APPLICATION OF SOME LACTOBACILLUS STRAINS PRODUCT FOR CONTROL OF SALMONELLA TYPHIMURIUM INFECTION IN DIARRHOEIC NEONATAL CALVES

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ABSTRACT

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Salmonella enterica serovar typhimurium (S. typhimurium) was isolated from both dead and clinically diarrhoeic beef calves, which had history of severe diarrhoea. Another two serovars ((Salmonella enterica serovar dublin (S.dublin) and *Salmonella enterica* serovar muenster (S.muenster)) were demonstrated from both clinically diarrhoeic calves as well as from the contact apparently healthy ones. Out of 77 diarrhoeic cattle calves, 34 were proven positive for *Salmonella* isolates (44.2%) whereas the apparently healthy contact calves showed lower rate of isolation 12 out of 97 (12.9%). *S.typhimurium* was the most dominant serovar as revealed from the isolation pattern. In clinical diarrhoeic cases S. *typhimurium* constituted 21 out of 34 isolates (61.8%) and 9 out of 12 (75%) in apparently healthy calves. *Salmonella dublin* and *muenster* were isolated in lower patterns, as 11 out of 34 isolates (32.3%) and 2 out of 12 (16.7%) in case of S.dublin whereas 2 out of 34 (5.9%) and 1 out 12 (8.3%) in case of S.muenster were detected in diarrhoeic and apparently healthy calves respectively. Lipopolysaccarid (LPS) ElISA demonstrated higher antibodies titer in the diarrhoeic animals (1:2400 to 1:9600) than apparently healthy calves (1:400 to 1:7200). After administration of *Lactobacillus casei (L.casei*) (10^{10}cfu) to clinical diarrhoeic calves, the diarrhoea stopped and the shedding of *Salmonella* ceased. *Coliform* counts were also reduced with remarkable increase in the *Lactobacillus* counts were determined ($6.47\pm2.2\log_{10}$). The humoral as well as the cellular immune responses were also boosted. *Salmonella* antibodies levels were significantly increased and inhancement of the macrophages activity was demonstrated (from 4.4 ± 1.2 to 33.2 ± 5.1 cell. macrophage). Serum biochemical analysis of diarrhoeic calves showed significant decrease in total proteins, albumin, globulins, A/G ratio as well as glucose levels. The enzyme activity of ALT, AST, alkaline phosphates as well as the values of creatinine, urea and uric acid were significantly increased. Serum minerals profiles were also altered where calcium, phosphorus, magnesium, zinc, cupper, iron, sodium and chloride were decreased, whereas potassium was significantly increased. After treatment with *L. casei* significant improvements of certain biochemical parameters were observed.

استخدام بعض عترات اللاكتوباسلس في الوقاية من مرض الإسهال في العجول الرضيعة المصابة بميكروب السالمونيللا تيفيميوريم

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تم عزل ميكروب السالمونيللا المعوية النوع تيفيميوريم من عجول تعانى من اسهالات حادة ولها تاريخ مرضى للاصابة، كما تم عزل انواع اخرى منها سالمونيللا دوبليم وسالمونيللا مونيستر من عجول مصابة بالاسهال واخرى سليمة ظاهريا ومخالطة للعجول المصابة. كان اجمالى نسب العزل للعترات المختلفة من ميكروب السلمونيللا ٣٤ من اصل ٧٧ حالة مصابة بالاسهال (**٤٤.٢%**) **و ١٢** ميكروب من اصل ٩٧ حالة سليمة ظاهريا (١٢.٩%). سجلت النتائج اعلى نسب عزل من اعداد الميكروبات المعزولة من نصيب السالمونيللا المعوية النوع تيفيميوريم ، ٢١ ميكروب من اصل ٣٤ (٦١.٨ %) فى الحالات المصابة بالاسهال بينما كان العدد ٩ من اصل ١٢ ميكروب فى الحالات السليمة ظاهريا والمخالطة للمصابة (٧٥ %). كانت الاعداد المعزولة من السالمونيللا دوبليم والسالمونيللا مونيستر قليلة مقارنة بالتيفيموريم فتم عزل ١١ من اصل ٣٤ ميكروب (٣٢.٣ %) و ٢ من اصل ١٢ (١٦.٧ %) للسلمونيللا دوبليم ، بينما كانت الاعداد ٢ من اصل ٣٤ ميكروب (٥.٩ %) و ١ من اصل ١٢ (٨.٣ %) للسالمونيللا مونيستر فى الحالات المصابة بالاسهال الحاد والسليمة ظاهريا والمخالطة للمصابة بالتتابع. تم تطبيق اختبار الاليزا على عدد ٢٠ عينة بلازما دم من عجول تعانى من إسهال حاد ومصابة بالسالمونيللا ومقارنتها بعينات من عجول تسمين تبدو سليمة صحيًا. اظهرت النتائج ارتفاع ملموس فى مستوى الاجسام المضادة لميكروب السلمونيللا فى العجول المصابة (١: ٢٤٠٠ - ١:٩٦٠٠) عنها فى العجول السليمة ظاهريا والمخالطة للمصابة (١:٤٠٠ - ١ : ٧٢٠٠) بالتتابع. تم تحليل نفس العينات بيوكيميائيا، وقد اظهرت التحاليل للعجول المصابة وجود إنخفاض ملحوظ فى نسب كلا من :- البروتين الكلى ، الألبيومين ، الجلوبيولين ، نسبة الألبيومين / الجلوبيولين ، كذلك نسبة الجلوكوز. بينما كان هناك إرتفاع معنوى فى نشاط إنزيمات الكبد وإنزيم الألكالين فوسفاتاز ونسبة الكرياتينين واليوريا وحامض البوليك. أيضا تغيرت نسب عناصر الأملاح المعدنية ، حيث انخفضت نسبة كل من الكالسيوم ، الفوسفور ، الماغنسيوم ، الزنك ، النحاس ، الحديد ، الصوديوم والكلوريد. أما عنصر البوتاسيوم فقد زاد زيادة ملحوظة. تعزى جميع النتائج الى التأثير المباشر لحالات الاسهال الشديدة والتى تؤثر بدورها تأثير معنوى على مكونات الدم المختلفة. لوحظ أنه بعد إستخدام بكتيريا اللاكتوباسيلس كازياى فى العلاج ، ظهر تحسن ملحوظ فى بعض نسب ومعدلات مكونات بلازما الدم البيوكيميائية حيث تساوت تقريبا مع المعدلات الطبيعية.

