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Modulation of Anti-Microbial Resistant *Salmonella heidelberg* Using Synbiotics (Probiotics and Prebiotics) in Two *In-Vitro* Assays (Cross-Streaking and Agar Wells Diffusion)

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Abstract

Salmonellosis is the most prevalent bacterial foodborne disease in many countries worldwide. Utilization of probiotics is one of the most accepted ways to reduce *Salmonella*, especially lactic acid bacteria, as it has proven to reduce the enteric pathogens in monogastric and ruminant livestock animals through different mechanisms such as antimicrobials production, competitive adhesion to the gastrointestinal tract, and immune stimulation. Prebiotics could be utilized solely for health benefits as an alternative to probiotics or in addition to probiotics for a synergistic effect known as synbiotics. The aim of this study was to compare effects of different probiotic strains (*Lactobacillus acidophilus* (La-14), *Lactobacillus paracasei* (Lpc-37), *Streptococcus thermophiles* (St-21), *Bifidobacterium bifidum* (Bb-06), and *Aspergillus niger* (ATCC®16888™) and without prebiotics (Mannose; Xylose; Galactooligosaccharides GOS; Inulin; and Dandelion extract) on lowering *Salmonella heidelberg* CFU *in vitro*. Different inhibition levels probiotic strains were assessed and compared in the presence and absence of 2.5% prebiotic compounds using cross-streaking and agar well diffusion assays. Recommendations for the growth of selected microorganisms such as temperature and oxygen conditions were taken into consideration. All the analysis was conducted in triplicates. The results showed that all the probiotics strains except *S. thermophiles* were able to significantly ($P < 0.05$) inhibit the growth of *S. heidelberg* in at least one of the assays. The difference in inhibition percentage confirms that probiotic strains have multiple inhibition mechanisms, such as production of antimicrobials, lower pH by producing organic acids

(acetic acid, lactic acid, etc.), and inhibition of pathogen's virulence factor expression, and production of lipopolysaccharide solubilizing compounds.

Keywords

Salmonella, Synbiotics, Cross-Streaking, Agar Well Diffusion

1. Introduction

Probiotics are live microorganisms that have been proven to induce many health benefits and prevent diseases in the human body [1]. In the last decade, human clinical and animal research reported many benefits and functionality of probiotics such as, oxalate degradation [2], restoration of healthy oral flora [3] [4]; alleviating the symptoms of lactose intolerance [5]; production of folic acid [6]; reduction colon cancer [7]; providing antioxidant activity [8]; lowering inflammatory bowel disease and diarrhea [9]; inhibition of *Escherichia coli*, *Listeria monocytogenes*, *Candida*, and *Staphyloccocus aureus* growth [10] [11] [12]. The most known common positive health impacts are lowering the pH of the gastrointestinal (GI) tract, inhibit pathogens growth and motility, producing short-chain fatty acids (SCFA), lowering cholesterol, and preventing or reducing the risk of colon cancer [1]. As defined by Gibson [13], prebiotics are beneficial substances for human and animal health, acting as selectively utilized substrates by host microorganisms to confer a health benefit. Prebiotics are different from other dietary fibers because of their abilities not to be digested in the upper GI tract, and they resist absorption in the small intestine. Synbiotics are another term that describes the symbiotic relationship between probiotic microorganisms and prebiotic fibers. Synbiotics were firstly introduced in 1995 by Gibson and Roberfroid as a "mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare".

In the USA, approximately 1.6% of adults (3.9 million) consume probiotics and prebiotics in the form of natural products and dietary supplements [14] [15]. The top frequently used probiotic species in food and nutraceuticals industries are *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*.

