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Identification of Antiadhesive Fraction(s) in Nonimmunized Egg Yolk Powder: In Vitro Study

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The presence of antiadhesive component(s) in the hen egg yolk against foodborne pathogens was anticipated from results of a previous animal study conducted by the authors. The previous work showed egg yolk powder without specific antibodies is effective in controlling *Salmonella enteritidis*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7 colonization in laying hens. Therefore, this study was necessary to locate the activity and identify the effective component(s). In vitro experiments were conducted using confluent Caco-2 cell monolayers. *S. enteritidis*, *S. typhimurium*, and *E. coli* O157:H7 were investigated against the various extracted granule and plasma fractions in three different assays: adhesion elimination, adhesion prevention, and antimicrobial. This study revealed original findings and identified the protective yolk fraction against the foodborne pathogens as the granule component, high-density lipoproteins (HDL). The protective activity conveyed by HDL was confirmed to remain intact despite peptic and tryptic enzymatic digestion and to have antiadhesive but not antimicrobial effect.

KEYWORDS: **Egg yolk; granule; high-density lipoproteins; *Salmonella enteritidis*; *Salmonella typhimurium*; *Escherichia coli* O157:H7; antiadhesive**

INTRODUCTION

Chickens may represent a reservoir of *Salmonella* and *Escherichia coli* O157:H7 that can potentially infect humans (*1*). Therefore, the prevention or the reduction of these pathogenic colonies in the intestines of laying and broiler chickens may greatly reduce the contamination of eggs and poultry meat products during processing.

Previous in vivo work conducted by the authors (*2*, *3*) unveiled a novel functional application of egg yolk powder derived from eggs not containing specific antibodies against the foodborne pathogens *Salmonella* spp., *E. coli* O157:H7, and *Campylobacter jejuni*. Sufficient evidence was provided to suggest that nonimmunized egg yolk possesses protective abilities in laying hens either by preventing colonization by the aforementioned pathogens or by eliminating bacteria established in the gut. Furthermore, results from the in vivo studies confirmed and concluded that the inhibitory activity exhibited by egg yolk powder against the pathogens was not due to the immunoglobulin fraction, IgY, but another novel component. Many studies have demonstrated antimicrobial activity of hen egg yolk (*4*−*6*) specifically attributed to the IgY fraction. However, this is the first known study to demonstrate an important and novel antiadhesive activity associated with the egg yolk components other than the IgY fraction.

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The major portion of egg yolk exists as lipoproteins, which are separated into two main fractions, the plasma (supernatant on centrifugation) and the granule (precipitate on centrifugation) (*7*). The functional properties of egg yolk are attributed primarily to the phospholipid and protein components (*8*). The egg yolk proteins include the low-density lipoptoteins (LDL), high-density lipoproteins (HDL), phosvitin, and livetin. In fact, until recently, despite their important functional properties and rich composition, egg yolk proteins had not been investigated to a great extent (*9*).

The process of adherence and invasion of pathogenic microorganisms to the host epithelium during infection can be mimicked in vitro by infecting tissue culture cells of epithelial origin (*10*). Caco-2 cells have been exploited by many researchers in regard to a broad spectrum of intestinal and epithelial parameters (*11*). Consequently, they resemble the fetal colon cells and intestinal epithelia (*12*), especially that of the ileum, the primary site of *Salmonella* invasion (*13*). For this reason, the vast majority of bacterial cell interaction studies with *Salmonella* species and other enteropathogens have been carried out in vitro with such cultured cell lines (*14*). This cell line, thus, was selected to be used in the in vitro experiments of the present work.

The primary goal of this in vitro work was to identify, for the first time, the hen egg yolk fraction(s) that is (are) most likely contributing to the protective effects observed in the prior in vivo experiments (*2*, *3*) against *S. enteritidis*, *S. typhimurium*, and *E. coli* O157:H7. Another important objective was to confirm that the activity exhibited by those distinguished egg

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yolk fraction(s) against the investigated enteropathogens was an antiadhesive effect and not an antimicrobial one.

MATERIALS AND METHODS

Bacterial Strains. The isolates used for infection were *S. enteritidis*, PT4 SA992212 from chickens (Dr. Cornelius Poppe, Health Canada, Guelph, ON, Canada), *S. typhimurium* var. Copenhagen PT 10 SA992416 from turkey (Dr. Cornelius Poppe), and *E. coli* O157:H7 920005 from chickens (CRIFS, Guelph, ON, Canada).

Prior to preparation, the isolates were stored at −80 °C in 30% glycerol (Fisher Scientific, Whitby, ON, Canada). The *S. enteritidis* and *S. typhimurium* challenge inocula were prepared from an overnight culture in tryptic soy (TS) broth (BD Diagnostic System, Oakville, ON, Canada) and were then serially diluted to obtain ∼1.0 × 10^5 bacteria in the inoculum. The viable cell concentrations of *S. enteritidis* and *S. typhimurium* challenge inocula were estimated by spectrophotometry (Shimadzu UV-12013, Kyoto, Japan) at an optical density setting of 660 nm and were confirmed by colony counts on brilliant green (BG) agar (BD Diagnostic System) plates containing 20 μg of novobiocin (NO) (BD Diagnostic System) and xylose lysine deoxycholate (XLD) agar (BD Diagnostic System) plates, respectively.

E. coli O157:H7 was prepared from an overnight culture in LB Lennox broth (BD Diagnostic System), which was incubated at 37 °C for 24 h. After incubation, serial dilutions were prepared from the bacterial inoculum to obtain ∼1.0 × 10^5 colony-forming units (CFU)/mL. The viable cell concentration in the inoculum was also estimated by spectrophotometry as before and was confirmed by colony counts on tellurite−Cefixine−sorbitol MacConkey (TC-SMAC) agar (BD Diagnostic System) plates, which were incubated overnight at 37 °C.

