Survival of *Salmonella typhimurium* in Simulated Intestinal Fluids

by

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ABSTRACT

Salmonella species are among the major foodborne intestinal pathogens that are of public concern with respect to food safety. The ability of intestinal pathogens to resist gastric acidity corresponds to their oral infective dose (ID). The survival and lipopolysaccharide (LPS) profiles of *Salmonella typhimurium* grown at different pH values and to different phases of growth were examined in simulated gastric fluid (pH 1.5), ileal fluid (pH 7.0), colon fluid (pH 8.0). The survival and growth of *S. typhimurium* were also examined during sequential passage through all three fluids. Viable cells were rapidly reduced from 10^6 $CFU.ml^{-1}$ to <10 $CFU.ml^{-1}$ within 4 min in gastric fluid. Cells inoculated directly into ileal and colon fluids survived and multiplied extensively. When low numbers of viable cells of *Salmonella* in contact with gastric fluid (0.5 min of contact) were transferred sequentially to ileal and colon fluids, only the early and late stationary phase cells were capable of recovery and growth to high numbers. The harsh environment of the gastric fluid did not change the LPS profiles of the inoculated *Salmonella* cells. Entrapment of *S. typhimurium* in calcium alginate beads and chocolate increased its survival in gastric fluid. This implies that *Salmonella* cells are protected from killing when ingested with food. These results may explain why *Salmonella* species have a very low ID when consumed as part of some contaminated food sources.

RÉSUMÉ

Les espèces de *Salmonella* sont parmi les plus importants pathogènes intestinaux pouvant causer la contamination alimentaire. L'habileté des pathogènes intestinaux à résister l'acidité de l'estomac est relié à leur dose infective (DI). La croissance de *Salmonella typhimurium* prit place en différent pH, puis la survie de l'organisme, ainsi que l'expression de lipopolysaccharides (LPS) furent examinés lorsque différentes phases de croissance furent exposées à des fluides gastro-intestinales synthétiques, soit le fluide gastrique (pH 1.5), le fluide de l'iléon (pH 7.0) et le fluide du colon (pH 8.0). La survie et la multiplication de *Salmonella typhimurium* furent aussi examinés à la suite de passages séquentiels d'un fluide à l'autre. Le nombre de cellules viables fut réduit rapidement de 10^6 CFU·ml^{-1} à < 10 CFU·ml^{-1} en 4 min d'exposition au fluide gastrique. Les cellules directement transférées au fluides de l'iléon et du colon survécurent et se sont multipliées de manière extensive. Après 0.5 min d'exposition au fluide gastrique, un petit nombre de survivants furent transférés au fluide de l'iléon et du colon de façon consécutive. Seules les cellules du début et de la fin de la phase stationaire furent en mesure de recouvrir et de croître en grand nombre. Par ailleurs, le rude environnement gastrique n'a pas modifié l'état des LPS. Le temps de survie dans le fluide gastrique fut augmenté lorsque *S. typhimurium* fut présenté en compagnie de chocolat et de billes d'alginate de calcium. Cela suggère que *Salmonella* est protégée lorsque ingérée avec un aliment. Ces résultats peuvent expliquer la raison pour laquelle *Salmonella* a une très basse DI lorsque consumé avec un aliment contaminé.

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INTRODUCTION

The genus, *Salmonella*, consists of a variety of types and can be classified according to their pathologic properties. They are among the major foodborne pathogens which are of public concern with respect to food safety (Kwon and Ricke, 1998). Generally, the disease caused by *Salmonella* spp. is called salmonellosis. This ranges from typhoid fever caused by *Salmonella typhi* to the less severe gastroenteritis caused by species like *Salmonella typhimurium*. In Canada, the most frequently reported serotype is *S. typhimurium*, followed by *Salmonella enteritidis*, *Salmonella heidelberg*, and *Salmonella hadar*. These four serovars continue to be the most frequently isolated salmonellae from 1993 to 1998 (Anonymous, 1998).

Salmonellae have simple growth requirements and are able to survive a wide variety of systems, including most environmental systems and food production and processing systems. The mouth is the port of entry for salmonellae and the small intestine is the principal reservoir and site of pathological action. Salmonellae, therefore, have to survive the low pH of approximately 1.5 of the stomach to cause an infection. The high acidity of gastric fluid as the first line of defense against bacterial enteric pathogens has long been recognized (Gianella *et al.*, 1972). The ability of enteric pathogens to resist killing by acid during transit through the stomach increases their likelihood of colonizing the intestines and causing an infection (Waterman and Small, 1998) and the ability of pathogens to resist this pH corresponds to their oral infective dose (ID) (Lin *et al.*, 1995).

The minimum number of ingested *Salmonella* organisms necessary to produce clinical symptoms in humans remains a controversial issue. Earlier studies showed that it ranges

between 10^5 to 10^9 cells, depending on the *Salmonella* strain (McCullough and Eisele, 1951a, b, c). In contrast, the ID of salmonellae in foods, including hamburger, milk chocolate and cheddar cheese, was found to be less than 10^3 cells (Blaser and Newman, 1982). There have been other reports of ID of less than 100 cells in chocolate (D'Aoust *et al.*, 1975; Greenwood and Hooper, 1983) and between 1 to 6 cells in cheddar cheese (D'Aoust, 1985). Foodborne outbreaks characterized by a low ID are often associated with consumption of bacteria with food, which implies that food may protect enteric pathogens against the acidity of the stomach (Waterman and Small, 1998). The acidity of the human stomach depends on physiological factors that include previous food intake (Waterman and Small, 1998). Reduction of gastric acidity has been associated with an increase in the survival rates of some foodborne pathogens (Peterson *et al.*, 1989) and with lowering of ID (Schlech *et al.*, 1993).

This study was done to determine the effects of initial growth pH, initial number of cells, physiologic age of cells, and cell entrapment in calcium alginate and chocolate on the survival of *S. typhimurium* in simulated gastric, ileal and colon fluids. The LPS profiles in Tricine-SDS-PAGE from *Salmonella* cells exposed to gastric fluid were also analyzed.

1.0 LITERATURE REVIEW

1.1 EFFECT OF pH ON GROWTH AND SURVIVAL OF *SALMONELLA*

Pathogens may be exposed to dramatic pH fluctuations in nature, such as in foods and during pathogenesis in animal or human hosts (Abee and Wouters, 1999). The acidification of foods is an age-old means of preservation, and is still used as the principal barrier to the outgrowth of pathogens and spoilage bacteria. *Salmonella typhimurium*, *Escherichia coli* 0157:H7, *Shigella flexneri*, and *Listeria monocytogenes* grow best at neutral pH.

The optimum pH for growth of salmonellae is between 6.5 and 7.5, although they can grow in the pH range of 4.0 to 9.0 (Doyle and Cliver, 1990). Experiments performed on *S. typhimurium* showed that growth is inhibited in media of pH 3.4 and killing requires a pH <1.5 to 2.5. There is a complete destruction of the organism in a few minutes in lemon juice and lime juice at pH 2.3 to 2.5 (Prost and Riemann, 1967). Roering *et al.* (1999) reported a decrease in the populations of *S. typhimurium* DT104, *L. monocytogenes* and *E. coli* 0157:H7 in pasteurized and unpasteurized apple cider of pH 3.3 to 3.5 after 21 days at 4°C and 10°C.

The minimum pH for growth depends on several factors, one of which is the strain of the organism. Some strains are more acid tolerant than others (Doyle and Cliver, 1990). Effect of low pH also depends on the type of acid present in food or media. Organic acids are intrinsic to some foods. They may also be added as preservatives or can be present as a consequence of microbial fermentation processes. The effect of acids on bacteria depends on the nature of acid, the final pH of the food, as well as other environmental factors (Hill *et al.*, 1995). When inorganic acids, such as hydrochloric acid (HCl), are used to adjust the pH of

media, salmonellae can grow at a pH as low as 4.0. Organic acids such as acetic, propionic and butyric acids are more bacteriostatic than inorganic acids. Acetic acid is very effective in the destruction of salmonellae (Prost and Riemann, 1967). When the pH of foods is adjusted by acetic acid, e.g., the addition of vinegar in preparing mayonnaise, salmonellae will not grow below pH 5.0 (Doyle and Cliver, 1990). Destruction of salmonellae is complete in 2 h at 40°C, or 4 days at 15°C if the pH of egg yolk is adjusted to 4.5 with acetic acid (Prost and Riemann, 1967).

The growth temperature is also important. The more optimal the temperature for growth, the more tolerant is the organism to adverse pH conditions (Doyle and Cliver, 1990). Water activity (A_w) also affects bacterial growth at different pHs. The further the A_w is from the optimum for salmonellae growth, the more difficult it is for the organism to grow at the lower pH values. The rate of destruction of salmonellae is higher at 20°C in meat and bone meal at a A_w of 0.9 than at a higher A_w when acidified with lactic acid (Banwart and Ayres, 1957). The number of organisms present is also a factor. Survival at a low pH is more likely if the initial number is 10^7 cells.g^{-1} rather than 10 cells.g^{-1}. The pH at which bacteria can grow also depends on the composition of food. The organism can grow at a lower pH, than it might otherwise, where more nutrients are present (Doyle and Cliver, 1990).

1.2 ACID ADAPTATION

Many bacteria have the ability to withstand small changes in an environmental parameter. Larger changes away from the optimal values can cause the induction of a more elaborate stress response. These adaptive strategies are generally directed towards survival

rather than growth. *Salmonella* species are known to induce adaptive responses to various stresses, including acids, salts and temperature, and these responses may enhance survival in deleterious environments (Leyer and Johnson, 1992).

Acidic pH is one of the most frequent stress conditions encountered by microbial systems. The ability to survive and flourish during these encounters is crucial to the perpetuation of the species. Some organisms (acidophiles) prefer an extreme acid environment. However, other organisms, e. g. salmonellae, with optimal pH values for growth in the neutral range (neutrophiles) must cope with frequent encounters with potentially lethal levels of acid.

Bacteria can survive acidic environments because of their ability to regulate their cytoplasmic pH in a process referred to as pH homeostasis. This process is primarily driven by the controlled movement of cations across the membrane (Hill *et al.*, 1995). pH homeostasis describes the ability of an organism to maintain the cytoplasmic pH at a value close to neutrality despite fluctuations in the external pH. For example, in *E. coli*, the cytoplasmic pH changes by less than 0.1 unit per pH unit change in external pH in the range of external pH 4.5 to 7.9 (Hill *et al.*, 1995).

Acidic foods such as apple cider have been implicated in food-borne illnesses caused by *Salmonella* spp. Several dairy foods, including cheese, have also been the causative agent of *Salmonella* diseases. Leyer and Johnson (1992) showed that acid adaptation promotes the survival of *S. typhimurium* in fermented milk and cheese by enhancing its tolerance for organic acids. Acid adaptation was induced in *S. typhimurium* LT2 by transferring cells grown at a pH of 5.8 into a buffer acidified to pH 3.85 with 125mM lactic, acetic or propionic acid.

Non-adapted cells were used as a control. With each acid, the adapted cells survived better than non-adapted cells. Lactic acid was found to be the most lethal, however, the acid-adapted cells were about 10^5 times more tolerant than the non-adapted cells. The results of Leyer and Johnson (1992) also showed that acid adaptation enhances survival in cheese and milk during active fermentation, in which several organic acids and other inhibitors are produced.

Short-chain fatty acids (SCFA), such as acetate, propionate and butyrate are produced as fermentation products by intestinal microflora. In humans, the concentrations of SCFAs are 35 mmol/kg in the small intestine and 134 mmol/kg in the large intestine (Cummings *et al.*, 1987). Salmonellae may also encounter acetate and propionate in food products, such as meat carcasses, salad dressing and mayonnaise, where they are widely used as preservatives (Kwon and Ricke, 1998). In an attempt to understand the role of SCFA in the pathogenesis of *S. typhimurium*, Kwon and Ricke (1998) examined the effects of SCFA on the acid resistance of the organism. Cells of *S. typhimurium* were subjected to acid challenge at pH 3.0 after exposure to SCFA at various concentrations, times and pHs. The percent survival was found to vary, depending on the type of SCFA, the concentration used, the acid adaptation time and pH. Under aerobic conditions, 42% of *S. typhimurium* cells survived as a result of prior exposure to 100mM of propionate at pH 7.0 for 1 h, while <1% of cells, not exposed to propionate, survived. They also reported that the SCFA-induced acid resistance was enhanced by anaerobiosis, reduced pH conditions and increase in incubation time during exposure to propionic acid.

1.2.1 Acid Tolerance Response

The ability to proliferate in fluids or within cells of a living host is a key attribute of pathogenic bacteria. Their primary objectives, like all bacteria, are survival and multiplication. They encounter changes in temperature, osmolarity, oxygen tension and nutrient deprivation in their environment (Mahan *et al.*, 1996), inside the host as well as in foods. In order to survive, they have evolved a number of adaptive responses to the various environmental stresses.

The most extensively studied response to acidity is the acid tolerance response (ATR) of *S. typhimurium*. This involves the increased expression of some proteins and/or the synthesis of new ones, termed acid-shock proteins (ASPs), which protect cells from acidification at low pH. At least three systems are reported to be involved, including a pH-dependent system induced in log phase, a pH-dependent system induced in the stationary phase and a pH-independent system induced in stationary phase (Foster, 1993). *S. typhimurium* can survive severe acid conditions of up to pH 3.0 if it is allowed to induce one of these acid tolerance response systems (Foster and Hall, 1990; Foster and Spector, 1995). Acid adaptation has also been observed in *E. coli* and species of *Listeria*, *Streptococcus* and *Enterococcus* (Tsai and Ingham, 1996).