In previous studies, probiotics showed a significant inhibition effect on pathogens such as *Salmonella*, *E. coli*, etc. *Salmonella* contamination in food products may occur at multiple steps along the food chain, which includes production, processing, distribution, retail marketing, handling, and preparation [16]. Although the major signs and symptoms of salmonellosis such as diarrhea, headache, fever and abdominal pain are not life-threatening and the mortality occurrences are rare, some risk groups may suffer fatally from salmonellosis in-

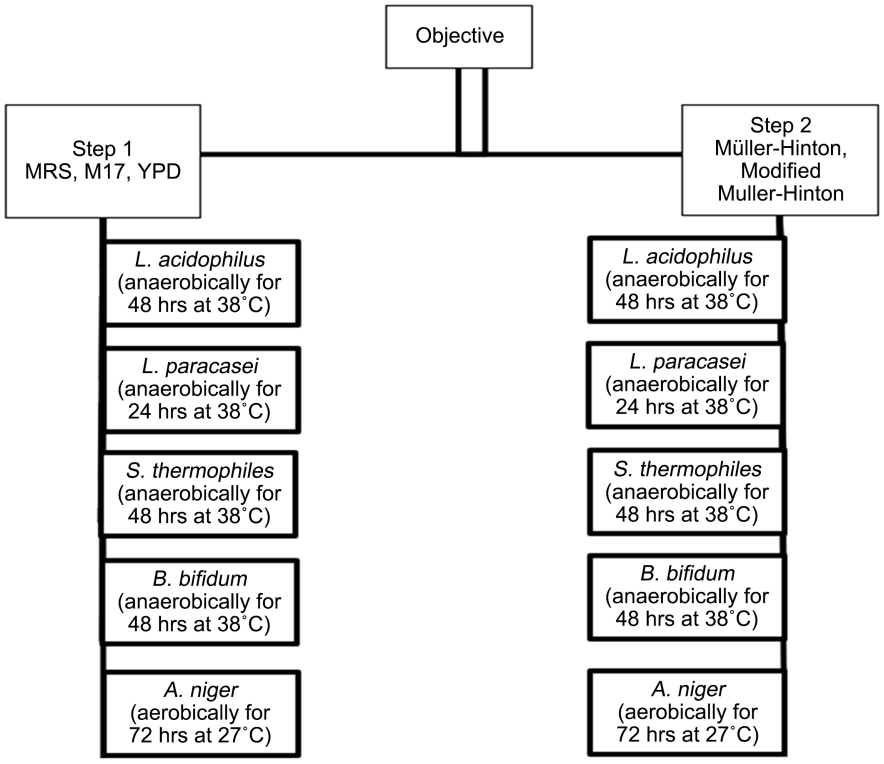
fection such as, senior adults, children, and insufficient immune system patients [17]. Food products that do not undergo processing such as ready to eat foods (RTE) are a potential target for food pathogens such as *salmonella* and coliforms [18], especially that the majority of these foods contain the minimum requirements for pathogens grows (carbon, nitrogen, and B vitamins). Food pathogens could also be transmitted between raw foods causing contamination of one item with a pathogen that is not typically associated with, for example, contamination of beef or pork with *Salmonella* due to contact with chicken juice during cutting [18]. RTE foods could be controlled by other non-processing preservatives such as essential oils, organic acids, and antibacterial peptides. However, those preservation factors may affect the organoleptic properties of the food, and accordingly its acceptability [19].

Antimicrobial agents such as antibiotics are usually used in severe and emergency cases. However, in some cases, some *Salmonella* strains have shown antibiotic resistance [17] [20] [21]. The combination of prebiotics and probiotics (synbiotics) could be an efficient alternative for antibiotics, as their use may decrease the risk of salmonellosis and enhance the whole production process. Observing that inhibition was conducted in a couple of studies, however many of these studies did not factor the effect of the media used on the inhibition induced by the probiotics. The aim of this *in-vitro* research is to examine the inhibition of different probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Streptococcus thermophiles*, *Bifidobacterium bifidum*, and *Aspergillus niger*) on the growth of *Salmonella heidelberg* using two different methods, growth media, and in the presence and absence of prebiotics (mannose; xylose; galactooligosaccharides (GOS); Inulin; and dandelion extract).