In the assays, 100 μL of each bacterial incoculum was applied to the cell monolayers described below.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells of passage 21−40 were maintained in confluent layers in flasks in DMEM/F-12 (Gibco Invitrogen Corp., Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (FCS) (Gibco Invitrogen Corp.). For routine culture, penicillin (40 units/mL) and streptomycin (40 μg/mL) were added. Flasks were treated with trypsin−EDTA (Gibco Invitrogen Corp.) to split cells. Following two washes with complete medium, the concentration of the cells was adjusted by counting in a hemocytometer, and 2 × 10^5 cells was applied into 24-well tissue culture plates (Corning Costar, Cambridge, MA). Cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2 in a Sanyo CO_2 incubator (Sanyo Canada Inc., Concord, ON, Canada). Cells were fed with fresh medium every other day for 2 weeks. Three hours before the experimental assays were performed, the growth medium was removed from each well and replaced with a fresh medium devoid of antibiotics.

Preparation of Egg Yolk and Egg Yolk Fractions. Plasma, granule, HDL, and LDL fractions along with the nonimmunized egg yolk sample were prepared from eggs obtained from the University Research Station (Arkell Poultry Research Unit, Guelph, ON, Canada). The eggs were cracked after the disinfection of the exterior shell surface, and the egg yolks were aseptically separated from albumen. Pooled egg yolks were freeze-dried and crushed into a fine powder. All egg yolk samples were tested for antibodies to *S. enteritidis*, *S. typhimurium*, and *E. coli* 0157:H7 by the enzyme-linked immunosorbent assay (ELISA) using formalin-killed whole cells as coating antigens. Samples were negative for the aforementioned antibodies. The egg immunoglobulin, IgY, and phosvitin were the other fractions used in these in vitro assays. The whey protein isolate, as a control, was a commercial sample obtained from Protein Fractionation Inc. (Toronto, ON, Canada).

In the extraction procedure (*15*), granule, the source of phosvitin and HDL, was prepared by diluting fresh egg yolk at a ratio of 1:4 with 0.05 M NaCl. The mixture was then stirred for 30 min at room temperature. Centrifugation at 70000*g* for 2 h at 10 °C in a Beckman Coulter ultracentrifuge (Optima LE-80K, Beckman Coulter Canada Inc., Mississauga, ON, Canada) formed a firm pellet of granules on the bottom and an opaque orange supernatant, which was kept for further fractionation. The granule pellet was additionally washed two times with 0.05 M NaCl as before and stored in airtight vials at 4 °C.

HDL was obtained from the above granules by the dissociation of 10 g in 30 mL of 0.45 M $MgSO_4$ (*16*). After 1 h of centrifugation at 10 °C and 95000*g*, a floating layer of crude HDL and a lower phase of crude phosvitin were formed. The floating layer was removed and precipitated by adding 25−30 mL of 0.45 M $MgSO_4$. This was stirred at room temperature for 30 min and then centrifuged again as before and dialyzed against distilled water, using a tubing dialysis of 6−8-kDa molecular weight cutoff (Fisher Scientific) until HDL precipitated. Finally, the HDL precipitate was centrifuged for another 30 min at 10 °C and 8000*g* and stored at 4 °C.

The phosvitin fraction, which was isolated from the lower phase after removal of the HDL fraction, was prepared in previous work at our laboratory (*17*).

The supernatant remaining after granule precipitation was the plasma and the source of the LDL (18) and γ-livetin (IgY) fractions. The supernatant was treated with 45% $(NH_4)_2SO_4$ added gradually, and the resultant precipitated solution was stored at 4 °C for 24 h. After centrifugation for 1 h at 10 °C and 30000*g*, a precipitate of IgY and a supernatant of crude LDL were formed. The supernatant of the LDL fraction was then dialyzed in an aqueous solution of 0.05 M NaCl, using the dialysis tubing of 6−8-kDa molecular weight cutoff (Fisher Scientific), at 4 °C for 2 days. The resultant LDL fraction was filter sterilized, purged with nitrogen, and stored in aluminum-wrapped bottles at 4 °C. The IgY fraction was obtained from previous preparations according to the method of ref *19*.

Protein Concentration. The protein concentration of the above extracted plasma and LDL egg yolk fractions was determined according to the modified Lowry method as described in ref *20*, whereas that of granule and HDL was determined according to the Bio-Rad D_C protein assay method (Bio-Rad Laboratories, Hercules, CA).

The protein concentrations of the granule, HDL, LDL, and plasma, which were obtained from the above assays, were then used to determine the concentration yield of the fractionated samples, milligrams of fraction per milligram or milliliter of total sample fractionated. The concentration yield was determined on the basis of the total percentage of protein in each sample as quoted in the literature (granule, 60%; HDL, 80%; LDL, 12.5%; and plasma, 18%) (*21*). Dilutions were then made to prepare a concentration of 5.0 mg of each fraction in 1 mL of 100 mM phospahate, pH 7.2, containing 100 mM NaCl (PBS). In the assays, 100 μL of each prepared sample was added per well of a cell culture plate that contained 0.9 mL of assay solution to obtain a final concentration of 0.5 mg/mL of each fraction per well.

Enzymatic Digestion of Egg Yolk Fractions. To mimic the in vivo conditions of the gastrointestinal tract, enzymatic digestions of egg yolk and its fractionated samples with pepsin and trypsin were undertaken prior to assay testing. This step was also essential to assist in dissolving the fractions that were sterilized by filtration (0.45 μm, Acrodisc, Fisher Scientific). In addition, the source of activity is a large lipoprotein and requires degradation in order to release maximal levels of the active component(s). In the procedure, 20 μL of pepsin solution prepared by dissolving 10 mg of porcine pepsin (P-6887 Sigma) in 1 mL of solution A (0.15 N HCl, 0.03 M NaCl) was added to the sample solution (5.0 mg of sample in 0.98 mL of solution A). The mixture at an enzyme/substrate ratio of 1:25 (w/w) was incubated for 4 h at 37 °C; the pepsin was then inactivated by neutralization of the solution with 0.5 M Na_2CO_3. Solution A (0.15 N HCl, 0.03 M NaCl) from above was also used in the adhesion−inhibition assays below as a control.