The pH-dependent ATR of bacteria has been demonstrated by several investigators (Foster and Hall, 1991; Leyer and Johnson, 1992, 1993; Ryu *et al.*, 1999). Studies with nonpathogenic *E. coli* and *S. typhimurium* demonstrate that prior exposure to or growth in a moderately acidic medium enhances survival at low pH (Goodson and Rowbury, 1989; Foster and Hall, 1991). If cells in the exponential phase of the growth cycle are interrupted

by a shift from neutral pH to pH 4.0-4.5 (called acid shock), there is an induction of 50 ASPs, of which approximately 20 are induced only by acids. This leads to the development of profound acid tolerance to extremely low pH (Foster, 1991; 1993). In contrast, stationary-phase cells shifted to pH 4.3 only induce 15 ASPs, 4 of which are also log-phase ASPs, that can still provide resistance to extreme acid. Thus the pH-dependent ATR induced in the log phase is different from that induced in the stationary phase because mutations that dramatically affect one system have only a small effect upon the other (Foster and Spector, 1995).

Tsai and Ingham (1996) determined the effects of storage temperature (5°C and 23°C) and acid adaptation on the survival of *E. coli* 0157:H7, non-pathogenic *E. coli* and three strains of *Salmonella* species in ketchup (pH 3.6), mustard (pH 3.1) and sweet pickle relish (pH 2.8). Acid-adapted cells were prepared by incubating cells in Trypticase Soy Broth of pH 5.0 at 37°C for 4 h. They found that acid adaptation enhanced the survival of all the *Salmonella* and *E. coli* 0157:H7 strains, although the degree of adaptation varied with the strain and storage temperature. Acid adaptation also enhanced the survival of the non-pathogenic *E. coli* strain at 5°C but not at 23°C. Cells of all strains survived longer at 5°C than at 23°C.

Acid-shocked cells develop significant cross-protection to heat, oxidative stress and osmotic stress (Foster and Spector, 1995). The relationship of acid adaptation and tolerance to other environmental stresses was examined in *S. typhi* by Leyer and Johnson (1993). They determined that acid-adapted cells have increased tolerance to heat, osmotic stress, lactoperoxidase system, crystal violet and polymyxin B. The ability of *L. monocytogenes*

L028 to survive at pH 3.5 is enhanced by prior induction, for 90 min, at pH 5.0 (Hill *et al.*, 1995). The mechanism of acid-induced cross-protection involved changes in cell surface properties as well as enhancement of intracellular pH homeostasis (Leyer and Johnson, 1993).

The pH-independent ATR has also been demonstrated in many bacteria. This type of response is highly affected by the growth phase of the organism. Maximal acid resistance is exhibited at stationary phase (Gordon and Small, 1993). The stationary phase of some organisms such as *E. coli* and *S. typhimurium* brings about significant physiological changes that allow the cells to survive a wide variety of environmental stresses including starvation, hydrogen peroxide, heat, high salt concentration (Abee and Wouters, 1999), as well as low pH. The pH-independent ATR of stationary phase cells is highly controlled by the *rpoS* gene. This encodes the protein, rpoS {referred to as the alternate sigma factor (δ^s)}. This is the sigma subunit of the enzyme RNA polymerase and it is the master regulator of the general stress response in *S. typhimurium* and other enteric bacteria, including *E. coli* and *Shigella flexneri* (Small *et al.*, 1994). A common regulatory mechanism involves the modification of sigma factors whose primary roles are to bind core RNA polymerases thus conferring promoter specificity (Haldenwang, 1995). Alternate sigma factors have different specificities, directing expression of specialty regulons involved in general stress response (Abee and Wouters,1999). Levels of rpoS are low in log-phase cells not exposed to any particular stress, whereas it is induced in response to a variety of environmental stresses (Abee and Wouters, 1999).

RpoS has been shown to be an acid shock protein (ASP) that controls the expression of at least 8 other ASPs in *S. typhimurium* (Lee *et al.*, 1995). It controls the expression of

more than 35 genes involved in general stress response in *E. coli* (Abee and Wouters, 1999). Inactivation of rpoS renders cells sensitive to heat shock, oxidative stress, starvation, acid, ethanol and near UV light (Farewell *et al.*, 1998). The direct relevance of rpoS to food microbiology has been the discovery that bacteria defective in *rpoS* are highly sensitive to food processing conditions (Rees *et al.*, 1995).

1.3 GASTRIC BACTERICIDAL BARRIER

About 2.5 l of gastric juice (pH 2) are secreted into the stomach and about 0.7 l l of pancreatic juice (pH 8) is secreted into the proximal small intestine each day (Keele and Neil, 1965; Hill, 1990). These secretions present a pH and enzymatic barrier to the survival of ingested microorganisms during digestion and act in concert with bile and peristalsis to ensure that the resting small intestine is only heavily colonized in conditions of stasis (Charteris *et al.*, 1998). A relationship between gastric acid secretion and enteric infections has been suspected for over 100 years. Enteric bacteria usually do not survive in acidic environments and gastric acidity was thought to act as a barrier to ingested microorganisms by regulating the number of ingested bacteria that enter the small intestine, where they proliferate and cause enteritis (Gianella *et al.*, 1973). So the concept of the "gastric bactericidal barrier" was enunciated in the early 20th century.

Many investigators believe that patients with reduced or absent gastric acid secretions are more susceptible to enteric bacterial infections (Arnold, 1927; Hurst, 1934; Garrod, 1939), but there have been some contradictions over attempts to define the components of the "gastric barrier" to microorganisms. Other factors, as well as acid, have been suggested.

Many investigators, including Hurst (1934) and Teale (1934), in their studies done *in vitro*, concluded that the free acid content of gastric juice was the sole antibacterial factor. Garrod (1939), using a similar method, concluded that gastric juice was more bactericidal than HCl of equivalent acidity. Many *in vivo* studies have also lead to the conclusions that the bactericidal activity of gastric juice was not pH-dependent. Dack and Petran (1934) introduced cells of *Serratia marcescens* into the stomach of a single monkey and observed no death of the organisms after 5 h. However, the pH of the gastric content was above 4.5. Gianella *et al.* (1972) reported that the lack of controls for the effects of gastric dilution and gastric emptying as well as the use of diseased or surgically altered stomachs made these studies non-conclusive. They also noted that the contribution of other constituents, including organic acids, mucous, lysozyme and antibodies to the bactericidal effect of gastric juice suggested by several investigators was unconvincing. They demonstrated, using both *in vivo* and *in vitro* methods, a strict pH-dependence of the bactericidal activity of gastric juice. In studies done *in vitro*, the survival of *E. coli*, *S. typhimurium*, *Salmonella paratyphi*, *Salmonella enteritidis* and *S. marcescens* was monitored in normal gastric juice at various pH levels (from 2.0 to 7.0). As controls, trypticase soy broth and saline at the same pH levels were also tested. They observed that saline and nutrient broth had the same bactericidal activity as that of gastric juice at comparable pH levels. They also demonstrated that the lack of nutrient in gastric juice did not account for the death of bacteria they observed since an inoculum of 10^3 organisms increased to $10^8/10^9$ organisms.ml^{-1} in 18 h when the pH of gastric juice was greater than 5.0. *In vivo*, there was a prompt reduction in the number of non-pathogenic *S. marcescens* in the normal stomach (pH <2.0) but no reduction in the

hypochlorhydric (pH 6.0) and the achlorhydric (pH 6.8) stomach. Survival in acid has clinical relevance, as enteric pathogens must pass through the stomach of pH <3.0 for up to 2 h before colonization of the intestine (Gianella *et al.*, 1972).

The bactericidal effect of the stomach in addition to gastric acid secretion, is influenced by other factors, including previous food intake (Gianella *et al.*, 1972). Snepar *et al.* (1982) reported that under fasting conditions, the mean pH of healthy stomach is about 2.0, ranging from 1.5 to 5.5 and ingestion of a meal characteristic of a Western diet raises the pH to about 6.0. Peterson and others, in 1989, determined the effect food has on the antibacterial activity of gastric juice. The survival of *S. typhimurium* ATCC 14028, *E. coli* 078:H11 and *Shigella flexneri* were determined in gastric juice obtained from volunteers after the infusion of only a beverage (preprandial gastric juice) and a beverage plus a meal (postprandial gastric juice). Gastric juice was also obtained from fasting individuals (fasting gastric juice). They demonstrated that the respective proportions of surviving *S. typhimurium*, *E. coli*, and *S. flexneri* were, 25%, 31% and 33% (in fasting juice), 22%, 34% and 37% (in beverage-only juice), and 62%, 66% and 78% (in postprandial juice). The survival of each strain was significantly higher in postprandial gastric juice than survival in either fasting or beverage-only gastric juice. They, therefore, concluded that food helps the survival of enteric bacteria in gastric juice.

Similar results were observed by Waterman and Small (1998) when they did an *in vitro* acid resistance assay to determine if enteric pathogens could survive under normally lethal acidic conditions when inoculated onto foods. Ground beef, boiled rice and egg white were inoculated with bacteria and the bacteria were allowed to dry on foods' surfaces for 10

min at room temperature. The inoculated foods were then placed into acidified Luria-Bertani (LB) of pH 2.5 and incubated at 37°C for 2 h. They found that all of the different enteric bacteria they tested survived at pH 2.5 when inoculated onto ground beef. *S. typhimurim* was also protected from killing at pH 2.0 when inoculated on egg white but was not protected when inoculated onto boiled rice. Many of the organisms could not survive when assayed in acidified LB broth at the same pH.

The protective effects of food has also been demonstrated for some probiotics. Charteris *et al.* (1998) demonstrated the effects of adding milk proteins and hog gastric mucin on the gastrointestinal transit tolerance of 15 isolates of *Lactobacillus* and *Bifidobacterium* species. In general, milk proteins increased the survival rates of the organisms. Two of the isolates showed 100% tolerance to gastric acid in the presence of milk proteins. Hog gastric mucin had no effect on the tolerance of lactobacilli but increased that of bifidobactria. However, *B. bifidum* and *B. animalis* were negatively affected. They postulated that milk proteins and mucin may act as both buffering agents and inhibitors of digestive protease activity to protect ingested bacteria in the upper gastrointestinal tract.

1.4 INFECTIOUS DOSE

The ability of pathogens to establish a successful invasive infection is dose-dependent and relative resistance to gastric acid may be responsible in defining the infectious dose of a particular enteric pathogen. The minimum number of ingested salmonellae (infectious dose, ID) necessary to produce clinical symptoms in humans remains a contentious issue. Several factors affect the ID of salmonellae, including the type and quality of food, the strain of

Salmonella, and other factors (Doyle and Cliver, 1990). This, as well as variability in individual tolerances or susceptibility to infection, makes it difficult to determine the minimum number of organisms to cause illness.

Two main types of studies, feeding and retrospective studies, have been done to estimate the infectious dose of salmonellae. Earlier work involved feeding studies done with normal, healthy volunteers from penal institutions (McCullough and Eisele, 1951a, b, c). Subjects were fed with different doses (10^4 to 10^{10} cells) of *S. meleagridis*, *S. anatum*, *S. newport*, *S. derby*, *S. bareilly* and *S. pullorum* and monitored for more than two weeks for any clinical symptoms. It was observed that there was a difference in virulence of the different strains. There was also a difference between individual's susceptibility to infection by the same strain of *Salmonella*, likely because of differences in immunocompetence and antibody titres to salmonellae (Doyle and Cliver, 1990). The number of salmonellae necessary to produce symptoms varied from strain to strain. Generally, more than 10^5 cells were required to produce illness, of which the lowest was 1.3×10^5 cells of *S. bareily*. Hornick *et al.* (1970) determined that no one became ill when 14 persons were each tested with 10^3 cells of *S. typhimurium*, but 32 out of 116 persons became infected when given 10^4 cells and 16 of 32 infected with 10^8 cells.

Some data have been derived from retrospective studies (Doyle and Cliver, 1990). After an outbreak of salmonellosis, the number of *Salmonella* cells in the implicated food is determined. An estimate of the number of salmonellae that were consumed is determined by calculating the average amount of food eaten by each person. For exampie, Armstrong *et al.* (1970) determined that 1.11×10^4 cells of *S. typhimurium* were consumed by the people who

became ill from an outbreak linked to imitation ice-cream.

The studies mentioned above suggest that a large number of salmonellae is needed to cause illness. According to Bergey's Manual, the infectious dose of *Salmonella* is 10^8 to 10^9 cells (Doyle and Cliver, 1990). Prost and Rieman (1967) also reported that *Salmonella* foodborne disease develops if a comparatively large number of *Salmonella* cells are present in the consumed food. Results of other studies, however, have demonstrated that very few *Salmonella* cells can produce clinical symptoms. A large outbreak involving *S. eastbourne* contamination of chocolate candy in 1973 and 1974, that affected 95 persons mainly in eastern Canada and about a similar number in the northeast United States, was reported and the number of cells constituting an infective dose was estimated to be less than 100 (D'Aoust *et al.*, 1975; Todd, 1983).

Another outbreak, again involving contaminated chocolate, was reported by Hockin *et al.* (1989) and the number of *S. nima* that caused clinical symptoms in individuals affected ranged from 1 to 6 cells. In a report by D'Aoust (1985), the infective dose of *S. typhimurium* in an outbreak involving cheddar cheese was estimated based on the amounts of cheese consumed. According to this report, 1 to 6 cells were required to cause illness in 6 patients infected. In a 1994 outbreak of illness caused by *S. enteriditis* contamination of ice-cream, the number of cells that caused illness was not more than 25 (Vought and Tatini, 1998).

D'Aoust (1985) concluded that the fat content of contaminated foods, including cheddar cheese, may protect salmonellae from gastric acidity, thereby promoting salmonellosis. According to him, organisms trapped in hydrophobic lipid molecules may readily survive the acidic conditions of the stomach and pass into the intestinal tract where

they cause illness.

