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Effect of Physiological factors on molecularly identified Campylobacters.

[1]Adekunle O.C.[2] Onilude A.A, [3]Adesiji Y.O
[1,3] Department of Medical Microbiology and Parasitology of Ladoke Akintola University of Technology, Osogbo, Nigeria.[2] Department of Microbiology, University of Ibadan, Nigeria.
Corresponding Author: Adekunle O.C

Abstract: *Campylobacter jejuni and C. coli have the highest rate of foodborne-related clinical Campylobacteriosis. In this study twenty-five Campylobacter (C) isolates identified by PCR comprising of 18 C. jejuni and7 C. coli were tested for physiological factors such as adhesion potential, bile tolerance, bacteriocin tolerance and ability to synthesise proteolytic enzymes on solid medium. These were determined by standard methods. Campylobacter adhered mostly to wood (0.82 nm), followed by plastic (0.5 nm), metal(0.4 nm) and glass (0.2 nm). Campylobater coli adhered to the surfaces more than C. jejuni. Campylobacter isolates survived at different concentrations of bile (2.1 -6.8%), low pH (7.1-3.2) and in the presence of bacteriocin (3.8-6.8 AU/mL) with the production of proteolytic enzymes in the range of 16.2-15.2 mm. The occurrence of Campylobacter species in the faecal samples and the ability of the isolates to survive in the presence of bacteriocin, different concentrations of acid, bile salt are indicative of virulence of the strains.*

***Keywords**: Campylobacter species, adherence, bacteriocin, bile, proteolytic enzymes*

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I. Introduction

Campylobacter is the most common bacterial cause of gastroenteritis in most populations especially in children, elderly and immunocompromised people (1). One of the species Campylobacter jejuni is most common cause of community acquired inflammatory enteritis. It causes enteric infection which produces an inflammatory, sometimes watery or bloody diarrhoea or dysentery, headache and abdominal pain (2). Campylobacter causes tissue injury in the jejunum, ileum and the colon. C. jejuni appears to achieve this by invading and destroying epithelial cells (3). The organism produces diffuse, bloody, oedematous and exudative enteritis. In a small number of cases, the infection may be associated with haemolyticureamic syndrome and thrombotic thrombocytopaenic purpura. During the course of human disease numerous proinflammatory cytokines are produced. The pathology includes severe inflammation of the intestinal mucosa with an influx of professional phagocytes (4). Histological analysis of biopsy samples from patients with C. jejuni colitis has shown that the bacteria invade the colonic mucosa. Campylobacter can invade human epithelial cell monolayers (5), causing disruption to the epithelium and gaining access to its basal side (6).

Virulence factors in C. jejuni and C.coli are a useful tool to assess the potential risk of Campylobacter infection in human especially in one the vulnerable groups: children. This study intends to determine the virulent properties of Campylobacter isolated from children stool in Osun State, Nigeria. Adhesion potential, bile tolerance, bacteriocin tolerance and ability to synthesise proteolytic enzymes on solid medium were determined using standard methods.

II. Materials and Methods

Twenty-five Campylobacter (C) isolates obtained from children stool samples identified by PCR comprising of 18 C. jejuni and 7 C. coli were tested for physiological factors:adhesion potential, bile tolerance, bacteriocin tolerance and ability to synthesise proteolytic enzymes on solid medium.

Ethics Statement

This work was performed according to University ethics committee code of conduct.

Test for adhesion potential

This was carried out by studying the growth of the isolates on four different surfaces. Preparation of surfaces: Two hundred and twenty four chips (56 each (4x2x1 cm) for glass (Easy way medical, England) metal (Type 304, #4, Ajaokuta Steel Co., Nigeria) and wood (Softwood, Oak) and plastics were used. The chips were washed with detergent (Unilever, Lagos, Nigeria), rinsed with sterile distilled water and air-dried. The chips

were then wrapped in aluminum foil and placed in hot air oven (Elektro-Helios, Sweden) at 75°C for 30 min for sterilization before use.

Assessment of Campylobacter jejuni and Campylobacter coli adhesion: A discrete colony of a 24- h test cultures on nutrient agar (Fluka 7014, Germany) was used for adherence assessment. One colony each for Campylobacter jejuni and Campylobacter coli on nutrient agar slope was transferred into each of 20 sterile glass jars with lids containing 150 mL of nutrient broth (Fluka 7014, Germany). An uninoculated nutrient broth was used as control.

Quantification method was by Crystal violet binding assay.

The technique used was the method of Stepanovic et al. (7). Each set of chips was washed 3 times with 5 mls of sterile distilled water. The remaining adhered bacteria were fixed with 2.5 mL of methanol per chip. Each chip was stained with Crystal violet for 15 minutes and then the excess stain washed off under running tap water. After the chip was air-dried, the dye bound to the adhered cells was re-solubilized with 2.5 mL of 33% glacial acetic acid for each chip. The re-solubilised liquid for each chip was poured into a cuvette. The absorbance (optical density) of each re-solubilised liquid was measured against the optical density of blank reading without inoculation (control) at wavelength of 620 nm and 520 nm for Campylobacter coli and Campylobacter jejuni respectively using a spectrophotometer (Springfield, UK). The absorbance of negative control was subtracted from the absorbance values to determine the actual values (8).

