Refined functional carbohydrates reduce adhesion of *Salmonella* and *Campylobacter* to poultry epithelial cells in vitro

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ABSTRACT The development of interventions to reduce human foodborne pathogens in the gastrointestinal (**GI**) tract of chickens will be important for improving the microbial food safety of poultry. *Saccharomyces*-derived prebiotic refined functional carbohydrates (**RFC**), composed primarily of β-glucans, mannanoligosaccharides (**MOS**), and D-mannose have been demonstrated to reduce GI colonization of *Salmonella* and *Campylobacter* when administered to poultry. Although they are presumed to inhibit adhesion of pathogens to the GI epithelium, this functionality of RFC has not been well characterized. In this study, we investigated the effects of RFC and other prebiotics on the adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* to the LMH chicken epithelial cell line in vitro. The reduction of adherent pathogens was observed to be dose-dependent with *C. jejuni* being more sensitive than *Salmonella* to inhibition by RFC. Comparison of the primary constituent carbohydrates of RFC found D-mannose to inhibit both pathogens less effectively than β-glucan and MOS, suggesting that it contributes less to inhibition of pathogen adhesion than the other carbohydrates. Finally, the reduction of adherent pathogens by RFC was compared with that of fructooligosaccharides (**FOS**), galactooligosaccharides (**GOS**), and raffinose. All 4 prebiotics inhibited adhesion of both pathogens to chicken epithelial cells. Reduction of adherent *Salmonella* was greatest with FOS and lowest with GOS, whereas reduction of adherent *C. jejuni* was greater with RFC and raffinose than with FOS and GOS. These results will inform future research elucidating mechanisms important to adhesion inhibition of pathogens by RFC and other prebiotics.

Key words: prebiotics, bacterial adhesion, *Salmonella*, *Campylobacter jejuni*, epithelial cells

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INTRODUCTION

The Centers for Disease Control and Prevention has estimated there are approximately 48 million cases of foodborne illness in the United States annually (Elaine et al., 2011a, 2011b). *Salmonella* and *Campylobacter* are the most frequently reported bacterial causes of poultry-related foodborne illness (Heyndrickx et al., 2002; Mead, 2002) because of their association with the gastrointestinal (**GI**) tract of chickens (Duong and Konkel, 2009). Because the pathogen load in the GI tract at the beginning of processing is an important factor contributing to the pathogen load at the end of processing (Lahellec and Colin, 1985; Rouger et al., 2017), the development of interventions that reduce *Salmonella* and *Campylobacter* load pre-harvest will be important to improving the microbial food safety of poultry.

Defined by expert consensus from the International Scientific Association for Probiotics and Prebiotics (Gibson et al., 2017), a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit," and when administered orally are referred to specifically as dietary prebiotics (Bindels et al., 2015). Prebiotic feed additives commonly include indigestible carbohydrates that remain intact until reaching the lower portion of the GI tract where they interact with intestinal microbiota (Slavin, 2013). The administration of dietary prebiotics has been demonstrated to reduce bacterial pathogens including *Salmonella* (Spring et al., 2000; Fernandez et al., 2002), *Campylobacter* (Baurhoo et al., 2009; Huff et al., 2013), and *Clostridium perfringens* (Yang et al., 2008a; Allaart et al., 2013) in the GI tract of poultry. Refined functional carbohydrates (**RFC**), composed of mannanoligosaccharides (**MOS**), D-mannose, and β-glucan are derived from the cell wall of *Saccharomyces cerevisiae* (Moran, 2004; Walker et al., 2018). The cell wall of *S. cerevisiae* accounts for between 20 and 30% of the cell

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dry mass and is a readily available source of RFC for use as dietary prebiotics in humans and animals (Dallies et al., 1998).

Adhesion to mucosal surfaces is important to the colonization and persistence of pathogens in the GI tract (Rosenberg et al., 1983) because it allows bacteria to resist peristaltic movements and establish themselves in the GI environment (Granato et al., 1999). The inhibition of adhesion by prebiotics has been suggested to contribute to the reduction of pathogens in the GI tract of poultry. However, the inhibition of pathogen adhesion by prebiotics and the mechanisms responsible are not well characterized. A more complete understanding of this important functionality will be important to the continued development and application of prebiotics in poultry production.

