SPSD II

DEVELOPMENT OF DETECTION METHOD FOR NON-O157-PATHOGENIC-*E. COLI* IN FOOD: FIRST STEP IN PREVENTION

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PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

GENERAL ISSUES

AGRO-FOOD

ENERGY

TRANSPORT

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SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY
(SPSD II)

Part 1:

Sustainable production and consumption patterns

FINAL REPORT

"Development of detection method for non-O157-pathogenic-*E. coli* in food: first step in prevention"

CP/58

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1 Introduction

1.1 Context and summary

Food safety is a very important issue all around the world: several crises, associated with food products, broke out during the last decades resulting in a decline of consumption of certain foodstuffs and damaged consumer's confidence. Due to the immense economical implications concerned with global food safety, preventive actions are much needed: the development of a sustainable detection method for use in routine screenings is a first and indispensable step. The screening of food products (and their production sites and processing units) to detect possible pathogenic bacteria is an essential part of a solid preventive policy. Due to the specific characteristics and the high level of variability among bacterial species, the development of functional methods to detect the most important pathogens is both a national and international issue. Developing new detection methods will allow public authorities to compose a new preventive policy in order to improve food safety.

Escherichia coli typically colonize the gastrointestinal tract of human infants within a few hours after birth: it is the predominant facultative anaerobic species of the human colon. These Gram-negative rod-shaped bacteria usually remain harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "non-pathogenic" strains of *E. coli* can cause infection (21). Few micro-organisms are as versatile as *E. coli*: there are several highly adapted *E. coli* clones that have acquired specific virulence attributes, which confers an increased ability to adapt to new niches and allows them to cause a broad range of diseases. These virulence attributes are frequently encoded on genetic elements that can be mobilized into different strains to create novel combinations of virulence factors (12). Only the most successful combinations of virulence factors have persisted to become specific pathotypes of *E. coli* that are capable of causing disease in healthy individuals. In addition to their role in disease processes, virulence factors presumably enable the pathogens to exploit their hosts in ways unavailable to commensal strains, and thus to persist and spread in the bacterial community (5).

The association of Shiga toxin-producing *Escherichia coli* (STEC) strains, also called verotoxin-producing *E. coli* (STEC) strains (1, 15), and human infections was first described

in 1979 (16, 32, 34). Afterwards, STEC strains were increasingly isolated from humans with diarrhoea and haemolytic-uremic syndrome (HUS) (4, 21). Today, more than 200 different *E. coli* O:H serotypes are known to be associated with the production of Shiga toxins (Stx). Since its identification as a food borne pathogen in 1982, STEC O157:H7 has been identified as the cause of several outbreaks (14). Domestic ruminants, mainly cattle, sheep and goats, have been implicated as the principal reservoir (2).

Facilitated by the extreme low infectious dose required for infection (<100 cells), STEC has also caused numerous outbreaks associated with drinking water, food products, farm and petting zoo visitations (5, 12, 21). One study indicated a potential airborne transmission after exposure to a contaminated building (31). Enterohaemorrhagic *E. coli* (EHEC) were recognized as a distinct class of STEC. The term EHEC was originally coined to indicate strains that cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), express *Stx*, cause attaching and effacing lesions on epithelial cells, and possess a 60-Mda plasmid (17, 18, 21, 24). Thus, EHEC denotes a subset of STEC and includes a clinical connotation that is not implied with STEC; whereas not all STEC are believed to be pathogens, all EHEC strains are considered to be pathogenic to humans and can result in severe gastrointestinal illnesses. Besides O157, the World Health Organisation (WHO) (28, 33) considers serotypes O26, O103, O111 and O145 to be of great importance in respect to human infections and defined them as emerging pathogens.

Human pathogenic strains are characterized by the presence of Shigatoxins mostly together with additional virulence factors such as the outer membrane protein intimin, encoded by the *eaeA* gene involved in the attachment of the bacteria to enterocytes, and a plasmid encoded enterohaemolysin (*hlyA*). STEC are forming the fourth major group of food borne pathogens in Belgium in respect to amount of cases. In respect to the severity of the disease, it belongs together with *Listeria monocytogenes* to the most feared group. STEC cause a spectrum of clinical manifestations ranging from asymptomatic carriage to diarrhea, bloody diarrhea and the life-threatening haemolytic uremic syndrome (HUS), which affects especially children and the elderly.

In Belgium the most frequent isolated serotype is O157:H7 (STEC O157), accounting for 20% of all cases. This serotype is responsible for as much as 77% of the HUS cases, underlining the higher pathogenicity of this serotype (26, 27). The pathogenicity of STEC belonging to other serotypes (non-O157 STEC) is probably lower but is variable for each strain. The Shigatoxin (*stx*) subtype is one of the factors susceptible to affect the pathogenicity (25). Antibiotic therapy is contraindicated and no alternative therapy for

treatment of STEC infections is available. Moreover, most patients require prolonged clinical and follow-up outpatient treatment. Healthcare costs associated with such infections are very significant. Prevention of primary infection in humans is therefore a crucial, but difficult, goal.

The most important infection sources of STEC for humans are besides inter human transmission, all related to cattle. Food products such as raw minced beef, hamburgers, salami, milk and dairy products but also water, fruit and vegetables, contaminated with cattle feces, direct contact with contaminated cattle and contact with contaminated environment are reported. The prevalence of the O157 STEC in cattle is estimated at 2 to 10% (29). Up to now there are no prevalence data available about the presence of non-O157 STEC in Belgian farm animals. The ecology of STEC at farm level is complex and only very limited information is available in the literature. At the moment it is impossible to prevent the entrance of positive animals in the slaughterhouses. Slaughtering positive animals increases the risk for contamination of the carcasses considerably even when a decent HACCP system is operational. Furthermore, almost no information can be found about the prevalence of *E. coli* non-O157 STEC strains in food products. The limited data about STEC in animals and food products is partly due to the lack of efficient diagnostic methodology allowing the detection of very small numbers of STEC cells in a background of other bacteria, including other non-pathogenic *E. coli*. Detection and isolation of *E. coli* O157:H7 is based on its biochemical characteristics as non-sorbitol fermenting and lack of β-glucuronidase activity. In human clinical samples sorbitol fermenting *E. coli* O157:H7 strains were recently isolated (1, 13). STEC of other serogroups than O157 and sorbitol positive *E. coli* O157 have no reliable biochemical, serological or morphological characteristics to distinguish them from commensal *E. coli*. Thus to detect STEC other than O157 and phenotypic variants of *E. coli* O157 in food, a new detection methodology has to be defined.

This project aims to develop detection and isolation methodologies for non-O157 *STEC* and for sorbitol positive *E. coli* O157 in food products and animal related farm samples. The availability of reliable methodologies is the first necessary step in the development of a program for prevention of human STEC infection due to contaminated food products in Belgium.

The detection and isolation methodology for non-O157:H7 STEC will be based on recent available scientific information concerning biochemical characteristics of the human clinical isolates which can be used to improve differentiation from other *Enterobacteriaceae* and from non-pathogenic *E. coli*. In this respect the hemolytic property identified on blood agar, the higher acid tolerance and the specific resistance to certain antibiotics will be applied.

Differentiation from other *Enterobacteriaceae* can be performed on recently developed selective *E. coli* media such as Rapid *E. coli*. For the most important serotypes (O26, O111, O103, O145) magnetic beads coated with specific antibodies are recently commercially available. These magnetic beads will be used to improve the isolation efficiency of these serotypes. Identification of non-O157:H7 STEC isolates, pathogenic for human, will be performed by PCR based methodology. Multiplex PCR's will be developed and optimized for this purpose.

The detection and isolation methodology will be evaluated on milk, raw milk cheese, minced beef, Belgian dry sausage and cattle feces. For this purpose artificially contaminated samples will be applied in a first phase.

1.2 Objectives

The project aims at developing a detection methodology for all STEC (Shigatoxin producing *Escherichia coli*) pathogens, including non-sorbitol fermenting *E. coli* O157. At this moment there is no effective method available to detect these hazardous organisms. In Belgium no detection of these extreme severe pathogens (except typical *E. coli* O157) is performed in food and farm related samples. Consequently, no information exists about the prevalence and the contamination sources. Nevertheless, human clinical samples collected in Belgium indicate that non-O157 STEC cause 80% of hospitalised STEC infections and 23% of the extreme life-threatening forms of the disease. This project aims at consolidating the Belgian scientific potential in this field of research. The project generates data and methods which will allow public authorities, represented by an extended users committee, to develop tools that will provide a reliable scientific basis for working out an appropriate strategy for prevention of human infections with pathogenic STEC.

1.3 Expected outcomes

In this project a routine detection and isolation methodology for non-O157 *E. coli* STEC and for sorbitol positive O157 in food products and animal related farm samples will be developed. The project will result in an isolation and detection scheme for non-O157 *E. coli* STEC and for sorbitol positive O157 strains in food products such as raw milk, raw milk cheese, minced meat and in farm related samples as faeces and overshoes. This project will offer the molecular biological tools for routine identification and characterization of STEC

strains. During the developmental process primary information about the prevalence in Belgium of different pathogenic STEC types in critical food products and on the farm will be collected. Primary information will be transferred to the public authorities in order to allow them to take the first steps in an effective plan to prevent human infections.

The detection methodology for non-O157 STEC and its availability in Belgium will form the first and necessary step in a programme for Project CP/58 "Development of detection method for non-O157-pathogenic *E. coli* in food: first step in prevention" SPSD II - Part I - Sustainable production and consumption patterns - *Agro-Food* 10 prevention of human infection due to contaminated food products. Development of a sensitive multiplex PCR which is able to detect the most important virulence genes as well as genes which allow identification of the most important human pathogenic serotypes will offer a valuable tool for routine screening of both food and farm related samples.

1.4 Research team

In order to reach the goals of this project, a multidisciplinary team of two different scientific institutions has been composed. Coordinator of this team, Prof. Dr. L. De Zutter, is staff member of The University of Ghent (Faculty of Veterinary Sciences), and experienced in conventional microbiological methods and in field research concerning meat contamination and slaughterhouse sampling.
As a partner of the multidisciplinary team, the research group led by Dr. L. Herman and Dr. M. Heyndrickx (ILVO, T&V), is experienced in molecular microbiology and bacterial taxonomic methods, including molecular typing tools. The institute has also a broad experience in the quality of milk and dairy products.

2 Results

The results, conclusions and recommendations are presented according to the different tasks of the project.

2.1 Task 1: Optimization of separate steps in the detection methodology of non-O157 STEC

Subtask 1.1: collection of STEC and commensal strains and characterization

- Partners:
    - Ghent University: collection of pathogenic and non-pathogenic *E. coli*
    - ILVO: strain characterization

- Objective:

Main objective was to compose a collection of pathogenic and non-pathogenic STEC strains emphasising serotypes O26, O103, O111, O145 and O157. Metabolic and genetic characterisation of these strains will provide data for optimization of the different parts of the STEC isolation method this project aims at.

- Results:

    - Collection of strains

Human clinical STEC isolates of different serotypes and commensal *E. coli* strains have been collected. Human pathogenic strains isolated from clinical samples were donated kindly by the Belgian reference laboratory Prof. Dr. D. Pierard (member of the user committee and head of the reference lab in Belgium), Prof. Dr. H. Karch (Institute for Hygiene, university of Münster) contributed, amongst other human pathogenic strains, several sorbitol positive O157 EHEC strains. In order for the collection to contain the full range of genetic variants of some mayor virulence genes for human pathology, strains containing these variants were donated by Prof. Dr. E. Möller-Nielsen (Denmark) and Prof. Dr. J. Osek (Poland). From cattle

faeces, milk and beef samples 42 E. coli strains were isolated and tested for virulence factors. Those strains which are *stx*-negative are considered as commensal *E. coli* in respect to this project. Because the latter can mask the presence of STEC strains, elimination or reduction of their growth in the culture for STEC is advisable.

A total of 146 *E. coli* isolates were included in this study. 104 strains belonging to several EHEC serotypes were isolated from hospitalized patients diagnosed for (bloody) diarrhea, HC or HUS. Forty-two commensal *E. coli* strains isolated from beef carcasses were also included; see Table 1 for an overview of strains. Commensal strains were isolated using Rapid *E. coli* medium (REC, Bio-Rad). Isolated *E. coli* strains found PCR negative for both *stx1* and *stx2* are considered to be non-pathogenic strains. Control strains used for *stx2* subtyping were 137/98 (O157:H7, *stx*2c), 551/98 (O62:H-, *stx2d*), 107/86 (O139:K2, *stx2e*) and 214/125 (O?:H18, *stx2f*) (23). Reference strains E 2348/69 (*eae* α, O?) , TB154A (*eae* ε, O?) and DVI797 (*eae* ζ, O?) (22) were used for intimin subtyping. For all results presented below, strains indicated "PHxx" cover pathogenic strains, while strains indicated "PCxx" cover non-pathogenic strains.

Serotype	**# strains**
O26	15
O91	2
O103	14
O111	15
O121	3
O128	2
O145	15
O157 sorbitol negative	11
sorbitol positive	16
O172	2
Commensal strains	42

Table 1. Collection of strains

- Genetic characterisation

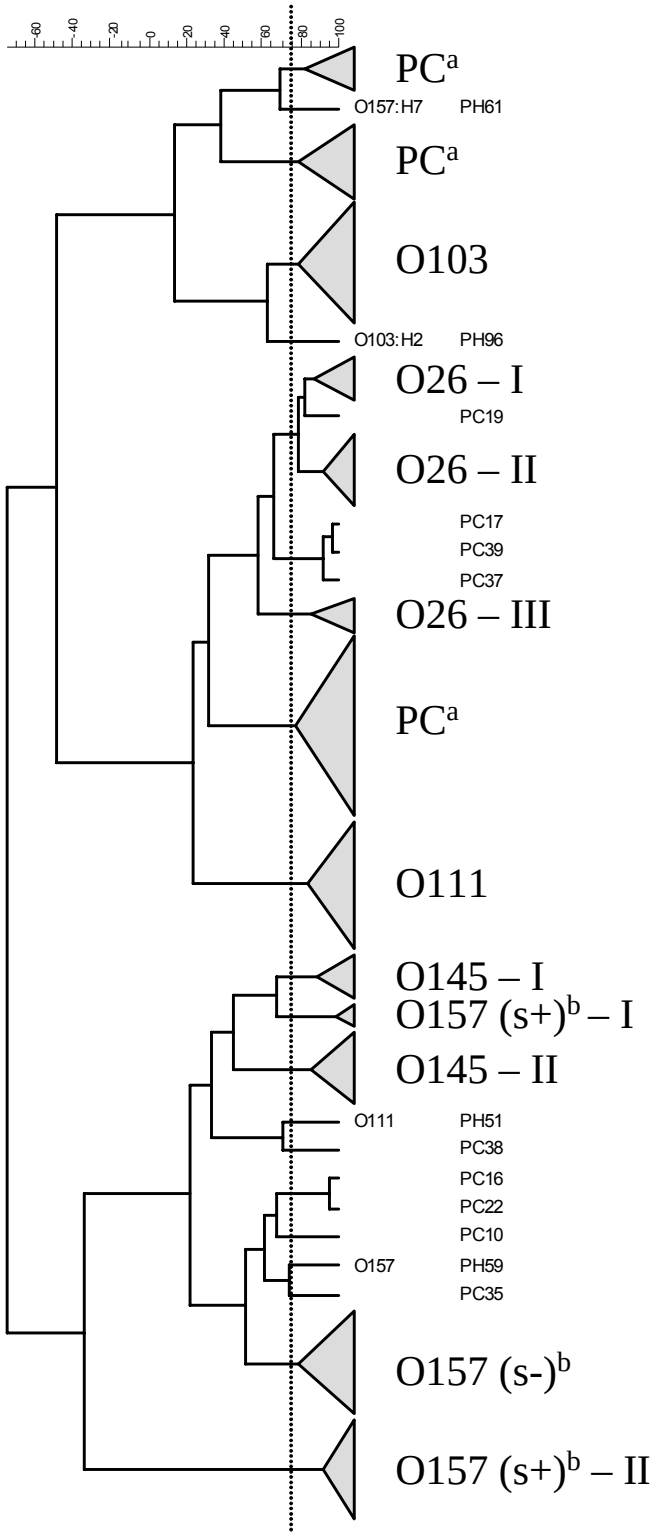
All collected strains were typed by means of random amplification of polymorphic DNA (RAPD) to unravel their genetic diversity. Primers were selected based on previously published data (Akopyanz, 1992). All selected primers were tested to define an optimised RAPD-PCR method using two RAPD primers together in one PCR. This genetic information is primary used as a selection criterion for the further analysis to ensure that a genetic diversity of strains is included; secondly this information functions as an internal control for the serotyping, performed in external laboratories.

Primers used in this protocol:
1247 (5' AAGAGCCCGT 3') and 1254 (5' CCGCAGCCAA 3')

Molecular fingerprints were processed using Bionumerics software (Applied Maths, Kortrijk, Belgium). After inverting the colour-scale, images were analysed based on peak-intensities, allowing the construction of a specific intensity profile representing the genetic fingerprint of a strain. In order to analyse a large collection of genetic fingerprints, all fingerprints were normalised using a composed marker profile as shown in Figure 1. To obtain a clear pattern, a background subtraction with an average of 12% was performed. To reduce the number of artefacts in the agarose gels an arithmetic average was calculated using these settings: least square filtering was set to a cut off value of 0.64% and power was 2.0 to increase the discriminatory capacity of the algorithm. A rooted tree was constructed using Bionumerics software: clustering algorithm was Ward and similarity coefficient was set to Cosine correlation to optimize clustering based on band intensity profiles (10, 19). The most relevant clusters in the dendrogram were determined by calculating the similarity cut-off value that produced the highest point-bisectional correlation (Bionumerics manual, Applied Maths). Jack-knife analysis was used to determine how accurately isolates could be assigned to serogroups based on maximum-similarity coefficients.

