

THE ENTEROBACTERIACEAE AND THEIR SIGNIFICANCE TO THE FOOD INDUSTRY

REPORT

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Han Joosten and Andy Davies*

REPORT

COMMISSIONED BY THE ILSI EUROPE EMERGING MICROBIOLOGICAL ISSUES TASK FORCE

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CONTENTS

| | |
|--|----|
| EXECUTIVE SUMMARY | 4 |
| 1. INTRODUCTION | 5 |
| 2. CHANGES IN TAXONOMY | 6 |
| 3. ENTEROBACTERIACEAE AND THEIR ROLE AS INDICATOR AND INDEX ORGANISMS IN FOODS | 9 |
| 4. DETECTION AND ENUMERATION METHODS FOR ENTEROBACTERIACEAE IN FOODS | 13 |
| 5. ENTEROBACTERIACEAE AS FOODBORNE PATHOGENS | 17 |
| 5.1 <i>Salmonella</i> | 19 |
| 5.2 <i>Yersinia enterocolitica</i> | 21 |
| 5.3 <i>Yersinia pseudotuberculosis</i> | 21 |
| 5.4 Pathogenic <i>Escherichia coli</i> | 22 |
| 5.5 <i>Cronobacter</i> spp. | 25 |
| 5.6 <i>Shigella</i> spp. | 26 |
| 5.7 Opportunistic and emerging pathogenic Enterobacteriaceae | 26 |
| 5.8 Extended-spectrum β -lactamase (ES β L)-producing Enterobacteriaceae | 28 |
| 6. ENTEROBACTERIACEAE IN FOOD SPOILAGE | 29 |
| 7. GROWTH, INACTIVATION AND SURVIVAL | 31 |
| 7.1 Temperature | 31 |
| 7.2 Water activity (a_w) | 32 |
| 7.3 pH | 32 |
| 7.4 Gaseous atmosphere | 33 |
| 8. FURTHER READING | 35 |
| 9. REFERENCES | 36 |
| GLOSSARY OF TERMS | 42 |
| ABBREVIATIONS | 47 |

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EXECUTIVE SUMMARY

The Enterobacteriaceae is a family of Gram-negative, non-spore-forming bacteria and is one of the most important groups of bacteria known to man. The introduction of genetic methods, in particular the analysis of 16S rRNA gene sequences, has revolutionised our understanding of these bacteria and the relationship that exists between the different genera and species. Consequently, there have been many changes in bacterial taxonomy resulting in the introduction of new genera and species and the reclassification of some existing bacteria belonging to the Enterobacteriaceae. There are currently 48 genera and 219 species recognised within the Enterobacteriaceae and these numbers are likely to increase in the future.

This family includes a number of important foodborne pathogens such as *Salmonella*, *Yersinia enterocolitica*, pathogenic *Escherichia coli* (including *E. coli* O157:H7), *Shigella* spp. and *Cronobacter* spp. Other members of the family are regarded as opportunistic pathogens, especially in clinical settings (e.g., *Klebsiella* spp., *Serratia* spp. and *Citrobacter* spp.). It is not the intention of this report to provide detailed information on specific pathogens that belong to the Enterobacteriaceae. These have either been covered by separate ILSI reports or may instead be the topic of future publications.

In addition to their aetiology in foodborne illness, some members of the family are also associated with food spoilage and therefore contribute to significant economic losses for the agricultural and food industries. For example, *Erwinia* spp., and the more recently introduced *Pectobacterium* spp. and *Brenneria* spp., have long associations with plant and fruit diseases. Many other members of the Enterobacteriaceae are responsible for spoilage of a variety of foods including fruit and vegetables, meats, poultry, eggs, milk and dairy products, as well as fish and other seafoods.

Different numbers of Enterobacteriaceae can be cultured from a variety of raw materials, depending both on the origin of the raw material and on the control of hygiene. Raw materials entering the food chain are often subjected to further manipulations that impact on the microbial ecology. The impact of processing, product formulation and storage on Enterobacteriaceae in food will be discussed. It is important to note that the precise conditions required to support the growth and survival of a particular Enterobacteriaceae member can differ depending on several factors. These include prior exposure to intrinsic factors (i.e., acidity (pH), water activity and natural antimicrobial substances), extrinsic factors (i.e., temperature, relative humidity and gaseous atmosphere), and implicit conditions (i.e., interactions with other microbial populations, associated with a particular food product and the particular strain of bacterium). It is well-established that pathogenic strains of *E. coli*, *Salmonella* and *Cronobacter* demonstrate prolonged survival under adverse conditions. Thus Enterobacteriaceae demand particular attention both in perishable food and in processed foods with a long shelf-life.

The Enterobacteriaceae family includes genera with the ability to ferment lactose (termed coliform bacteria) and that have long been used as indicator organisms by the food and water industry. Nowadays, both Enterobacteriaceae and coliforms are isolated from foods to indicate evidence of poor hygiene or inadequate processing (especially heat-treatment), process failure and post-process contamination of foods. *E. coli* is commonly used to provide evidence of potential faecal contamination in certain foods and is used as an index organism for the presence of enteric pathogens such as *Salmonella*.

Methods for the detection and enumeration of Enterobacteriaceae have changed little since they were first introduced and many still rely on the growth of the bacterium in selective media along with the use of carbohydrate (e.g. glucose) as an energy source. In contrast, several rapid methods are now available for detection of specific pathogenic members of the Enterobacteriaceae found in foods including *Salmonella* and *E. coli* O157.

1. INTRODUCTION

The family Enterobacteriaceae comprises a large group of Gram-negative non-spore-forming bacteria typically 1-5 μm in length. They are facultative anaerobes and with the exception of *Saccharobacter fermentans* and some strains of *Yersinia* and *Erwinia*, they share the ability to reduce nitrate to nitrite. These bacteria are generally motile by peritrichous flagella except for *Shigella* and *Tatumella* and some other non-motile members of this family. For example, *Salmonella* are typically motile, notable exceptions being the *Salmonella* serotypes Pullorum and Gallinarum. A common feature of the Enterobacteriaceae, which helps to differentiate them from other closely related bacteria, is the lack of cytochrome C oxidase, although there are exceptions such as *Plesiomonas* spp. Enterobacteriaceae are catalase-positive with the exception of *Shigella dysenteriae* 1 and *Xenorhabdus* species. Enterobacteriaceae ferment a variety of carbohydrates, but their ability to produce acid and gas from the fermentation of D-glucose is one characteristic that remains an important diagnostic property and is commonly used as a basis for their detection and enumeration. Some members of the Enterobacteriaceae (e.g., *Enterobacter* spp., *Escherichia coli*, *Citrobacter* spp. and *Klebsiella* spp.) can be recognised using methods that exploit their ability to ferment lactose rapidly (usually within 24-48 h) producing acid and gas. These are collectively termed coliform bacteria and are often used as (faecal) indicator organisms by the food and water industry (see below), because their normal habitat is the gastrointestinal tract of mammals, birds etc. However, unlike the Enterobacteriaceae family, this is not a well-defined taxonomic group.