The chicken LMH epithelial cell line, derived from a hepatocellular carcinoma (Kawaguchi et al., 1987), has been used widely to investigate host–microbe interactions in the GI tract of poultry. The use of this chicken epithelial cell line has enabled the characterization of *C. perfringens* NetB toxin's role in necrotic enteritis (Keyburn et al., 2008) and identification of genes important to *Campylobacter jejuni* colonization (Flanagan et al., 2009; Quiñones et al., 2009) and *Salmonella Enteritidis* virulence (Shah et al., 2012) in the GI tract of chickens. In addition, competitive inhibition between *C. jejuni* F38011 and *C. jejuni 02-833L* for adherence to GI epithelia was modeled in vitro using LMH cells and demonstrated in broiler chicks in vivo by Konkel et al. (2007), and adhesion of *Lactobacillus* spp. to poultry epithelia was characterized in vitro using LMH cells and demonstrated in vivo using broiler chicks by our own research group (Spivey et al., 2014). Thus, the value of the LMH cell line in modeling host–microbe interactions in the GI tract of poultry has been well established.

In this study, we characterized the effect of RFC and other prebiotics on the adhesion of *Salmonella* Typhimurium and *C. jejuni* to poultry epithelial cells in vitro using the LMH chicken epithelial cell line to develop an improved understanding of this potentially important functionality of prebiotics.

MATERIALS AND METHODS

Culture of LMH Cells

Chicken LMH hepatocellular carcinoma epithelial cells (ATCC CRL-2117) were cultured in 0.1% gelatin (MilliporeSigma, Burlington, MA) coated flasks using Waymouth MB 752/1 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Bacterial Strains

Primary poultry isolates of *Salmonella* Typhimurium (TDC 100) and *C. jejuni* (TDC 130) were obtained from the USDA-ARS Southern Plains Agricultural Research Center (College Station, TX). *Salmonella* was cultured using the tryptic soy broth (BD, Franklin Lakes, NJ) or xylose-lysine-tergitol 4 agar (**XLT-4**; BD) incubated aerobically at 37 °C. *Campylobacter* was cultured using Mueller-Hinton broth (BD) or campy cefex agar (**CCA**; Hardy Diagnostics, Santa Maria, CA) incubated in 10% CO_2 at 42 °C. For adhesion inhibition assays, 18-h broth cultures of bacteria were harvested by centrifugation, washed 3 × using assay medium (Waymounth's + 1% fetal bovine serum), and resuspended in the assay medium using absorbance ($OD_{600\ nm}$). Counts of resuspended *Salmonella* and *C. jejuni* were confirmed by enumeration using XLT-4 and CCA, respectively.

Prebiotics RFC and Oligosaccharides

The commercially purified prebiotic oligosaccharides used in this study appear in Table 1. Crude yeast–extracted RFC (Arm and Hammer Animal and Food Production, Princeton, NJ) and purified prebiotic oligosaccharides including β-glucans (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), D-mannose (MilliporeSigma), fructooligosaccharides (**FOS**; Beneo GmbH, Mannheim, Germany), galactooligosaccharides (**GOS**; Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), MOS (MilliporeSigma), and raffinose (**RAF**; MilliporeSigma) were suspended in the assay medium (1% w/v) and diluted as appropriate before addition to LMH cells.

LMH Cell Adhesion Inhibition Assays

Inhibition of *Salmonella* and *C. jejuni* adhesion to LMH cells by prebiotics was investigated using methods adapted from Spivey et al. (2014). Gelatin-coated 24-well plates were seeded with LMH cells (3.0×10^5 cells $well^{-1}$) and incubated for 18 h. Wells were rinsed 3 × with assay medium to remove nonadherent LMH cells. Approximately 3.0×10^7 cfu bacteria, a multiplicity of infection of 100:1, and prebiotics were added to the wells simultaneously. Plates were centrifuged at 600× *g* for 5 min at 20°C to promote bacterium–host cell contact and then incubated for 30 min at 37 °C in a

Table 1. Structure and composition of commercially purified prebiotics used in this study.

