QTL detected in various resource populations and comparative sequence analysis to identify functional polymorphisms.

Key Words: Swine, Growth, QTL

W268 Mapping and genetic variation within porcine 70 kiloDalton heat shock protein 2 (HSPA2). M. Grosz*[1] and G. Rohrer[2], [1]*Monsanto Company, West Chesterfield, MO,* [2]*USDA-ARS US Meat Animal Research Center, Clay Center, NC.*

70 kiloDalton heat shock protein 2 (HSPA2) is expressed in the testis during the meiotic phase of spermatogenesis in humans and mice. Mutations in HSPA2 have been shown to cause sterility in male mice, while not affecting female reproductive capacity. Based on these comparative observations, HSPA2 is a candidate gene for influencing porcine male reproductive phenotypes. To initiate research assessing this possible relationship, the human HSPA2 sequence was used to identify a putative porcine HSPA2 sequence from public EST databases. This EST was used to isolate a Bacterial Artificial Chromosome (BAC) clone containing the porcine homolog of HSPA2. BAC DNA was subcloned, and the porcine HSPA2 locus was defined by sequence assembly and analysis. Extant genetic variation was identified by amplification and re-sequencing of HSPA2 (and flanking regions) with a series of overlapping primer pairs. In total, 11 Single Nucleotide Polymorphisms (SNPs) and 1 single base insertion/deletion were identified in the region spanning from 1062 bases upstream of the start codon to 672 bases downstream of the stop codon. None of the identified SNPs alter the amino acid sequence of the peptide. Two SNPs were converted into PCR-RFLP assays and used to genetically map the HSPA2 locus to chromosome 7, 87 cM, consistent with the syntenic relationship between Hsa14 and Ssc7. Future research can now be directed toward detecting associations between genetic variation and swine reproductive performance and other phenotypes.

Key Words: Swine, HSPA2, SNP

Food Safety

W269 Correlation of genomic changes with morphological dimorphism of *Campylobacter jejuni*. H. Wang*[1] and M. Slavik[1], [1]*POSC University of Arkansas, Fayetteville.*

Campylobacter jejuni is one of the leading causes of human bacterial gastroenteritis. Previous research has shown that variation in pathogenicity of *C. jejuni* may be associated with its polymorphism. The spiral form of *C. jejuni* has been shown to be more highly pathogenic than the coccoid cells of the same strain. The objective of this research was to investigate the possibility of genomic changes associated with the polymorphism in *C. jejuni* using pulsed-field gel electrophoresis (PFGE) and DNA sequencing analysis. *Campylobacter jejuni* isolated from pre-chill, post-chill, and retail chicken carcasses and human stool samples of enteritis patients were cultured on Campylobacter enrichment agar for 18 hours (spiral form) and 72 hours (coccoid form) under microaerobic conditions at 42 °C. All isolates were confirmed as *C. jejuni* positive by using polymerase chain reaction (PCR). The isolates then were embedded in agarose plugs and the DNA was analyzed by PFGE (CHEF-DR III system) after digestion using either *Sma* I and *Sac* II restriction endonucleases. After an ethidium bromide staining, the DNA patterns were analyzed using the molecular analyst fingerprinting software (BIO-RAD). The molecular fingerprint of each isolate in the spiral form was compared to the fingerprint of the same isolate in the coccoid form. No genomic variation in the overall restriction patterns associated with polymorphism was observed in the strains tested. Both forms of *C. jejuni* PFGE profiles showed 100% genetic similarity following *Sma* I or *Sac* II digestion. At same time, PCR products of *fla*A gene from the isolated *C. jejuni*were purified by using Wizard PCR Preps DNA Purification System (Promega Corporation. Madison, WI). The purified DNA samples were tested for DNA sequencing analysis and the DNA sequences of each isolate in both forms were computer analyzed by using SeqMan (DNAstar inc., Madison, WI). For two forms of same isolate, the nucleic acid sequences of *fla*A gene showed 90-100% similarity. It is concluded that the morphological dimorphism of *C. jejuni* is not associated with genetic modification.