Members of the Enterobacteriaceae are widely distributed. Although strains of some species are harmless commensals, such as some strains of *E. coli*, others are important human and animal pathogens, and some are pathogenic to plants and insects. Their ubiquitous distribution means that it is inevitable that some members of the Enterobacteriaceae will enter the food chain. Members of the family are responsible for causing foodborne disease and some also cause food spoilage and therefore contribute to substantial economical losses and food wastage. The initial Enterobacteriaceae contamination level in the raw materials is predominantly governed by Good Agricultural Practices (GAP) during primary production and subsequently during slaughter of livestock at the abattoir. Further along the food supply chain, contamination by Enterobacteriaceae, including pathogens, must be prevented or controlled by the application of one or more of the acknowledged quality assurance systems including Hazard Analysis and Critical Control Point (HACCP) systems and Good Manufacturing Practices (GMP).

2. CHANGES IN TAXONOMY

The name Enterobacteriaceae was first proposed by Rahn (1937). The type genus is *Escherichia*. Enterobacteriaceae comprise a large group of genetically and biochemically related bacteria. Phylogenetic studies place them in the phylum Proteobacteria, Class Gammaproteobacteria and Order Enterobacteriales (Brenner *et al.*, 2005). Until the early 1960s bacterial classification was largely based on phenotypic characteristics and culture-based observations. The introduction of genetic methods, such as DNA-DNA hybridisation and guanine plus cytosine (G+C) determination revolutionised bacterial taxonomy and classification. More recently analysis of 16S rRNA gene sequences has been used to elucidate further the genetic relationships between members of the Enterobacteriaceae and their similarity to other closely related bacteria. Consequently, there have been recent changes to the classification of members of this family. Indeed, the number of genera and species of Enterobacteriaceae increased from 12 genera and 36 species in 1974 to at least 34 genera, 149 species and 21 subspecies in 2006 (Baylis, 2006). By the time of writing, these numbers had grown to at least 48 genera, 219 species and 41 sub-species in the family Enterobacteriaceae (Table 1). This revised list excludes some additional genera that have been proposed but have yet to be validated and approved for inclusion. The situation is likely to continue to evolve as unpublished and as yet undescribed genera and species are added.

Table 1. Genera, species and sub-species belonging to the Enterobacteriaceae

| Genus | Species | Sub species |
|--------------------------------|---|--|
| <i>Alterococcus</i> | <i>agarolyticus</i> | |
| <i>Arsenophonus</i> | <i>nasoniae</i> | |
| <i>Brenneria</i> | <i>alni, nigrifluens, quercina, rubrifaciens, salicis</i> | |
| <i>Buchnera</i> | <i>aphidicola</i> | |
| <i>Budvicia</i> | <i>aquatica</i> | |
| <i>Buttiauxella</i> | <i>agrestis, brennerae, ferragutiae, gaviniae, izardii, noackiae, warmboldiae</i> | |
| <i>Candidatus Phlomobacter</i> | <i>fragariae</i> | |
| <i>Cedecea</i> | <i>davisae, lapagei, neteri</i> | |
| <i>Citrobacter</i> | <i>amalonaticus, braakii, farmeri, freundii, gillenii, koseri (diversus), murlinae, rodentium, sedlakii, werkmanii, youngae</i> | |
| <i>Cosenzaea</i> | <i>myxofaciens</i> | |
| <i>Cronobacter</i> | <i>dublinensis, malonaticus, muytjensii, sakazakii, turicensis, Genomospecies I</i> | <i>dublinensis</i> subsp. <i>dublinensis</i> , <i>dublinensis</i> subsp. <i>lactardi</i> , <i>dublinensis</i> subsp. <i>lausannensis</i> |
| <i>Dickeya</i> | <i>chrysanthemi (Pectobacterium), dadantii, dianthicola, dieffenbachiae, paradisiaca, zae</i> | |
| <i>Edwardsiella</i> | <i>anguillimortifera, hoshinae, ictaluri, tarda</i> | |
| <i>Enterobacter</i> | <i>aerogenes, amnigenus, arachidis, asburiae, cancerogenus (Ent taylorae/Erwinia cancerogena), cloacae, (Erwinia disslovens/Ent disslovens), cowanii, gergoviae, helveticus, hormaechei, kobei, ludwigii, mori, nimipressuralis (Erwinia nimipressuralis), oryzae, pulveris, pyrinus, radicincitans, soli, turicensis</i> | <i>cloacae</i> subsp. <i>cloacae</i> , <i>cloacae</i> subsp. <i>disslovens</i> |
| <i>Erwinia</i> | <i>amylovora, aphidicola, billingiae, carnegieana, mallotivora, papayae, persicina, psidii, pyrifoliae, rhapontici, tasmaniensis, toletana, tracheiphila</i> | |

| | | |
|-----------------------------------|--|--|
| <i>Escherichia</i> | <i>albertii, coli, coli inactive, fergusonii, hermannii, vulneris</i> | |
| <i>Ewingella</i> | <i>americana</i> | |
| <i>Hafnia</i> | <i>alvei, paralvei</i> | |
| <i>Klebsiella</i> | <i>granulomatis, oxytoca, pneumonia, singaporensis, variicola</i> | <i>pneumoniae</i> subsp. <i>ozaenae</i> , <i>pneumoniae</i> subsp. <i>pneumoniae</i> , <i>pneumoniae</i> subsp. <i>rhinoscleromatis</i> , |
| <i>Kluyvera</i> | <i>ascorbata, cryocrescens, georgiana, intermedia</i> | |
| <i>Leclercia</i> | <i>adecarboxylata (Escherichia)</i> | |
| <i>Leminorella</i> | <i>grimontii, richardii</i> | |
| <i>Moellerella</i> | <i>wisconsensis</i> | |
| <i>Morganella</i> | <i>morganii psychrotolerans</i> | <i>morganii</i> subsp. <i>Morganii</i> <i>morganii</i> subsp. <i>sibonii</i> |
| <i>Obesumbacterium</i> | <i>proteus</i> | |
| <i>Pantoea</i> | <i>agglomerans, allii, ananatis, anthophila, brenneri, calida, conspicua, cypridiedii, deleyi, dispersa, eucalypti, eucrina, gaviniae, stewartii, vagans</i> | <i>stewartii</i> subsp. <i>indologenes</i> , <i>stewartii</i> subsp. <i>stewartii</i> |
| <i>Pectobacterium</i> | <i>atrosepticum, betavasulorum, cacticida, carotovorum, cypridiedii, wasabiae</i> | <i>carotovorum</i> subsp. <i>carotovorum</i> , <i>carotovorum</i> subsp. <i>odoriferum</i> |
| <i>Photorhabdus (Xenorhabdus)</i> | <i>asymbiotica, luminescens temperata</i> | <i>asymbiotica</i> subsp. <i>asymbiotica</i> , <i>asymbiotica</i> subsp. <i>australis</i> , <i>luminescens</i> subsp. <i>Akhurstii</i> <i>luminescens</i> subsp. <i>caribbeanensis</i> <i>luminescens</i> subsp. <i>hainanensis</i> <i>luminescens</i> subsp. <i>Kayaii I</i> <i>uminescens</i> subsp. <i>kleinii</i> <i>luminescens</i> subsp. <i>Laumondii</i> <i>luminescens</i> subsp. <i>Luminescens</i> <i>luminescens</i> subsp. <i>thracensis</i> <i>temperata</i> subsp. <i>Cinerea</i> <i>temperata</i> subsp. <i>Khanii</i> <i>temperata</i> subsp. <i>Stackebrandtii</i> <i>temperata</i> subsp. <i>Tasmaniensis</i> <i>temperata</i> subsp. <i>temperate</i> <i>temperata</i> subsp. <i>thracensis</i> |
| <i>Plesiomonas</i> | <i>shigelloides</i> | |
| <i>Pragia</i> | <i>fontium</i> | |
| <i>Proteus</i> | <i>hauseri, inconstans, mirabilis, penneri, vulgaris</i> | |
| <i>Providencia</i> | <i>alcalifaciens, burhodogranariea, heimbachae, rettgeri, rustigianii, sneebia, stuartii, vermicola</i> | |
| <i>Rahnella</i> | <i>aquaticus</i> | |
| <i>Raoultella</i> | <i>ornithinolytica, planticola, terrigena</i> | |
| <i>Saccharobacter</i> | <i>fermentatus</i> | |
| <i>Salmonella</i> | <i>enterica, bongori</i> | <i>enterica</i> subsp. <i>enterica</i> , (Group I) <i>enterica</i> subsp. <i>salamae</i> , (Group II) <i>enterica</i> subsp. <i>arizonae</i> , (Group IIIa) <i>enterica</i> subsp. <i>diarizonae</i> , (Group IIIb) <i>enterica</i> subsp. <i>houtenae</i> , (Group IV) <i>enterica</i> subsp. <i>indica</i> , (Group V) |
| <i>Samsonia</i> | <i>erythrinae</i> | |
| <i>Serratia</i> | <i>entomophila*, ficaria, fonticola, glossinae, grimesii*, liquefaciens, marcescens, marinorubra, nematodiphila, odorifera, plymuthica, proteamaculans*, quinivorans, rubidaea, symbiotica, ureilytica * liquefaciens-like</i> | <i>marcescens</i> subsp. <i>marcescens</i> , <i>marcescens</i> subsp. <i>sakuensis</i> <i>proteamaculans</i> subsp. <i>proteamaculans</i> |