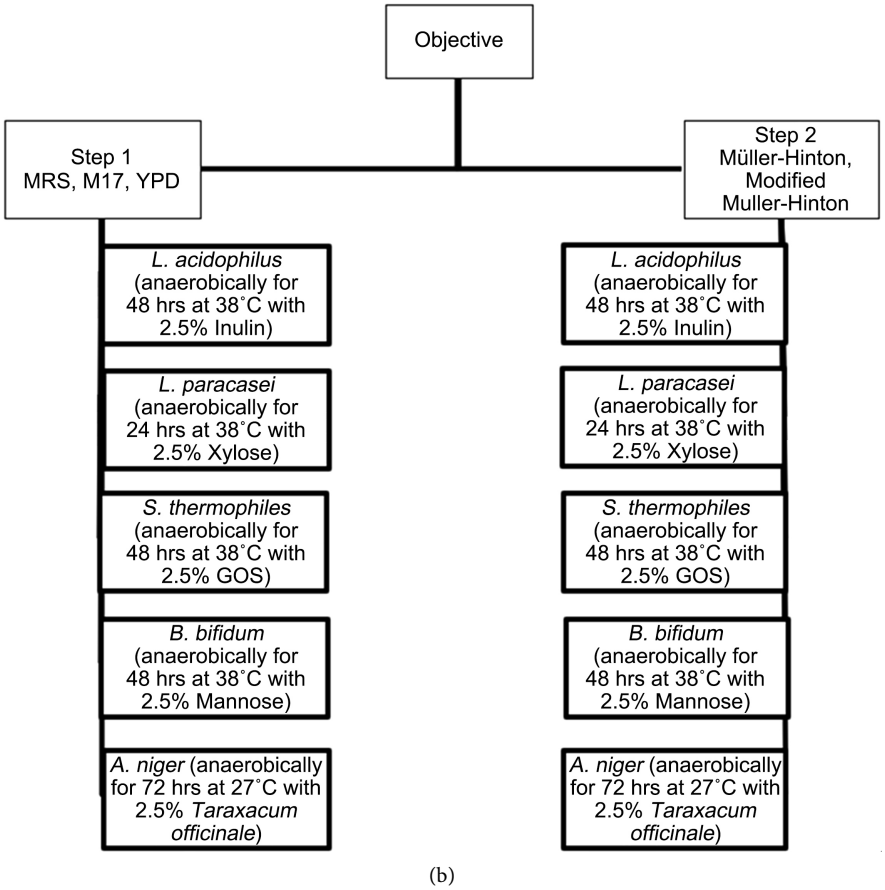
2. Materials and Methods

2.1. Study Design

The presented *in-vitro* research is a factorial design that was divided into without prebiotics and with prebiotics (**Figure 1(a)** & **Figure 1(b)**) and was performed at the food biochemistry lab at Alabama A&M University (Normal, Alabama). The work in **Figure 1(a)** was conducted to serve as a positive control as a means to know that the organisms would grow on the different media. **Figure 1(b)** work was conducted as the treatments of probiotics with prebiotics grown together (synbiotics) data was to be compared to data generated from **Figure 1(a)**. Ten treatments were composed of **Figure 1(a)** and **Figure 1(b)** (probiotics alone and synbiotics) with possible competitive inhibition for *S. heidelberg* through the different media (De Man, Rogosa and Sharpe agar (MRS) and Mueller-Hinton for *Lactobacillus acidophilus*, *Lactobacillus paracasei*, and *Bifidobacterium bifidum*; M17 and Mueller-Hinton for *Streptococcus thermophiles*; and modified Muller-Hinton (Mueller Hinton Agar, 2% Glucose with Methylene blue) and Yeast Peptone Dextrose (YPD) for *Aspergillus niger*).



(a)



(b)

Figure 1. (a) Study design (part 1); (b) Study design (part 2).

2.2. Agar and Broth Media

De Man, Rogosa and Sharpe agar (MRS) media were purchased from BD Difco™ and BD BBL™, Luria-Bertani (LB) media from bioWORLD, Yeast Extract Peptone Dextrose (YPD) media from BD Difco™, M17 media from Difco™ and BD Oxoid, and Mueller-Hinton agar from Difco™ and BD BBL™. All media were handled and stored according to manufacture recommendations.

2.3. Prebiotics Preparation

Dehydrated prebiotic powders, mannose (Acros Organics); xylose (Fisher Scientific); galactooligosaccharides GOS (VITAGOS™); inulin (MP Biomedicals); and Dandelion extract *Taraxacum officinale* (Florida Herbs) were purchased and prepared to be mixed with each strain's broth (2.5% of total volume).