1.5 BACTERIAL LIPOPOLYSACCHARIDE

Lipopolysaccharides (LPSs) are a class of macromolecules that form the major constituent of the outer membrane of gram-negative bacteria. They are of great compositional and structural diversity and biologically highly active substances. LPSs are called endotoxins due to their toxic properties and termed O-antigens because of the antigenic properties they confer to the surface of the bacterial cell (Luderitz *et al.*, 1983). LPS consists, generally, of three regions which are covalently attached to one another and of contrasting chemical and biological properties - the lipid A, rough core oligosaccharide and O-antigenic side chain.

1.5.1 Biologic Functions of LPS Regions

1.5.1.1 Lipid A

The hydrophobic component, lipid A, is made up of diglucosamine phosphate with five or six attached fatty acyl chains (Nikaido and Nakae, 1979). It contains the endotoxic principle of LPS which is responsible for the induction of the various endotoxin effects such as fever and lethal toxicity. It activates the classical antibody-independent complement cascade, binds to serum proteins such as lipoproteins, interacts with recognition proteins (receptors) exposed on macrophages and other host cells, and it is essential for the function of the outer membrane in growth and survival of gram-negative bacteria (Rietschel *et al.*, 1992).

1.5.1.2 The Core Region

The rough core oligosaccharide consists of about 10 to 12 sugars and contains most of the cellular octose and heptose present in LPS. It is characterized by the presence of an unusual sugar, 2-keto-3-deoxyoctonate (KDO). The core is of great significance for many of the biological activities and functions of the LPS molecule. It carries a common LPS epitope for antibodies and serum factors (Brade and Brade, 1985). The KDO-containing inner core, being a common structural element of LPS, is considered a target for antibodies which cross-react with LPS of different bacterial origin and which possibly provide cross-protection against pathogenic gram-negative bacteria and their endotoxins. Bacteria with a defect in KDO residue biosynthesis do not grow. An R mutant of *H. influenzae* containing just one KDO residue shows that only one KDO residue, in addition to lipid A, is enough for the survival of a gram-negative bacterium. Although lipid A is essential for endotoxin effect *in vivo* and *in vitro*, the degree of lipid A bioactivity may be modulated by the polysaccharide portion, particularly the KDO-containing inner core (Helander *et al.*, 1988).

1.5.1.3 The O-Antigenic Side Chain

The O-antigenic side chain is made up of repeating units, consisting of up to 50 oligosaccharides, which are, in general, glycosyl residues extending from the cell surface (Wyk and Reeves, 1989). The nature, ring form, type of linkage and substitution of the individual monosaccharide residues, as well as their sequence within a repeating unit, is characteristic and unique for a given LPS and the parental bacterial serotype (Rietschel *et al.*, 1992). An enormous number of O-chain structures exist in nature because of the diversity of

sugar constituents and their possible linkage sites. The lipid A and core regions do not vary greatly within species of the *Enterobacteriaceae*, whereas O-antigens are extremely polymorphic and unique to each bacterial serotype (Luderitz *et al.*, 1983). O-specific chains are known which contain repeating units consisting of only one type of sugar, such as D-mannose in *E. coli* O8 and *E. coli* O9, the "repeating units" being characterized as containing identical sequences of specific linkages (Rietschel *et al.*, 1992).

The O-specific chain determines the serological specificity of LPS and the parental bacterial strain (serotype). The immunogenic and antigenic properties of LPS are determined by O-factors which are located within the repeating units. In many cases, correlations between the structural and serological features of the defined LPS or its parental bacterial strain have been established. For example, O-factor 4 present in the O-specific chain of *Salmonella abortus equi* LPS is determined by abequose (3,6-dideoxy-D-galactose), whereas O-factor 9 of *Salmonella typhi* LPS is represented by tyvelose (3,6-dideoxy-D-mannose) (Rietschel *et al.*, 1992). The O-specific chain functions as a receptor for bacteriophages. The structure and the conformation generated by more than one repeating unit determines the phage receptor site.

In *Salmonella* spp., 40 major forms of O-antigen are recognized (Wyk and Reeves, 1989). Some bacteria in nature lack the O-antigenic side chain. Such mutants are easily obtained by selecting for resistance to certain bacteriophages. Bacteria which contain LPS that lacks the O-antigenic side chain often are referred to as 'rough' (R-form) owing to their colonial morphology, whereas bacteria which have this LPS component are referred to as 'smooth' (S-form, Darveau and Hancock, 1983).

1.5.2 Role of LPS in Bacterial Virulence

LPS of gram negative bacteria constitutes a chemical and physical barrier for the cell. The latter function is carried out by the O-antigen polysaccharide (O-Ag) subunit of LPS. O-Ag plays an important role in the interaction of the cell with its environment. The O-Ag chain represents the major heat-stable antigen, whereas lipid A is the primary agent responsible for the endo-toxicity of gram-negative bacteria (Craven, 1994). O-Ag confers resistance to outer membrane protein-specific bacteriophages, blocks access of core-specific antibodies to their cognate epitopes and leads to partitioning of the cell in the hydrophilic phase of two-phase system (Grossman *et al.*, 1987).

The O-chain is also important in complement activation and the virulence of bacteria. It is known that LPS activates the alternative complement pathway through the O-specific chain, but the efficiency of activation is dependent on the structure of repeating units. Bacteria expressing O-specific chains with factors 4 and 12 are significantly more virulent than those expressing 9 and 12. This difference in virulence is related to the relative inability of factors 4- and 12-expressing bacteria to activate complement by the alternative pathway and to bind C3b, thereby resisting phagocytic uptake and killing (Rietschel *et al.*, 1992). The O-chain also determines the susceptibility of bacterial cells to complement-mediated killing. Thus, bacteria lacking O-Ag are directly killed by complement; whereas, bacteria possessing O-Ag resist this killing. According to Joiner *et al.* (1982), this is due to the fact that bactericidal C5b-9 complex is bound to smooth cells by weak hydrophobic interactions. As a result, it is released from the cell surface without carrying out its bactericidal action. They suggested that C5b-9 complexes are shed because complement activation takes place on long O-Ag side chains and

that C5b-9 complexes formed are stearically inhibited from inserting into hydrophobic membrane domains. Grossman *et al.* (1987) examined the effect of LPS composition on the resistance of salmonellae to direct complement-mediated killing. Their result showed that the cells were resistant to serum killing when the average number of O-Ag units per LPS was more than 5 and when more than 20% of the LPS contained more than 14 units per molecule.

Chart *et al.* (1989) reported a correlation between LPS synthesis and virulence in strains of *S. enteritidis*. They showed that, regardless of plasmid possession, strains of *S. enteritidis* were virulent only when able to synthesize LPS. They showed that the loss of ability to synthesize LPS was responsible for the conversion of highly virulent strains to avirulent strains. Intact LPS is also required for infection by certain rhizobia. The requirement for O-polysaccharide by *Rhizobium leguminosarum* during infection of bean plant is well known. *R. leguminosarum* mutants deficient in O-polysaccharide are incapable of complete infection of the legume host. Infections on *Phaseolus vulgaris* (bean) by such mutants abort early in nodule development, usually within root hairs (Noel *et al.*, 1986).

Several studies have suggested that the LPS layer of *E. coli* and *S. typhimurium* might play a role in the adhesion of these enteric bacteria to components of intestinal mucus and in their colonization of the intestinal tract of mice (Cohen *et al.*, 1985; Nevola *et al.*, 1985). The ability of *S. typhimurium* strains to colonize the large intestines of mice decreased as their LPS structure became more defective (Nevola *et al.*, 1985). Craven (1994) reported that LPS also contributes to the colonization of the avian intestinal tract by *S. typhimurium* strain 333/O. In his experiment with broiler chicks, he noted that the colony-forming unit (CFU) of cells recovered from chicks given the LPS-deficient strains was significantly lower

than the CFU from chicks given the wild-type strain. He suggested that LPS side-chain components may shield the bacterial cell from entrapment in the chicken mucus. Further, Guard-Petter *et al.* (1996) determined that the O-antigen of *S. enteritidis* LPS is involved in invasiveness. Their results suggested that O-antigen in association with several proteins facilitates invasiveness. They found that a phenotypic variant producing a high-molecular weight (HMW) O-antigen was more organ invasive in 5-day-old chicks than that producing low molecular weight O-antigen. Thus they postulated that the role of HMW O-antigen could be to facilitate the secretion of virulence factors.

Antibiotic resistance is one of the factors that defines bacterial virulence in the host. Several investigators have reported that a relationship exists between the O-chain of LPS and antibiotic resistance. Laub *et al.* (1989) showed a correlation between increased susceptibility of *Salmonella* to rifamycin and the deletion in LPS chain at pH 7.2. They also reported that LPS mutants of *Salmonella* were more susceptible to β-lactam antibiotics than the wild type.

Several environmental factors such as temperature, pH and osmolarity, as well as the physical and chemical characteristics of foods affect the growth and virulence of pathogenic bacteria, including *Salmonella*. Idziak and Crossley (1973) found that the virulence, in day-old chicks, of *S. typhimurium* grown in barbecue sauce (pH 5.2) increased with age of cells, whereas there was no significant change in virulence with age of cells grown in ground, cooked chicken meat (pH 7.0). Idziak and Suvanmongkol (1971) also demonstrated that, virulence of the same organism grown in Brain Heart Infusion Broth (BHIB) at pH 5.5 increased with age of cells while it decreased with age at pH 7.5. There was no change in virulence of cells grown at pH 9.5.

The LPS expression of bacteria is also affected by growth or environmental conditions. LPS undergoes modifications as part of the adaptation to several environmental stresses, including, low pH, phosphate starvation, and high temperature (Tao *et al.*, 1992). Studies done by Aguilar *et al.* (1997) showed that strains of *Aeromonas hydrophila* grown at high osmolarity showed more virulence (lower LD_{50}) in mice and fish than strains grown at low osmolarity. They demonstrated the presence of O:34 antigen LPS in this strain when grown at high osmolarity and its absence at low osmolarity, at the same growth temperature. However, it was not concluded whether the higher virulence at high osmolarity was due to the change in the sub-population of LPS or whether the LPS change allowed a higher extracellular activity of some products (toxins) that rendered the cells more virulent at high osmolarity. They postulated that it might be due to the activation of a regulator that increases expression of genes involved in O-side chain synthesis as well as synthesis of other virulence factors.

Benedi *et al.* (1991), using ELISA experiments, demonstrated that *Klebsiella pneumoniae* C3 cells grown at low pH have less LPS on their surface, as indicated by their lower A_{405}, than those grown at pH 7. They proposed that this is the reason why LPS-specific phage FC3-1 less efficiently infects bacterial cells grown under conditions of low pH. This is because LPS is no longer available on the bacterial cell surface for reception of these LPS-specific phages. On the other hand, acid pH stimulates LPS biosynthesis in *Helicobacter pylori* (McGowan *et al.*, 1998). *H. pylori* cells grown at pH 5 exhibited altered LPS profiles compared with cells grown at neutral pH. Immunoblots of LPS preparations using anti-*H. pylori* LPS immune sera showed two high-molecular-weight bands present in cells grown at

pH 5 that were absent in pH 7 LPS preparations. There was also an over expression of a low-molecular-weight band in LPS from cells grown at pH 5.

Idziak and Abdullah (unpublished data) determined the effects of growth temperature and pH on the LPS expressions of three species of *Salmonella* at different phases of growth. They found, generally, that the change in LPS profiles varied with the type and age of cells of the different species. At 20°C and 37°C, more low molecular weight (LMW) LPS was detected in log phase *S. typhimurium* at pH 9.5 than at pH 7.5; in contrast, growth temperature, and not pH, affected the LPS profile from stationary phase cells. At pH 5.5, 7.5 and 9.5, more LPS was observed at 37°C than at 20°C.

McGroarty and Rivera (1990) reported that high temperature, high concentrations of salt, sucrose, or glycerol, low phosphate concentration, and low pH altered the size heterogeneity of the serotype-specific LPS produced by *Pseudomonas aeruginosa* and allowed the exposure of the common antigen LPS on the cell surface. They showed that the production of long-chain LPS from cells grown at pH 5.5 was low compared to LPS production from cells grown at pH 6.9 and 7.8. Pettit *et al.* (1995) noted that *Neisseria gonorrhoeae* strain F62 exhibited different lipooligosaccharide (LOS) patterns when grown in broth cultures of pH 5.8, 7.2 and 8.2. The strain expressed a 4.9-kDa band at the three different pH values but more was expressed by cells grown at pH 5.8 and 7.2. Certain gonococcal strains exhibit an acquired form of serum resistance due to the covalent transfer of host sialic acid to the galactose in the terminal lactoside of LOS. The 4.9-kDa band does not accept sialic acid (Mandrell *et al.*, 1990) thereby rendering the cells serum-sensitive. The 4.9-kDa LOS component also contains the binding site for bactericidal immununoglobulin M

in normal human serum (Mandrell *et al.*, 1990). So gonococci growing at alkaline pH might be less susceptible to killing by normal human serum than cells grown at neutral pH (Pettit *et al.*, 1995).

2.0 MATERIALS AND METHODS

2.1 ORGANISM AND GROWTH CONDITIONS

The organism used in this study was *Salmonella typhimurium* (ES 878). Pure cultures were stored on Microbank beads containing a cryoprotectant (Pro-lab Diagnostics, Ontario) and stored at -70°C. These constituted the stock cultures. When needed, one bead was transferred into 50 ml of Brain Heart Infusion Broth (BHIB, pH 7.5, Difco) in 125 ml Erlenmeyer flasks and incubated (stationary culture) at 37°C for 24 h. Two consecutive transfers of one loopful of culture and incubation at 37°C for 24 h followed. Finally, a working culture was obtained by inocuiating (one loopful) into 50 ml of BHIB (pH 7.5 and 5.5) and incubating at 37°C with shaking at 200 rev. min^{-1} (New Brunswick Scientific Company, NB. Model G-25) for variable periods of time to reach different growth phases. The pH of BHIB was adjusted to 5.5 with 1M HCl.

