JOURNAL OF INTERNATIONAL ACADEMIC RESEARCH FOR MULTIDISCIPLINARY
Impact Factor 4.483, ISSN: 2320-5083, Volume 5, Issue 10, November 2017

SEROLOGICAL STUDIES ON LACTOBACILLUS ACIDOPHILUS EFFECTS AGAINST *E. COLI* INFECTION IN CALVES

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Abstract

The aim of this study was to investigate the diversity of the *Escherichia coli* population, focusing on the occurrence of pathogenic *E. coli* incalves and the effects of Lactobacillus acidophilus against *E. coli* infection in calves. In this study a total of 76 fecal samples and 54 internal organs (18 intestines, 18 livers and 18 spleens) were collected from diarrheic and dead calves, for the isolation of *E. coli*. Different serological patterns were detected, but only serotype O: 115 and O: 119 were consistent in their isolation, in both fecal and organ specimens. There was no correlation between the serotype and the virulence factors studied (haemolysis of sheep RBCs, haemaglutination (HA), mannose-resistant-haemaglutination (MRHA) and HeLa cell invasion).Only all MRHA isolates were invasive to HeLa cells. In challenge experiments, mice that were fed on 10% skim milk containing L. acidophilus (1.5 x 10^8 c.f.u.) for 1 week, then challenged with *E. coli*O:115 or O:119 (2.5 x 10^9c.f.u.) showed 100% survival, whereas control unprotected mice showed 53.3% and 73.3% survival rate respectively when challenged with the same dose of *E. coli*. The viable counts of *E. coli*O: 115in the liver and spleen of L. acidophilus protected mice were significantly lower ($P<0.05$) than the corresponding counts in the control unprotected mice. Furthermore, *E. coli*O: 115 appeared in these organs only in the second day post challenge, and disappeared by fifth day in the protected mice, whereas *E. coli*persisted in a high counts in the control unprotected mice up to eighth day. Meanwhile *E. coli*antibody titres in the serum as well as in the intestinal fluid of the L. acidophilus protected mice were significantly higher ($P<0.05$) than the titres in the control unprotected mice. These results confirmed the probiotic effect L. acidophilus against the colonization of *E. coli*in the animal tissues as well as enhancing their immune response.

Introduction

Escherichia coli have been associated with a wide range of infections in man and animal. Calf diarrhea caused by *E. coli*was considered as one of the most tenacious field problems, as some epidemiological studies has revealed that 30% of the new born calves were hypo-gammaglobulinemic and highly susceptible to opportunistic *E. coli*infection **(Gay**

et al., 1983 and Girardeau et al., 1988). Due to certain virulence characteristics and variable interactions with the host cells, *E.coli*pathogenesis was categorized as Enteroinvassive (EIEC), Enterotoxigenic (ETEC), Enteropathogenic (EPEC), Enterohemorrhagic (EHEC) and Enteroadherent (EAEC) (Finalay and Falkow 1997).*E. coli* is able to acquire resistance genes from other microorganisms or from the environment, and can represent a potential reservoir for the horizontal transmission of these genes to different bacterial species (Trobos et al., 2008; Ahmed et al., 2009; Marshall and Levy, 2011; EFSA-ECDC, 2012).

Many virulence factors were encountered in initiating the infections, such as fimbriae, adhesions and toxins. This made it difficult to develop a collective or sub-unit vaccines to control such problem that might be intensified by the failure of serospecific antibodies to cross protect against heterogenous challenges (Levine 1987 and Finlay and Falkow 1997). Antimicrobial agents used in the treatment of infectious diseases, and is actually considered an emerging and serious public health concern (Martinez and Baquero, 2002; Ramos et al., 2013).

Recently many authors resorted to the use of probiotics and biological preparations of Lactobacilli and other lactic acid bacteria to combat diarrhea in different animals (Nemcova et al, 1997, Jensen 1998 and Nemcova et al, 1999). L. acidophilus has shown antagonistic effect against many pathogens as *E. coli*, Salmonella, Shigella and Clostridium; furthermore it was considered as one of the major health products and immune potentiators(Sato 1984, Fernandez et al., 1988 Hillman et al., 1993, Nemcova 1997, Jensen 1998, Roberfroid 1998 and Nemcova 1999).

The purpose of this study was to determine the serological and virulence patterns of *E. coli* isolated from calves and to evaluate the protective and immune stimulating effect of L. acidophilus against *E. coli* infection experimentally in mice.