كذلك انخفضت الاعداد البكتيرية انخفاضا ملحوظا للانواع المختلفة من السالمونيللا المعوية وكذلك الميكروب القولونى، مع زيادة ملحوظة فى اعداد بكتيريا اللاكتوباسلس. لذلك يوصى باستخدام مستحضرات بيولوجية طبيعية من بكتريا اللاكتوباسيليس كازياى كعلاج بديل للإسهال.

Key words: *Diarrhoeic calves, Lipopolysaccarid (LPS), ALT, AST.*

INTRODUCTION

Salmonella infection occurs throughout the world and has a hazardous effect on human and animal health as well as great impact on farm economics. In calves the infection appears in the form of septicemia and diarrhoea and in pregnant cows many abortion cases were often manifested (Santos *et al.,* 2001; Barrington *et al.,* 2002). *Salmonella enterica* serovar *typhimurium* and *dublin* where the commonest serovars isolated from cattle. Other serovars as anatum, enteritidis, cerro, montevedio, saint paul, infantis rostock, newport and newington were also reported, but in lower incidences. Calves are highly susceptible to *Salmonella* infection especially when the pregnant dames were not vaccinated once or twice before parturition and if the neonatal did not receive colostrum (Visser *et al.,* 1990; Konrad *et al.,* 1994 and Santos *et al.,* 2002).

Enzyme-linked immunosorbent assays (ELISAs) based on lipopolysaccharide (LPS) for different salmonella serovars has been evaluated by many researchers as a highly specific test for diagnosing *Salmonella* infection in bovine.

Combination Not only serum was tested in these ELISA but also milk samples and other body fluids were investigated (Hoorfar *et al.,* 1995; Hoorfar and Wedderkoppe 1995; Smith *et al.,* 1995; Seleim, 1999; Galland *et al.,*

2000; Radke *et al.,* 2002 and Veling *et al.,* 2002).

Some authors declared, that serum biochemical profiles of diarrhoeic calves were affected as,total proteins, albumin, globulin, A/G ratio and glucose were significantly decreased (Manaa *et al.,* 1993 and Kaneko *et al.,* 1997). Liver and kidney profiles were on the contra`ry increased, whereas macro and microelements decreased significantly except for potassium which was significantly increased (Ragab *et al.,* 1986; Aly *et al.,* 1996 and Kaneko *et al.,* 1977).