2.2 PREPARATION OF SIMULATED ENVIRONMENTS

Simulated gastric (pH 1.5), ileal (pH 7.2) and colon (pH 8.0) fluids were prepared as described by Beumer *et. al.* (1992), except that bile salts no3 (Difco) was used instead of porcine bile (Appendix, Tables 1-3). Ingredients were dissolved in de-ionized water. Gastric fluid was adjusted to pH 1.5 with 1M HCl. The enzymes were filter-sterilized (Millipore membrane filter, type HA, 0.45μm pore size) and the other components were autoclaved.

2.3 *SALMONELLA* SURVIVAL IN SIMULATED FLUIDS

2.3.1 Cells in culture fluid

2.3.1.1 Survival in individual simulated fluid

Both high (10^5 to 10^6 CFU ml^{-1}) and low (10^2 to 10^3 CFU ml^{-1}) inocula were used to inoculate simulated ileal and colon fluids. Only a high inoculum was used to inoculate the simulated gastric fluid. One ml of the working culture (section 2.1) was added to 9 ml of the different fluids in test tubes and incubated (stationary) at 37°C. After 0.5, 1, 2 and 4 min contact in gastric fluid, 1 ml aliquots were removed to 9 ml of 0.05M phosphate buffer (pH 7.8) to neutralize the acid. It was previously determined that 9 ml of the buffer neutralized 1 ml of gastric fluid. Subsequent dilutions were made in 0.85% (wt/vol) saline. Survival in ileal and colon fluids were determined after 1, 2, 4, 8, 16 and 24 h contact. One ml aliquots were removed to 9 ml of 0.85% (wt/vol) saline with subsequent dilutions in saline. Dilutions were plated (pour plate) in Brain Heart Infusion Agar (BHIA, Difco) for total viable counts and Brilliant Green Sulfa (BGS) Agar (Difco) for non-injured cell counts. The plates were incubated at 37°C for 24 h.

2.3.1.2 Survival during sequential exposure to simulated fluids

Transfers of cells from one simulated environment to the other were done to represent the passage of cells through the gastrointestinal tract (GIT). One ml of culture was added to 9 ml of gastric fluid. After 0.5 min of contact, surviving organisms were determined. At the same time, 1 ml of the inoculated gastric fluid was transferred into 9 ml of ileal fluid. After 1, 4 and 10 h of contact, surviving populations were determined. At 1 and 10 h, 1 ml from

the inoculated ileal fluid was also transferred into 9 ml of colon fluid. One ml aliquots of inoculated colon fluid were taken after 1, 4 and 10 h, diluted, and viable cells enumerated.

2.3.2 Entrapped cells

2.3.2.1 Cells entrapped in calcium alginate beads

Two grams of sodium alginate powder (Fisher Scientific Company, USA) were dispersed in 100 ml of de-ionized water by gentle stirring. The solution was then autoclaved at 121°C for 15 min and cooled to room temperature. One ml of cells from working cultures (grown at pH 7.5), was diluted to 10^6 - 10^7 CFU ml^{-1}. The cells were added to 5 ml of sterile sodium alginate solution in 100 x 125 mm test tubes and vortexed. The cell-sodium alginate mixture was added, dropwise, from a 1 ml pipette, to sterile $CaCl_2$ solution (5% wt/vol) in a beaker, to form calcium alginate beads (4mm in diameter, Figure 7A) with entrapped cells. The formed beads were left in the $CaCl_2$ solution at room temperature for about 20 min to solidify and then removed from the solution. Ten beads, equivalent to 0.5 ml and containing 10^6 - 10^7 CFU, were added to each of several tubes containing 9 ml of gastric fluid and incubated at 37°C. To simulate the effect of mastication, beads with entrapped cells were crushed in a sterile mortar with a sterile pestle (Figure 7B) prior to introduction to the gastric fluid. After 0, 0.5, 1, 2, 4, 8, 16 and 24 min respectively, the contents of one test tube were emptied into a 125 ml Erlenmeyer flask containing 81 ml of 0.05M phosphate buffer (pH 7.8) and mixed. The suspension was then macerated for 1 min, using the stomacher (Lab-blender 400 , Seward Laboratory UAC House, London). Viable cells were enumerated on BHIA and BGS agar.

2.3.2.2 Cells entrapped in chocolate

Ten grams of chocolate (Baker's unsweetened chocolate, Kraft Canada Inc., Ontario) in a beaker were melted in a water bath at 100°C and cooled to 45°C. One ml of working culture (containing *ca.* 10^{10} CFU.ml^{-1}) grown at pH 7.5 was added and the suspension mixed vigorously. The mixture was then spread thinly on a sterile tray and left to solidify at 10°C for 20 min. The solidified chocolate mixture was then chopped into small pieces, ranging in size from 0.8 to 2.8 mm in diameter (Figure 7C), with a sterile knife. One gram of chopped chocolate was added to 9 ml of gastric fluid in different test tubes. Incubation took place at 37°C and aliquots for viable cell determinations in BHIA and BGS were removed after 0, 0.5, 1, 2, 4, 8, 16 and 24 min.

2.4 ANALYSIS OF LIPOPOLYSACCHARIDE (LPS) PROFILES

2.4.1 Extraction of LPS

LPS extraction from cells of working cultures and after contact with simulated gastric fluid, was done according to the method described by Hitchcock and Brown (1983). Working cultures were centrifuged (12 100 x g, for 10 min), washed with PBS (pH 7.2) and re-suspended, in PBS (pH 7.2), to an OD_{600} of 0.5. From this suspension, 1.5 ml were centrifuged (10 000 x g) in a micro-centrifuge for 3 min. The pellets were re-suspended in 100 µl of lysing buffer, containing 2% (wt/vol) SDS, 4% (vol/vol) mercaptoethanol, 10% (vol/vol) glycerol and 0.2% (wt/vol) bromophenol blue in 1M Tris-HCl buffer (pH 6.8), and boiled at 100°C for 10 min. For protein digestion, 100µg of proteinase K (Sigma Chemical Co., USA), solubilized in 100µl of de-ionized water were added to the lysate after cooling

and incubated at 60°C for 2 h.

For extracting LPS from cells exposed to gastric fluid, working cultures were first centrifuged. Sediments were resuspended in 2 ml of BHIB and 1 ml of the suspension transferred into 9 ml of simulated gastric fluid in different test tubes, mixed vigorously and incubated. After 0.5 and 4 min respectively, the contents of each test tube were neutralized with 81 ml of phosphate buffer, centrifuged (12 100 x g, for 10 min), washed with PBS (pH 7.2) and re-suspended to an OD_{600} of 0.5. LPS extraction was carried out as above. Crude LPS samples were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.2 Electrophoresis and Silver Staining

LPS preparations were subjected to electrophoresis using a discontinuous Tricine-SDS-PAGE system in a Protean Vertical Electrophoresis Cell (Bio-Rad, Richmond, California) (Laemmli, 1970; Lesse *et. al.*, 1990). The separating gel (12 x 20 cm) had a final concentration of 16.5% *T*, 6% *C*, where *T* represents the total percentage of acrylamide (both acrylamide and bisacrylamide) and *C* represents the percentage of bisacrylamide to the total concentration of acrylamide. The stacking gel (4 x 20 cm) had a final concentration of 4% *T*, 3% *C*. Ten µl of each sample were loaded under cathode buffer (0.1 M Tris-HCl, 0.1 M tricine, and 0.1% (wt/vol) SDS, pH 8.25) and run at a constant current of 10 mA/gel. Gels were removed 1 h after the bromophenol blue front had left the gel and then fixed overnight in a solution of 40% (vol/vol) ethanol and 5% (vol/vol) acetic acid. Bands were visualized by silver staining with shaking (70 rev min^{-1}) according to the method described by Tsai and

Frasch (1982). A molecular weight marker, 26.6, 16.9, 14.4, 6.5, 3.5 and 1.4 kDa (Bio-Rad, California, USA) was included.

The residual protein in each crude LPS extract was measured according to the method of Bradford (1967).

3.0 RESULTS

3.1 GROWTH OF *SALMONELLA* IN BHIB AT pH 7.5 AND pH 5.5

The growth of *Salmonella typhimurium* in BHIB adjusted to pH of 7.5 and 5.5 is shown in Figure 1. Growth was monitored for 36 h. At pH 7.5, cells reached their mid-log, early stationary and late stationary phases after 4 h, 12 h and 36 h, respectively; whereas, at pH 5.5, the respective growth phases were reached after 4 h, 12 h and 18 h. Viable cells were not isolated after 24 h in BHIB adjusted to pH 5.5.

3.2 *SALMONELLA* SURVIVAL IN SIMULATED INTESTINAL FLUIDS

3.2.1 Cells in culture fluid

3.2.1.1 Survival in individual simulated fluid

The number of mid-log phase cells grown in BHIB adjusted to pH 7.5 and 5.5 was rapidly reduced in gastric fluid. The number of viable cells isolated after 0.5 min of exposure was <10 CFU.ml^{-1} (Figure 2). With pH 5.5-grown cells, there was a highly significant difference ($p<0.006$) in the number of survivors of mid-log and early stationary, and early stationary and late stationary phase cells after 0.5 min of exposure to gastric fluid. The viable cell number of early stationary phase cells grown at pH 7.5 and exposed for 2 min to gastric fluid decreased by 6 logs; similar cells grown at pH 5.5, by 5 logs. After 2 min of contact with the gastric fluid, late stationary phase cells grown at pH 7.5 were not markedly different in sensitivity than similar cells grown at pH 5.5. The number of injured *S. typhimurium* cells grown at pH 7.5 and pH 5.5 to the various phases of growth, in contact with gastric fluid for 4 min was minimal (data not shown).

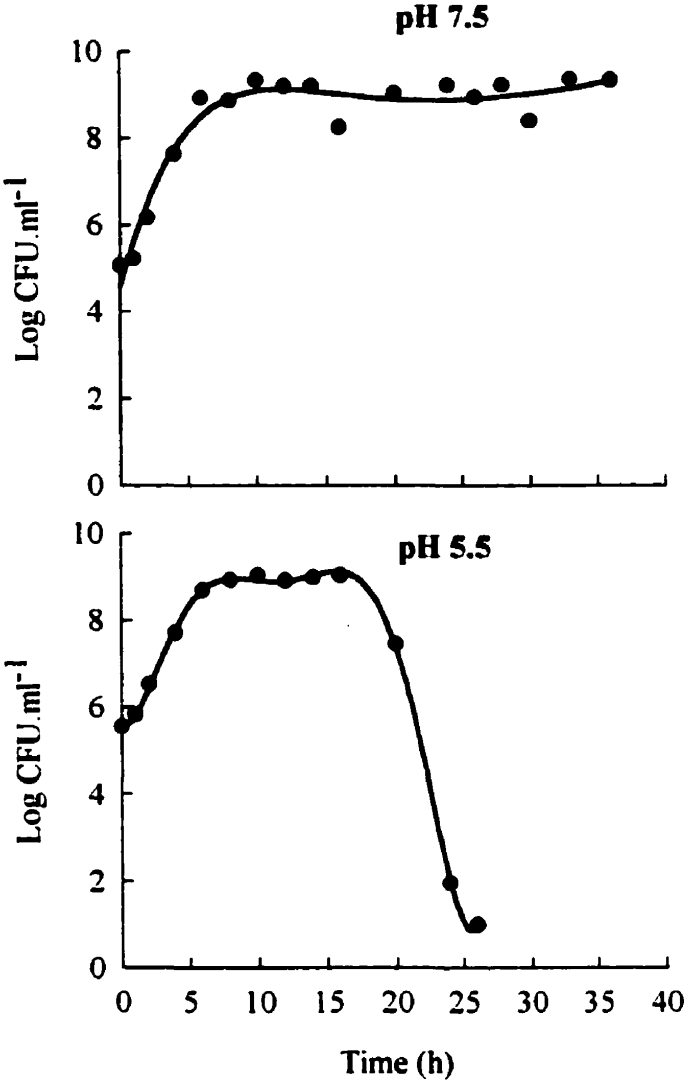

Figure 1. Growth of *S. typhimurium* in BHIB adjusted to pH 7.5 and 5.5. Cultures were grown at 37°C with shaking at 200 rev.min^{-1}.

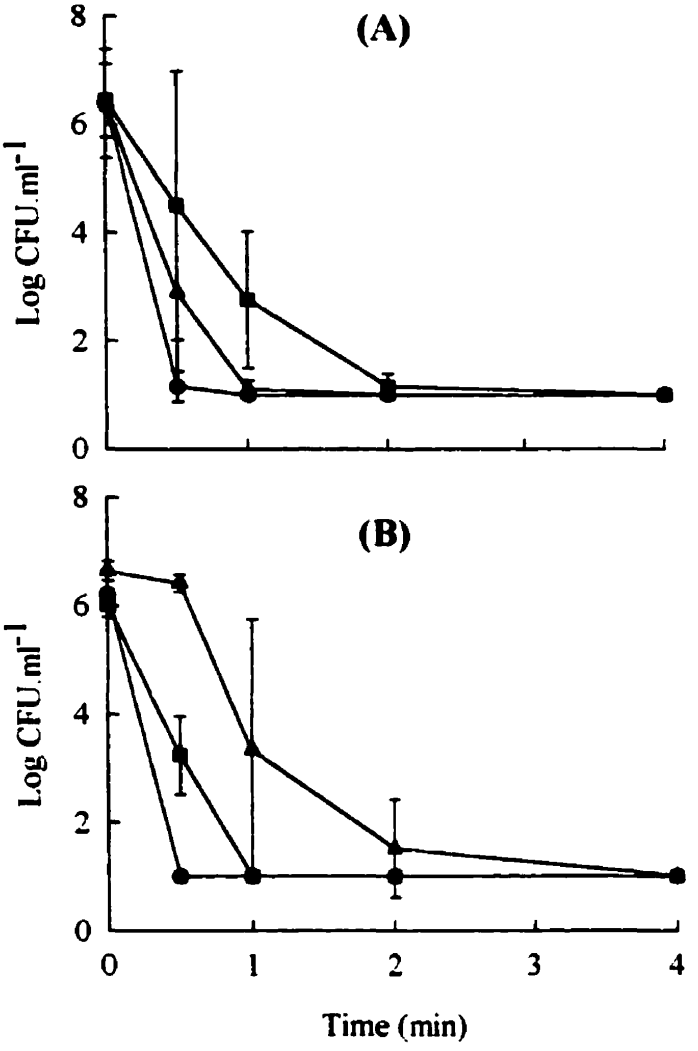

Figure 2. Survival of *S. typhimurium* cells grown in BHIB pH 7.5 (A) and pH 5.5 (B) to mid-log —●—, early stationary—■— and late stationary phase—▲—, in contact with simulated gastric fluid for various periods of time.
Data points: average of triplicate results ± SD (95% confidence limit).