The trypsin digestion was performed by adding 25 μL of trypsin solution (2.0 mg of TPCK−trypsin T-1426, Sigma, in 1 mL of 0.1 M Tris-HCl, pH 8.0, + 0.03 M NaCl) to the sample solution (5.0 mg of sample in 0.9 mL of 0.1 M Tris-HCl, pH 8.0, + 0.03 M NaCl) at an enzyme/substrate ratio of 1:100 (w/w). The mixture was incubated at 37 °C for 24 h, and trypsin was inactivated by heating at 80 °C for 15 min.

Experimental Design. The antiadhesive activity of the hen egg yolk fractions was studied in vitro against the bacterial strains *S. enteritidis*, *S. typhimurium*, and *E. coli* O157:H7 in three separate procedures. The first was an adhesion−elimination and the second an adhesion−prevention; the term "adhesion−inhibition" will be used in the text when referring to both combined. The third procedure, on the other hand, was an antimicrobial assay. It is necessary to note that the assays

performed did not differentiate between the bacteria that adhered to the surface of the cells and those that invaded the monolayer cells. Furthermore, except for the inoculum broth and the plating agar media, the procedures applied with respect to the three different organisms were essentially the same.

Adhesion−Elimination Assay. Confluent Caco-2 cell monolayers that were grown in 24-well plates were used. Three hours prior to the assay, the growth medium was replaced with 0.8 mL of the same medium (DMEM + 20% FCS), but without antibiotics. After the cells had been stabilized for 3 h at 37 °C in 5% CO_2 (Sanyo CO_2 incubator, Sanyo Canada Inc.), 100 μL of the bacterial inoculum at a concentration of 1.0×10^5 CFU/mL was added to all of the wells. After incubation for 1 h with shaking in a 37 °C incubator to allow for bacterial adhesion, 100 μL of the various samples prepared above was added to the designated wells in duplicate, whereas 100 μL of sterile PBS was added to the negative control wells. A final volume of 1 mL and a final concentration of 0.5 mg/mL of each sample were attained per well.

The samples examined in these assays were the whey protein isolate (after pepsin and trypsin digestion) and solution A as controls; nonimmunized egg yolk powder, IgY, phosvitin, granule, and HDL (before and after pepsin and trypsin digestion); and plasma and LDL. Following the addition of the samples, the assay plates were incubated with shaking on an orbital shaker (Cole-Parmer Instrument Co., Vernon Hills, IL) for an hour at 37 °C. The effect of each of the tested fractions on the colonization of the various pathogens was determined after the monolayers were washed three times with 1 mL of PBS per wash to remove nonadherent bacteria.

The bacteria-associated monolayers were then released with 1 mL of 1% (v/v) Triton X-100 (Sigma, St. Louis, MO) in 0.1 M PBS (pH 7.2) by gentle agitation on the orbital shaker for 10 min at room temperature. Adherent bacteria were counted by plating 100 μL from each of the serial 10-fold dilutions prepared from the suspensions onto the selective agar plates. BG containing 20 μg of NO, XLD), and TC-SMAC (BD Diagnostic System) agar plates were used for *S. enteritidis*, *S. typhimurium*, and *E. coli* O157:H7, respectively. Selective growth and differential enumeration of each targeted bacterium on each medium were verified according to the methods described in ref *22*.

Adhesion−Prevention Assay. These assays were adopted to determine which of the various hen egg yolk fractions is/are most effective in preventing or inhibiting bacterial adhesion and/or invasion of the culture cells. The basic procedure was similar to that of the adhesion−elimination assay except that the sequence of bacterial/sample additions was reversed. The test samples were first added to the monolayer cellsm and, after incubation, the bacterial incula were introduced.

Antimicrobial Assay. The purpose of these assays was to confirm that the inhibitory activity inflicted by the hen egg yolk fractions was an antiadhesive rather than an antimicrobial one.

These assays were performed in the same 24-well plates that contained 0.8 mL of antibiotic-free DMEM + 20% FCS growth medium, but without the Caco-2 cell monolayers. In the procedure, 100 μL of each bacterial inoculum at a concentration of 1.0×10^5 CFU/mL was added to all of the wells. After incubation for 1 h with shaking in a 37 °C incubator, 100 μL of the various yolk samples in PBS was added to the designated wells in duplicate, whereas 100 μL of sterile PBS was added to the negative control wells to obtain a 1 mL final volume and a final concentration of 0.5 mg/mL of each sample. Following the addition of the samples, the assay plates were incubated with shaking for an hour at 37 °C. The effect of each tested fraction on the various pathogens was determined after the plated bacteria were counted on the selective agar plates as before. Selective growth and differential enumeration of each targeted bacterium on each medium were also verified.

Statistical Analysis. Differences between treatments were examined for the level of significance by Student's *t* test after analysis of variance. A *P* value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The breakdown of the polypeptides into smaller fragments in each fraction was confirmed by SDS−electrophoresis (data not shown). Pepsin well digested almost all of the egg yolk components, whereas trypsin retained large fragments.

