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GROWTH, SURVIVAL AND ADAPTATION OF *Shigella flexneri* UNDER ACIDIC CONDITIONS RELEVANT TO THOSE APPLIED DURING DAIRY PROCESSING

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ABSTRACT

Shigella flexneri is an important fooborne pathogen that has been associated with dairy products. This study aimed to assess the growth, survival and adaptation of *S. flexneri* under acidic conditions relevant to those adopted in dairy processing. *S. flexneri* could grow at mildly acidic pH values of 6.0 and 5.0 adjusted by HCl, lactic acid, or acetic acid, but at slower growth rates, compared to unacidified conditions of pH 7.3. Lower pH values ranging from 3.0 to 4.5 caused declines in the initial numbers of *S. flexneri*, but the pathogen could survive exposure to pH 4.0 and 3.5 for variable times depending on the acidifying agent. The order of sensitivity of *S. flexneri* to acids at those lower pH values were acetic acid > lactic acid > HCl. The survival of *S. flexneri* under the acidic conditions of yoghurt prepared from full fat (6%) and half fat (3%) buffalo's milk was also studied. The viable numbers of *S. flexneri* were generally higher in full fat yoghurt than half fat yoghurt on milk coagulation and during the cold storage of yoghurt. *S. flexneri* could survive for at least 14 days in full fat yoghurt, compared with 7 days in half fat yoghurt. This could be attributed to a potential protective effect of fat. *S. flexneri* exhibited an ability to adapt to acidic conditions, since its previous exposure to pH 5.5 increased its tolerance to pH 3.5. The pattern of acid tolerance in pH 3.5 by acid-adapted cells of *S. flexneri* depended upon the adaptation time that ranged from 30 min to 120 min.

Keywords: *Shigella flexneri,* milk, low pH, lactic acid, acetic acid, yoghurt, acid adaptation.

INTRODUCTION

Shigella is a major cause of foodborne gastrointestinal illness that is estimated to be responsible of 80–165 million disease cases and 600,000 deaths worldwide every year (Warren *et al*. 2006; Bowen *et al*. 2012). While *Shigella* has been classically presented as a waterborne pathogen, several foodborne outbreaks have been also linked to this pathogen and involved diverse foods including potato salad, ground beef, raw vegetables, and dairy products (Smith 1987; Mead *et al.* 1999; Wu et al. 2000). For instance, a large outbreak involving 200 persons who consumed cheese contaminated with *Shigella* was reported in Murcia Region in Spain in the winter of 1995 – 1996 (Garcia-Fulgueiras *et al*. 2001). All four species of the genus *Shigella*: *S. dysenteriae, S. fexneri, S. boydii* and *S. sonnei* can cause gastrointestinal illness (Todd 1989; Archer and Kvenberg 1985). However, *S*. *sonnei* and *S*. *flexneri* are more frequently associated with forborne illness than other *Shigella* species. These two species have been shown to be involved in 70% and 25% of foodborne shigellosis, respectively (Uyttendaele *et al*. 2001). *S. sonnei* is more commonly associated with gastrointestinal disease in industrialized countries, whereas *S. flexneri* is more frequent in developing countries (Bowen *et al*. 2012).

Transmission of *Shigella* via foodstuffs would relay, at least in part, upon its ability to tolerate preservation factors of foods, including low pH, heat, cold, salt, low water activity, etc.. However, little is known about the behavior of *Shigella* under food acidic conditions. Bagamboula *et al*. (2002) studied acid tolerance of *Shigella* under acidic conditions of fruit and vegetable juices, but did not include low pH conditions relevant to dairy processing. The present study was therefore designed to assess the growth, and survival of *S. flexneri* under conditions of low pH adjusted by organic acids developed during milk fermentation. It also examined the survival of *S. flexneri* under practical acidic conditions of yoghurt and considered the ability of the pathogen to adapt to acidic pH that it may encounter during the manufacture and/or storage of fermented milk products.

MATERIALS AND METHODS

Cultures and growth conditions

Shigella flexneri ATCC 12022 was used throughout the whole study. *S. flexneri* was maintained on tryptone soya agar (TSA) at 4°C (Oxoid, Basingstoke, UK). Prior to each use, *S. flexneri* was inoculated at 1% (v/v) into tryptone soya broth (TSB) (Oxoid), followed by incubation at 37°C for 24 h.