127 strains were characterized by RAPD-PCR to analyse the genetic diversity (Figure 1): strains belonging to serotypes O26, O103, O111, O145, O157 and 42 commensal strains. The RAPD analysis protocol, allowed a clear separation of different serotypes with amplified fragment sizes ranging from 0.4 to 6.0 kb. Jack-knife analysis was performed using Bionumerics to assess the possibility of group violations: strains serotyped as O26, O103, O111, O145 and O157 were placed in a specific RAPD cluster for 81.8 %, 97.7 %, 76.3 %, 92.7 %, 77.5 % of the cases respectively.

Similarity cut-off values were calculated to identify the relevant clusters. Similarity indices for the serotypes were: 93.53 %, 91.45 % and 85.74 % for O26 I to III, 79.36 % for O103 and 83.45 % for O111. Strains serotyped belonging to serotype O145 were placed into two clusters: similarity indices were 88.11 % and 85.55 % for cluster I and II respectively. Strains of serotype O157 were mainly clustered into two groups, corresponding to the ability to ferment sorbitol: the sorbitol negative group showed a similarity index of 79.48 %, while the sorbitol positive group was split into 2 clusters: calculated similarity index were 97.91 % and 92.21 % for I and II respectively. The sorbitol positive subgroup can be distinguished from the sorbitol negative group based on the presence of a single 690bp fragment in the RAPD pattern.



All major clusters were collapsed. Aberrant pathogenic strains are indicated by the collection number listed in table 2 and start with "PH". All aberrant non-pathogenic strains, indicated as PCxx, belong to serotypes other than O26, O103, O111, O145 and O157.

[a] PC labelled clusters contain non-pathogenic strains

[b] (s-) indicates sorbitol negative O157 STEC strains, while (s+) indicates sorbitol positive O157 strains.

Figure 1. Clustered RAPD patters after Bionumerics processing

Polymerase Chain reactions (PCR) were used to screen strains for virulence genes. Several virulence genes were combined to compose one virulence profile: Shigatoxin encoding genes (*stx1, stx2, stx2c, stx2d, stx2e* and *stx2f*), intimin encoding genes (*eaeA, eae α, eae β, eae γ, eae θ, eae ε* and *eae ζ*,), shiga-toxin auto agglutinating adhesin gene *saa*, enterohaemolysin gene *hlyA*, translocated intimin receptor gene *tir*, katalase antiperoxidase gene *katP*, and extra cellular serine protease *espP*. Strains positive for the *eae* gene, as determined using general *eae* primers as described by Nielsen and Andersen (22), were analyzed afterwards with 5 pairs of primers detecting the most important *eae*-variants for human infections (α, β, γ, θ and ε) (2). Strains positive for the *stx2* gene, as determined using general *stx2* primers as described by Botteldoorn *et al.* (3), were subtyped as variants c, d, e or f using 4 pairs of primers described previously (23). An overview of virulence profiles is shown in Table 2.

strain	serotype	source	*stx1*	*stx2*	*stx2* subt.	*eae*	*eae* subt.	*saa*	*ehly*	*tir*	*katP*	*espP*	Diagnosis [b]
PH45	O26	Belgium[c]	-	+	c	+	β	-	+	+	+	+	BD
PH46	O26	Belgium [c]	+	-	-	+	β	-	+	+	+	+	D
PH63	O26	Slovenia[d]	-	+	c	+	β	-	+	+	-	+	U
PH64	O26	Slovenia[d]	-	+	c	+	β	-	-	+	+	+	U
PH67	O26	Slovenia[d]	-	+	-	+	β	-	+	+	-	+	U
PH11	O26:H-	Belgium [e]	+	-	-	+	β	-	+	+	+	+	D
PH22	O26:H-	Belgium [e]	-	+	c	+	β	-	-	+	-	-	D
PH25	O26:H-	Belgium [e]	+	-	-	+	β	-	+	+	+	+	D
PH09	O26:H11	Belgium [e]	+	-	-	+	β	-	+	+	+	+	AP
PH10	O26:H11	Belgium [e]	+	-	-	+	β	-	+	+	+	+	BD
PH15	O26:H11	Belgium [e]	+	-	-	+	β	-	+	+	+	+	D
PH16	O26:H11	Belgium [e]	+	-	-	+	β	-	+	+	+	+	AP
PH20	O26:H11	Belgium [e]	-	+	c	+	β	-	-	+	-	-	D
PH26	O26:H11	Belgium [e]	-	+	c	+	β	-	+	+	+	+	HUS
PH13	O26:H11 (?)[g]	Belgium [e]	+	-	-	-	–	-	-	-	-	-	AP
PH49	O103	Belgium [c]	+	-	-	+	θ	-	+	+	+	+	D
PH50	O103	Belgium [c]	+	-	-	+	θ	-	+	+	-	+	BD
PH97	O103:H-	Germany[f]	+	+	-	+	-	-	+	+	+	+	HUS
PH02	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	+	D
PH03	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	+	D
PH04	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	+	D
PH05	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	-	NA
PH07	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	-	+	D
PH12	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	-	D
PH17	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	-	D
PH19	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	+	D
PH23	O103:H2	Belgium [e]	+	-	-	+	θ	-	+	+	+	+	D
PH24	O103:H2	Belgium [e]	+	-	-	+	ε	-	+	-	+	-	D
PH96	O103:H2	Germany[f]	+	+	c	+	ε	-	+	+	+	+	HUS
PH51	O111	Belgium [c]	+	-	c	+	ε	-	+	-	+	+	BD
PH65	O111	Slovenia[d]	+	-	-	+	γ	-	+	-	-	+	NA
PH29	O111:H-	Belgium [e]	+	-	-	+	ε	-	+	-	+	+	D
PH37	O111:H-	Belgium [e]	+	-	-	+	γ	-	+	-	-	+	NA
PH39	O111:H-	Belgium [e]	+	-	-	+	γ	-	+	-	+	+	D

PH93	O111:H-	Germany[f]	+	+	c	+	γ	-	+	-	+	+	HUS
PH94	O111:H-	Germany[f]	+	-	-	+	γ	-	+	-	+	+	HUS
PH95	O111:H-	Germany[f]	+	+	-	+	γ	-	+	-	+	+	HUS
PH30	O111ac	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	D
PH31	O111ac	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	NA
PH32	O111ac	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	NA
PH33	O111ac	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	D
PH35	O111ac	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	D
PH36	O111ac	Belgium[e]	+	+	-	+	γ	-	+	-	+	+	D
PH41	O111ac	Belgium[e]	+	+	c	+	ε	-	-	-	+	+	HC
PH01	O145:H-	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	BD
PH06	O145:H-	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	U
PH08	O145:H-	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	NA
PH27	O145:H-	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	HUS
PH34	O145:H-	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	NA
PH40	O145:H-	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	AP
PH43	O145:H-	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	AP
PH88	O145:H-	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH89	O145:H-	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH90	O145:H-	Germany[f]	+	+	-	+	γ	-	+	-	+	+	HUS
PH91	O145:H-	Germany[f]	+	+	c	+	γ	-	+	-	+	+	HUS
PH92	O145:H-	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH21	O145:H?	Belgium[e]	+	-	-	+	γ	-	+	-	+	+	AP
PH14	O145:H16 (?)[g]	Belgium[e]	+	-	-	+	β	-	+	-	+	+	D
PH18	O145:H16 (?)[g]	Belgium[e]	+	-	-	-	-	-	-	-	-	-	NA
PH54	O157:H7 (s-)[a]	Belgium[c]	-	+	-	+	γ	-	+	-	+	+	HUS
PH55	O157:H7 (s-)[a]	Belgium[c]	-	+	c	+	γ	-	+	-	+	+	HUS
PH56	O157:H7 (s-)[a]	Belgium[c]	-	+	c	+	γ	-	+	-	+	+	HUS
PH59	O157:H7 (s-)[a]	Slovenia[d]	-	+	-	+	γ	-	+	-	+	+	NA
PH61	O157:H- (?)[a, g]	Slovenia[d]	-	+	-	-	–	-	-	-	-	-	NA
PH69	O157:H7 (s-)[a]	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	NA
PH71	O157:H7 (s-)[a]	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	NA
PH73	O157:H7 (s-)[a]	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	NA
PH60	O157:H7 (s-)[a]	Slovenia[d]	-	+	c	+	γ	-	+	-	+	+	NA
PH70	O157:H7 (s+)[a]	Belgium[e]	+	+	c	+	γ	-	+	-	+	+	NA
PH72	O157:H7 (s+)[a]	Belgium[e]	-	+	c	+	γ	-	+	-	+	+	NA
PH44	O157:H7 (s+)[a]	Belgium[e]	-	+	-	+	γ	-	+	-	-	+	HUS
PH74	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH75	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH76	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH77	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH78	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH79	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	U
PH80	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH81	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	-	-	-	+	D
PH82	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	-	+	HUS
PH83	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH84	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	U
PH85	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH86	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS
PH87	O157:H7 (s+)[a]	Germany[f]	-	+	c	+	γ	-	+	-	+	+	HUS

[a] *(s+) indicates a group of strains belonging to serotype O157 able to ferment sorbitol within 24 hours, (s-) indicates strains unable to ferment sorbitol within 24 hours.*

[b] *abbreviations used indicating clinical diagnose: D – diarrhea; BD – bloody diarrhea; AP – abdominal pain; HC – hemorrhagic colitis; HUS – hemolytic uremic syndrome; NA – data not available; U – unclear.*

[c] *Veterinary and agrochemical research centre, Ukkel, Belgium*

[d] *strains kindly provided by the institute for microbiology, Ljubljana, Slovenia*

[e] *strains kindly donated by the Belgian national VTEC reference laboratory (D. Piérard)*

[f] *strains kindly donated by H. Karch, Germany*

[g] *"(?)" represent presumably wrongly serotyped strains.*

Table 2. Virulence profiles grouped by serotype

- Metabolic characterisation

The minimal inhibitory concentrations (MIC) towards a set of antibiotics, such as those used in selective media for *E. coli* O157 as well as in other microbiological culture media were determined. For this purpose a serial dilution method in microtiterplates was applied. Resistance for other inhibitory substances was determined using the same method as described for the antibiotics. Results of these extended experiments are shown in Table 3.

Antibiotic	**PH**	**PC**
Vancomycin	>256	>256
Cefoperazone	0.125-4	0.125-1
Nalidixic acid	4-16	4-8
Enrofloxacine	<0.0156	<0.0156
Trimethoprim	0.25-1	0.25-2
Rifampicine	8-32	8-32
Tobramycine sulfate salt	0.25-1	0.25-1
Phosphomycine	4-32	2-64
Cefsulodine sodium salt	128-256	64-256
Piperacilline sodium salt	2-8 *	2-8
Colistine sulfaat	4-16	4-16
Novobiocine	128- >256	128- >256
Lincomycine	>256	>256
Azithromycine	2-4 *	2-8
Nitrofurantoine	128-256	128-256
Ticarcilline	4-32 *	2-16
Cefixime	2-4	2-4
Inhibitor	**PH**	**PC**
Acriflavine	16-64	16-64
Briljant green	2-8	2-16
Na azide	>256	>256
Irgasan	0.015625-1	0.015625-1
Cetrimide	32-64	32-64
Fusidine	>256	>256
Malachietgroen	8-16	8-16
Potassium tellurite	>256	32-256
Cycloserine	16-64	32-64

** Some strains > 256*

Table 3. MIC values for PH (pathogenic) and PC (non-pathogenic) strains in mg/L

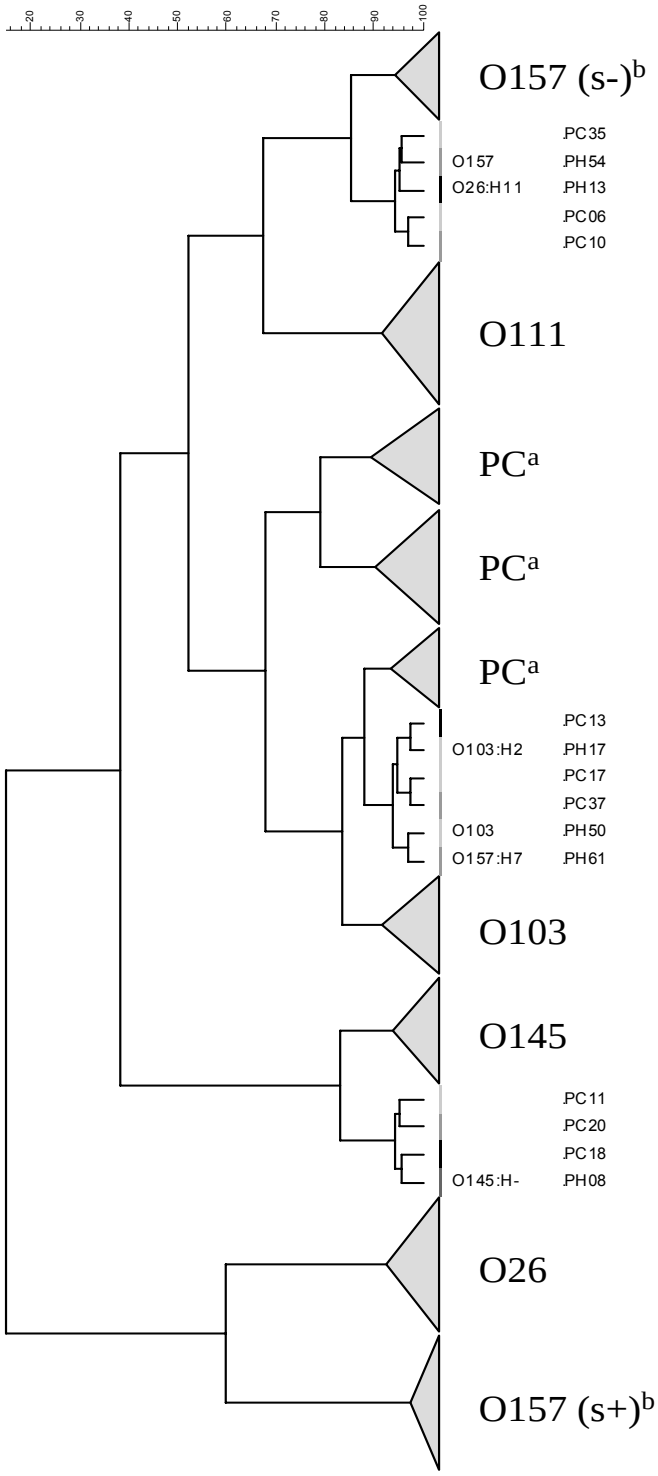
No significant differences were found between pathogenic and non-pathogenic strains: antibiotics will be applied in selective isolation and enrichment media to reduce background bacteria. No additional inhibitory components can be added to these media to reduce growth potential of non-pathogenic *E. coli* favouring growth of pathogenic strains: a selection of antibiotics/inhibitory components will be evaluated for maximum growth of STEC during subtask 1.2.

Utilization of chemical substances like a range of sugars: pathogenic *E. coli* strains generally express a reduced carbohydrate metabolism. In order to asses the differences between pathogenic and commensal *E. coli* strains and between different pathogenic serotypes the carbohydrate fermentation was analyzed using API 50 tests (Biomérieux, France). Fermentation patterns were digitalized and analyzed using Bionumerics software 3.0 (Applied Maths, Kortrijk, Belgium).

Table 4 indicates which carbohydrates were evaluated using API50 galleries. As all *E. coli* strains have been found to be metabolically very similar, only minor differences between serotypes were found. The research team has chosen to concentrate research efforts on distinguishing strains belonging to serotypes O26, O103, O111, O145 and O157. As indicated in Figure 2, metabolic profiles obtained after marking incubated API50 galleries, can be imported into Bionumerics software, and subsequently be used to construct dendrograms. As all main serotypes are placed into different branches of these dendrograms, several elements from these metabolic profiles are responsible for these differences. Data-analysis indicated 7 carbohydrates possibly able to discriminate serotypes O26, O103, O111, O145 and O157. These carbohydrates were studied in a more detailed way: results will be discussed in subtask 1.4.

STRIP 0-9 tube / substrate	STRIP 10-19 tube / substrate	STRIP 20-29 tube / substrate	STRIP 30-39 tube / substrate	STRIP 40-49 tube / substrate
0 CONTROL	10 GALactose	20 α-Methyl-D-Mannoside	30 MELibiose	40 D TURanose
1 GLYcerol	11 GLUcose	21 α-Methyl-D-Glucoside	31 Sucrose	41 D LYXose
2 ERYthritol	12 FRUctose	22 N-Acetyl-Glucosamine	32 TREhalose	42 D TAGatose
3 D ARAbinose	13 MaNnosE	23 AMYgdalin	33 INUlin	43 D FUCose
4 L ARAbinose	14 SorBosE	24 ARButin	34 MeLeZitose	44 L FUCose
5 RIBose	15 RHAmnose	25 ESCulin	35 RAFfinose	45 D ARabitoL
6 D XYLose	16 DULcitol	26 SALicin	36 Starch	46 L ARabitoL
7 L XYLose	17 INOsitol	27 CELlobiose	37 GLYcoGen	47 GlucoNaTe
8 ADOnitol	18 MANnitol	28 MALtose	38 XyLiTol	48 2-Keto-Gluconate
9 β Methyl-D-Xyloside	19 SORbitol	29 LACtose	39 GENtiobiose	49 5-Keto-Gluconate

Table 4. Carbohydrates evaluated using API50 galleries



[a] *PC labelled clusters contain non-pathogenic strains*

[b] *(s-) indicates sorbitol negative O157 STEC strains, while (s+) indicates sorbitol positive O157 strains.*

Figure 2. Dendrogram constructed using API50 data

– Conclusion:

Molecular fingerprinting was applied to assess genetic variations between pathogenic serotypes and non-pathogenic strains. PCR using previously published primers sequences were used for virulence profile definition. Our results confirmed information found scattered in various international journals, indicating a strong correlation between some virulence genes and serotypes can be defined.

Besides molecular fingerprints, metabolic carbohydrate fermentation profiles were used to construct detailed dendrograms: using cosine correlation coefficient and Ward clustering algorithm, serotypes O26, O103, O111, O145 and O157 were grouped into different branches of these dendrograms indicating some elements in the metabolic profile can differentiate between these serotypes. Data-analysis was used to define these elements and there use in specific isolation media will be discussed in subtask 1.4.