Recently many probiotic bacteria as lactic acid bacteria (LAB), *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and some fungi as *Aspergillus oryzae* and yeast were used as health promoters for humans and animals. Certain preparations were used to boost immune status, increase resistance to infectious diseases, particularly of the intestine, decrease duration of diarrhea (Romond *et al.,* 1998; sreekumar and hosono 1998; Tannock 2002; Ibnou-Zekri *et al.,* 2003; Makras *et al.,* 2006; Gratz *et al.,* 2010). The mechanism of action of probiotics is not fully understood, either they migrate through the gut wall as viable cells and multiply to a limited extent or antigens released by the alive or dead organisms, that can be absorbed and stimulate the immune system directly. A third school of thought suggested that the *Lactobacillus* species acted indirectly through an effect on the other microbial components (as Coliform) of the gut flora. It was the product of this change which induced the immune response. Moreover, it appeared to be some relationship between the ability of *Lactobacillus* strain to translocate and the ability to be immunogenic (Fuller, 1989; Gibson, 1995; Roberfroid, 1998; Ibnou-Zekri *et al.,* 2003).

The objective of this study was to determine the different *Salmonella enterica* serovars causing fatalities and diarrhoea in calves and to apply lipopolysaccaharide-ELISA for diagnosis. Moreover, the study included investigations on the serum biochemical profiles of diarrhoeic calves before and after competitive exclusion treatment with *L.casei*, as well as assessment of this biological treatment in curbing *Salmonella enterica*-induced diarrhoea in beef calves.

MATERIALS and METHODS

Specimens:-

Internal organs from 5 recently dead calves (5 Intestines, 5 gall bladders, 5 livers, 5 spleens, 5 lungs and 5 kidneys) aged from 1 week to 6 months, had a history of anorexia, pyrexia and sever diarrhoea sometimes tinged with blood, were examined bacteriologically. Another 174 faecal samples (77 from diarrhoeic calves and 97 from apparently healthy contact animals) and 174 blood samples (from the same animals) were examined bacteriologically for *Salmonella* and serologically for *Selmonella* antibodies respectively. Samples were collected during the period of one year from august 2010 to august 2011 form calves aged (1 week – 6 months) at governmental and private farms in Giza, Gharbia and Dakahlea governorates. All specimens were transferred to the laboratory in ice box with minimum delay.

Animals:-

In the second phase of investigation 20 diarrhoeic beef calves that were proven positive for *S.typhimurieum* were selected for treatment with *L. casei* as competitive exclusion treatment. Faecal and blood samples were collected from these calves on weekly bases and assessed for *Salmonella*, *Coliform* and *lactobacillus* content, while blood samples were assessed for humoral and cellular immune response as well as the serum biochemical analysis was carried out. Another 15 calves were apparently healthy, had no history of diarrhoea and proven negative for *Salmonella* were selected as control.

Isolation and identification of salmonella enterica:-

Faecal samples were cultured into selenit-f. broth, and incubated at 37^0C for 18hrs. Loopful from these broth cultures were then streaked onto MacConkey and S. S. agar plates, incubated at 37^0C for 24 and 48hrs. Suspected colonies were identified morphologically, biochemically by the API

20E System (BioMereaus, France) and serologically according to the Kauffman-White Scheem by slide agglutination test using polyvalent and monovalent somatic (O) and flagellar (H) antisera (Wellcome Research Laboratories, UK.) according to (Edwards and Ewing 1972).

Isolation and identification of Lactobacillus casei for oral administration to calves:-

L. casei was isolated from the intestine of healthy calves on Togosa agar medium at 37^0C and 10% CO_2. The isolation and identification was carried out according to Qin *et al.* (1995). The selected *L. casei* isolate was tested for bile and acid tolerance (growth at 1% bile salt rogosa agar and at pH5) then adjusted photometreally at (10^{10} CFU/ml PBS pH 7.4). One ml of adjusted L. casei was mixed into 250ml of 2% sterilized skim milk immediately before oral inoculation of the calves. The bacterial population was confirmed by enumeration of serial dilutions on rogosa agar plates in duplicate. The administration of the L. casei was carried out every other day for a period of 3 weeks.

Salmonella, *Coliform* as well as *Lactobacilli* counts were determined in the faeces before and after oral administration of the *Lactobacilli*. *Salmonella* count was determined by direct inoculation onto S.S. agar, Mac Conkey agar (for coliform) and Rogosa agar (for *Lactobacillus*). If the number of *Salmonella* was less than 500/g, enrichment in selenit-f. broth (Difco) could detect those samples which were negative in direct plating (Zaho *et al.,* 1998).