Assessment of the growth and survival of *S. flexneri* under acidic conditions

A 24 h culture of *S. flexneri* grown in TSB was inoculated at 1% (v/v) into unacidified TSB (pH 7.3) (control) and TSB, acidified by HCl, lactic acid or acetic acid to pH values of 6.0, 5.0, 4.5, 4.0, 3.5 and 3.0. Cultures were incubated at 37°C and samples were taken after 0, 2, 4, 6, 24 h for the assessment of viable counts.

Assessment of acid adaptation of *S. flexneri*

S. flexneri was grown in TSB for 24 h and inoculated at 1% (v/v) into unacidified TSB (pH 7.3) and TSB, acidified to pH 5.5 by HCl. Both cultures were incubated at 37°C for 30 min, 60 min, 90 min, and 120 min and challenged in pH 3.5 at each of those times. Acid challenge was achieved by inoculating 1% of *S. flexneri* into TSB acidified to pH 3.5 by HCl, followed by incubation for up to 120 min. Samples were taken during acid challenge for the assessment of viable counts.

Assessment of the survival of *S. flexneri* during the preparation and cold storage of *S. flexneri*

Yoghurt was prepared from full fat and half fat buffalo's milk containing 6% and 3% fat, respectively. Milk was heated at 95°C for 10 min, rapidly cooled to 42°C, and inoculated with 24 h *S. flexneri* culture and a yoghurt starter culture (Yoghurt culture YO-Mix^{tm} 495 LYO 100 DCU, Danisco, France) at 0.1% (v/w%) and 2% (w/w%) of milk weight, respectively. Inoculated milk was then incubated at 42°C till obtaining satisfactory coagulum. The resultant yogurt was kept in the refrigerator at 6°C, and samples were taken after coagulation, and 1 d, 3 d, 7 d, and 14 d of cold storage to assess the viable numbers of *S. flexneri* and pH.

Assessment of the viable counts of *S. flexneri*

Serial dilutions cultures containing *S. flexneri* were prepared in sterile saline solution (0.85% NaCl) and plated onto TSA or MacConkey agar (Oxoid, Basingstoke, UK). Inoculated plates were counted after incubation at 37°C for 24 h.

RESULTS AND DISCUSSION

Growth and survival of *S. flexneri* under acidic conditions

Food preservation by organic acids has been an effective means in reducing the populations and controlling the growth of many spoilage and pathogenic microorganisms (Tetteh and Beuchat 2003). Organic acids are produced by lactic acid bacteria and other industrial microorganisms during the preparation of fermented dairy products, where they greatly contribute to the preservation of these products. However, other inhibitory components produced by milk fermenting bacteria can also contribute to their inhibitory effect against food spoilage and pathogenic microorganisms (Abdel-Bar and Harris 1984; Nassib et al. 2006). To elucidate the effect of organic acids on *S. flexneri,* the present part of this study examined the growth and survival of *S. flexneri* under different pH values adjusted by lactic acid and acetic acid. This was compared with the growth and survival of the same pathogen in pH values adjusted by HCl. To pursue this, a 24 h culture of *S. flexneri* grown in TSB was inoculated at 1% (v/v) into unacidified TSB (pH 7.3) (control) and TSB acidified by HCl, lactic acid or acetic acid to pH values of 6.0, 5.0, 4.5, 4.0, 3.5 and 3.0. Cultures were incubated at 37°C and samples were taken after 0, 2, 4, 6, 24 h for the assessment of viable counts.