Acid tolerance studies

Acid tolerance of Campylobacter isolates was studied by incubating 0.2 mL suspension of each isolate in Butzler broth adjusted with hydrochloric acid (HCl) to different pH values 1.5, 3.0, 4.5, 5.5 and the value determined with digital pH meter (Θ 240pH/Temp Meter, Beckman, USA) and cultures were incubated at 37°C in an incubator (Sanyo CO_2 incubator Model MCO-15A, Japan) for 48 hours. Bacterial survival in the different pH values was monitored by use of Multiskan Ascent VI. 2 (Ascent software version 2.6) at 600 nm. The absorbance values obtained were plotted against the different pH values. To determine the number of surviving cells in the different pH preparation after 48 hours of incubation, 10-fold serial dilutions were made using MRS broth. Ten micro-liter was taken out from each dilutions and plated onto Butzler-type agar in duplicate and the plates were incubated in microaerophilic condition. Acid tolerance was determined by comparing the colony forming unit (CFU mL^{-1}) on the plates from the absorbance values from the Multiskan.

Bacterial cultures were obtained on Butzler-type agar medium. After an incubation time of 48 hours at 37 ºC are suspended in sterile (0.5%, w/v) (10^5 CFU/mL= colony forming units/mL). Gastric juice is prepared by regulation of pH at 1.5, 2.5, 3.5, 4.5 and 5.5 values with hydrochloric acid using a pH metre-ruler.

One aliquot (0.2 mL) from each suspension of bacterial cellules is transferred in a 2 mL sterile Eppendorf tube and mixed with 0.3 mL (0.5%, w/v) sterile sodium chloride solution and 1.0 mL simulated gastric juice (pH 1.5, 2.5,3.5,4.5 and pH 5.5). This mixture was vortexed for 10s and incubated at 37ºC. Viability of strains was analysed by determination of CFU/mL after different periods of incubation (1 hour, 2 hours, 3 hours, 4 hours and 5 hours in the simulated gastric juice) by inoculation on Butzler-type agar solid media.

Bile tolerance

Two milliliter aliquot of bacterial cultures containing 10^5 CFU= colony forming units/mL obtained on Butzler-type agar media after an incubation time of 48 hours at 37ºC were suspended in sterile sodium chloride 0.5 %; w/v solution.This was performed for each isolate. Intestinal juice was prepared by resolving in sterile sodium chloride solution (0.5%, w/v) bile salts in different concentrations (1.5%, 3.0%, 4.5, 6.0 %), pH is regulated at 8.0.

Point two millilitre (0.2 mL) aliquot of each suspension of bacterial cell was transferred into a 2 mL sterile Eppendorf tube and mixed with 0.3 mL 0.5%, w/v sterile sodium chloride solution and 1.0 mL simulated intestinal juice (0.3%, 0.5%, 1.0% bile salt). This mixture was vortexed for maxim 10s and incubated at 37ºC.Viability of strains was analysed by determination of CFU/mL after different periods of incubation (0 hour, 6 hours, 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours and 48 hours in the simulated intestinal juice) by inoculation on Butzler type agar solid media, after an incubation time of 48 hours at 37ºC.

Bacteriocin tolerance

The bacteriocin utilised in this work were from Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus casei, and Lactobacillus lactis. They were supplied by the Group Leader, Microbial Physiology and Biochemistry Research, University of Ibadan, Ibadan. Ten microlitres(10 µL) of each bacteriocin type was introduced into sterile Butzler-type broth medium in several tubes which were already inoculated with 0.2 mL (containing 1.0×10^5 cfu/ml) aliquot of 12 hour growing

cells of Campylobacter coli. This was repeated for bacteriocin from other sources.The tubes were incubated in a cool Air incubator (Phillip Harris 3710) at 32 ± 5 ^{o}C. At regular intervals specifically after 24, 36, 48, 60,72, 84 and 96 hours of incubation, triplicates tubes were taken and inoculated onto Butzler-type agar medium using the 10-fold serial dilution. Plates were incubated at 32 ± 5°C for 48 hours after which colony counts were carried out. The count number obtained is taken as the number of survivors on each occasion.

Screening for proteolytic enzyme

A loopful culture of Campylobacter coli was inoculated into a 50 ml nutrient broth (g/L): peptic digest of animal tissue, 5; beef extract, 1.5; yeast extract, 1.5, Sodium Chloride, 5, pH 8.0. The culture was incubated for 72 hours at 37°C under shaking condition (150 rpm). The crude enzyme was obtained by centrifugation at 10000 rpm, at 4 °C for 10 min.