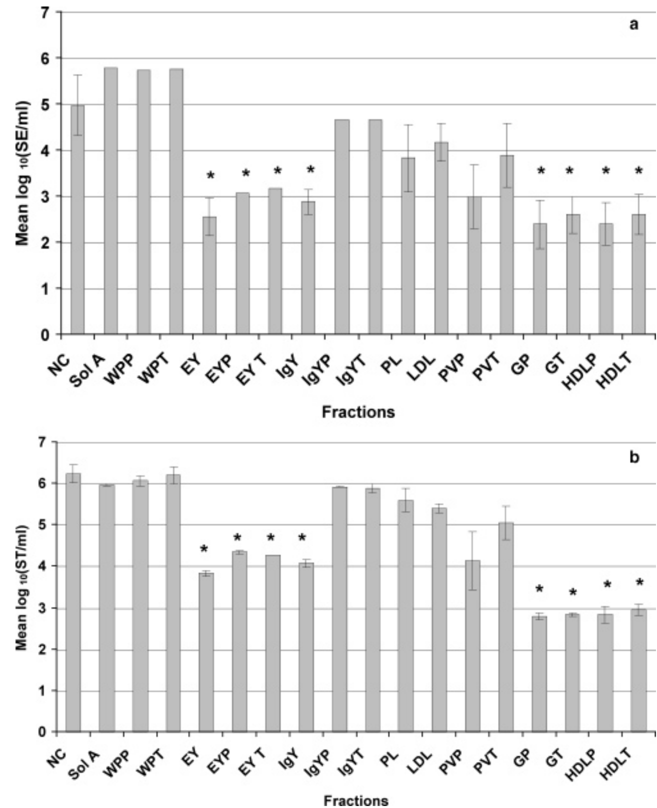

Figure 1. Eliminating effects of various yolk fractions against (**a**) *S. enteritidis* and (**b**) *S. typhimurium* in vitro. Monolayer cells were inoculated with 10^5 CFU/mL of *S. enteritidis* or *S. typhyimurium* and then treated with the fractions. NC, negative control; Sol A, 0.15 N HCl + 0.03 M NaCl; WPP, whey protein pepsin digested; WPT, whey protein trypsin digested; EY, egg yolk; EYP, egg yolk pepsin digested; EYT, egg yolk trypsin digested; IgY, yolk antibody; IgYP, pepsin digested; IgYT, trypsin digested; PL, plasma; LDL, low-density lipoprotein; PVP, phosvitin pepsin digested; PVT, phosvitin trypsin digested; GP, granule pepsin digested; GT, granule trypsin digested; HDLP, high-density lipoprotein pepsin digested; HDLT, high-density lipoprotein trypsin digested. The data are presented as the mean ± standard deviation of 12 replicates. * Values of significant reduction (P < 0.05) in mean log *S. enteritidis* (or *S. typhimurium*)/mL compared with the negative control. Error bars incidate the standard error of the mean.

The effects of the several extracted egg yolk fractions, granule, HDL, phosvitin, plasma, LDL, and IgY, in addition to the nonimmunized egg yolk, were examined in vitro against *S. enteritidis* and *S. typhimurium*. Although *S. enteritidis* and *S. typhimurium* were studied in separate assays, the results obtained regarding the reduction levels in the counts and the yolk fraction(s) which were inducing the activity were statistically identical. Therefore, the term "*Salmonella* spp." is used in the text when referring to both *S. enteritidis* and *S. typhimurium*.

Salmonella spp. *Adhesion−Elimination Assay.* The inhibitory effect of the egg yolk and its fractions on the binding of *S. enteritidis* and *S. typhimurium* to Caco-2 cells is shown in parts **a** and **b** of **Figure 1**, respectively. These assays were aimed to determine the yolk fractions that convey the highest capability in eliminating or reducing *Salmonella* spp. that had already bound to and/or invaded the cells.

To exclude the possibility that the inhibitory effects observed by the egg yolk fractions were the result of a typical physical barrier that may be induced by any other protein, the whey

protein isolate was used as a control. Furthermore, solution A (0.15 N HCl, 0.03 M NaCl), which was used in the enzymatic digestion of the yolk proteins, was selected as another control in the assays. Finally, a negative control, where the pathogens were introduced into the Caco-2 cell monolayers without the treatment with the yolk fractions, was also used in all assays. The mean log counts of *Salmonella* spp. acquired from each tested sample were compared with this control, and differences were considered to be statistically significant when $P < 0.05$.

When the bacteria-associated monolayers were released and the adherent/invaded pathogens were counted by plating, the mean log counts of *S. enteritidis* and *S. typhimurium* from the negative control wells were 4.98 ± 0.66 and 6.25 ± 0.21 CFU/mL, respectively. However, the number of bacteria from those treated with the enzymatically digested granule and HDL fractions was significantly lower ($P < 0.05$) than the above negative controls (**Figure 1**). Consequently, the bacterial-reducing effect associated with these fractions was the highest among all others in the study. This suggested that the granule and HDL fractions were capable of significantly interfering with the attachment and/or invasion of *Salmonella* spp. to epithelial cells even if their proteins were subjected to enzymatic alterations. From the results also, the undigested nonimmunized egg yolk and IgY showed a similar significant reduction. However, the IgY activity was completely lost after it was enzymatically digested.

The egg yolk fraction also showed a slightly diminished activity after the enzymatic digestions. Nevertheless, the mean log counts of *Salmonella* spp. remained significantly lower, 3.08 ± 0.12 and 4.35 ± 0.05 CFU/mL, than that of the negative controls. This also indicates a reducing effect by the hen egg yolk against the established bacterial adherence and/or invasion. Furthermore, phosvitin, the second major fraction besides HDL in the yolk granule, seemed to reduce the mean bacterial counts relative to that of the negative controls. However, the extent of the activity could not be considered to be statistically significant ($P < 0.05$).

Because the plasma and its LDL fraction (**Figure 1**) did not show any significant reducing effect ($P < 0.05$), whereas the granule and its HDL fraction significantly decreased the bacterial adhesion and/or invasion ($P < 0.05$), the activity may most likely lie in the granule fraction of the hen egg yolk. The tendency seems inclined toward HDL of the granule, in particular, because phosvitin did not exhibit a similar degree of inhibition. Furthermore, a synergistic effect may be less likely to exist because the activity induced by the granule containing both the HDL and phosvitin was not higher than that of either one.