It could be seen in Figure 1 that *S. flexneri* showed exponential growth under unacidified conditions (pH 7.3) reaching viable numbers of approximately 10^9 cfu/ml after 6 h of incubation at 37ºC. Acidification with HCl to pH values of 6 and 5 did not prevent the growth of *S. flexneri* as it also showed exponential growth at these pH values, but at slower rates compared with pH 7.3 (Figure 1). After 6 h of incubation in pH 6 and 5, the viable numbers of *S. flexneri* were approximately 10^8 cfu/ml. However, further acidification with HCl to pH values of 4.5, 4.0, 3.5 and 3.0 were associated with declines in the viable numbers of *S. flexneri*, where the death rate of the organism increased with lowering the pH value (Figure 1). At pH 4.5, 4.0, and 3.5, *S. flexneri* could not be detected after 24 h of incubation at 37ºC, but they were able to survive in these acidic pH values for at least 6 h. Acidification to pH 3.0 was even more detrimental since *S. flexneri* could not be detected after 2 h. These results showed that *S. flexneri* was able to grow in pH 6.0 and 5.0, but at slower rates, compared to unacidifife conditions of pH 7.3. *S. flexneri* could not grow at lower acidic pH values of 4.5, 4.0, and 3.5, but could survive in those acidic environments for at least 6 h. The organism could not survive exposure to pH 3.0 for 2 h.

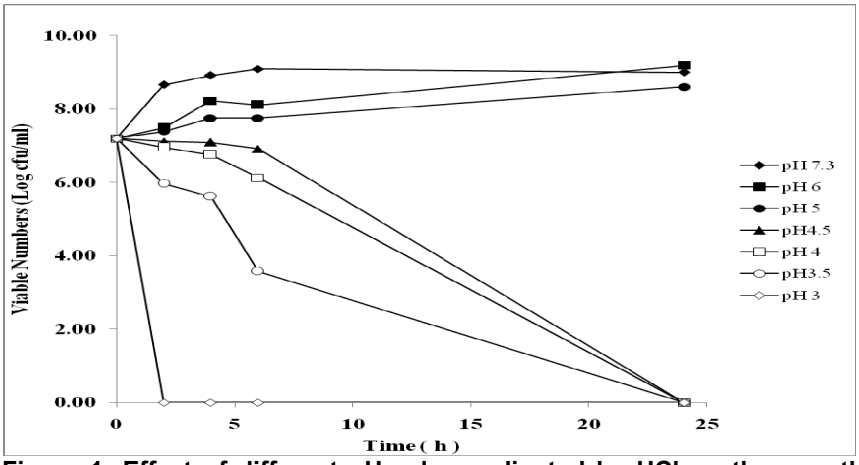

Figure 1: Effect of different pH values adjusted by HCl on the growth and survival of *S. flexneri.*

As with HCl, acidification with lactic acid to pH 6.0 and 5.0 did not prevent the growth of *S. flexneri* but slowed it, as compared to pH 7.3 (unacidified conditions) (Figure 2). Here too, lower pH values caused decreases in the initial viable numbers of *S. flexneri* that could not be detected after 24 h, 6 h, 4 h, and 2 h at pH values of 4.5, 4.0, 3.5, and 3.0, respectively (Figure 2). However, lactic acid was generally more detrimental to *S. flexneri* than HCl, since the organism was undetectable after shorter times of exposure to pH values of 4.0, and 3.5 adjusted by lactic acid compared to HCl. For example, *S. flexneri* could not be detected after 4 h of exposure to pH 3.5 adjusted by lactic acid (Figure 2), but could survive the same pH for at least 6 h when HCl was used as an acidifying agent (Figure 1).

Similarly to HCl and lactic acid, acidification with acetic acid to pH values of 6.0 and 5.0 resulted in slower growth of *S. flexneri*, compared to pH 7.3 (Figure 3). Lower pH values were associated with declines in the viability of the pathogen as observed with HCl and lactic acid (Figure 3). Nonetheless, acetic acid was more damaging to *S. flexneri* than HCl at pH 4 and 3.5. It was even more detrimental to cells than lactic acid at pH 3.5, since the pathogen could not be detected after 2 h with acetic acid.The above results showed that acidification with either a mineral acid (HCl), or organic acid (lactic acid or acetic acid) to pH values of 6.0 and 5.0 permitted the growth of *S. flexneri* but at slower rates, compared to the optimum growth pH value of 7.3. This was consistent with previous reports showing that the minimum pH for the growth of *Shigella* was 4.9 – 5.0 (ICMSF 1996) and 4.75 – 5.5 for *S. flexneri* (Jay et al. 2005). The present results also showed that lower pH values ranging from 3.0 to 4.5 caused declines in the initial numbers of *S. flexneri*, but the pathogen could survive exposure to pH 4.0 and 3.5 for variable times depending on the acidifying agent.