In ileal fluid, the number of viable cells did not vary much within 24 h, regardless of the physiologic age of the cells, when the initial cell concentration was 10^6 CFU.ml^{-1} (Figures 3A and 3B). The cell concentrations were always 1 or 2 logs higher than the initial cell concentration after 24 h of incubation.

In colon fluid, cells at the different phases of growth reached the highest concentration (>10^8 CFU.ml^{-1}) after 8 h, except early stationary phase cells grown at pH 7.5 which reached the highest concentration (10^8 CFU.ml^{-1}) after 16 h of incubation (Figures 4A and 4B). In both instances, a slight decrease in viable cell number followed.

Since reduction of viable cell number in gastric fluid was >5 logs within 2 min, the survival of low number of organism was tested only in ileal (Figures 3C and 3D) and colon fluids (Figures 4C and 4D). Cell numbers increased by *ca.* 5 logs within 16 h and then remained stationary or decreased slightly.

The number of viable cells counted on BHIA and BGS Agar when surviving cells were determined in the ileal and colon fluids (using both high and low inocula), was essentially similar, regardless of growth phase or initial growth pH (data not shown).

3.2.1.2. Survival during sequential exposure to simulated fluids

The survival of *S. typhimurim* during its sequential transfer from one simulated environment to the other is shown in Figures 5 and 6. Cells grown in BHIB at pH 7.5 (Figure 5) and pH 5.5 (Figure 6) were first incubated for 0.5 min in gastric fluid and then transferred into ileal fluid. After 0.5 min in gastric fluid, the number (pH 7.5 and pH 5.5) of mid-log phase cells was reduced from 10^5 to *ca.* 10 CFU.ml^{-1} as previously observed (Figure 2). The

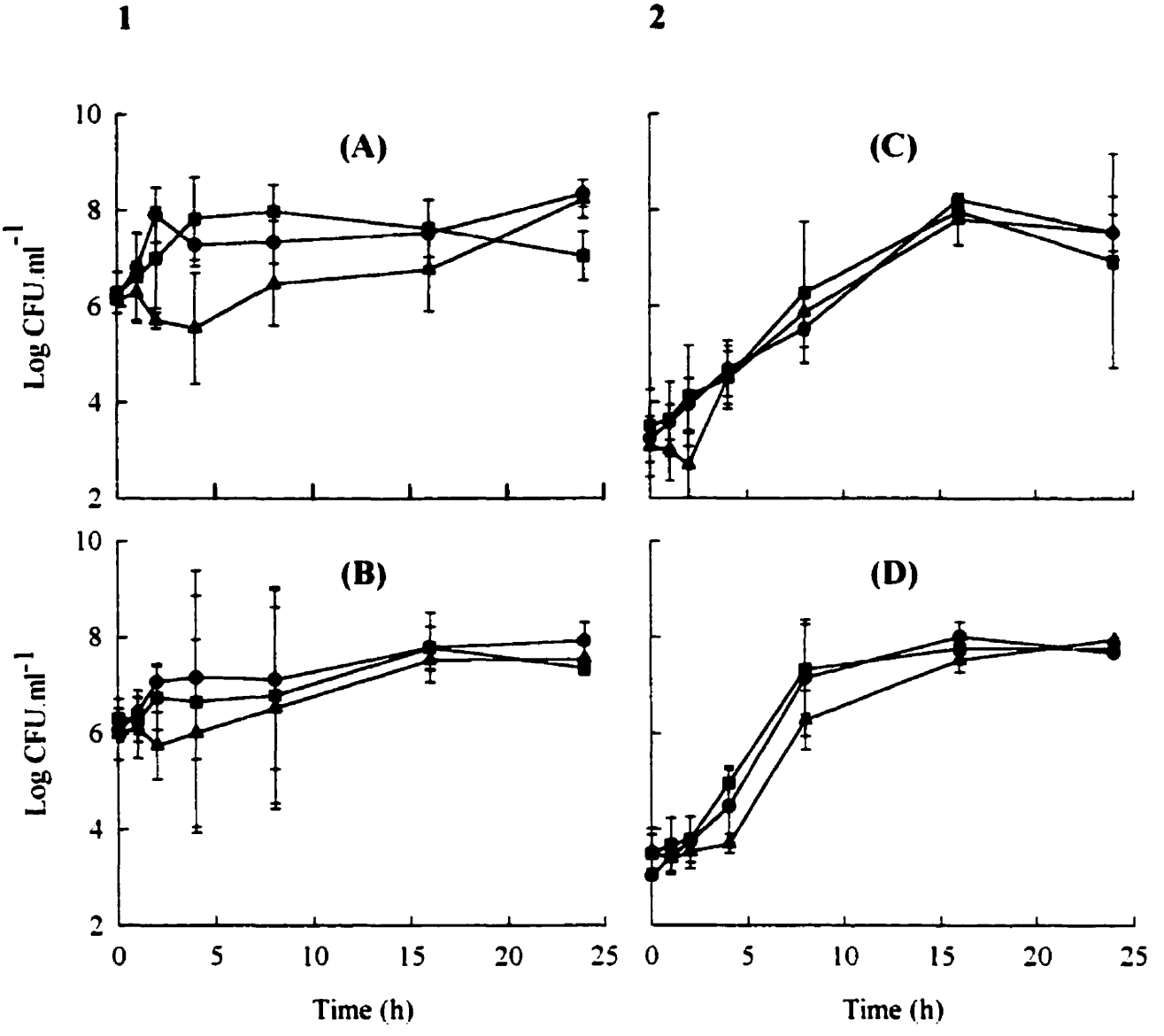

Figure 3. Survival of *S. typhimurium* cells grown in BHIB pH 7.5 (A and C) and pH 5.5 (B and D), to mid-log —●—, early stationary —■— and late stationary phase —▲—, in contact with simulated ileal fluid for various periods of time. High (1) and low (2) initial number of cells were used to inoculate ileal fluid. Data points: average of triplicate results ± SD (95% confidence limit).

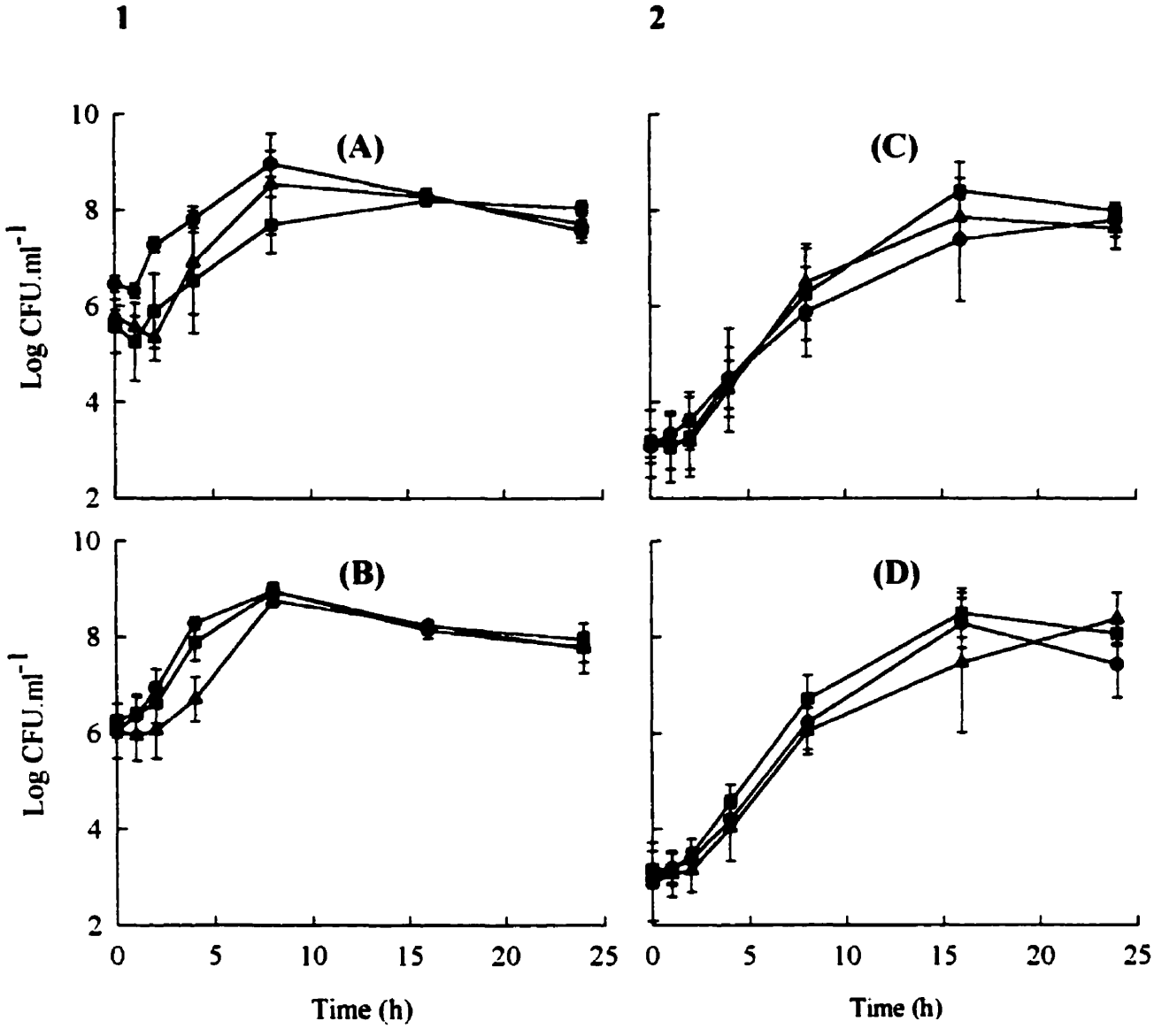

Figure 4. Survival of *S. typhimurium* cells grown in BHIB pH 7.5 (A and C) and pH 5.5 (B and D), to mid-log —●—, early stationary —■— and late stationary phase —▲—, in contact with simulated colon fluid for various periods of time. High (1) and low (2) initial number of cells were used to inoculate colon fluid. Data points: average of triplicate results ± SD (95% confidence limit).

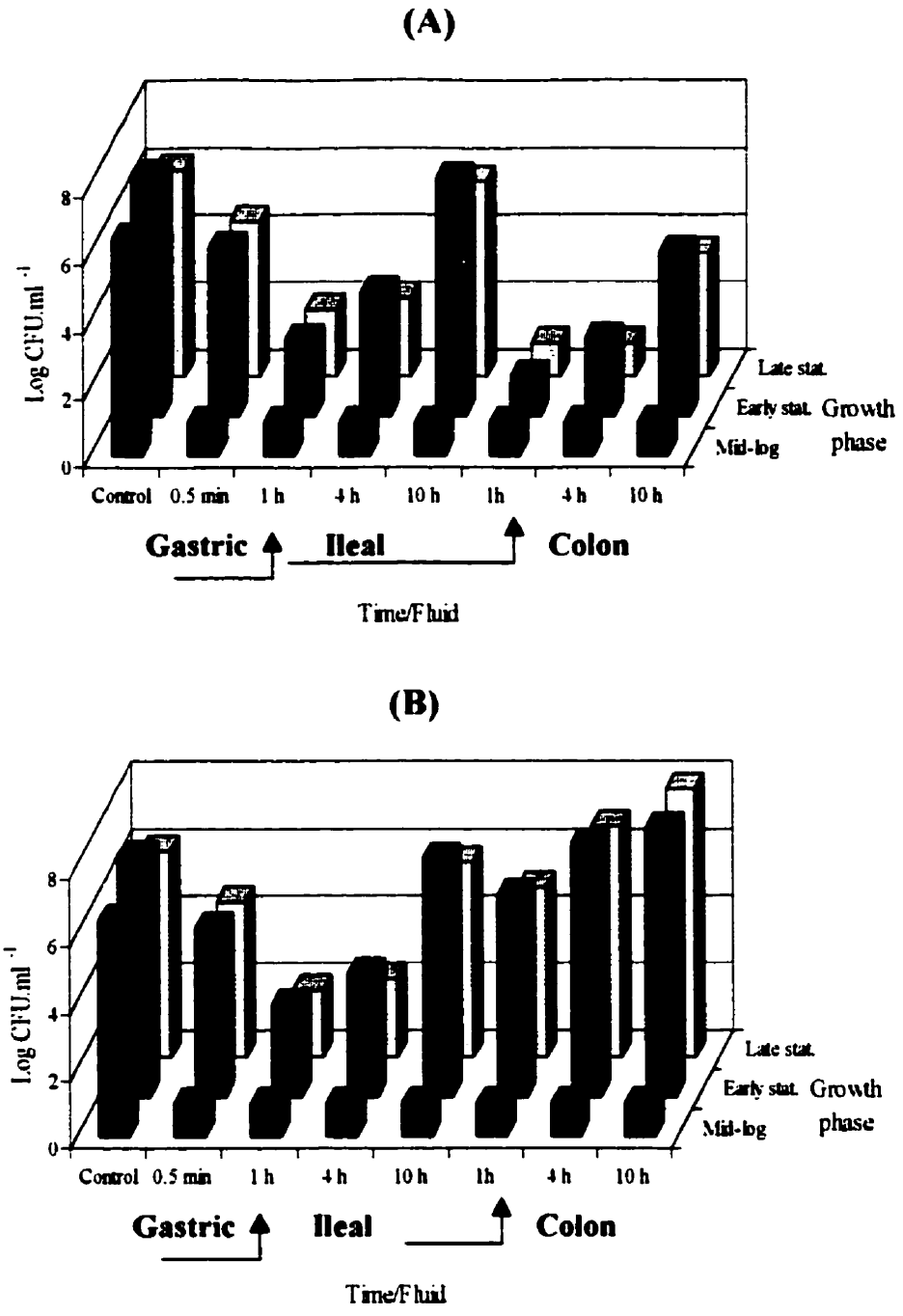

Figure 5. Survival of *S. typhimurium* (grown at pH 7.5) during sequential exposure to simulated fluids. (A), 1 ml of culture was added to 9 ml of gastric fluid. After 0.5 min of contact, surviving organisms were determined. At the same time, 1ml of inoculated gastric fluid was transferred from gastric fluid to ileal fluid. After 1, 4 and 10 h of contact, surviving populations were determined. At 1 h, 1 ml from the inoculated ileal fluid was transferred into 9 ml of colon fluid. Viable cells in colon fluid were enumerated after 1, 4 and 10 h. (B), Same as in A except that 1 ml of inoculated ileal fluid was transferred into colon fluid after 10 h. Arrows indicate transfer points.
Data points: average of triplicate results.

