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Research Article
Ability of Select Probiotics to Reduce Enteric *Campylobacter* Colonization in Broiler Chickens

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Abstract

Background and Objective: *Campylobacter* is the leading cause of foodborne enteritis worldwide and is primarily caused by consumption/mishandling of contaminated poultry. Probiotic use in poultry has been an effective strategy in reducing many enteric pathogens, but has not demonstrated consistent reduction against *Campylobacter*. This study was conducted to screen probiotic isolates that could eliminate or reduce cecal *Campylobacter* counts in poultry. **Materials and Methods:** As *Campylobacter* resides and utilizes intestinal mucin for growth, isolates selected on the basis of mucin utilization might be a strategy to screen for probiotic candidates with efficacy against *Campylobacter*. In this study, bacterial isolates demonstrating increased growth rates in the presence of mucin in media, in vitro were selected for their ability to reduce *Campylobacter* colonization in 14 day old broiler chickens. In replicate trials, 90 days-of-hatch chicks were randomly divided into 9 treatment groups (n = 10 chicks/treatment) and treated individually with one of four bacterial isolates (Bacillus spp.) grown in media with or without mucin prior to inoculation or a *Campylobacter* control (*Campylobacter*, no isolate). In both the trials, all the birds except control were orally gavaged with individual isolates at day-of-hatch. On day 7, all the birds were orally challenged with a four strain mixture of *C. jejuni* and ceca were collected on day 14 for *Campylobacter* enumeration. **Results:** Results from these two trials demonstrated two individual isolates, one isolate incubated with mucin in the media and another isolate incubated without mucin prior to inoculation, consistently reduced cecal *Campylobacter* counts (1.5-4 log reduction) compared to controls. **Conclusion:** These results support the potential use of mucin to pre-select isolates for their ability to reduce enteric colonization of *Campylobacter* in broiler chickens.

Key words: *Campylobacter*, probiotic, chicken, pre-harvest, mucin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Campylobacter is one of the leading causes of bacterial gastroenteritis in humans worldwide[1,2]. In the United States alone, 1.3 million cases of human *Campylobacter* infections have been reported annually[3]. More than 17 *Campylobacter* spp. have been identified[4,5], of which, *Campylobacter jejuni* is responsible for approximately 95-99% of cases of human campylobacteriosis[6-8]. Most of the human campylobacteriosis cases are self-limiting; however, some severe post-infectious sequelae such as Guillain-Barré syndrome and reactive arthritis have been reported[9]. Various sources of *Campylobacter* have been identified; among them, poultry is regarded as the principal source of infection for humans[3,10-12]. It has been reported that more than 90% of the US poultry flocks are contaminated with *C. jejuni*[13], which potentially present a serious threat for humans[14,15]. Hence, reduction or elimination of *Campylobacter* in poultry flocks would significantly reduce the human incidence of campylobacteriosis[12]. Several pre-harvest intervention strategies have been evaluated to eliminate/reduce *Campylobacter* prevalence in poultry flocks with varying degrees of success[16-22]. Unfortunately, none of them are successful in completely eliminating *Campylobacter* from poultry[23]. Application of probiotic bacteria is one strategy that may potentially inhibit or reduce *Campylobacter* colonization in poultry. Probiotics are "Live micro-organisms which when administered in adequate amounts can confer beneficial effects on host health[24]". Probiotics have effectively reduced foodborne pathogens such as *Salmonella*, *E. coli*, *Listeria*, *Clostridium*, etc., *in vivo*[25-30]. Although effective *in vitro*, administration of probiotics produced inconsistent reductions in *Campylobacter* colonization in broiler chickens[21,31,32]. Such inconsistent results against *Campylobacter* colonization suggest the need of better screening methods of probiotic bacteria. It has been reported that supplementation of porcine intestinal mucin in broth media induces the cell surface proteins in *Lactobacillus reuteri* strains and improve the mucus-binding properties *in vitro*[33]. Since *Campylobacter* colonizes in intestinal mucus and uses mucin as a source of carbon and energy[34-36], selection of probiotic isolates which utilize mucin could be an effective approach to competitively inhibit the enteric colonization of *Campylobacter*.

