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Report 742

The adhesive capacity of coffee-related products and fractions for E. coli K99, a pathogen in calves and piglets

December 2013

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Publisher
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Internet http://www.livestockresearch.wur.nl

Editing
Communication Services

Wageningen UR Livestock Research and Central Veterinary Institute of Wageningen UR, both part of Stichting Dienst Landbouwkundig Onderzoek (DLO Foundation), together with the Department of Animal Sciences of Wageningen University comprises the Animal Sciences Group of Wageningen UR (University & Research centre).

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Abstract
An *in vitro* study called “the adhesive capacity of coffee products” was performed.
From a former study (Becker and Galletti, 2008) there were indications that residues from the coffee processing industry could potentially contribute to the prevention of diarrhoea in farm animals, by acting as bacterial receptor analogues.
The objective of the present study was to test *in vitro* the adhesive capacity of coffee-related products and fractions, especially for enterotoxigenic *E. coli* (ETEC) that causes diarrhoea in calves and piglets.
It was concluded from the present study that treatment of ETEC diarrhoea in neonatal calves by bacterial receptor analogues, such as coffee products, looks promising but needs further research *in vivo*.

Keywords
Coffee, *E coli* K99 (F5), receptor analogues, diarrhoea, calves, piglets

Reference
ISSN 1570 - 8616

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Preface

This research topic “adhesive capacity of coffee products” was performed in the framework of the Knowledge base theme “Sustainable Agro-chains” of the Ministry of Economic Affairs, under project number KB-12-006.04-002, “Food Waste for Feed”. The study started in 2012. The authors are grateful to the Ministry of Economic Affairs for the financial support.
Paola Del Serrone of the Research Centre of Meat production and Genetic Improvement (CRA-PCM) in Monterotondo (Italy), acknowledges also the financial support by the Agricultural Research Council (CRA) in Rome (Italy).The authors are grateful to all the suppliers of test products (Table 4 and 5). Furthermore authors acknowledge Dr. D. Carminati of The Fodder and Dairy Productions Research Centre (CRA FLC) of Lodi, Agricultural Research Council (CRA), Italy and Dr. H. Vu Khac of the Institute of Veterinary Research and Development of Central Vietnam, Vietnam, and Ank van Zijderveld-Bemmel for kindly providing *E. coli* isolates.

Teun Veldkamp

Project leader

Summary

From a former study (Becker and Galletti, 2008) there were indications that residues from the coffee processing industry could potentially contribute to the prevention of diarrhoea in farm animals, by acting as bacterial receptor analogues.
The objective of the present study was to test the adhesive capacity of coffee-related products and fractions, especially for enterotoxigenic *E. Coli* K99 (ETEC) that causes diarrhoea in neonatal calves and very young piglets. Different *E. coli* isolates that possess different fimbriae (F4, F5, F6, F18 and F41) were tested. It appeared that only *E. coli* isolates that contained K99 (F5) fimbriae showed adhesion to the tested coffee products. Probably galactose plays a role in the attachment of K99 (F5) fimbriae to the tested coffee products. This was in agreement whit Mouricout and Julien (1987), who found that galactose appeared to be at least partly responsible for the attachment of K99 (F5) fimbriae.
The degree of roasting both in the coffee grounds as well in the coffee brews (mild, dark and extra dark) had no effect on the binding results in the used adhesion test. The remark has to be made that the used test was probably not the most appropriate test to study differences in 'degree of roasting'. To find differences in 'degree of roasting' solubility of polysaccharides should be studied and this was not included in the used adhesion test (test products are always used in excess).
It was concluded from the present study that treatment of ETEC (K99) diarrhoea in neonatal calves and young piglets by bacterial receptor analogues, such as coffee products, looks promising but needs further research *in vivo*.

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1 Introduction

The project "Food Waste for Feed", part of the Knowledge base theme "Sustainable Agro-chains", has the goal to reduce food waste by upgrading using conservation and valorisation. Manufacturing nutrient rich and healthy ingredients from residual side-streams for animal feed may create added value for the food production chain.

Many strains of *Escherichia coli* are part of the non-pathogenic facultative flora of intestinal tract of humans and other mammals. Some of them are capable of inducing diseases of the gastrointestinal and urinary tracts or may affect the central nervous system (Dargatz *et al*., 1997). Such effects are ascribed to enterotoxigenic *E. coli* (ETEC, EPEC) which cause serious economic losses in farm animal herds and are widespread in new-borns (Kenny *et al*., 1988) in both developed and developing countries. This is a consequence of a wide range of transmission possibilities of these pathogens including direct contact, food, drinks, environment, and others (Jarvis *et al*., 1997). Epidemiology and clinical symptoms of the disease are similar in various animal species but the majority of strains are species-specific (Wang *et al*., 2003). They differ particularly in the type of the expressed surface “adherence” antigen (adhesin or pilus). These microorganisms produce two main types of virulence factors, fimbrial adhesins and enterotoxins (Kaper *et al*., 2004).