Materials and Methods

Specimens: A total of 76 faecal samples and 54 internal organs (18 intestines, 18 livers and 18 spleens) were collected from diarrhoeic and dead calves aged 1-8 weeks. The specimens were collected from private and governmental farms in Kafr-El-Sheikh and Fayoum governorates and examined for *E. coli*. Other intestine specimens were collected from healthy calves at El-Basatein slaughterhouse for isolation of L. acidophilus. All the specimens were labeled and transported to the laboratory in ice box with minimum delay.

Animals: Two groups of albino mice weighing 20-30g were kept in plastic cages at room temperature. The first group was fed for one week 10 % skim milk containing L acidophilus (1.5 xl0^{8}c.f.u.) isolated from the intestine of healthy calves. The other group of mice was kept as control and only fed on 10% skim milk.

Isolation and identification of *E. coli*: Faecal and organ samples were cultured directly on blood agar (containing 7-10% sheep blood), Mac-Conkey agar and indirectly on nutrient broth. Plates were incubated at 37°C for 24-48 hrs. Suspected colonies were identified biochemically by the API 20E kit system and serologically by the slide agglutination test using poly and monovalent antisera **(Edward and Ewing 1972).**

Isolation and identification of L. acidophilus: L. acidophilus was isolated from the intestine of healthy calves on Rogosa agar medium at 37°C and 10% CCh. The isolation and identification was carried out according to **Qin et al., (1995).**

Hemaggulatination (HA) and Mannose resistant Hemaggulatmation test (MRHA): The test was earned out according to Evans et al, (1980) using bovine RBCs. 1 ml of 3.8% citric acid was added to 9 ml bovine blood, then diluted with Phosphate buffer saline (PBS) pH 7.2 to test HA, or diluted with 1:4 with 1% D-mannose in PBS to test MRHA. All *E. coli* isolates were grown on colonization factor agar (CFA). Colonies were mixed with a drop of diluted blood solution on a glass slide to check the HA or MRHA activity (Evans et al, 1980).

Invasiveness to HeLa cells: The test was carried out according to the method of **Jones et al., (1981) and Seleim (1997).** Briefly, the HeLa cells were grown in Eagle's minimal essential medium over glass slips. The *E. coli* isolates were added at 3xl0^7 bacteria/ml and incubated at 37°C for 3 hrs. The extra cellular bacteria were rinsed and the internalized bacteria were counted. The invasion index was calculated according to **Jones et al., (1981).**

Challenge procedures: Mice which were fed for seven days on 10% skim milk containing **L. acidophilus** 1.5xl0^{8}c.f.u., as well as the control unprotected mice (fed on 10% skim milk) were challenged with ***E. coli*** 0:115 (2.5xl0^{9}c.f.u) and ***E. coli*** 0:119 (2.5x10^{9}c.f.u) separately and observed for 20 days. The daily mortality was recorded in each group and the percentage of survival was calculated.

***E. coli* counts in tissue homogenates:** The number of viable *E. coli* counts in the livers and spleens of the mice challenged with *E. coli* 0:115 (previously received L. acidophilus 1.5 x10^{8}c.f.u for 7 days), as well as the control unprotected mice were not cropped on 1st, 2nd, 5th and 8th days post challenge. Liver and spleen were removed, homogenized in 5ml PBS

with glass homogenizer, then 1ml of the 10-fold serial dilution was plated on Mac-Conkey agar and the viable counts of *E. coli* was determined as reported by **Rebucci et al. (2007).**

Detection of antibodies in serum and intestinal fluid: The two challenged groups of mice (L. acidophilus protected and control unprotected) were bled from retro-orbital venous plexus before the necropsy (previously mentioned). Sera were harvested and diluted, then antibody titers were determined by agglutination test against *E. coli* 0:115 bacterial suspension (adjusted at 3xl09 c.f..u./ml). For detection of *E. coli* antibodies in the intestinal fluid, the full length of the small intestines was removed (from the gastric-duodenal junction to ileal-caecal junction). The contents were flushed out with 1ml cold PBS pH 7.2, centrifuged at 2000g for 30min. The supernatant was collected, and if. coli antibody titers were determined by diluting the supernatant in PBS and testing for agglutination with *E. coli* as mentioned before. Antibodies were measured on the same day the bacterial counts were carried out.

Statistical analysis: The *E. coli* counts in livers and spleens of the **Lacidophilus**protected mice as well as the control unprotected mice were subjected to statistical analysis using the two way ANQVA test. Also the antibody titers in the serum and intestinal fluid of both challenged groups of mice were also subjected to the same statistical analysis test.

