^{2+}, Mg^{2+}, and Na^{+} were identified in well water and

artesian water, comparative to World Health Organization drinking water guidelines (7). Silicon also appears with interesting values, but due to the higher levels of Ca^{2+}, Mg^{2+}, and Na^{+} relative to Si, and due to the biological role of these cations within cellular processes, this study focused on these cations, primarily Ca^{2+}.

KOMBUCHA CULTURE (SCOBY)

Kombucha cultures were purchased from Anahata Balance at Organic-Kombucha.com (Buckley, MI USA). The primary strains used in these cultures were *Acetobacter xylinum* and *Saccharomyces boulardii*. The composition of the disks was specially constructed by Anahata Balance to fit the fermentation containers used, to prevent cross-contamination. Each culture was then selected to ferment in only one water-type. A total of five cultures were assessed, one for each water-type in the experimental batches (as control batches did not have any cultures).

PREPARATION OF TEA FERMENTS

The recipe provided by Anahata Balance (Fig. 1) was used to prepare solutions. Kombucha was prepared by infusing 75.0g of organic sucrose (10% w/v) with 3.30g of hibiscus tea (0.44% w/v) into 750mL of boiling water in a 1L beaker, and allowed to steep for 20 min. Organic sucrose and hibiscus tea were purchased from The Wedge food co-op (Minneapolis, MN USA). The contribution of the tea's antimicrobial activity to Kombucha ferments is shown to be insignificant (13) and as such, hibiscus tea was selected. Five experimental Batches (EB) and five Control Batches (CB) were prepared, each relative to the corresponding water-type. CBs were identical to the EBs except no Kombucha culture was added (only water, sugar, and tea). After homogeneity of the sugar tea solutions in the EBs and CBs, the tea solutions were cooled to ambient temperature (20°C) to allow for the addition of Kombucha cultures to the EBs, in order to prevent harming the cultures. Necessary pH sensor measurements were taken to ensure all EBs and CBs started the fermentation process at pH 4.5. EBs and CBs were then placed into a sterilized Thermolyne Type 4200 Incubator at 30°C for 8 – 9 days (pH dependent) until a pH of approximately 3.0 was reached in the EBs (as no change in pH would be noted in CBs). CBs pH was then adjusted to 3.0 with 1.0M acetic acid, which later acts as the pH controls. All pH determinations for the EBs and CBs were measured using a Vernier pH Sensor and Vernier LabQuest Palm Pilot. EBs and CBs were covered with Parafilm and then placed in a walk-in cooler for later experimentation.

EXPERIMENTAL TEST MICROORGANISMS

Two bacterial species were chosen to test against the antimicrobial activity of Kombucha. One Gram-positive bacteria: *Staphylococcus aureus ATCC 27661* and a Gram-negative bacteria: *Escherichia coli ATCC 11303*. Both were provided by MCTCs Department of Microbiology teaching stocks. *S. aureus* and *E. coli* were cultivated on Nutrient agar (NA) at 37°C for 24h.

A common human pathogenic yeast strain was chosen to test against the antimicrobial activity of Kombucha. *Candida albicans* SN76 was provided by Dr. Kirkpatrick at the University of Minnesota.

OPTICAL DENSITY DETERMINATION

Optical density (OD) determinations were measured with a Vernier Spectro Vis-plus at 600nm, on a Vernier LabQuest

Instructions: To make a 1 gallon batch of Kombucha tea

A. Bring 1 gallon of distilled or filtered water to a boil. After the water starts to boil, slowly add 1 to 1 ½ cups of organic cane sugar.
Stir & cover solution, then simmer for another 10 min. or until sugar is completely dissolved. Tea/sugar solution may be boiled in metal or glass container. It is suggested not to use non-organic sugar, honey, or maple syrup, agave, etc. when brewing kombucha. Alternative sugars may not provide the kombucha yeast cultures with the correct food source and is not recommended.

B. Remove tea solution from heat. Add 6 tea bags (6 teaspoons) worth of green tea or black tea and allow solution to steep for 10 minutes. After 10 min. remove tea bags, cover, and allow solution to cool to room temperature. Always keep tea solution securely covered with a tight weave cloth.

C. Transfer tea solution to a glass container for brewing the Kombucha culture, never allow metal or ceramic to have long term contact with the culture. *IMPORTANT NOTE: ADD KOMBUCHA S.C.O.B.Y. MUSHROOM and STARTER LIQUID ONLY AFTER TEA HAS COOLED TO ROOM TEMPERATURE.*
Notes: Make sure not to fill the brewing jar to full (it should be level with the straight side of the jar not up into the rounded edge. Make sure that the smoother white side of mushroom faces upwards, it may float or sink, and this is acceptable. However, by gently placing the culture on top of the tea it should stay towards the top. If it does sink, the culture should start to rise to the surface as gases are forming in the fermenting tea. Securely cover container with cloth (the culture needs to breath) and allow the inoculated tea solution to set undisturbed in a warm place out of direct sunlight.

D. Tea will be ready to drink after 5 to 14 days of fermentation, depending on temperature (around 75 –85 deg F is ideal) and amount of sugar added (more sugar = more time). If checking pH of a finished batch of tea, it should be around 3; a reading of 4 is to high and 2 too low. Checking pH can greatly reduce the chance of contamination of the culture and is highly recommended.

E. To make new batch of tea, make sure to save 10–15% of the old tea solution (as a starter to lower pH below 4.6) and one or two layers of the kombucha mushroom. When it is time for a new batch, just follow the directions above to start the process again.

Figure 1. Kombucha Mushroom Tea Culture Starter Recipe (AnahataBalance.com)

Palm Pilot. Distilled water was used for calibration and for blank samples. A McFarland Standard of 0.5 was prepared using ASM McFarland Standards SOP 5.14.1 (2). Bacterial and yeast colonies were transferred to 10mL of sterile distilled water and vortexed. After vortexing, OD_{600} determinations were measured until each sample reached the target density at or around 0.20. Each run included 2 blanks, 2 standards, 1 *S. aureus*, 1 *E. coli*, and 1 *C. albicans*. One run per zone of inhibition assay, three runs total, were performed.

AGAR DIFFUSION METHOD

The antimicrobial activity of Kombucha was determined using an agar diffusion method. Kombucha EBs and CBs were removed from the cold storage and allowed

to equilibrate to ambient temperature (20°C). Agar plates (100mm) were purchased "pre-poured" from Teknova (Hollister, CA USA). Mannitol Salt Agar (MSA) plates were used for *S. aureus* inoculations, Luria broth (LB) agar plates were used for *E. coli* cultivation, and Yeast Mannitol (YM) agar plates were used for *C. albicans*. Agar plates were inoculated using a spreading technique to achieve lawn growth throughout with use of sterile cotton wool swabs instead of glass spreaders. Thirty Millipore absorbent disks (25mm) were then sorted appropriately, three disks per EB water-type, and three disks per CB water-type (hence 30 total) with 1mL of the appropriate correlating batch. This was to ensure duplicate and triplicate measurements were taken. All appropriate general lab practices were followed to prevent cross-contamination of one batch to another during disk saturations. Once disks became saturated they were placed in the center of the inoculated agar plate, one disk per plate (3 inoculated plates per batch type, 30 total). Plates were then covered and placed into a NorLake Scientific Incubator at 37°C for 72 h. Agar plates were then removed from the incubator and zone of inhibitions were measured with a standard metric ruler. Measurements included the diameter of the clearings end-to-end, disregarding the disk in the center. All zone of inhibition assays were carried out in triplicate and analyzed statistically using 1-way ANOVA ($p<0.05$).

RESULTS AND DISCUSSION

CATIONIC RELATIONSHIPS

Results of the ICP-OES water-type analysis are shown in Table 1. High levels of Ca^{2+} (104 ppm), Mg^{2+} (56 ppm) and Na^{+} (77 ppm) were observed in well water samples relative to the other water-type samples (Fig. 2). Of the 11 major cation concentrations determined in all five water-types, the three cations noted above (Ca^{2+}, Mg^{2+}, Na^{+}) demonstrated the largest variance throughout; in addition a gradient trend of Ca^{2+} concentration levels was observed in the water-types and seemed to correlate with the zone of inhibition measurements in the *S. aureus* plates. Due to Ca^{2+} exhibiting the highest concentration levels, this study will focus on that cation.