Enterotoxigenic *E. coli* (ETEC) can be one of the causes of diarrhoea in young piglets and calves. The majority of ETEC strains isolated from neonatal pigs, calves, or lambs possess one of the adhesive antigens-K88 (F4), K99 (F5), or 987P (F6) (Gaastra and De Graaf, 1982). Mouricout *et al.*(1990) showed that experimentally infected animals can be successfully treated by agents, which act on the attachment sites of bacterial fimbriae. Treatment of ETEC diarrhoea in neonatal calves by bacterial receptor analogues provides a new and promising approach to early therapy, either alone or in combination with other treatments. Residues from the coffee processing industry could potentially contribute to the prevention of diarrhoea in farm animals (Becker and Galletti, 2008). The research on this topic started in 2012, in collaboration with Paola Del Serrone of the Research Centre of Meat production and Genetic Improvement (CRA-PCM) in Monterotondo (Italy).The aim of this research was to test the adhesive capacity of coffee-related products and fractions, especially for *E. coli* that causes diarrhoea in neonatal calves and very young piglets. A collection of isolates of *E. coli* was considered. They had different geographical origin; they were from different source of isolation, and they showed different pathogenic characteristics. The future goal is to prevent intestinal colonization by this pathogen.

2 Material and methods

2.1 Microorganisms collection

Forty-eight strains of *Escherichia coli* were considered in the study.
Seventeen strains were from the microorganisms collection of The Fodder and Dairy Productions Research Centre (CRA FLC) of Lodi, Agricultural Research Council, Italy. They were isolated from milk and cheese. All Strains were typed both phenotypically and genotypically. Phenotyping was made by the PhenePlateTM system for *E. coli* (PhP-EC, PhPPlateMicroplate Techniques AB, Stockholm, Sweden) and genotyping by the RAPD PCR technique (Zago *et al*., 2007).
Seventeen strains were from the microorganisms collection of the Institute of Veterinary Research and Development of Central Vietnam, Vietnam. They were isolated from faeces of calves affected by diarrhoea. They were examined for the presence of genes coding adhesins, and toxicity activity by PCR.
Ten strains were collected in The Netherlands. They were from the microorganisms collection of the Central Veterinary Institute of Wageningen UR. They were antigenically different and detectable by using specific Mabs towards different fimbria antigens in an *in vitro* agglutination test (Van Zijderveld *et al*., 1989).
They were grew on LB agar

2.2 Molecular biology analysis for *Escherichia coli* isolates: detection and characterisation

Detection and characterisation were made by PCR. Fourteen primer pairs were selected to specifically amplify target genes coding for virulence factors (fimbrial adhesins and toxins) as described in Table 1. The DNA templates were prepared from overnight LB agar-grown cultures by suspending 2–3 colonies in 100 µL of double-distilled (dd) H2O, boiling for 5 min and centrifuging the suspension (1 min, 13,000 x *g*). Amplification of DNA was performed using 5 µL of the supernatant of lysed bacteria, 90 ng oligonucleotide primers, 2 mmol/L of each dATP, dGTP, dCTP, and dTTP, 1 U of AmpliTaq DNA polymerase (Perkin Elmer) and buffer according to producer of DNA-polymerase including $MgCl_2$. The mixture was adjusted with dd H2O to a total volume of 50 µL. Control DNA-samples from reference strains were included in each reaction.
PCR-amplified DNA products were shown by agarose gel electrophoresis, using 1.5 or 2 % agarose gel in electrophoretic TAE buffer. DNA was stained with ethidium bromide (0.5 mg/L) and visualized under UV light. A 100 bp ladder (Invitrogen Life Technologies ItaliaFil. Life Technologies Europe BV Monza MB Italy) was used as molar-mass standard. Dried gels were photographed with a Kodak Digital Science DC120 camera (Eastman-Kodak, Rochester, NY) and analyzed with a Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Eastman-Kodak).

Thirteen strains among the microorganisms collection, with different type of fimbriae and enterotoxin genes, were chosen after characterisation to carry out this study (Fig.1a, b. Table 1). The strains are maintained in MicrobankTM vials at −70 °C.

Table 1 Primer pairs used to specifically amplify target gene coding for virulence factors (1-8 = toxins; 9-14 fimbriae) of *E. coli*.