2.4. Probiotic Cultures

The freeze-dried bacterial probiotics (*Lactobacillus acidophilus* (La-14), *Lactobacillus paracasei* (Lpc-37), *Streptococcus thermophiles* (St-21), *and Bifidobacterium bifidum* (Bb-06)) were provided by DuPont™ Danisco® Food Ingredients and stored according to the manufacturer description. The *Aspergillus niger* freeze-dried cultures were purchased from ATCC (ATCC®16888™) and stored according to the manufacturer's description.

2.5. *Salmonella heidelberg*

Salmonella heidelberg cultures were enumerated from an already prepared 50% glycerol stock into several LB agar Petri dishes and LB broth media glass bottles.

2.6. Media Preparation

For *Lactobacillus acidophilus* and *Lactobacillus paracasei*, MRS agar and broth were prepared and stored according to manufacture recommendations. For *Bifidobacterium bifidum*, the MRS media were prepared according to manufacture recommendations with modification by adding 0.5 grams/liter of L-cysteine hydrochloride to the media powder. For *Streptococcus thermophiles*, the M17 broth and agar were prepared and stored according to manufacture recommendations. The LB agar and broth were prepared and stored according to manufacture recommendations. For the zones of inhibition experiment, the Mueller-Hinton agar was prepared according to manufacturer's description with the addition of 2% glucose and 0.0005 grams methylene blue for *A. niger* zones of inhibition.

2.7. Bacterial Probiotics Preparation and Enumeration

For freeze-dried bacterial probiotic cultures, one gram of the powder was measured and transferred aseptically to a sterilized test tube containing the recommended media for each microorganism for rehydration. The tube was vortex mixed for one minute, and the entire solution was added to a 125 mL bottles

containing the same broth. Since that all the bacterial probiotics to be used are anaerobic, the culture was left to rehydrate in an anaerobic environment at 38˚C for 24 - 48 hours using Oxoid Anaero Gen 2.5L sachets anaerobic atmosphere generation system (GasPak, Thermo Scientific, Hampshire, UK). The cultures were then spread on the respective agar plates to be used two days after they are made for the competitive inhibition. All bacterial probiotics were sub-cultured until they reached an average of 10^6 CFU before being used in the inhibitory experiments.

2.8. *Aspergillus niger* Preparation and Enumeration

One mL of sterilized distilled water was pipetted to the vial containing the freeze-dried culture. Then, the entire content was drawn up into the pipette and transferred; to a test tube with a 6 mL sterilized distilled water. The mold was left to rehydrate for a 24 hour aerobically at 25˚C then kept in 50% glycerol stocks, YPD broth, and Potato Dextrose Agar (PDA) slants. *A. niger* was sub-cultured until it reached an average of 10^4 CFU before being used in the inhibitory experiments.

2.9. Prebiotic and Probiotic Mixing

For synbiotic treatments, each prebiotic was mixed with the broth before probiotics were added, where 2.5% of inulin solution was mixed with MRS broth for *L. acidophilus*; 2.5% of xylose solution was mixed with MRS broth for *L. paracasei*, and 2.5% of mannose solution was mixed with *B. bifidum*; and 2.5% of *Taraxacum Officinale* solution was mixed with YPD broth for *A. niger*.

2.10. Cross-Streak Method

The cross-streaking method was conducted by streaking in opposite directions by a modified cross-streaking method according to [22]. *S. heidelberg* was streaked first on the agar plate, starting from one side to another, forming a Z shape. Then the treatment was streaked perpendicular to the first streak forming another Z shape. The plates were then incubated according to the conditions recommended for each treatment (anaerobic for 24 - 48 hours at 38˚C for bacterial probiotics and aerobic for 96 hours at 27˚C for fungal probiotics). *S. heidelberg*'s CFU reduction was calculated by subtracting CFU in cross-streaked plates from control plates.

