Novel Method of Culturing Bacteria using Soil Extract and Nutrient-rich Broths

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Abstract

The misuses of antibiotics and the lack of development for novel antimicrobial compounds have culminated in an antibiotic resistance crisis that threatens the life of human. Various strategies have been proposed to overcome this crisis, one of which is to find compounds in unknown bacteria by adopting novel techniques of bacterial culturing. With the same goals in mind, we investigated our method of combining soil extract with nutrient-rich broths to create compound agars. We designed a multifactorial experiment, blocked by temperatures of incubation and pathogens in the soft agar overlay, to assess the efficiency of these agars in growing more diverse bacterial colonies and colonies with antimicrobial characteristics. Our results indicated that compound agars provided a suitable environment for a wide range of bacteria but original soil extract agar produced more colonies with antimicrobial characteristics. In the future, more advanced techniques such as gene sequencing should be included for better assessment of diversity and future study should narrow on specific pathogens to potentially explore other antimicrobial mechanisms.

1) Introduction

The widespread introduction of antibiotics, beginning with penicillin in the 1930s (Flemming, 1929; Kardos & Demain, 2011) and streptomycin in the 1940s (Schatz et al., 1944), has transformed the field of infection treatment. However, the misuses of antibiotics in human therapy and animal husbandry, combined with the lack of development for novel antimicrobial compounds from the pharmaceutical industry, have culminated in an antibiotic resistance crisis that threatens the lives of humans (Ventola, 2015). According to a report by the Centers for Disease Control and Prevention in 2019, antibiotic-resistant bacteria are responsible for approximately 2.8 million infections and 35 thousand deaths each year in the United States (CDC, 2019). Recognizing the serious implications of this crisis, different strategies have been developed to tackle the problem, one of which is to find new antibiotic compounds by the culture of unknown soil bacteria (Hamamoto et al., 2014; Ling et al., 2015). However, this process is significantly hindered by the paradox of Great Plate Count Anomaly: approximately 1% of bacteria from the environment can be cultured with laboratory context by conventional culture techniques due to the failure in replicating specific environmental and nutritional factors. To overcome this challenge, we investigated a novel method of making agar, which is built upon the soil extract agar/soft agar overlay research of Hamamoto et al. (2021). We combined the soil extract with nutrient-rich broths (Tryptic Soy broth and Luria broth) to create compound agars. We hypothesized that the nutrient similarity from the soil extract and the nutrient supply from the broths would allow the compound agars to support growth of more bacterial colonies that demonstrate antimicrobial characteristics.

2) Material and Methods

2.1) Agar Preparation

To prepare the soil extract agar, we suspended approximately 400 g of soil in 500 ml of deionized water (DI water). The suspension was then autoclaved and the supernatant was collected after centrifugation at 5000 rpm for 10 minutes. The supernatant was autoclaved again after the addition of 9 g Bacto Agar (Becton, Dickinson, and Company, Sparks, MD, USA). The resulting aliquot was allowed to cool in a water bath at 50℃ before 2 ml of cycloheximide was added to prevent fungal growth. Around 25 ml of the aliquot was then poured into standard petri dishes for solidification. With the compound agars, the procedure was mostly similar, except we added 1.5 g of Tryptic Soy broth (Becton, Dickinson, and Company, Sparks, MD, USA) for the Tryptic Soy broth (TSB) compound agars and 1.25 g of Luria broth (Thermo Fisher Scientific, MA, USA) for the Luria broth (LB) compound agars to achieve one tenth strength of nutrients.

2.2) Bacteria Culture and Test of Antimicrobial Activity

To culture bacteria, we use the conventional microbiological techniques of serial dilution and plate streaks. One gram of the same soil used for the agar was suspended in 10 ml of DI water and serially transferred in 100 μl aliquots to new Eppendorf tubes that contain 900 μl DI water to reach the dilution factor of 10^{-4}. A 100 μl aliquot of this dilution was plated on each agar plate and streaked using glass beads. The plates were incubated at designated temperature (20℃, 25℃, 30℃) in a random order and the number of bacterial colonies was assessed after 24 hours.