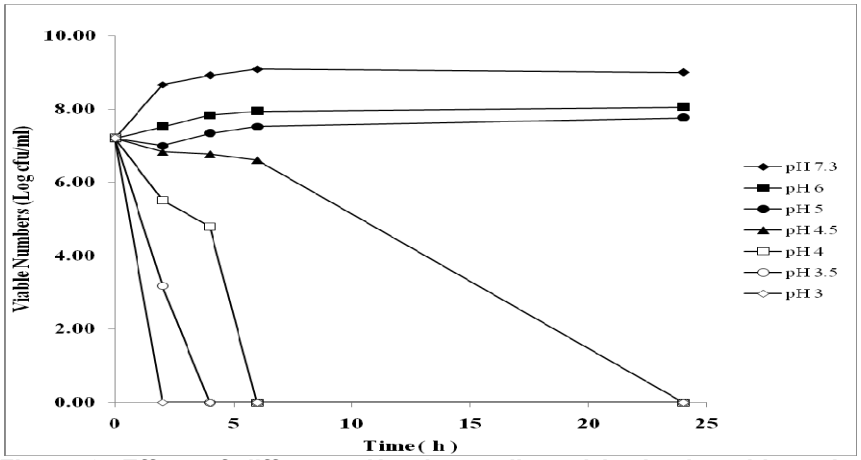

Figure 2: Effect of different pH values adjusted by lactic acid on the growth and survival of *S. flexneri.*

For instance, it survived pH 4.0 and 3.5 for at least 6 h with HCl, but survived pH 4.0 for less than 6 h with lactic acid and acetic acid and pH 3.5 for less than 4 h with lactic acid and less than 2 h with acetic acid (Figures 1-3). Previous studies have also reported that *S. flexneri* and *S. sonnei* strains could survive pH 4.0 and 4.5 for 4 h, but died rapidly at pH values lower than 4.0 (Fehlhaber 1981). Zaika (2001) also showed that *S. flexneri* could variably survive acidic pH values from 2.0 and 5.0, depending on the incubation temperature. It was found that the pathogen's survival generally increased with increasing incubation temperature from 4ºC to 37ºC and with increasing the pH value from 2.0 to 5.0. Furthermore, Small *et al*. (1994) observed that *S. flexneri* strains could survive several hours at pH 2.0 to 3.0.

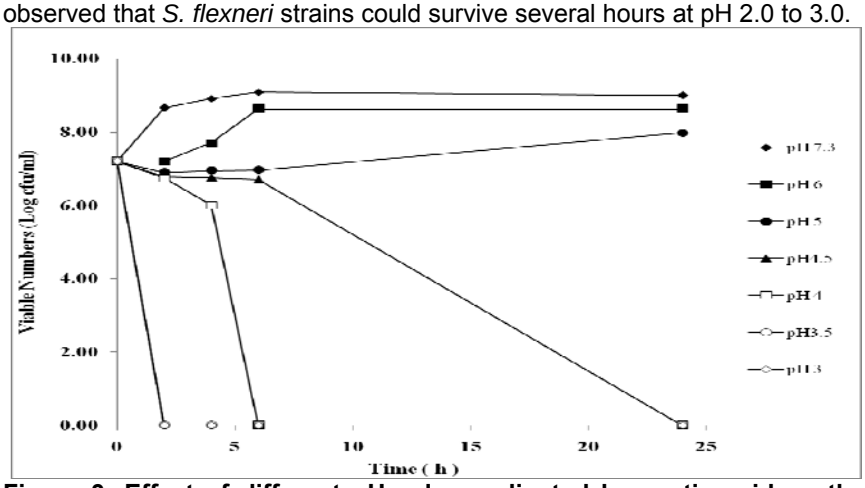

Figure 3: Effect of different pH values adjusted by acetic acid on the growth and survival of *S. flexneri.*

Taken together, these findings report the ability of *S. flexneri* to survive in acidic environments, which could aid its transmission via fermented dairy products. Acid tolerance also helps *S. flexneri* initiate gastrointestinal infection since this allows the pathogen to overcome the acidic environment barrier in the stomach (Martinic *et al*. 2011). It may also account for the low infective dose associated with shigellosis since even low numbers of *Shigella* could survive stomach's acidity and cause illness (Waterman and Small 1996). The composition of lipopolysaccharides in the outer membrane of *Shigella flexneri* was recently suggested to contribute to its ability to resist acidity (Martinic *et al*. 2011).