| | | |
|-----------------------|--|--|
| <i>Shigella</i> | <i>boydii, dysenteriae, flexneri, sonnei</i> | |
| <i>Shimwellia</i> | <i>blatae, pseudoproteus</i> | |
| <i>Sodalis</i> | <i>glossinidius</i> | |
| <i>Tatumella</i> | <i>citrea, morbirosei, pyseous, punctata, terrea</i> | |
| <i>Thorsellia</i> | <i>anophelis</i> | |
| <i>Trabulsiella</i> | <i>guamensis, odontotermis</i> | |
| <i>Wigglesworthia</i> | <i>glossinidia</i> | |
| <i>Xenorhabdus</i> | <i>beddingii, bovienii, budapestensis, cabanillasii, doucetiae, ehlersii, griffiniae, hominickii, indica, innexi, japonica, koppenhoeferi, kozodii, mauleonii, miraniensis, nematophila, poinarii, romani, stockiae, szentirmaii, vietnamensis</i> | |
| <i>Yersinia</i> | <i>aldovae, aleksiciae, bercovieri, enterocolitica, frederiksenii, intermedia, kristensenii, massiliensis, mollaretii, nurmii, pekkanenii, pestis,, pseudotuberculosis, rohdei, ruckeri, similis</i> | <i>enterocolitica</i> subsp. <i>enterocolitica</i> , <i>enterocolitica</i> subsp. <i>palaearctica</i> |

Among the recent changes are the addition of new genera such as *Consenzaea*, *Shimwellia*, the insect symbionts *Arsenophonus*, *Buchnera* and *Wigglesworthia* and the creation of the genus *Cronobacter* which includes the pathogen previously known as *Enterobacter sakazakii* (Iversen et al., 2007; 2008). In addition to *Cronobacter sakazakii*, this new genus includes five other species (*malonaticus*, *turicensis*, *mytjensii*, *dublinensis* and *genomospecies I*) along with 3 sub-species of *Cronobacter dublinensis* (subsp. *dublinensis*, subsp. *lactaridi* and subsp. *lausannensis*). Other new genera and species have also been created to accommodate changes in taxonomy. *Erwinia* spp. have always been important because of their association with plant diseases and this genus once contained a large number of species. More recently, some *Erwinia* spp. were re-assigned to either the genus *Brenneria* or *Pectobacterium*, which encompass necrotic phytopathogenic species and soft rotting phytopathogenic species, respectively.

The genus *Raoultella* includes three former *Klebsiella* spp, including two (*ornithinolytica*, and *planticola* species), which are associated with histamine formation. Some strains of *R. planticola* were previously identified as histamine producing strains of *K. pneumoniae* or *K. oxytoca* (Kanki et al. 2002). Another former *Klebsiella* spp. (*K. trevisanii*) is now re-classified as a *R. planticola*. Following comparison of DNA relatedness data, the genus *Photorhabdus* was created to accommodate strains of *Xenorhabdus* and both genera now include several new species of Enterobacteriaceae.

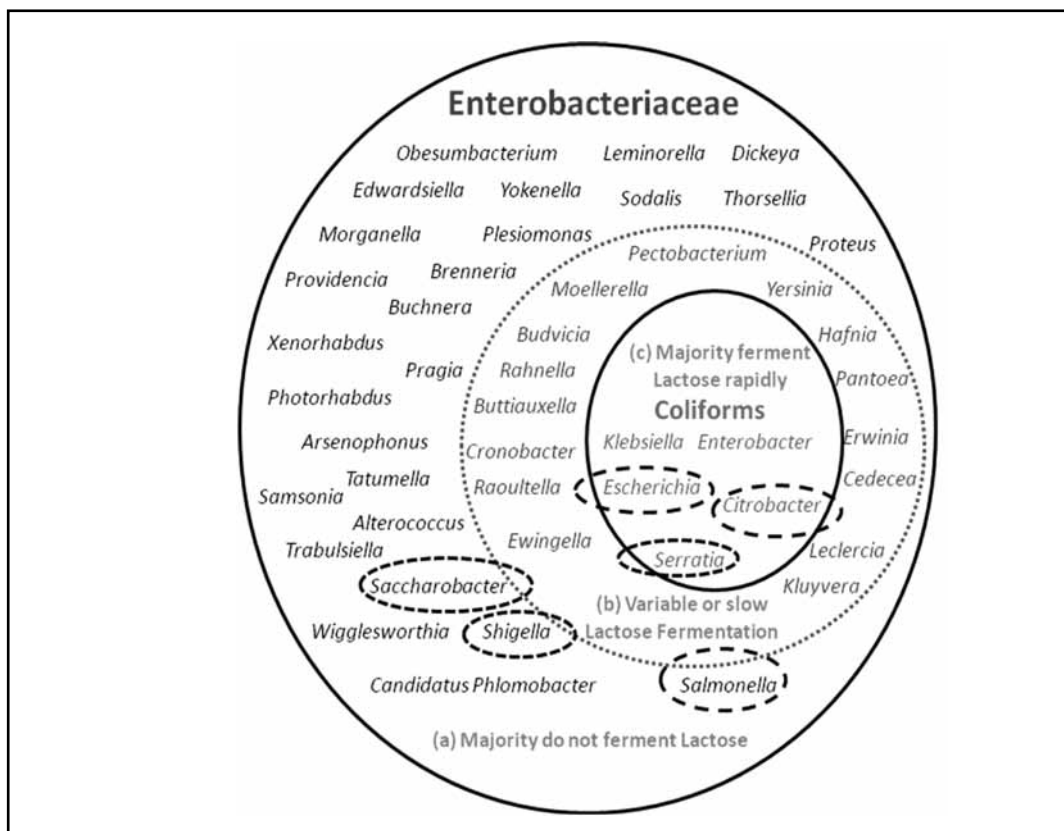
Although genetic evidence reveals that *Shigella* spp. can be considered to represent metabolically inactive biogroups of *E. coli*, this genus has been retained because of the clinical importance associated with *Shigellae* and to avoid confusion that any reclassification would cause. Furthermore, comparison of 16S rRNA sequences has revealed a close phylogentic relationship between *Salmonella*, *E. coli* and *Shigella* (Christensen et al., 1998).