2.11. Agar Well Diffusion Method

The agar well diffusion assay was conducted according to [23] method with the following modifications. One mL of a 10^{-2} dilution of an overnight culture of *Salmonella heidelberg* was added to Mueller-Hinton media agar plates and was left to dry at 37˚C for a couple of minutes. After that, five wells were made with a diameter of 20 mm in each agar plates of the triplicate. Each well contained 80 ml of the medium-plus 100 ml of the probiotic cultures, except for the fifth well

(control well) in the center that only contained the original sterile broth media for each treatment (MRS for *Lactobacillus* and *Bifidobacterium*, and M17 for *Streptococcus thermophiles*). The plates were left for 48 hours of anaerobic incubation at 37˚C - 38˚C. The presence of an inhibition zone of more than 1 mm was used as an inhibition criterion.

2.12. Statistical Analysis

S. heidelberg inhibition (reduction in CFU and diameters of zones of inhibition) was determined by ANOVA using mixed model in SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Significance was tested ($P < 0.05$). Each treatment was repeated three times. *S. heidelberg* CFU and agar well diameters were transformed to satisfy the assumption of equal variances (homoscedasticity). T-test on Microsoft Office Excel 2007 was used to determine differences between single strains control and treatment effect at levels of significance of $P < 0.05$ and to compare the pH change due to different probiotic treatments.

3. Results

3.1. *Salmonella Heidelberg* Controls

There were no statistical differences ($P > 0.05$) between the control groups of *S. heidelberg* for the cross-streaking and the agar well diffusion methods (data not shown).

3.2. Effect of Probiotics on pH of the Media

Table 1 shows the change in the pH of all the media after and before fermentation. All the treatments were able to decrease the pH of media significantly with except to *A. niger* in YPD supplemented with dandelion root. The highest rate of reduction was observed in *L. acidophilus* (3.02-fold reduction). The reduction in the pH of the media was found to be due to the interaction between both the probiotic and prebiotic factors (data not shown).

Table 1. Effect of probiotics growth (with/without prebiotics) on pH of media (25˚C).

Probiotics	Media pH Before	Media pH After	*P* Value
L. acidophilus	6.45 ± 0.06	3.42 ± 0.23	<0.01
L. acidophilus with Inulin	6.31 ± 0.02	3.61 ± 0.35	<0.01
L. paracasei	6.48 ± 0.04	3.92 ± 0.20	<0.01
L. paracasei with Xylose	6.45 ± 0.04	3.76 ± 0.18	<0.01
S. thermophilus	7.07 ± 0.01	4.43 ± 0.16	<0.01
S. thermophilus with GOS	6.94± 0.08	4.37 ± 0.16	<0.01
B. bifidum	6.24 ± 0.03	4.49 ± 0.01	<0.01
B. bifidum with Mannose	6.27 ± 0.08	4.42 ± 0.05	<0.01
A. niger	5.52 ± 0.005	4.11 ± 0.09	<0.01
A. niger with Dandelion Root	5.53 ± 0.14	5.42 ± 0.06	0.34

Results are expressed as Least Squares Mean (LSM) ± Standard Deviation.

3.3. Cross Streak Method

All of the probiotic strains with/without prebiotics with except to *S. thermophilus* were able to significantly ($P < 0.05$) decrease the growth of *S. Heidelberg* (Table 2). The level of reduction was highest by *A. niger* with and without prebiotic (both 100% reduction) than *B. bifidum* with and without prebiotic (Respectively, 95.29% and 97.09% reduction) than *L. paracasei* with prebiotic (82.69% reduction) than *L. acidophilus* without prebiotic (77.08% reduction) than *L. paracasei* without prebiotic (71.31% reduction), lastly *L. acidophilus* with prebiotic (67.90% reduction). In order to compare the reduction effect of each treatment the separation of the means using ANOVA Design was done and it was shown that the most successful treatments (*B. bifidum* w/o prebiotics and *A. niger* w/o prebiotics) and the treatments with moderate reductions, each shared statistically similar levels of reduction ($P > 0.05$) (Figure 2).

3.4. Agar Well Diffusion Method

All of the probiotic strains with and without prebiotics with except to *S. thermophilus* and *A. niger* (*L. acidophilus*, *L. paracasei*, and *B. bifidum*) showed significant inhibitory activity against *S. heidelberg* in the agar well diffusion assay (Table 3). After separation of the means using ANOVA, the wells' level of inhibition between the treatments was shown to be similar ($P > 0.05$) between *S. thermophilus* and *A. niger* without prebiotics. All the other treatments that showed a statistically significant reduction exhibited the same levels of well's diameter increase ($P > 0.05$) (Figure 3).