Lipopolysaccaharide (LPS) ELISA:-

ELISA to detect antibodies to LPS prepared from *Salmonella enterica* serovar typhimurium was carried out by extraction of LPS by phenol-chloroform-petroleum ether (extraction mixture) as described by Demarco de Hormacche *et al.* (1988). Each well of microtiter 96-well plates (Falcon) was coated with 5µg LPS/ml in carbonate bicarbonate buffer (pH 9.6). After overnight incubation at 37^0C, the plates were incubated with blocking buffer, consisting of 3% bovine serum albumin (BSA-Sigma) in phosphate buffer saline pH 7.4 and 0.05% Tween 20 (PBS-T) to coat the unoccupied sites on the plates. After 1 hr at 37°C. The plates were then washed with PBS-T and 100µl goat anti-bovine horseradish peroxidase conjugate (Dako) diluted 1:1000 in 0.3% BSA in PBS-T, was added to each well and incubated for 1 hr at 37°C. The plates were washed with PBS-T then 100 µl 3,3',5,5'-tetramcthylbenzidine (ICN), prepared according to the manufacturer's instructions, were added to each well. After 10 min, 25 µl 5 M H_2SO_4 were added to each well and plates were read at 450 nm in ELISA reader. The cut off value was calculated as the average optical density (OD) value of the negative control values plus 2 standard deviations (SD). The antibody titer was calculated as the highest serm dilution that gives OD value above the cut off point (Ramos *et al.,* 2000).

Estimation of cellular immunity (Macrophage activity):-

Calves leucocytes were harvested from the whole blood by density centrifugation on Histopaque 1077 (Sigma) according to, Lammler and Ding `(1994). Histopaque can separate the leucocytes in a buffy coat layer over the erythrocytes. After the separation of the leucocyte cell fraction over the histopaque surface, the RBCs among the harvested cells were lysed by adding 0.87% ammonium chloride solution pH 7.2 (1:5 v/v) with gentle shaking.

The leucocytes were then washed with Minimal Essential Medium (MEM, Sigma), and were finally adjusted to 10^5 cells/ml MEM using a hemocytometer. *Salmonella* cultures were adjusted photometrically to 10^9 bacteria /ml in MEM medium, then equal volumes of leucocytes and S.typhimurium isolates were incubated at 37°C for 1hr with gentle shaking. The leucocytes-S.typhemeurium mixtures were then spread on a microscope slide, fixed and stained with acridine orange and examined under the microscope. The phagocytosis index was measured according to (Shoshani *et al.,* 2000)

Serum Biochemical Profile:-

Collected serum samples from 20 clinically diarrhoeic calves before and after administration of *L. casei* (10^{10} CFU/calf), as

well as 15 control apparently normal calves were analysed biochemically for determination of total proteins (Hoffmann and Richterrich 1990), albumin and globulins (Doumas *et al.,* 1971), ALT and AST aminotransferases (Reitman and Frankel 1957). Glucose (King and Woottin 1959), alkaline phosphatase (Kilchling and Fraiberg 1951), Magnesium (Neil and Nelly, 1956), sodium and potassium by using flame photometer (Oser, 1989), iron cupper and zinc were estimated spectrophotometrically by Fernandez and Kohn (1991) and chloride (Varley *et al.,* 1980).

Statistical Analysis:-

Statistical analysis of obtained serum values were carried out using the "t" test according to the method of (SSPS 14, 2006)

RESULTS

Only one serovar, *Salmonella enterica* serovar typhimurium, 11 isolates were dected from the 30 collected organs of 5 dead calves. No haemolytic *E. coli* was isolated from these 5 dead calves. Three different *Salmonella* serovars were demonstrated from both clinical cases with diarrhoea as well as from the contact apparently healthy ones. Out of 77 diarrhoeic 34 were proven positive for *Salmonella* isolation (44.2%) whereas the apparently healthy contact calves demonstrated lower rate of isolation (12 out of 97, 12.4%).

Salmonella enterica serovar typhimurium (S.typhimurium) was the most dominant serovar as revealed from the pattern of isolation. In clinical cases S.t.constituted 21 out of 34 isolates (61.8%) and 9 out of 12 (75%) in apparently healthy calves**.** *Salmonella enterica* serovar dublin (S.dublin) and muenster (S.muenster) were revealed in lower isolation pattern, as 11 out of 34 (32.3%) and 2 out of 12 (16.7%) in case of S.dublin and 2 out of 34 (5.9%) and 1 out of 12 (8.3%) in case of S.muenster were detected in both diarrhoeic and apparently healthy calves respectively. All *Salmonella* serovars were confirmed for its somatic (O) and flagellar (H) antigenic structure (Table 1). The cut off value for the LPS ELISA was calculated as 0.33 OD at 450 nm. Diarrhoeic calves recorded higher OD values (range 0.89 to 1.68) than apparently healthy (0.34 to 1.32). The high OD reading was expressed in high antibody titers in the diarrhoeic animals that ranged from 1:2400 to 1:9600, whereas the antibody titer ranged in the apparently healthy calves from 1:400 to 1:7200. LPS-ELISA testing generally revealed higher incidences of *Salmonella* infection than the conventional culture method as 53 out of 77 (68.8%) and 37 out of 97 (38.1%) in diarrhoeic and apparently healthy calves respectively were tested positive for *Salmonella* antibodies (Table-2).