The present results also showed that organic acids were more inhibitory to *S. flexneri* than HCl. This is due to the lipophilic trait of organic acids that allows these acids to traverse the cellular membranes in their undissociated form (Booth and Kroll 1989; El-Sharoud 2004). On entering the cytoplasm, organic acids dissociate and liberate hydrogen ions that interfere with metabolic activities in the cell. Whereas HCl, as a strong mineral acid, is highly dissociated and not membrane-permeant, which decreases its inhibitory effect, compared to organic acids (El-Sharoud 2004). Previous studies have shown that lactic acid was more inhibitory against foodborne pathogens than HCl at the same pH (Ray and Sandine 1992). It was reported that lactic acid was more effective in liberating lipopolysaccharides from the outer membrane of Gram-negative bacteria than HCl, which allows lactic acid to act as a permeabilizer agent of the cellular membranes (Al Akomi *et al*. 1999). Zaika (2002) also reported that lactic acid and acetic acid were more inhibitory against *S. flexneri* at pH 4.0 than HCl. At pH 3.5, acetic acid was more effective against *S. flexneri* than lactic acid (Tetteh and Beuchat 2001). These results agree with the present study since both organic acids were more inhibitory to *S. flexneri* at pH 4.0 than HCl, and the pathogen survived for a shorter time at pH 3.5 adjusted by acetic acid, compared to lactic acid (Figures 1 - 3). This could be explained based on the p*Ka* value of each acid that is inversely proportional to the degree of acid dissociation. The p*Ka* value of acetic acid and lactic acid are 4.74 and 3.86, respectively indicating that acetic acid is less dissociated than lactic acid (Presser *et al*. 1997). To adjust the culture medium to a certain pH value, higher concentrations of acetic acid than lactic acid are therefore required, which leads to higher inhibition (Eklund 1983; Salmond *et al*. 1984).

Survival of *S. flexneri* during the preparation and cold storage of yoghurt

To examine the effect of acidity developed during the preparation of fermented dairy products, the survival of *S. flexneri* during the preparation and cold storage of yoghurt was studied. Yoghurt was prepared from full fat and half fat buffalo's milk containing 6% and 3% fat, respectively. Milk was heat treated and inoculated with *S. flexneri* and a commercial yoghurt starter culture, followed by incubation at 42°C till coagulation. The resultant yogurt was kept in the refrigerator at 6°C, and samples were taken at intervals to assess the viable numbers of *S. flexneri*, and pH.

Figure 4 shows the viable numbers of *S. flexneri* during the preparation and cold storage of yoghurt. It could be seen that there was a slight decrease in the initial numbers of *S. flexneri* on the coagulation of full fat milk, compared with a higher drop in viability in coagulated half fat milk. *S. flexneri* continued to decline in numbers during cold storage of both full fat and half fat yoghurt. However, the viable numbers of *S. flexneri* were generally higher in full fat yoghurt than half fat yoghurt over the whole cold storage time. After 14 days of cold storage, *S. flexneri* could not be detected in half fat yoghurt, but was still viable at more than 10^2 cfu/g in full fat yoghurt (Figure 4). Assessment of acidity development in full fat and half fat yoghurt showed similar pH values in both treatments at corresponding observation times with half fat yoghurt having slightly higher pH values (Table 1). This suggests that the higher survivability of *S. flexneri* in full fat yoghurt than half fat yoghurt can not be attributed to lower acidity in full fat yoghurt, but to a potentially protective effect of milk fat. It has been suggested that food contaminating bacteria may be trapped within the fat moieties in food, and this may aid their survival under the acidic conditions in the stomach (D'Aoust 1985). The present results support this hypothesis and show that fat content could prolong the survival of *S. flexneri* within the acidic environment of yoghurt.

The survival of *S. flexneri* for up to 7 d and 14 d in full fat and half fat yoghurt, respectively is higher than that reported by Lefoka (2009) for *Shigella* spp. that survived in plain and fruit yoghurt for 4 d and 3 d, respectively. This could be attributed to the use of lower initial inoculum of *Shigella* in that study: 10^4 cfu/g versus 10^6 cfu/g in the present work.