Subtask 1.2: Enrichment media

– Partners: Ghent University and ILVO

– Objective:

During this task several enrichment broths were evaluated on their use for STEC isolation methods: both previously published and well known enrichment broths as newly modified enrichment broths, based on the results of subtask 1.1, will be selected for testing the growth of pathogenic and non-pathogenic *E. coli*. To simulate *in vivo* conditions, the effect of stress on the resuscitation rate was also evaluated.

– Results:

Based on the characterisation described above different broths for the enrichment of *E. coli* O157 and other STEC were evaluated for the growth of the collected strains (STEC and commensal E. coli strains). Enrichment media tested: tryptic soy broth (TSB), Brain Heart Infusion broth (BHI), modified TSB containing bile salts (1.5g/l) and K2HPO4 (1.5g/l), mTSB supplemented with vancomycin, cefixime and cefsulodin, TSB supplemented with cefixime, potassium tellurite and vancomycin, modified E. coli broth (E. coli broth with 1.12g/l instead of 1.5g/l bile salts) supplemented with 25 or 10mg/l novobiocine, Buffered Peptone Water

(BPW), Gram Negative Broth (GN), *E. coli* broth (ECB), Luria-Bertani broth (LB), Mueller-Hinton Broth (MHB) and MacKonkey broth (MAC). Some modifications were made to these media based on the results of the MIC assessment described above. Enrichment was evaluated after 3, 6 and 24 hours at 37°C and 42°C. Figure 3 shows some growth curves of pathogenic STEC strains (PH03 – O103:H2) in TSB, BPW, BHI, ECB, LB, MHB and MAC broth.

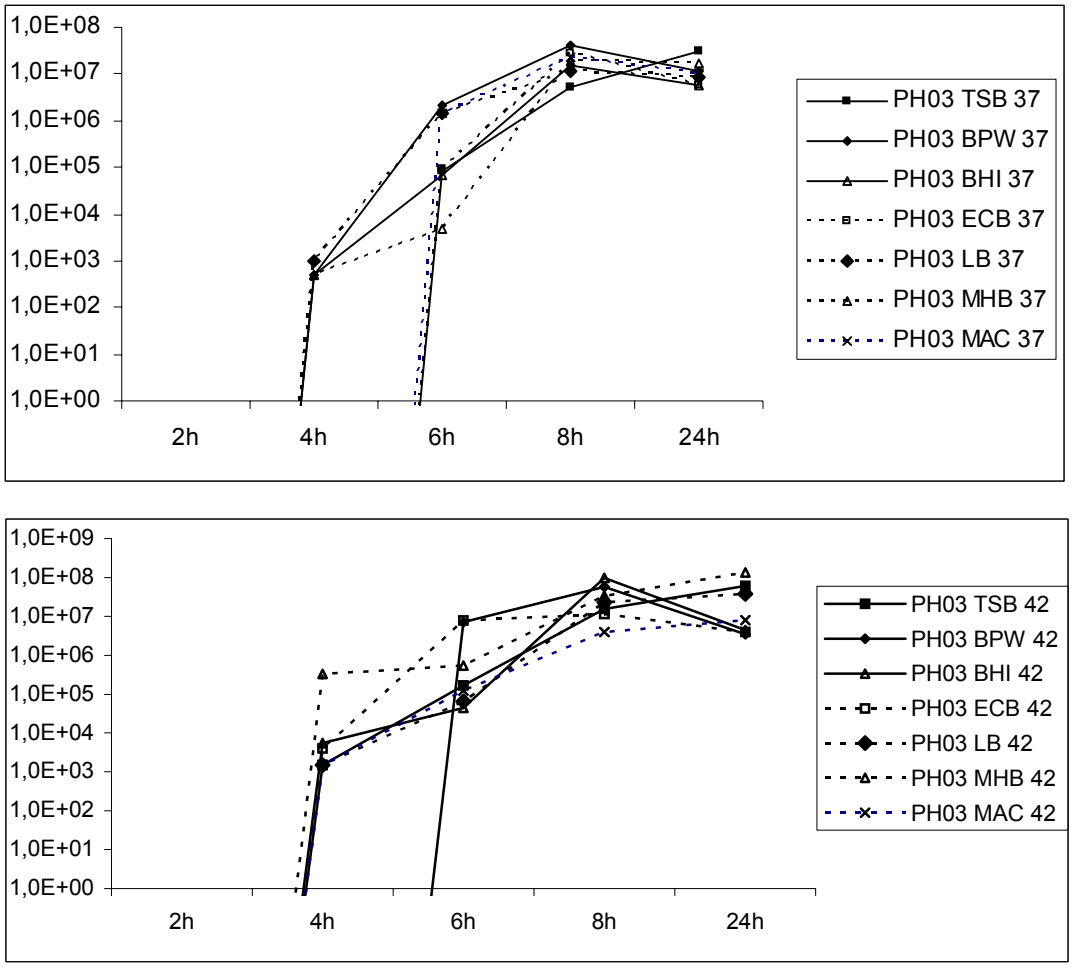


Figure 3. Growth rates in some commercially available enrichment broths

From each STEC serotype at least 2 isolates were tested against a mixture of commensal *E. coli* types (commensal strains were selected based on their genetic profile obtained after RAPD analysis and differences in MIC values and carbohydrate metabolism). In order to evaluate the effect of stress on the growth of STEC in the enrichment broths both unstressed and stressed target strains (cool stressed: cultures stored at 2-4°C during 7 days and acid stressed: bacteria exposed to acetic acid at pH 3.0 during 30 minutes) were tested.

Enrichment broths showing a good growth of STEC were also evaluated for the growth inhibition capacity of commensal *E. coli*.

Adding antibiotics to an enrichment broth to a concentration based on MIC values as described above, implies a certain risk. In order to quantify this risk, a selection of antibiotics and inhibitory components was added to enrichment media in various concentrations. The charts below (Figure 4) show some example data from these experiments. Table 3 indicates a MIC value of 128 mg/L for novobiocine, but when this antibiotic was added below this level, reduced growth potential was observed when incubating at 37 °C. This hampered growth was less obvious when incubating at 42 °C; a normal growth rate was observed when adding this antibiotic at lower concentrations. When adding K-tellurite at 16 mg/L, no viable cells were detected after 24 hours of incubation at 42 °C, while incubating at 37 °C did not show this problem. Moreover, STEC are know to grow abundantly up to 42 °C, which was confirmed during extensive experiments using a range of commercially available enrichment broths. Both TSB and BPW showed excellent growth potential for STEC, with a slightly higher growth rate for TSB after adding antibiotics. Based on our lab-results and in accordance to ISO standards for using mTSB in O157 STEC detection, TSB was defined as the most appropriate basic enrichment broth.

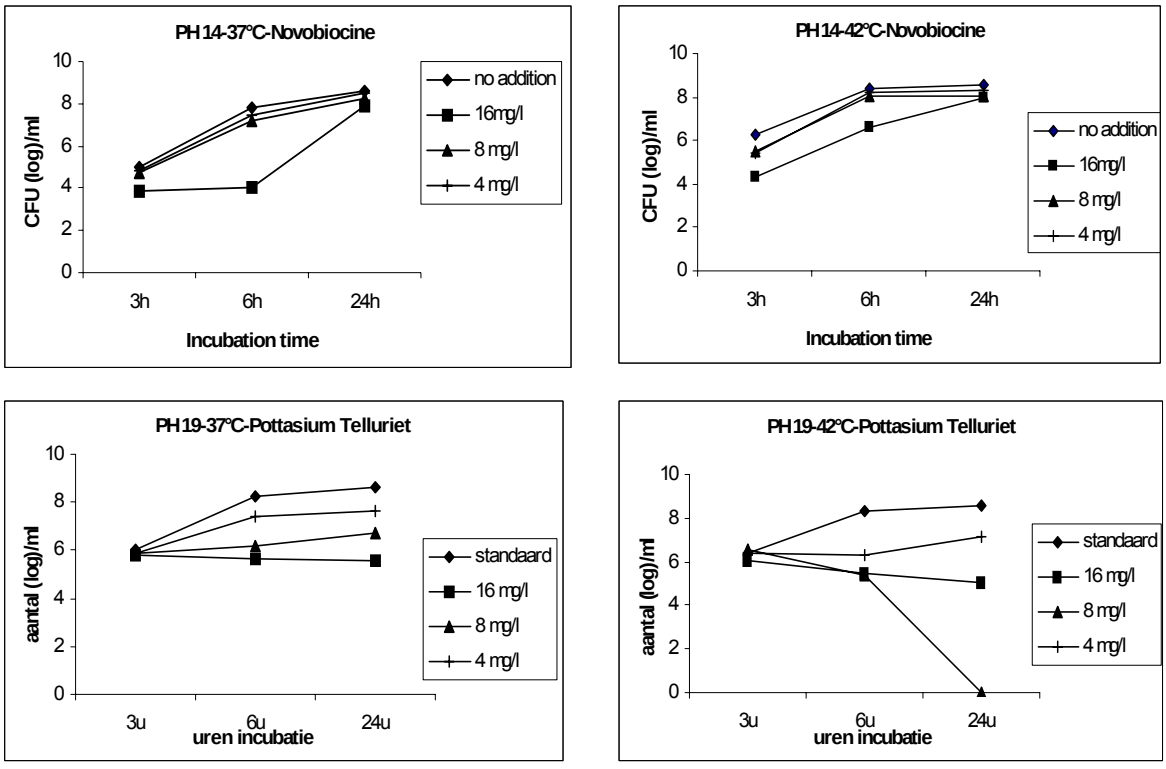


Figure 4. Effect of adding antibiotics at MIC value for some PH (pathogenic) STEC strains

Different methods for bacterial stress induction were applied to simulate *in vivo* situations in food samples. Refrigerating pathogenic and non-pathogenic strains at 4° for 1 to 3 days did not result in a reduced growth at 37 and 42°C. However, freezing these strains at -20 °C for 1 to 3 days caused a delayed logarithmic growth as shown in Figure 5: the effect of freezing is mostly undone after 24 hours of incubation, but when applying shorter incubation periods, results will be affected.

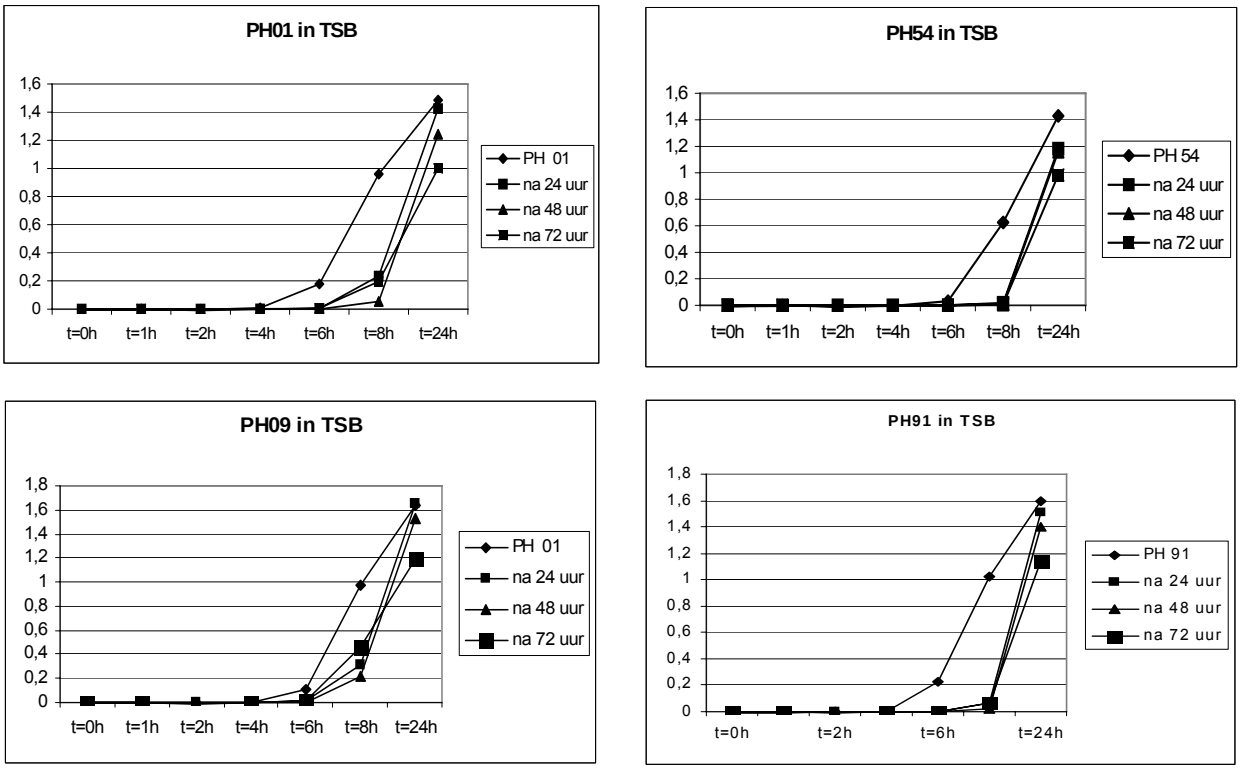


Figure 5. Shift in growth curve for some STEC strains after freezing episode (based on OD data)

Like chilled storage at 4 °C, osmotic stress does not significantly influence growth or recovery potential of STEC strains: only minor changes in growth rate could be observed when strains were grown TSB containing 6.5 to 8.5% NaCl. Overnight storage of bacterial cultures (lag-phase, log-phase and stationary phase) at 4°C in TSB containing 6.5% NaCl, did not affect growth rate when these stressed cells were inoculated into normal enrichment broth. As food samples will always require an enrichment procedure (see below), viability on selective isolation media (task 1.4) of these different stressed bacteria was not evaluated.

– Conclusion:

Based on the observations of reduced growth due to the combination of adding antibiotics/inhibitory components, incubating at 37 or 42 °C and the possibility of sub-lethally injured or stressed cells in food samples, the research team had to find a compromise. This compromise was designed balancing out both reduction of background bacteria (implying higher concentrations of antibiotics and elevated incubation temperature) and recovery of possibly injured/stressed pathogenic STEC strains. The specific nature and application of this compromise will be presented below (task 2).

Subtask 1.3: Post-enrichment treatment

– Partners:
  - Ghent University: IMS treatment
  - ILVO: Acid treatment

– Objective:

IMS and acid treatment have often been cited as additional tools for enhancing selectivity. During this task, the effectiveness of a post-enrichment acid treatment was evaluated and serotype-specific immunomagnetic beads applied to enrichment media.

– Results:

  - IMS treatment

Magnetic beads coated with antibodies for *E. coli* serotypes like O26, O103, O111 and O145 (Dynal, Oslo, Norway) were recently becoming commercially available (immunomagnetic separation, IMS). These beads will also be used to enhance selectivity of the enrichment procedure (6, 30).

Specific beads and equipment were acquired for IMS treatment, but the research team faced some unexpected problems, forcing us to postpone these activities to task 2. A "Bead retriever" was used for automated IMS treatment of samples. The preliminary experiments conducted using this piece of lab-equipment, identified a serious problem: at low levels of STEC present in an enrichment broth, the revenue of STEC after automated IMS treatment

was remarkably low. The table below (Table 5) shows some data on these experiments, comparing manual IMS and automated IMS using the Bead retriever.

	before IMS	**IMS manual, 10 minutes**	**IMS manual, 30 minutes**	**IMS bead retriever**
***E. coli* 0157 Reference strain**	355 CFU/ml	270 CFU /100 µl	313 CFU /100 µl	18 CFU /100 µl
***E. coli* 026 reference strain**	295 CFU/ml	65 CFU/100 µl	290 CFU /100 µl	23 CFU /100 µl

Table 5. Comparing manual IMS treatment to automated treatment

Data indicated in Table 5 shows no increased isolation efficiency when using automated IMS treatment as similar CFU counts are observed prior and after IMS treatment. Manual IMS treatment increases the selectivity of the procedure: 10 times more STEC cell were found after IMS treatment. However, 30 minutes of manual shaking is required for achieving this level of selectivity as 10 minutes of shaking only resulted in a minor increase of cell counts when non-O157 beads are applied. Because of the high level of bench work required for manual IMS treatment, the research team concentrate resources on optimizing the enrichment and isolation procedures. Further details on these procedures and circumstances where IMS treatment is indispensable, regardless of the amount of bench work required, are discussed under task 2.

- Acid treatment

Some scientific papers mention the use of an acid treatment or an acid shock on the enrichment media to enhance the selectivity of the isolation of STEC strains prior to plating on selective media (7-9). This selectivity is thought to be closely related to acid resistance in clinical isolates of STEC strains. Several methods were evaluated to determine the possibilities of using this post enrichment procedure to enhance enrichment selectivity. Experimental design comprised the use of both organic and inorganic acid at pH values ranging from 1.5 up to 3.5.

To evaluate this hypothesis several acid treatments following an enrichment phase were evaluated. Acid treatment using hydrochloric acid at pH 1.5 to 2.5 showed poor reproducibility while acid treatment using HCl at pH 3.0 and 3.5 showed too little selectivity. Using organic acids like lactic and acetic acids showed more comprehensive results. After numerous experiments, lactic acid at pH 3.0 showed to be most suitable for this kind of acid treatment. Survival rates were measured at several fixed times during acid treatment. Sampling started after 30 minutes of acid treatment and lasted up to 3 hours after start of treatment. Using this method, a large number of pathogenic and non-pathogenic *E. coli* were tested for their resistance to acid conditions. Some results are presented in Figure 6.