Prebiotic[1]	Chemical structure[2]	Purity (%)[3]
β-glucan	$[\beta\text{-Glc}(1\rightarrow3)/(1\rightarrow6)]_n$[4]	81
FOS	β-Fru-(1→2)-$[(\beta\text{-Fru-}(1\rightarrow2)]_{1\text{-}10}$ and α-Glc-(1→2)-$[(\beta\text{-Fru-}(1\rightarrow2)]_{2\text{-}10}$	95
GOS	α-Glc-(1→4)-$[\beta\text{-Gal-}(1\rightarrow6)]_{2\text{-}4}$	55
MOS	$[\alpha\text{-Man-}(1\rightarrow6)]_n$	99
RAF	α-Gal-(1→6)-α-Glc-(1→2)-β-Fru	99

[1]FOS, fructooligosaccharides; GOS, galactooligosaccharides; MOS, mannanoligosaccharides; RAF, raffinose.
[2]Fru, fructose; Gal, galactose; Glc, glucose; Man, mannose.
[3]As reported by the manufacturer.
[4]*n*, indicates the degree of polymerization not provided by the manufacturer.

humidified 5% CO_2 incubator. After incubation, wells were rinsed 5 × with PBS to remove nonadherent bacteria. LMH cells were lysed by the addition of 200 μL of 0.1% (w/v) Triton X-100 (MilliporeSigma) to each well and incubation for 10 minutes at 37 °C. Bacterial suspensions were diluted in PBS, and *Salmonella* and *Campylobacter* were enumerated using XLT-4 or CCA, respectively.

Calculations and Statistical Analysis

Counts of adherent bacteria were $\log_{10}$ transformed, $\log_{10}$ reductions were calculated as compared with untreated LMH cells, and the percent reduction was calculated using the following:

$$\%\ \text{reduction} = \left(1 - 10^{-l}\right) \times 100\ \%$$

where $l = \log_{10}$ reduction.

The dose response data were analyzed using four-parameter logistic (**4PL**) nonlinear regression (Gadagkar and Call, 2015). The minimum and maximum asymptotic responses were constrained at 0 and 100%, respectively; the half-maximal inhibitory concentration ($\mathbf{IC_{50}}$) was determined at the inflection point of the 4PL function; the Hill's slope coefficient ($\boldsymbol{n_H}$) is the slope of the curve at the IC_{50}; and the coefficient of determination ($\boldsymbol{r^2}$) was used to establish goodness-of-fit for the regression. For nondose response assays, percent reductions were arcsine square root transformed and analyzed using ANOVA with $\alpha = 0.05$. Results from multiple independent assays were pooled for analysis using the assay as a blocking factor. Significantly different means were separated using Tukey's honestly significant differences test *post hoc*.

RESULTS AND DISCUSSION

The administration of dietary prebiotics has been demonstrated to reduce human foodborne pathogens, including *Salmonella* (Spring et al., 2000; Fernandez et al., 2002) and *Campylobacter* (Baurhoo et al., 2009; Huff et al., 2013), and poultry pathogens, such as *C. perfringens* (Yang et al., 2008a; Allaart et al., 2013), in the GI tract of poultry. Refined functional carbohydrates composed of MOS, β-glucans, and D-mannose account for 20–30% of the cell dry mass of *S. cerevisiae* and are a readily available source of prebiotics for human and animal use (Dallies et al., 1998). The administration of yeast-derived prebiotic RFC has been demonstrated previously to reduce *Campylobacter* in broiler chickens (Froebel et al., 2019, 2020), *Salmonella* in broilers chickens (Walker et al., 2018) and broiler breeder hens and their progeny (Walker et al., 2017), and both *Salmonella* and *Campylobacter* in turkeys during transport stress (Huff et al., 2013). Whereas positive effects have been reported when RFC and other prebiotics are administered in poultry production, the mechanisms by which they reduce pathogen colonization are not well characterized.

Adhesion to the GI mucosa is important to the colonization and persistence of bacterial pathogens in the GI tract (Rosenberg et al., 1983) because it allows resistance to the shear forces of GI peristalsis and facilitates establishment of bacterial colonization in the GI environment (Rosenberg et al., 1983; Granato et al., 1999). Although not a prebiotic functionality per se because it does not involve selective utilization by the host microbiota, prebiotics have been demonstrated to bind and agglutinate bacterial pathogens (Firon et al., 1987; Shoaf et al., 2006). Binding of bacteria by prebiotics is thought to block access of bacterial surface adhesins to host receptors and inhibit their adhesion to the GI mucosa (Ganan et al., 2012; Xu et al., 2017), resulting in their passage through the GI tract without the opportunity to colonize (Oyofo et al., 1989; Spring et al., 2000; Walker et al., 2017). Although it has been suggested to contribute to pathogen reduction in the GI tract of poultry, the inhibition of pathogen adhesion by prebiotics has not been well characterized. A more complete understanding of this functionality will be important to the continued development and application of prebiotics in poultry production.