Table 2. Percentage of *S. heidelberg* reduction by of each probiotic strain (*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Bifidobacterium bifidium*, *Streptococcus thermophilus*, and *Aspergillus niger*) in presence and absence of prebiotics (Inulin, Xylose, Mannose, Galactooligosaccharides (GOS), and Dandelion Root).

Treatment	Percentage of S. *heidelberg* CFU Reduction (%)	*P*-Value
L. acidophilus	74.41 ± 30.92	0.009
L. acidophilus with Inulin	69.03 ± 15.17	0.007
L. paracasei	71.16 ± 14.31	0.001
L. paracasei with Xylose	82.81 ± 2.11	0.01
S. thermophilus	18.23 ± 14.60	0.08
S. thermophilus with GOS	26.14 ± 16.68	0.053
B. bifidum	97.08 ± 2.85	0.02
B. bifidum with Mannose	95.17 ± 2.48	0.001
A. niger	100 ± 0	0.003
A. niger with Dandelion Root	100 ± 0	0.004

Results are expressed as Least Squares Mean (LSM) ± Standard Deviation.

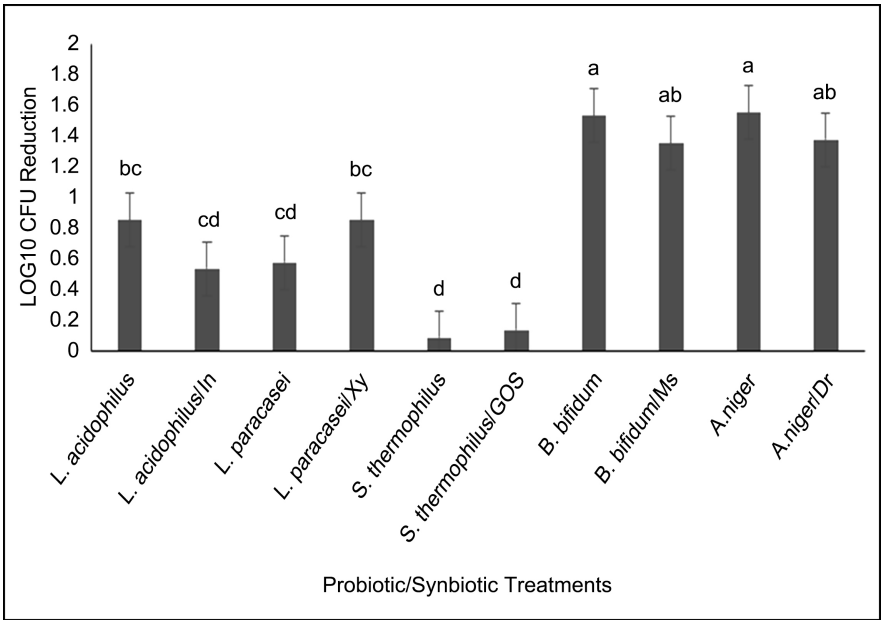


Figure 2. LOG10 reduction of *S. heidelberg* due to the growth of different probioitc treatments in the cross-streaking assay. Results are expressed as Least Squares Mean (LSM) ± Standard Error. Bars with different letters (a, b, c) differ significantly (P < 0.05). In: Inulin; Xy: Xyloe; GOS: Galactooligosaccharides; Ms: Mannose; and Dr: Dandelion Root.