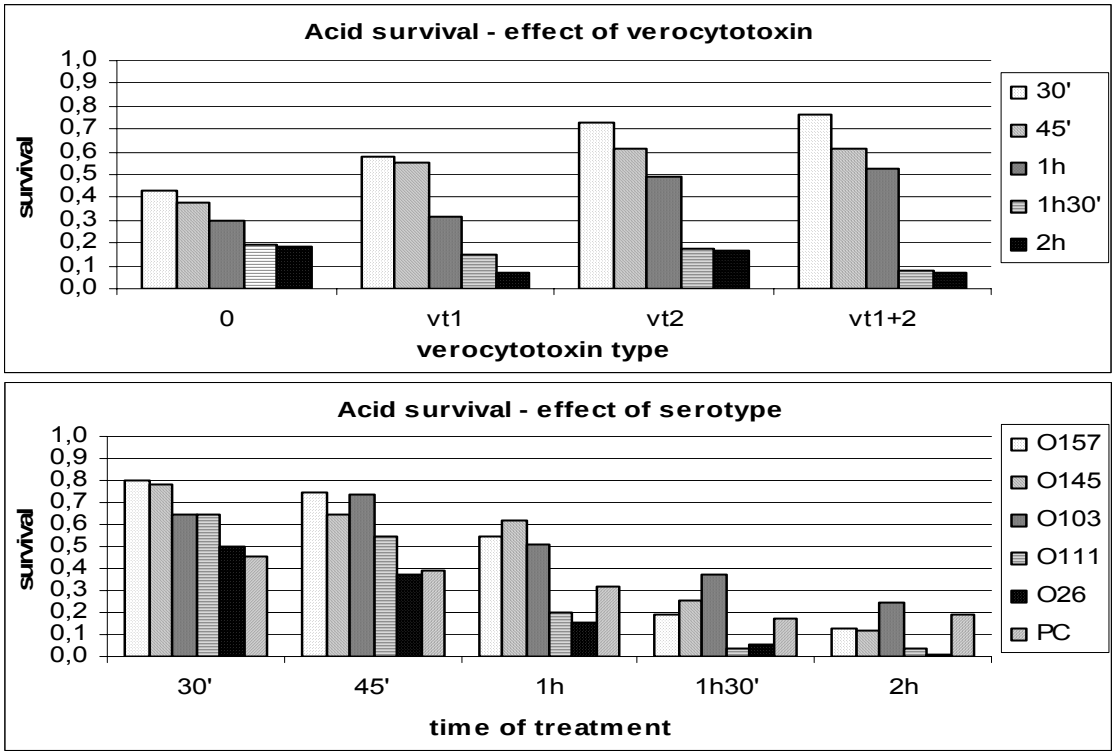


Figure 6. Effects of acid treatment and STEC strains grouped by toxins detected or grouped by serotype

After statistical analysis of the survival rates some general conclusions are presented here:

- Survival to acidic conditions is higher in strains where *stx1* (*vt1)*, *stx2* (*vt2)* or both genes are present. However, it must be noted that data for strains presenting no verocytotoxin are very heterogeneous: several strains show 100% survival after 2 hours, while other strains didn't show any survival, even after just 30 minutes of acid treatment.
- Some serotypes (like O157 and O145) show a slightly higher acid resistance compared to other serotypes, but these rates drop rather quickly after 1 hour of treatment.

– Conclusion:

Acid treatment of STEC cultures showed some interesting results, but due to in-group variance, these have proven to be not significant. Manual IMS treatment results a significance increase of isolation efficiency, but further optimizing of classical microbiological methods renders this procedure unnecessary in most cases. Post enrichment treatment is therefore restricted to application of IMS in specific cases as described below (task 2).

Subtask 1.4: Agar media

– Partners: Ghent University and ILVO

– Objective:

During this task commercially available isolation media will be evaluated. Based on data generated during subtask 1.1, serotype specific isolation and purification media will be designed to optimize non-O157 and sorbitol positive O157 STEC isolation from food products and feces. Results from this subtask will be combined with results obtained during subtask 1.3 to construct isolation protocols to be evaluated during task 2.

– Results:

Selective as well as elective media were designed previously for the isolation of STEC. During several stages, these media were tested for their sensitivity and specificity. Both commercially available media as custom designed media were evaluated. Table 6 shows some commercially available media evaluated and what modifications were examined to increase selectivity.

Commercial medium	Supplements added to the media
Chrom-Agar Peptone Tergitol X-gluc Trypton Bile X-gluc Fluorocult Agar Rapid *E. coli* Rainbow-Agar	sucrose, lactose, sorbitol, novobiocine, tellurite
EMB agar	
SMAC	Bile salts, tellurite
RMAC	X-gluc, K_2HPO_4, KH_2PO_4, bile salts, tellurite
Violet Red Bile agar	
Brilliant green agar	
Phenol Red Agar	carbohydrates, tellurite, novobiocine, bile salt
EHEC agar	ph 7.0 - 9.0, K_2HPO_4, KH_2PO_4

Table 6. Commercially available media evaluated

One of the media often mentioned is enterohaemorrhagic *E. coli* (EHEC) agar: this medium was designed to allow selection of EHEC strains amongst a mixture of other bacteria based on the activity of a special haemolysin: enterohaemolysin (11). EHEC agar is a variation of sheep blood agar in which the sheep red blood cells are washed 3 times in phosphate buffered saline, resuspended to the original volume and added at 5% to tryptic soy agar (TSA) supplemented with 10 mmol/l (final concentration) CaCl2. As some STEC strains do no present enterohaemolysin activity, not all STEC will be detected using this medium as a single selective component. Furthermore, these blood-agar media have a very short shelf life and enterohaemolysin activity is often difficult to detect or very similar to normal haemolytic activity.

Finally, the application of media used for the counting of *E. coli* including chromogenic media like Rapid E. coli (BioRad), PTX, Chrom-agar, Rainbow agar, fluorocult and TBX (e.g. Oxoid) was examined for the isolation of STEC. These media contain a chromogenic compound: X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid – Figure 7). This is a substrate for beta-glucuronidase (GUS): upon reduction, X-gluc produces a localized colour allowing easy identification of suspected colonies. Unfortunately, not all STEC are beta-glucuronidase positive: about 60% of O26 strains present in our collection and almost all O103 strains showed to be negative using X-Gluc. Several supplements for beta-glucuronidase detection were evaluated and results varied between products. However, some exceptions not included, most strains were positive using specific PCR methods designed for beta-glucuronidase detection (20). Most STEC strains have the *uidA* gene present but some (typically O157 strains) do not exhibit beta-glucuronidase activity because they carry a

mutant gene. Therefore, no X-gluc was included in the custom designed selective isolation media. The commercially available media described above were all designed specifically for O157 detection. Non-O157 strains lack discriminating traits compared to non-pathogenic serotypes; sorbitol is a good discriminatory agent as far as only sorbitol negative strains are to be distinguished, but this is insufficient for detection of other serotypes amongst a mixture of commensal *E. coli* and other background bacteria. The research teams combined data from different subtask above to design a novel isolation protocol able to distinguish five main serotypes as cited above. Functioning and composition of these media are described below.

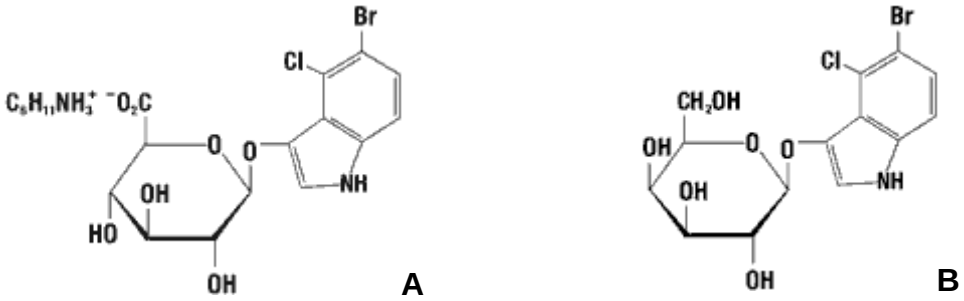


Figure 7. X-gluc (A) and X-gal (B)

Several elective and selective components have to be used for isolation of STEC from a mixture of other bacteria and in order to decrease the incidence of false positive results, a range of secondary purification media were developed. Figure 8 shows a schematic overview of these media and the selective/elective components involved. Briefly, a sample is plated onto a first isolation medium containing X-Gal, IPTG, a mixture of carbohydrates and selective components (also Task 2 for details). On this first medium, suspected colonies can be identified based on X-gal hydrolyzation and carbohydrate usage. Suspected colonies are purified using one or two additional agar media containing a specific carbohydrate. Colonies still suspected after this second plating procedure are to be confirmed using molecular techniques as described below (subtask 1.5).

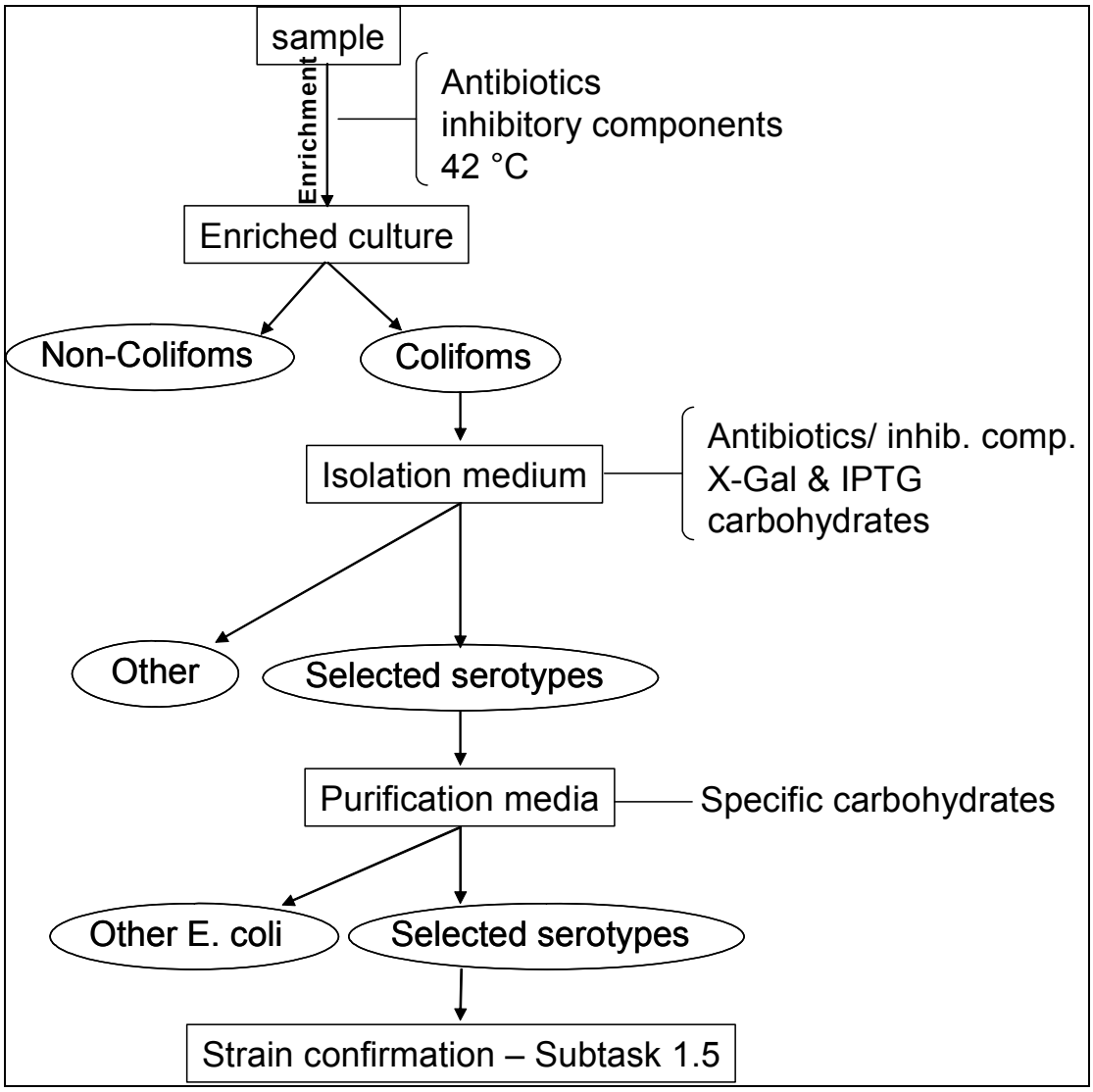


Figure 8. Schematic overview of selective/elective isolation media

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) is a non-inducing chromogenic substrate for beta-galactosidase, which hydrolyzes X-Gal forming an intense blue precipitate (Figure 7). X-Gal is most frequently used in conjunction with IPTG (Isopropyl β-D-1-thiogalactopyranoside) in blue/white colony screening: This compound is used as a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon. As all coliforms are beta-galactosidase positive, X-Gal and IPTG are added to the isolation medium as a first elective component. Additional inhibitory components and antibiotics were added based on results obtained during task 1.1: detailed composition and functioning of this isolation medium is discussed below (Task 2).

Carbohydrate fermentation data obtained during task 1.1 were analysed using Bionumerics software as described above: some carbohydrates have been combined into one single isolation medium to allow a more extended discrimination based on colony colour than when using only sorbitol. The first steps in designing these media made use of a minimal medium

containing Phenol red. Differentiation of different serotypes based on contrasting colours worked fine, but some problems occurred when large numbers of colonies were present on the plates. With fewer colonies present on the plates (max 100 colonies per plate), colonies with different carbohydrate fermentation abilities can clearly be distinguished. If more colonies are present on the plates a problem can occur: the agar medium contains a pH indicator which causes the agar to change colour in case of carbohydrate fermentation takes place. Due to this change of colour, neighbouring colonies can be falsely accounted for. With low numbers of colonies present on the plates, this is not a major problem, but if colony numbers rise (e.g. when testing meat or feces) the number of false positive and negative colonies rises. As a consequence, some modifications had to be made. The aim of these modifications was to incorporate the change of colour inside the colony itself, instead of the nutrient agar changing colour. This problem was bypassed using minimal MacConkey agar as a base for our custom designed isolation medium: this medium, containing neutral red and crystal violet, causes the colony itself to change colour upon carbohydrate fermentation. More details on this topic will be presented below (Task 2). For detection of O157 strains a modification of the commercially available SMAC medium was designed and combined with an optimized enrichment procedure and additional purification medium for suspected sorbitol positive colonies. Figure 9 shows some pictures of both types of selective isolation media:

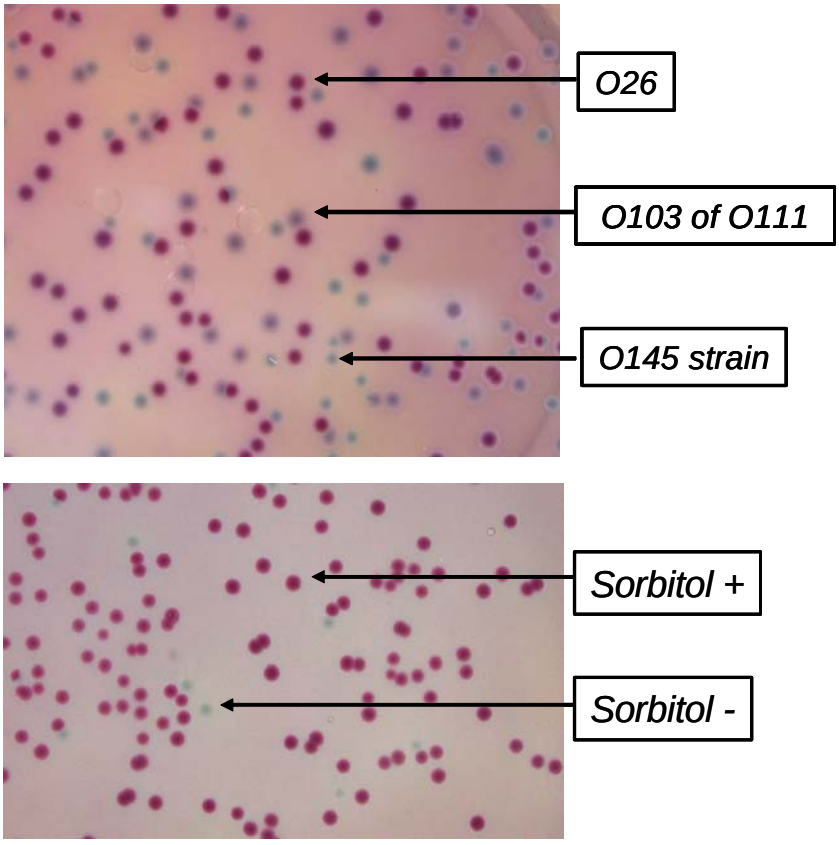


Figure 9. Colour differentiation on isolation medium for non-O157 serotypes (top) and O157 isolation medium (bottom)

Suspected colonies, based on X-gal hydrolyzation and carbohydrate fermentation, can be picked off from the first isolation medium and streaked out onto one or two purification media. These media are essentially minimal media containing Phenol red and a single carbohydrate. Figure 10 shows basic inoculation instructions: colour shifts documented from these purification media allows identification of the serotype aimed at with low chance of false positive results.