The objective of this study was to screen probiotic isolates that could eliminate or reduce cecal *Campylobacter* counts in poultry. In this study we used selected bacterial isolates that are Generally Regarded as Safe (GRAS) and possess efficacy against *Campylobacter in vitro*. These isolates were further screened for their ability to utilize mucin. Isolates which demonstrated increased growth in the presence of mucin *in vitro* were selected and tested *in vivo*.

MATERIALS AND METHODS

Probiotic isolates: In this study, we used previously isolated GRAS bacterial isolates (*Bacillus* spp.) with efficacy against *Campylobacter in vitro*[21,22].

Screening of mucin utilizing probiotic bacteria: A total of 38 isolates were screened for increased growth in the presence of mucin. The procedure involved growing selected bacterial isolates separately in Tryptic Soy Broth (TSB, Becton Dickinson and Company, Sparks, MD, USA) and in TSB supplemented with 3% porcine gastric mucin (Sigma-aldrich, St. Louis, MO, USA). The isolates were incubated aerobically at 37°C for 24 h. The cultures were then serially diluted with Butterfield's Phosphate Diluent (BPD, Becton Dickinson and Company, Sparks, MD, USA) and plated on Tryptic Soy Agar (TSA, Becton Dickinson and Company, Sparks, MD, USA) for enumeration of each bacterial isolate. Bacterial counts were logarithmically transformed ($\log_{10}$ CFU mL^{-1}). The four isolates which demonstrated greatest increase in counts in the presence of mucin were selected and evaluated for their efficacy to reduce *Campylobacter* colonization in broiler chickens.

Experimental animals and housing: For all the *in vivo* trials, day of hatch broiler male chicks were procured from a local commercial hatchery. Chicks were weighed at the beginning and at the end of each trial. Birds were raised in floor pens with pine shavings, with *ad libitum* access to feed and water throughout the 14 days trial period.

Experimental design: A total of 2 birds trials were conducted at the University of Arkansas Poultry Research Farm and all the experiments were approved by the Institutional Animal Care and Use Committee of the University of Arkansas. Four probiotic isolates which had demonstrated increased growth in the presence of mucin in the broth media were selected for *in vivo* studies. In each trial, a total of 90 male chicks were randomly divided into 9 treatment groups (n = 10 chicks/treatment). The treatment groups included a *Campylobacter* control (*Campylobacter*, no isolate) and 8 treatment groups each receiving a separate bacterial isolate grown in the presence or absence of mucin prior to oral administration.

Bacterial dosing in chicks: In each trial, at day of hatch, chicks from all the treatment groups except *Campylobacter* control were orally gavaged individually with 0.25 mL of specific probiotic isolate containing approximately 10^6-10^8 CFU mL^{-1} as previously described[21]. On day 7, all the chicks were orally gavaged with a cocktail of 4 strains of wild type *C. jejuni* containing approximately 10^7 CFU mL^{-1} organisms as previously described[37]. Briefly, four-strains of wild type *C. jejuni* were successively sub-cultured twice at 42°C for 48 h under microaerophilic conditions. The strains were then pooled, centrifuged at 3000 rpm for 10 min and re-suspended in appropriate volume of BPD for oral challenge. On day 14, ceca were aseptically collected, cecal contents were serially diluted with BPD and each dilution were plated on *Campylobacter* Line Agar[38] for direct enumeration.

Statistical analysis: To achieve homogeneity of variance, cecal *Campylobacter* counts were logarithmically transformed (log_{10} CFU g^{-1} of cecal material) before analysis of data[39]. Data were analyzed using the PROC GLM procedure of SAS[40]. Treatment means were partitioned by least square means (LSMEANS) analysis and a probability of $p<0.05$ was required for statistical significance.

RESULTS

A total of 38 GRAS isolates were tested *in vitro* in this study and the four isolates (Isolate 1, 2, 3 and 4) which showed a greatest increase in counts when grown in media supplemented with mucin compared with the un-supplemented media (Fig. 1) were selected for the *in vivo* studies.