As a result of using genetic-based classification methods, the nomenclature of *Salmonella* has changed. The genus *Salmonella* comprises over 2,500 serotypes, which were once considered separate species based on a combination of somatic O and flagellar H antigens expressed by these bacteria. DNA-DNA hybridisation studies revealed that all *Salmonella* form a single DNA homology group comprising two species. The first species *S. enterica* comprises six groups or subspecies including *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (V). The seventh group, *Salmonella bongori* has become the second species.

3. ENTEROBACTERIACEAE AND THEIR ROLE AS INDICATOR AND INDEX ORGANISMS IN FOODS

Indicator organisms are bacteria that are used to provide evidence of poor hygiene, inadequate processing or post-process contamination of foods. They are often chosen because they are relatively quick and simple to detect. Their absence in food provides a degree of assurance that the hygiene and food manufacturing process has been carried out appropriately, whereas their presence usually indicates that a potential problem or failure in the process has occurred. The Enterobacteriaceae and coliform bacteria within this family represent two of the most common groups of indicator organism used by the food industry. Historically, coliforms were the most common indicator group tested for by the food industry, especially within the dairy sector. In some countries, depending on regulatory requirements, the food industry has moved towards testing for Enterobacteriaceae. The ability of coliforms to ferment lactose rapidly is often employed by conventional culture methods for their detection and enumeration. Consequently coliforms are often defined by the method used. The genera normally regarded as coliforms include *Enterobacter*, *Klebsiella*, *Citrobacter* and *Escherichia*, particularly *E. coli*. However, others may include *Hafnia alvei* and strains belonging to genera such as *Buttiauxella*, *Leclercia*, *Pantoea*, *Serratia*, *Yersinia* etc. (Figure 1). Bacteria outside the Enterobacteriaceae, notably *Aeromonas* spp., can also ferment lactose and these can be falsely detected as coliforms by some methods, if no additional confirmatory tests are performed.

Figure 1 Diagram showing the relationship between genera within the Enterobacteriaceae and those in the coliform group.



(a) Most species do not ferment lactose. Some exceptions exist. (b) Some species ferment lactose (variable or slowly). Not typical coliforms but some may be regarded as coliforms (depending on the method used). (c) High proportion ferment lactose (rapidly). Traditionally regarded as typical coliforms.

Dotted circles show genera that include species or strains which commonly cross between two categories.

Moreover, species of other genera such as *Erwinia* and *Serratia* can also ferment lactose, albeit slowly, whereas some strains of *Citrobacter* and *Klebsiella*, as well as some strains of *Salmonella*, notably *Salmonella enterica* subsp. *arizonae* (IIIa), and *Hafnia alvei* show delayed or variable lactose fermentation.

Currently there is no consensus view as to how the coliform group should be defined or which genera or species should be included. The coliforms remain useful as indicators, even though there is no taxonomic basis for this grouping; this lack of definition, however, can present problems for international trade. Microbiologists examining water attempted to improve the definition of coliforms by making the expression of β -galactosidase activity a requirement. Although this definition was intended as a practical working definition of coliform bacteria, it has no taxonomic value (Anon 1994). The criterion simply seeks to provide a working definition that is not dependent on methods that rely on rapid lactose fermentation. It now implies that slow lactose fermenting strains, which may not produce acid or gas by traditional methods, and those that demonstrate β -galactosidase activity when using chromogenic media can be regarded as coliforms.

Testing foods and water for coliforms has remained popular, not least because specific guidelines and regulations demand coliform testing. Whether testing foods for coliforms or Enterobacteriaceae, the significance of the results obtained must be put into context with the type of food matrix being analysed. This is especially important with foods of plant origin because of the natural associations that can exist (Baylis and Petitt, 1997). The ability of some Enterobacteriaceae to multiply in certain foods, even during chilled storage, can make interpretation of results more difficult because the numbers present may not always reflect the initial level of contamination. These psychrotrophic Enterobacteriaceae are widely distributed and can be found in a variety of foods including milk, meat and poultry and other foods. Therefore, high levels of Enterobacteriaceae in some chilled foods may not necessarily indicate temperature abuse or improper storage. For these reasons Enterobacteriaceae provide a good indicator of overall GMP on the day of production but not throughout the shelf-life or at the end of shelf-life of some (refrigerated perishable) products.

Undoubtedly Enterobacteriaceae provide a valuable role as indicator organisms in processed foods, particularly those subjected to heat-treatment. Depending on the initial contamination level and treatment, they can provide a reliable indication of process failure, under-processing or post-process contamination Table 2 (page 11). In European legislation there are designated sampling plans and limits for the level of Enterobacteriaceae in certain foods for food business operators. These are laid down in Commission Regulation (EC) No. 2073/2005 on microbiological criteria for food-stuffs, as part of the process hygiene criteria. Examples of the sampling plans and the criteria for Enterobacteriaceae applied to specific food categories in this regulation are given in Table 3 (page 12).

With certain foods Enterobacteriaceae can also provide a measure of food quality and spoilage potential. However, because the Enterobacteriaceae is such a large and diverse group they may be useful indicators of overall GMP, but not necessarily faecal contamination, and their relevance in foods should be assessed and interpreted carefully. Some Enterobacteriaceae are commonly found in the gastrointestinal tract of animals including humans. These bacteria can be used as indicators of potential faecal contamination, although *E. coli* strains are perhaps the most common bacteria used for this purpose. Methods exist, which use elevated incubation temperatures (e.g. 44°C), to preferentially isolate these so-called faecal coliform bacteria, but the term is misleading and the term thermotolerant coliforms is therefore a more appropriate description for these bacteria.

As well as using indicator organisms for the above purposes, some groups or individual species of bacteria can be used to provide evidence of potential contamination of food or water by closely related pathogens. Such organisms have been given the term "index" or "marker" organisms (Mossel, 1978; 1982). This term should not be confused with "indicator organism", which has a different

purpose. Bacteria such as *E. coli* can have a dual purpose in the same food – as an indicator of faecal contamination and as an index organism for enteric pathogens such as *Salmonella*. Regulation EC 2073/2005 initially required testing of dried infant formulae for *Salmonella* and *Cronobacter* spp. (*E. sakazakii*) if Enterobacteriaceae were detected. However, it was concluded by the Scientific Panel on Biological Hazards of the European Food Safety Authority that it was not always possible to establish a correlation between Enterobacteriaceae and *Salmonella* and that no universal correlation between Enterobacteriaceae and *Cronobacter* spp. exists. Therefore, EC Regulation 2073/2005 was subsequently revised (Commission Regulation No 1441/2007).

Despite some limitations, testing foods for index organisms rather than pathogens is simple and relatively cheap, with results often available in 24 h. By comparison, traditional culture methods for pathogens such as *Salmonella* can take 3-7 days to obtain a result. Furthermore, pathogenic bacteria in food are often heterogeneously distributed and present in low numbers making detection difficult. Many food production sites also prefer not to isolate enteric pathogens in their on-site laboratory, but elect instead to have testing performed externally by an approved laboratory. In contrast, testing for indicator and index organisms is done routinely by most laboratories, including those on food manufacturing sites. Some Enterobacteriaceae can also cause spoilage of certain foods and this aspect is discussed further in Section 6.