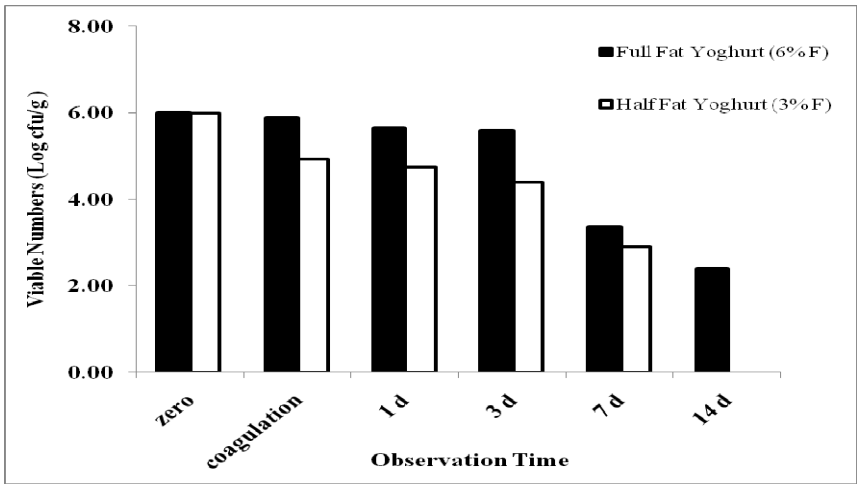

Figure 4: Survival of *S. flexneri* during the preparation and cold storage of yoghurt prepared from full fat (6%) and half fat (3%) buffalo's milk.

Table 1: Acidity development during the preparation and cold storage of yoghurt prepared from full fat (6%) and half fat (3%) buffalo's milk

Observation Time	pH Values	
	Full Fat Yoghurt (6% F)	Half Fat Yoghurt (3% F)
Zero	6.60	6.60
Coagulation	5.06	5.06
1 d	4.35	4.50
3 d	4.25	4.36
7 d	4.20	4.28
14 d	4.02	4.17

There could be also species and strain-dependent variations between test organisms used in both studies.

Acid Adaptation of *S. flexneri*

To assess the ability of *S. flexneri* to adapt to acidic conditions, a 24 h culture of *S. flexneri* was exposed to pH 5.5 for up to 120 min, followed by acid challenge in pH 3.5. The tolerance of this acid-adapted culture to pH 3.5 was compared to that of non-adapted culture of the same strain pr-exposed to unacidified conditions (pH 7.3) for the same time length. The viable numbers of both *S. flexneri* cells adapted for 30 min in pH 5.5 and non-adapted cells pre-subjected to pH 7.3 for the same time length during acid challenge in pH 3.5 are presented in Figure 5.A. It could be seen that there was an initial drop in the viable numbers of both acid adapted and non-adapted cells after 10 min of exposure to pH 3.5. This continued to occur during the following observation times with non-adapted *S. flexneri* that was undetectable after 90 min. However, no substantial decline in the viable numbers of acid adapted cells could be noticed for up to 120 min, where the viable count was approximately10^4 cfu/ml (Figure 5.A). Extending the adaptation time in pH 5.5 to 60 min also increased the tolerance of *S. flexneri* to pH 3.5, compared to non-adapted cells (Figure 5.B). Non-adapted *S. flexneri* could not be detected after only 10 min of exposure to pH 3.5, whereas acid-adapted cells survived this lower pH for up to 60 min (Figure 5.B). Further increase of the adaptation time for up to 90 min elevated the tolerance of acid adapted *S. flexneri* cells to pH 3.5, compared to non-adapted cells (Figure 5.C). However, more apparent differences in survivability in pH 3.5 between acid-adapted and non-adapted cells could be only noticed at later stages of acid challenge: after 90 min and 120 min (Figure 5.C). After 120 min of exposure to pH 3.5, non-adapted *S. flexneri* cells could not be detected, compared with the acid-adapted cells being viable and detected at more than 10^4 cfu/ml. *S. flexneri* continued to show higher tolerance in pH 3.5 with extending the adaptation time to 120 min, compared to non-adapted cells grown in pH 7.3 for the same time length (Figure 5.D). Significant differences in survivability between acid-adapted and non-adapted cells could be noted after 60 min, 90 min, and 120 min of exposure to pH 3.5 (Figure 5.D). At those times, non-adapted *S. flexneri* could not be detected, whereas, acid-adapted cells were viable at approximately 10^3 - 10^4 cfu/ml (Figure 5.D).