Key Words: C. Jejuni, PFGE, DNA Sequence

W270 Risk assessment for antibiotic resistance in foodborne pathogens isolated from poultry products. N. Kotrola and R. Roy*, *Auburn University, AL.*

Our initial goal of this study was to determine the prevalence of antibiotic resistant foodborne pathogens in poultry product samples at the retail level. 160 samples of chilled raw poultry meat (thighs, drumsticks, and breasts) and fully cooked turkey hot dogs were sampled from selected stores. One strain from each pathogen-positive sample was selected for susceptibility testing with the E-test method (AB Biodisk North America, Inc.). The E-test was performed for ciprofloxacin, tetracycline, and erythromycin according to the manufacture's instructions. Inocula were prepared by incubating the campylobacter strains for 24 h at 42°C under microaerobic conditions in brucella broth or incubating for 24 h at 37°C in BHI broth for Listeria monocytogenes, Salmonella and E.coli strains. After application of the E-test strips, campylobacter plates were incubated in microaerobic conditions at 42°C for 24 h or 37 C for all other strains. The minimal inhibition concentration (MIC) was read directly from the test strip at the point where the elliptical zone of inhibition intersected the MIC scale on the strip. Our preliminary results indicated that the overall prevalence of tetracycline, ciprofloxacin, and erythromycin resistance in Campylobacter strains was 65.52%, 7.69%, and 7.41% respectively. The overall prevalence of tetracycline, ciprofloxacin, and erythromycin resistance in Listeria monocytogenes strains was 100%, 0%, and 50% respectively. The overall prevalence of tetracycline, ciprofloxacin, and erythromycin resistance in Salmonella strains was 100%, 0%, and 100% respectively. And finally, the overall prevalence of tetracycline, ciprofloxacin, and erythromycin resistance in E. coli strains was 56.25%, 0%, and 100% respectively. These initial results confirm the notion of multiresistant strains in Campylobacter, Listeria monocytogenes, Salmonella and E. coli.

Key Words: Antibiotic Resistance, Foodborne Pathogens, Poultry Products

W271 Risk Assessment of stress factors and Listeria monocytogenes Biofilm formation. B. Dean*, P. Mohyla, R. Roy, and N. Kotrola, *Auburn University, AL.*

Differential adherence capabilities and reaction to sanitizers for biofilm removal among tetracycline resistant, quaternary ammonium compound (quat), and sodium hypochlorite (bleach) stressed, acid and alkali adapted, and non- stressed Listeria monocytogenes (LM) were tested by the microtiter bio- screening assay. Cell turbidity and biofilm formation were assessed using a microtiter plate reader at the wavelength of 630 nm. The quantitative analysis of biofilm production was performed by adding 200 ul of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate and optical densities (OD) of the crystal violet present in the destaining solution was evaluated. Viable counts were also assessed to determine the efficacy of several sanitizers for the removal of LM biofilm. Results showed significant differences in both biofilm formation and cell turbidity at 630 nm between stressed and non-stressed LM isolates ($p<0.001$). At 630nm, bleach stressed and tetracycline resistant LM formed stronger biofilm compared to the unstressed LM with OD of -2.75 x 10-3, -5.70X10-3 and 3.31x10-3, respectively. There were significant difference in cell turbidity at a 630 nm, between the stressed cells compared to the positive control ($p<0.05$), but there were no difference among the cells subjected to the various stressors. Results of the viable cell counts of the stressed LM in biofilm were significantly higher ($p<0.05$); than the positive controls (average means of 1.8 x 107 +/- 1.6 and 4.5 x 104 +/- 1.16, respectively) when treated with sanitizers. In conclusion, stressed LM cells in biofilm appear to exhibit higher tolerance to sanitizer treatment when compared to unstressed LM cells and this tolerance may influence the efficacy of the sanitizer for biofilm removal.

Key Words: Biofilm, L. Monocytogenes, Stressed Cells

W272 Fumonisin B1 absorption study in Ussing Chamber model reveals a possibile active transport. G. Casadei[1], F. Galvano[2], N. Chiulli[1], G. Biagi[1], and A. Piva*[1], [1]*University of Bologna, Italy,* [2]*University of Reggio Calabria, Italy.*