Screening for protease activity by well diffusion method.

Agar was prepared along with 1% (w/v) casein, skimmed milk, gelatin and poured in petri dishes. The plates were solidified for 30 min and holes (3 mm diameter) were punched. A crude culture supernatant from Campylobacter coli was loaded into the holes. These plates were incubated overnight in temperature (32±5°C). These plates were flooded with BCG reagent and incubated for 20-30 minutes at room.

Statistical analysis

All tests were carried out in duplicate and results presented in Mean±Standard Deviation (Mean±SD). A one-way Analysis Of Variance (ANOVA) was used to determine significant differences between Means for each surface and strains. Student-test was used to assess significant differences between strains. Statistical significance was evaluated at $p<0.05$.

III. Results

Table 1 shows the comparison of bacterial adhesion produced by the Campylobacter jejuni and Campylobacter coli strains on four surface types. On all surfaces, higher adhensions were recorded with increasing time for both Campylobacter coli and Campylobacter jejuni. The greatest adhesion was recorded on wood surface ($0.7832 \leq x \leq 0.9262$) while the least was on glass being as low as 0.16 at 24 hours. The variation of time did not significantly affect the adhesion on plastic surfaces over the 72 hours.

It also shows variations in adhesion of Campylobacter coli and Campylobacter jejuni (Cj) isolates on four different surfaces (wood, metal, glass and plastic) at different time intervals of 24-72hours. At 24 hours, there was significant different ($P<0.05$) in adhesion between Cc (0.78 ±0.02) and Cj (0.62 ± 0.06) on wood. However, there was no significant difference ($P>0.05$) in adhesion between Cc and Cj on metal, glass and plastic.

Also at 24 hours, Cc exhibited significant difference ($p<0.05$) in adhesion on wood (0.78 ±0.02) and metal (0.29 ±0.01); glass (0.16 ±0.01) and plastic (0.45 ±0.01).

At 24 hours, assessment of adhesion potential of Campylobacter jejuni (Cj) showed significant difference ($P<0.05$) in adhesion on wood (0.61 +0.06) and metal (0.35 ± 0.02); glass (0.18 ±0.02) and plastic (0.46 ±0.02).

At 24 hours, Campylobacter coli (Cc) had higher adhesion on wood when compared with Campylobacter jejuni (Cj), however, Cj had higher adhesion on metal, glass, and plastic when compared with Cc.

At 72 hours, there was significant difference ($p<0.05$) in adhesion between Cc (0.93 ±0.03) and Cj (0.82 ±0.01) on wood. However, there was no significant difference ($P>0.05$) in adhesion between Cc and Cj on metal, glass and plastic.

Also at 72 hours, Cc exhibited significant differences ($P<0.05$) in adhesion on wood (0.82 ± 0.01) and metal (0.33 ± 0.02); glass (0.17 ± 0.02) and plastic (0.52 ± 0.02).

At 72 hours, assessment of adhesion potential of Campylobacter jejuni (Cj) showed significant difference ($P<0.05$) in adhesion on wood (0.82 ± 0.02) and metal (0.39 ± 0.02); glass (0.21 ± 0.02) and plastic (0.52 ± 0.03).

At 72 hours, Campylobacter coli (Cc) had higher adhesion on wood when compared with Campylobacter jejuni (Cj), however, Cj had higher adhesion on metal, glass and plastic when compared with Cc.

Table 1: Comparison of adhension abilities in Campylobacter coli and Campylobacter jejuni strains on 4 different surfaces (abs 560nm).

Strains	Period	Surfaces (Mean ±SD)			
		Wood	Metal	Glass	Plastic
Cc	24	0.7832±0.0260	0.2984±0.0186	0.16±0.010	0.4594±0.0152
Cj	24	0.6192±0.0672	0.3554±0.0217	0.1898±0.020	0.4636±0.0243
Cc	72	0.9262±0.0317	0.3346±0.0262	0.1702±0.020	0.5166±0.0190
Cj	72	0.8173±0.0169	0.3927±0.0248	0.2070±0.0235	0.5249±0.0252

Cc Campylobacter coli
Cj Campylobacter jejuni

Table 2 shows an increase from 0-48 hour within the Oxgall bile concentrations tested (1.5 to 6%) and there was a corresponding increase in the viable cell counts. At 1.5% bile concentration there was a 42% growth increase while at 60% a corresponding 50% growth increment of the Campylobacter strain was observed.

This illustrates the effects of concentration of bile acid and extent of exposure to bile acid on growth of Campylobacter isolates CC5 and CC7.The results showed similar and regular growth pattern for the different isolates. There was increased growth with increased concentration at extended hours of exposure. The highest growth was observed at the highest bile concentration used i.e. 6.0% and at 48 hours.

In Table 3, the Campylobacter isolates were screened for the expression proteolytic activity. The measured zones of hydrolysis are described below.