Adhesion−Prevention Assay. In these assays, the ability of egg yolk and its extracted fractions to prevent *Salmonella* spp. from adhering and/or invading epithelial cells in vitro was assessed. Whereas in the above adhesion−elimination assay the Caco-2 monolayers were exposed to the bacteria first, in the present assays the yolk fractions were introduced prior to the bacterial infection.

The results from these experiments are represented by **Figure 2**. The respective mean log counts of *S. enteritidis* and *S. typhimurium* in the negative controls were 5.29 ± 0.32 and 5.87 ± 0.10 CFU/mL 2 h after their inoculation with the organism. However, the same fractions, namely, the granule fraction of egg yolk and its HDL component that demonstrated a decrease in the bacterial counts in the above adhesion−elimination assay, appeared also to deter the organism from infecting the cells in these assays. Therefore, the adhesion−inhibition occurred at the same level by similar fractions regardless of the infection procedure.

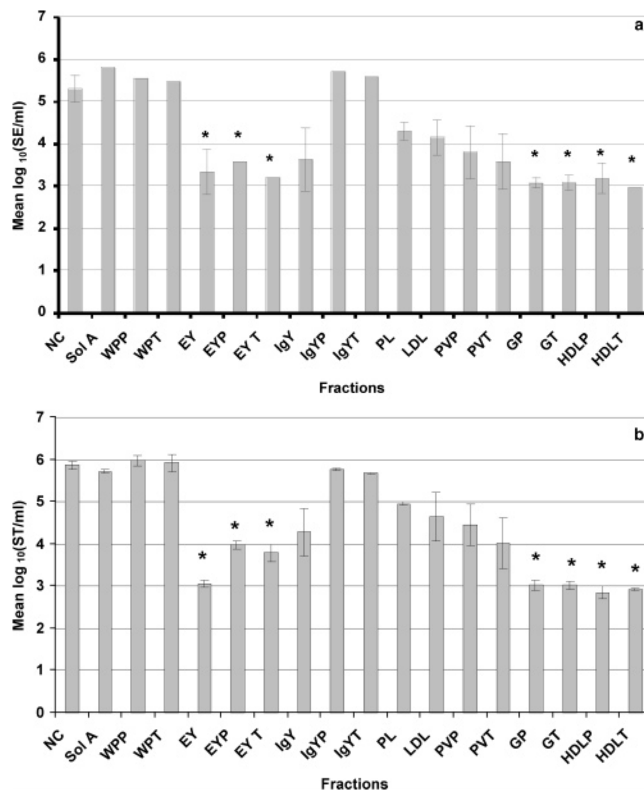

Figure 2. Preventive effects of various yolk fractions against (**a**) *S. enteritidis* and (**b**) *S. typhimurium* in vitro. Monolayer were cells treated with the fractions and then inoculated with 10^5 CFU/mL of *S. enteritidis* or *S. typhimurium*. * Values of significant reduction ($P < 0.05$) in mean log *S. enteritidis* (or *S. typhimurium*)/mL compared with the negative control. Error bars indicate the standard error of the mean of 12 trials.

Phosvitin seemed also to be successful to a certain extent in preventing the adhesion/invasion. Nevertheless, the effect was not statistically significant ($P < 0.05$) when compared with the negative control and that of HDL and granule. Egg yolk's preventive effect came next after the granule's and HDL's; and although slightly decreased after the enzymatic digestion, it was statistically significant (**Figure 2**).

IgY, on the other hand, failed to prevent bacterial adhesion in these assays both before and after it was enzymatically digested. Such observations agree with the reported results (*23*) even if in the present assay's nonspecific IgY was used. In the cited in vitro study of adhesion (*23*), the anti-*S. enteritidis* antibodies were unable to completely block the adhesion of *Salmonella*, and the antibody binding did not suppress the multiplication of bacteria inside the cells.

The results from the present assays, interestingly, suggest that the same fractions exhibit both reducing and preventive effects. The most probable explanation for this is that the mechanism(s) by which egg yolk and/or its fractions directly interfere with *Salmonella* spp. infection is (are) always the same without the host effect(s), regardless of the sequence of events or introductions.

The findings of this study highlighted the HDL component of the granule as the possible candidate for such functional properties. Although these were preliminary experiments, they will lead and direct future research to HDL and not IgY as the fraction responsible for the beneficial effects exhibited by egg yolk both in vivo and in vitro.

Antimicrobial Assay. To assess if a direct antimicrobial effect exists by the fraction(s) against *Salmonella* spp., these assays were performed without the Caco-2 cell monolayers. The mean

log counts of *S. enteritidis* and *S. typhimurium* in the controls, which were not mixed with the extracted yolk fractions, were 5.23 ± 0.17 and 5.59 ± 0.42 CFU/mL, respectively, after incubation. These counts were identical to the mean log counts of *Salmonella* spp. that were treated with the various fractions including the active HDL and granule (data not shown). As anticipated, in the absence of Caco-2 cells, egg yolk and/or its components do not inhibit or kill the pathogens. On the contrary, the egg yolk and its active fractions (HDL and granule) provide the favorable inhibitory effects observed by blocking the adhesion of these same pathogens and inhibiting their infection to the epithelial cell surfaces in vitro.

The induction of de novo protein synthesis necessary for invasion appears to be regulated by the epithelial cell surface. If these bacterial molecules are not induced, the bacteria neither adhere avidly nor invade. These up-regulated proteins have yet to be characterized genetically and functionally (*24*). Alterations in the epithelial cell surfaces, which may be caused by the introduction of the active egg yolk fractions from above, might hinder the protein synthesis process necessary for bacterial adherence and/or invasion.