Target gene coding for virulence factors	Oligonucleotide sequences of primers	Reference
1) LT	5'-ATT TAC GGC GTT ACT ATC CTC-3' 5'-TTT TGG TCT CGG TCA GAT ATG-3'	Osek *et al*., 1999
2) STa	5'-TCC GTG AAA CAA CAT GAC GG-3' 5'-ATA ACA TCC AGC ACA GGC AG-3'	Ojeniyi *et al*., 1994
3) STb	5'-GCC TAT GCA TCT ACA CAA TC-3' 5'-TGA GAA ATG GAC AAT GTC CG-3'	Ojeniyi *et al*., 1994
4) Stx1all	5'-CGC TGA ATG TCA TTC GCT CTG C-3' 5'-CGT GGT ATA GCT ACT GTC ACC-3'	Blanco *et al*., 2004
5) Stx2all	5'-CTT CGG TAT CCT ATT CCC GG-3' 5'-CTG CTG TGA CAG TGA CAA AAC GC-3'	Blanco *et al*., 2004
6) Stx2e	5-ATG AAG AAG ATG TTT ATA GCG-3' 5'-TCA GTT AAA CTT CAC CTG GGC-3'	Osek *et al*., 1999
7) EAST1	5'-CCA TCA ACA CAG TAT ATC CGA-3' 5'-GGT CGC GAG TGA CGG CTT TGT-3'	Yamamoto and Nakazawa, 1997
8) eae	5'-GGA ACG GCA GAG GTT AAT CTGCAG-3' 5'-GGC GCT CAT CAT AGT CTTTC-3'	Blanco *et al*., 2004
9) F4 (K88)	5'-GCT GCA TCT GCT GCA TCT GGTATG G-3' 5'-CCA CTG AGT GCT GGTAGT TAC AGC C-3'	Vu Khac *et al.*, 2006
10) F5 (K99)	5'-TGC GAC TAC CAA TGC TTC TG-3' 5'-TAT CCA CCA TTA GAC GGA GC-3'	Ojeniyi *et al*., 1994
11) F6 (P987)	5'-TCT GCT CTT AAA GCT ACT GG-3' 5'-AAC TCC ACC GTT TGT ATC AG-3'	Ojeniyi *et al*., 1994
12) F17	5'-GGG CTG ACA GAG GAG GTG GGGC-3' 5'-CCC GGC GAC AAC TTC ATCACC GG-3'	Vu Khac *et al.,* 2006
13) F18	5'-GTG AAA AGA CTA GTG TTT ATT TC-3' 5'-CTT GTA AGT AAC CGC GTA AGC-3'	Imberechts*et al*., 1994.
14) F41	5'-GAG GGA CTT TCA TCT TTT AG-3' 5'-AGT CCA TTC CAT TTA TAG GC-3'	Ojeniyi *et al*., 1994

2.3 Culturing of bacteria

The *E. coli* isolates that were tested are listed in Table2. Isolates A, B, E, and H (= E) were provided by the Institute of Veterinary Research and Development of Central Vietnam, Vietnam and isolates C, D, G (=C), I, L, M, N, O, P, Q and R by the Bacterial Culture Collection of the Central Veterinary Institute of Wageningen UR. The *E. coli* isolates that were tested possessed different fimbriae (F4, F5, F6, F18 and F41). Prior to testing, the *E. coli* isolates were cultured in Brain Heart Infusion (BHI) broth (BD Difco, Sparks, Maryland, USA), or cultured in Minca-IsoVitaleX(BBL Microbiology Systems, Cockeysville, MD, USA), as described by Becker and Galletti (2008).

Bacterial cells were harvested after culturing by centrifuging 2 ml broth volume (5 min, 1,700 x *g*; Eppendorf 5415R centrifuge; Eppendorf A.G., Hamburg, Germany).

The supernatant was removed and the pellet was washed with 2 ml PBS buffer (phosphate buffered saline: 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 with HCl and sterilized by autoclave) and centrifuged again. Then the pellet was re-suspended and diluted in PBS to an optical density of 0.02 at 600 nm and a path length of 1 cm.

Table 2 *E. coli* (ETEC) tested for adhesive capacity in the study.

***E. coli* isolates code**	**Isolate collections designation**	**Host**	**Medium**
1. A	10B	Calf	BHI
2. B	KH10	Calf	BHI
3. C (=G)	K99	Pig	BHI
4. D	P987	Calf	BHI
5. E (=H)	12b	Calf	BHI
6. I	K99-1	Calf	Minca
7. L	K99-3	Calf	Minca
8. M	K99-5	Calf	Minca
9. N	K99-7	Calf	Minca
10. O	K99-9	Calf	Minca
11. P	K99-11	Calf	Minca
12. Q	K99-15	Calf	Minca
13. R	K99-19	Calf	Minca

BHI = Brain heart infusion; Minca = Minca-IsoVitaleX; nr = not revealed

2.4 Adhesion test

The coffee products that were tested for binding capacity of *E. coli* (ETEC) with different fimbriae and *E. coli* (ETEC) with F5 fimbriae are listed in respectively Table 3 and 4. The different coffee products were applied as coating materials in the adhesion test along with Bovine Serum Albumin (BSA) (Table 3) as a control. Microplate adhesion experiments were performed as described by Becker *et al*.(2007) and Becker and Galletti (2008).

The different test products derived from coffee pads were prepared in a Senseo coffee apparatus by letting the machine preparing five cups of coffee through one pad (to remove most of the soluble parts of the coffee ground). The different coffee grounds from the pads were dried in an oven at 40 °C during 24 hours and then used in the adhesion test. The coffee brew (first cups) from the different pads were also used in the adhesion test. The test products were diluted in PBS buffer to a final concentration of 1% (w/v). After sonicating and centrifuging, the supernatants were pipetted into a polystyrene microplate (350 µl/well; high-binding Microlon F plate 655092;Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) using them as coating. The micro plates were then incubated overnight at 4°C. Non-coated wells were included as negative controls in each plate. After that the plates were washed with 350 µl PBS buffer to remove non-binding coating material. Blocking of the microplates was done by incubating the wells with 350 µl of 1% BSA in PBS (w/v) that contained 0.5% sodium azide at 4°C for 1 h. Then, plates were washed twice with 300 µl of PBS.

Table 3 Coffee products tested for binding capacity of *E. coli* (ETEC) with different fimbriae.

Description	Code	Supplier
Bovine serum albumin	BSA	A7906, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands
Coffee ground of 'Fresh Brew Select' for automatic dispensers	CG	Coffee producer A
Coffea ®(commercial product)	CP	Dr. Schaette ECOSTYLE GmbH, Bad Waldsee, Germany
Green bean	GB	Laboratory of Food Chemistry, Wageningen University, The Netherlands
Low roasted, LMW fraction	LR1	
Mild roasted, LMW fraction	MR1	
Dark roasted, LMW fraction	DR1	

LMW= Low Molecular Weight fraction.