Exposure of the Campylobacter isolates CC5 and CC7 to bile salts, bacteriocin and different pH caused slight reduction in their ability to produce proteolytic enzymes when compared with their respective controls. Exposure to 3% Oxgall, pH 2.5 and 1% bacteriocin caused 1.59%, 4.76%, and 19.05% reduction in proteolytic enzyme producing ability of CC5 isolates respectively. For the CC7 isolates, exposure to 3% Oxgall, pH 2.5 and 1% bacteriocin caused 3.70%, 3.70% and 6.17% reduction in their proteolytic enzyme producing ability respectively.

In all, 1% bacteriocin was most potent in inhibiting C. coli isolates from producing proteolytic enzyme while HCl at pH 2.5 had the least inhibitory potential.

In Table 4, the result of Campylobacter isolates screened for proteolytic enzymatic activities after being grown in the presence of Bile salt, bacteriocin and acidic pH are shown. At 1hour exposure to acid, Campylobacter isolate (CC5) survived most in acid concentration 1.5 and was most sensitive at pH 4.5. At 2 hour exposure, isolate (CC5) was least sensitive at pH 2.5 and most sensitive pH 4.5. From 3 to 5 hours exposure, C. coli (CC5) was most tolerant to acid at pH 1.5 and least survived (least tolerant) at pH 5.5.

Campylobacter isolate CC7 showed more regular pattern in their tolerance to acid. From 1 to 5 hours exposure to acid, the isolate showed least tolerance at pH 5.5. At 1, 3, and 4 hours acid exposure Campylobacter isolate was most tolerant at pH 2.5 while at 2 and 5 hours exposure, the highest tolerance was observed at pH 1.5.

Bacteriocin tolerance of Campylobacter coli isolates upon exposure at different time intervals is reported in Table 5 Source of bacteriocin were Lb. plantarum, Lb. delbrueckii, Lb. acidophilus, Lb. fermentum, Lb. casei, Lb. lactis.

At 24 hours exposure, Lb. acidophilus bacteriocin was most potent, while Lb. lactis was least potent against Campylobacter CC5 isolate. From 84 to 96 hours exposure Lb fermentum and Lb casei respectively were most potent against Campylobacter isolates. The results suggested that between 24 and 72 hours exposure to bacteriocin from these Lactobacilli, Campylobacter survived least in media containing Lb acidophilus and survived most in media containing Lb lactis.

However, for the Campylobacter isolate CC7, there were no regular patterns of survival on exposure to bacteriocins from Lactobacillus. At 24 hours exposure, Campylobacter isolate survived most in bacteriocin produced from Lb delbrueckii .On the contrary, the C.coli isolate survived least in Lb casei bacteriocin.

At 36 hour exposure, it has most tolerance and least tolerance in media containing Lb delbrueckii and Lb fermentum respectively. From 48 hours to 60 hours exposure, CC7 survived most in media containing Lb casei and had least survival in media containing Lb plantarum. From 72 to 84 hours exposure, C. coli CC7 was least sensitive to bacteriocin from Lb plantarum

Table 2: Survival of Campylobacter coli isolates in the presence of different concentrations of bile salt

		Bile Salt Concentration(%)/Survival count (log CFU/mL)			
Isolates	**Time (hr)**	**1.5**	**3.0**	**4.5**	**6.0**
CC5	0	2.05±0.02	2.05±0.02	2.05±0.02	2.05±0.02
	6	2.21±0.03	2.96±0.07	2.98±0.08	3.14±0.08
	12	2.64±0.06	2.98±0.06	3.14±0.07	3.44±0.12
	18	2.84±0.04	3.07±0.08	3.46±0.08	3.94±0.17
	24	2.92±0.09	3.42±0.13	3.54±0.10	4.17±0.16
	30	2.96±0.08	3.66±0.15	3.85±0.11	4.67±0.13
	36	3.04±011	4.15±0.22	4.67±0.17	5.53±0.15
	42	3.22±0.18	5.64±0.18	5.66±0.16	6.72±0.16
	48	3.52±0.15	6.12±0.16	6.38±0.18	7.13±0.21
CC7	0	2.05±0.02	2.05±0.02	2.05±0.02	2.05±0.02
	6	2.46±0.05	2.99±0.06	3.20±0.07	3.41±0.05
	12	2.56±0.04	3.15±0.07	3.34±0.10	3.58±0.08
	18	2.76±0.07	3.41±0.05	3.68±0.13	3.77±0.11
	24	2.91±0.05	3.74±0.08	3.76±0.12	3.92±0.11
	30	3.02±0.11	3.88±0.05	3.97±0.15	3.97±0.13
	36	3.18±0.14	4.51±0.12	4.43±0.17	4.84±0.11
	42	3.43±0.12	4.92±0.15	5.18±0.18	5.61±0.18
	48	3.66±0.13	5.83±0.17	6.17±0.15	6.81±0.16