Although the chicken LMH cell line is derived from the liver, its suitability for the investigation of host–microbe interactions in the GI tract of poultry has been well established previously. The LMH cell line has been used previously to characterize the role of *C. perfringens* NetB toxin in necrotic enteritis (Keyburn et al., 2008) and identify genes important to *C. jejuni* colonization (Flanagan et al., 2009; Quiñones et al., 2009) and *Salmonella* Enteritidis virulence (Shah et al., 2012) in the GI tract of chickens. In addition, adhesion of *Lactobacillus* spp. to poultry epithelia (Spivey et al., 2014) and competition between *C. jejuni* strains for binding to epithelial cells (Konkel et al., 2007) have been characterized in vitro using LMH cells and verified in vivo using broiler chicks. Thus, in the absence of a poultry-specific intestinal epithelial cell line, the LMH chicken epithelial cell line was used in this study as a model to investigate the inhibition of pathogen adhesion by RFC and other prebiotic oligosaccharides. Although in vitro cell culture–based models are limited because they cannot account for the effects of many in vivo factors including mucus, extracellular matrix components, host immune factors, and other microbiota, the important insights gained through their use have made significant contributions to the understanding of host–microbe interactions in the GI tract.

Dose Response of Adhesion Inhibition by RFC

In this study, we evaluated the effect of increasing concentrations of prebiotic RFC on the inhibition *Salmonella* Typhimurium and *C. jejuni* adhesion of to the LMH chicken epithelial cell line. Each bacterium was incubated with epithelial cells treated with 0, 0.025, 0.05, 0.1, 0.25, 0.375, 0.5, 0.625, 0.75, 1, and 2% (w/v)

RFC, and the reduction of adherent bacteria as compared with untreated (0%) cells was determined (Figure 1). The ability of prebiotic RFC to inhibit adhesion of *Salmonella* ($r^2 = 0.989$) and *C. jejuni* ($r^2 = 0.994$) to the epithelial cells in vitro was dose dependent and saturable with the reduction of both pathogens increasing with RFC concentration. This result suggests that inhibition of adhesion to epithelial tissues may be an important mode of action through which prebiotic RFC reduce *Salmonella* and *Campylobacter* colonization in the GI tract of poultry. In addition, the IC_{50} was lower ($P < 0.001$) and the Hill's slope was steeper ($P = 0.003$) for *C. jejuni* ($IC_{50} = 0.020\%$, $n_H = 2.143$) than for *Salmonella* ($IC_{50} = 0.048\%$, $n_H = 0.935$), suggesting that the adhesion of *C. jejuni* is more sensitive to inhibition by RFC than that of *Salmonella*. Prebiotic RFC have been demonstrated previously to inhibit adhesion of *Escherichia coli* O157:H7 E318N to a bovine colonic line in a dose-dependent manner (Baines et al., 2011). However, the IC_{50} was not reported in the previous study.