Table 2. Indicator functions associated with Enterobacteriaceae and *E. coli*

| Production process or application | Indicator function | Comments on choice of indicator group |
|--|--|--|
| Raw/unprocessed foods | | |
| Slaughter hygiene & processing meat (raw) and fish | GMP/GHP Faecal contamination | Both Enterobacteriaceae and <i>E. coli</i> acceptable indicator groups |
| Harvesting and processing of fruit and vegetables (raw) | GAP Environmental contamination | Can have high/variable numbers of Enterobacteriaceae compared to <i>E. coli</i> |
| Harvesting and processing of fruit and vegetables (raw) | GAP Faecal contamination | High levels of <i>E. coli</i> useful indicator of potential faecal contamination |
| Raw milk, fermented dairy products, ice cream | GMP/GHP Hygiene | Limitations if products mixed or seasoned with (dried) raw ingredients e.g. fruit. Some Enterobacteriaceae not associated with faeces (unlike <i>E. coli</i>) |
| Pasteurised milk and dairy products | GMP/GHP Post-process contamination | Both Enterobacteriaceae and <i>E. coli</i> acceptable indicator groups |
| Heat-treated foods | | |
| Cooked meats, Pasteurised milk and dairy products, milk powder, chocolate, egg products, REPFEDs etc | GMP/GHP Post-process contamination Insufficient heat-treatment | Limitations if products mixed or seasoned with (dried) raw ingredients |
| Canned foods Foods heated in package | Leakage or under-heating | Both Enterobacteriaceae and <i>E. coli</i> acceptable indicator groups |
| Food production environments | | |
| Hand hygiene Environmental swabs | GMP/GHP Faecal contamination | <i>E. coli</i> (preferred choice) |

REPFED: Refrigerated Processed Foods of Extended Durability; GMP: Good Manufacturing Practice; GHP: Good Hygiene Practices; GAP: Good Agricultural Practices

Table 3. Examples of Enterobacteriaceae limits stated in the process hygiene criteria of Commission Regulation 2073/2005 on microbiological criteria for foodstuffs and subsequent amendments (No. 1441/2007 and 365/2010)

| Food category | Sampling plan | | Limits | | Analytical method | Action in case of unsatisfactory* results |
|---|---------------|---|---|---|-------------------|---|
| | n | c | m | M | | |
| Meat and meat products | | | | | | |
| Carcases of cattle, sheep, goats and horses | – | – | 1.5 log cfu/m ² daily mean log | 2.5 log cfu/m ² daily mean log | ISO 21528-2 | Improvements in slaughter hygiene and review of process controls |
| Carcases of pigs | – | – | 2.0 log cfu/m ² daily mean log | 3.0 log cfu/m ² daily mean log | ISO 21528-2 | Improvements in slaughter hygiene and review of process controls |
| Pasteurised milk and other pasteurised liquid dairy products | 5 | 0 | 10 cfu/ml | | ISO 21528-2 | Check on the efficiency of heat-treatment and prevention of recontamination as well as the quality of raw materials |
| Milk powder and whey powder | 5 | 0 | 10 cfu/g | | ISO 21528-2 | Check on the efficiency of heat treatment and prevention of recontamination |
| Ice cream and frozen dairy desserts | 5 | 2 | 10 cfu/g | 100 cfu/g | ISO 21528-2 | Improvements in production hygiene |
| Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age | 10 | 0 | Absent in 10g | | ISO 21528- 1 | Improvements in production hygiene to minimise contamination. |
| Dried follow-on formulae | 5 | 0 | Absence in 10g | | ISO 21528- 1 | Improvements in production hygiene to minimise contamination. |
| Egg products | 5 | 2 | 10 cfu/g or ml | 100 cfu/g or ml | ISO 21528-2 | Checks on the efficiency of the heat treatment and prevention of recontamination |

The above criteria are applied to meat products at the carcass stage (after dressing but before chilling) and to dairy products at the end of the manufacturing process. n = number of units comprising the sample; c = number of sample units giving values between m= minimum and M=maximum. The analytical method to be used shall be the most recent edition of the standard. *Satisfactory, if all the values observed are < m. Acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are < m. Unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

4. DETECTION AND ENUMERATION METHODS FOR ENTEROBACTERIACEAE IN FOODS

Several published standardised methods exist for the detection and enumeration of Enterobacteriaceae, coliforms and *E. coli* in foods including international standard methods such as those published by the International Organization for Standardization (ISO) (Table 4). Most are quantitative because food manufacturers often impose specifications or limits for these bacteria in their products. Detection methods are commonly used for foods where the presence of low numbers needs to be confirmed, or for high-risk foods where zero tolerance is imposed.

Table 4.

a. Examples of Conventional Culture Methods used for the Detection/Enumeration of the Enterobacteriaceae in Foods

| Test and medium | Diagnostic property | Technique | Incubation | Appearance or positive reaction | Examples or standards |
|--|---------------------------------------|---|------------------------------|---|-----------------------|
| Enterobacteriaceae | | | | | |
| Enterobacteriaceae enrichment (EE) broth | Glucose fermentation | Detection or MPN: used in conjunction with VRBGA following pre-enrichment in BPW. | 30°C or 37°C for 24 h | Broth turns turbid & yellowish green (note: streak all broths irrespective of colour) | ISO 21528-1 (2004) |
| Violet Red Bile Glucose agar (VRBLA) | Glucose fermentation (pH indicator) | Pour plate + overlay | 30°C or 37°C for 24 h | Typical colonies: red/purple with halo | ISO 21528-2 (2004) |
| Coliforms | | | | | |
| Lauryl sulphate tryptose broth (LSTB) | Lactose fermentation (gas production) | Detection or MPN | 30°C or 37°C for 24-48 h | Gas collected in Durham tube (sub-cultured into BGBLB) | ISO 4831 (2006) |
| Brilliant Green Bile Lactose broth (BGBLB) | Lactose fermentation (gas production) | Detection or MPN | 30°C or 37°C for 24-48 h | Gas collected in Durham tube | ISO 4831 (2006) |
| Violet red bile agar (VRBA) | Lactose fermentation (pH indicator) | Pour plate + overlay | 30°C or 37°C 24 h | Typical colonies red/purple with halo | ISO 4832 (2006) |
| <i>Escherichia coli</i> | | | | | |
| Tryptone Bile Agar (TBA) | Indole from tryptophan | Spread plate (with 85 mm 0.45 µm membrane) | 4h at 37°C + 18-20 h at 44°C | Pink cerise colonies upon addition of indole detecting agent | BS 5763-13 (1998) |
| Tryptone bile X-glucuronide agar (TBX) | β-glucuronidase (BCIG) | Spread plate (with 85 mm 0.45 µm membrane) | 4h 37°C + 18-24 h at 44°C | Typical colonies: blue | ISO 16649-1 (2004) |
| Tryptone bile X-glucuronide agar (TBX) | β-glucuronidase (BCIG) | Pour plate (optional resuscitation 4 h at 37°C) | 44°C for 18-24 h | Typical colonies: blue | ISO 16649-2 (2004) |

b. Examples of Alternative Methods with Tests available for Enterobacteriaceae, Coliforms and E. coli