Every metal cation has its own unique system to support prokaryotic and eukaryotic cellular regulation and growth requirements. Of the most abundant cations required for these conditions (Ca^{2+}, Mg^{2+}, and Na^{+}), it is calcium that plays the universal role as a messenger, transmitting signals from the cell surface to the cytoplasm (6). Calcium is also associated with the regulation of many types of cellular processes relative to cell differentiation, transport, motility, gene expression, metabolism, cell cycle effects, cell division, and pathogenesis (11).

To date, a number of bacterial surface proteins have been implicated in various calcium transport mechanisms (11). In *S. aureus*, this includes a biofilm-associated protein (Bap), a *S. aureus* surface protein essential for biofilm formation. Studies have shown that *S. aureus* biofilm matrices are assembled by Bap proteins (16). Under controlled conditions, Bap proteins are also shown to become inhibited by

Table 1: Results of the ICP-OES analysis on the water-types evaluated in this study (+/- SD).

	Water type				
	Well Water	Artesian Water	City Water	Type-II Water	Distilled Water
Al (ppb)	4.3 ± 1.0	6.3 ± 0.8	8.8 ± .0.8	1.0 ± 1.5	6.5 ± 0.6
Ba (ppb)	42.5 ± 0.8	105.0 ± 1.2	7.2 ± 0.3	0.3 ± 0.5	0.0 ± 0.5
Ca (ppb)	103716.7 ± 231.4	72122.0 ± 214.3	22332.0 ± 54.4	117.9 ± 3.7	-1.7 ± 11.2
Fe (ppb)	13.1 ± 0.9	1.0 ± 0.9	10.0 ± 0.9	1.6 ± 0.7	0.0 ± 1.2
K (ppb)	1201.8 ± 11.7	1219.0 ± 7.8	2561.1 ± 9.9	4.0 ± 0.6	5.0 ± 4.6
Mg (ppb)	56462.0 ± 53.3	23091.0 ± 166.1	6518.4 ± 21.3	7.8 ± 0.7	3.1 ± 8.7
Mn (ppb)	8.3 ± 0.2	4.8 ± 0.1	0.3 ± 0.1	5.6 ± 0.1	0.2 ± 0.2
Na (ppb)	77140.0 ± 145.8	3546.2 ± 28.2	16581.3 ± 36.7	9.0 ± 71.8	-26.3 ± 9.1
P (ppb)	4.4 ± 3.8	0.2 ± 3.8	281.1 ± 5.6	2.3 ± 1.6	-0.1 ± 2.4
Si (ppb)	8398.4 ± 45.7	5778.7 ± 32.4	4352.1 ± 8.6	5.0 ± 1.4	59.0 ± 4.0
Sr (ppb)	88.2 ± 0.6	121.7 ± 0.8	54.9 ± 0.2	0.2 ± 0.0	0.2 ± 0.5

calcium treatments, thus preventing any Bap-mediated biofilm formation (4) and allowing S. *aureus* to become susceptible to any number of exposures that can lead to cellular complications. As the concentration of calcium increases, so do the zones of inhibition by S. *aureus*. This result seems to be a response specific to the calcium concentrations seen on the ICP-OES in the water-types, and not pH alone, as the CBs were used as pH controls. S. *aureus*, when exposed to the CBs, saw zero detectable inhibition, which tells us that a pH of 3.0 does not inhibit S. *aureus*. Calcium could be playing a direct role, either through a synergistic relationship with the pH or with the other cations present (Mg^{2+} & Na^{+}) or a combination of these effects.

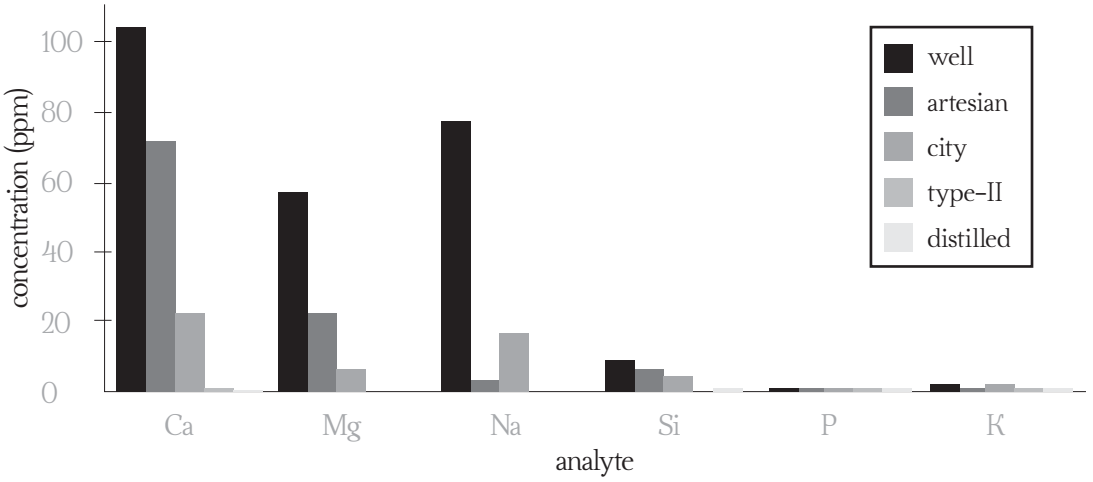
CHANGES IN PH DURING FERMENTATION

The changes in pH during fermentation for the CBs and EBs are shown in Fig. 3. In the first three days of fermentation, CBs observed the greatest change in pH, 4.5 to 3.3, a 1.2 pH difference, while the EBs maintained their initial pH throughout the 8-day period, suggesting no microbial contamination occurred. The initial CBs pH drop could be relative to the high availability of glucose for yeast and bacteria, which ultimately leads to the production of acetic acid and gluconic acid, decreasing the pH. As glucose gets consumed, the availability becomes limiting, thus resulting in a slower change of pH.

ANTIMICROBIAL ACTIVITY OF THE FERMENTS

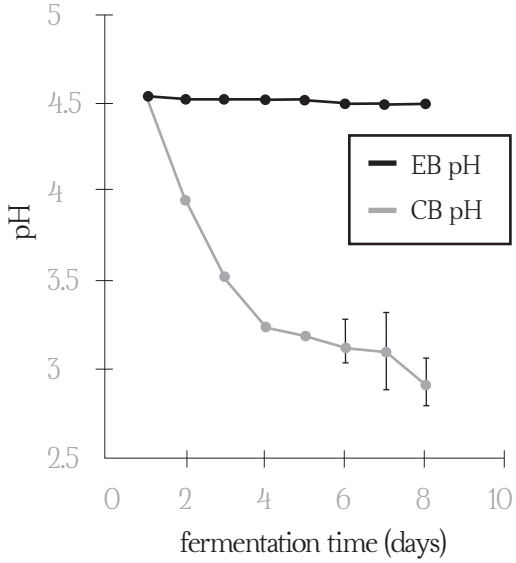
The antimicrobial activity of the EB and CB treatments against bacteria and yeast strains are shown in Figs. 4 and 5. Results indicate that high-ion (Ca^{2+}, Mg^{2+}, Na^{+}) content water (well water) resulted in the largest zones of inhibitions at 35.7 mm for S. *aureus*, with a 12.3 percent difference when compared to near zero ion content water

Figure 2: Comparison of the ICP-OES major cation results relative to water-types.