To test the antimicrobial activity, the technique of soft agar overlay was applied. We prepared the medium for the overlay with Luria broth (Thermo Fisher Scientific, MA, USA) with a lower agar concentration of 0.7%. After autoclaving, the medium was allowed to cool down to about 50℃ and this temperature was maintained in a water bath. The pathogens for our study, *Acinetobacter baylyi* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), were grown overnight in Luria broth (Thermo Fisher Scientific, MA, USA) and this broth was added to the soft agar medium at the ratio of 1:10. From this, approximately 5 ml aliquots were overlaid directly on the plates. We then incubated the plates at the designated temperatures for another 24 hours to judge the antimicrobial activity through the presence of inhibition zones (Figure 2).

2.3) Experimental Design

The number of colonies and colonies with antimicrobial characteristics can vary substantially between incubation temperatures and the pathogens in the overlay. To mitigate these variations, we designed our study with these 2 factors as blocks, with 2 levels for pathogens (*A. baylyi* and *S. aureus*) and 3 levels of incubation temperatures (20℃, 25℃, 30℃). For agar types, as mentioned above, we have 2 compound agars, TSB agars and LB agars, as treatments while we included normal soil extract agar as the control. Since all factors are crossed, we had 18 balanced groups with 7 randomly chosen plates in each block, totaling to 126 plates for the study. We summarize our experimental design in Table 1.

	S. aureus			***A. baylyi***		
	Control	***LB***	***TSB***	***Control***	***LB***	***TSB***
20℃	7	7	7	7	7	7
25℃	7	7	7	7	7	7
30℃	7	7	7	7	7	7

Table 1. The multifactorial, block design of the study with 2 blocking factors of incubation temperatures and pathogens in the overlay

3) Results

In our analysis with the number of bacterial colonies as a response , we analyzed the data using a two-way ANOVA with agar types and temperatures as our factors. We log-transformed the response variable to account for the skewness in the counts (Appendix C) and found that there were significant differences between the three types of agars ($F = 75.57$, $p < 0.0001$) as well as in the interaction between agar types and temperatures ($F = 4.10$, $p = 0.0038$). However, we did not see significant differences in temperatures alone ($F = 1.97$, $p = 0.1442$). Analyzing our results with Tukey's HSD using α of 0.05, we saw that all three agar types were significantly different, with LB compound agar having the highest mean colonies, followed by soil extract agar and TSB compound agar (Appendix A & D).

To analyze the colonies with antimicrobial characteristics, we used multifactorial ANOVA, with agar types, pathogens in overlay, and temperatures as our factors of interest (Table 1). Additionally, our model included interactions between agar types and pathogens in overlay as well as temperatures and pathogens in overlay. We also tested for interactions between agar types and temperatures, as well as the three-way interaction between all of the factors. However, these interactions were non-significant with p-value higher than the 0.05 threshold and therefore, they were pooled into the error term. We log-transformed our response variable, the number of antimicrobial colonies, to account for significant skewness in the data (Appendix C).

Our analysis of the model showed significant effects for all three of our factors, even when we included the number of colonies as covariate (Appendix D). We performed post-hoc Tukey's HSD with each individual factor and interaction to identify the difference. With agar types ($F = 4.32$, $p = 0.016$), we found that soil extract agar showed significantly more colonies with antimicrobial characteristics than TSB compound agar while LB compound agar was not significantly different than either of the other agar types. With temperatures of incubation ($F = 7.27$, $p = 0.0011$), plates at 30℃ had significantly higher numbers of colonies than plates at 25℃. Interestingly, there was no statistically significant difference in the number of colonies with antimicrobial characteristics between plates at 20℃ and plates at other two temperatures. Finally, with the pathogens in the

overlay ($F = 34.3$, $p < 0.0001$), we saw significantly more colonies displaying antimicrobial properties on plates overlaid with *S. aureus* than on plates overlaid with *A. baylyi*.

We also found significant effects for the interaction between agar types and pathogens in overlay ($F = 9.27$, $p = 0.0002$) as well as temperatures and pathogens in overlay ($F = 3.8$, $p = 0.0253$). Post-hoc Tukey's HSD analysis of the agar*overlay interaction showed that the Soil, S. a. group had significantly higher numbers of colonies with antimicrobial characteristics than any other group while the Soil, A. b. group had significantly lower numbers of colonies than both the Soil, A. a. and the LB, S. a. group. For the temperature*overlay interaction, the 30℃, S. a. group had higher numbers of colonies with antimicrobial characteristics than any other group. In addition, the 25℃, A. b. group showed significantly lower numbers of colonies with antimicrobial characteristics than any of the S. a. groups.