CC5 Campylobacter coli isolate 5
CC7 Campylobacter coli isolate 7

Table 3. Screening of selected Campylobacter coli isolates for proteolytic enzyme production in the presence of bile salt, bacteriocin and of different pH

ISOLATES	PARAMETERS	ZONE
CC5	Proteolytic enzyme	16.2 ± 0.04*
	3% Oxgall	15.6 ± 0.06
	pH 2.5	15.6 ± 0.05
	1% Bacteriocin	15.2 ± 0.07
CC7	Proteolytic enzyme	12.6 ± 0.05
	3% Oxgall	12.4 ± 0.07
	pH 2.5	12.0 ± 0.04
	1% Bacteriocin	10.2 ± 0.02

CC5 Campylobacter coli isolate 5
CC7 Campylobacter coli isolate 7

Table 4. Survival of Campylobacter coli isolates in the presence of different concentrations of HCl acid

pH/Viable Count(log_{10} cfu/mL)						
Isolate	Incubation Time (h)	1.5	2.5	3.5	4.5	5.5
	Control	7.14±0.02	7.18±0.01	7.20±0.04	7.12±0.02	7.10±0.02
CC5	1	7.12±0.01	7.05±0.05	6.85±0.05	6.64±0.01	6.80±0.03
	2	7.15±005	7.18±0.03	6.92±0.04	6.60±0.01	6.81±0.02
	3	7.10±0.02	7.08±0.03	6.68±0.07	5.63±0.04	5.14±0.02
	4	7.15±0.01	7.06±0.01	6.60±0.03	5.15±0.02	4.62±0.04
	5	7.12±0.02	7.04±0.02	6.45±0.02	5.10±0.08	3.24±0.09
CC7	Control	6.84±0.04	6.92±0.02	6.80±0.01	6.51±0.01	6.62±0.01
	1	6.80±0.02	6.81±0.04	6.74±0.03	6.62±0.02	6.5±0.03
	2	6.88±0.06	6.84±0.02	6.70±0.02	6.41±0.01	6.4±0.03
	3	6.68±0.02	6.84±0.01	6.70±0.02	6.35±0.02	6.2±0.01
	4	6.60±0.03	6.80±0.01	6.64±0,04	6.06±0.06	6.0±0.04
	5	6.60±0.03	6.55±0.04	6.42±0.01	5.62±0.02	4.28±0.01

CC5 Campylobacter coli isolate 5
CC7 Campylobacter coli isolate 7

Table 5: Bacteriocin Tolerance of Campylobacter coli isolates upon exposure at different time intervals.

Incubation Time (hrs)/ Survival (log_{10} cfu/mL)								
Isolate	Source of Bacteriocin	24	36	48	60	72	84	96
CC5	Lb plantarum	3.25±0.14	3.62±0.21	3.84±0.17	4.12±0.24	5.64±0.27	7.04±0.18	7.52±0.21
	Lb delbrueckii	3.66±0.18	3.88±0.17	4.18±0.21	4.20±0.22	5.26±0.22	6.18±0.18	6.66±0.26
	Lb acidophilus	2.86±0.12	3.04±0.24	3.64±0.26	3.82±0.21	4.42±0.19	6.08±0.36	6.75±0.31
	Lb fermentum	3.04±0.11	3.55±0.11	4.22±0.24	4.64±0.18	5.18±0.27	5.62±0.29	6.82±0.31
	Lb casei	3.62±0.22	3.82±0.18	4.16±0.20	4.86±0.24	5.24±0.21	5.84±0.28	6.4±0.30
	Lb lactis	3.84±0.26	4.28±0.16	4.74±0.18	5.60±0.21	5.88±0.27	6.15±023	6.82±0.32
CC7	Lb plantarum	2.82±0.16	3.12±0.21	3.44±0.27	3.92±0.18	5.04±0.17	6.56±0.27	6.88±0.32
	Lb delbrueckii	3.46±0.23	3.58±0.17	3.82±0.24	4.08±0.15	4.64±0.25	4.86±0.26	5.62±0.22
	Lb acidophilus	2.55±0.17	3.41±0.21	3.88±0.21	4.08±0.27	4.57±0.32	5.43±0.21	6.18±0.23
	Lb fermentum	2.82±0.30	2.82±0.30	vv3.91±0.26	4.17±0,34	4.38±0.26	5.13±0.11	6.15±0.19
	Lb casei	2.06±0.21	3.56±0.21	3.92±0.22	4.26±0.21	4.91±0.21	5.34±0.16	5.96±0.20