Administration of *L.casei* (10^{10}cfu) every other day for 3 weeks to 20 diarrhoeic calves elucidated significant improvement in the calves health conditions starting the first few days after administration. *Salmonella* count was greatly reduced from 6.37 ± 2.5 $\log_{10}$ to zero. The *Coliform* count was also reduced but still within a limitted range of 5.28 ± 2.1 to 6.21 ± 2.3 ($\log_{10}$). The remarkable increase in the *Lactobacillas* count was noticed immediately after administration from zero to 6.47 ± 2.2 ($\log_{10}$) (Table 3). The humoral as well as the cellular immune responses were also stimulated as the *Salmonella* antibody levels were increased which was monitored by the elevated OD values from 1.68 to 1.94 OD at 450nm. The cellular immunity manifested in the macrophages activity was significantly enhanced by almost eight folds from 4.4 ± 1.2 to 33.2 ± 5.1 cell/macrophange (Table – 3).

In diarrhoeic calves serum biochemical analysis showed significant decrease in total proteins, albumin, globulins, A/G ratio (hypoproteinemia), as well as glucose levels (Table 4 & 5). The enzyme activity of ALT, AST, alkaline phosphatase as well as the values of creatinine, urea and uric acid were significantly increased (Table 5). Minerals profiles were also changed, where serum calcium, phosphorus, magnesium, zinc, cupper, iron, sodium and chloride levels were significantly decreased. Potassium level was significantly increased (Table 6). After treatment with *L. casei* certain improvements of most serum biochemical parameters were recorded, though in some cases some

discrepancies were manifested compared to the control group (Table 4, 5, 6).

Table 1: Isolation and identification of different salmonella serovars from diarrhoeic and apparently healthy beef calves.

Animal condition and number	Organ/ sample	No of +ve	Salmonella serovar isolation pattern number & %	Serogroup and antigenic structure		
				O antigen	H antigen Phase I	H antigen Phase II
Dead animals (n=5)	Intestine	3/5	3 typhimurium	1,4,5,12	I	1,2
	Gall bladder	3/5	3 typhimurium	1,4,5,12	I	1.2
	Liver	3/5	3 typhimurium	1,4,5,12	I	1,2
	Spleen	1/5	1 typhimurium	1,4,5,12	I	1,2
	Kidney	0/5	Zero	-------	------	------
	Lung	1/5	1 typhimurium	1,4,5,12	I	1,2
Diarrhoeic animals (n=77)	Faeces	44/77 (44.2%)	21 typhimurium (61.8%)	1,4,5,12	I	1,2
			11 Dublin (32.3%)	1,9,12	g.p	-
			2 Muenster (5.9%)	3,10	e,h	1,2
Apparently healthy animals (n=97)	Faeces	12/97 12.4%)	9 typhimurium (75%)	1,4,5,12	I	1,2
			2 Dublin (16.7%)	1,9,12,Vi	g,p	-
			1 Muenster(8.3%)	3,10	e,h	1,2

Table 2: Serum from diarrhoeic and apparently healthy, contact animals tested with LPS ELISA.

Origin of serum	No. of positive Bacteriological Samples	No. of LPS-ELISA Positive (%)	Range of Antibody Titer to Salmonella LPS	Optical density (OD) range at 450 nm
Diarrhoeic animals	34/77 (44.2%)	53/77	1:2400-1:9600	0.89-168
Apparently health Animals	12/97 (12.4%)	37/97 (38.1%)	1:400-1:7200	0.34-1.32

Table 3: Effect of lactobacillus casei administration on the intestinal microbial content, on humoral and cellular immunity of 20 salmonella enterica serovar typhimurium infected calves.

Animal Condition	Symptoms	Log_{10} of Coliform count/g	Log_{10} of lactobacillus count/g	OD Range of LPS ELISA	Phagocytosis capacity
Before treatment	Severe diarrhea	6.37±2.5*	Nil	0.69-1.68	4.4±1.2**
1st week after treatment	Moderate recovery	4.3±2.1[a]	3.1 ±1.3[a]	0.71-1.71	12.3±3.7[a]
2nd week after treatment	Full recovery	{500/cfu[c]	5.47±2.4[b]	0.73-1.82	23.7+4.2[b]
3rd week after treatment	Stable and normal	Nil	6.47±2.2[c]	0.81-194[a]	33.5.1[c]

* log_{10}±SD

a) Significant : P< 0.05) b) Significant : P<0.01) c) Significant: P<0.001)

** Average number of cells inside the macrophage ± standard deviation.

Table 4: Proteinogram in sera of 20 infected calves before and after treatment with probiotics (L.casei) in comparison with apparently healthy group.