Table 4 Coffee products tested for binding capacity of *E. coli* K99/F5 (ETEC).

Product	Description	Code	Supplier
SENSEO Extra Dark Roast	Cup (1st)	ED C1	Coffee producer A
	Pad (5 x washed)	ED P1	
SENSEO Dark Roast	Cup (1st)	D C1	
	Pad (5 x washed)	D P1	
SENSEO Mild Roast	Cup (1st)	M C1	
	Pad (5 x washed)	M P1	
Coffea ®		CP	ECOSTYLE GmbH, Bad Waldsee, Germany

Bacteria that has been grown, washed and suspended in PBS conform 2.3 were added into the microplate wells (300 µl/well) and allowed to adhere at room temperature for 30 min. Afterwards, the wells were washed three times with 300 µl of PBS to remove non-adherent bacteria. Then the wells were filled with 300 µl growth medium (BHI or Minca).
The control wells were filled with 300 µl of a ten-fold dilution series in growth medium (BHI or Minca) with a known amount of the test bacterium. Then the microplate was placed in a microplate reader (SpectraMax 340; Molecular Devices Ltd., Wokingham, United Kingdom), incubated at 37°C and shaken at medium intensity for 3 sec prior to every reading. The OD was determined at a wavelength of 650 nm every 15 min during 24 hours. All readings were done in two independent assays and in quadruplicate per microplate.
The data generated by the photometer software (SoftMaxPro 2.2.1.; Molecular Devices Ltd., Wokingham, United Kingdom) were processed by non-linear regression analysis employing the Boltzmann sigmoidal equation to describe the kinetics of bacterial growth: Y=Bottom+(Top-Bottom)/(1+exp((V50-X)/Slope)).In this equation, V50(t) is the time at which half of the maximal yield has been reached. Analyses of variance were performed using GenStat (VSN International Ltd., Hemel Hempstead, UK).The V50s were also converted to adhering cell numbers of the test bacterium according to (Becker et al., 2007), using the V50s of the ten-fold dilution series of the test bacterium.

3 Results

3.1 Characterisation of *Escherichia coli* isolates

Amplicons of specific *E. coli* ETEC genes coding either fimbrial adhesins or enterotoxins of the expected size were obtained in fourteen isolates among the forty-eight tested as reported in Table 5 and shown if Fig 1a and Fig 1b. In table 6 is reported the characterization of the fourteen *E. coli* isolates considered in the study.

Table 5 Synopsis of amplicons obtained by PCR detection carried out with the forty-eight isolates of *E. coli* considered.

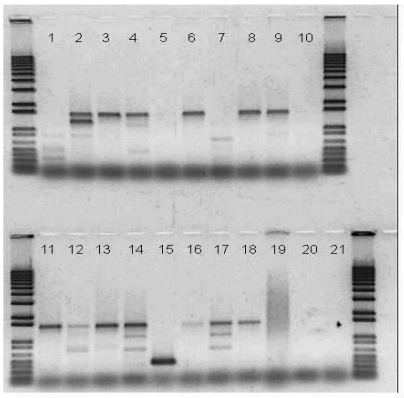
Target gene coding for virulence factors	Amplicon (bp)	Primer Coordinates	Accession number
1) LT	281	27–47, 287–307	S60731
2) STa	244	267–286, 492–510	M58746
3) STb	279	515–534, 773–793	AY028790
4) Stx1all	302	113–134. 394–414	M17358
5) Stx2all	516	50–69, 543–565	M59432
6) Stx2e	264	1176–1196, 1419–1439	M36727
7) EAST1	111	2–24, 94–114	S81691
8) eae	775	1441–1460, 2193–2215	AF022236
9) F4 (K88)	792	31–54, 798–822	M29374
10) F5 (K99)	450	45–64, 475–494	M35282
11) F6 (P987)	333	193–212, 506–525	M35257
12) F17	411	289–310, 677–699	AF055313
13) F18	510	1–23, 490–510	M61713
14) F41	431	154–173, 565–584	X14354

Table 6 Synopsis of characterisation of the eighteen isolates of *E. coli* studied in the present study.

E. coli isolate collection's designation	Surface antigen	Toxins	Fimbriae
10B	nd	STb,LT,EAST1	F4
KH10	nd	STa,STb	F18
K99	O8K85K99	nr1	F5
P987	O64:K;9877	STa+	F6
12b	nd	STa	F5, F41
K99-1	O8:K25:K99	nr	F5
K99-3	O101:K28:K99	nr	F5
K99-5	O9:K30:K99	nr	F5
K99-7	O101:K32:K99	nr	F5
K99-9	O9:K35:K99	nr	F5
K99-11	O9:K37:K99	nr	F5
K99-15	O20:K?:K99	nr	F5
K99-19	O101:K?:K99	nr	F5

nd = not detected; nr = not revealed

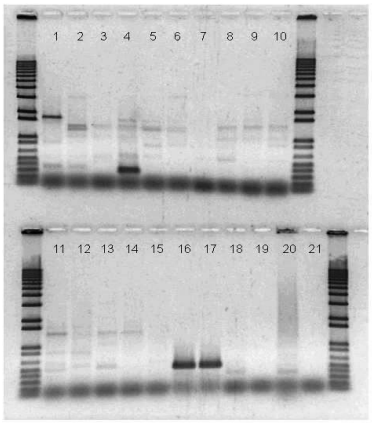
Fig 1a PCR-amplified DNA products of genes coding fimbriae visualized by agarose gel electrophoresis as above described.