IV. Discussion

The results showed that all strains of Campylobacter adhered to the wood, glass, plastic and metal surfaces. However, the extent of adhesion was different, depending on the surface and strain. At 24 hours, Campylobacter coli adhered more on wood, plastics, metal and glass than Campylobacter jejuni which was significant at ($p<0.05$) for wood but not for plastic, metal and glass. At 72 hours, Campylobacter coli also adhered more on wood, plastic, metal, and glass surfaces than Campylobacter jejuni. These values were not significant at ($p<0.05$) for all the surfaces considered. These observations were in agreement with previous studies by Hood and Zottola (1997) and Gulsun et al. (2005) in which the level of biofilm formation of the isolates increases with the virulence characteristics: i.e., strains with higher virulence produced more biofilms than strains with lower virulence. Biofilms are formed by adhesion of bacterial cells to surfaces and the level of biofilm production of isolates is a virulence characteristic of such isolates. Higher producers of this factor have higher virulence than lower producers. A significant difference ($p<0.05$) was observed between 24 hours incubation period for Campylobacter coli and Campylobacter jejuni. The assessment of Campylobacter coli and Campylobacter jejuni strains showed an increase in bacterial adherence with an extension of incubation time. Variations in bacterial adherence were also observed among the strains on all the four surfaces at 24 and 72 hours of incubation. In fact, adhesion occurred in great extent to the material with highest hydrophobicity (wood and plastic), being quite similar to a material moderately hydrophobic and other hydrophilic (glass and metal). In this study, the mean absorbance values (nm) of biofilms produced by Campylobacter showed that wood retained the greatest biofilms followed plastic, metal and glass. The attachment of the bacteria to the food product or the product contact surfaces leads to serious hygienic problems, this could lead to diarrheoa in which the children were experiencing. In addition to that, a number of reports have appeared on the persistence of several food-borne pathogens on food contact surfaces. For these reasons, it is considered that the presence of biofilms in the food systems is a serious public health risk.

Biofilm formation constitutes an efficient adaptive strategy, because such behavior offers four major advantages: (I) protection from adverse environmental factors, (II) increased availability of nutrients for growth, (III) increased binding of water molecules, reducing the possibility of dehydration, and (IV) proximity to progeny and other bacteria, facilitating higher rates of DNA transfer. All these circumstances can increase the survival of bacteria.

This result is in agreement with the report of Sinde and Carballo, (10) and Donlan (11) who reported that glass and stainless steel are hydrophilic materials while wood and plastic are hydrophobic materials thereby encouraging adhesion. Hydrophobic materials that provide a greater bacterial adherence (12).

The results indicated that Campylobacter coli were able to survive on different bile salt concentrations (1.5%, 3.0%, 4.5%, 6.0%), after 48 hours of incubation of cells with simulated intestinal juice, there cells were still viable even at higher concentration of bile salts. Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (13). This helped Campylobacter to reach the small intestine and colon and thus causing infection. For microorganism to survive in the human gastrointestinal tract microorganisms, it must endure numerous environmental extremes.

Variations in pH, low oxygen levels, nutrient limitation and elevated osmolarity all these constitute potential impediments to survival (14). Given that the liver secretes as much as a litre of bile into the intestinal tract each day, exposure to bile also represents a serious challenge.

Bile is a digestive secretion that plays a major role in the emulsification and solubilization of lipids. It has the ability to affect the phospholipids and proteins of cell membranes and disrupt cellular homeostasis. Therefore, the ability of pathogens and commensals to tolerate bile is likely to be important for their survival and subsequent colonization of the gastrointestinal tract. Bile is a yellow/green aqueous solution of organic and inorganic compounds whose major constituents include bile acids, sodium, potassium, cholesterol, phospholipids (mainly phosphatidylcholine) and the pigment biliverdin (bili = bile, verdi = green) (15). Immunoglobulin A and mucus are secreted into bile to prevent bacterial growth and adhesion, and the presence of tocopherol may prevent oxidative damage to the biliary and small intestinal epithelium (16).

Campylobacter are also considered to bile resistant and have been isolated from the gallbladder and directly from bile. The observed survival of Campylobacter spp in different concentrations of bile solutions in this study supported the findings of (13) Also, the slight decrease in Campylobacter spp proliferation in concentrations beyond 5% (w/v) bile were similar in these two studies. Microorganisms that inhabit the gastrointestinal tract are exposed to various toxic factors, one of which is bile salts. These potent antimicrobial can disrupt cell membranes, and different bacterial species have developed varying levels of resistance against bile salts. In this study, C. coli was shown to possess a level of tolerance for Oxgall above 4.5% (w/v). At Oxgall concentrations of up to 6% (w/v), the C. coli species was still capable of growing at 7.13 at 48 hours i.e 3.5 folds of the control culture with no bile. The ability of C .coli to survive on this study may be due to some factors. Certain proteins in Campylobacter have been implicated in bile tolerance (16). Flagellin proteins in Campylobacter have been shown to be an important virulence factor of the bacterium (Mshana et al., 2009). Studies have shown that bile acid concentration of about 2.5% (w/v) up regulate both flagellin A and flagellin B and this enables Campylobacter spp to survive in the presence of bile (13).