Biochemical Parameters of serum samples	Diarrhoeic animals		Control N=15
	Before treatment (n=20)	After treatment (n=20)	
Total protein (g/dl)	5.20 ± 0.21[a]	6.20 ± 0.18[b]	6.86 ± 0.24[c]
Albumin (A) g/dl	2.13 ± 0.05[b]	2.6 ± 0.05[b]	3.03 ± 0.18[c]
Globulins (G) g/dl	3.07 ± 0.13[a]	3.59 ± 0.19[b]	3.83 ± 0.12[a]
A/G ratio	0.69 ± 0.03[a]	0.72 ± 02[a]	0.79 ± 0.03[a]

Average value ± standard error
a, b, c, values with different letters are significant (P<0.05). values with the same letters are not significant.

Table 5: Liver and Kidny Function indices in Serum of Calves Before and After Treatment

Biochemical Parameters of serum samples	Biochemical Parameters of serum samples		Control N=15
	Before treatments (n=20)	After treatment (n=20)	
Glucose (mg/dl)	44.93 ± 3.33[a]	55.70 ± 2.32[b]	59.86 ± 1.98[b]
ALT (u/l)	28.66 ± 1.29[a]	19.69 ± 1.78[b]	17.93 ± 0.83[b]
AST (u/l)	59.63 ± 1.54[a]	47.65 ± 1.18[b]	43.50 ± 0.93[c]
Alkaline phosphatase (m.M/l)	2.27 ± 0.05[a]	2.00 ± 0.05[b]	1.6 ± 0.04[b]
Urea (mg/dl)	43.93 ± 1.96[a]	30.26 ± 1.73[b]	26.66 ± 0.93[b]
Uric acid (mg/dl)	2.64 ± 0.27[a]	1.96 ± 0.17[b]	1.65 ± 0.15[b]
Creatinine (mg/dl)	2.00 ± 0.06[a]	1.41 ± 0.04[b]	1.32 ± 0.05[b]

Average value + standard error
a, b, c, values with different letters are significant (P<0.05). values with the same letters are not significant.

Table 6: Serum Biochemical Analysis of Minerals Before and After Treatment.

Biochemical Parameters of serum samples	Biochemical Parameters of serum samples		Control N=15
	Before treatments (n=20)	After treatment (n=20)	
Total calcium (mg/dl)	10.57 ± 0.59[a]	12.35 ± 0.32[b]	12.50 ± 0.85[b]
Inorganic phosphorus(mg/dl)	4.90 ± 0.12[a]	5.81 ± 0.17[b]	6.25 ± 0.24[b]
Magnesium (mg/dl)	1.47 ± 00.3[a]	2.13 ± 0.08[b]	2.40 ± 0.05[c]
Zinc (mg/dl)	0.10 ± 0.01[a]	0.12 ± 0.03[a]	0.14 ± 0.02[a]
Cupper (µg/dl)	68.9 ± 1.54[a]	78.2 ± 1.82[b]	89.58 ± 1.68[c]
Iron (ug/dl)	80.8 ± 1.82[a]	90.88 ± 1.41[b]	97.28 ± 1.24[c]
Sodium (mEq/l)	106.37 ± 2.80[a]	128.65 ± 3.44[b]	135.53 ± 2.21[b]
Potassium (mq/l)	14.99 ± 1.36[a]	6.35 ± 0.35[b]	4.73 ± 0.17[c]
Chloride (mg/l)	325.50 ± 3.15[a]	354.87 ± 5.15[b]	360.10 ± 4.50[b]

Average value + standard error
a, b, c, values with different letters are significant (P<0.05). values with the same letters are not significant.