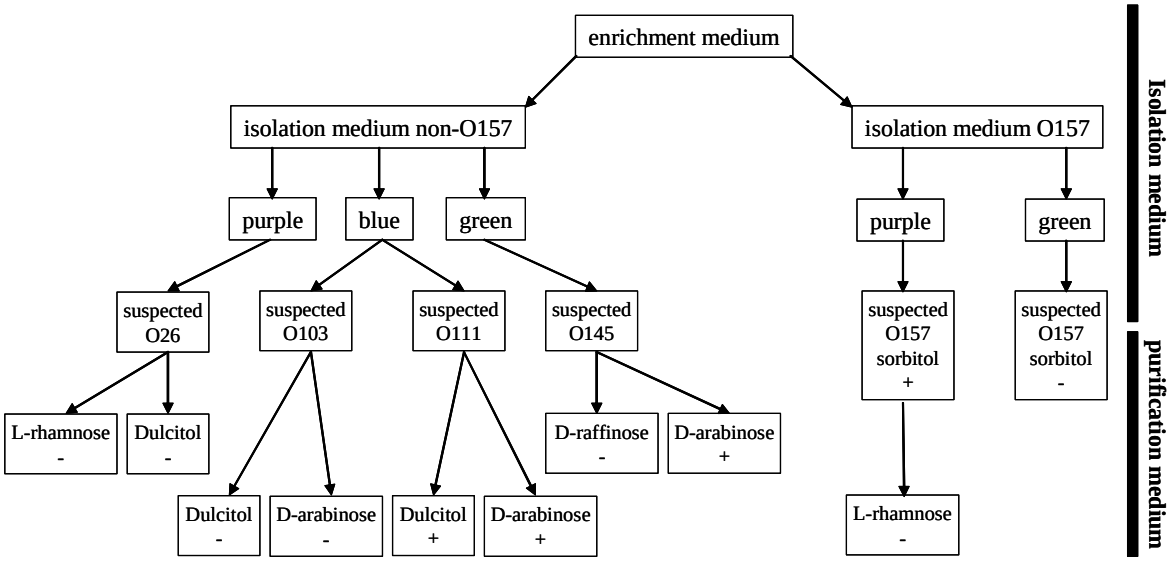


Figure 10. Schematic overview of isolation medium and purification media inoculations dependent on serotype aimed at

False positive ratios (FP %) were determined by calculating chance rates for isolating commensal strains expressing the exact same carbohydrate fermentation pattern for certain carbohydrates as the pathogenic strains – all data used for these calculations was generated by commensal and pathogenic strains present in our collection. Table 7 shows fermentation properties for the collection of strains used in this study. For example, isolation of O103 strains has a FP % of 45% on the first selective isolation medium: this ratio is calculated as 85% (85% of commensal strains able to ferment sucrose) * 53 % (53% of commensal strains not able to ferment sorbose) = 45%. False positive ratio for the purification medium is calculated using the FP ratio on the isolation medium. For O103 strains, FP % on the confirmation medium is calculated as: 45 %*(1-90%)*(1-93%) = 0.3%.

serotype	isolation medium				Confirmation medium				
	sucrose	sorbose	sorbitol	FP % [a]	arabinose	rhamnose	dulcitol	raffinose	FP % B2 [c]
O26	+	+	+	39	66% -	-	-	+	0.05
O103	+	-	+	45	-	+	-	+	0.3
O111	+	-	+	45	+	+	+	+	37.6
O145	-	-	+	8	+	+	86% -	-	1.008
O157 sorb –	+	55% +	-	2	-	+	+	+	2.0
O157 sorb +	+	+	+	98	-	-	+	+	1.96
O91 [d]	+	-	+		+	+	*50%* +	+	
O121 [d]	*66%* +	+	+		+	+	+	-	
O128 [d]	+	-	+		+	+	+	+	
O172 [d]	-	-	+		+	+	*50%* +	-	
PC [b]	85% +	47% +	98% +		90% +	98% +	93% +	86% +	

Table 7. Calculation of false positive ratios using both isolation and purification media. a – FP % = calculated false positive ratios on the isolation media; b – PC = collection of non-pathogenic E. coli strains; c – FP % B2 = calculated false positive ratio on the purification media; d – data on other serotypes not included in this specific isolation method.

For strains belonging to serotype O111 no additional discriminating carbohydrate could be identified decreasing the occurrence of false positive results to lower levels. The isolation method for O157 strains makes use of sorbitol fermentation to indicate presumably positive colonies on the specific isolation medium. As indicated in Table 7, low incidence of false positive results is obtained for sorbitol negative O157 (2 %). On the contrary, for sorbitol positive O157 isolation very high number of false positive colonies will be obtained using only sorbitol as a single discriminatory agent as 98% of commensal strains are able to ferment this carbohydrate. To eliminate high numbers of false positive results prior to final confirmation (using serotyping PCR or other typing methods) sorbitol positive colonies from the O157-specific isolation medium are streaked onto a purification medium containing D-rhamnose (6 g/L). After 24 hours of incubation at 37 °C this medium allows an increased detection of O157 strains among commensal coliforms.

– Conclusion:

Commercially available isolation media mainly focus on O157 detection: most media contain sorbitol as a single metabolic indicator and X-gluc to discriminate beta-glucuronidase negative O157 from background bacteria presumed beta-glucuronidase positive. New media were designed based on X-gal hydrolyzation and carbohydrate usage: combining two carbohydrates and X-gal in one medium allows a more detailed discrimination of different serotypes.

Subtask 1.5: Multiplex PCR for confirmation of isolates and screening samples

– Partners: Ghent University and ILVO

– Objective:

A full scale multiplex PCR was designed to allow a one-step genetic characterization of strains. Besides this multiplex, previously published primers can be applied for more easy to perform strain characterisation using standard PCR routines.

– Results:

In literature a variety of PCR tests have been published for the characterization of the different virulence genes: these methods are convenient, but lack the ability to perform a thorough screening of samples in just a few hours. One of the aims of this project is to design a broad-range multiplex PCR. In the early stages of this project, the research team was contacted by VIB (Flanders Interuniversity Institute for Biotechnology): using a novel mathematical algorithm this large multiplex PCR can be developed. Once finalized the PCR will be able to detect 25 genes simultaneously: the most important virulence genes, as well as genetic variants important in respect to human infections, and genes suited for serotyping 7 of the most important serotypes associated with human pathology.

Strains isolated from samples can be confirmed using this full scale multiplex PCR or using primers available in literature, as primer sequences and mix composition of the multiplex PCR designed by VIB remain confidential.

Genes screened for by the multiplex PCR are listed below:

- *VT1*
- *VT2*: c, d, e, f
- *eae*: a, b, g, e, q, z
- *saa*: STEC auto-agglutinating adhesin
- *ehly:* enterohemolysin
- *tir*: translocated intimin receptor
- *katP*: katalase peroxidase
- espP: extracellular serine protease

- specific genes for identification of 7 serotypes: O26, O103, O111, O157, O172, O91 and O121

– Conclusion:

Both advances technologies and standard PCR routines are available to characterize and/or confirm isolates. Straightforward detection of main virulence genes can be performed by most laboratories using standard PCR routines. Pure cultures can be fully genetically characterized using a one step multiplex PCR comprising 25 amplicons.

2.2 Task 2: Evaluation of defined protocols on artificially contaminated food and cattle feces

2.2.1 Protocol layout

After analysis of the different set of data (MIC, carbohydrates, growth curves …) a maximum of four different protocols will be defined. Each of these protocols will be composed of combination of steps: sample preparation, selective enrichment, post-enrichment treatment and isolation of presumptive colonies on serotype specific agar media.

In a first stage these protocols will be evaluated for the efficiency of detecting EHEC strains using artificially contaminated samples. Sampling material will consist of minced beef, Belgian dry sausage, milk, raw milk cheese and cattle faeces. After testing the samples for the natural presence of EHEC strains, sub samples will be artificially contaminated using different STEC strains from our collection. As a final result, estimation will be calculated in order to determine the best suited protocols for the different types of samples. Depending on the results, further identification of contaminating flora will take place.

A second stage in the process of evaluating the protocols, a maximum of two protocols will be selected instead of four. These two remaining protocols will be evaluated using artificially contaminated and naturally contaminated samples. Stressed cultures will be used for artificial contamination of samples to mimic *in vivo* condition of STEC in food products. Briefly, a stationary phase culture was diluted to 10^4; this diluted culture was added to TSB containing 6.5% NaCl and stored overnight at 10 or 20 °C prior to being used for sample contamination.

The figure below (Figure 11) shows a schematic view on the structure of the protocols to be evaluated. Briefly, two types of pre-enrichment procedures are combined with two types of enrichment procedures resulting in 4 different methods (abbreviations used refer to Figure 11).

- VA1 (non-selective): no additions
- VA2 (selective):
    - Novobiocine
    - Vancomycine
- SA1 (mild selective)
    - novobiocine
    - Bile salts
- SA2 (selective)
    - novobiocine
    - rifampicine
    - Bile salts
    - tellurite

Using these combinations of pre-enrichment and enrichment methods, 4 different methods were designed with increasing selectivity for STEC: VA1SA1 < VA1SA2 < VA2SA1 < VA2SA2.

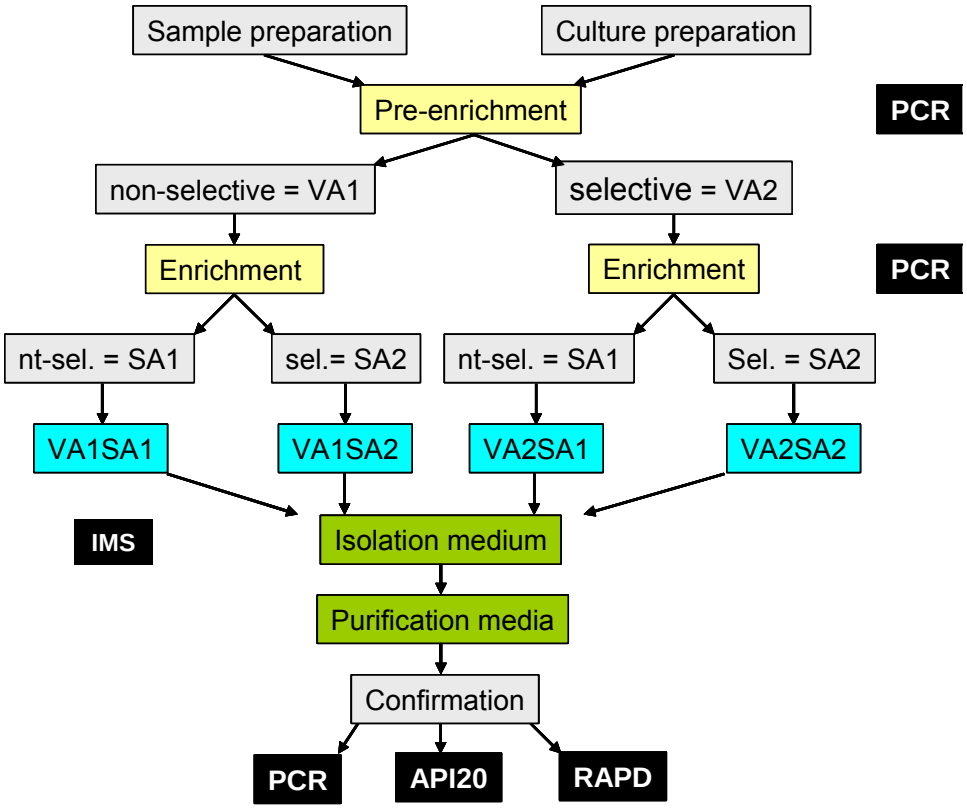


Figure 11. Schematic overview of the structure of isolation methods to be evaluated

Detailed data on the differences between these protocols when applied for STEC isolation from various sample matrices are described below. Because of the strong survival mechanism observed in STEC strains most bench work was focused on protocol VA2SA1 and VA2SA1: a non-selective pre-enrichment (VA1) did not show to be necessary to resuscitate sublethally injured cells.

2.2.2 Database design

Figure 11 showed all elements involved in STEC isolation. As every element in this figure generates data and all elements are joined to each other, every sample treated with one or more protocols described above yields large quantities of data. It is obvious all data should be stored and processed accurately in order to formulate useful recommendations regarding the most appropriate strategy for isolating STEC from different matrices. All isolates confirmed as pathogenic STEC at the end of the protocol should be traced back to the food sample: this implies all relations and all variables should be stored in the same accurate way. Using spreadsheets software, all data are stored in a single spreadsheet: no linkage of data is possible and duplication of data is hard to avoid.

Large quantities of linked data have to be stored in multiple tables. Using indexed data and primary key definitions, MS Access databases allow a large number of possibilities for data use and storage. A custom database was designed to store data generated by isolating STEC from both artificially and naturally contaminated samples (food and feces). All data are stored in separate tables and primary keys were defined to force referential integrity: this prevents duplication of data, allows data input using drop down menus and table linkage. Figure 12 shows database layout and relationships defined: these relationships allow an easy to apply end-user interface with automated report generation. Some automated reports will be discussed in the paragraphs below.

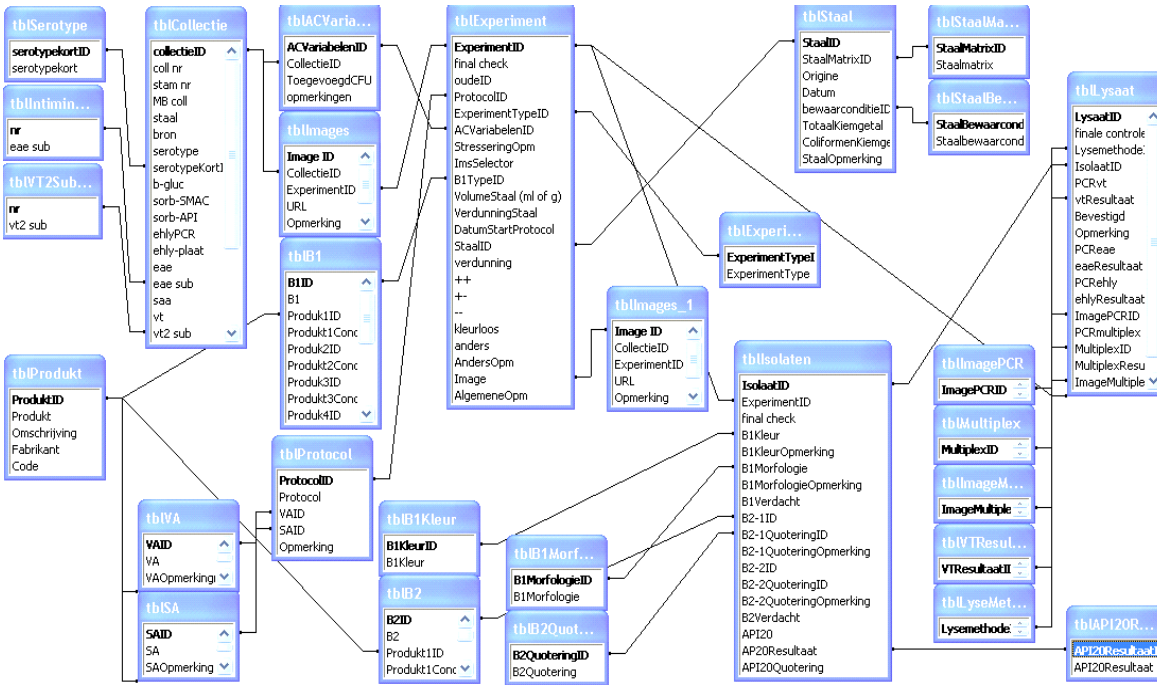
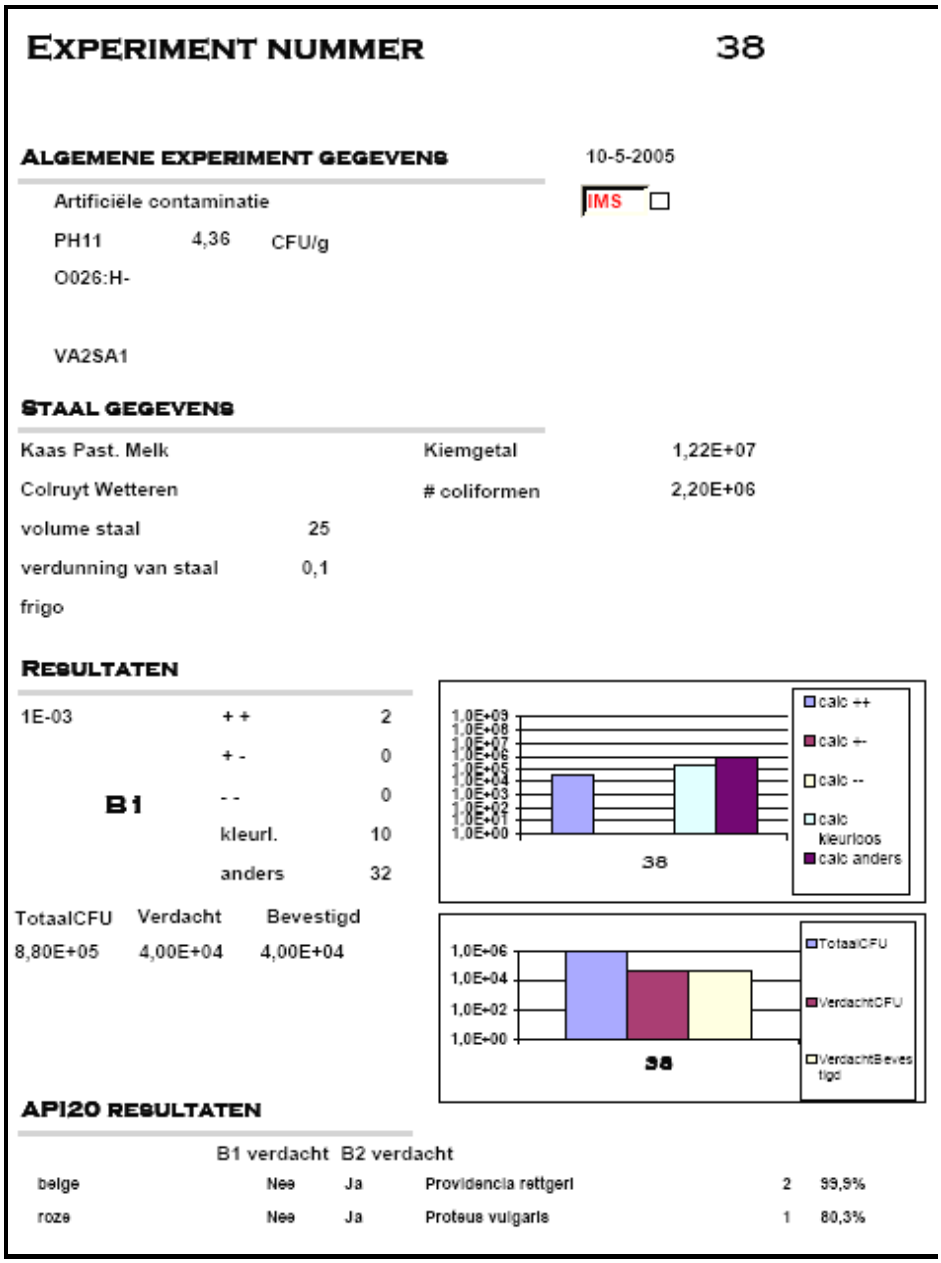


Figure 12. Database layout

As shown in the figure above, this database design is complex due to the many different types of data to be stored and to the different levels of data interrelation. An easy to use end-user interface was developed to facilitate data-input and to produce comprehensive reports. Using different forms all data obtained for each experiment entered in the corresponding tables: using automated indexing all data entered for a specific experiment is linked so every element can be related to other data from the same experiment. Using these indexes, data can be sorted as needed: different calculations or statistical analysis can be performed after data has been sorted by e.g. serotype, sample matrix…. For every experiment, an automated report produces a file card showing all relevant data (Figure 13): serial number,

sample information, type of experiment, CFU count, information on STEC found, protocol applied and information on background bacteria identified using API20 tests.