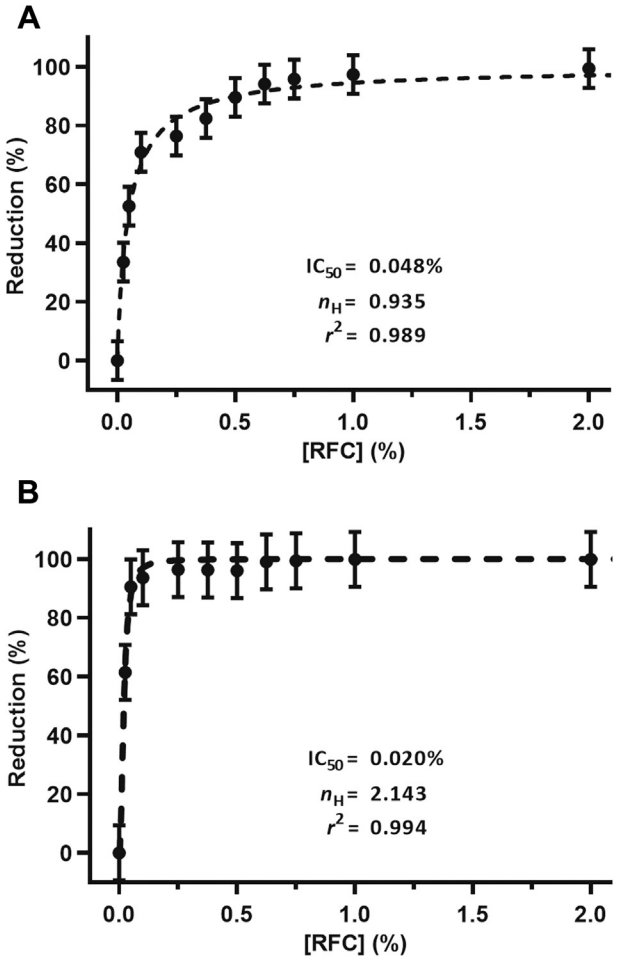

Figure 1. Dose response of adhesion inhibition by RFC. (A) *Salmonella* Typhimurium and (B) *Campylobacter jejuni* were co-incubated with LMH cells (MOI 100:1) treated with increasing concentrations of RFC, and the number of adherent bacteria was enumerated. The mean ± SEM% reduction of adherent bacteria from 3 independent wells is reported. Abbreviations: IC_{50}, half-maximal inhibitory concentration; MOI, multiplicity of infection; n_H, Hill slope coefficient; r^2, coefficient of determination; RFC, refined functional carbohydrates.

The suggested incorporation rate for RFC administration to broilers is 50–200 g t^{-1} (0.05–0.2% w/w) in-feed. Although this recommended incorporation rate was likely to have been determined based on results from growth performance studies and economic factors, the IC_{50} we observed for *Salmonella* and *C. jejuni* in this study fall within the expected concentration range at which the prebiotic would be present in the GI tract when administered in-feed at the recommended incorporation rate. Based on these results, a concentration of 0.1% (w/v) was selected for use in subsequent assays evaluating inhibition of *Salmonella* and *C. jejuni* adhesion to the LMH cell line by individual carbohydrates and other prebiotics. Although they would not be present in equimolar amounts because of differences in composition and degree of polymerization, the carbohydrates would be equivalent on a total monosaccharide basis because the molecular weights of the monosaccharides from which they are composed (fructose, galactose, glucose, and mannose) are identical (180.16 g mol^{-1}).

Comparison of Major RFC Constituents

Mannoproteins and glucans comprise approximately 85–90% of the dry mass of the *S. cerevisiae* cell wall (Fleet, 1991; Klis, 1994) and serve as a readily available source of prebiotics for human and animal use. Whereas the exact composition varies by strain and culture conditions (McMurrough and Rose, 1967; Catley, 1988), glucans are estimated to make up 55–60% of the cell wall, with the remaining content being mannan–protein complex and cell wall–linked and periplasmic glycoproteins (Phaff, 1971). Thermal and enzymatic processing of the cell wall releases β-glucans, MOS, and D-mannose (Hunter and Asenjo, 1988), which can then be extracted crudely for use as prebiotics. These individual constituents are not present in equal concentrations in the cell wall and, therefore, not presumed to be extracted at equal concentrations. However, understanding the effectiveness of the primary constituent carbohydrates individually will inform the continued development and application of RFC and other prebiotics in poultry production.

We evaluated inhibition of *Salmonella* and *C. jejuni* adhesion by commercially purified β-glucan, MOS, and D-mannose to understand the contribution of the individual constituent components to adhesion inhibition of the cruder yeast–derived extract. All three of the major constituent carbohydrates were observed to inhibit ($P < 0.001$) adhesion of *Salmonella* to the epithelial cells as compared with untreated cells (Figure 2A). Reduction of adherent *Salmonella* by β-glucan (95.80%) and MOS (90.90%) was greater than by D-mannose (32.14%). Similarly, each of the major component carbohydrates of prebiotic RFC also inhibited adhesion of *C. jejuni* ($P < 0.001$) (Figure 2B). Reduction of adherent *C. jejuni* by β-glucan (98.57%) and MOS (97.02%) was