Secondly, acquisition of iron by bacterium have been shown to represent a major determinant in the development of a pathogen within its host (17) and exposure to bile leads to higher expression of ferritin which is very important in the metabolism and storage of iron. Hence, bile may act as an environment signal for the bacterium to increase its iron storage capacity. Campylobacter jejuni is exposed to bile as it colonizes and proliferates in the bovine gut, and therefore needs to tolerate the bactericidal effects of bile acids. The response regulator CbrR has been shown to modulate bile resistance and, as such, chicken colonization ability (18). The bacterium uses the multidrug efflux pump CmeABC as a mechanism of bile resistance (19), the expression of which is modulated by a transcriptional repressor factor encoded by cmeR (19). It has been determined that C. jejuni shows complex interactions with bile and its constituents, for example, chemoattractive behaviour and upregulation of important virulence factors, such as the flagellin FlaA and the Campylobacter invasion antigens Cia (20). Nonetheless, the current knowledge about the molecular responses of C. jejuni to bile remains very limited. Many genes and proteins are known to participate in the interactions of other bacterial species with bile; thus, a more global study of C. jejuni protein expression when exposed to bile will serve to increase our knowledge about the adaptation of this bacterium to the intestine. Campylobacter jejuni is exposed to bile as it colonizes and proliferates in the bovine gut, and therefore needs to tolerate the bactericidal effects of bile acids. The response regulator CbrR has been shown to modulate bile resistance and, as such, chicken colonization ability (21). The bacterium uses the multidrug efflux pump CmeABC as a mechanism of bile resistance (Layton et al., 2011), the expression of which is modulated by a transcriptional repressor factor encoded by cmeR (22). It has been determined that C. jejuni shows complex interactions with bile and its constituents, for example, chemoattractive behaviour and upregulation of important virulence factors, such as the flagellin FlaA and the Campylobacter invasion antigens Cia (23). Nonetheless, the current knowledge about the molecular responses of

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On the basis of the results obtained after 5 hours at pH 1.5, 2.5, 3.5, 4.5, 5.5 Campylobacter coli survive very well at low pH in gastric juice, there were viable cells at pH of 1.5. Before reaching the intestinal tract, Campylobacter survived transit through the stomach where the pH can be as low as 1.5 to 2 (24). The results pointed out that the Campylobacter coli at different pH (1.5 to 3.5), survived more at 1 hour to 3 hours. However, from pH 3.5 to 5.5, there were significant reductions in cell count or viability. This may be an indication that pH and time of exposure are critical factors in determining patency/mainteinance of the cell morphology. This finding is in agreement with what has been reported by Kelly et al., (24) who indicated that the resistance of the cells to different stress was greater during exponential-phase (growing period) and that there is a flunctuation in resistance during the early and late stationery-phases. The results in this study showed that whereas, the viable cell counts decreased upon exposure to acidic conditions, this decrease paralleled with an increase in time of exposure. The results presented demonstrated that acidic stress, particularly 4.5 and 5.5 could cause obviously greater reduction in cell viability. These higher pH 4.5 and pH 5.5 caused greater morphological changes on cell surface of Campylobacter and allowed H^+ to gain access to cytoplasmic components of the cell. Such changes in the cell envelope could subsequently induce lysis and an obvious reduction in viable cell counts.

Enteric pathogens try to keep the internal pH close to 7.6 to 7.8 during growth (25). The difference between internal pH and outside pH is known as delta pH (Δ pH). The internal pH is maintained by pumping the protons into the cell in an alkaline environment outside pH, or by extruding the protons in an acidic environment (26). Two systems are critical to maintaining the internal pH homeostasis in gram negative bacteria. These systems are potassium-proton antiporters (27) and sodium-proton antiporters (26). Briefly, shifting to acidic condition causes the potassium-proton antiporter system to alkalinize the cytoplasm, whereas shifting to alkaline media result in acidification of the cytoplasm through sodium-proton antiporter (27). In addition to the above mentioned systems, adaptive acid stress response systems are “emergency” systems which enable the enteric pathogens to induce certain regulatory pathways to cope with the damage caused by severe acidic conditions.

Another possible explanation for the survival of Campylobacter in low pH as observed in this study may be due to possible synthesis of certain proteins which may have conferred protection on the organism in acidic condition. Some of these proteins may include SodB, AhpC, and Dps which have associated with oxidative stress (28) and these proteins have been shown to be acid induced (29) suggesting multiple protective mechanisms. This link has further been supported by a recent Campylobacter transcriptomic study where upregulation of numerous genes including ahpC, sodB and p19 during HCl exposure were reported (30). Dps had been said to play a role in in acid tolerance response response and has been established to have multi-functional properties such as DNA binding, iron sequestration, ferrioxidase activity, and a central role for several stress responses-including acid stress (31). Oxidative stress and free iron are closely connected (31), and it has been shown that decreasing pH results in enhanced iron-mediated lipid peroxidation processes (32). Via the Fenton reaction, free iron can react with H_2O_2 and generate cell-damaging hydroxyl radicals (•OH) (30). Regulation of free Fe2+ is therefore essential for cellular activities. Iron storage proteins indirectly contribute to oxidative stress defence by storing iron in an inactive form thereby preventing formation of harmful hydroxyl radicals.