(type-II water) at 31.3 mm. *E. coli* maintained a constant zone of inhibition regardless of water-type or batch-type, while *C. albicans* showed no zone of inhibition outcomes. Six different sets of the antimicrobial activity of Kombucha EB treatments against S. *aureus*, (Table 2) were used to calculate percent change, with the presumption that well water EBs = 100 percent zone of inhibition. Relative to well water, type-II water had the lowest standard deviation value of +/-2.07. EBs and CBs had zero detectable effect on *C. albicans*, as aggressive growth was seen throughout the plate, even on the absorbent disks. These results indicate a direct relationship may exist between Kombucha prepared in high-Ca^{2+} content water and the antibacterial activity of Kombucha ferments against S. *aureus*. It is also suggestive of further study to determine how this relationship applies to other pathogenic strains. This behavior is indicative of a gradient trend, with an R^2 value of 0.9604, which seems to correlate well with the Ca^{2+} concentration levels identified within these water-types. The *E. coli* strain displayed constant zone of inhibitions regardless of batch-type, with a mean variance of 43-49mm. Both S. *aureus* and *E. coli* results

Figure 3: After fermentation, CBs were adjusted to a pH of 3.0 for comparative purposes against EBs.



were pH independent for EBs and CBs. We can conclude that something other than the acidic conditions must be inhibiting S. *aureus*. One possibility could be the varying levels of cations, especially Ca^{2+}, in the water-types. Distilled water is the gold standard source of water when making Kombucha, and as such, understanding the

Table 2. Comparison of six different EB treatment sets of the antimicrobial activity against *S. aureus*.

	S. aureus zone-of-inhibition %-change								
Water type	set-1	set-2	set-3	set-4	set-5	set-6	Mean	Standard deviation	Standard error of mean (SEM)
Well water	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
Artesian water	0.00	5.70	0.00	12.50	14.60	7.70	6.75	6.13	1.02
City water	11.1	5.7	13.9	5.4	11.0	5.1	8.7	3.8	0.6
Type-II water	11.1	11.4	13.9	10.2	12.2	7.7	11.1	2.1	0.4
Distilled water	13.9	20.0	19.4	5.4	2.4	2.6	10.6	8.2	1.4

Figure 4. Antimicrobial activity of Kombucha ferments versus controls.

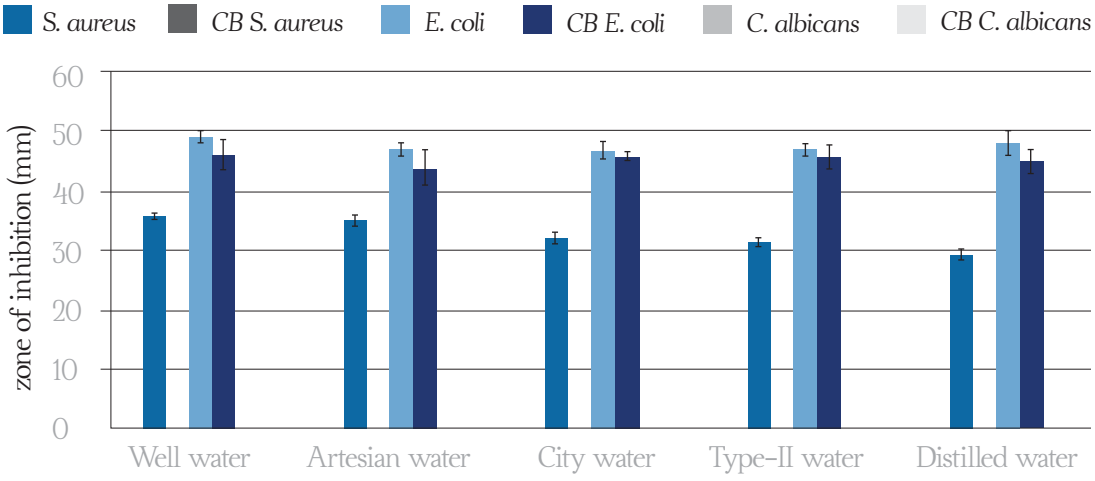
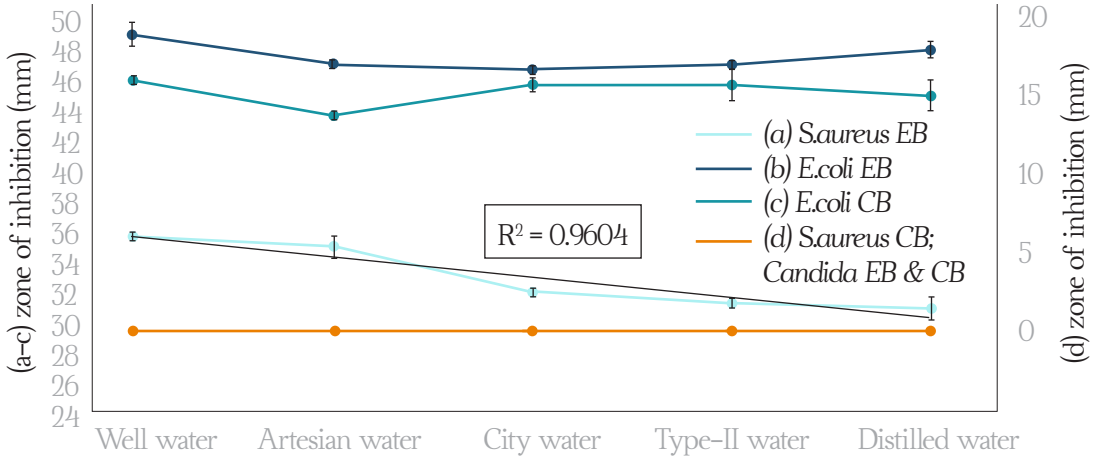


Figure 5: Zone-of-inhibition results of EB and CB treatments against *S. aureus*, *E. coli* and *C. albicans*



antimicrobial properties of Kombucha in response to the addition of metal ions is still yet unknown and unidentified.

Since no detectable zone of inhibitions were observed in S. *aureus* CB treatments and in *C. albicans* EB and CB treatments, statistical data only represent the remaining treatment scenarios. Because Ca^{2+} concentrations were much higher in well water and artesian water relative to the other water.

CONCLUSION

The antimicrobial effects of Kombucha are reflected as having no inhibition for *C. albicans*, and a constant zone of inhibition for *E. coli* regardless of EB or CB treatments. S. *aureus* displayed the greatest potential, with zone of inhibitions decreasing in size linearly relative to the cationic concentrations measured by the ICP-OES in each of the five water-types. This is indicative that a Ca^{2+} relationship may exist due to the same linear fashion it displayed in its concentration levels decreasing in each of the water-types (well water > artesian water > distilled water). It should also be noted that Mg^{2+} and Si also exhibited linear drops in concentration levels relative to water-types, but due to being less than the Ca^{2+} levels and not playing as much a role within cellular systems as Ca^{2+}, they were not the focus of this paper. Every metal cation has its own unique system to support prokaryotic and eukaryotic cellular regulation and growth requirements. However, it is calcium that plays the universal role as a messenger, transmitting signals from the cell surface to the interior of the cell (6). Calcium is also identified as regulating many types of cell processes relative to cell differentiation, transport, motility, gene expression, metabolism, cell cycle, cell division and pathogenesis (11). Within that, a number of bacteria, surface proteins, many number of these factors have been identified and shown to be involved in various calcium transport mechanisms (11). On S. *aureus* one such component has been identified as a biofilm-associated protein (Bap), a S. *aureus* surface protein that is essential for biofilm formation. One particular study shows that S. *aureus* biofilm matrices assembled by Bap proteins protect the bacteria from antimicrobial treatments (15), and under controlled conditions, these Bap proteins become inhibited by calcium treatments, thus preventing any Bap-mediated biofilm formation (4) and in return allowing S. *aureus* to become susceptible to various environmental stressors. As the concentration of Ca^{2+} increases, so does the zone of inhibitions in S. *aureus*. This result appears to be a response specific to the calcium concentrations measured in the water-types, and not pH alone, as the CBs were used as pH controls, and as such, when S. *aureus* was exposed to the CBs, zero inhibition took place. This tells us that a pH of 3.0 is not enough to inhibit S. *aureus*. There must be a direct role that these cations are having on the Kombucha during fermentation to allow inhibition, possibly calcium, either through synergistic relationships with the Kombucha SCOBY, pH or with the other cations (Mg^{2+}, Na^{+}, Si). No Ca^{2+} control was conducted, so it is difficult to say with certainty how much of a role Ca^{2+} is really having on S. *aureus*. It is suggested that if this study is replicated, that cationic controls be inserted.

S. *aureus* is a major opportunistic human pathogen, able to cause a wide range of

diseases in humans and in animals (6), which is why it is important to further investigate these relationships. Further study is needed to determine how these cations are affecting Kombucha fermentation, how it is interacting with S. *aureus*, and then whether or not the same results apply to other S. *aureus* strains, specifically methicillin resistant S. *aureus* (MRSA).