4) Discussion and Conclusion

Soil extract agar and media supplemented with soil extract have been used successfully to isolate previously uncultured microbes (Taylor, 1951; James, 1958; Nguyen et al., 2018). Inspired by the works of Nguyen et al. (2018) and Hamamoto et al. (2021), we decided to incorporate Tryptic Soy and Luria broth, which provide nutrients for a wide range of microbes, into soil extract to create different compound agars. We expected that nutrient abundance of the broths would complement the soil extract, resulting in more diverse colonies and more colonies with antimicrobial characteristics within the laboratory settings. However, our experimental results did not support our hypothesis: the compound agars provided a better environment for diverse bacterial colonies to thrive, both in terms of pigmentations and morphologies, but the original soil extract agar could culture more colonies with antimicrobial characteristics. Among the agar types, LB compound agar was the most suitable for bacterial growth, with significantly more colonies than the other two agars, while the number of colonies on TSB compound agar was slightly inferior than on the soil extract agar. It is also important to note that the colonies on compound agars were at least 3 – 10 times larger and demonstrated a wider variety of colors and shapes than colonies on soil extract agar, which could serve as evidence for diversity. For colonies with antimicrobial characteristics, there were clear inhibition zones against both *S. aureus* and *A. baylyi* on all 3 types of agars, suggesting that the antimicrobial compounds created by the bacterial colonies worked with both Gram-positive and Gram-negative bacteria. In particular, compound agars seemed to produce more colonies against Gram-negative bacteria while soil extract agar worked better with Gram-positive bacteria, although there were no statistically significant data to prove this. Future research can focus on this direction as it is generally more difficult to find antimicrobial compounds against Gram-negative bacteria due to the complex structure of the cell walls of this bacterial group.

Our study bears several limitations that need to be addressed. Firstly, due to time constraint and a lack of budget, we assessed the diversity of colonies using pigmentations and morphologies, which would not be precise because under the influence of different nutrients, colonies of same species might exhibit distinctive characteristics. Therefore, we suggest the use of polymerase chain reaction (PCR) and sequencing techniques in future research to determine the diversity. Secondly, despite the use of cycloheximide, the presence of fungus could be observed in a few plates, especially in compound agars. This would affect the final result of our study as fungus can compete with the bacteria. 2 sources could cause this problem: the amount of cycloheximide was insufficient as the nutrients are excessively abundant or the soft agar with LB broth can promote the growth of fungus. Regardless of the sources, we propose pilot studies to be performed in order to decide the adequate amount of cycloheximide and the choice of agar for the soft overlay. Lastly, we chose S. aureus and A. baylyi as broad representatives of bacterial classification based on cell walls (Gram-positive and Gram-negative). However, the antimicrobial compounds might have other mechanisms than cell wall lysis so future research should narrow down on the pathogens of interest.

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Appendix

A) Exploratory Data Analysis

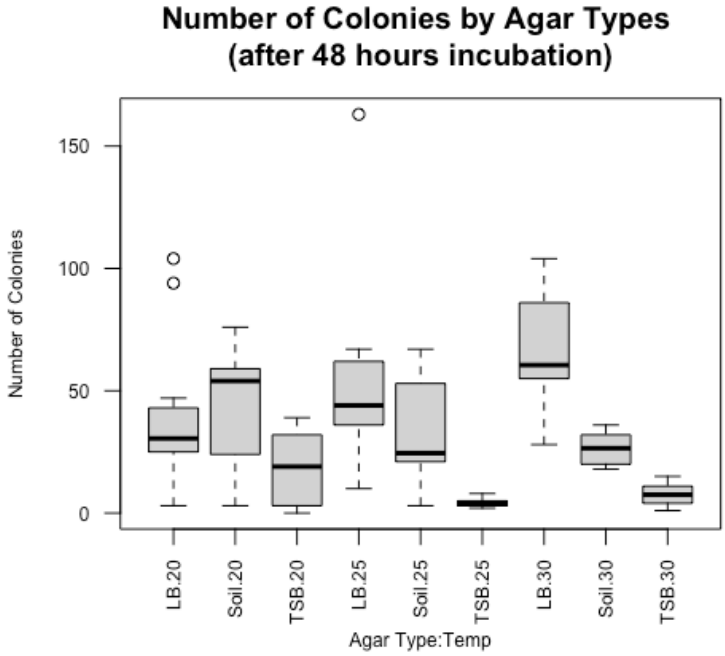

Figure 1. Boxplot of the number of colonies by agar types after 48 hours of incubation at designated temperatures.