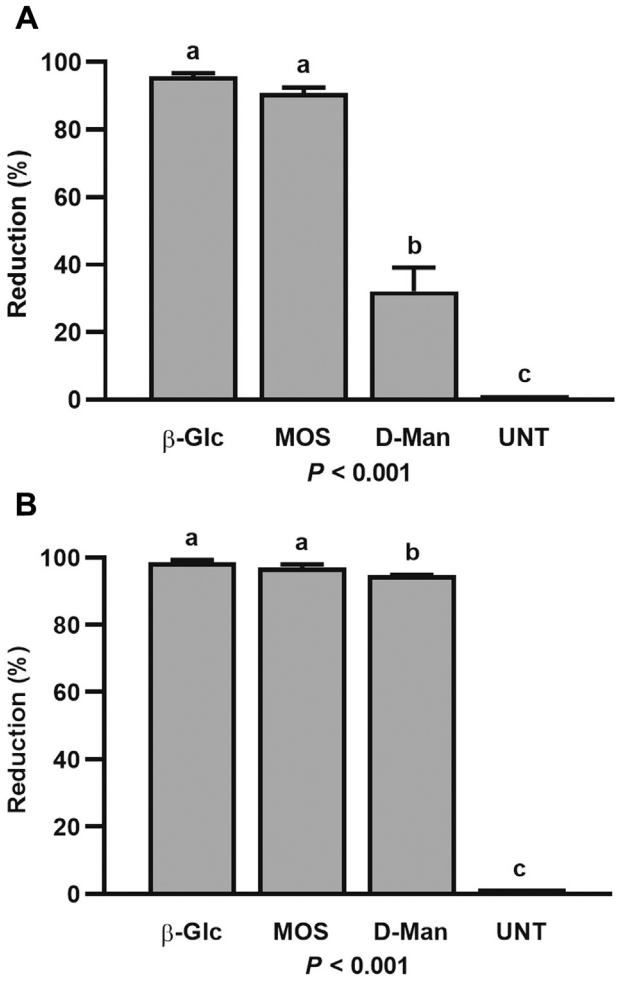

Figure 2. Inhibition of pathogen adhesion to chicken epithelial cells by carbohydrate components of prebiotic RFC. (A) *Salmonella* Typhimurium and (B) *Campylobacter jejuni* were co-incubated with LMH cells (MOI 100:1) treated with β-glucans (β-Glc), mannanoligosaccharides (MOS), or D-mannose (D-Man) and untreated (UNT) cells, and the number of adherent bacteria was enumerated. The mean ± SEM% reduction of adherent bacteria as compared to UNT cells from 3 independent wells from 3 independent assays is reported. The means not sharing common letters are significantly different ($P \leq 0.05$). Abbreviations: MOI, multiplicity of infection; RFC, refined functional carbohydrates.

greater than by D-mannose (94.67%). Similarly to the cruder extract, adhesion of *C. jejuni* is more sensitive to inhibition by these individual commercially purified components than *Salmonella*.

Studies evaluating the administration of crude yeast cell wall extracts as prebiotics typically report MOS as the primary active component (Yang et al., 2008b; Morales-Lopez and Brufau, 2013; Santos et al., 2013), and any observed reductions in pathogen reduction are attributed to MOS exclusively (Hooge, 2004; Baurhoo et al., 2009). The results of our study suggest that β-glucans and other cell wall–derived carbohydrates are also likely to contribute to reduced pathogen colonization in poultry through their inhibition of adhesion to the GI epithelium. Thus, characterization of other cell wall components, in addition to MOS, will be important to the understanding of this and other important functionalities.