Table 1: Effect of selected bacterial isolates on cecal *Campylobacter* counts (log_{10} CFU g^{-1} of cecal contents) in 14-day old broiler chicks (Mean±SEM)*

	Trial 1	Trial 2
Treatments	----------log_{10} CFU g^{-1}----------	
Campylobacter control	7.95±0.23^{a}	9.19±0.15^{a}
Isolate 1	5.61±0.93^{bcd}	4.98±0.81^{d}
Isolate 2	7.11±0.33^{ab}	6.94±0.54^{c}
Isolate 3	5.79±0.95^{abcd}	7.78±0.40^{bc}
Isolate 4	4.55±1.16^{cd}	8.56±0.14^{ab}
#Isolate 1 incubated with mucin	6.80±0.85^{abc}	8.37±0.23^{ab}
#Isolate 2 incubated with mucin	5.62±0.95^{bcd}	8.36±0.27^{ab}
#Isolate 3 incubated with mucin	4.47±1.08^{d}	9.11±0.20^{ab}
#Isolate 4 incubated with mucin	5.28±0.43^{bcd}	7.89±0.24^{bc}

$^{a-d}$Means within columns with no common superscript differ significantly ($p<0.05$), *Chicks were orally challenged on day 7 with 0.25 mL of approximately 1×10^7 CFU mL^{-1} of a 4 strain mixture of wild type *Campylobacter jejuni* in each trial (n = 10/treatment group), #Isolates incubated with mucin prior to oral challenge in chicks. All *Campylobacter* data were log_{10} CFU g^{-1} transformed for statistical analysis

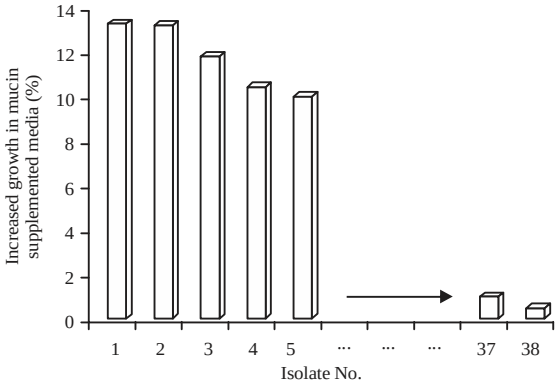

Fig. 1: Probiotic isolates demonstrating increased growth in the presence of media supplemented with porcine mucin. Values represents average of percentage increased growth of select probiotic isolates in mucin supplemented media from two separate replicate trials. Isolates were ranked in decreasing order of growth from highest to lowest. Isolates 1 through 4 were selected for *in vivo* trials 1 and 2

In trial 1, isolate 1 and isolate 4 grown in media without mucin prior to inoculation reduced cecal *Campylobacter* counts (approximately 2-3 log CFU g^{-1}) whereas isolates 2, 3 and 4 incubated with mucin prior to inoculation reduced *Campylobacter* counts (approximately 2-3 log CFU g^{-1}, Table 1) when compared with the controls. In trial 2, isolates 1, 2 and 3 grown without mucin reduced *Campylobacter* counts by approximately 1.5-4 log CFU g^{-1} in the ceca whereas only isolate 4 incubated with mucin reduced *Campylobacter* counts compared to controls (Table 1). When compared across trials, isolate 1 grown without mucin or isolate 4 incubated with mucin prior to inoculation consistently reduced *Campylobacter* counts in two separate trials (Table 1).

DISCUSSION

Campylobacter is a flagellated, highly motile, microaerophilic bacterium able to colonize heavily in cecal crypt mucus[35,36]. One theory of why probiotics are ineffective against enteric *Campylobacter* colonization is because *Campylobacters* are sequestered in the intestinal mucus laden crypts and the probiotic bacteria are not able to penetrate and inhibit their colonization in these locations[41]. In an effort to overcome this issue, four bacterial isolates demonstrating the ability to inhibit *Campylobacter* and increased growth in the presence of mucin, *in vitro* were selected to evaluate their ability to inhibit *Campylobacter* colonization in chickens