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RECOVERY AND ENUMERATION OF *STAPHYLOCOCCUS AUREUS* BY THE SELECTIVE AGAR OVERLAY METHOD

JILL BANGE*, EMILY BRUMFIELD, AND ALYSHA L ELLISON
DEPARTMENT OF BIOLOGY, BALL STATE UNIVERSITY
MUNCIE, IN USA

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ABSTRACT

Staphylococcus aureus is an example of a commensal bacterium responsible for emesis, acute diarrheal syndrome, and sepsis. S. *aureus* often must be isolated from patient samples in a clinical setting or from food samples during food processing in an industrial setting, although these bacterial cells may be injured by the human immune system or by food processing measures. Therefore, injured cells may not be fully recovered on media selective for S. *aureus* and enumeration (e.g., CFU/mL) may not reflect the true concentration of the original sample. The objective of this study was to determine whether the selective agar overlay method of recovery is more sensitive, selective, and time-effective for enumeration of artificially injured S. *aureus* cultures when compared to more traditional techniques. The selective agar overlay method involves pour plating S. *aureus* in non-selective medium, allowing the sample to incubate for a four hour recovery period, and then overlaying selective medium over the non-selective medium. Artificial injury of S. *aureus* cells was accomplished by treatment with carvacrol, an extract from oil of oregano. Our results indicated that carvacrol-injured S. *aureus* cells were recovered by the selective agar overlay at the same concentration as recovery on non-selective media, and at a significantly higher concentration than recovery on selective media. This method allows for more rapid and accurate diagnoses, and may be more cost-effective due to the reduction or elimination of false negative results.

CORRESPONDING AUTHOR

*Jill Bange
jillsbange@gmail.com

KEYWORDS

- *Staphylococcus aureus*
- Overlay
- Sublethally injured
- Carvacrol

INTRODUCTION

Staphylococcus aureus is a Gram-positive, ubiquitous, coccoid bacterium, which is a prevalent organism in the food and clinical microbiology arenas (1,10). This commensal organism is an opportunist and an emerging pathogen for both nosocomial and community-acquired infections (6).

Staphylococcus aureus enterotoxins, exotoxins, and hemolysin are responsible for emesis, acute diarrheal syndrome, and sepsis in humans (6,10). In addition, methicillin-resistant and vancomycin-resistant strains are troublesome because they are difficult to treat effectively (14).

A variety of media can be used to grow S. *aureus*, using both non-selective and selective techniques. However, the human immune response as well as food processing techniques may injure S. *aureus*, limiting growth and detectability in lab cultures, particularly if bacterial cells are not allowed to repair. Using a non-selective medium for isolating a pure bacterial culture is not warranted, as other species may grow (7). Employing a selective medium alone may be an improvement, but unfortunately, cells may not repair well enough to grow visible colonies on the medium (7), and may result in a false negative for presence of bacteria in the original sample. To solve this problem, enrichment of bacterial samples in broth is needed before plating onto selective media. However, enrichment in broth will not allow for accurate calculation of the original sample's bacterial cell concentration because only cells enriched in agar form countable colony forming units (CFU); each CFU represents one cell from the original sample. Therefore, the selective agar overlay method allows isolation of a pure strain and recovery of injured cells as well as calculation of the bacterial cell concentration from the original sample (13).

Unfortunately, methodology on the use of Mannitol Salt Agar (MSA; Hardy Diagnostics, Santa Maria, CA) as a differential and selective medium with clinical relevance (3) in the selective agar overlay method of enumerating S. *aureus* has not been documented. Others have found that Gram-positive Agar (GPA) was the most effective selective medium component of the overlay method for S. *aureus* (10). However, GPA is selective for all Gram-positive bacteria, not just S. *aureus*. Furthermore, the selective agar overlay method has been studied for S. *aureus* in the context of food microbiology but not for clinical microbiology (13).

Several stressors have the potential to sublethally injure S. *aureus* cells prior to recovery by the selective agar overlay method. Carvacrol, an extract from oil of oregano, increases the bacterial cell membrane permeability to potassium, which alters potassium and hydrogen concentration gradients. This disrupts ATP synthesis and leads to a decreased pool of ATP available within the cell (15). Carvacrol has the potential to be incorporated into the clinical arena in conjunction with antibiotics and, therefore, has been chosen as a clinically relevant stressor for this study.

Ultraviolet (UV) radiation injures bacterial cells by damaging DNA. UV light initiates a reaction between thymine bases in DNA to create a thymine dimer; two thymine bases are bonded together, which is not a normal base pairing. This mutation is stable and difficult for the cell to repair; therefore, the cell cannot transcribe its DNA normally and a loss of function occurs (11). UV light is used as a disinfecting measure in clinical settings and is, therefore, considered a clinically relevant stressor.

Hydrogen peroxide acts at the bacterial cell membrane as an oxidizer, which would alter the activity of certain bacterial membrane proteins (9). Hydrogen peroxide produced by immune cells can be used to combat bacterial infection; therefore, hydrogen peroxide is relevant in the clinical arena.

By stressing S. *aureus* cultures with carvacrol, UV light, and hydrogen peroxide, a stressor which is most useful for testing the selective agar overlay method can be chosen. In order to be effective for this assay, the selected stressor needed to sublethally injure cells and therefore produce significantly lower cell counts on non-selective media than counts for non-stressed cells. This stressor also needed to allow for consistent

recovery of enough injured cells in order to conduct reliable statistical analyses.

The goal of this study was to develop a time-efficient selective agar overlay method of enumerating S. *aureus* which is sensitive and selective in the clinical arena. The objectives of this study were: 1) to show the selective agar overlay method is significantly more sensitive to enumerate S. *aureus* than plating directly onto selective media, such as MSA, and 2) to show the selective agar overlay method is equally as sensitive, but more selective than plating S. *aureus* onto non-selective Tryptic Soy Agar (TSA; Teknova, Hollister, CA). We hypothesize that if selective overlay plating (TSA-MSA) allows for recovery of injured S. *aureus* and selects for S. *aureus*, then selective overlay CFU/mL will be significantly higher than direct selective plating (MSA-MSA) CFU/mL alone and comparable to direct non-selective plating (TSA-TSA) CFU/mL.

MATERIALS AND METHODS

BACTERIA

Staphylococcus aureus strain ATCC BAA-977 was obtained from Microbiologics (St. Cloud, MN). Cultures were incubated in Tryptic Soy Broth (TSB; Alpha Biosciences, Baltimore, MD) for 24h at 37°C. Optical density (OD) was measured as 0.46 using a Beckman Coulter DU 530 spectrophotometer at 550 nm (Beckman Coulter, Brea, CA). All laboratory work was performed at Ball State University.