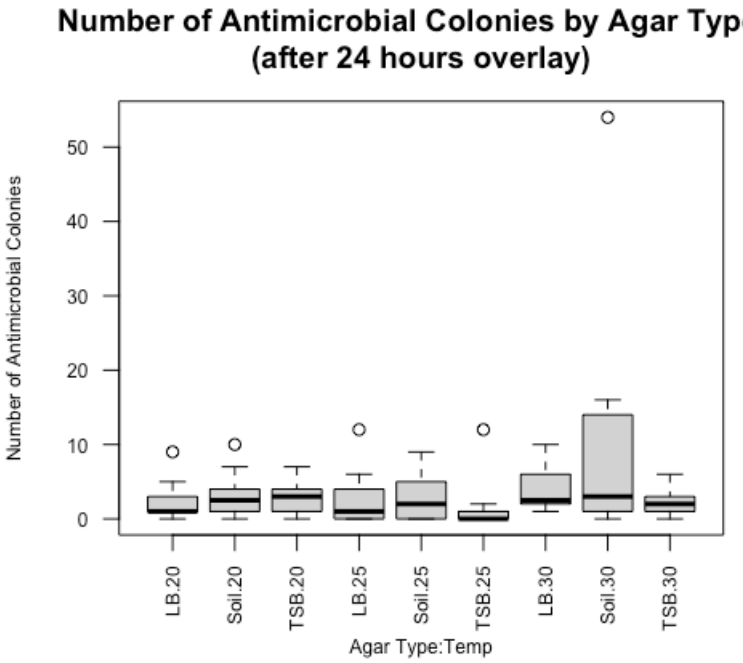

Figure 2. Boxplot of the number of colonies with antimicrobial characteristics by agar types after 24 hours of overlay at designated temperatures.

Response variable	Agar type	Temp	Overlay	Min	Max	Mean	Standard deviation
The number of colonies after 48 hours of incubation at designated temperatures	Soil	20	A.b	3	67	42.29	23.22
	LB	20	A.b.	20	43	31.29	7.63
	TSB	20	A.b.	1	39	17.57	16.93
	Soil	25	A.b.	19	67	28.71	17.00
	LB	25	A.b.	26	163	66.00	45.37
	TSB	25	A.b.	3	6	4.14	1.21
	Soil	30	A.b.	18	32	24.57	5.35
	LB	30	A.b.	28	104	74.14	27.75
	TSB	30	A.b.	1	11	6.43	3.60
	Soil	20	S.a.	19	76	47.86	23.51
	LB	20	S.a.	3	104	48.00	37.89
	TSB	20	S.a.	0	36	19.57	12.19
	Soil	25	S.a.	3	57	41.57	21.49
	LB	25	S.a.	10	52	36.14	14.76
	TSB	25	S.a.	2	8	4.29	2.06
	Soil	30	S.a.	18	36	27.71	7.89
	LB	30	S.a.	52	73	60.29	7.11
	TSB	30	S.a.	4	15	9.43	3.95
The number of colonies that demonstrate antimicrobial characteristics after 24 hours of overlay	Soil	20	A.b.	0	7	1.86	2.41
	LB	20	A.b.	0	3	1.57	1.13
	TSB	20	A.b.	0	7	2.82	2.48
	Soil	25	A.b.	0	3	0.71	1.11
	LB	25	A.b.	0	4	1.57	1.40
	TSB	25	A.b.	0	2	0.57	0.79
	Soil	30	A.b.	0	2	1.14	0.90
	LB	30	A.b.	1	3	1.86	0.69
	TSB	30	A.b.	0	3	1.43	1.27
	Soil	20	S.a.	2	10	4.57	2.70
	LB	20	S.a.	0	9	2.86	3.34
	TSB	20	S.a.	0	6	3.00	2.24
	Soil	25	S.a.	1	9	5.43	2.82
	LB	25	S.a.	0	12	3.43	4.53
	TSB	25	S.a.	0	12	2.14	4.41
	Soil	30	S.a.	4	54	17.29	16.68
	LB	30	S.a.	1	10	5.43	3.05
	TSB	30	S.a.	1	6	3.29	1.80