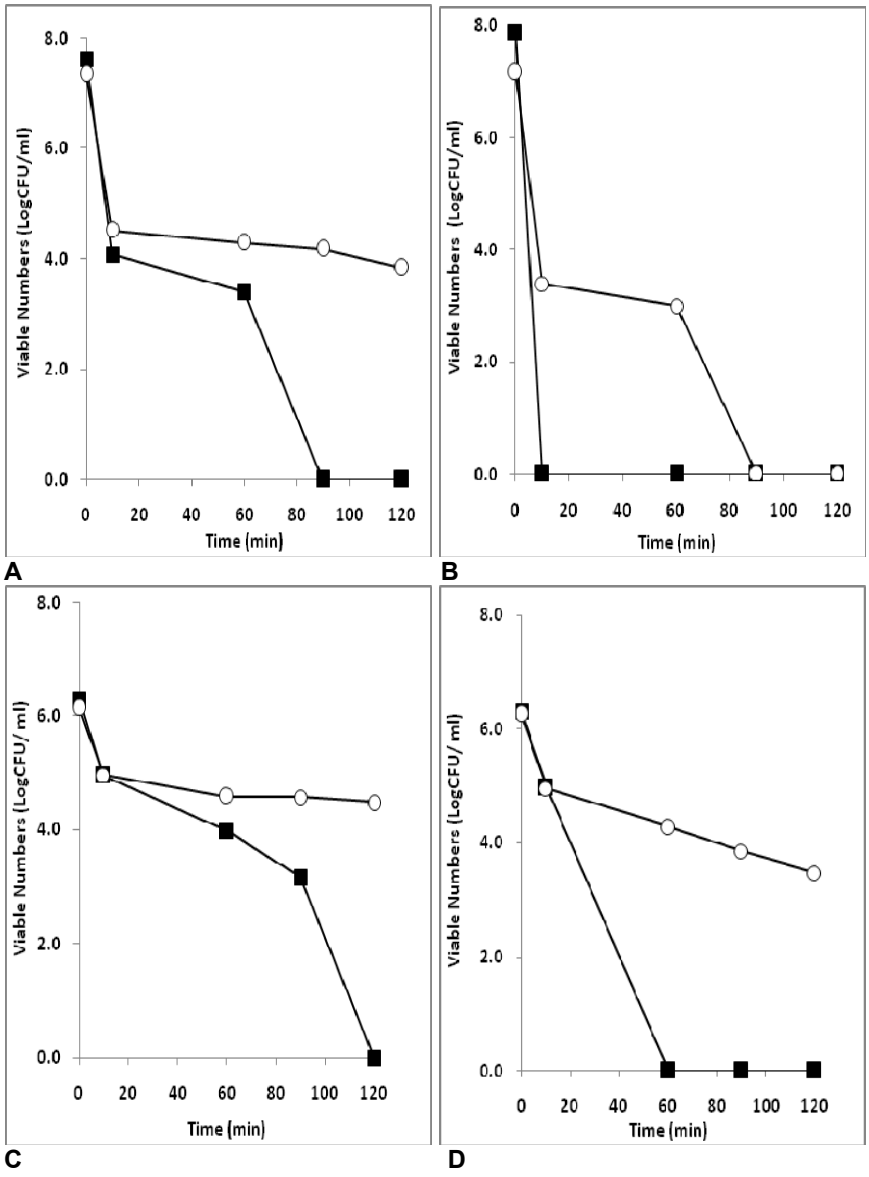

Figure 5: Tolerance of acid adapted (○) and non-adapted (■) *S. flexneri* to pH 3.5 following exposure to pH 5.5 and 7.3, respectively for 30 min (A), 60 min (B), 90 min (C), and 120 min (D).