The finding of survival of C. coli in bacteriocin is in agreement with report by Hoang et al., 2011 who reported C. coli strain tolerated bacteriocin synthesized Lactobacillus spp. In this study, all the Campylobacter coli exposed to bacteriocin from Lactobacillus spp. survived. Studies have shown that only C. coli could develop bacteriocin resistance in vitro, although it is still unknown why only bacteriocin resistance in C .coli develops more quickly than in C. jejuni (31). The observed wide range of resistance by Campylobacter coli to bacteriocin in this study indicates the possibility of a critically high level of arising resistance. Although, this study recorded continous growth in C .coli in the presence of bacteriocin, series of studies have shown contradiction results. For instance, Line et al. (19) reported that bacteriocin reduced growth of Campylobacter and thereby reduce their extent of colonization. Since most studies have used only one or only a few strains, it appears likely that some of these conflicting results are to strain or isolate specific variations in the frequency of bacteriocin resistance development.

Bacteriocin – endocing plasmid may give the bacteria that harbour them a competitive advantage in the microbial world. Bacteriocins are proteins that destroy other closely related bacteria. Bacteriocin is also an example of microbial toxin which is used to control the population of other micro-organisms. Campylobacter coli produced bacteriocin which was able to destoy other bacteria, thereby allowing Campylobacter to thrive. Notably, recent breakthroughs in the discovery and characterization of potent anti-Campylobacter bacteriocins (BCNs) may lead to an effective measure for on-farm control of Campylobacter in poultry (20). BCNs are short

antimicrobial peptides (AMPs) produced and exported by most bacterial species examined to date for the apparent purpose of destroying their competitors (21). Many BCN-producing bacteria (e.g., lactic acid bacteria) are commensals in the intestine. Therefore, the intestinal BCN-producing bacteria may achieve competitive advantage and function as an innate barrier against pathogens in the host. The natural and low-toxicity BCNs have been proposed as promising candidates for novel antimicrobials (18).

Survey have indicated that BCN Campylobacter strains are rarely detected among clinical, poultry, and environmental isolates, suggesting that development of BCN resistance in Campylobacter is uncommon, even though Campylobacter strains frequently encounter various BCNs produced by commensal bacteria in the intestine (21).

Proteolytic enzymes were produced by Campylobacter. Casein or skimmed milk agar plate assays allow principally for qualitative determinations of protease activity. The hydrolysis zone produced on the casein agar could be related to the amount of protease produced by the organism (33). But some exceptions have been reported, such as the protease produced by Bacillus licheniformis, which produces very narrow zones of hydrolysis on casein agar plates inspite of large enzyme production by submerged culture (34). Although protease activity could be readily observed easily, in some cases, without the use of a developing agent, it was very difficult to detect and photograph the narrow zone. BCG dye was effective in determining the narrow zone, and this method is very simple. In the present study, the clear distinct zone was observed after the addition of BCG reagent on the casein agar plate. The zone was distinct and the surrounding was greenish-blue in colour, but the colour of the plates strongly depended on the pH value of the agar medium (35). The zone was not clear in the control. A zone of proteolysis was detected on the casein/skimmed milk agar plates. The proteolytic activity was determined as the clear zone where as the rest of the plates were greenish-blue in colour. During transmission from the primary reservoirs to the consumer, Campylobacter is exposed to a changing environment, such as fluctuations in temperature and availability of oxygen, to which the bacterium has to adapt to in order to survive. One of the key consequences that environmental changes impose on living cells is the production of nonnative, misfolded proteins, which tend to unfold and form toxic protein aggregates (36). The cell responds by increasing the synthesis of a set of highly conserved chaperones and proteases, which play important roles in bacterial survival by either refolding or degrading stress-damaged proteins. Thus the increased concentration/synthesis of proteolytic enzymes (protease) observed in this study might have been due to degrade nonnative, misfolded proteins in the cell possibly formed as a result of stressed environmental condition occasioned by high pH (presence of Oxgall), presence of degrading enzyme (bacteriocin) and acidic condition (pH 2.5).

V. Conclusion

Determination of adherence potential test shows that both Campylobacter jejuni and Campylobacter coli adhered well to the intestinal mucosa of the host but Campylobacter coli adhered more. Campylobacter spp. grew in acid condition implies that they were able to withstand HCl in the hosts' stomach, they survived in the presence of bile salts in the small intestine, also survived low oxygen content of the gut proves that they are microaerophilic in nature. The ability to survive acidic conditions, presence of bile, tolerate bacteriocin shows that Campylobacter spp isolated are likely to be virulent ones.

The authors declare that there is no competing interests, the article will include a statement to this effect: The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article

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