(Fig. 1). Each of these isolates were separately grown in media with or without mucin prior to inoculation to determine if this would enhance efficacy, possibly due to changes in gene expression associated with mucin co-incubation[42]. In the first bird trial, two out of four isolates grown without mucin prior to inoculation reduced cecal *Campylobacter* counts (approximately 2-3 log CFU g^{-1}) whereas three out of four of these isolates (isolates 2, 3 and 4) incubated with mucin prior to inoculation reduced *Campylobacter* counts (approximately 2-3 log CFU g^{-1}, Table 1). In trial 2, many of these isolates also reduced *Campylobacter* counts by approximately 1.5-4 log CFU g^{-1} in the ceca. When compared across trials, two isolates consistently reduced *Campylobacter* counts in two separate trials (Table 1). Isolate 4 was more efficacious when grown in mucin prior to inoculation with an approximate 1.5-2.5 log reduction in *Campylobacter* counts whereas isolate 1 produced a greater reduction when not incubated with mucin prior to inoculation with an approximate 2-4 log reduction in *Campylobacter* counts. None of these isolates adversely affected body weight gains at 14 days of age when compared with controls. Some of the isolates grown in mucin prior to inoculation demonstrated a significant reduction in trial 1, however, these isolates (isolates 2 and 3) did not reduce *Campylobacter* colonization in trial 2. Previous study conducted in our laboratory demonstrates that probiotic isolates can maintain their efficacy when administered directly into the lower intestinal tract, as they bypass the acidic environment in the upper intestinal tract[22]. The gastrointestinal tract also contains a large, dynamic and complex microflora[43], which makes the gut an extremely competitive environment. The interaction between the various types of bacteria in gut lumen is complex[44] and these interactions may also inhibit or reduce the efficacy of probiotic isolates within the GI tract. The pre-selected bacterial isolates administered in the current study did not eliminate *Campylobacter* colonization in chickens possibly due to a reduction in the number of isolates reaching or penetrating the cecal crypts containing *Campylobacter*. Results from these trials suggest the need of additional isolates to be tested to verify the utility of this strategy. Also, the efficacy of probiotic bacteria can be enhanced by adhesion to GI tract, which may increase the residence time *in vivo*[45] and understanding the molecular mechanisms behind probiotic adhesion in the mucus could help determining the efficacy of the probiotic isolates. In addition to the current strategy presented in this study, screening of probiotic isolates on the basis of their adhesive potential in mucus may also be considered as Ouwehand *et al.*[46] demonstrated a significant variation (3-43%) in adhesion between the lactobacillus strains. Even though these isolates did not eliminate *Campylobacter* colonization, they did reduce *Campylobacter* counts by 1.5-4 log. Risk assessment studies conducted by Rosenquist *et al.*[12] predicted that a 2 log reduction of the *Campylobacter* on chicken carcasses can reduce the human incidence by 30 times. Therefore, bacterial isolates demonstrating the reduction in counts produced in the current study could significantly reduce the incidence of this disease in humans.

CONCLUSION

In conclusion, this study supports the use of probiotic bacteria in reducing/eliminating *Campylobacter* in broiler chickens. However, detailed knowledge on *Campylobacter* colonization characteristics in the chicken gut should be explored, which would be helpful in selecting an effective strategy in controlling *Campylobacter* in broiler chickens.

Results from these trials demonstrated one isolate grown in mucin prior to inoculation consistently reduced cecal *Campylobacter* counts (1.5-3 log reduction). These results support this screening method could be part of a multifaceted approach in evaluating bacterial isolates with the ability to reduce enteric *Campylobacter* colonization. However, more isolates need to be tested to verify this screening strategy. Further study in probiotics is warranted to reduce or eliminate *Campylobacter* colonization in broiler chickens.

SIGNIFICANCE STATEMENTS

- In the present study, specific probiotic isolates consistently reduced cecal *Campylobacter* counts up to 4 $\log_{10}$ CFU g^{-1} when compared to controls
- Combining the probiotic isolates with a prebiotic supplementation in feed or protecting the isolates and facilitating targeted release of the probiotic isolates (e.g., encapsulation) may be effective in reducing cecal colonization of *Campylobacter*
- The presented strategy could be used as part of a multi-faceted approach to reduce enteric *Campylobacter* counts in broiler chickens

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