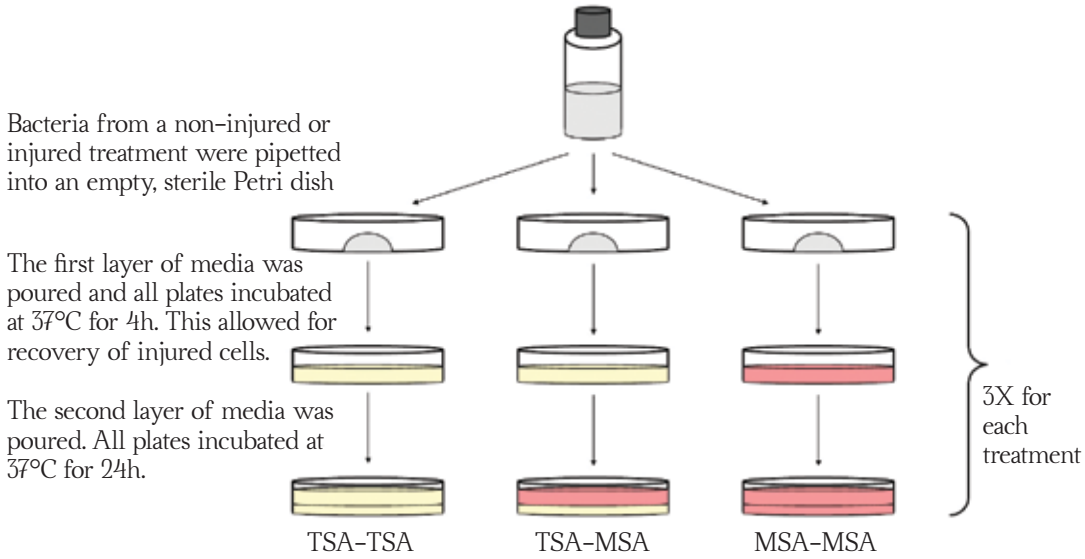
BACTERIAL INJURY

Staphylococcus aureus cells from TSB cultures were serially diluted 1:100 in sterile saline before undergoing stress treatments. Three stressors were tested: carvacrol (SAFC Supply Solutions, St. Louis, MO), UV light, and 3% hydrogen peroxide (VWR International, LLC, Radnor, PA). Our intent was to find a stressor that caused injury, but did not completely kill all cells. Carvacrol was added to the 10^{-2} diluted S. *aureus* cells at a volume of 100 uL, and these cells incubated for 5 min at room temperature. Serial dilutions up to 10^{-6} CFU/mL were then performed on the carvacrol-injured cells to determine efficacy. Injury by UV light was achieved by pouring cells from the 10^{-2} dilution to a layer of 5 mm thick into two separate, sterile Petri dishes. This allowed for injury by UV light for either 60 s or 90 s at room temperature, with agitation at every 30 s. Three percent hydrogen peroxide was added to the 10^{-2} diluted S. *aureus* cells at a volume of 5 mL, and these cells incubated for 5 min at room temperature. Serial dilutions up to 10^{-6} CFU/mL were then performed on the hydrogen peroxide-injured cells.

STRESSOR ANALYSIS

Carvacrol-injured, UV-injured (60s and 90s), hydrogen peroxide-injured, and 10^{-6} serially diluted non-injured (control) S. *aureus* cells from TSB cultures were pour plated with non-selective media (TSA) in accordance with methods used by Leboffe and Pierce (8). Plates were incubated 4 h at 37°C, and then overlaid with 20 mL of TSA. Plates were then incubated for 24 h at 37°C. Each of the five treatment groups was inoculated in triplicate. Colonies were counted and converted to CFU/mL.

Figure 1. Schematic of the methods used for recovery of *Staphylococcus aureus* cells by nonselective (TSA–TSA), selective (MSA–MSA), and selective overlay (TSA–MSA) plating techniques.



MEDIA ANALYSIS

Three base layer–top layer test media combinations were used in this study: non-selective (TSA–TSA), selective (MSA–MSA), and selective overlay (TSA–MSA). Non-selective plates and selective overlay plates used TSA as the base layer medium for pour plating, while selective plates used MSA as the base layer (Fig. 1). Non-injured *S. aureus* cells from TSB cultures were serially diluted to 10^{-6} CFU/mL and pour plated with the base layer of each test media (non-selective, selective, and overlay) and labeled. Carvacrol-injured *S. aureus* cells were pour plated at a 10^{-6} dilution with the base layer of each test media (non-selective, selective, and overlay). All plates were incubated 4 h at 37°C. Plates were then overlaid with a top layer of 20 mL of either TSA (non-selective plates) or MSA (selective overlay and selective plates). Plates were then incubated at 37°C for an additional 24 h after which colonies were counted and converted to CFU/mL. Each test media group for both non-injured and carvacrol-injured treatments was inoculated in triplicate.

STATISTICS

Two sets of statistical analysis were run. First, recovered *S. aureus* CFU/mL (response variable) were compared by a general linear model to determine how they were impacted by the four stressors and control (predictor variables). Once the most appropriate stressor was identified (i.e., carvacrol), *S. aureus* was subjected to the stressor and grown using non-selective, selective, and selective overlay media (predictor variables). Second, comparison of *S. aureus* CFU/mL (response variable) was done using a general linear model and a Tukey's honest significant difference test (Minitab 17, State College, PA). Significance was set at $p = 0.05$.

RESULTS

Concentration of recovered *Staphylococcus aureus* cells on non-selective media (TSA-TSA) varied depending upon the stressor type used (GLM, $F = 445.81$, $N = 15$, $P < 0.001$). Hydrogen peroxide-injured cell recovery was not statistically different from non-injured recovery, but was higher than carvacrol and UV-injured cell recovery. Carvacrol-injured were recovered at significantly lower concentrations than non-injured cells, but were recovered at significantly higher concentrations than UV-injured cells. UV-injured cells for both 60 and 90 seconds of exposure were recovered at significantly lower concentrations than all other treatment groups (Fig. 2).

Differences were shown in the recovery of carvacrol-injured *S. aureus* cells based on the media type. Cells were recovered from selective media at significantly lower concentrations than non-selective media and selective overlay (GLM; $F = 11.90$, $N = 9$, $P = 0.008$). Carvacrol-injured *S. aureus* cell recovery from selective overlay was not significantly different than recovery from non-selective media (Fig. 3).

Figure 2. *Staphylococcus aureus* density (CFU/mL) recovered on non-selective media (TSA-TSA) for five stressor treatments: non-injured, carvacrol-injured, hydrogen peroxide-injured, UV-injured for 60 sec, and UV-injured for 90 sec. Bars are 2 standard errors from the mean. Letters indicate significant difference as indicated by Tukey's honest significant difference test.

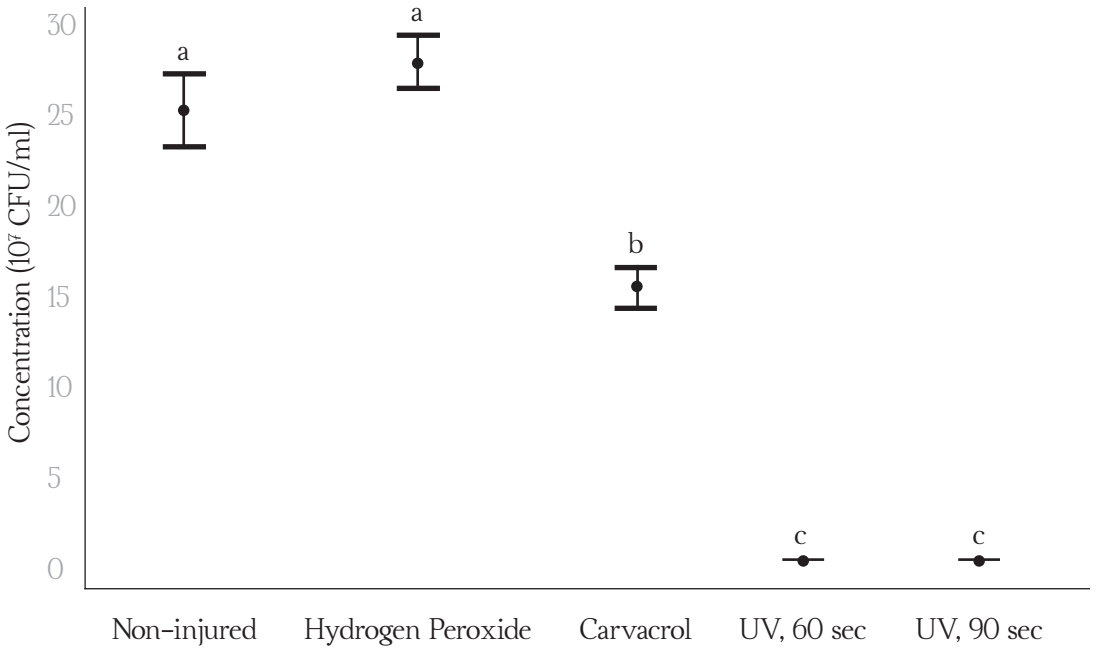
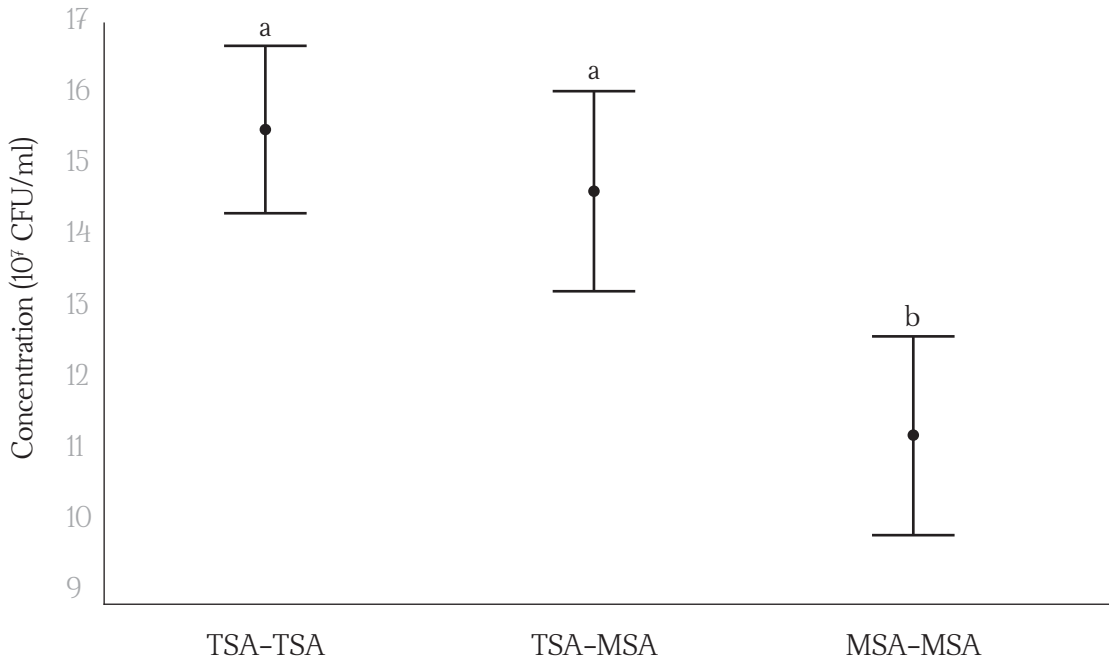