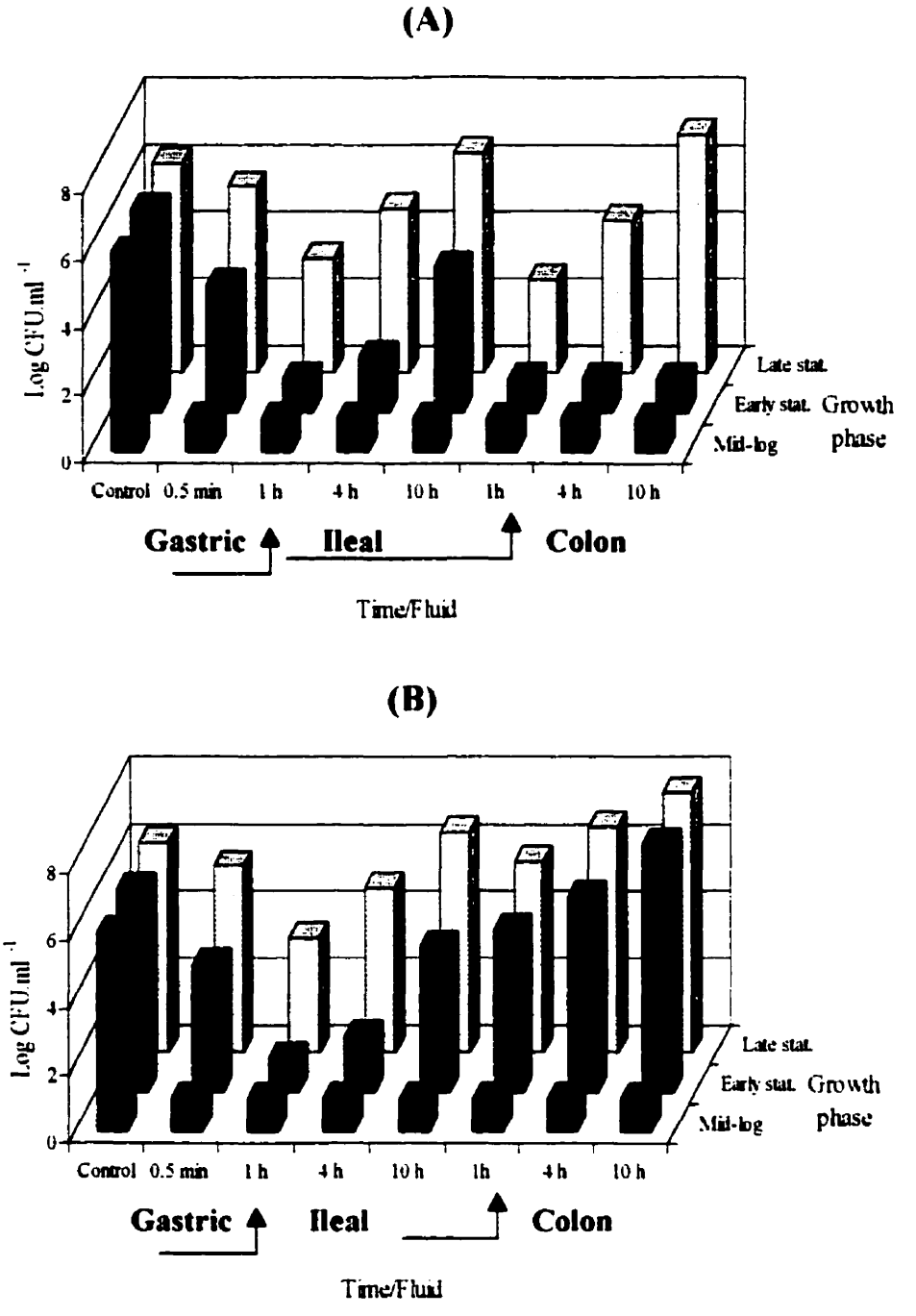

Figure 6. Survival of *S. typhimurium* (grown at pH 5.5) during sequential exposure to simulated fluids. (A), 1 ml of culture was added to 9 ml of gastric fluid. After 0.5 min of contact, surviving organisms were determined. At the same time, 1ml of inoculated gastric fluid was transferred from gastric fluid to ileal fluid. After 1, 4 and 10 h of contact, surviving populations were determined. At 1 h, 1 ml from the inoculated ileal fluid was transferred into 9 ml of colon fluid. Viable cells in colon fluid were enumerated after 1, 4 and 10 h. (B), Same as in A except that 1 ml of inoculated ileal fluid was transferred into colon fluid after 10 h. Arrows indicate transfer points.
Data points: average of triplicate results.

cells did not recover after 10 h of incubation in ileal fluid and a further 10 h incubation in colon fluid. With early and late stationary phase cells (pH 7.5), 0.5 min of contact in gastric fluid resulted in an *ca.* 2-log reduction in number. There was a further 2-log decrease after I h of incubation in ileal fluid, part of which was due to the dilution factor of transferring 1 ml of inoculated gastric fluid into 9 ml of ileal fluid. However, the cells recovered and increased by *ca.* 5 logs within 10 h in ileal fluid. An initial reduction in cell number (*ca.* 1-log reduction) was observed when early and late stationary phase cells were transferred from ileal to colon fluid after 1 h (Figure 5A) and 10 h (Figure 5B) in ileal fluid. However, incubation for 10 h in colon fluid resulted in cell increase from *ca.* 10^1 to 10^5 CFU.ml^{-1} and *ca.* 10^6 to 10^8 CFU.ml^{-1} following 1 h and 10 h prior exposure to ileal fluid, respectively.

The same pattern of growth was observed with cells grown at pH 5.5 (Figure 6), except that early stationary phase-cells did not show any increase in cell population within10 h of incubation in colon fluid, after being in contact with ileal fluid for 1 h.

3.2.2 Survival of *Salmonella* entrapped in calcium alginate beads

The effect of encapsulation (Figure 7A) on the survival of *S. typhimurium* in contact with gastric fluid can be seen in Figure 8. Since pH 7.5 and pH 5.5 grown cells were found to be equally sensitive to gastric fluid (Figure 2), only pH 7.5 grown cells were encapsulated and exposed to the gastric fluid. The viable counts of cells encapsulated in calcium alginate beads decreased only 1-2 logs after 8 min of contact in gastric fluid (Figure 8A). After 24 min of contact, viable cells were still recovered. The survival rate of these entrapped cells in gastric fluid is about 25 times that of cells in culture.

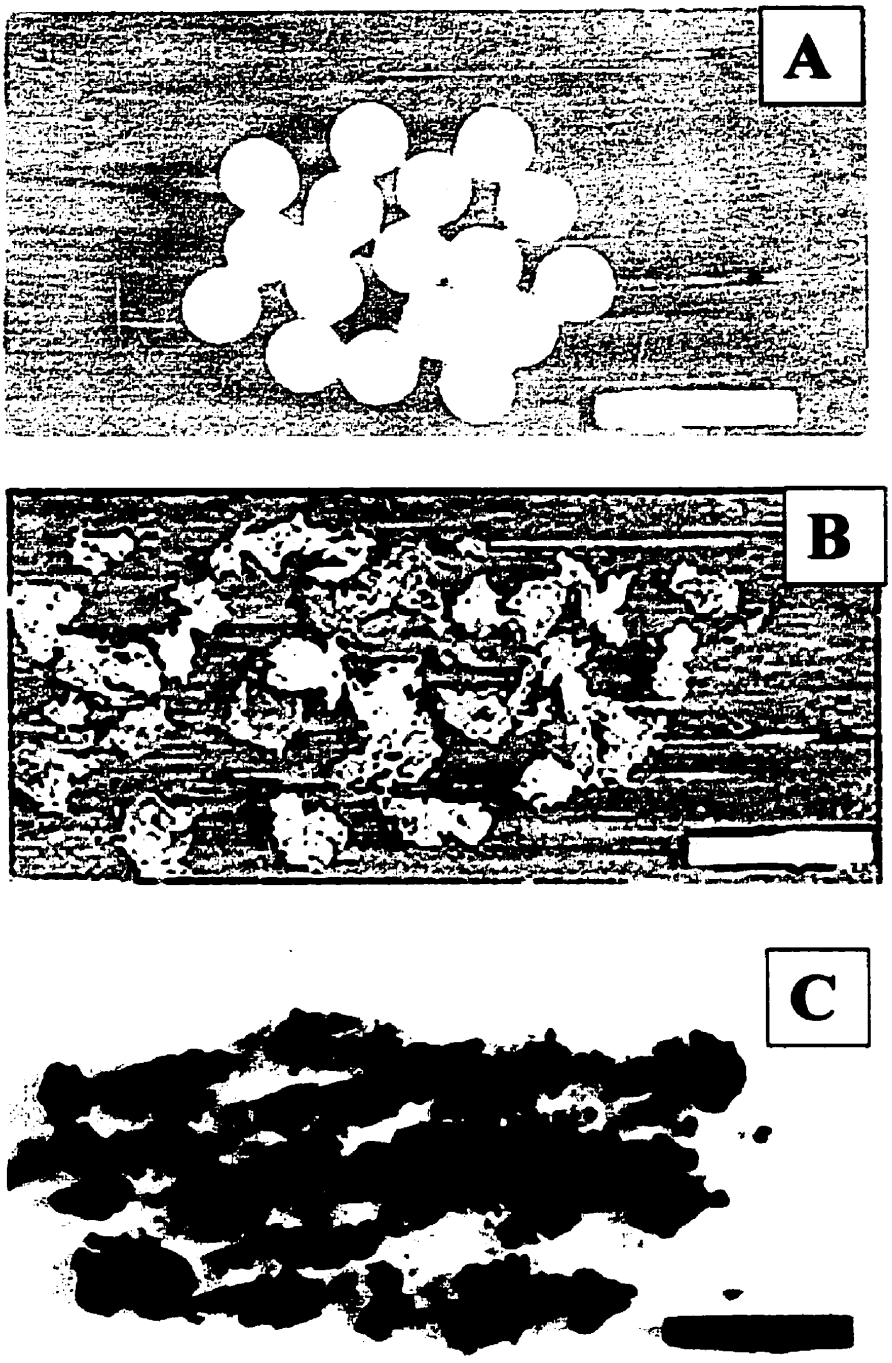

Figure 7. Whole calcium alginate beads (A), crushed calcium alginate beads (B), and chopped chocolate (C). Bar = 1 cm.