Table 2. The summary of the data.
Descriptive analysis was performed based on each level of every factor, including agar types, temperatures of incubation, and the pathogens in the overlay. For the overlay, S.a is the abbreviation of *Staphylococcus aureus* while A.b. is the abbreviation of *Acinetobacter baylyi.*

B) Examples of Antimicrobial Characteristics

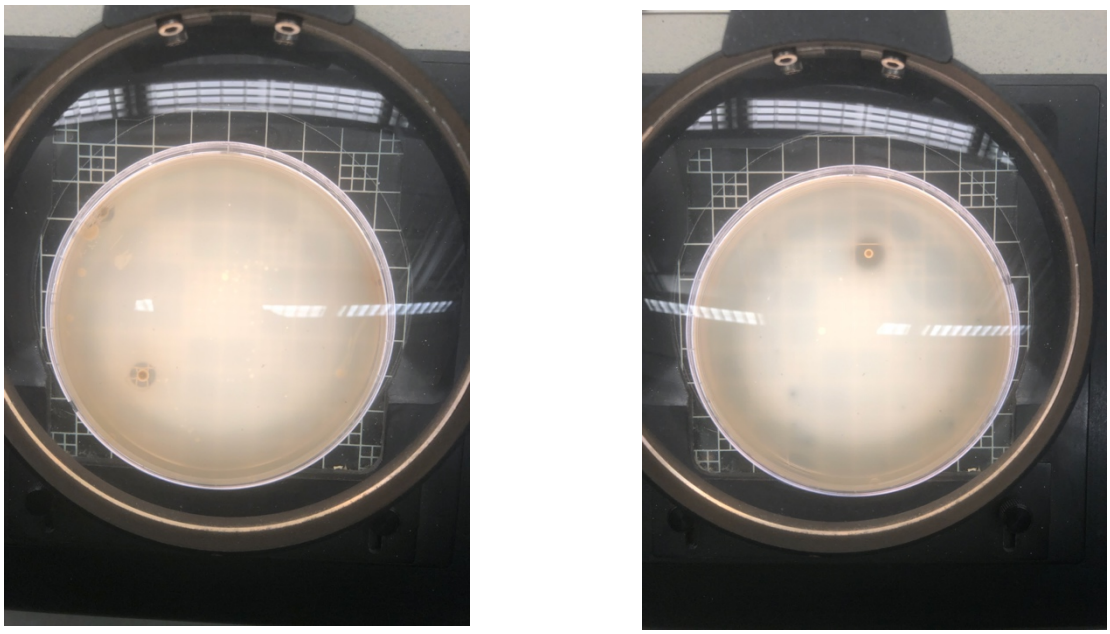

Figure 2. The clear zones of inhibition created by colonies with antimicrobial characteristics