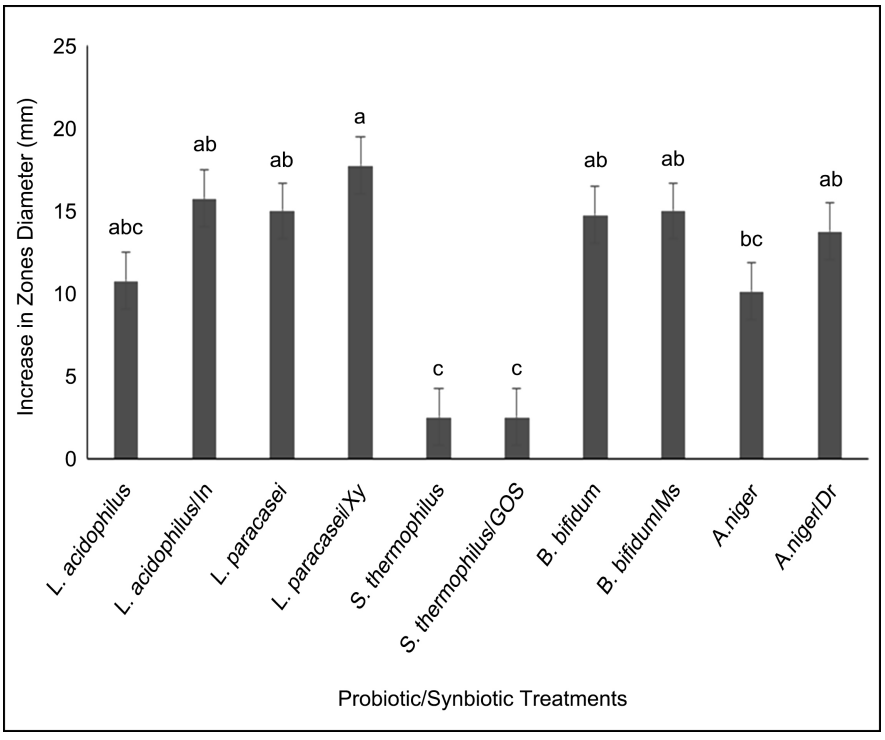


Figure 3. Inhibition zones of *S. heidelberg* due to the growth of different probiotic/synbiotic treatments in the agar well diffusion assay. Results are expressed as Least Squares Mean (LSM) ± Standard Error. Bars with different letters (a, b, c) differ significantly ($P < 0.05$). In: Inulin; Xy: Xyloe; GOS: Galactooligosaccharides; Ms: Mannose; and Dr: Dandelion Root.

Table 3. Zones of inhibition of each probiotic strain (*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Bifidobacterium bifidium*, *Streptococcus thermophilus*, and *Aspergillus niger*) in presence and absence of prebiotics (inulin, xylose, mannose, galactooligosaccharides (GOS), and dandelion root).

Probiotic Strains	Prebiotic	Zones of Inhibition (Millimeter)	*P*-value
L. acidophilus	No Prebiotic	33.25 ± 5.67	0.01
L. acidophilus	Inulin	35 ± 0.81	0.00004
L. paracasei	No Prebiotic	35 ± 5.77	0.01
L. paracasei	Xylose	37.75 ± 2.62	0.0008
B. bifidum	No Prebiotic	34.75 ± 3.68	0.004
B. bifidum	Mannose	35.5 ± 0.57	0.00001
S. thermophilus	No Prebiotic	22.5 ± 2.88	0.1
S. thermophilus	GOS	22.5 ± 5	0.3
A. niger	No Prebiotic	22 ± 2.82	0.2
A. niger	Dandelion Root	33.75 ± 17	0.2

Results are expressed as Least Squares Mean (LSM) ± Standard Deviation; Zones of Inhibition Diameter (Millimeter) as Compared to Control.

4. Discussion

Salmonella heidelberg is a non-typhoidal serotype of *Salmonella*. It is among the top five serovars associated with human foodborne illness [24] [25] and is typically linked to the consumption of poultry products and contact with dairy calves [26]. The CDC has estimated more than one million salmonellosis foodborne cases in the USA annually [17]. *S. heidelberg* was resistant to more than one drug such as amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline; making it a Multi-Drug Resistant (MDR) pathogen [27]. A review by [20] suggested that the mechanism of resistance of *Salmonella* towards antimicrobial agents is changing continuously, and observational studies and adequate research are mandatory for creating the optimum treatment for the infected cases. This study showed that four of the tested commercial probiotic strains were able to inhibit the growth of all tested *S. heidelberg* strains in the two inhibition assays. The application of a specific concentration of 2.5% prebiotics in each experiment was chosen based on our lab preliminary study regarding the best concentration and time for probiotics growth. The inhibition of the pathogen could be attributed to many factors including but not limited to pH, which was assessed in the study and was found to be reduced as a result of probiotic fermentation [28]. The pH reduction was significant in most of the strains that caused inhibition to the pathogen, knowing that *S. heidelberg* isolates have only moderate growth around 4.4 - 5.2 pH with abundant growth around 6.8 pH [29].