Figure 3. Carvacrol-injured *Staphylococcus aureus* concentration (CFU/mL) recovered on non-selective media (TSA-TSA), selective overlay (TSA-MSA), and selective media (MSA-MSA). Bars are 2 standard errors from the mean. Letters indicate significant difference as indicated by Tukey's honest significant difference test.



DISCUSSION

We hypothesized that using a selective agar overlay method would result in more accurate enumeration of viable cell counts of S. *aureus* compared to direct plating onto a selective agar. As hypothesized, the recovery of injured cells *via* the selective agar overlay method was comparable to recovery on non-selective media, but greater than recovery on selective media.

We intended to choose a stressor which would result in significantly less recovery of S. *aureus* cells on non-selective media than non-injured cells, and would allow for high enough recovery to ensure reliable colony counts. Hydrogen peroxide and UV light were not chosen as stressors for assessing the selective agar overlay because they did not meet this requirement. Hydrogen peroxide treatment allowed for recovery of enough cells to perform statistical analyses, but did not stress cells significantly from control, which is a necessity for ensuring the presence of sublethally injured S. *aureus*. UV-injured cells were recovered at a significantly lower concentration than non-injured cells, but viable cell counts from cells treated with this stressor were too low (fewer than 100 colonies per plate) to ensure consistency upon replication of the experiment. Carvacrol significantly stressed S. *aureus* cells compared to control, but not

to the extreme degree as seen in the UV treatments. In recent studies, carvacrol has been used to cease the synthesis of proteins that are important in bacteria such as *Escherichia coli* (2). Therefore, the selection of carvacrol to sublethally injure S. *aureus* was ideal for determining the effectiveness of the selective agar overlay method.

The selective agar overlay assay was effective because recovered carvacrol-injured viable cell counts on the selective agar overlay were not statistically different from non-selective counts. In addition, the selective agar overlay counts were statistically higher than selective media cell counts. This indicates that the four hour recovery period in the selective overlay method promotes sensitivity. Our findings and others (12) support the conclusion that the selective agar overlay method does allow for sensitive recovery of injured cells in the same manner that TSA would, but the overlay method does not compromise the selectivity of MSA for S. *aureus*. This was concluded because the recovered CFU/mL for the overlay method are comparable to non-selective CFU/mL but higher than selective CFU/mL.

The selective agar overlay method is a relevant detection method for the clinical microbiology arena. This assay is more sensitive than direct selective plating, but maintains the selective and differential nature of selective media. These traits together minimize the potential for false negative diagnoses of S. *aureus* infection and allow for effective enumeration of S. *aureus* in samples with multiple bacterial species. The selective agar overlay assay is also time-efficient, which is necessary to expedite detection of S. *aureus* cells in patients and to prevent long-term damage resulting from illness. This may reduce financial burdens that long-term hospital stays can place on patients and families. The selective agar overlay method has advantages compared to current clinical methods of S. *aureus* isolation, which are direct plating on selective media containing antibiotics and multiplex polymerase chain reaction (PCR; 4,5). As shown in our results, recovery of S. *aureus* on selective media results in lower enumeration compared to the original sample. Multiplex PCR is both rapid and sensitive, but it is extremely expensive and is a difficult technique to execute. The selective agar overlay method is inexpensive and can be performed correctly with minimal training.

Future studies may use alternative methods of stressing cells, including higher concentrations of hydrogen peroxide or less exposure time to UV. Additional *in vitro* and *in vivo* studies, using clinical samples and/or animal models, are required before application of this assay to a clinical setting.

ACKNOWLEDGMENTS

We thank Dr. Thomas Lauer for guidance and support in manuscript writing and editing. We also thank Dr. John McKillip for assisting with experimental design and for supplying materials. In addition, we acknowledge the Ball State University Department of Biology for funding the project.

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LETTER

LETTER TO THE EDITOR

ELIZABETH A. B. EMMERT
CHAIR, *ASM TASK COMMITTEE ON LABORATORY BIOSAFETY*
PROFESSOR OF BIOLOGICAL SCIENCES,
SALISBURY UNIVERSITY, SALISBURY, MD USA

I commend *Fine Focus* for its article highlighting the importance of biosafety for microbiology undergraduate researchers (1). Biosafety in the microbiology laboratory should be of utmost concern to all involved in undergraduate microbiology education including student researchers, students enrolled in microbiology lab courses, faculty research mentors and teaching faculty. The article by Schwartz *et al.* included much valuable biosafety material, but I believe the article omitted two significant pieces of information. One was the laboratory-associated infections most relevant to undergraduate microbiology students and the other was the American Society for Microbiology (ASM) biosafety guidelines specifically developed for the undergraduate microbiology laboratory (2).

The article by Schwartz *et al.* emphasizes the importance of laboratory biosafety through its description of two laboratory accidents with unfortunate and deadly outcomes for the researchers involved who did not practice all aspects of proper biosafety (1). Although these cases are tragic, neither involved an undergraduate student in the laboratory. Unfortunately, infections and hospitalizations linked to microbiology teaching laboratories are all too common. The Centers for Disease Control and Prevention (CDC) has documented numerous cases of undergraduate students and their close contacts developing infections from exposures to organisms in the teaching laboratory. Between November 2013 and May 2014, a total of 41 cases of *Salmonella* Typhimurium infection with strains known to be used in laboratory settings were identified (http://www.cdc.gov/salmonella/typhimurium-labs-06-14/index.html). These cases came from thirteen different states and 86% of the affected individuals interviewed were enrolled in a biology or microbiology course. Thirty-six percent of affected individuals were hospitalized. In 2011 another multi-state outbreak of *Salmonella* Typhimurium linked to clinical or teaching microbiology laboratories involved 109 cases and one death (http://www.cdc.gov/salmonella/2011/lab-exposure-1-17-2012.html).