EXPERIMENT NUMMER 38

ALGEMENE EXPERIMENT GEGEVENS 10-5-2005

Artificiële contaminatie IMS ☐

PH11 4,36 CFU/g

O026:H-

VA2SA1

STAAL GEGEVENS

Kaas Past. Melk		Kiemgetal	1,22E+07
Colruyt Wetteren		# coliformen	2,20E+06
volume staal	25		
verdunning van staal	0,1		
frigo			

RESULTATEN

1E-03		+ +	2
		+ -	0
	B1	- -	0
		kleurl.	10
		anders	32

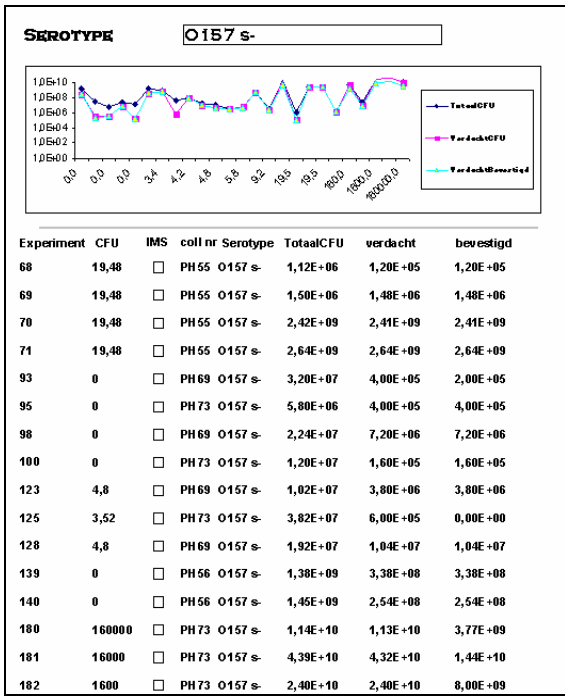
TotaalCFU	Verdacht	Bevestigd
8,80E+05	4,00E+04	4,00E+04

API20 RESULTATEN

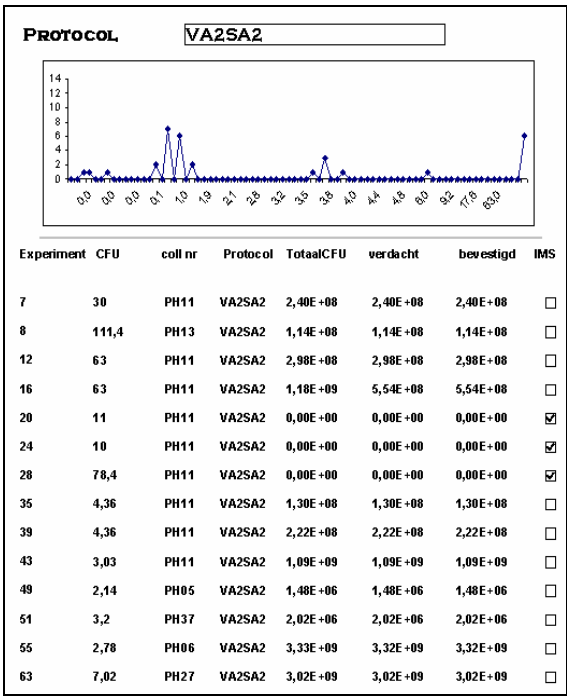
	B1 verdacht	B2 verdacht			
beige	Nee	Ja	Providencia rettgeri	2	99,9%
roze	Nee	Ja	Proteus vulgaris	1	80,3%

Figure 13. Experiment file card example

The end-user reports allow users to apply various grouping levels for data analysis. Some typical analyses performed comprise calculation of the efficiency of different protocols, calculation of detection limit for each serotype or protocol etc. Some screenshots of these reports are shown in Figure 14: more charts generated by these reports will be shown to support results presented in the paragraphs on the subtasks below.



Experiment	CFU	IMS	coll nr	Serotype	TotaalCFU	verdacht	bevestigd
68	19,48	☐	PH55	O157 s-	1,12E+06	1,20E+05	1,20E+05
69	19,48	☐	PH55	O157 s-	1,50E+06	1,48E+06	1,48E+06
70	19,48	☐	PH55	O157 s-	2,42E+09	2,41E+09	2,41E+09
71	19,48	☐	PH55	O157 s-	2,64E+09	2,64E+09	2,64E+09
93	0	☐	PH69	O157 s-	3,20E+07	4,00E+05	2,00E+05
95	0	☐	PH73	O157 s-	5,80E+06	4,00E+05	4,00E+05
98	0	☐	PH69	O157 s-	2,24E+07	7,20E+06	7,20E+06
100	0	☐	PH73	O157 s-	1,20E+07	1,60E+05	1,60E+05
123	4,8	☐	PH69	O157 s-	1,02E+07	3,80E+06	3,80E+06
125	3,52	☐	PH73	O157 s-	3,82E+07	6,00E+05	0,00E+00
128	4,8	☐	PH69	O157 s-	1,92E+07	1,04E+07	1,04E+07
139	0	☐	PH56	O157 s-	1,38E+09	3,38E+08	3,38E+08
140	0	☐	PH56	O157 s-	1,45E+09	2,54E+08	2,54E+08
180	160000	☐	PH73	O157 s-	1,14E+10	1,13E+10	3,77E+09
181	16000	☐	PH73	O157 s-	4,39E+10	4,32E+10	1,44E+10
182	1600	☐	PH73	O157 s-	2,40E+10	2,40E+10	8,00E+09



Experiment	CFU	coll nr	Protocol	TotaalCFU	verdacht	bevestigd	IMS
7	30	PH11	VA2SA2	2,40E+08	2,40E+08	2,40E+08	☐
8	111,4	PH13	VA2SA2	1,14E+08	1,14E+08	1,14E+08	☐
12	63	PH11	VA2SA2	2,98E+08	2,98E+08	2,98E+08	☐
16	63	PH11	VA2SA2	1,18E+09	5,54E+08	5,54E+08	☐
20	11	PH11	VA2SA2	0,00E+00	0,00E+00	0,00E+00	☑
24	10	PH11	VA2SA2	0,00E+00	0,00E+00	0,00E+00	☑
28	78,4	PH11	VA2SA2	0,00E+00	0,00E+00	0,00E+00	☑
35	4,36	PH11	VA2SA2	1,30E+08	1,30E+08	1,30E+08	☐
39	4,36	PH11	VA2SA2	2,22E+08	2,22E+08	2,22E+08	☐
43	3,03	PH11	VA2SA2	1,09E+09	1,09E+09	1,09E+09	☐
49	2,14	PH05	VA2SA2	1,48E+06	1,48E+06	1,48E+06	☐
51	3,2	PH37	VA2SA2	2,02E+06	2,02E+06	2,02E+06	☐
55	2,78	PH06	VA2SA2	3,33E+09	3,32E+09	3,32E+09	☐
63	7,02	PH27	VA2SA2	3,02E+09	3,02E+09	3,02E+09	☐

Figure 14. Some automatically generated reports showing detection limit for O157 sorbitol negative strains and incidence of false positive results when using protocol VA2SA2

Results of STEC isolation from different matrices will be presented in the paragraphs below. All charts printed in this document were generated using database reports and show inoculation level and resulting colony counts after specific enrichment procedures and plating onto the first isolation medium. Results printed in different subtasks, will emphasize detection limits for different sample matrices while more general conclusions on defining most optimal protocol and serotype dependent variations will be discussed at the end of this chapter.

Subtask 2.1: Minced beef and Belgian dry sausage

– Partners: Ghent University

– Objective:

Protocols described above will be evaluated using artificially contaminated minced beef and Belgian dry sausage. Samples will be contaminated with various concentrations of pathogenic strains, both stressed and non-stressed cultures, belonging to serotypes O26, O103, O111, O145 and O157 to define best available STEC isolation method.

– Results:

The main objective of this subtask was to define best suited protocols for isolation of various STEC serotypes from minced meat and Belgian dry sausage. Samples of both matrices were bought on several occasions and in different shops, both small retail shops and supermarkets.

For fresh samples of minced meat, exact counts are available: total CFU, suspected CFU and confirmed CFU. Suspected colonies on the first isolation medium (described above), are defined based on the serotype-specific carbohydrate fermentation properties as described in subtasks 1.1 and 1.4. Confirmed CFU counts were obtained after PCR confirmation of suspected colonies as described in subtask 1.5.

Figure 13 shows some data on experiments using artificially contaminated minced meats samples. Contamination levels ranged from 2 CFU to 17.6 CFU per gram of sample. As indicated on the chart, high numbers of suspected colonies (~10^8) could be confirmed by the purification media as described above and by PCR, even at very low contamination levels. Very low numbers of background bacteria were found on the first isolation medium.

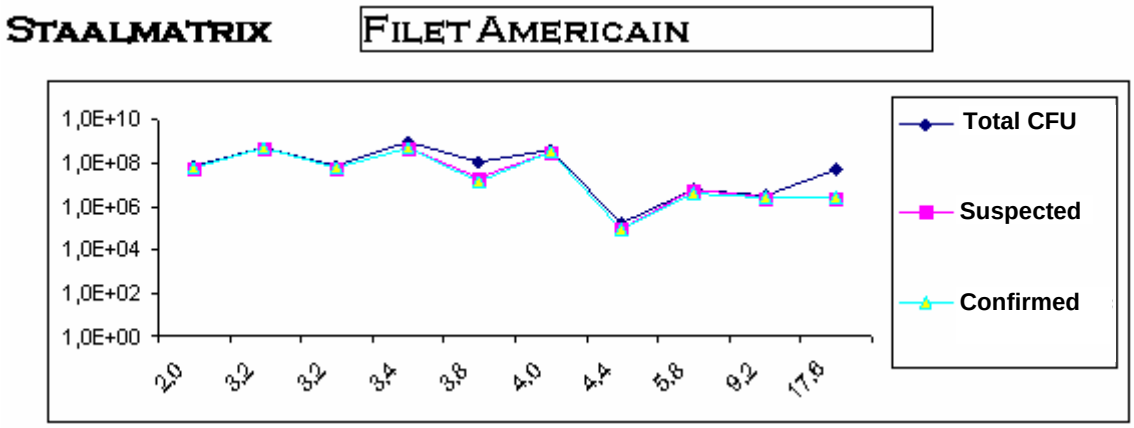


Figure 15. Detection limit for STEC in minced meat

All minced meat samples were treated with protocol VA2SA2 based on results obtained from preliminary tests on raw milk (subtask 2.2): high levels of pathogenic STEC strains were isolated from enriched samples even after inoculation at very low levels. Minced meat samples were contaminated with 17 to 2 CFU per gram of sample; three sample conditions were contaminated: fresh meat, minced meat refrigerated at 4 °C for 7 days and minced meat frozen at - 20 °C. Comparing results obtained after enrichment of these three sample

conditions did not show significant differences: inoculated STEC strains were recovered during each experiment with similar CFU count. Figure 15 shows minor variations in total CFU and number of colonies confirmed as STEC strain used for artificial contamination: no background bacteria were found after incubation of the first isolation medium due to elective/selective properties of enrichment procedure and isolation medium composition.

Belgian dry sausage samples (pure pork meat) were contaminated using healthy stationary phase cultures with 11 to <1 CFU per gram of sample. Figure 16 shows CFU counts of experiments using this sample matrix: no differences were observed between total CFU counts and the number of suspected/confirmed colonies. As was the case for minced meat: growth of all background bacteria was inhibited due to selective/elective isolation properties of the protocols used. For all experiments using Belgian dry sausage, protocol VA2SA2 was applied.

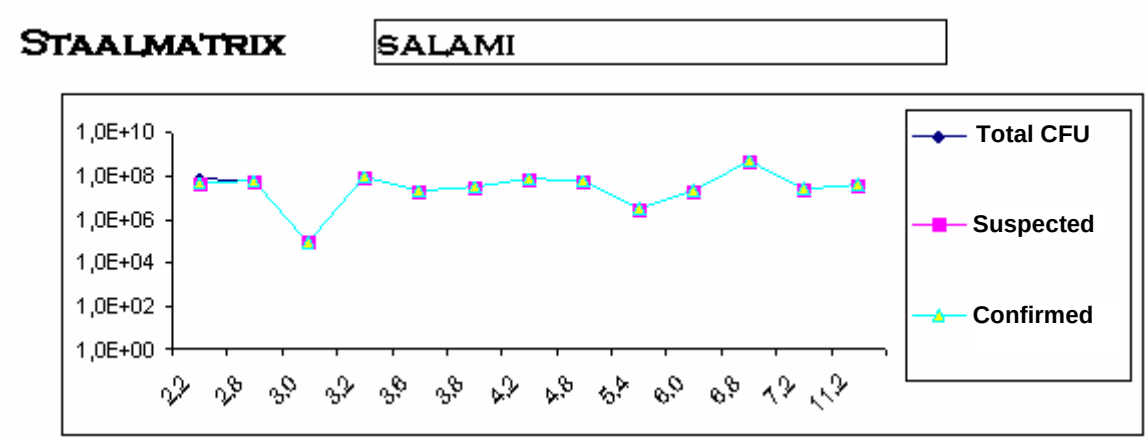


Figure 16. Artificial contamination of Belgian dry sausage

– Conclusion:

Minced meat and Belgian dry sausage samples, using pathogenic STEC strains for artificial contamination, were processed using protocol VA2SA2. High levels of suspected colonies observed and all were confirmed using PCR; very little to no background bacteria were found after incubation of the enrichment broth on the first isolation medium. Detection limit for both sample matrices is estimated at < 1 CFU per gram.

Subtask 2.2: Raw milk and raw milk cheese

– Partners: ILVO

– Objective:

Protocols described above will be evaluated using artificially contaminated raw milk and raw milk cheese. Samples will be contaminated with various concentrations of pathogenic strains, both stressed and non-stressed cultures, belonging to serotypes O26, O103, O111, O145 and O157 to define best available STEC isolation method.

– Results:

For preliminary studies on the efficiency of STEC isolation methods used during task 2, scald milk cheeses were used as these cheeses contain far less background bacteria. The research team made use of the additional sample matrix to map possible flaws in the protocols described above and to define most favourable protocols as full application of all four protocols in all experiments requires large quantities of bench work and resources.

Figure 17 shows CFU counts after application of STEC isolation protocols on scald milk cheese samples: most experiments yielded nothing but the pathogenic strain used for sample contamination. All scald milk samples used for these experiments had comparable total cell counts (~10^7 CFU) and most samples did not present any coliforms. Experiments yielding substantial numbers of background bacteria on the isolation medium also showed high numbers of coliforms presents in the sample prior to inoculation (~10^5 to 10^6).

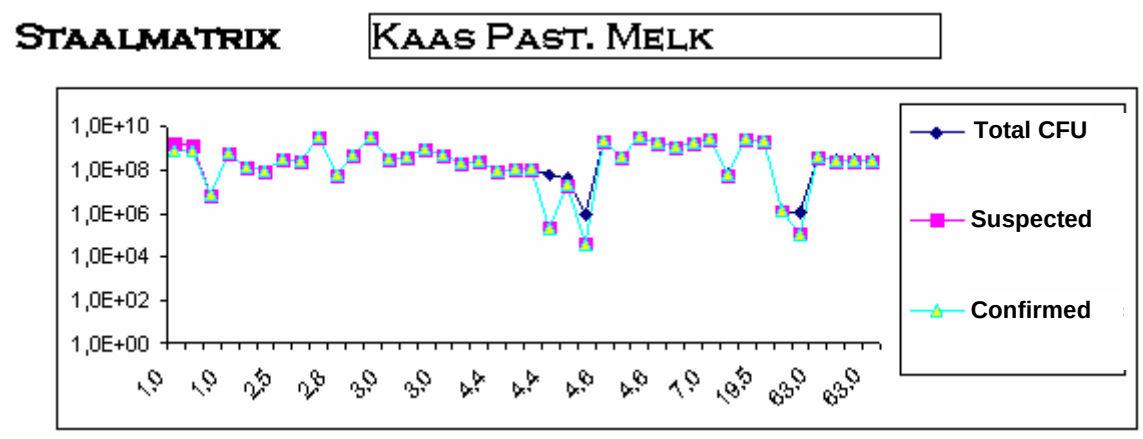


Figure 17. Artificial contamination of cheese made from scald milk

Some experiments presented in the figure above, show a significant portion of strains present on the first isolation medium to lack the morphology of the suspected strain. These non-suspected isolates were characterized using API20 test: all isolates were confirmed as *Providencia rettgeri* or *Proteus vulgaris*. Nevertheless, from all experiments conducted using artificially contaminated scald milk cheese, the pathogenic STEC strain used for inoculation could be isolated afterwards. Figure 18 shows more detailed data on these experiments: charts indicate results after artificially contamination of scald milk cheeses using strains of serotypes O26, O145 and sorbitol positive O157. Isolation protocols perform well on isolating all serotypes from scald milk, showing very high revenues for O145 and O157 s+ contaminated samples. Some O26 contaminated samples did show some remaining background bacteria on the isolation medium, but colonies lacked typical morphology of O26 strains both on the isolation medium and the purification media. These morphological and metabolical assumptions were confirmed using PCR. During some experiments samples were artificially contaminated using stresses STEC cultures as described above (3.2.1); no difference in detection limit could be observed compared to inoculation of samples using unstressed cultures.