F5 450 bp

M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

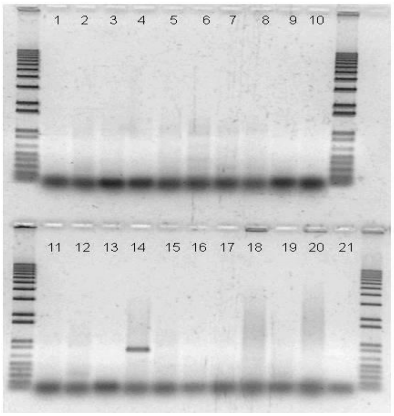
M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 ATCC 11775
21 C-
M



F18 510 bp

M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

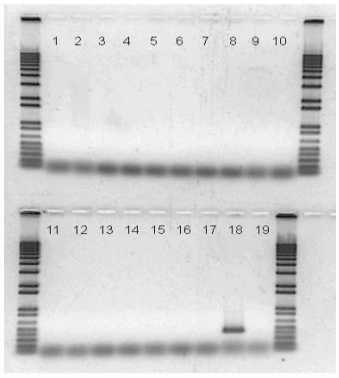
M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 ATCC 11775
21 C-
M



F4 792 bp

M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 ATCC 11775
21 C-
M

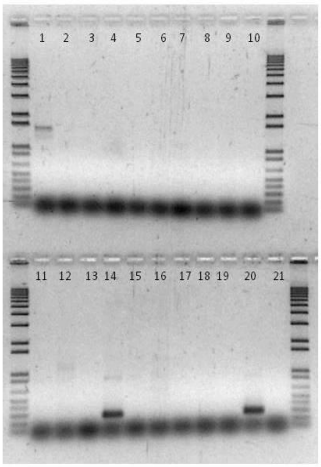


F41 431 bp

M
1 1056
2 1247
3 1059
4 1243
5 1048
6 1167
7 1249
8 1055
9 1054
10 1085
M

M
11 1244
12 1165
13 1086
14 1053
15 1095
16 1219
17 1235
18 12b
19 C-
M

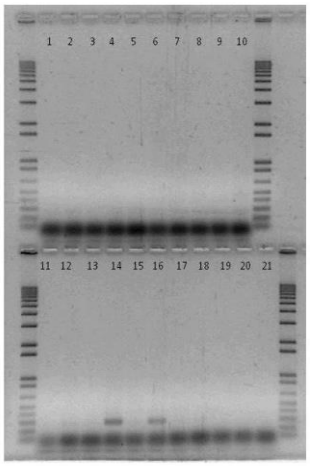
Fig 1b Agarose gel electrophoresis of PCR-amplified DNA products of genes coding enterotoxins (LT, STb, STx2e and EAST1)*.



LT 280 bp

M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

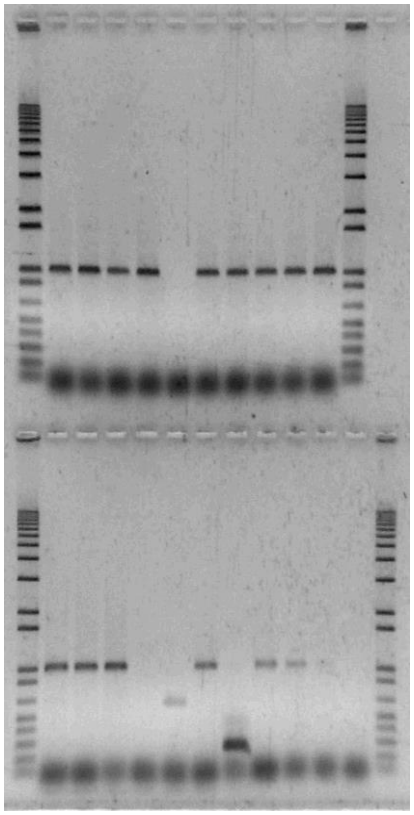
M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 DSM 10973
21 C-
M



STb 278 bp

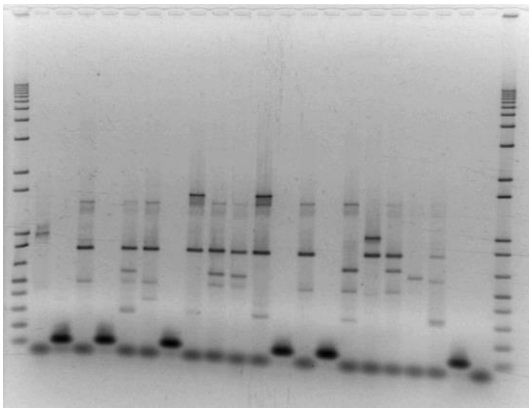
M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 DSM 10973
21 C-
M



Stx2e 267 bp
M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
M

M
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH
436
18 ATCC
35150
19 DSM
9025
20 DSM
10973
21 C-
M