RESULTS

The isolation of ***E. coli*** from the feces and internal organs of diarrheic calves revealed different serological patterns (Table 1 and 2). Only two serotypes 0:115 and 0:119 were detected, from the faeces as well as the intestines, livers and spleens. Studying some virulence characteristics of the isolates revealed no correlation between the serotype isolated and the virulence factor studied, except for a direct correlation between maimose resistance haemagglutinating (MRHA) strains and invasiveness to HeLa cells. It was found that, all MRHA isolates were highly invasive to HeLa cells. In the protective-challenge experiment, mice which were fed for 1 week with 10% skim milk containing L. acidophilus ($1.5xl0^8$c.f.u.) showed 100% survival rate when challenged with *E. coli* 0:115 and 0:119 ($2.5\ xlO^9$c.f.u.), whereas control unprotected mice showed 53.3% and 73.3% survival rates respectively (Table 3). The invasion of the liver and spleen by the ***E. coli*** 0:115 challenge strain (detected by viable bacterial count) in **L. acidophilus** -protected mice was significantly lower ($p<0.05$) than the control-unprotected group. Further, the!?, coli appeared in the liver and spleen of protected mice only in the 2nd day post challenge (in relatively low counts) and disappeared completely by the 5th day, whereas the control-unprotected mice maintained relatively high

counts up to the 8th day post challenge (Table 4). The anti ***E. coli*** antibody titers in the serum and intestinal fluid of the previously mentioned groups of mice showed significant increase in the antibody titers of the **L. acidophilus** protected mice ($P<0.05$) compared to the unprotected groups (Table 5).

Table (1): Serotype and virulence characteristics of *E. coli* isolated from feces of diarrchoeic calves.

Serotype	No. of positive	Haemolysis of sheep RBCS	Haemaggluti nation. (HA)	MRHA	HeLa cell invasion
O: 115	7 (29.2%)	3/7	4/7	2/7	2/7
O:119	4 (16.7%)	2/4	2/4	1/4	1/4
O :126	4 (16.7%)	1/4	2/4	1/4	1/4
O: 166	3 (12.5%)	1/3	2/3	0/3	0/3
Untypable	6 (25%)	2/6	3/6	2/6	1/6
Total	24/76 (31.6%)	9/24 (37.5%)	13/24 (42.2%)	6/24 (25%)	5/24 (20.8%)

MRHA = Mannose Resistant Haemagglutination

Table 2:- Serotype and virulence characteristics of *E. coli* isolated from (Intestines , livers , spleens) of dead calves with history of diarrhoea.

Organ	Intestine	Liver	Spleen	Haemolysis of sheep RBCs	HA	MRHA	HeLa cell invasion
0: 115	2/18	1/18	1/18	4/14	4/14	2/14	3/14
0:119	2/18	1/18	1/18	1/14	3/14	1/14	2/14
Untypable	2/18	2/18	2/18	1/14	2/14	1/14	2/14
Total	6/18 (33.3%)	4/18 (22.2%)	4/18 (22.2%)	6/14 (42.9%)	9/14 (64.3%)	4/14 (28.6%)	7/14 (50%)

Table (3):-Survival rate of mice protected with L. acidophilus and control unprotected when challenged with *E. coli* 0:115

Challenge experiment	Survival up to 4 days	Up to 8 days	Up to 12 days	Up to 16 days	Up to 20 days	Survival rate	
						No.	%
Unprotected control challenged with 0: 115(2.5 xl09)	14/15	10/15	9/15	8/15	8/15	8/15	53.3%
Unprotected control challenged with 0:119 (2.5xl09)	14/15	12/15	12/15	11/15	11/15	11/15	73.3%
L acidophilus protected (l.oxl08) and challenged with *E. coli* 0:115 or 0:119	15/15	15/15	15/15	15/15	15/15	15/15	100%

Table (4):- *E. coli* counts in liver and spleen of L. acidophilus protected and control unprotected mice.

Challenge experiment	2nd Log c.f.u ±S.D. (Average c.f.u)		5th day Log c.f.u +S.D. (Average c.f.u)		8th day Log c.f.u ±S.D. (Average c.f.u)	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
L. acidophilus (1.5 × 108) for 7 days and challenged with *E. coli* 0:115 (2.5×l09)	2.67±1.64 (4.56×l02)	2.4±1.76 (2.53×102)	Nil	Nil	Nil	Nil
Not protected with L. acidophilus only challenged with *E. coli* (2.5×l09)	2.7+1.92 (5.05×102)	2.5±1.8 (3.33×l02)	4.13±1.9 (1.3×l04)	3.51+2.09 (3.2×l03)	3.44+2.08 (9.78×l03)	2.37+1.86 (2.35×l03)

*Logarithm of colony forming units ± standard deviations.