| Method | Manufacturer | Diagnostic property | Tests available |
|---|-------------------------------|---|---|
| Direct plate/alternative plate count methods | | | |
| Petrifilm™ | 3M Health Care | Carbohydrate fermentation (with/without gas production) and enzyme activity | Coliforms, <i>E. coli</i> /coliform, Enterobacteriaceae |
| Compact Dry™ | Nissui Pharmaceutical Co. Ltd | Carbohydrate fermentation and enzyme activity | Coliforms, <i>E. coli</i> /coliform, Enterobacteriaceae |
| RIDA® Count | r-BioPharm-Rhône | Carbohydrate fermentation and enzyme activity | Coliforms, <i>E. coli</i> /coliform, Enterobacteriaceae |
| SimPlate® Coliforms/ <i>E. coli</i> | BioControl Inc | Binary detection technology (MPN principle) using 100 well plate and fluorogenic/chromogenic substrates | Total Coliforms and <i>E. coli</i> |
| Automated methods | | | |
| Soleris® | Neogen Inc | Optical assay measuring changes in pH and other metabolic activity | Enterobacteriaceae, Coliforms and <i>E. coli</i> |
| TEMPO® | bioMérieux | Metabolic activity and enzyme activity. Automated MPN procedure. | Enterobacteriaceae, Coliforms and <i>E. coli</i> |
| Bactometer | bioMérieux | Impedance/conductance | Enterobacteriaceae, Coliforms and <i>E. coli</i> |
| Rapid automated bacterial impedance technique (RABIT) | Don Whitley Scientific | Impedance | Enterobacteriaceae, Coliforms and <i>E. coli</i> |
| BacTrac 4300 Microbiological Analyser | SY-LAB | Impedance | Enterobacteriaceae, Coliforms and <i>E. coli</i> |

Enzyme activity is commonly detected using chromogenic or fluorogenic substrates e.g. β -galactosidase activity (coliforms) and β -D-glucuronidase activity (*E. coli*). The above table excludes tests (including immunoassays, PCR test etc) for specific members of the Enterobacteriaceae e.g. *E. coli* O157, *Cronobacter* spp and *Salmonella* spp. Various chromogenic media specifically for the detection/enumeration of Enterobacteriaceae (including coliforms, *E. coli*, *E. coli* O157, *Cronobacter* spp (*Enterobacter sakazakii*) and *Salmonella* are available from culture media manufacturers.

Enumeration methods often involve direct plating on solid selective media prepared using a pour or spread plate technique. Liquid media can be used for detecting Enterobacteriaceae, coliforms and *E. coli* and for enumeration using the most probable number (MPN) technique (typically 3 or 5 tube MPN). The latter is particularly useful when low levels of these bacteria are present. Fermentation of glucose and lactose is the most common diagnostic feature exploited by traditional culture methods for Enterobacteriaceae and coliforms, respectively. Both carbon sources yield acid that is subsequently detected by a colour change in the medium. In liquid media, gas is collected using an inverted Durham tube placed in the tube containing the medium. Tubes are inspected after 24 h and if necessary after a further 48 h incubation. Bile salts are commonly used to inhibit Gram-positive and other non-bile tolerant bacteria, including some Gram-negative bacteria, especially those that do not belong to the Enterobacteriaceae. Alternative selective agents include detergents such as sodium dodecyl sulphate (lauryl sulphate).

Violet red bile glucose agar (VRBGA) and violet red bile lactose agar (VRBLA) are common media used to isolate Enterobacteriaceae and coliforms, respectively. Both are compositional variants of the MacConkey agar, developed for the detection of bile tolerant Gram-negative bacteria. Both VRBGA and VRBLA are used along with a pour plate technique with an overlay of the same medium to ensure fermentation of

the carbohydrates and to reduce the likelihood of oxidation. This approach improves the specificity of these media and reduces interference from motile strains or background flora. Plates are incubated aerobically for 24 h at 37°C and inspected for purple-red colonies surrounded by a purplish halo. If the purpose of the test is to include psychrotrophic coliforms or Enterobacteriaceae the incubation temperature may be lowered to 30°C, but 37°C is the preferred temperature if the test is being used as a hygiene indicator.

Bacteria other than Enterobacteriaceae can also grow successfully on VRBLA and VRBGA, e.g., *Aeromonas* spp. and some *Bacillus* spp., although an oxidase test can be employed to distinguish these from Enterobacteriaceae. These bacteria are often smaller and exhibit atypical colony morphologies. If necessary further confirmatory tests can be performed including the production of gas at 37°C in liquid media and growth in brilliant green bile lactose broth (BGBLB) (ICMSF, 1978). The ISO standard methods 21528-2 (Anon, 2004a) and ISO 4832 (Anon, 2006a) are colony-counting methods designed for Enterobacteriaceae using VRBGA and coliforms using VRBLA, respectively. ISO 21528-2:2004 stipulates biochemical identification of typical colonies whereas ISO 4832:2006 requires no confirmation of typical colonies, only confirmation of gas production by atypical colonies in BGBLB.

Several types of culture media and diagnostic tests have been developed specifically for *E. coli* detection and enumeration, whilst others involve testing for coliforms followed by additional tests to confirm the presence of *E. coli*. Many traditional tests use acid or gas-production at 44°C and production of indole from tryptophan to indicate the presence of *E. coli*. Indole production is a feature of biotype I *E. coli*, which represent about 98% of *E. coli* strains (Farmer *et al.*, 1985).

There are standard methods for the detection and enumeration of presumptive *E. coli*, e.g., ISO 7251 (Anon 2005), and coliforms, e.g., ISO 4831 (Anon, 2006b), in foods using a MPN approach. For the detection and enumeration of Enterobacteriaceae, Mossel (1963) developed a method using pre-enrichment in buffered peptone water followed by enrichment in buffered brilliant green-bile-glucose broth (EE broth) and subsequent streaking onto VRBGA. This method has become ISO standard 21528-1 (Anon 2004b) and is now a mandatory analytical method in European legislation that uses Enterobacteriaceae in process hygiene criteria for a range of food products. However, there is evidence that the combination of dyes and bile salts used in the EE broth can inhibit the growth of some Enterobacteriaceae, including certain strains of *Cronobacter* (Joosten *et al.*, 2008). Consequently, this standard is likely to be revised.

Incorporating chromogenic and fluorogenic substrates into existing media compositions can greatly improve their specificity for identification of Enterobacteriaceae, especially coliforms and *E. coli*. These substrates detect enzyme activity unique to the target bacterium and are commonly used in many alternative and more rapid methods. Enzymes acting on chromogenic substrates generate chromophores, which are subsequently absorbed into the bacterial cell, producing colonies of a particular colour that can be distinguished easily from others. Fluorogenic substrates yield free fluorophores that diffuse into the surrounding medium and which fluoresce under ultra-violet light at a defined wavelength.

For coliforms, β -D-galactosidase, the enzyme that hydrolyses lactose to galactose and glucose, is a common target. The target enzyme for many *E. coli* methods is β -D-glucuronidase (GUD), which catalyses the hydrolysis of β -D-glucopyranosiduronic acids to their corresponding aglycones and D-glucuronic acid. This enzyme activity is reported to be present in about 97% of *E. coli* (Kilian and Bülow, 1976). However, GUD activity is not exclusive to *E. coli*; other members of the Enterobacteriaceae, notably some *Salmonella* and *Shigella* as well as *Hafnia alvei* and other genera, also possess this enzyme activity (Hartman, 1989; Baylis and Patrick, 1999). Furthermore, the majority of *E. coli* O157:H7 strains are GUD negative (Baylis *et al.*, 2006). Incorporating chromogenic substrates for both of these enzymes enables simultaneous enumeration and differentiation of *E. coli* and total coliforms using the same culture medium.