Consequently, the decrease in the mean log *Salmonella* spp. counts observed in the present in vitro assays, most prominently from the yolk, granule, and HDL-treated bacterial-associated monolayers, may be the result of such interference. At this point, it could not be exactly determined if the yolk fractions' interference affected the bacterial adherence and/or the bacterial invasion. Further experiments are in progress to characterize the nature and the mechanism(s) of the beneficial activity exhibited by the yolk fractions identified above. Presumably, when *Salmonella* species bind to the host cell surface, they transduce an "uptake signal" to the host cell that, in turn, recruits the cytoskeletal elements to provide the endocytic functions necessary to internalize whole bacterium (*24*). Any factor disrupting this process disrupts adhesion and invasion. For instance, it appears that tyrosine protein kinases are involved in *Salmonella* internalization. Tyrphostins, which are specific inhibitors of tyrosine protein kinases, block invasion of *S. typhimurium* into Madin−Darby canine kidney (MDCK) cells (*24*).

In addition, data and information so far compiled from studies against the enteric tested pathogens allow us to deduce that egg yolk and/or its fractions may act in vitro via at least one route. This may be by directly interfering with the epithelial surface-bacterial adherence/invasion interactions. It had been stated (*13*) that *Salmonella* spp. in vitro interact with the tips of microvilli found on the apical surface of Caco-2 cells, presumably, by a pilus-like structure. They, then, locally disrupt the microvilli and enter into the cells within a membrane-bound inclusion.

The exact mechanism(s) by which *Salmonella* adhere to epithelial surfaces has (have) not been identified. Nevertheless, the several bacterial surface proteins necessary for adherence and invasion are induced by epithelial cell surfaces. Therefore, changes to these surfaces, for instance, by the introduction of egg yolk and/or its fractions, may alter the protein production and induction, hindering bacterial adherence (*25*). In a previous study (*26*), researchers reported that when the surface proteins of the Caco-2 cells were disrupted by an external fraction, a significant decline in the number of internalized bacteria occurred. Furthermore, *Salmonella* spp. invasion was also studied (*27*). The researchers demonstrated that *Salmonella* spp. activates a protein hormone receptor on the surface of tissue culture cells that may trigger actin arrangements in the plasma membrane to promote internalization.

It has also been indicated that when *Salmonella* adheres to Caco-2 cells, several fimbriae-like structures extend between the bacterium and host cell surface (*13*). On the other hand, it has been shown that dietary lipids can modify the functions of enzymes and nutrient transporters present in the mucosal membrane (*28*) leading to changes in the physiochemical properties of the membrane (*29*). Therefore, the inhibitory activity against *Salmonella* infections may involve not only direct negative effects on bacterial metabolism but also modification of the epithelial cell receptors needed for bacterial adhesion and invasion. Hence, the hen yolk fractions may have interfered with other previously cited and still undetermined mechanism(s).

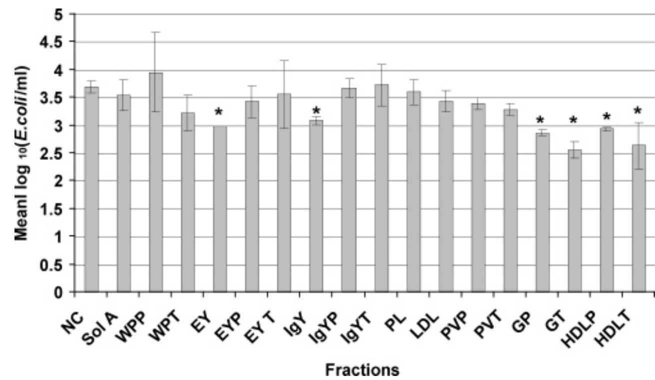

Figure 3. Eliminating effects of various yolk fractions against *E. coli* 0157:H7 in vitro. Monolayer cells were inoculated with 10^5 CFU/mL of *E. coli* 0157:H7 and then treated with the fractions. * Values of significant reduction ($P < 0.05$) in mean log *E. coli*/mL compared with the negative control. Error bars indicate the standard error of the mean of 12 trials.

***E. coli* O157:H7.** *Adhesion−Elimination Assay.* The same egg yolk components, concentrations, controls, and conditions as those used in the *Salmonella* spp. assays were also applied in the following *E. coli* O157:H7 experiments. **Figure 3** shows the protective effects of egg yolk and its different fractions against *E. coli* O157:H7 in the Caco-2 cell monolayers. With *E. coli* O157:H7 being reported as noninvasive by the known means, internalization into the Caco-2 cells was not expected at this point, especially because the incubation period was relatively short. The mean log counts of *E. coli* O157:H7 (**Figure 3**) obtained from each tested sample were compared with the negative controls. Differences in the *E. coli* O157:H7 counts were considered to be statistically significant when $P < 0.05$. The mean log counts of the organism in the control monolayer cells were 3.69 ± 0.11 CFU/mL. However, the number of bacteria in the monolayer cells infected and treated with the enzymatically digested granule and HDL was significantly lower ($P < 0.05$). The respective mean log *E. coli* O157:H7 counts from the above treated wells with both the granule and HDL−pepsin and trypsin-digested fractions were 2.86 ± 0.05/2.56 ± 0.16 and 2.94 ± 0.03/2.84 ± 0.41 CFU/mL, respectively.

Granule and HDL exhibited the highest degree of inhibition among all of the other tested fractions in the study. Evidently, these results suggest that the granule fraction and its HDL component were capable of significantly reducing the bacterial infection of epithelial cells by not only *Salmonella* spp. but also *E. coli* O157:H7. From results in these and the previous assays, both undigested nonimmunized egg yolk and IgY showed similar significant inhibitory effects, but the IgY activity was completely lost after the enzymatic digestion. This indicates that any inhibitory activity which IgY may possess against this particular pathogen could not overcome the gastrointestinal tract conditions if the egg yolk was orally introduced in the diet as a supplement.