Fumonisins are mainly produced by *Fusarium verticillioides*, *F. moniliforme* and *F. proliferatum* that grow on cereals. As their pathogenic activity depends on bio-availability, fumonisin B1 (FB1) absorption was studied *in vitro* with Ussing chamber technique. Male rats (n=9), *Rattus norvegicus* Wistar strain, weighing 170-230g underwent laparotomy under ether anesthesia. Jejunum, starting 10cm from the ligament of Treitz was removed, cut into 2.5cm segments, and immediately immersed in a modified Krebs-Ringer buffer, oxygenated with 95% O2 and 5% CO2 at room temperature. Each piece was cut along the mesenteric border and mounted in Ussing chambers. The experiments started (t=0), within 30 min after the induction of anesthesia, when the buffer in the mucosal reservoirs was exchanged with 5ml buffer containing FB1 at 30ppm. At 0, 20, 40, 60, 80, 100 and 120 minutes, a 1ml sample was withdrawn from serosal reservoir for FB1 analysis, and replaced with fresh media. Of the 150mg FB1 dissolved in the 5ml mucosal solution, serosal recovery at sampling times was: 7.6%, 12.1%, 18.1%, 25.2%, 30.2%, 36.0% respectively. The apparent permeability coefficient Papp [cm/sec*10^{-6}]=dc/dt*V/(A*C0), where dc/dt is the change in FB1 concentration on the mucosal side per unit time, V is the volume of the chamber ($5cm^3$), A the area of exposed intestine ($1.78cm^2$), and Co is the initial FB1 concentration in the mucosal reservoir (30ppm), was estimated to be as high as $134.3\pm3.7cm/sec*10^{-6}$. Regression of FB1 concentration over time revealed a linear correlation represented by the equation: y=0.412x+0.816; r^2=0.9807, where y=FB1 total absorbed μg and x=minutes. According to this model, FB1 absorption in rat small intestine averages $0.232\pm0.007\mu g/min$ per cm^2 of exposed mucosa. The linearity of FB1 absorption rate seems to obey an apparent zero-order kinetic involving a carrier-mediated transport. Other studies are required to better describe intestinal FB1 uptake, so far considered to be very low.

Key Words: Ussing Chambers, Fumonisin B1, Rat

W273 Effects of fumonisin B1 on pathological and immunological parameters in pigs consuming diets with or without the addition of activated charcoal. A. Piva*[1], D. E. Diaz[4], G. Casadei[1], E. Cabassi[2], and G. Piva*[3], [1]*University of Bologna, Italy,* [2]*University of Parma, Italy,* [3]*Catholic Univeristy of the Sacred Heart, Italy,* [4]*Fondazione Parco Tecnologico Padano, Italy* .

Fumonisins are mycotoxins found primarily in cereals and that are produced primarily by *Fusarium verticillioides*, *Fusarium moniliforme* and *F. proliferatum*. The effects of fumonisin B1 (FB1) on weaned piglets were evaluated. Fifty-six male weanling pigs (mean initial weight of 6.9kg) were randomly assigned to pens and one of four treatments diets according to their initial weight. The treatment diets were: 1) Corn-soybean basal diet with < 2ppm FB1 (Control) 2) BD + Activated Carbon at 1% of the diet DM (AC), 3) Control+FB1 (FU) (30ppm FB1), and 4) Control+AC+FU (AC+FU). A total of 4 replicates of 3 pigs/pen for treatments 3, and 4 and 4 piglets/pen for treatments 1, and 2 were utilized. Tissue and blood samples were collected at the end of the 42-days experimental period. Pigs fed FB1 contaminated diets showed distinct lesions in lungs, heart, pancreas, spleen, small intestine and liver and had marked pulmonary edema. Animals fed the AC+FU diet had the most severe lesions in these organs and the lowest CD14 (2.61 vs 14.09; P<0.01), CD8 (4.48 vs 27.68; P<0.01) CD4 (2.29 vs 16.22; P<0.01) values compared to control. Animals fed only FB1 at 30ppm showed a reduced functionality only relative to CD14 compared (6.32 vs 14.09; P<0.01). CD4 to CD8 ratio was never influenced by the addition of FB1 (30ppm) with or without activated carbon. The consumption of diets contaminated with 30ppm fumonisin B1, from cultured material, significantly affected organ pathology and macrophage functions in post-weaned piglets. The addition of an activated carbon added at 1% of the diet was not effective in protecting, against the detrimental effects of fumonisin consumption.