In response to the 2011 *Salmonella* outbreak, ASM developed a set of biosafety laboratory guidelines that address the unique aspects of biosafety in the undergraduate microbiology laboratory (2). Unlike the universal and extensive BMBL guidelines (3), the ASM biosafety guidelines are valuable to students and faculty because they are explicit to the undergraduate microbiology laboratory experience. The guidelines address personal protection requirements, laboratory physical space requirements, stock culture requirements, standard laboratory practices, training practices, and document practices. Biosafety guidelines were developed for work at both biosafety level one (BSL-1) and biosafety level two (BSL-2). A risk assessment including the microbes to be used and the laboratory procedures to be performed should determine the appropriate biosafety level. Although Schwartz *et al.* state, "An undergraduate microbiology teaching laboratory may be considered a BSL-1 laboratory," many undergraduate microbiology labs should be operating at BSL-2. If organisms such as *Staphylococcus aureus*, *Proteus vulgaris*, or *Salmonella enterica* are used or if students are subculturing unknown, environmental microbes, then the lab should be operating under BSL-2 conditions. An extensive appendix accompanies the ASM biosafety guidelines to clarify and expand on the guidelines. (http://www.asm.org/index.php/microbelibrary/laboratory-safety-guidelines).

Although these guidelines were published in 2013, it is likely that many faculty are

still unaware of them. The fact that an NIH researcher in the BioRisk Management Program failed to mention these guidelines in an article about biosafety for microbiology undergraduates demonstrates the unfortunate obscurity of the guidelines. While faculty want to keep their students safe in the lab, they may be ignorant of the best biosafety practices. The reasons why faculty are uninformed about the ASM biosafety guidelines are numerous. At many colleges and universities, faculty without expertise in microbiology are called upon to teach microbiology labs. Faculty who are not microbiologists occasionally do laboratory activities in their courses (Introductory Biology, Cell Biology, etc) that use microorganisms. Older faculty trained in an era with a cavalier attitude toward biosafety may not view biosafety as a significant concern. Even conscientious microbiology faculty whose students follow commonly accepted biosafety practices (washing hands, disinfecting benches, no food or drink, etc.) may not realize that students should only use institution-provided pens or pencils that are kept in the lab. When students use their own pens in the lab, they can become contaminated, allowing students to unwittingly take microbes out of the lab that could then infect them or others.

The article by Schwartz *et al.* focuses on undergraduate microbiology researchers (1). Although the ASM biosafety guidelines were written for the microbiology teaching laboratory, the information is also applicable to the undergraduate research laboratory. Certainly undergraduates performing microbiology research should be just as concerned with biosafety as undergraduates enrolled in microbiology lab courses. Ideally such students have already been instructed in the best biosafety practices while enrolled in a microbiology lab course. If a student researcher is working with microbes and has not taken a microbiology course, the student should be sufficiently trained in biosafety before beginning the research project. Faculty advisors should be aware of the biosafety training their student researchers have received – especially if a student took a microbiology course at a different institution – and tailor the biosafety training for each student accordingly. As faculty we set the example for our students when we stress the importance and required diligence of consistently following best biosafety practices.

I wholeheartedly agree that good science is safe science! Students and faculty should work together to make sure the best biosafety practices are being followed at all times in all undergraduate laboratories. As readers interested in undergraduate microbiology education, I urge you to do your part to spread the word about the ASM biosafety guidelines and the importance of following the best biosafety practices.

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PERSPECTIVE

UNDERGRADUATE PERSPECTIVES FROM THE *FINE FOCUS* STUDENT TEAM, FALL TERM 2015, UNIVERSITY OF AKUREYRI, ICELAND

BIRTA LÍF FJÖLNISDÓTTIR
BIOTECHNOLOGY

As an undergraduate science student, I feel like I have the constant urge to expand my knowledge in every possible way. With *Fine Focus* I've reached an unknown area of science, the other side.

When referring to the other side, I'm talking about the process of how things are handled after completing the actual experiments; that is, submitting a paper, based on those experiments and up to the point where it is accepted or rejected. Having said that, I've gotten the opportunity to understand the amount of work spent by each scientist out there performing experiments that leads to the expression of their thoughts and discoveries in articles, which then go through the exhausting review process.

After being a part of *Fine Focus*, I feel like I've realized how much mutual respect is among people from each of these sides, where the scientist depend on, and trust the reviewers to judge their work with a positive perspective. At the same time, reviewers depend on the scientists to do their work in such a manner that their free time is not poorly spent.

With that new realization, I also feel like my future work as a scientist, or a reviewer, will be better spent since I now know that genuine people out there are doing the same things. I also really believe that these people do their job with good intentions, to make science accessible. *Fine Focus* has not only expanded my knowledge on and respect for people in journalism but it has also opened an expression frame for undergraduate students to publish their work, which otherwise might be lost in the chaotic field of science.

Thank you *Fine Focus*, and last but not least, thank you John McKillip.

PÁLÍNA HARALDSDÓTTIR
BIOTECHNOLOGY

I didn't know much about the *Fine Focus* journal until I started the class, although I knew it was a journal for undergraduate students. I decided to take the *Fine Focus* class because I figured, since I plan on submitting papers in the future, I was able to understand the process of journal submissions and principles of how to write a good paper.

The *Fine Focus* class has taught me a lot! I was not aware of the process of journal systems, when journals receive manuscripts or papers until it gets submitted, re-submitted or rejected, I had questions like; "How can I submit a paper?", "Who decides whether my paper gets submitted or rejected?" and "Am I allowed to resubmit?". This class has allowed me to be a part of this

whole process, from receiving a manuscript or paper until the final decision has been made. This takes a lot of time and hard work for editors, reviewers and everybody involved.

I think each and every person in this class has improved the *Fine Focus* journal in many ways, by spreading our knowledge, opinions and suggestions. I highly recommend students to take the *Fine Focus* class, it is fun, instructive and improves your writing in so many ways.

In addition, I think the *Fine Focus* journal has great potential and will become a platform for many undergraduate students who are taking their first step into the world of scientific publication.

SIGRÍÐUR KRISTINSDÓTTIR
FISHERIES SCIENCE

This course has opened up for me the importance of cooperative working and international communication in a world of rapid change in science. The launching of this many-sided *Fine Focus* project is an intriguing initiative. It is a healthy and meaningful way of schooling undergraduates in scientific writing, reading and reviewing a paper. You are reminded of the importance of keeping up with the latest published material.

> “[*FINE FOCUS*] IS A HEALTHY AND MEANINGFUL WAY OF SCHOOLING UNDERGRADUATES IN SCIENTIFIC WRITING, READING AND REVIEWING A PAPER”

The course is probably a reflection of how things work out in the field, a combination of teamwork and taking on different tasks with feedback from other scientific milieus. When selecting three persons from an external worldwide Editorial Board, I came to realize, being a student of fisheries science, the various subdisciplines of microbiology and how they can relate to other expertise, even outside of biology.

As a product-based course it challenges our integrity with being held responsible as lead editors for one paper throughout the whole scenario from submission to decision-making, publishing and promotion. You have to work professionally from start and have your research and writing support each other along the process.

This is good training in how to summarise the content of a paper. Peer reviewing taught me the importance of clarity. It all revolves around the hypothesis when structuring a research paper. You adjust to the language and it is vital for your career to learn from start the rules of ethics, to handle citations and

references with respect. It also puts focus on originality and how good work adds to the body of knowledge.

I am more familiar with how to rate a paper for publishing, the scope of journals, their policies, rate of rejections, regulations and checkboxes. Rejection of a paper is something scientists will have to learn to live with, and we had to rationalize our final decision on student papers as for what to improve.

The situation of this student group is probably distinct from others in this field of study, with exchange students and people of different ages, some with previous work experience. My special contribution to the journal was updating the website. Despite my presence through Skype, with a few burls, being a distance student, I felt amazing as part of the group. John is definitely the right person to run the complicated tasks of this course and keeping up the spirit.

KRISTÝNA TRONEČKOVÁ
JOURNALISM/PSYCHOLOGY

Fine Focus is a great opportunity for me to get to know a real experience with marketing plans and raising funds. The big advantage is the fact that everything is real and not a training of hypothetical situations. *Fine Focus* is an unique opportunity for improving my soft skills, too.

I used my psychological and journalistic knowledge for creating a website called First Giving for potential donors, and for improvement of our existing marketing plan. It was also helpful to share information in the classes; we have made many useful decisions, thanks to brainstorming.

As I am not a microbiology student, I could not develop the ideas regarding this particular field, but I appreciated interesting discussion about this topic as well as proofing the manuscripts, with interesting microbiological information. It has opened the doors to the new world. Also, I liked taking photos for the First Giving website in a lab because students showed us their tools in examining microbes. I liked that the lessons were open discussion and a friendly atmosphere.