Processed or dry foods are likely to contain sub-lethally injured cells; therefore, direct use of selective media or elevated temperatures are inappropriate. Some methods include a resuscitation step typically involving pre-incubation at a lower temperature or in a medium with limited or no selective agents. Anderson and Baird-Parker (1975) developed a method to aid recovery of *E. coli* from frozen foods, which involved plating a homogenised sample onto a 0.45 µm membrane laid onto the surface of a non-selective or recovery medium (e.g., Minerals Modified Glutamate Agar) followed by incubation at 30°C or 37°C for 4 h. This step facilitates recovery of injured cells with the membrane then being transferred to Tryptone Bile agar (TBA), containing bile salts, and incubated at 44°C. Tryptophan in this medium allows indole producing colonies (*E. coli*) to be counted. This protocol became standard method BS5763-13 in the UK (Anon, 1998). A chromogenic medium (Tryptone Bile X-glucuronide agar) can now be used instead of TBA, thus enabling *E. coli* (GUD-positive) to be identified and enumerated on the membrane or this medium. These approaches became international standard methods ISO 16649-1 (Anon 2001a) and ISO 16649-2 (Anon 2001b), respectively.

Various proprietary media and tests are now available for the detection and enumeration of Enterobacteriaceae Table 4 (page 14). The introduction of new chromogenic media and alternative methods have had a marked effect on testing for Enterobacteriaceae. Colony recognition is made easier thus improving method specificity and reducing the amount of confirmation required. Traditional media such as VRBGA rely on the use of dyes and bile salts to make them selective for enteric bacteria. Consequently, these media can be inhibitory to sub-lethally injured cells and Enterobacteriaceae that are sensitive to these compounds. In contrast, the composition of many new chromogenic media and those used with the alternative methods has been optimised to improve the successful recovery of these bacteria. They are also less dependent on the selective agents to provide the desired specificity. The results from these newer media compositions can therefore yield higher and more accurate results compared to some standard conventional methods that employ older traditional culture media designs. Greater specificity is offered by molecular methods targeting the Enterobacteriaceae 16S rRNA gene. Methods have been developed to detect total Enterobacteriaceae in foods using the polymerase chain reaction (PCR) technique (Nakano *et al.*, 2003; Martinon *et al.*, 2011) and to quantify coliforms (lactose-fermenting Enterobacteriaceae) using real time quantitative PCR (Martin *et al.*, 2010) and total Enterobacteriaceae using specific 16S rRNA probes and the fluorescent *in situ* hybridisation (FISH) technique (Ootsubo *et al.*, 2003). However, despite the advances, these methods are not yet widely used.

5. ENTEROBACTERIACEAE AS FOODBORNE PATHOGENS

The Enterobacteriaceae includes some of the most intensively studied microorganisms, including several important foodborne pathogens. Notable examples are typhoid and non-typhoid *Salmonellae*, *Shigella dysenteriae*, *Yersinia enterocolitica* and a range of pathogenic *E. coli*, including *E. coli* associated with traveller's diarrhoea and *E. coli* O157:H7, which has become one of the most important foodborne pathogens. Moreover, some Enterobacteriaceae have emerged or could potentially become pathogenic as a result of the acquisition of virulence associated genes (toxins, colonisation factors) carried on mobile genetic elements such as transposons, plasmids, insertion sequences and bacteriophages. Mechanisms of virulence are summarised in Table 5.

Table 5: Summary of virulence characteristics of pathogenic Enterobacteriaceae

| Pathotype | Pathogenicity associated genes or factors | Mechanism | Clinical features |
|---------------------------------------|---|--|--|
| Enteroinvasive <i>E. coli</i> (EIEC) | 140 MDa plasmid (pInV) <i>lpa</i> encodes invasion plasmid antigens (IpA, IpB, IpC, IpD and IpH) Chromosomal genes | Bacterial attachment and invasion of colonic enterocytes via endocytosis, multiplication causing host cell death and inflammation accompanied by necrosis and ulceration of large bowel. | Ulceration of bowel, watery diarrhoea, dysenteric stools (bacillary dysentery) |
| Enterotoxigenic <i>E. coli</i> (EPEC) | Plasmid encoded Colonisation Factor Antigens (CFAs) CFAI (rigid rod-like fimbriae), III (bundle forming group), II & IV (flexible fimbriae) and type IV related Longus pili | Colonisation of surface of small bowel mucosa (CFA I-IV) and production of enterotoxins LTI, LTII, STa, STb). | Acute watery diarrhoea, usually without blood mucus or pus |
| | Labile toxins: LTI, LTII (plasmid encoded) | ADP ribosylation of G proteins → adenylate cyclase activation → increased cAMP secretion → reduced Na ⁺ absorption/Cl ⁻ secretion → diarrhoea. | |
| | Heat stable toxins: STa, STb (plasmid and transposon encoded) | Guanylate cyclase (G C-C) activation → increased cGMP secretion → chloride secretion and/or inhibition of NaCl absorption → diarrhoea. | |
| Enterotoxigenic <i>E. coli</i> (EPEC) | Plasmid (60 MDa) encoded: Aggregative adherence fimbriae (AAF/I & AAF/II), transcriptional regulator (AggR) | Adherence and colonisation of intestinal mucosa facilitated by AAF/I & AAF/II. | Aggregative adherence (AA) phenotype |
| | <i>E. coli</i> heat stable-like toxin-1 (EAST1) Plasmid encoded toxin (Pet) | Release of toxins and damage to host epithelial cells. | Persistent diarrhoea |