EAST1 111 bp
M
1 E10
2 E16
3 E48
4 E77
5 E98
6 E130
7 E148
8 E173
9 E210
10 E215
11 E273
12 E298
13 E320
14 10B
15 12b
16 KH10
17 KH 436
18 ATCC 35150
19 DSM 9025
20 DSM 10973
21 C-
M

(*)**LT,** heat labile exotoxin, causes hyper secretion of water and electrolytes within gut.
STb, heat-stable **enterotoxin** b, alters fluid and electrolyte transport in the gut
Stx2e, also known as edema disease factor, is the cause of lesions associated with **edema** disease in pigs
East 1, enteroaggregative heat-stable enterotoxin 1.
All of them act after fimbria interaction with a mucosal receptor

3.2 Binding capacity of different coffee products for ETEC with different fimbriae

Table 7 gives an overview of the results, presenting V_{50}, the time at which half of the maximal growth yield was reached as a measure for adhesion of the different *E. coli* isolates.
Products with the lowest V_{50} (= the fastest appearance of bacterial growth) bound most cells of *E. coli* isolates.

Table 7 Time [h] at which half of the maximal growth yield was reached (V50) as a measure for adhesion of *E. coli* isolates. The data represent least squared means. Data followed by different letters within one column are significantly different (P<0.05). Products with the lowest V50 (= the fastest appearance of bacterial growth) bound most cells of *E. coli* isolates.

	E. coli isolates[(2)]				
Coffee Products[(1)]	A	B	C	D	E
BSA (control)	7.8^{ab}	7.3^{a}	5.6^{cd}	6.7^{a}	7.5^{b}
Coffee ground	8.8^{c}	7.6^{ab}	5.2^{abc}	6.9^{a}	7.4^{b}
Coffea ®	7.7^{ab}	7.6^{ab}	4.8^{a}	7.0^{ab}	7.1^{a}
Green bean	8.0^{b}	7.5^{ab}	5.1^{ab}	6.7^{a}	7.4^{b}
Dark roasted, LMW fraction	7.8^{ab}	7.5^{ab}	5.2^{abc}	7.3^{b}	7.5^{b}
Low roasted, LMW fraction	7.7^{a}	7.6^{b}	5.8^{d}	6.7^{a}	7.2^{ab}
Mild roasted, LMW fraction	7.8^{ab}	7.5^{ab}	5.4^{bcd}	6.8^{a}	7.5^{b}
Lsd[(3)]	0.3	0.4	0.5	0.4	0.3

[(1)]Product specifications are given in Table 3; [(2)]Bacteria specifications are given in Table 1;
[(3)]Least significant difference

Table 8 gives an overview of the results, presenting the number of bacteria adhered at the start as a measure for adhesion of the different *E. coli* isolates. Products with the highest amount of bacteria bound most cells of *E. coli* isolates.

Table 8 Number of adhering bacteria (^{10}Log count.ml^{-1}) to the plate coatings. The data represent least squared means. Data followed by different letters within one column are significantly different (P<0.05). Products with the highest ^{10}Log count.ml^{-1} bound most cells of *E. coli* isolates.

	E. coli isolates[(2)]				
Coffee Products[(1)]	A	B	C	D	E
BSA (control)	2.8^{ab}	3.9^{a}	4.0^{cd}	1.6^{a}	1.6^{b}
Coffee ground	2.0^{c}	3.6^{ab}	4.4^{abc}	1.4^{a}	1.8^{b}
Coffea ®	2.9^{a}	3.6^{ab}	4.9^{a}	1.2^{ab}	2.2^{a}
Green bean	2.6^{b}	3.7^{ab}	4.6^{ab}	1.6^{a}	1.7^{b}
Dark roasted, LMW fraction	2.8^{ab}	3.7^{ab}	4.5^{abc}	1.0^{b}	1.7^{b}
Low roasted, LMW fraction	2.9^{a}	3.6^{b}	3.9^{d}	1.6^{a}	2.0^{ab}
Mild roasted, LMW fraction	2.8^{ab}	3.7^{ab}	4.3^{bcd}	1.5^{a}	1.7^{b}
Lsd[(3)]	0.3	0.3	0.5	0.4	0.4

[(1)]Product specifications are given in Table 3; [(2)]Bacteria specifications are given in Table 1;
[(3)]Least significant difference

Means of V50s (Table 7) and means of adhering bacteria amounts (Table 8) are followed by superscripts indicating significant differences between the readings and the ranking position of the

products per bacterial strain, starting with an ‘a’ for best performance and continuing in alphabetical order.

The test products ‘Coffea ®’ and ‘Green bean’ were binding to respectively two (C and E) and one (C) of the five tested *E. coli* (Table 7 and 8) The two binding bacteria C and E, respectively *E. coli* K99 (O8K85K99/F5) from pig and *E. coli* 12b (STa/F5,F41) from calf, possessed both K99 (F5)-fimbriae. *E. coli* 12b possessed beside F5-fimbriae also F41-fimbriae. The three non-binding *E. coli* isolates A, B and D possessed respectively the fimbriae F4, F18 and F6. It was concluded from these tests that F5-fimbriae were playing a role in binding of *E.coli* to coffee products. Therefore only *E. coli* isolates with F5-fimbriae were used in the further tests. Also different coffee products were used in the tests (except Coffea ®).