The utilization of probiotics, prebiotics, and/or synbiotics can be considered a

great alternative to standard antimicrobial agents currently used against food pathogens. Several mechanisms of actions have been proposed for the capability of probiotics to reduce pathogen load and activity *in-vitro*, in animals, and human studies [30] [31]. Probiotics can produce antimicrobial substances that can alter the growth environment of the pathogen or inhibit its growth. Among those substances are lactic and acidic acids, which alter the growth of pathogens by reducing the medium's pH and the intracellular pH of the microorganism [32]. Additionally lactic acid bacteria can synthesize bacteriocins, which are proteinaceous peptides that have the ability to prevent the growth of other bacteria, including foodborne pathogens [33]. Different bacteriocins can be produced by different lactic acid bacteria (*L. acidophilus*: acidocin; *L. paracasei*: lactocin; *S. lactis*: nisin; and *B. bifidum*: bifidin and bifidocin). These can be utilized for food quality control and preservation [33] [34]. In an unpublished preliminary experiment in our laboratory, a small concentration of nisin from *Lactococcus lactis* completely inhibited the growth of *S. heidelberg* when co-cultured together in LB broth. Secondly, competitive exclusion is another mechanism by which it could reduce pathogen load by competing for nutrients. In a study [35] inhibitory and exclusion abilities of probiotics (*Lactobacillus acidophilus*, *L. casei*, *L. paracasei* and *L. rhamnosus*) against pathogens (*Salmonella typhimurium* and *Listeria monocytogenes*) were assessed. After conducting auto-aggregation (cell-to-cell interactions), bacterial adhesion to solvent assay (cell surface properties), and pathogenic biofilm inhibition (competition, exclusion and displacement assays) experiments; it was found that *L. paracasei* and *L. rhamnosus* were able to competitively exclude *L. monocytogenes* biofilm cells by more than 3 logs. Thirdly, probiotics induce immunomodulatory effects that assist in pathogen destruction and other health benefits to the host, such as lactase production and reduction of autoimmune diseases such as lactose intolerance [31]. The immune modulation occurs through the adhesion of probiotics to the epithelial cells and initiation of signaling cascades [1]. A review by Kang & Im [36] suggests that probiotics can keep the balance between pro-inflammatory and anti-inflammatory cytokines, which is vital during pathogenesis by different toxins produced by different pathogens. Lastly, the inhibitory effects of probiotics could also be attributed to the ability of the probiotic microorganisms to block pathogen adhesion sites, preventing its growth and/or biofilm formation [31]. Adhesion of probiotics and its effect of pathogens can be non-specific (Van der Waals and electrostatic forces) or specific (lock and key) between the cell and the adhesion surface [37]. Probiotics can prevent adhesion of foodborne pathogens such as *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* [37], which is useful for human's health and food preservation. The inability of *A. niger* to inhibit *S. heidelberg* through the agar well diffusion could be attributed to the fact that *A. niger* was not able to grow and/or diffuse in the agar, although the medium was supplemented with glucose for better fungal growth and methylene blue for better zone edge definition [38].

5. Conclusion

Salmonella antimicrobial resistance has been reported in many cases and outbreaks, especially poultry-associated foodborne diseases. *Salmonella heidelberg*, the target pathogen for this study, was found to be antibiotic-resistant in more than one CDC outbreak; its prevalence was significantly high among poultry facilities and production. Probiotics have positive effects on human and animal health when consumed at adequate amounts, either as food or feed. This study observed that probiotic strains such as *L. acidophilus*, *L. paracasei*, and *B. bifidum* were able to significantly ($P < 0.05$) reduce *S. heidelberg* growth in both i*n-vitro* assays whereas *A. niger* was able to reduce the pathogen only in agar well diffusion assay. *S. thermophilus* was the only strain that failed to reduce *S. heidelberg* in any of the two assays. The prebiotic utilization was useful for improving the reduction of *S. heidelberg*; however after statistical analysis, it was found that this improvement was not statistically significant.

Acknowledgment

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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