These results showed that previous exposure of *S. flexneri* to mildly acidic conditions of pH 5.5 increased its tolerance to a more severely acidic pH of 3.5, which is termed "acid adaptation". However, the pattern of acid tolerance in pH 3.5 by acid-adapted *S. flexneri* depended upon the adaptation time. Acid adaptation has been described before in other fooborne bacteria including *Escherichia coli* (Rowbury *et al*. 1989), *Listeria monocytogenes* (Kroll and Patchett 1992), *Salmonella* ser. Typhimurium (Foster 1993), *Aeromonas hydrophila* (Karem *et al*. 1994) and root nodule bacteria (O'Hara and Glenn 1994). Acid adaptation was also described in *S. flexneri* but with the use of an adaptation procedure different from that presented in this work. In that procedure, *S. flexneri* was acid-adapted by overnight growth in TSB supplemented with 1% glucose, which allowed exposure of *Shigella* cells to acid produced by glucose fermentation (Tetteh and Beuchat 2003). In agreement with the present study, acid-adapted *S. flexneri* cells showed higher tolerance of acidic pH than unadapted cells. Acid adaptation of *S. flexneri* could have important consequences regarding the safety of fermented dairy products and the pathogen's virulence as it could increase its survival in those products and in the acidic environment of the human stomach.

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نمو ومقاومة البقاء وتأقلم بكتريا الشيجلا فليكسنري تحت ظروف حامضية ذات علاقة بظروف تصنيع المنتجات اللبنية

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بكتريا *الشيجلا فليكسنري* (*Shigella flexneri*) هي أحد الميكروبات الممرضة المهمة المرتبطة بالمنتجات اللبنية، وقد أستهدفت الدراسة الحالية تقييم قدرة هذا الميكروب علي النمو ومقاومة البقاء والتأقلم تحت ظروف حامضية ذات علاقة بتلك المستخدمة عند تصنيع المنتجات اللبنية. إستطاعت *الشيجلا فليكسنري* النمو في درجات pH حامضية وهي ٦ و ٥ تم ضبطها بإستخدام حامض الهيدروكلوريك أو اللاكتيك أو الاستيك، ولكن كان معدل نموها أبطأ بالمقارنة بالنمو تحت ظروف غير حامضية (pH 7.3). لم يستطع الميكروب النمو بل وحدث إنخفاض في أعداده المبدئية عند تعرضه لدرجات pH تتراوح ما بين ٣ إلي ٤.٥، إلا أن الميكروب أمكنه البقاء حياً عند تعرضه لدرجات pH ٤ و ٣.٥ وذلك لمدد زمنية مختلفة إعتماداً علي نوع الحامض المستخدم في ضبط pH الوسط، حيث كانت *الشيجلا فليكسنري* أكثر حساسية لحامض الاستيك يليه حامض اللاكتيك ثم الهيدروكلوريك. تم دراسة مقاومة *الشيجلا فليكسنري* للبقاء حية تحت الظروف الحامضية في اليوجورت المصنع من لبن جاموسي كامل الدسم (٦% دهن) ولبن جاموسي نصف دسم (٣% دهن). كانت الأعداد الحية من *الشيجلا فليكسنري* في اليوجورت كامل الدسم أكبر بصفة عامة من أعدادها في اليوجورت نصف دسم وذلك عند تجبن اللبن وخلال التخزين البارد. وإستطاع ميكروب *الشيجلا فليكسنري* البقاء حياً لمدة ١٤ يوم علي الأقل في اليوجورت كامل الدسم، ولمدة ٧ أيام في اليوجورت نصف دسم. وتم تفسير ذلك علي أساس أن زيادة نسبة الدهن تؤدي إلي حماية الميكروب في اليوجورت. أظهر ميكروب *الشيجلا فليكسنري* قدرةً علي التأقلم مع الظروف الحامضية حيث أدي تعرضه لدرجة pH ٥.٥ إلي زيادة مقاومته لدرجة pH أقل وهي ٣.٥. إلا أن نمط تحمل خلايا *الشيجلا فليكسنري* التي تم أقلمتها مع الظروف الحامضية لتأثير درجة pH ٣.٥ أعتمد علي المدة الزمنية التي تعرضت فيها الخلايا للأقلمة والتي تراوحت من ٣٠ إلي ١٢٠ دقيقة.

قام بتحكيم البحث

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