The above IgY-related observations are in agreement with those of other studies (*30*). They have demonstrated that IgY was found to be significantly sensitive to heat and acidic conditions. In addition, they stated that there was a rapid inactivation of IgY at low pH values (pH <4) and that the activity was fairly sensitive to pepsin, especially at pH values of <4.5 (*30*). Consequently, a significant loss in antigen-binding activity was detected after digestion with pepsin at pH <4.5.

The inhibitory activity of nonimmunized egg yolk against *E. coli* O157:H7, unlike that against *Salmonella* spp., significantly decreased after the enzymatic digestion. This result was evident when the mean log counts of *E. coli* O157:H7 were compared with those of the controls and the undigested egg yolk fraction, granule, and HDL. Several interpretations can be inferred from such a result. The previous findings obtained from the *Salmonella* spp. assays showed that egg yolk whether digested or not retained its protective activity against *Salmonella* spp. However, the same inhibitory activity against *E. coli* O157:H7 was significantly diminished after the digestion of the supplement. Therefore, this can be attributed to several factors. First, the adhesion/infection mechanism(s) and the targeted sites are different between the two organisms. Second, the sites on the epithelial cell surfaces, which may be targeted by egg yolk and/or its fractions, may be different for each pathogen. In addition, the bacterial−epithelial cell interaction processes that might be altered or affected by egg yolk and/or its fractions are different for different organisms.

Nevertheless, the essence of the observed diversities may lie within the egg yolk fractions themselves. Different pathogens may be susceptible to different microcomponents within the same active yolk fraction. Consequently, these microcomponents may interfere differently with different bacterial cell surface attachment processes, which may result in varying responses. The HDL fraction of the granule consistently appeared to be the component contributing most to the inhibitory activity observed against all of the investigated organisms. Therefore, no doubt, the activity, later to be further confirmed antiadhesive, lies in this particular egg yolk fraction. Moreover, **Figure 3** shows that none of the other tested fractions, not even phosvitin, were able to reduce the *E. coli* O157:H7 adhesion to the cell monolayers in the same magnitude as the granule fraction and its HDL component did.

Adhesion−Prevention Assay. The preventive effect of egg yolk and its fractions against *E. coli* O157:H7 is shown in **Figure 4**. The mean log counts of *E. coli* O157:H7 in the control monolayers were 3.39 ± 0.84 CFU/mL after the unbound bacteria were washed and the bacteria-associated monolayers were released. Persistently, the same analyzed fractions that demonstrated an inhibitory activity against *E. coli* O157:H7 and *Salmonella* spp. in the previous assays appeared to possess an equal preventive activity against *E. coli* O157:H7 in these experiments. However, the one difference was detected in the activity of the phosvitin fraction. The bacterial counts in the monolayers, which were treated with the pepsin-digested phosvitin fraction then infected, were shown to be statistically ($P < 0.05$) lower than that of the negative control.

A similar activity, however, was not detected with the tryspin-digested fraction. Nevertheless, neither the pepsin nor the trypsin-digested forms of phosvitin revealed any bacterial reducing activity against *E. coli* O157:H7 in the previous adhesion−elimination assays. The reason(s) and/or the nature of the difference cannot be understood at this point, but these results may suggest that the sequence of events can affect both the bacterial attachment and the ability of the fraction(s) to interfere with the process. Consequently, these results also show the importance of conducting both assay procedures to present all of the aspects of the activity that may be exhibited. Despite the above observations, the phosvitin effect was not as significant as that encountered by the HDL or the granule fraction itself.

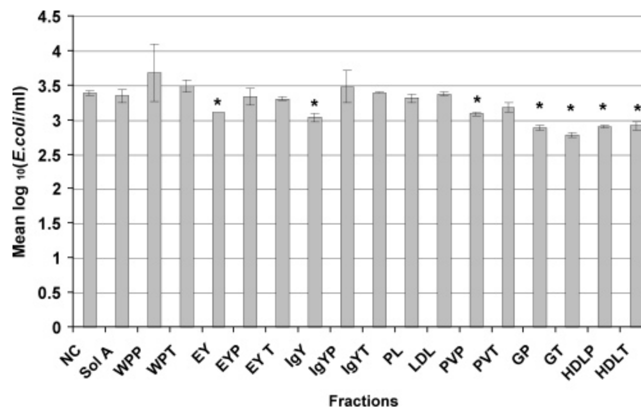

Figure 4. Preventive effects of various yolk fractions against *E. coli* 0157:H7 in vitro. Monolayer cells were treated with the fractions and then inoculated with 10^5 CFU/mL of. *E. coli* 0157:H7. * Values of significant reduction ($P < 0.05$) in mean log *Salmonella* spp./mL compared with the negative control. Error bars indicate the standard error of the mean of 12 trials.

Once again, the granule fraction of egg yolk and its HDL component, in particular, showed the highest preventive activity against *E. coli* O157:H7 adhesion. They appear to be the egg yolk components most likely interfering with bacterial infection in vitro. The activity was persistent regardless of the bacterial species and/or their relative varying infection mechanism(s). A synergistic effect between the two granule components HDL and phosvitin may exist, but it was not clearly reflected in the mean log counts of *E. coli* O157:H7. Even though the bacterial counts in the granule-treated monolayers seemed to be lower than that in either the HDL or phosvitin, the difference was not significant ($P < 0.05$) enough to suggest that such a synergistic effect may, indeed, exist between the two granule components. In addition, the variations observed in the activity of phosvitin between the two different assays (adhesion−elimination versus adhesion−prevention) also suggest that alterations in the sequence of events may result in varying consequences. Therefore, the implementation of preventive measures against bacterial infection is as important as the enforcement of eliminating or reducing means.

In the present in vitro assays, egg yolk and IgY, in their nondigested forms, also appeared to deter the bacterial adhesion significantly ($P < 0.05$) when the mean log bacterial counts were compared with that of the negative controls. However, the degree of inhibition was not as considerable as the one resulting from the recognized active fractions, HDL and granule. Consequently, the protective effects were intangible with the enzymatically digested egg yolk and IgY fractions.