I hope our effort, when we tried to improve the marketing and funding issue, will provide background and better conditions for student's work and manuscripts. I really like the fact that *Fine Focus* is a real journal where I can practice my knowledge. As it is not only theoretical schema, everything that I did was discussed and I could see the changes regarding *Fine Focus* during this semester.

I learned something from marketing and from the system of getting donations, as well as the steps which are necessary for success in this area. I find interesting the communication with administrators of the First Giving site, too. Finally, we could see our result and we can wait what new our effort will bring to *Fine Focus*. I am glad I could be a part of the *Fine Focus* team. Hopefully we did something worthwhile!

SIGÞÓRA BRYNJA KRISTJÁNSDÓTTIR
BIOTECHNOLOGY

My involvement in the *Fine Focus* course has been rewarding and fun. Before I signed up for this course, I didn't know much what I was going into and I was not sure if this course would benefit me and if it would be useful to my study. But really soon I changed my mind. It was really fun to be a participant and learn how the real peer-reviewed journal is produced from start to finish. The *Fine Focus* course has also helped me a lot to step considerably outside my comfort zone. I was trained in critical thinking, I improved a lot reading English scientific articles and my English vocabulary has increased considerably.

I think without a doubt that this course will be useful to me in many ways after my graduation. In my opinion, a subject like this should be taught in almost all courses that revolve largely about writing and publish articles and it would be a good preparation for students when they start working. I hope that my contribution will help so we will publish a good and well-made peer-reviewed journal, by helping review articles and other important projects within this course.

There was also a large part of the course devoted to marketing and presentation of the journal, and it was very informative, though I was not directly involved in that part. I hope this course will thrive and grow with each period and I look forward to watching this project develop for the better.

SAARA-MARIA KALLIO
INTERNATIONAL BUSINESS

I am a third-year International Business student, and being part of a group of student editors of *Fine Focus* has been an engaging way to relate my theoretical studies to real life, as well as to learn more about the world of microbiology, which used to be like an undiscovered land for me before. Through *Fine Focus*, my interest towards working in multinational teams, challenging myself and gaining experience from multiple fields, such as microbiology, has increased notably.

During *Fine Focus* Autumn 2015, I was mainly responsible for improving the marketing plan to increase the awareness of *Fine Focus*, especially in Europe, in order to increase the visibility of the journal and thereby the number of submitted undergraduate papers. I enjoyed the most the process of innovative thinking for bringing in fresh ideas for improving the marketing of *Fine Focus*, knowing that out of which eventually the most creative ideas would be selected and applied to the actual Marketing Plan after discussing as a group with the rest of the student editors.

Fine Focus has not only been a truly

interesting course completed as a part of my international studies, but also it will be part of my life again in the near future. As a marketing student, I got offered an opportunity to represent *Fine Focus* at the British Conference of Undergraduate Research (BCUR) in March 2016 in the UK. Through this exciting opportunity I will get to develop my skills in a real world context and gain further practical experience on international marketing.

I have found it truly fascinating to participate in running the development of an international journal and being part of the growth of *Fine Focus*. Attending the conference in Manchester will not only be a great opportunity for networking but also it will be one step closer to reaching our goals in expanding *Fine Focus* to Europe.

HELGA HELGADÓTTIR
BIOTECHNOLOGY

Taking part in *Fine Focus* has made me better at representing scientific work, both according to structure and content. Being good at representing my work will save time both for me as a writer and others that will read my work in the future. Another important thing that I have learned is to make my material interesting from the beginning and let it stay that way throughout. Keeping material interesting is essential to the readers so they don't loose interest and give up on reading and perhaps miss the good part of the paper.

I have also improved my skills in recognising a good paper, not only a well-written one but also one that involves good scientific practises. Recognising a good paper is critical when searching for good information on given topic. Learning how the peer review process works in detail, from submission to publication, is a valuable lesson and will make me more confident when publishing in the future.

> “FINE FOCUS HAS GIVEN ME VALUABLE INSIGHT INTO THE SCIENTIFIC WORLD OF REPRESENTING AND PUBLISHING DATA, AN INSIGHT THAT, UNFORTUNATELY, MANY UNDERGRADUATES DON'T GET.”

Participating in this course also gave me a glance of both marketing and graphics, two fields that I don't know much about but are highly connected to the scientific world. For example, it's useful to know how to promote my work after it has been published.

Overall, participating in *Fine Focus* has given me valuable insight into the scientific world of representing and publishing data, an insight that, unfortunately, many undergraduates don't get.

POST/BIOTICS

Vidhi Mehta
Theresa Schachner

www.post-biotics.com
Find more about Post/Biotics on BBC, Wired, FastCompany & ScienceAlert

What if the next antibiotic is in your backyard?
Post/Biotics is a platform that facilitates finding new antibiotics by integrating citizen scientists in the process of identifying natural substances with antimicrobial properties.

Opportunity
Most known antibiotics are developed from natural extracts, implying a potential new antibiotic could be anywhere. Post/Biotics distributes empirical research to the public by creating a platform and the according tools for participating in this process. The pop-up lab allows anyone to discover substances with antimicrobial values by testing locally available plants, vegetables, fruits, fungi, mushrooms & soil. With the help of the corresponding app, the results are stored on an online platform to create a library of antimicrobials. The value of this “microbial fingerprint” of our environment is significantly important for developing new drugs.

By partnering with universities & pharmaceutical companies, the massive costs for finding new drugs can be diminished when giving access to this platform.

Impact
The coming cost of antimicrobial resistance is 10 million deaths per annum by 2050. This is estimated to cost about 3% of the global GDP. Antibiotic resistance is growing faster than pharmaceutical companies are finding new ones. Corporate Social Responsibility of a pharma company can largely benefit from an educational initiative that promotes growth of young minds in STEM careers in research.

Method
Users test their samples like a crushed bark of a tree in their backyard with the toolkit that contains E.coli bacteria. If the tested tree bark has antimicrobial properties, it will be recognized by the platform and the user is encouraged to submit the original platform. Using gamification, users are incentivized to test different samples, geographical areas, and to use their peers for verifying their findings.

Vision:
Post/Biotics is a vision for revolutionizing drug discovery and raising awareness for the problem of antibiotic resistance.

The Spring 2016 Fine Focus team at Ball State University, Muncie, IN.

FINE FOCUS TEAM

Professor John McKillip
Kyla Adamson
Jill Bange
Mallory Beaty
Danielle Behrens
Tyson Bird
Cassandra Bormann
Breanna Brenneman
Bryce Buchanan
Adam Carr
Christa Castillo
Derek Chiles
Amie Cipolla
Lexie Crump
Jake Davis
Cory Diemler
Alysha Ellison
Emma Kate Fittes
Birta Líf Fjölnisdóttir
Catherine Galbraith
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Jack Hesser
Nick Holt
Kevin Jewett
Edward Jones
Saara-Maria Kallio
Sigþóra Brynja Kristjánsdóttir
Sigríður Kristinsdóttir
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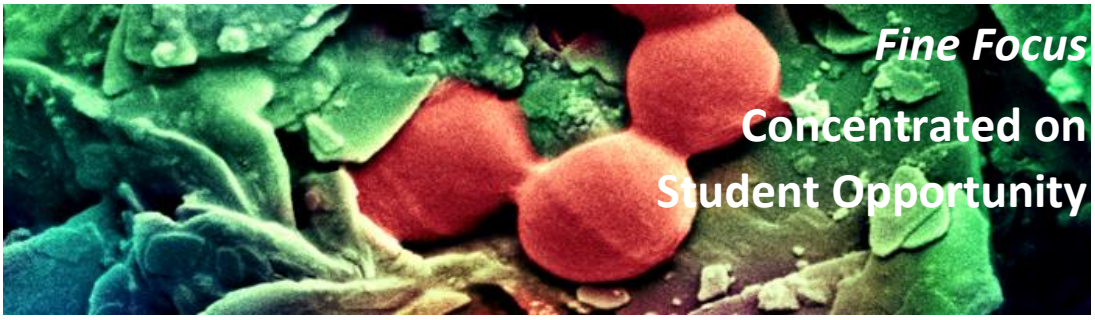

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