C) Model Diagnostic Plots

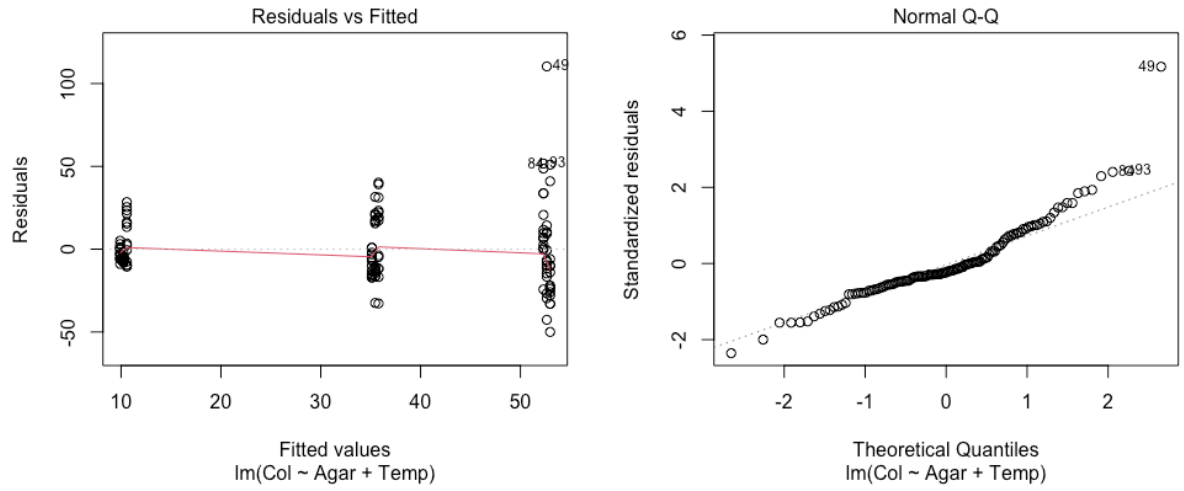

Figure 3. Diagnostic Plots for number of colonies after 48 hours of incubation
The residuals versus fitted plot (left) indicates that the variance is not constant across all groups. In particular, the spread of the residual becomes more noticeable when the fitted values become larger. The normality plot (right) also shows that the data are not normally distributed, with significant curvatures and several outliers at both ends of the plot. These plots inform that the data need to be transformed. Using the Box-Cox method, we found the transformation power of 0 and transformed our data with log function.

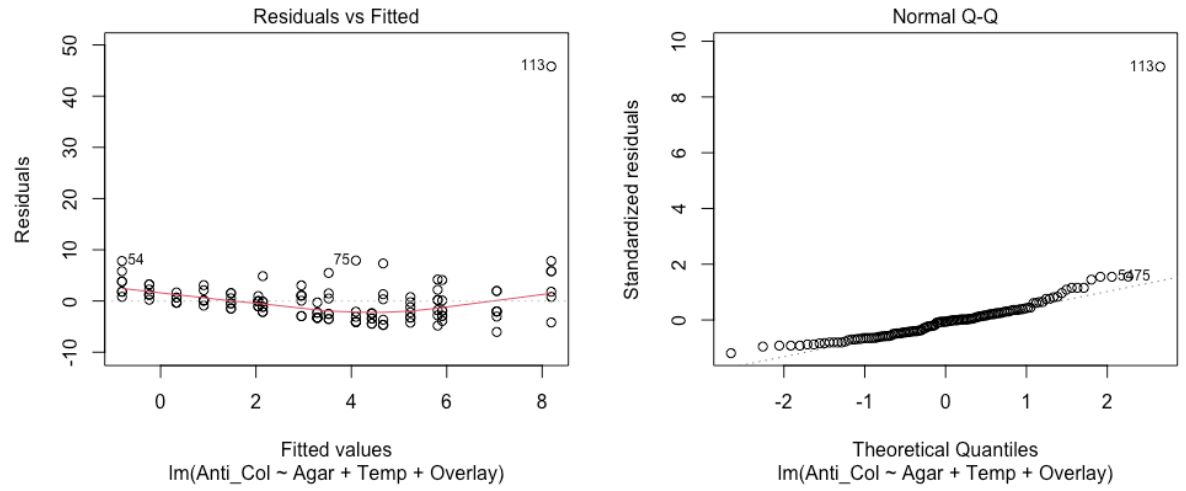

Figure 4. Diagnostic Plots for number of colonies with antimicrobial characteristics after 24 hours of overlay The residuals versus fitted plot (left) indicates that the variance between groups is not constant, with more significant spread at both ends of the plot. The normality plot (right), however, shows that the data are normally distributed, with slight curvatures and one outlier at both ends of the plot. These plots inform that the data need to be transformed. Using the Box-Cox method, we found the transformation power of 0 and transformed our data with log function.

D) Results of ANOVA F-test

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Agar	2	71.95669862	35.97834931	75.57	< 0.0001
Temp	2	1.87495659	0.93747829	1.97	0.1442
Agar* Temp	4	7.81395947	1.95348987	4.10	0.0038

Table 2. ANOVA Table for the number of colonies after 48 hours of incubation at designated temperatures

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Agar	2	3.67687539	1.83843770	4.32	0.0156
Temp	2	6.19589676	3.09794838	7.27	0.0011
Overlay	1	14.61322288	14.61322288	34.30	< 0.0001
Agar*Overlay	2	7.90111730	3.95055865	9.27	0.0002
Temp*Overlay	2	3.23394681	1.61697341	3.80	0.0253

Table 3. ANOVA Table for the number of colonies with antimicrobial characteristics after 24 hours of overlay at designated temperatures