Key Words: Fumonisin B1, Swine, Activated Carbon

W274 Comparison of rectoanal mucosal swabs (RAMS) and fecal culture for determining prevalence of *Escherichia coli* O157 in feedlot cattle. M. A. Greenquist*[1], J. S. Drouillard[1], T. G. Nagaraja[2], J. M. Sargeant[2], B. E. Depenbusch[1], X. Shi[2], and K. F. Lechtenberg[3], [1]*Department of Animal Sciences and Industry, Kansas State University, Manhattan,* [2]*Department of Diagnostic Medicine and Pathobiology , Kansas State University, Manhattan,* [3]*Midwest Veterinary Services, Inc, Oakland, NE.*

The lymphoid tissue in the rectoanal junction (RAJ) of the gastrointestinal tract has been suggested as the principal site of colonization of *E. coli* O157 in cattle. Samples collected by swabbing the rectoanal mucosa have been shown to be superior to fecal grab samples for detection of *E. coli* O157 in a study involving experimentally inoculated cattle and in a small number of dairy heifers. Our objective was to compare the utility of the two sampling techniques for determining prevalence of *E. coli* O157 in feedlot cattle (n = 747) fed high-grain diets. Isolation procedures included enrichment of RAMS or fecal samples in Gram negative broth with cefixime, cefsulodin, and vancomycin, followed by immunomagnetic bead separation and plating on sorbitol MacConkey agar with cefixime and potassium tellurite. Sorbitol-negative colonies were identified as *E. coli* O157 based on indole production, positive latex agglutination for O157 antigen and API 20E test strip results. Of the 82 animals that tested positive for *E. coli* O157, 87% were detected by the RAMS method, but only 45% by the fecal culture method. Genomic fingerprints of isolates were analyzed by pulse-field electrophoresis (PFGE) to compare clonal identity between RAMS and fecal isolates from the same animal. Of the 24 pairs of isolates evaluated, 20 had 100% similarity and 4 had >95% similarity in PFGE banding patterns, suggesting that strains colonizing the RAJ are the same as those isolated from feces. RAMS culture appears to be more sensitive than the traditional fecal culture method for determining prevalence of *E. coli* O157 in feedlot cattle.

Number of positive samples (% in parenthesis)

	RAMS	Fecal	RAMS of Fecal	RAMS and Fecal
Cattle, n=747	71 (9.5)	35 (4.7)	82 (11)	24 (3.2)

Chi Square test for RAMS vs. Fecal culture P < 0.01

Key Words: Rectoanal Mucosal Swabs, *E. coli* O157, Feedlot Cattle

W275 Effects of nitrate adaptation and chlorate supplementation on fecal *escherichia coli* conentrations in Holstein steers. J. T. Fox*[1], R. C. Anderson[2], G. E. Carstens[1], R. K. Miller[1], and D. J. Nisbet[2], [1]*Texas A&M University, College Station,* [2]*USDA-ARS, FFSRU, College Station, TX.*

In studies with broilers and pigs, bactericidal activity of an experimental chlorate product (ECP) against enteropathogens was enhanced by nitrate adaptation. Objectives of this study were to examine effects of nitrate adaptation on bactericidal activity of ECP against *E. coli* in cattle. Holstein steers (BW = 210 ± 5.1 kg) were trained to use Calan feeders and assigned to one of six treatments (n = 8) in a 2 x 3 factorial arrangement that included three ECP doses (0, 1.2 or 2.3 g chlorate ion equivalence/kg diet), with or without nitrate adaptation (0.95 g NO_3 ion equivalence/kg diet). The nitrate product (with 0 or 11% NO_3 ion equivalence) was added to the experimental diet (66, 15 and 19% corn, alfalfa hay and supplement) for 3 d immediately preceding administration of ECP (with 0, 12.5 or 25% chlorate ion equivalence), which was added one d prior to termination of the study. Fecal samples were collected prior to nitrate administration and one d following ECP administration, and cultured quantitatively for generic *E. coli*. Supplementation with nitrate and(or) ECP did not affect ADG, DMI or feed:gain during the 28-d study, which averaged 1.3 ± 0.1 kg/d, 6.4 ± 0.3 kg/d and 5.2 ± 0.3, respectively. ECP did not affect DMI during the last d of the study. Initial fecal *E. coli* concentrations were not different among treatments and averaged 5.4 ± 0.2 $\log_{10}$ CFU/g. Final fecal *E. coli* concentrations were not affected by nitrate adaptation (P = .26) or nitrate x chlorate interaction (P = .20). ECP supplementation reduced (P < .001) final fecal *E. coli* concentrations (5.2, 2.7 and 2.6 ± 0.3 $\log_{10}$ CFU/g for 0, 1.2 and 2.3 g chlorate ion equivalence/kg, respectively), but not in a dose-dependent manner. ECP supplementation effectively reduced fecal *E. coli* by 48%, but in contrast to previous results with