The dose-dependent and saturable nature of adhesion inhibition by RFC in our cell culture model suggests specific receptor–ligand binding reactions may contribute to reduced adhesion of bacteria to epithelial cells. Mannose binding of FimH-like adhesins on type 1 fimbriae has been demonstrated to inhibit epithelial cell adhesion of gram-negative bacteria, including *Salmonella* and *E. coli* (Oyofo et al., 1989; Spring et al., 2000) and has been suggested to reduce GI colonization (Fomentini et al., 2016). Although similar adhesins have not been identified in *Campylobacter* spp., mannose-binding lectins have been observed in *C. jejuni* 11168 (Day et al., 2009), suggesting analogous binding interactions may also be involved in *Campylobacter* reduction. In addition, the reduction of adherent *Salmonella* was significantly greater when cells were treated with MOS than when compared with D-mannose, suggesting inhibition of adhesion may be related to the branched-chain structure of oligosaccharides rather than simply the saturation of binding sites by monosaccharides. A previous study reported that inhibition of *E. coli* E2348/69 adhesion to the HEp-2 and CaCo-2 human epithelial cells by FOS was more effective when the degree of polymerization was greater (Shoaf et al., 2006), suggesting that higher molecular weight oligosaccharides may obstruct a greater surface area for adhesion to epithelial surfaces. β-Glucan has also been reported to bind bacterial pathogens including *Streptococcus*, *Salmonella*, and *E. coli* (Mattos-Graner et al., 2001; Ganner et al., 2013), whereas β-glucan secretion by beneficial bacteria including *Lactobacillus* spp. and *Pediococcus* spp. has been reported to increase their adhesion to epithelial surfaces as compared to nonsecreting strains (Garai-Ibabe et al., 2010). These studies suggest that binding and occupation of both host-mucosal binding sites and bacterial adhesins is likely to contribute to the ability of yeast-derived RFC to inhibit pathogen adhesion and colonization of the host GI tract.

Comparison of Prebiotic Oligosaccharides

Although pathogen reduction and other benefits of their administration are reported widely, the overall effectiveness of prebiotics when administered to poultry is mixed, and the beneficial effects of their administration are often inappropriately attributed broadly across all prebiotic products as a general class of functional feed additives. Because the ability to confer specific benefits is dependent on its composition, research investigating the functionality of specific prebiotics is required (Askelson and Duong, 2015). In addition, other prebiotic oligosaccharides including fructans and galactans must be either refined or synthesized from vegetable and dairy foods diverted from human consumption (Contesini et al., 2019), whereas *Saccharomyces*-derived prebiotic RFC are produced from spent yeast produced as co-products from industrial processes including brewing and fuel ethanol production (Gómez et al., 2012). Therefore, in addition to their effectiveness in reducing adhesion and

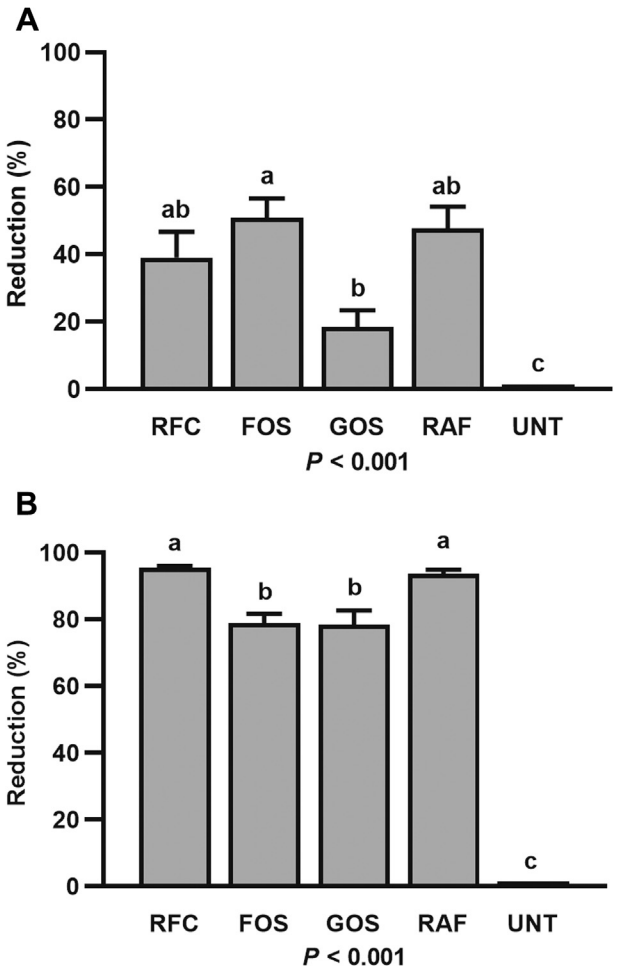

Figure 3. Inhibition of pathogen adhesion to chicken epithelial cells by prebiotic oligosaccharides. (A) *Salmonella* Typhimurium and (B) *Campylobacter jejuni* were co-incubated with LMH cells (MOI 100:1) treated with refined functional carbohydrates (RFC), fructooligosaccharides (FOS), galactooligosaccharides (GOS), or raffinose (RAF) and untreated (UNT) cells, and the number of adherent bacteria was enumerated. The mean ± SEM% reduction of adherent bacteria as compared with UNT cells from 3 independent wells from 3 independent assays is reported. The means not sharing common letters are significantly different ($P \leq 0.05$). Abbreviations: MOI, multiplicity of infection.