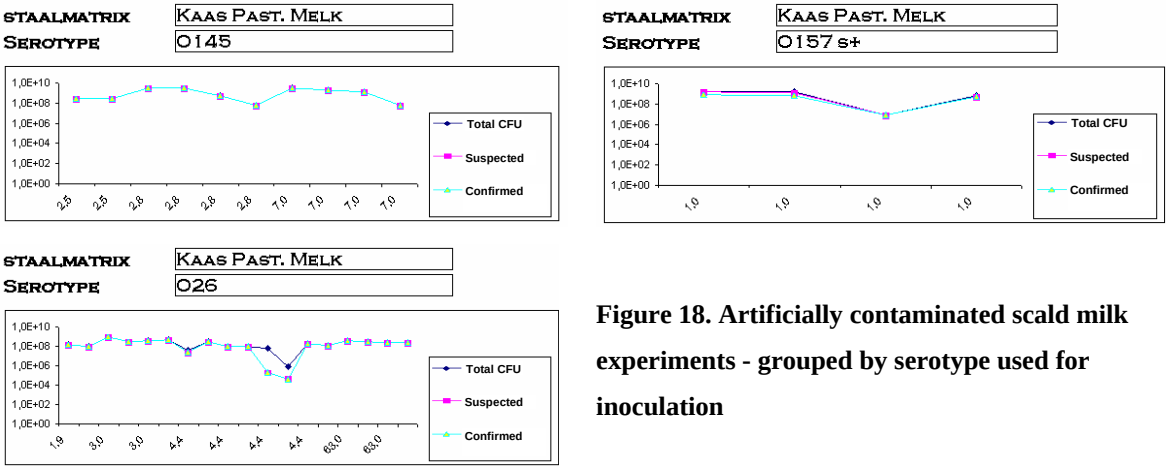


Figure 18. Artificially contaminated scald milk experiments - grouped by serotype used for inoculation

Protocols defined at the end of task 1 were also evaluated on artificially contaminated raw milk as indicated below (Figure 19). Total cell counts prior to contamination ranged from 10^3 to 10^6 and coliform counts ranged from 0 to 10^5. No experiments showed any growth of background bacteria on the isolation media: all colonies had STEC-like metabolic and morphologic traits and were confirmed using purification media and PCR.

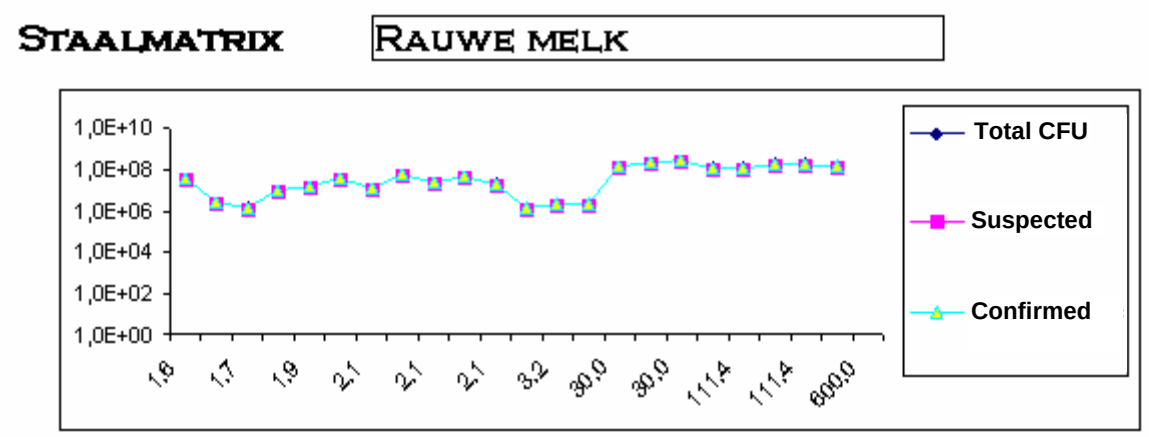


Figure 19. Results of STEC isolation after artificial contamination of raw milk samples.

Figure 20 shows charts of experiments using O103 and O26 strains (other serotypes resulted in similar charts): Strains used for inoculation of the samples reach counts up to 10^8 for all serotypes, even with very low inoculation levels used.

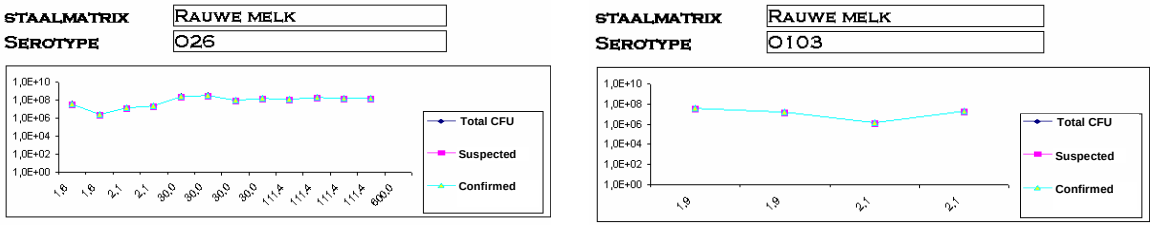


Figure 20. STEC isolation from artificially contaminated raw milk - results grouped by serotype used for inoculation

Raw milk cheese is a more complex matrix, as many samples contain coliform bacteria and various parameters are involved in the induction of bacterial stress. As shown in Figure 21, on most isolation media background bacteria can be identified. This is not uncommon as total cell counts and coliform counts were both estimated at 10^6 to 10^7. STEC was detected even at inoculation levels below 1 CFU per gram. During some experiments, the research team did not succeed in detecting STEC: this was probably caused by the statistical uncertainty of successful sample inoculation when using very diluted bacterial cultures. Experiments using <1 CFU for sample inoculation were performed repeatedly to bypass this uncertainty.

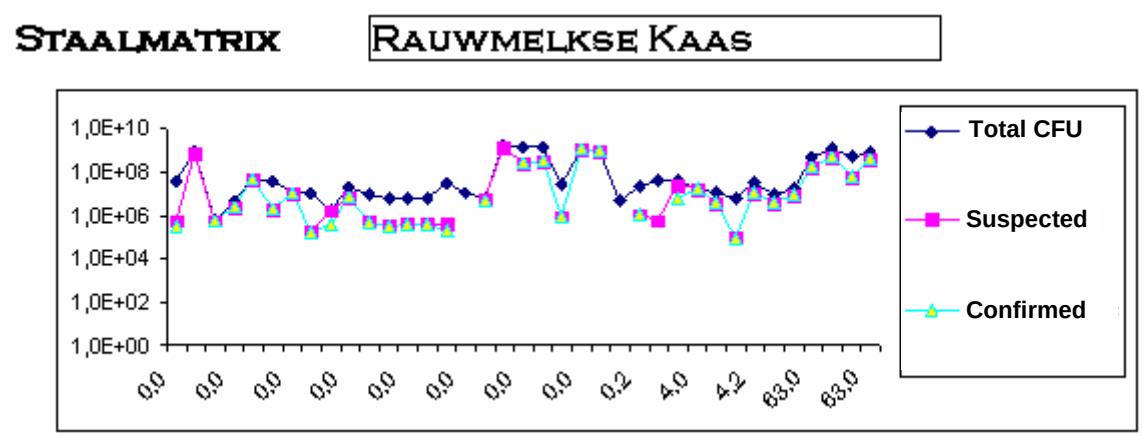


Figure 21. Isolation of STEC from artificially contaminated samples of raw milk cheeses.

The charts below (Figure 22) show results of these experiments grouped by serotype of strains used for artificial contamination of samples. For most serotypes, little to no background bacteria were found on the isolation media. Experiments using O157 strains for artificial contamination are plated onto a specific isolation medium after incubation stages (as described during subtask 1.4). This medium contains fewer inhibitory components (e.g. tellurite) more background bacteria were found during these experiments. However, high levels of suspected colonies could be identified on these media and were subsequently confirmed using PCR. During some experiments samples were artificially contaminated using stresses STEC cultures as described above (3.2.1); no difference in detection limit could be observed compared to inoculation of samples using unstressed cultures.

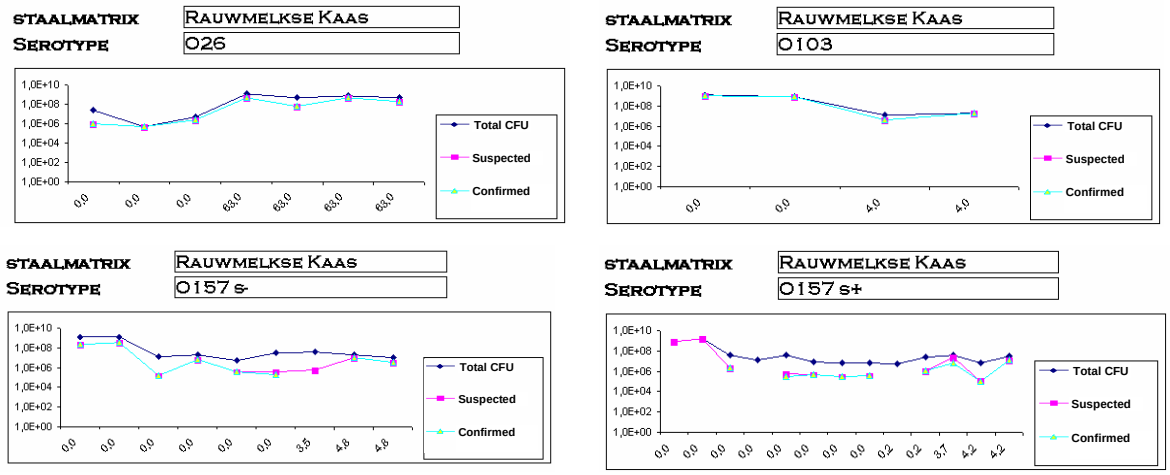


Figure 22. STEC isolation from artificially contaminated raw milk cheeses - results grouped by serotype used for inoculation.

– Conclusion:

Raw milk, scald milk and raw milk cheeses were inoculated using pathogenic STEC strains for artificial contamination. Contaminated samples were processed using all 4 protocols. High levels of suspected colonies were observed and all were confirmed using PCR; very little to no background bacteria were found after incubation of the enrichment broth on the first isolation medium. Detection limit for all three sample matrices is estimated at < 1 CFU per gram for all dairy products.

Subtask 2.3: Cattle feces

– Partners: Ghent University and ILVO

– Objective:

Protocols described above will be evaluated using artificially contaminated minced cattle feces. Samples will be contaminated with various concentrations of pathogenic strains belonging to serotypes O26, O103, O111, O145 and O157 to define best available STEC isolation method.

– Results:

As isolation methods described above have proven to be effective for in vitro isolation of selected STEC serotypes of artificially contaminated samples, these methods were also applied on cattle feces. This is a much more challenging sample matrix as it contains high levels of coliform bacteria. Several samples were used for artificial contamination experiments and total bacteria counts ran up to 10^9. Similar counts were documented for coliform bacteria. Coliform bacteria can survive in faecal material without suffering from stress conditions as was the case in cheese or fermented meat samples. Both the high number of coliform bacteria in faecal samples and the healthy state of bacteria in this matrix allowed the research team to increase selectivity when dealing with faecal samples: as no resuscitation period is required, enrichment is limited to SA2 without any pre-enrichment stage. All results presented below were obtained after application of this reduced protocol.

Figure 23 shows all experiments using cattle feces as sample matrix. Contamination of samples with very low numbers of STEC (<10 CFU per gram) does not allow identification of

suspected/confirmed colonies on the isolation media. Two possible explanations for this reduced detection efficiency are listed below:

- As samples contain more coliform bacteria, the incidence of similar morphologic/metabolic traits possibly increases. This can account for the high number of suspected bacteria found in some experiments shown below, failing subsequent confirmation: only a limited number of suspected colonies are transferred from the isolation medium to the purification media. With many similar colonies present there is a significant chance of missing STEC colonies when transferring to the purification media, resulting in no confirmed colonies.
- Inoculation of very low numbers holds many risks, some of which have been already cited above. More specifically, in cattle feces competitive exclusion or other niche-related effects can influence bacterial fitness during selective enrichment. These effects can not be defined as many sample-related factors influence bacterial competition.

Briefly, the reduced detection efficiency observed for cattle feces can possibly be explained using either statistical uncertainty or ecological influences. Results presented here were achieved without application of IMS: more information on this topic will be presented below.

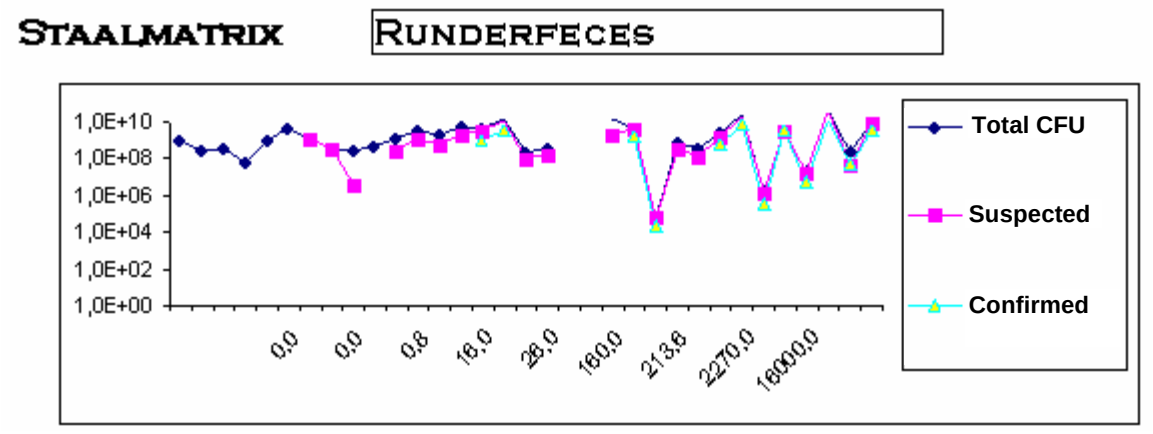


Figure 23. Isolation of STEC from artificially contaminated samples of cattle feces.

Figure 24 shows some results on artificially contaminated cattle feces grouped by serotype used for inoculation. Most serotypes do not present problems other than those cited above; however, O111 and O157 sorbitol positive strains have a significantly higher chance for false positive identifications as indicated in subtask 1.4. STEC was isolated from all fecal samples inoculated with >10 CFU per gram, except for these two serotypes: some non-pathogenic *E.*

coli present in fecal material have the same carbohydrate fermentation abilities concerning the isolation media used. As only a limited number of "suspected" colonies are transferred to the purification media, this routine is susceptible to experimental errors due to the statistical chance of missing the STEC strain used for sample inoculation. This is shown in the chart below covering experiments using O111 strains for sample contamination: regardless of the CFU used for inoculation, high numbers of "suspected" colonies were found on the isolation medium. However, confirmation of these "suspected" strains has proven to be difficult due to non-pathogenic strains with the same metabolic/morphologic traits on the isolation media. This problem should be resolved by transferring more suspected colonies to the purification media as non-pathogenic strains are likely to react different to carbohydrates used in these media.

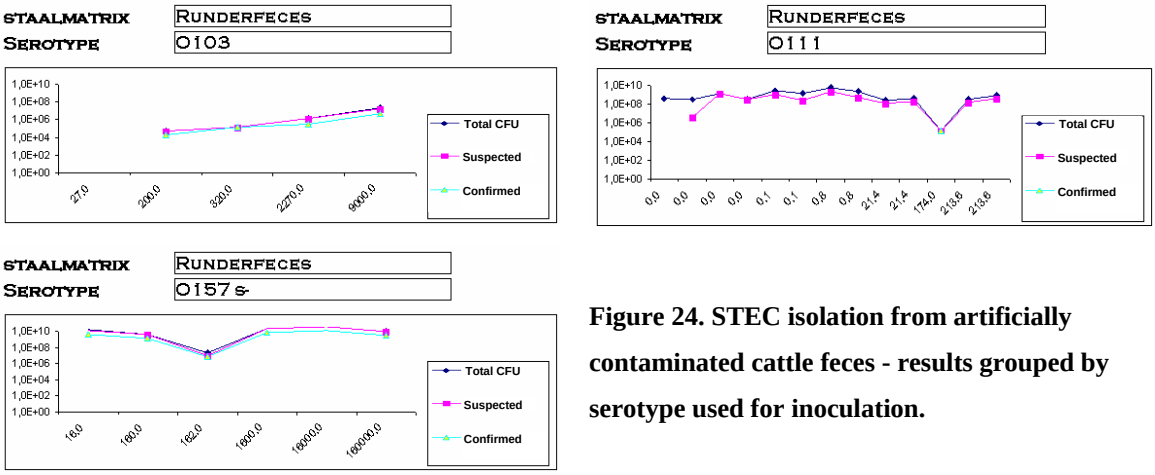


Figure 24. STEC isolation from artificially contaminated cattle feces - results grouped by serotype used for inoculation.

– Conclusion:

Cattle feces were inoculated using pathogenic STEC strains for artificial contamination. Contaminated samples were processed using a restricted SA2 protocol. Due to high levels of non-pathogenic *E. coli* present in fecal samples, confirmation of suspected colonies is complicated as some non-pathogenic *E. coli* strains have the same morphological/metabolical traits as the STEC strains used for inoculation. This has forced researchers to define STEC detection limit in cattle feces at >10 CFU per gram when no IMS is applied.

Subtask 2.4: PCR-screening

PCR typing was applied for strain confirmation as described above (subtask 1.5). For all strains used for sample inoculation, full virulence profiles and genetic fingerprints (RAPD) are available (subtask 1.1). Every sample used for experiments listed above was also evaluated without STEC culture added to check for natural STEC contamination. No sample was found to be contaminated. This allowed researchers to limit PCR identification to *stx1* and/or *stx2* detection. If required, this confirmation was completed using PCR based detection of eae and construction of RAPD fingerprints of suspected isolates. RAPD analysis of isolates was also used for identification of possible cross contamination between different experiments.