broilers and pigs, nitrate adaptation did not further enhance the bactericidal effects of ECP. Rapid reduction of ruminal nitrate may account for specie differences. Discovery of nitro-compounds that are more resistant to ruminal degradation may enhance the efficacy of ECP against enteropathogens in cattle.

Key Words: *E. coli*, Chlorate, Cattle

W276 Effect of caffeine on inactivation of *Escherichia coli* O157:H7 in laboratory media. S. A. Ibrahim*, *North Carolina A&T State University, Raleigh*.

Escherichia coli O157:H7, a leading cause of bacterial food borne disease outbreaks, is responsible for approximately 73,500 cases of foodborne illness per year. Recent research has shown that caffeine has the ability to inhibit DNA repair in bacteria and therefore could be mutagenic compound. The objective of this research was to determine the effectiveness of caffeine on inactivation of (*E. coli* O157:H7 in Brain Heart Infusion (BHI) broth. Overnight samples of six *E. coli* O157:H7 strains (E 1730, E 4546, E 0019, Cider, 380 and 944) were used in this study. These strains were inoculated individually at an initial inoculum level of 2 log CFU/ml into BHI broth containing caffeine with different concentrations of 0.0, 0.25, 0.5, 0.75, 1.00, 1.25, 1.50, 1.75, and 2.00%. Samples were then incubated at 37 C for 24hrs. Samples were withdrawn at different time intervals to determine turbidity using spectrophotometer at 575nm. Results revealed that the addition of caffeine inhibited the growth of *E. coli*. Significant growth inhibition was observed with concentration levels 0.50% and higher. These results indicate that caffeine has potential as an antimicrobial agent and should be investigated further as a food additive to increase the biosafety of consumable food products.

Key Words: Caffeine, *Escherichia coli* O157:H7

W277 Using origanox in combination with sodium lactate and sodium acetate to inhibit the growth of *Escherichia coli* O157:H7. S. A. Ibrahim* and S. R. K. Dharmavaram, *North Carolina A&T State University, Raleigh*.

Escherichia coli O157:H7, a leading cause of bacterial food borne disease outbreaks, is responsible for approximately 73,500 cases of foodborne illness per year. Origanox, a commercial spice, has been shown to have antimicrobial properties. It is believed that the effectiveness of origanox can be enhanced by the use of organic acids. The objective of this research was to determine the effectiveness of origanox alone and in combination with chemical preservatives; sodium acetate and sodium lactate on inactivation of *E. coli* O157:H7 in Brain Heart Infusion (BHI) broth. Overnight samples of five *E. coli* O157:H7 strains (E 1730, E 4546, E 0019, Cider and 944) and a mixture of the five strains were added to BHI broth at an initial inoculum level of 2 log CFU/ml. Several combinations of sodium lactate (0,1, and 2 % w/v), sodium acetate (0 and 1 % w/v), and origanox (0, 0.05 and 0.1 % w/v) were used as treatments. The samples were stored at 37 °C for 12 hrs and population changes of *E. coli* O157:H7 were followed using optical density (O.D. 610 nm) measurements and CFU techniques every two hours. Our results indicated that origanox was effective in controlling the growth of *E. coli* O157:H7 at concentration of 0.1 % in BHI broth. Sodium lactate alone was found to be effective at 3% concentration. Sodium lactate at 1-2% in combination with 0.05% origanox or sodium acetate at 1% in combination with 0.05% origanox was found to be the most effective in controlling the growth of *E. coli* O157:H7, $>$ 4 log reduction. Treatments containing a combination of 1% sodium lactate, 1% sodium acetate and 0.05% origanox showed significant reduction of *E. coli* O157:H7, $>$ 5 log reductions. Use of origanox at 0.05% could reduce the usage of chemical preservatives such as sodium lactate and sodium acetate to inhibit the growth of *E. coli* O157:H7.