DISCUSSION

Salmonella infectious diarrhoea is an important cause of neonatal calf morbidity and mortality in different parts of the world including Egypt, which results in huge economic losses in the beef and dairy industries. Many risk factors were encountered with the occurrence of infection that was related to the calf, the pathogens involved and to the surrounding environment. The immune status of calves, specifically the level of passively acquired immunity through colostrum, is the major risk factor related to the calf and the occurrence of diarrhea (Abouzeed *et al.,* 2000; Santos *et al.,* 2001 and Barrington *et al.,* 2002). Although numerous pathogens have been implicated in the occurrence of neonatal diarrhea as *Salmonella*, *E. coli*, *Yersinia enterocolitica*, *Campylobacter* and many others, only relatively limited numbers are commonly involved. Most should be viewed as secondary opportunists rather than primary pathogens, with the exception of *Salmonella* (Konrad *et al.,* 1994; Barrington *et al.,* 2002 and Santos *et al.,* 2002). The isolation of *Salmonella* from the internal organs of 5 dead calves which had an episode of severe diarrhoea revealed the isolation of only one serovar (S.typhimureium), wheras 11 isolates were detected from the 30 collected organs and no haemolytic *E. coli* were detected in these dead calves. The further bacteriological examination of specimens collected from clinical cases with diarrhoea and the contact apparently healthy revealed isolation of 3 *Salmonella* serovars. Out of 77 diarrhoeic calves 34 were proven positive for *Salmonella* isolation (44.2%) whereas the apparently healthy contact calves demonstarted lower incidence of isolation, as 12 out of 97 (27.9%). S.typhimureium was the most dominant serovar as revealed in the pattern of isolation (Table 1). In clinical cases S.typhimureium constituted 21 out of 34 isolates (61.8%) and 9 out of 12 (75%) in apparently healthy calves. *Salmonella enterica* serovar dublin (S.dublin) and muenster (S.muenster) were also isolated in lower pattern, 11 out of 34 (32.3%) and 2 out of 12 (16.7%) in case of S.dublin and 2 out of 34 (5.9%) and 1 out of 12 (8.3%) in case of S.muenster were detected. Many researchers recorded similar isolation patterns and many other *Salmonella* serovars as enteritidis, infantis, Rostock, Saint paul, Newington, cerro, newport and muenster were incriminated in the induction of diarrhoea in calves with different incidences and clinical severalties of infection (Visser *et al.,* 1990, Konrad *et al.,* 1994; Abouzeed *et al.,* 2000; Bishpham *et al.,* 2001; Santos *et al.,* 2002 and Ostad *et al.,* 2009).

All *Salmonella* serovars were confirmed for its somatic (O) as well as flagellar (H) antigenic structure (Table 1). The serological examination of blood samples for detection of *Salmonella* antibodies by LPS-ELISA, revealed a cut off value of 0.33 OD at 450nm. Diarrhoeic calves recorded higher OD values (range 0.89 to 1.68 OD) than apparently healthy (range 0.34 to 1.32 OD). These results were in agreement with other authors who employd different ELISAs in monitoring *Salmonella* antibodies in the serum. In some cases the OD values which usually reflect the antibody titers in the samples did not match the severity of the clinical status of the animal as some severely diarrhoeic lethargic animals has low OD values and vice versa. (Hoorfar *et al.,* 1995 Hoorfar and Wedderkoppe 1995; Smith *et al.,* 1995; Galland *et al.,* 2000; Radke *et al.,* 2002 Veling *et al.,* 2002 and Fayol-Messaoudi *et al.,* 2007).

The high OD reading was expressed in high antibody titer in the diarrhoeic animals that ranged from 1:2400 to 1:9600, whereas the antibody titer ranged in the apparently healthy calves from 1:400 to 1:7200. LPS-ELISA revealed higher incidences of *Salmonella* infection than the conventional culture method as 53 out of 77 (68.8%) and 37 out of 97 (38.1%) in diarrhoeic and apparently healthy calves respectively were detected with LPS-ELISA positive (Table 2.). These discrepancies between the culture and serological methods were explained due to the intermittent sheding of the micro-oganism as well as the eliciting of antibodies not instant with the onset of infection (Hoorfar and Wedderkoppe 1995; Smith *et al.,* 1995; Radke *et al.,* 2002; Chart *et al.,* 2002; Veling

et al., 2002; Ibnou-Zekri *et al.,* 2003 and Galland *et al.,* 2000).

Administration of *L. casei* was based on its resistance to culture on 1% bile salts media as well as its acid tolerance (pH 5). Diarrhoeic calves administered *L.casei* (10^{10} cfu) every other day for 3 weeks elucidated significant improvement in the health conditions starting the first few days after administration. *Salmonella* counts were greatly reduced from 6.37±2.5 $\log_{10}$ to zero. The *Coliform* counts were also reduced but stayed in the range of 5.28+2.1 to 6.21± 2.3 ($\log_{10}$). The remarkable increase in the lactobacillus counts was noticed immediately after administration from zero to 6.47±2.2 ($\log_{10}$) (Table 3). The change in the bacterial counts after the administration of *L. casei* could be attributed to the competitive exclusion of the *Salmonella* on the enterocytes receptors, production of lactic acid and many other metabolites which shift the pH in the intestine to acidic. This acidity was considered crucial in colonization of *L. casei* and hindering the growth of *Salmonella* and other enteropathogenic bacteria. Some researchers used the organic acids and other probiotic substances as lactulose and lactitol (synthetic disaccharides) to produce prophylactic effect against enteropathogenic bacteria (Fuller, 1989; Roberfroid 1998; Zaho *et al.,* 1998; Tannock, 2002 and Ibnou-Zekri *et al.,* 2003).