Task 2 conclusions

As indicated above, the research team focussed bench work on two protocols (VA1SA2 and VA2SA2) because preliminary data indicated the level of selectivity achieved during the second enrichment stage did not hamper growth of pathogenic STEC strains. Application of the less selective enrichment stage (SA1) was therefore scaled down during experiments using artificially contaminated samples. Charts printed below (Figure 25) show some general data on experiments using VA1SA2 and VA2SA2 protocols for STEC isolation. It is clear similar results are achieved by using both methods independently: even with very low CFU used for sample inoculation, high levels of STEC were isolated using both methods.

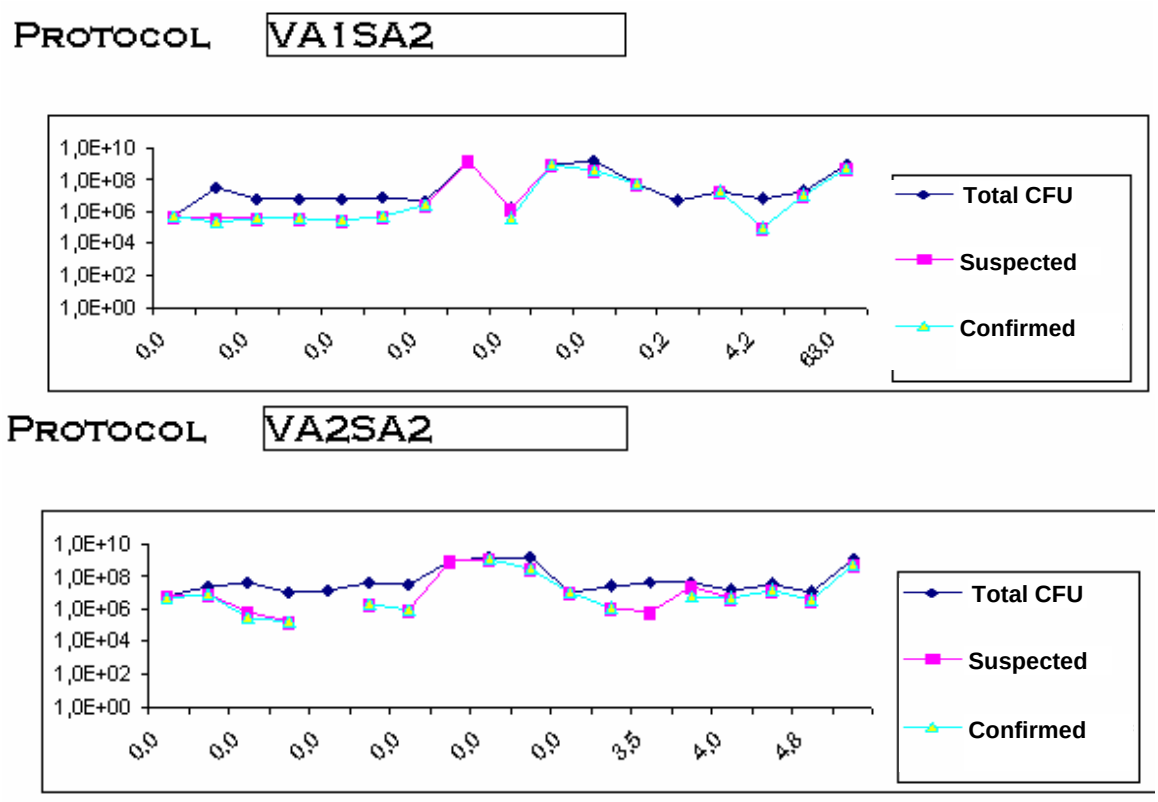


Figure 25. Comparison of VA1SA2 and VA2SA2

At the end of both enrichment phases, both methods produce similar results. The main difference is to be found after the pre-enrichment stage, originally designed for optimal recovery of sub lethally injured cells. Figure 26 shows growth levels of pathogenic STEC strains after these pre-enrichment stages (6 hours of incubation at 37°C). Application of "VA2", which was more selective compared to "VA1" (also see subtask 1.2), produced a log1 reduction of growth after 6 hours of enrichment. Data shown on this chart again confirm previous indications on similar growth potential after full enrichment procedure. Based on both Figure 25 and Figure 26, we conclude both protocols to be appropriate for sensitive STEC isolation from various food products. However application of "VA1" has greater recovering potential with regard to stressed cells, experimental data using in vitro stress induced cells, did not show significant differences at the end of both pre-enrichment and selective enrichment stages. Using both methods detection limit of <1 CFU per gram was achieved for all food matrices evaluated. For cattle feces this detection limit was defined at >10 CFU, without IMS applied.

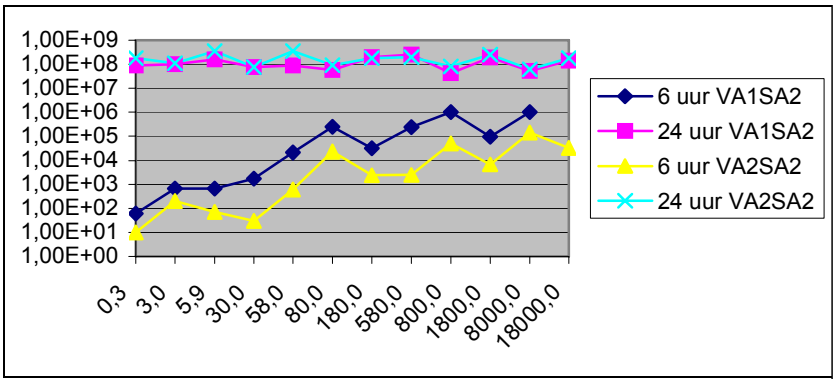


Figure 26. VA1SA2 and VA2SA2 pre-enrichment stages

For isolation of STEC from cattle feces, the application of serotype specific IMS beads is advised. This will improve selectivity on the isolation medium, reducing statistical issues discussed above. Studies indicate that application of IMS improved selectivity up to 40%. As only limited data on this topic is available from the research project discussed in this report, no adequately substantiated recommendations can be presented at this time.

Figure 27 summarizes results presented in the paragraphs below. Four charts present results obtained in isolation of O26 strains from various samples using all 4 protocols defined at the start of task2. In addition results on five remaining serotypes included in the scope of the STEC isolation method (O103, O111, O145 and both sorbitol positive and negative O157) are shown using protocols VA1SA2 and VA2SA2. Based on results shown in these charts, the most selective isolation method (VA2SA2) is advised for isolation of STEC from food samples. Using a mild pre enrichment procedure allows sub lethally injured cells to resuscitate sufficiently in order to survive more stringent conditions used during the second enrichment stage. Data also indicates a detection limit of <1 CFU is achieved for all serotypes and all sample matrices, except for cattle feces for which a detection limit of >10 CFU is defined.

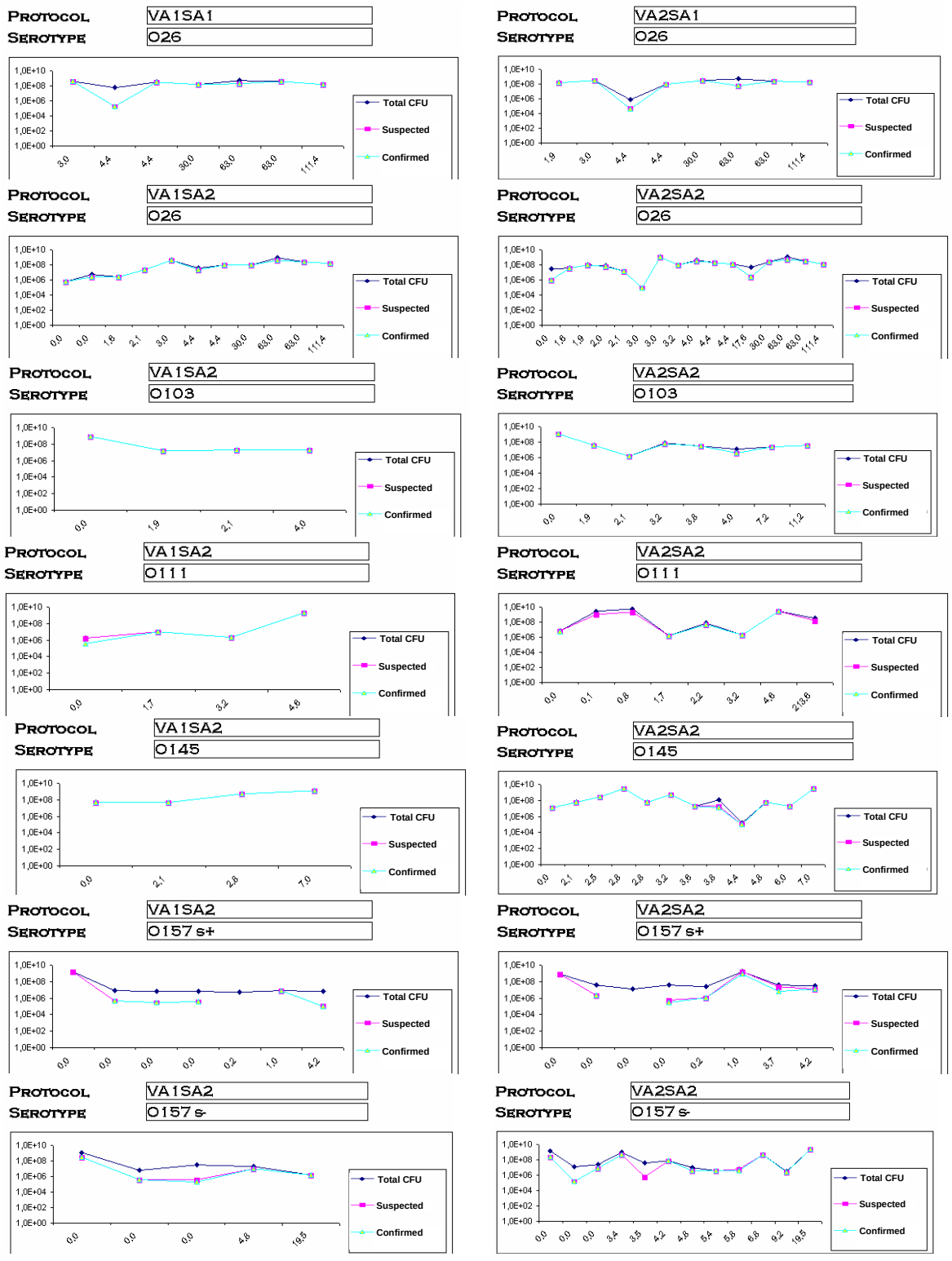


Figure 27. Summary of revenues produced using different protocols grouped by serotype used for sample contamination

3 Adjustments made to original project planning

Table 8 indicates the project planning realized compared to the planning originally states in the project description. This research project was framed in a tight schedule of 24 months. Time required for a preparatory study was included in this schedule, taking up the first two months. After this period of mainly literature study, bench work was started as planned. Results were obtained as planned. Subtask 1.3 however has turned out to be laborious and time consuming: as described above, various components were evaluated for their use in post enrichment acid treatment. In order to substantiate our decision to remove this procedure from STEC isolation protocols, many strains were evaluated under different conditions, as acid resistance of *E. coli* is known to be dependent of culture and preservation conditions. These experiments took up more time as originally scheduled, and as a consequence all subsequent task were pushed forward.

	Year 1		Year 2	
	1-6	7-12	13-18	19-24
Task 1	**Optimization of detection methods**			
1.1 – Collection of strains and characterization				
1.2 – Enrichment media				
1.3 – Post-enrichment treatment				
1.4 – Agar media				
1.5 – Multiplex PCR				
1..6 – Colony immunoblot and –hybridization				
Task 2	**Artificially contaminated samples**			
2.1 – Minced beef and Belgian dry sausage				
2.2 – Raw milk and cheeses				
2.3 – Cattle feces				
2.4 – PCR screening				
Task 3	**Naturally contaminated samples**			
3.1 – Slaughterhouse samples				
3.2 – Farm samples				
3.3 – Raw milk and beef carcasses				
3.4 – PCR screening				
3.5 - Reproducibility				

Table 8. Project planning overview

A literature study was conducted in preparation of subtask 1.6: several techniques are discussed in scientific papers and many manufacturers offer detailed technical information on various websites and in manuals. Colony immunoblot and –hybridization techniques are known to be labour consuming and require trained technicians to apply these techniques

accurately. The research team was contacted by VIB during the early stages of the project to construct a broad range multiplex PCR as described above. As more preliminary results of this multiplex PCR routine became available, this method became a promising alternative to classical immunoblot and hybridization methods. As a consequence, these methods planned for strain confirmation, were abandoned.

The remainder of task 1 was completed as planned and results were presented above: 4 protocols for isolation of selected STEC serotypes from different food matrices and cattle feces were designed.

During task 2, STEC strains were isolated from artificially contaminated samples. Because of the bulk data generated by these experiments, a database was custom designed to allow identification of the most favourable isolation method to be used for various matrices. Setting up, designing and testing of this database was not included in the time schedule from the original project planning. Preliminary experiments using artificially contaminated samples produced some promising results. As a consequence, all research efforts focussed on the detailed analyses of application of different protocols for STEC isolation, rather than rushing into task 3 without full completion of task 2.

This choice forced the research team to abandon task 3: no data is yet available on naturally contaminated samples using techniques designed during this project.

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5 Publications

Two publications have been prepared and will be submitted shortly. A first manuscript deals with the metabolic and genetic profiling of different STEC serotypes. Briefly, A collection of Shiga-toxin producing *E. coli* strains, mainly belonging to serotypes O26, O103, O111, O145 and O157 was characterised by a polyphasic approach including virulence typing, carbohydrate fermentation variations and RAPD fingerprinting. The RAPD protocol allows a clear separation of different STEC serotypes. A similar kind of separation was achieved after a large scale evaluation of carbohydrate fermentation abilities of a collection of STEC strains belonging to a range of important serotypes regarding human pathology. The determination of the virulence profile indicates some virulence types as a severe threat to human health, but also stresses the possibility of yet unknown virulence factors. Clinical diagnosis of strains did not correlate with either RAPD profiles or carbohydrate fermentation patterns, while the virulence profile of strains did show a strong correlation with the strain serotype. Data described in this paper indicates that 5 serotypes of great importance in respect to human health can be discriminated on the basis of genetic fingerprinting using RAPD and differences in carbohydrate fermentation abilities.

The second manuscript describes the composition and functioning of the isolation and purification media developed during this study. Also, a detailed description on the FP % is presented to support the effectiveness of the method at hand.

Some other publications have already been planned: one manuscript will present detailed results on STEC isolation from artificially contaminated samples, while another manuscript will focus on MIC analysis results and post-enrichment acid treatment of STEC strains.

6 Presentations / Lectures

During several meetings and conferences in Belgium and abroad, the development of the isolation method discussed in this publication was presented. The paragraphs below contain some short descriptions on key lectures/presentations on STEC isolation.

– COST920 meeting in Vienna (2004): (*http://www.cost920.com/Abstract_Binder.pdf*)
During a lecture some preliminary results and certain aspects of the methodology used were presented.

– CEN/TC 275 WG 6 STEC ad'hoc group meeting in Paris (2005): during a lecture current research was presented. Some aspects of the methodology used possibly will be used for the CEN method for STEC detection currently being developed.

– 10th Conference on Food Microbiology in Liege (2005): An informative poster presentation was used to inform all participants on the current research on STEC isolation methods.

– CEN/TC 275 WG 6 STEC ad'hoc group meeting in Copenhagen (2005): during a lecture current research was presented. Some aspects of the methodology used possibly will be used for the CEN method for STEC detection currently being developed.

– International Satellite Congress – Platform for Scientific Concertation: Food Safety (Antwerp - 2006): An informative poster presentation was used to inform all participants on the current research on STEC isolation methods.

Some scheduled presentations / lectures:

– September 2006: 11th Conference on Food Microbiology (Ghent) – invited lecture: methodology and some results will be presented during this lecture to inform a broad audience on new STEC isolation methods.

– November 2006: 6th International Symposium on STEC infections (Melbourne – Australia): 2 informative poster presentations will be used to inform all participants on the current research on STEC isolation methods and will present some key results obtained using the new isolation method developed during this project.

7 General conclusions

Assembling and characterizing a collection of both pathogenic and non-pathogenic STEC strains generated useful data. Analysis of MIC data combined with a profound study on growth characteristics in various enrichment media, allowed definition of the most appropriate enrichment broth for selective STEC enrichment balancing out resuscitation of possibly sub lethally injured STEC cells and reduction of commensal background bacteria. Application of a post-enrichment acid treatment did not prove to be suitable method for enhancing specificity as data showed significant inter- and intra-serotypic variation.

Using Bionumerics software, detailed dendrograms were constructed from digitalized carbohydrate fermentation data. This allowed the research team to identify a selection of carbohydrates suited for serotype discrimination. Results of this carbohydrate fermentation study were combined with data on both enzyme and pH indicators to formulate serotype specific isolation media. One isolation medium was developed for serotypes O26, O103, O111 and O145 while another isolation medium allowed isolation of both sorbitol positive and negative O157 strains. To enhance specificity, secondary purification media were designed: this reduced the theoretical incidence of false positive results to below 1% for most serotypes.

Isolation procedures defined during the first phase of this research project were evaluated using artificially contaminated samples from various matrices: raw milk, scald milk cheeses, raw milk cheeses, minced beef, salami sausage and cattle feces. Both healthy and stressed STEC cultures from serotypes cited above were used at concentrations ranging from <1 to 500 CFU / gram for artificial sample contamination.

Detection limit of STEC serotypes was < 1 CFU / gram in all dairy and meat samples evaluated. A detection limit of 15 CFU / gram was found when isolation STEC from cattle feces. Application of IMS will further improve this detection limit.

8 Acknowledgements

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