3.3 Binding capacity of different coffee products for a selection of *E. coli* K99/F5

Table 9 gives an overview of the results, presenting V50, the time at which half of the maximal growth yield was reached as a measure for adhesion of the different *E. coli* isolates. Products with the lowest V50 (= the fastest appearance of bacterial growth) bound most cells of *E. coli* isolates.

Table 9 Time [h] at which half of the maximal growth yield was reached (V50) as a measure for adhesion of *E. coli* isolates. The data represent least squared means. Data followed by different letters within one column are significantly different (*P<0.05*). Products with the lowest V50 (= the fastest appearance of bacterial growth) bound most cells of *E. coli* isolates.

Coffee Products[1]	*E. coli* isolates[2]									
	G	H	I	L	M	N	O	P	Q	R
BSA (control)	11.2^{bc}	11.5^{d}	12.6^{d}	11.8^{d}	12.0^{c}	9.5^{b}	9.9^{b}	12.1^{e}	10.0^{c}	12.4^{e}
Coffea ®	12.0^{cd}	9.7^{c}	10.6^{cd}	11.2^{cd}	12.2^{c}	8.1^{a}	8.2^{a}	11.0^{d}	9.6^{bc}	11.0^{d}
Dark Cup	12.5^{de}	9.7^{c}	10.9^{cd}	10.4^{bc}	10.7^{b}	8.0^{a}	9.8^{b}	10.7^{cd}	9.6^{bc}	11.0^{d}
Dark Pad	10.5^{ab}	8.8^{ab}	9.4^{a}	8.6^{a}	9.9^{ab}	8.2^{a}	7.7^{a}	9.9^{abc}	9.0^{a}	9.2^{b}
Extra Dark Cup	13.2^{e}	9.6^{c}	10.1^{abc}	9.9^{abc}	9.5^{a}	7.8^{a}	7.8^{a}	10.5^{bcd}	9.4^{b}	10.6^{cd}
Extra Dark Pad	9.9^{a}	8.7^{a}	9.6^{ab}	9.9^{abc}	9.6^{a}	7.8^{a}	7.3^{a}	9.5^{a}	8.8^{a}	8.4^{a}
Mild Cup	12.2^{cde}	9.3^{bc}	10.5^{bc}	10.4^{bcd}	10.7^{b}	7.7^{a}	7.8^{a}	10.3^{bcd}	9.5^{b}	10.2^{c}
Mild Pad	10.0^{a}	8.4^{a}	9.2^{a}	9.4^{ab}	9.1^{a}	8.1^{a}	7.6^{a}	9.8^{ab}	8.9^{a}	9.1^{ab}
Lsd[3]	1.1	0.6	1.0	1.4	1.0	0.7	1.0	0.8	0.4	0.8

[1]Product specifications are given in Table 4; [2]Bacteria specifications are given in Table 1 ;[3]Least significant difference

Table 10 gives an overview of the results, presenting the number of adhering bacteria at the start as a measure for adhesion of the different *E. coli* isolates. Products with the highest number of bacteria bound most cells of *E. coli* isolates.

Table 10 Number of adhering bacteria (^{10}Log count.ml^{-1}) to the plate coatings. The data represent least squared means. Data followed by different letters within one column are significantly different (*P<0.05*). Products with the highest ^{10}Log count.ml^{-1} bound most cells of *E. coli* isolates.

Coffee Products[1]	*E. coli* isolates[2]									
	G	H	I	L	M	N	O	P	Q	R
BSA (control)	2.2^{bc}	2.4^{e}	0.7^{d}	1.0^{d}	0.8^{c}	5.5^{bc}	2.5^{b}	3.4^{e}	1.7^{c}	1.1^{e}
Coffea ®	1.6^{cd}	3.5^{cd}	2.0^{c}	1.4^{cd}	0.7^{c}	5.6^{a}	3.8^{a}	4.1^{d}	2.0^{bc}	2.0^{d}
Dark Cup	1.3^{de}	3.4^{cd}	1.8^{c}	2.0^{bcd}	1.7^{b}	5.4^{c}	2.8^{b}	4.3^{cd}	2.0^{bc}	2.0^{d}
Dark Pad	2.5^{ab}	4.0^{ab}	2.8^{a}	3.2^{a}	2.2^{ab}	5.2^{d}	4.1^{a}	4.7^{abc}	2.4^{a}	3.2^{b}
Extra Dark Cup	0.9^{e}	3.2^{d}	2.3^{abc}	2.3^{abc}	2.5^{a}	5.4^{c}	4.0^{a}	4.4^{bcd}	2.1^{b}	2.3^{cd}
Extra Dark Pad	2.9^{ab}	4.0^{ab}	2.7^{ab}	2.3^{abc}	2.5^{a}	5.3^{d}	4.4^{a}	5.0^{a}	2.6^{a}	3.7^{a}
Mild Cup	1.5^{de}	3.8^{bc}	2.1^{bc}	1.9^{bcd}	1.7^{b}	5.5^{ab}	4.1^{a}	4.5^{bcd}	2.1^{b}	2.6^{c}
Mild Pad	2.9^{a}	4.2^{a}	2.9^{a}	2.6^{ab}	2.8^{a}	5.2^{d}	4.2^{a}	4.7^{ab}	2.5^{a}	3.3^{ab}
Lsd[3]	0.7	0.4	0.6	1.0	0.7	0.1	0.6	0.5	0.3	0.5

[1]Product specifications are given in Table 4; [2]Bacteria specifications are given in Table 1 ;[3]Least significant difference

Means of V50s (Table 9) and means of adhering bacteria numbers (Table 10) are followed by superscripts indicating significant differences between the readings and the ranking position of the products per bacterial strain, starting with an ‘a’ for best performance and continuing in alphabetical order.