The humoral as well as the cellular immune responses were also stimulated after the *L casei* administration as the *Salmonella* antibody levels were increased as monitored by the elevated OD values from 0.68 to 1.94 OD. The cellular immunity manifested in the macrophages activity was also significantly enhanced by almost eight folds from 4.4+1.2 to 33.2 + 5.1 cell macrophage. All these signs of health and immune status improvement were due to the direct or indirect action of the probiotic *L.casei,* that stimulated B-lymphocytes to produce different immunoglobulins isotypes. Other cytokines, interleukines and interferon were also produced due to direct stimulation of certain cell receptors triggered by the *L. casei* (Roberfroid 1998; Zaho *et al.,* 1998; Tannock, 2002; Ibnou-zekri *et al.,* 2003 and Pengcheng *et al.,* 2011).

On the other hand when investigating the serum biochemical analysis of diarrhoeic calves, significant decrease in total proteins, albumin, globulins, A/G ratio (hyprproteinemia) as well as glucose was manifested due to the action of *Salmonella* enterotoxines (Table 4 & 5). These toxines activated the adenyl cyclase enzyme, which lead to producation of cyclic adenosine monophosphate (cAMP). This cAMP instantly increased the intestinal fluid secretion from the systemic circulation resulting in varying degrees of dehydration, electrolyte imbalance and acidosis. These results were supported with many other authors (Blood *et al.,* 1983; Manna *et al.,* 1993 and Kaneko *et al.,* 1997). Also the enterotoxines induced intestinal secretion may be blocked by cycloheximide which is an inhibitor of protein synthesis Serebro *et al.* (1969). The significant decrease in glucose level (Table 5) was due to decrease in glycogenesis and increase an aerobic glycolysis which was induced by the effect of diarrhoea as (Tennant *et al.,* 1968). It was also manifested in diarrhoeic calves, that the enzyme activity of ALT, AST, alkaline phosphatase as well as the values of creatinine, urea and uric acid were significantly increased (Table 5), that could be explained due to the direct damaging effect of *Salmonella* toxines on hepatic and renal cells. These results were also confirmed by Manaa *et al.* (1993); Aly *et al.* (1996).

The serum minerals profiles were also altered in diarrhoeic calves, where serum calcium, phosphorus, magnesium, zinc, cupper, iron sodium and chloride levels were significantly decreased, whereas potassium level was significantly increased (Table 6). The decrease in calcium and magnesium levels could be due to secondary nutritional and metabolic disturbances, that was caused by excessive faecal losses, malabsorption that results from vairous types of bowel diseases including *Salmonella* infection, or due to intrinsic biochemical effect in the mucosal cells that interfere with digestion and absorption (Kaneko *et al.,* 1997). Hypomagnesimia may also be exacerbated by the server diarrhoea (Groutides and Michell 1990). The significant decrease in serum

sodium, chloride, iron, zinc and cupper (Table 6) was due to the increased intestinal secretion of water and electrolytes (Kaneko *et al.,* 1997). Moreover, diarrhoea is a common cause of metabolic acidosis due to direct loss of bicarbonate via faeces (Lewis and Phillips, 1972; Ragab *et al.,* 1986) and therefor, during diarrhoea the increase in hydrogen ions were buffered by intracellular and extracellular buffers. In exchange for the intracellular movement of the hydrogen and potassium ions to the extracellular compartment predisposing to hyperkalemia (Robinson and Hauxtable 1988; Aly *et al.,* 1996). This hyperkalemia continued due to the increased movements of cellular potassium into the extra cellular fluid and decreased renal excretion (Fisher, 1965).

The oral administration of *L. casei* could also amelurate the damaging effect of *Salmonella* toxines on the microvilli, entrocytes as well as the liver and kidney cells. Significant improvement was noticed on the serum biochemical parameters (Table 4,5,& 6) though in some cases the values did not reach those of the control apparently healthy calves. *L. casei* could optimize the permeability of the mucosal epithelium as well as colonizing on the intestinal mucosa (Zaho *et al.,* 1998; Tannock, 2002; Ibnou-Zekri *et al.,* 2003 and Musa *et al.,* 2009). Though *Salmonella* infection in calves could produce heavy economic losses in the animal wealth, yet we could conclude, that by regular monitoring of animals with bacteriological examination of faecal samples as well as serological (by LPS-ELISA) and biochemical analysis of serum, we could control *Salmonella* infection in calves. Also oral administration of *L. casei* could improve the health and the immune status of diarrhoeic calves and could be considered as value-added to the farmers and breeders economics if they widely use it.

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