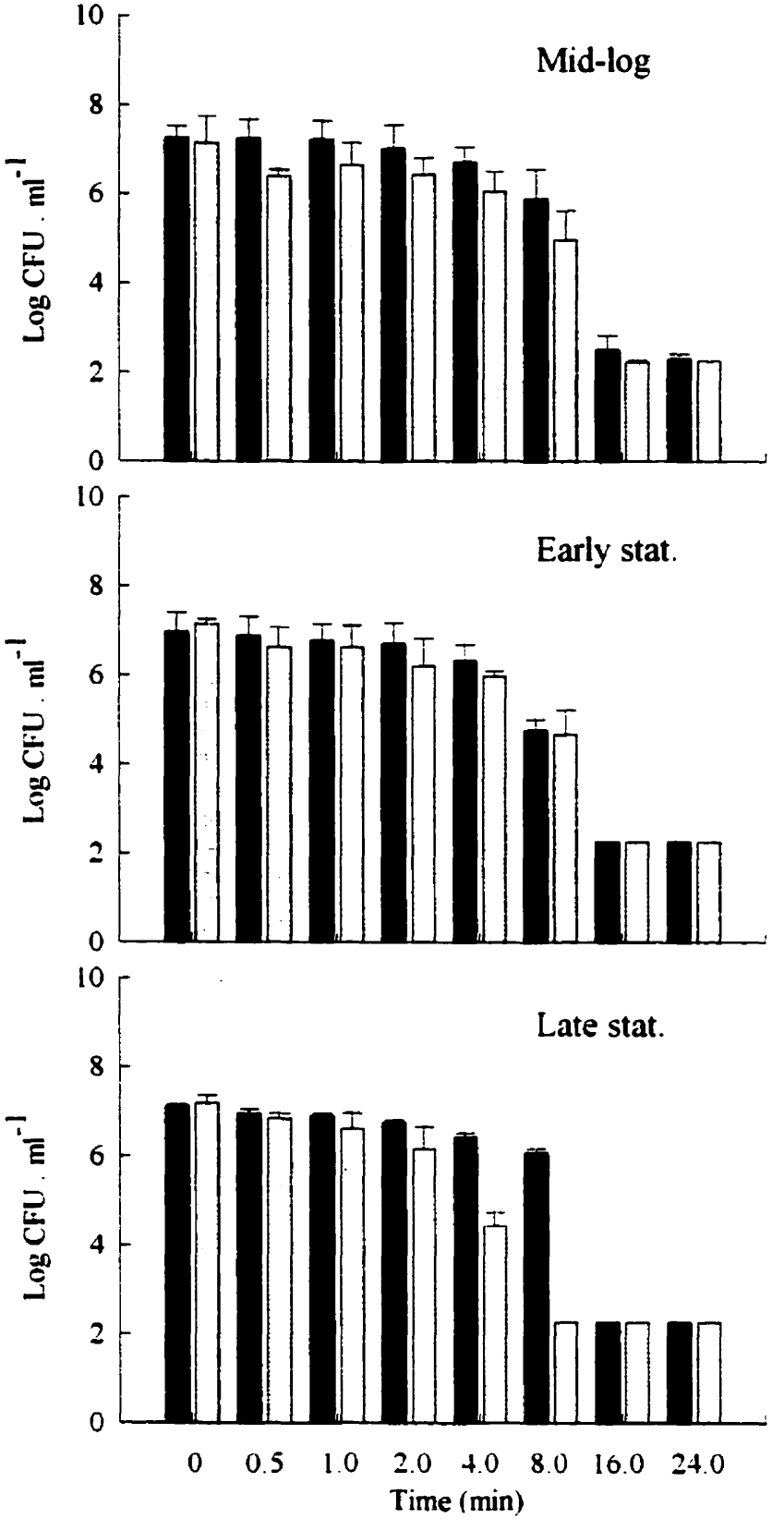

Figure 8. Survival of *S. typhimurium* cells grown to mid-log, early stationary, and late stationary phases of growth embedded in whole calcium alginate beads (■) and crushed calcium alginate beads (□) in contact with simulated gastric fluid for various periods of time.
Data points: average of triplicate results ± SD (95% confidence limits).

Crushing of the beads to particles of sizes 1 to 2.5 mm (Figure 7B) had very little effect on the survival of mid-log- and early stationary-phase cells in gastric fluid (Figure 8B). The cells were reduced by *ca.* 2 logs after 8 min of exposure. However, late stationary-phase cells were reduced by *ca.* 2 logs only after 4 min and by 5 logs after 8 min. The difference between the number of viable late stationary-phase cell embedded in whole and crushed calcium alginate beads (after 4 min and 8 min of exposure to gastric fluid) was found to be highly significant ($p<0.001$). Survivors were still present after 24 min. Essentially, very low or no number of injured cells was isolated from gastric juice when cells were embedded in whole or crushed calcium alginate beads (data not shown).

3.2.3 Survival of *Salmonella* entrapped in chocolate

Cells embedded in chocolate chips were essentially not affected by exposure to gastric fluid (Figure 9). There was only *ca.* 1 log decrease in the viable cell population after 24 min of contact. There was no evidence of injured cells (data not shown).

3.3 EFFECTS OF GASTRIC ACIDITY, GROWTH pH AND GROWTH PHASE ON THE LPS PROFILE OF *SALMONELLA*

Silver-stained PAGE profiles of LPS from *S. typhimurium* of different physiological ages grown in BHIB (pH 7.5 and 5.5) are shown in Figure 10. The analysis revealed a "ladder" pattern typical of LPS. *S. tyhimurium* showed the same LPS profile, regardless of initial growth pH or growth phase. Cells that were exposed to simulated gastric fluid for 0.5 min and 4.0 min also showed similar LPS profiles as unexposed cells.

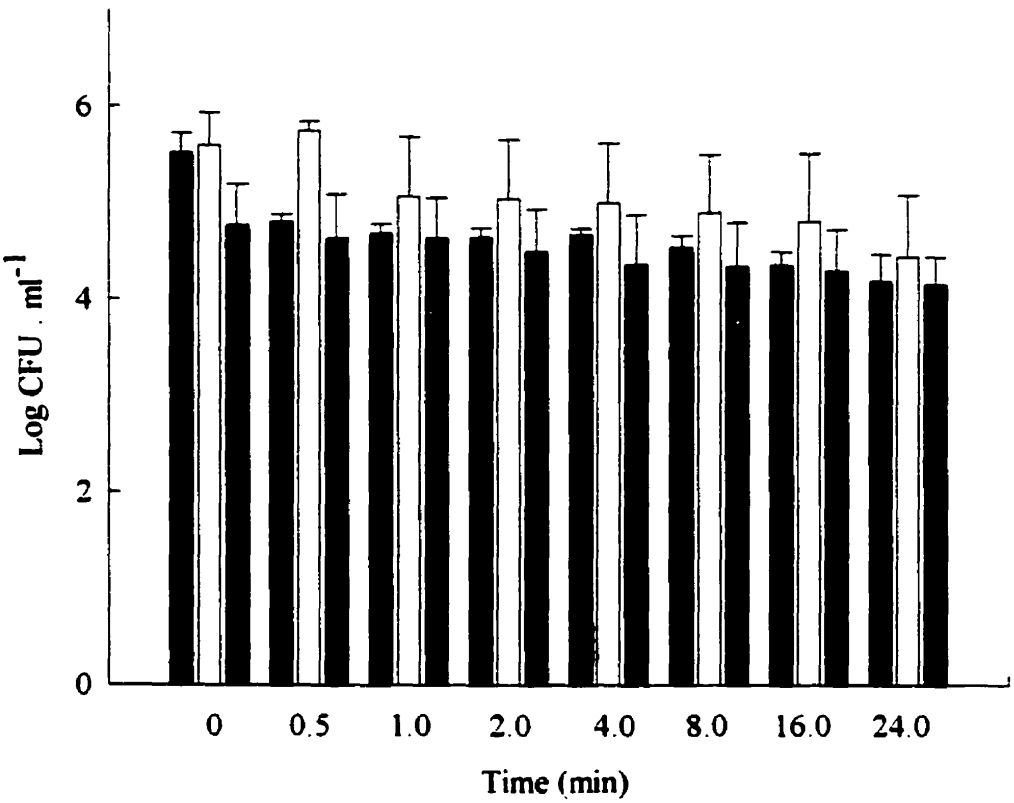

Figure 9. Survival of *S. typhimurium* cells grown to mid-log (▬) early stationary (▭), and late stationary (▬) phases of growth embedded in crushed chocolate and exposed to simulated gastric fluid for various periods of time.
Data points: everage of triplicate results ± SD (95% confidence limits).

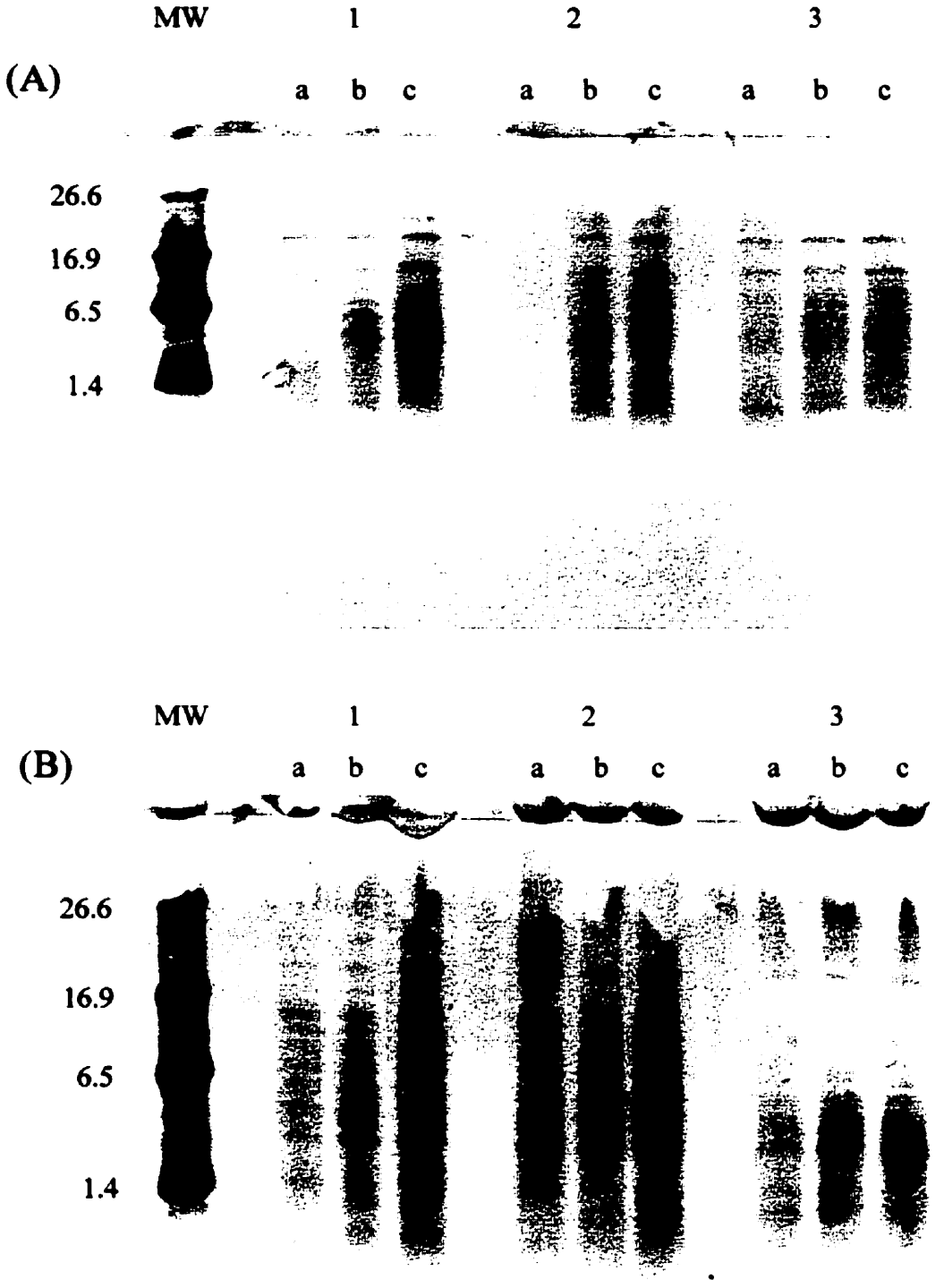

Figure 10. Silver stained Tricine-SDS-PAGE of LPS from *S. typhimurium* grown at 37°C in BHIB at pH 7.5 (A) and pH 5.5 (B) with shaking (200 rev.min^{-1}). Cells were grown to mid-log phase (1), early stationary phase (2), and late stationary phase (3) and exposed to simulated gastric fluid for 0.5 min (b) 4.0 min (c). Cells that were not exposed to gastric fluid served as controls (a)
MW is the Protein Molecular Weight Marker in kDa.

The amount of residual protein detected in the various crude LPS preparation was negligible (data not shown).

4.0 DISCUSSION

Research on the survival of ingested pathogenic microorganisms in the GIT is important in order to analyse the risk of foodborne pathogens (Notermans *et al.*, 1994). Ingested *Salmonella* cells need to overcome the bactericidal action of the acidic pH of the gastric micro-environment before entering the lumen of the small intestine where they precipitate an inflammatory reaction (D'Aoust, 1990). While travelling through the GIT, they must endure the extremely low pH in the stomach, as well as volatile fatty acids present in the intestine.

Several factors influence the survival of pathogens in the stomach, including pH, the number of organisms ingested, the vehicle in which they are ingested, physical protection of bacteria by food, buffering of gastric content and the rate of gastric emptying (Gianella *et al.*, 1972). In our study, the effects of growth pH, physiologic age of cells, initial cell population and entrapment in calcium alginate beads and chocolate, on the survival of *S. typhimurium* in simulated GIT juices were investigated. Cells in their mid-log, early stationary, and late stationary growth phases were destroyed within 4 min in simulated gastric fluid of pH 1.5. This result is consistent with that of Idziak and Boray (unpublished data) in similar experiments done in our laboratory. In gastric fluid survival experiments done by Roering *et al.* (1999), with three phage types of *S. typhimurium*, *L. monocytogenes*, and *E. coli*, none of the *Salmonella* strains survived after 5 min in simulated gastric fluid at pH 1.5. They noted that the cell concentration decreased by 5.5 log CFU during this period. Schlech *et al.* (1993) demonstrated the killing of *L. monocytogenes* serotype 4B in saline at pH 2.0 whereas, over 80% of the inoculum survived the effect of exposure to acid at pH 6.0 after 15 min. The same

result was also obtained by Peterson *et al.* (1989). In their experiment, the growth of *E. coli*, *S. flexneri* and *S. typhimurium* strains were inhibited at pH ≤3.4 to 4.3, and the cells were killed at pH <1.5 to 2.5. Gianella *et al.* (1972) reported that there was no reduction in the number of *S. marcescens* introduced into fasting stomachs of three patients with pernicious anaemia (gastric pH greater than 6.8). In contrast, cells were reduced by 99% within 15 min in the normal stomach (gastric pH less than 3.0) and no viable cells were recovered after 1h.