All tested *E. coli* isolates possessed F-5 fimbriae. All tested coffee products showed binding, but some more than others (to more *E. coli* isolates). The coffee grounds from the different coffee pads (Mild Pad, Dark Pad and Extra Dark Pad) showed the best binding results (to the most *E. coli* isolates). The coffee brews from the different coffee pads (Mild Cup, Dark Cup, and Extra Dark Cup) were second best and the commercial product ‘Coffea ®’ was third best in binding to the different *E. coli* isolates. The degree of roasting in the coffee grounds and coffee brews (mild, dark and extra dark) had no effect on the binding results.

4 Discussion

Enterotoxigenic *Escherichia coli* (ETEC) can be the cause of diarrhoea in young piglets and calves. Besides the production of enterotoxins, the ability of ETEC to adhere to the intestinal epithelial mucosa is important. ETEC binds to specific receptor structures in the intestine by using fimbriae, long filamentous polymeric protein structures located at the surface of bacterial cells. The majority of ETEC strains isolated from neonatal pigs, calves, or lambs possess one of the following fimbriae: K88 (F4), K99 (F5), or 987P (F6) (Gaastra and De Graaf, 1982). In the present study *E. coli* with F4, F5, F6 and F41 fimbriae were tested. But only *E. coli* isolates that contained K99 (F5) fimbriae showed adhesion to the coffee products that were tested. In other research K99 (F5) fimbriae from ETEC were found to bind specifically *N*-Glycolylsialyl residues that are found in the glycoproteins and glycolipids of young piglets and calves (Gaastra and De Graaf, 1982). Mouricout *et al.*(1990) have shown that agents which act on the attachment sites of bacterial fimbriae mimic the glycan moieties of natural receptors. Sialic acids and galactose appeared to be at least partly responsible for the attachment of K99 (F5) fimbriae (Mouricout and Julien, 1987). In a former study we demonstrated that *E. coli* K99 CIDC10 bound well to coffee, but also to other products, like Artichoke and Willow (Becker and Galletti, 2008). It is not known which sugars in these products mimic the glycan moieties of the natural receptor of K99 (F5), but galactose looks a good candidate. The main storage carbohydrate of tubers of Japanese artichoke (*Stachys sieboldii)* is stachyose (α-Gal-[1→ 6]-α-Gal-[1→6]-α-Glc-[I↔2]-β-Fru)(Greutert and Keller, 1993). And a water-soluble galactan has been isolated from the pectic material of the bark of the white willow (*Salix alba* L) (Toman *et al*. 1972).Galactomannans and arabinogalactans are present in green coffee beans. Galactomannans are mainly present as unextractable polymers, but they are solubilised to a large extent with increasing degrees of roast. The arabinogalactans in the roasted bean are highly soluble. The arabinose as present as side-chains in the arabinogalactans is more susceptible to degradation at more severe roasting conditions than the galactans. (Oosterveld *et al.*, 2003).

In our experiments the effect of ‘degree of roasting’ on the binding results of the different *E. coli* isolates was also studied. It was concluded that the degree of roasting both in the coffee grounds as well in the coffee brews (mild, dark and extra dark) had no effect on the binding results.
During coffee roasting brown polymers, called melanoidins, are formed, through Maillard reaction. Polysaccharides are involved in the formation of melanoidins. Arabinogalactans seem to be relatively more involved in melanoidin formation than galactomannans and galactomannans are continuously incorporated in arabinogalactan proteins -melanoidins upon roasting (Bekedam *et al.*, 2008).The degree of roasting seems to play a role in the solubilisation of arabinogalactans. During roasting the extractability of arabinogalactans, pectins and galactomannans increase significantly (Oosterveld *et al.*, 2003). To find differences in ‘degree of roasting’ solubility of polysaccharides should be studied and this was not included in the used adhesion test (test products were always used in excess).

Sharon (2006) suggested that anti-adhesive agents, like the coffee products used in our experiments, do not act by killing or suppressing the growth of the pathogens. Therefore the formation of resistance to such agents will probably emerge slower than formation of resistance to antibiotics.

Mouricout *et al.*(1990) showed that calf diarrhoea due to infection by enterotoxigenic *Escherichia coli* could be treated by administration of glycoprotein glycans derived from bovine plasma. The glycan moieties of the non-immunoglobulin fraction of plasma mimicked the oligosaccharide moiety of intestinal receptors recognized by K99 (F5) fimbriae. This receptor analogue reduced bacterial adherence *in vivo*. Adhesion of *E. coli* K99 to the intestines (duodenum, jejunum, and ileum) was significantly reduced (100 fold) in treated new-born calves. It was concluded from the study of Mouricout *et al.*(1990) and our study that treatment of ETEC diarrhoea in neonatal calves by bacterial receptor analogues, such as coffee products, looks promising but needs further research *in vivo*.

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