Antimicrobial Assay. This activity of the HDL component needed to be further characterized and confirmed as antiadhesive rather than antimicrobial. The mean log bacterial counts in the negative and solution A controls, which were not mixed with the fractions, were 4.23 ± 0.17 and 4.69 ± 0.65 CFU/mL, respectively, after the incubation period. As anticipated, in the absence of Caco-2 cells, egg yolk and/or its fractions do not convey any inhibitory activity against *E. coli* O157:H7 (data not shown). On the basis of these and previous results, a stronger conclusion can be drawn about the nature of the activity inflicted by the HDL and the granule fractions of hen egg yolk. The favorable effects are associated with blocking the adhesion of these same pathogens and inhibiting their infection to the

epithelial cell surfaces in vitro. The egg yolk fractions, HDL and granule, do not seem to possess a functional antimicrobial (bacteriostatic and/or bactericidal) activity against, at least, those pathogens investigated in the present work.

The desirable effects conveyed by egg yolk and notably by its HDL fraction appear to be more considerable against *Salmonella* spp. than against *E. coli* O157:H7. This, at this point, can be explained only on the basis of differences in the infection mechanism(s) by the pathogens, particularly, in the targeted sites for adhesion and/or invasion.

Against noninvasive bacteria such as *E. coli* O157:H7, regardless of the inhibitory mechanism(s) involved, the main protective effect lies in the inhibition of the bacterial adherence to intestinal epithelial cells (*31*). Egg yolk fractions in vitro may have inhibited interbacterial cell associations, which are responsible for the localized adherence phenotype (*32*), without affecting significantly the bacterial cell−epithelial cell interactions. This inhibition of localized adherence may explain why the number of *E. coli* O157:H7 microcolonies was decreased, but the organism could not be abolished after the incubation period. *E. coli* O157:H7 does not produce type-I fimbriae or curli fimbriae (*33*, *34*). However, the presence of other putative adhesins (*35*) may play a role in the adherence of *E. coli* O157:H7 to the Caco-2 cells. The first step in infection is, hence, adherence and growth of *E. coli* O157:H7 on the surface of the enteric epithelium.

From the assays in the present study, novel functional properties for hen egg yolk granule and its HDL components were identified for the first time. These fractions were never before targeted for such purposes, and to the best of our knowledge, this is the first report of the preparation of egg yolk fractions for the specific use in studies against foodborne pathogen infection.

Significant ($P < 0.05$) reduction and prevention in adhesion were equally observed against *Salmonella* spp. and *E. coli* O157:H7 when HDL or granule was added. Indeed, among the various fractions tested, the best inhibitors of localized adhesion were the granule and its fraction, HDL. However, the exact mechanism(s) should be identified in the future. The HDL lipoproteins are quite unlike the more familiar lipoproteins. They represent a unique example of protein−lipid interactions, in which the phospholipids are well hidden inside the protein structure where they are not accessible to enzymes (*36*) unless the structure has been disrupted. Therefore, the enzymatic digestion of the protein structure might have exposed the hidden phospholipids that could have contributed to the enhanced activity of HDL observed throughout the study. The HDL from hen egg yolk contains two apoproteins that enclose protein-bound carbohydrates of 0.75% polysaccharides (*37*). HDL also contains mannose, galactose, and glucosamine. Protein−carbohydrate interactions appear to play a critical role in the adherence of pathogens to epithelial surfaces (*38*).

Furthermore, the relatively acidic nature of HDL is due to the predominance of sialic acid (*39*). Moreover, several sialyloligosaccharides are contained in the egg yolk. Sialylconjugates such as gangliosides, sialyloligosaccharides, and sialylglycoproteins have been reported to play important roles in animal and human tissue cells. For instance, they act as receptors of several microorganisms, the most studied of which are the influenza virus (*40*, *41*) and rotavirus (*42*). Sugita-Konishi et al. (*43*) also suggested that egg yolk plasma derived sialyloligosaccharides (YDS) and their derivatives are useful in preventing *Salmonellae* infection when ingested continuously.

Egg yolk is recognized as an excellent emulsifying agent. However, egg yolk may have novel functions beyond the known agglutination and neutralization of toxins. The new functions may include the interference in the metabolic activity of infected cells and the neutralization of bacterial enzymes. There is still uncertainty about whether the proteins and lipoproteins are independent of each other in intact yolk or are present in complexes. HDL, a lipoprotein with higher protein content than lipid, showed a significant antiadhesive activity against the enteric pathogens, whereas LDL, a lipoprotein with higher lipid content, did not.

However, the antiadhesive activity cannot be attributed to the proteins of the yolk as such. Phosvitin and IgY, the proteins in the granule and plasma, respectively, did not show a protective activity of the same magnitude as that enforced by HDL or granule, which are protein−lipid complexes. Therefore, the activity must be the result of an association between the proteins and the lipids but enhanced by a higher protein content rather than that of the lipid. An antibacterial activity of fractionated hen egg yolk (LDL) from nonimmunized egg yolk against two pathogenic *Streptococcus* strains in vitro had been earlier reported (*44*). However, in the present study the activity by the various egg yolk fractions against the specific enteric pathogens in question was only granules or HDL fraction.

In conclusion, the present work confirms that the granule fraction and, in particular, the HDL component most likely are responsible for the anti-infectious effects observed against the enteric pathogens investigated. Furthermore, by using a cultured intestinal epithelial cell layer, the nature of the activity encountered against *Salmonella* spp. and *E. coli* O157:H7 was emphasized as antiadhesive and not antimicrobial as has been believed. It is imperative to note that these in vitro experiments, although preliminary, revealed significant and novel results, which would lead the way for more fundamental future work.

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