colonization of pathogens and other benefits provided to the host, economic factors including the availability of raw materials and cost will also likely be important factors for the selection of prebiotics.

In this study, we compared the ability of *Saccharomyces*-derived prebiotic RFC to inhibit adhesion of *Salmonella* and *C. jejuni* to the chicken LMH cell line with that of FOS, GOS, and RAF. All 4 prebiotics evaluated were observed to significantly inhibit adhesion of *Salmonella* Typhimurium ($P < 0.001$) to the LMH cells (Figure 3A). The reduction of adherent *Salmonella* was greatest when cells were treated using FOS (50.79%) and lowest when cells were treated using GOS (18.44%). Inhibition of *Salmonella* adhesion by RAF (47.70%) and RFC (39.09%) was not significantly different than inhibition by FOS or GOS. In addition, all 4 prebiotics evaluated were also observed to inhibit adhesion of *C. jejuni* ($P < 0.001$) to the chicken LMH cell line (Figure 3B). Reduction of adherent *C. jejuni* was greater when cells were treated with RFC (95.43%) and RAF (93.66%) than when compared to FOS (78.79%) and GOS (78.41%).

These results demonstrate inhibition of pathogen adhesion by a relatively crude extract of yeast-derived prebiotic RFC to be comparable to that of highly purified (55–99% pure) synthetic prebiotic oligosaccharides. Fructooligosaccharides, GOS, and RAF have been demonstrated previously to inhibit adhesion of enteropathogenic *E. coli* to the HEp-2 and CaCo-2 human epithelial cell lines (Shoaf et al., 2006). Using 1.6% (w/v) of prebiotic, GOS was reported to more effectively inhibit pathogen adhesion than FOS and RAF, whereas in our study, using 0.1% (w/v) prebiotic, GOS was either less or similarly effective as RAF and FOS. In addition to the different results, the many differences between the previous study and ours demonstrate the importance of specificity in host cell lines, pathogens, and products in the evaluation of the effectiveness of interventions. To our knowledge, our study is the first of its kind evaluating inhibition of *Salmonella* and *C. jejuni* adhesion by prebiotics using a poultry-specific cell line.

In this study, we investigated inhibition of pathogen adhesion to chicken LMH epithelial cells by RFC in vitro. We have demonstrated in vitro inhibition of *Salmonella* and *C. jejuni* adhesion to epithelial cells by prebiotic RFC to be dose-dependent and saturable, suggesting specific ligand-binding interactions are likely to contribute to the adhesion-inhibiting functionality of RFC. Whereas adhesion inhibition by yeast-derived prebiotics is often attributed exclusively to MOS, by characterizing the major carbohydrate constituents of RFC individually, we have demonstrated β-glucan is also likely to contribute to this functionality. We have also demonstrated the inhibition of pathogen adhesion by crudely extracted yeast-derived prebiotic RFC in vitro to be comparable to that of highly purified synthetic prebiotic oligosaccharides used more commonly in human health and nutrition. Although adhesion is critical to bacterial colonization (Rosenberg et al., 1983), the results of our study are limited by the many complex factors not included in this model, including mucus, extracellular matrix components, host immune factors, and additional GI microbiota, which will need to be taken into account to understand the effects of prebiotics on *Salmonella* and *C. jejuni* colonization in poultry. In addition, we have further demonstrated the utility of the chicken LMH epithelial cell line in the in vitro investigation of the complex interactions at the host–microbe interface in the GI tract of poultry, and the assay we have described is expected to provide a platform for the elucidation of mechanisms important to the functionality of prebiotics in the poultry GI tract. The results of our study are expected to inform future research investigating prebiotic functionality in poultry and the continued development of prebiotics for and their applications in poultry production.

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DISCLOSURES

The authors declare no conflicts of interest.

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