Key Words: Origanox

W278 Selection of anti-bacterial peptides against *E. coli*O157:H7 and UTI from f88-4/15 library. C. J. Fu*, F. J. Schmidt, S. A. Mounter, and M. S. Kerley, *University of Missouri-Columbia*.

Phage display technology was used to select anti-bacterial peptides against pathogenic *E. coli* O157:H7 (isolates PA 1 and PA 2 from human clinical case and ground beef, respectively) and UTI (isolate PA 3 from a urinary tract infection case). After 4 rounds of affinity selection, 40 phage clones (PC 1 to 120) bearing colonies selected against each pathogen were examined. The purified phage clones were used to test their function of inhibition/killing the pathogenic *E. coli*. DNA sequencing indicated that only 2 phage sequences were repeated in 16 colonies from the PA 1 and PA 2 selection. A single clone dominated the PA 3 selection (12/16). No similar peptide sequences were found from published databases by BLAST search. Several PC (PC 5, 16, 41, 42, 46, 84, 94, and 95) inhibited or killed the pathogens (40 to 85% within 2 hours). Phage clones selected against either PA 1 or PA 2 inhibited both strains but not PA 3. However PCs selected against PA 3 inhibited PA 1 and PA 2.

Key Words: Pathogenic *E. coli*, Peptide, Phage Display

Dairy Foods: Microbiology

W279 Lactic acid fermentation by *Lactobacillus reuteri* in laboratory medium supplemented with various nutrients. S. Phetsomphou* and S. A. Ibrahim, *North Carolina A&T State University, Raleigh*.

Lactic acid is a product that has numerous applications in the chemical, pharmaceutical, and food industries. Lactic acid bacteria have been used widely for the production of lactic acid. However, certain nutrients are needed for the maximum production of lactic acid. Therefore, objective of this research was to investigate the effect of nutrient supplements and carbohydrate substrates on lactic acid production using free and calcium alginate immobilized *Lactobacillus reuteri*. *L. reuteri* MM 2-3 in a free cell form and calcium alginate beads (immobilized) was used to determine lactic acid production in laboratory medium supplemented with different nutrients: yeast extract, beef extract, tryptone peptone, and proteose peptone at 0, 10 and 20% concentrations or carbohydrate substrates: maltose, lactose, glucose, sorbitol and sucrose at 10% concentration. Fermentation experiments were conducted in 500 ml flasks with 300 ml final volume at 37 °C for 24 hrs. At different time intervals (2 hrs), samples were withdrawn, and analyzed for pH values and lactic acid concentrations. Fermentations of immobilized *L. reuteri* in samples containing yeast extract, phytone and proteose peptone at 20% produced the highest concentrations of lactic acid after 24 hrs with pH measurements (3.20, 3.41, and 3.61, respectively) as compared to the control (4.70). Lactic acid concentration ranged between 9.00 and 12.50. Regarding carbohydrate substrate (sugar) fermentation, maltose produced the lowest pH (3.32) followed by glucose, lactose and sorbitol (3.47, 4.00, and 6.11 respectively). Results indicated that immobilized cells of *L. reuteri* MM2-3 produced higher lactic acid and in a shorter time when compared to free cells. The results show that immobilized *L. reuteri* could be used for the production of high lactic acid concentrations in a laboratory media supplemented with both yeast extract and maltose.

Key Words: *L. reuteri*, Lactic Acid

W280 Influence of an *Arthrospira (Spirulina) platensis* biomass on acid production of lactococci. N. Molnár, L. Varga*, J. Szigeti, and B. Gyenis, *Institute of Food Science, Faculty of Agricultural and Food Sciences, University of West Hungary, Mosongyarovar, Hungary*.

Arthrospira (Spirulina) platensis is a planktonic cyanobacterium belonging to prokaryotic algae. Its dried biomass typically contains 3% to 7% moisture, 55% to 60% protein, 6% to 8% lipids, 12% to 20% carbohydrate, 7% to 10% ash, 8% to 10% fiber, 1% to 1.5% chlorophyll *a*, and a wide range of vitamins. *A. platensis* has recently been marketed and consumed as a safe human food and has been approved for human nutrition by many governments, health agencies, and associations of some 80 countries, including the United States. The effect of