Although our studies showed essentially no difference in survival rate of cells of different physiologic age, there have been reports that bacteria in their stationary phase, as compared to cells in their log phase of growth, are more resistant to various environmental stresses, including low pH. Arnold and Kaspar (1995) demonstrated the growth-dependence of acid tolerance in *E. coli* 05157:H7. They determined that mid-log phase cultures were more acid sensitive than stationary phase cultures. Pagan and Mackey (2000) also showed that stationary phase cells of *E. coli* 0157:H7 were more resistant to high pressure treatment than log phase cells. The ability of *S. flexneri* to survive low pH was dependent on time, pH and growth phase. Maximal acid resistance is exhibited at stationary phase and is dependent on *rpoS* which encodes a stationary-phase sigma factor (Gorden and Small, 1993).

S. typhimurium was grown in BHIB at pH 5.5 before introduction into gastric fluid to determine if previous exposure to a moderately low acidity would enhance its survival in gastric fluid at pH 1.5. No enhancement was observed. Arnold and Kaspar (1995) also reported that the acid tolerance of *E. coli* 0157:H7 was not dependent on prior exposure to a low pH. On the other hand, several other investigators have reported that a prior exposure to moderate acid pH's enhances the survival of *Salmonella* species at a low pH. Tsai and

Ingham (1996) reported that acid-adaptation at pH 5.0 enhanced the survival of salmonellae in ketchup at pH 3.6. Acid adaptation also prolonged the survival of *S. typhimurium* in cheeses and during milk fermentation (Leyer and Johnson, 1992).The reason why there was no acid tolerance observed in cells grown at pH 5.5 may be due to the possibility that acid tolerance works to a certain extent. In our study, cells were shifted from BHIB of pH 5.5 to gastric fluid of pH 1.5. Perhaps acid tolerance would have been observed if the cells were shifted to pH of *ca.* 3.0. Most studies on acid adaptation reported a shift of bacterial cells from media of pH 5.0 to that of *ca.* 2.8 to 3.8 (Leyer and Johnson, 1992; Tsai and Ingham, 1996; Kwon and Ricke, 1998).

Survival of *S. typhimurium* was also monitored in ileal and colon fluids. Both high and low inocula were used to determine the effect of initial cell population on survival in these environments. As shown in Figures 3 and 4, cells survived and grew rapidly in both fluids, regardless of previous growth pH, growth phase and initial cell number. Cells increased to 10^8 $CFUml^{-1}$ in 24 h when about 10 to 100 cells were added to these fluids. This indicates that the survival of *S. typhimurium* in the ileum and colon is not affected by growth pH, growth phase or the number of ingested cells.

The high survival rate of salmonellae in ileal and colon fluids was further illustrated when cells were transferred from one fluid to another. Although other authors have studied the effects of gastric, ileal and colon fluids on *Salmonella* spp., this is the first time, to our knowledge, that one has studied the result of sequential passage through gastric, ileal and colon fluids. The passage of salmonellae through these fluids was done to determine the ability of salmonellae to recover in the ileum and colon after exposure to high acidity in the

stomach. Results obtained show that the ileal and colon environments highly favour the growth and multiplication of salmonellae. In these environments, as long as cells survive the high acidity, cell numbers will be reached sufficient to cause illness. This suggests that the number of surviving bacteria reaching the intestines does not necessarily affect the severity of the disease, but perhaps does affect the time of onset of the disease, once the pH barrier of the stomach has been breached. Schlech *et al.* (1993) reported that the ability of virulent *L. monocytogenes* to establish an invasive infection in Sprague-Dawley rats is dose-dependent.

In general, results obtained in our experiments correlate with those of other investigators that described gastric pH as the barrier to intestinal pathogens (Garrod, 1939; Gianella *et al.*, 1972, 1973; Charteris *et al.*, 1998).

It has been reported that the micro-encapsulation of bacteria can ensure a greater survival in gastric and intestinal environments (Lee and Heo, 2000; Rao *et al.*, 1989). Immobilized cells exhibit many advantages over free cells, including the maintenance of stable and active biocatalyst, and the protection of cells against damage (Scott, 1987). Many methods have been developed to immobilize or encapsulate cells. Rao *et al.* (1989) described a method to encapsulate freeze-dried *Bifidobacterium pseudolongum* using cellulose acetate phthalate (CAP). CAP is an enteric coating material used for control of drug release in the intestine (Sun and Griffiths, 2000). In our study, calcium alginate was used to encapsulate *Salmonella* cells before exposure to gastric acidity to determine the effect of encapsulation on their survival. Alginate is non-toxic to cells being immobilized, and it is an accepted food additive (Sheu and Marshall, 1993). The beads were prepared to be spherical and to be

relatively uniform in size. The cells used here were grown at pH 7.5. Cells grown at pH 5.5 were not used since survival in gastric fluid was previously shown not to be different from that of cells grown at pH 7.5. As shown in Figure 8, viable cells were recovered even after 24 min of exposure to gastric acidity. The entrapped cells survived a lot longer than cells in culture fluid. Results suggest that calcium alginate protects salmonellae against the acidic effect of gastric fluid. In all probability, the acid partially penetrates the calcium alginate beads but not completely to reach the inner core where the bacteria would remain untouched by the acid. Similar observations have been made by several investigators. Lee and Heo (2000) showed that encapsulated *Bifidobacterium longum* in calcium alginate beads were more resistant than unencapsulated *B. longum* against the effect of simulated gastric fluid (pH 1.55). Sheu *et al.* (1993) also reported that calcium alginate provided protection of *Lactobacillus bulgaricus* L2 from freezing damage, although their data did not show whether protection was due to chemical properties of alginate (such as water binding or interaction between the polymer and cell wall), the gel structure, or both.

Similar results were obtained from survival determinations of salmonellae in crushed calcium alginate beads. This was done to determine the effect of mastication, as well as reduced bead size, on *S. typhimurium* survival in gastric fluid. No difference was observed between the gastric acid tolerance of cells entrapped in crushed beads and that in whole beads except that of late stationary-phase cells which survived a little longer in whole beads. This implies that mastication might not have any effect on the protection food offers ingested microorganisms. This was a bit surprising because crushing the bead reduced the bead size as well as the distance between entrapped cells and the gastric fluid environment. Perhaps

gastric acid would have had more effect on the entrapped cells if the crushed calcium alginate particles were smaller in size. The survival of bifidobacteria is strongly dependent on various factors, including alginate concentration and bead size. The survival of cells in beads is higher with larger beads (Lee and Heo, 2000). Sheu *et al.* (1993) indicated that larger bead (diameter, 102 μm) provided more protection for *L. bulgaricus* in frozen desserts than smaller-sized beads (diameter, 30μm) although, according to the authors, the difference in the level of protection provided by large- and small-sized beads was not significant.

Salmonella cells were also entrapped in chocolate to investigate whether a food source high in fat would have a protective effect on its survival in gastric fluid. Preliminary studies were done to determine the survival of *Salmonella* cells in chocolate. Results obtained (data not shown) indicated a 4 log-decrease in viable cell counts during the cooling of the inoculated chocolate from 45°C to 10°C (20 min). This result was unexpected as salmonellae are capable of growth at 45°C and have been known to survive temperatures as high as 90°C in milk chocolate (Goepfert and Biggie, 1968). This decrease in microbial numbers was probably due to the inability of a portion of the population to adjust to the low A_w environment of the chocolate. Because of this large reduction in viable cell counts, a very high number of cells (10^9 CFU.g^{-1}) was used to inoculate chocolate so that enough viable cells were available for the survival studies in gastric fluid. Compared to results in Figure 2, chocolate, in one way or another, promotes the survival of *S. typhimurium* in gastric fluid. There was little, if any, reduction in the viable population in the chocolate chips suspended in the simulated gastric fluid. *S. typhimurium* inoculated onto ground beef and boiled egg white was also protected from killing in Luria-Bertani (LB) broth at pH 2.5 whereas, cells

inoculated onto boiled rice were not protected (Waterman and Small, 1998). The authors suggested that the survival of acid-sensitive bacteria on the surface of ground beef is most probably the result of the ability of ground beef to raise the pH of the acidified medium at the microenvironment occupied by the bacteria. Reduction of gastric acidity has been associated with an increase in the survival rates of some food-borne pathogens resulting in a lowering of infective dose (Schlech *et al.*, 1993). In our study, the pH of simulated gastric fluid did not vary when chocolate chips or calcium alginate beads were added to gastric fluid. At present, it is not clear exactly how food protects bacteria from acidic conditions. It has been speculated by D'Aoust (1985) that *Salmonella* outbreaks with low infectious dose are often associated with a food source with a high fat content such as chocolate and cheese. This led to the hypothesis that the fat content of contaminated foods may play a significant role in human salmonellosis. The rationale behind this hypothesis is that organisms trapped in hydrophobic lipid moieties may readily survive the acidic conditions of the stomach and pass on to the intestinal tract. However, Waterman and Small (1998) observed that boiled egg white, which is low in fat protected *S. typhimurium* from killing by acid.

The role food plays in enhancing the survival of ingested pathogens might explain the controversy regarding the minimum number (infective dose, ID) of salmonellae necessary to produce illness in humans. Several authors have reported an ID of $\geq 10^5$ CFU of *Salmonella meleagridis* or *Salmonella anatum* when cells in culture were fed to volunteers (McCullough and Eisele, 1951a, b, c). Other authors have reported a lower ID of salmonellae when ingested with food. Greenwood and Hooper (1983) reported a case where 50 CFU of *S. napoli* in chocolate caused illness. D'Aoust (1985) reported 1 to 6 CFU of *S. typhimurium*

in cheddar cheese. These reports indicate that *Salmonella* spp. can cause infection at a much lower ID if they are ingested with a food source.

The envelope macromolecules of gram negative bacteria undergo considerable variation at different growth rates and when specific nutrients are limiting (Brown and Williams, 1985) and bacterial LPS has been shown to vary with changes in environmental and growth conditions, including temperature, osmolarity and pH (Idziak and Suvanmongkol, 1971; Benedi *et al.*, 1991; Tao *et al.*, 1992). Abdullah and Idziak (unpublished data) determined that the level of LPS expressed by *Salmonella* spp. is dependent on growth phase and pH. They also showed that LPS profiles of both log phase- and early stationary phase-*S. typhimurium* at pH 7.5 and pH 9.5 remained the same. In our study, the LPS from *S. typhimurium* cells, which were grown at pH 7.5 and 5.5 to different phases of growth, were analysed to determine the effect of growth phase and growth pH on LPS profile. LPS from cells that were exposed to gastric fluid were also analysed to determine the effect, if any, of gastric acidity on the LPS profile of *S. typhimurium.* It was observed that the LPS from cells exposed to gastric fluid had the same profiles as unexposed cells. Perhaps the exposure time of these cells to gastric fluid was too short to cause a significant change in LPS profile. The results also indicated that the LPS profiles from cells grown at pH 7.5 and 5.5 and at different growth phases did not vary. Leyer and Johnson (1993) reported that the LPS components from S. *typhimurium* cells grown at pH 5.8 were the same as those from cells grown at pH 7.6. However, McGowan *et al.* (1998) determined that *H. pylori* cells exhibited altered LPS profiles when grown at pH 5.0 compared with cells grown at neutral pH. Gonococcal cells also produced an additional LPS band when cells were transferred from pH 8.2 to pH 5.8,

indicating pH regulation of LPS profiles (Pettit *et al.*, 1995).

A negligible amount of residual protein was detected when Bradford's assay was used to analyse the crude LPS extract. Darveau and Hancock (1983) also detected a low level of protein when they examined the LPS preparations for protein by the method of Lowry *et al.* (1951). They suggested that the results obtained with the protein assay may be due to other compounds, including EDTA, hexosamines, and lipids known to interfere with protein determinations in this type of assay.

CONCLUSION

S. typhimurium was killed in simulated gastric fluid of pH 1.5 within 4 min of exposure. The initial growth pH and the growth phase of this organism did not affect its survival in this environment; however, only the surviving early and late stationary phase cells were capable of initiating growth when transferred to the ileal fluid. When *S. typhimurium* cells were added directly to the ileal and colon fluids, growth ensued irrespective of whether low (10^2 to 10^3 CFU. ml^{-1}) or high (10^5 to 10^6 CFU.ml^{-1}) number of cells served as inocula and of the physiological age of the cells. *S. typhimurium* also survived longer in gastric fluid when it was entrapped in calcium alginate beads and chocolate chips. These protected the bacteria from the lethal effect of gastric acidity. This may help explain the low infective dose of *Salmonella* and the rapid appearance of symptoms after the consumption of contaminated food. *S. typhimurium* LPS composition (profile) remained unchanged, regardless of initial growth pH, growth phase or exposure to gastric acidity.

APPENDIX

Table 1. Composition of simulated gastric fluid.

Compound	Concentration (l^{-1})
Proteose-peptone	8.3 g
D-Glucose	3.5 g
KH_2PO_4	0.6 g
$CaCl_2.H_2O$*	0.2 g
KCl	0.37 g
Bile salts no 3*	0.05 g
Lysozyme	0.1 g
Pepsin	13.3 mg

*Modified from Beumer *et al.* (1992), pH = 1.5, adjusted with 1M HCl.

Table 2. Composition of simulated ileal fluid.

Compound	Concentration (l^{-1})
Proteose-peptone	5.7 g
D-Glucose	2.4 g
NaCl	6.14 g
$KH_2PO_4.H_2O$*	0.68 g
NaH_2PO_4	0.5 g
$NaHCO_3$	1.01 g
Bile salts no 3*	5.6 g
Lysozyme	0.2 g
α-amylase	1 000 U
Lipase	960 U
Trypsin	110 U
Chymotrypsin	380 U

*Modified from Beumer *et al.* (1992), pH = 7.2.

Table 3. Composition of simulated colon fluid.

Compound	Concentration (l^{-1})
Proteose-peptone	6.25 g
D-Glucose	2.6 g
NaCl	0.88 g
KH_2PO_4	0.43 g
$NaHCO_3$	1.7 g
$KHCO_3$	2.7 g
Bile salts no 3*	4.0 g

*Modified from Beumer *et al.* (1992), pH = 8.0

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