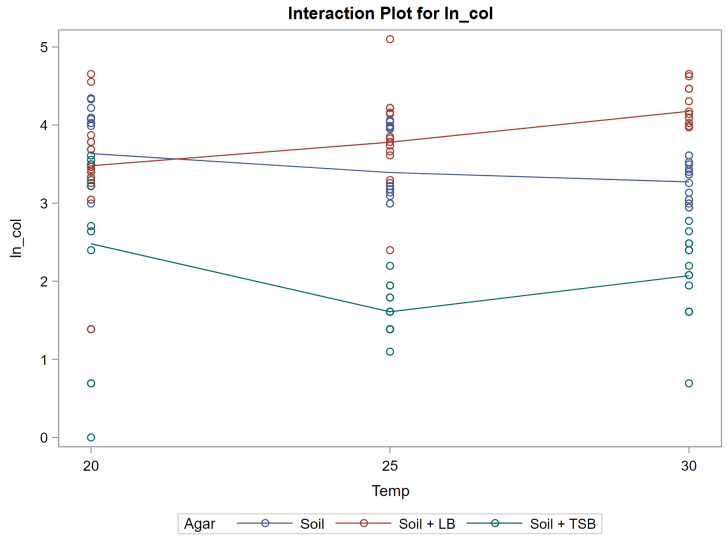

Figure 5. The interaction plot with log-transformed data of the number of colonies by agar types after 48 hours of incubation at designated temperatures

E) Multiple Comparisons (Tukey's HSD with LSMeans)

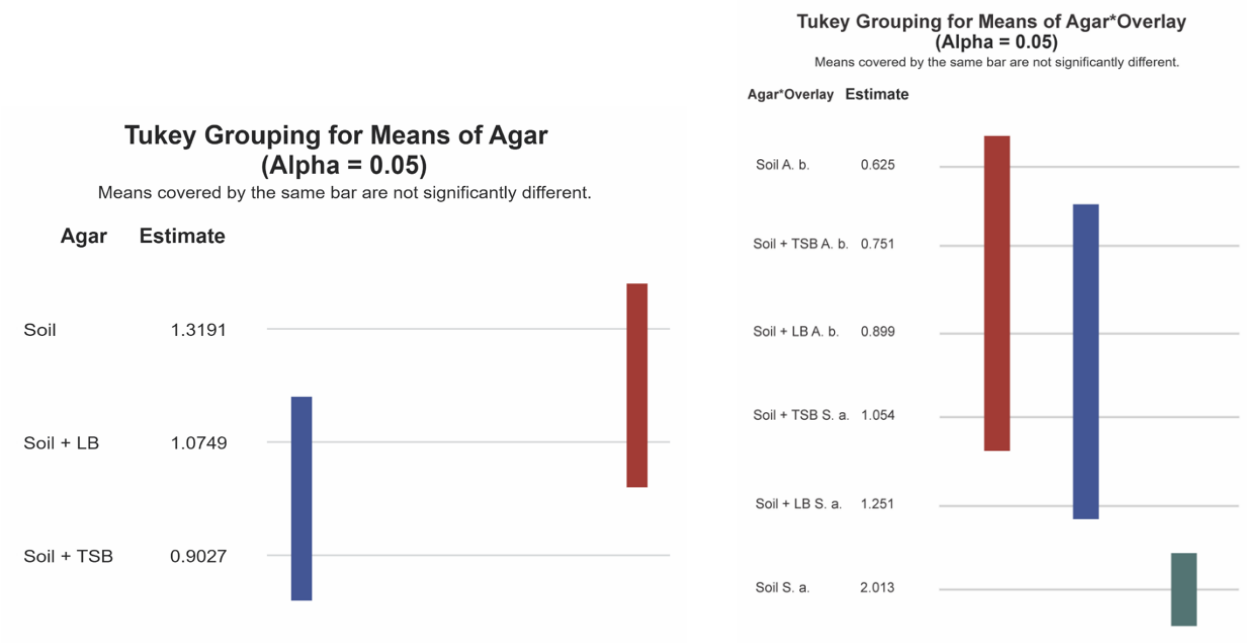

Figure 6. Tukey's HSD comparisons of colonies with antimicrobial characteristics between the agar types (left) and between the combinations of agar types and pathogens in overlays (right)
(S.a. = S. aureus; A.b. = A. baylyi)