Table (5):- *E. coli* antibody titers (inverse dilution) in the serum and the intestinal fluid of the L. acidophilus protected and control unprotected mice

Challenge experiment	2nd day		5th day		8th day	
	Serum	Intestine	Serum	Intestine	Serum	Intestine
L. acidophilus protected and challenged with *E. coli* 0:115	53.3+20.7*	46.7+16.3	1173.3+261.3	533.3+165.2	586.3+130.6	146.7+32.7
Control mice not protected with L.acidophilus and challenged with *E. coli* 0:115	16.7±5.2	15.0+5.5	140.8+62.4	79.2+17.5	51.43+29.3	33.3+10.3

*Inverse dilution of *E. coli* 0:115 antibody titers and standard deviarins.

DISCUSSION

Examination of faecal and internal organs from diarrhoeic calves revealed different serological and virulence patterns of *E. coli* isolates. The serotype 0:115 and 0:119 were the most prevalent and virulent whereas 0:126 and 0:166 were isolated in lower or less virulent frequencies (table 1&2). These findings were found in agreement with Orskov and Orskov (1978) and Fair brother et al., (1986) ,Matthew et al., (2009) who isolated both serotypes among other serotypes from diarrhoeic calves and other animals. The results indicated that, there was no correlation between the serotype isolated and the virulence factors investigated, except for the direct correlation between man- nose resistant hem agglutination (MRHA) and invasion of HeLa cells. All MRHA isolates were highly invasive to HeLa cells which were also supported by Fairbrother et al., (1986) and Finlay and Falkow (1997) who suggested that these isolates might not have possessed the type I fimbriae which was mannose sensitive.

In the protection-challenge experiments on mice the L. acidophilus protected mice showed 100% survival percentage whereas, the unprotected mice showed 53.3% and 73.3% survival rates when challenged with *E. coli* 0:115 and 0:119 respectively. The invasion of liver and spleen by *E. coli* 0:115 in the L. acidophilus-protected and unprotected-control mice showed different invasion patterns. The *E. coli* counts, in the liver and spleen of the L. acidophilus - protected mice were detected in a lower rate in the second day only then disappeared completely by the fifth day post challenge, whereas the control unprotected mice showed significantly higher *E. coli* counts ($P<0.05$) which maintained up to the eighth day post

challenge (Table 4). These results elucidated the protective effect of L. acidophilus which encompassed cell-associated and cell-free components e.g. S-layer hydrophobic proteins, polysaccharides and lipoteichoic acid polymers. These components enhanced the adherence of L. acidophilus to intestinal mucosa, and hindered the colonization of *E. coli* and other pathogens (Hillman et aL,1993, Nemcova et a!.,1997 and Nemcova et al.,1999). Other lactobacillus cell-free products were acetate, lactate, H2O2, butyric acid, butyrates as well as lactoperoxidasethiocynate system (LPT), all these components had an inhibitory effect on the *E. coli* (Nemcova et al, 1997, Jensen 1998,Roberfroid 1998 and Oyetayo et al, 2003).

The antibody titers against the *E. coli* 0:115 in the serum as well as in the intestinal fluid of the L. acidophilus-protected and unprotected-control mice, showed significant increase ($P<0.05$) in the antibody titers in the former groups by 4-8 times (Table 5). This increase in antibody titers was elucidated by many workers (De Macias et al.,1993, Nemcova et al., 1999, Guktepe,2006 ,El-Kholyet al., 2014)who confirmed the immune stimulating effect of L. acidophilus on the cell-mediated as well as the humoral immunity. They also noticed that the L. acidophilus had a strong adjuvanicity and initiated the induction of lymphokines and immunoglobulines. The Lactobacillus -primed-T- cells enhanced the formation of epitheloid granulomas in the liver and spleen which was accompanied by activation of migratory macrophages and monocytes (Sato 1984 and Fairbrother et al,1998) This finding also explained the disappearance of *E. coli* 0:115 from the liver and spleen of the L. acidophilus-protected mice. Such potential probiotic effect of L. acidophilus indicated a realistic route for using such biological preparations in the prevention and control of *E. coli*entropathies in calves and other animals as previously suggested by Fernandez et al,(1988), Roberfroid (1998) and Nemcova (1999) (Ripamonti et al., 2011). Zhang et al. (2016) found that Lactobacillus spp. were good probiotic candidates, help to promote health of hosts, protect hosts from intestinal pathogens and maintain the natural balance of intestinal microflora during antibiotic treatments.

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