| | | | |
|---|--|--|--|
| Diffusely Adherent <i>E. coli</i> (DAEC) | Afa/Dr family adhesins (AIDA adhesins) EAST-1 Set genes (enterotoxins) Possible TTSS involvement | DA phenotype facilitated by surface fimbriae, e.g., F1845 encoded via <i>daaC</i> gene, or by other related adhesins, which are plasmid or chromosomally encoded. Events in pathogenesis remain unclear. | Diffusely adherent (DA) phenotype Watery diarrhoea, usually without blood |
| Enteropathogenic <i>E. coli</i> (EPEC) | | | |
| Typical EPEC (tEPEC) | 50-70 MDa plasmid (pEAF) encodes: Bundle forming pilus (BFP) and Per (plasmid encoded regulator). Virulence factors mainly encoded by the pEAF and the LEE. | Localised adherence (LA) via BFP | Acute diarrhoea (especially in children <1 year old) |
| Atypical EPEC (aEPEC) | Various virulence genes (some found in EAEC, VTEC and others of unknown function). Various adhesins. | LA Like (LAL), diffuse adherence, aggregative adherence and no adherence patterns | Diarrhoea (often milder than caused by tEPEC) |
| Both tEPEC and aEPEC | Chromosomal PAI: locus for enterocyte effacement (LEE) TTSS comprising intimin (<i>eaeA</i>), secreted proteins: Tir, EspA, B, D, F, G & MAP EAST-1 Cytotoxic distending toxin (CDT) in O86:H34 strains | A/E histopathology; cytoskeletal rearrangement of host epithelial cells involving TTSS. Intimate effacing adherence mediated by intimin. Destruction of microvilli and interference with host cell signalling cascades. | A/E lesions |
| Enterohaemorrhagic <i>E. coli</i> (EHEC) | Large (60 MDa) plasmid encodes: enterohaemolysin (Ehx), LCT, EspP | A/E histopathology and intimate adherence similar to EPEC. Alternative adherence mechanisms (besides <i>eae</i>) known. TTSS aids pathogenesis, toxins (Stx/VT) inhibit protein synthesis of host cells and mediate different pathological effects. | Bloody diarrhoea (haemorrhagic colitis) |
| Verocytotoxin-producing <i>E. coli</i> (VTEC) | Chromosomal PAI (LEE) Chromosomal (prophage) encoded Shiga toxins (Stx)/Verocytotoxin (VT) VT1, VT2 & VT2 variants | | Haemolytic uraemic syndrome (HUS) TTP |
| <i>Shigella</i> spp. | Virulence plasmid encodes 2 loci (<i>ipa</i> and <i>mxi-spa</i>). <i>ipa</i> encodes invasion plasmid antigens (IpaA, IpaB, IpaC, IpaD and IpaH) <i>mxi-spa</i> operon encodes components of a TTSS and <i>mxi-spa</i> Shiga toxin (<i>S. dysenteriae</i> 1) | Invasion of the host's epithelial cells via the host's M cells involves <i>ipa</i> and <i>mxi-spa</i> . IpaB and IpaC produce pores in epithelial cells and IpaC and IpaD facilitate uptake of the bacterium into a vacuole by the epithelial cell. The TTSS delivers Ipa proteins from the bacterium to the host cell. Uptake of the bacterium and replication induces the host inflammatory response and the epithelial cells ultimately lyse. Shiga toxin (see EHEC). | Diarrhoea, fever, nausea, vomiting, stomach cramps, flatulence. Dysentery: stool may contain blood, mucus, or pus Seizures in children (rare) and reactive arthritis |

| | | | |
|-------------------------------------|---|---|---|
| <i>Salmonella</i> (non-typhoid) | <p><i>Salmonella</i> plasmid virulence (spv) genes (invasive salmonellae)</p> <p><i>Salmonella</i> pathogenicity island-1 (SPI-1) encodes a TTSS</p> <p>SPI-1 encoded proteins: SopE, SipA, SipC, StpP</p> | Translocation through M cells of Peyer's patches resulting in destruction of M cells and adjacent epithelium enables the <i>Salmonella</i> to cross the small intestine epithelium. A TTSS exports proteins into the host cells to initiate cytoskeletal rearrangement and bacterial internalisation by macrophages. | Nausea, diarrhoea and vomiting, fever and abdominal pain. |
| <i>Salmonella</i> (Typhi/Paratyphi) | Vi antigen (Vi polysaccharide) | Multiplication in the intestine. Transportation from the small intestine to the mesenteric lymph nodes via M cells of the Peyer's patch. Dissemination is via the lymphatic system and blood stream. | Fever, diarrhoea, secondary bacteraemia and reinvasion of gut via the liver and gall bladder. Inflammation, ulceration and necrosis of the Peyer's patches can lead to perforation or haemorrhage. |
| <i>Yersinia enterocolitica</i> | <p>pYV or Lcr plasmid (64-75 kb) chromosomally encoded invasion genes (<i>inv</i>, <i>ail</i>)</p> <p>Large outer membrane protein (YadA) and OMP</p> <p>Plasmid encoded Yop virulon</p> <p>TTSS (Ysc), yersiniabactin, heat stable enterotoxins.</p> | Invasion of host cells is facilitated by pYV in conjunction with <i>inv</i> and <i>ail</i> gene. Bacteria colonise intestinal tract, transverse intestinal lumen, attach to and penetrate mucus layer of mucosal epithelial cells, and adhere to intestinal brush border membranes. YadA is involved in bacterial adherence. <i>Yersinia</i> type III secretion system and Yop proteins inhibit phagocytosis. | Wide range of symptoms dependent on strain, dose and susceptibility and age of host. Gastrointestinal syndromes: enteritis with fever, enterocolitis, inflammation of the lymph glands, diarrhoea, abdominal disorders (pseudoappendicitis). Although rare, reactive arthritis can occur following infection. |
| <i>Cronobacter</i> spp. | Secretory factors: glycopeptides, elastases, collagenases and other proteases Endotoxins | Translocation of the bacterium through the chordus plexus. Cellular invasion aided by secretory factors increases permeability of the blood-brain barrier enabling access to the brain. | Neonatal meningitis (ventriculitis, brain abscess/cyst formation and development of hydrocephalus). Other clinical manifestations: neonatal necrotising enterocolitis. Bacteraemia. |

pYV, plasmid associated with *Yersinia* virulence; Lcr, low calcium response; Yop, *Yersinia* outer membrane protein; OMP, outer membrane protein; *inv*, invasion (encodes *Yersinia* invasion); *ail*, attachment invasion locus.

5.1 *Salmonella*

In many developed countries *Salmonella* is the second most common cause of bacterial foodborne illness after *Campylobacter*. *Salmonella* are widely distributed in nature with a diverse range of host species including mammals, birds, fish and reptiles. Consequently, there are many recognised animal reservoirs and *Salmonella* remains an important zoonotic pathogen. *Salmonella* has recently been associated with contamination of fresh produce (e.g., tomatoes, lettuce, fresh basil, melons). Two *Salmonella* species are now recognised; *S. enterica*, which includes serotypes commonly associated with the majority of food-related infections, and *S. bongori*, which is commonly associated with reptiles. *Salmonella* cause two distinct forms of disease, these being non-typhoid salmonellosis and typhoid and paratyphoid disease as explained below.

Non-Typhoid Salmonellosis

Salmonella generally cause illness by localised infection of the gastrointestinal tract. Infection is characterised by colonisation and attachment of the bacteria to epithelial cells, and subsequent invasion of the intestinal tissue. During this invasive process, a heat-labile enterotoxin is secreted by the bacterium that precipitates a net efflux of water and electrolytes into the intestinal lumen resulting in diarrhoea. During infection by *Salmonella* a strong innate immune/inflammatory response is induced in the host.

Humans infected by *Salmonella* typically develop nausea, vomiting, fever, abdominal cramps and diarrhoea. The severity of the symptoms will depend on several factors such as the serotype, numbers of bacterial cells ingested and the age and susceptibility of the human host. Symptoms generally appear within 12 to 72 h and the duration of the illness is usually 4-7 days. Most healthy people recover without specific treatment, although occasionally the bacterium can enter the blood stream or the lymphatic system resulting in systemic infection and more severe illness or even death.

Typhoid fever

Typhoid is caused by *Salmonella enterica* subsp. *enterica* serotype Typhi and is common in developing countries, although the incidence of the disease is falling worldwide. Consumption of contaminated food and water or contact with a patient or carrier of the disease are common vehicles of infection. Unlike other salmonellae, there is no known animal reservoir so the host range is restricted to humans. Once ingested the bacteria pass through the stomach and enter the intestine where multiplication occurs. Transportation of the bacteria from the small intestine to the mesenteric lymph nodes is via microfold cells (also termed M cells) of the Peyer's patches (aggregations of lymphoid tissue usually found in the lowest portion of the ileum). The bacteria perforate and enter through the intestinal wall and are phagocytosed by macrophages, where they can exist and avoid destruction by neutrophils, complement and the immune response. While inside the macrophages, the bacteria spread via the lymphatic system and blood stream. This gives them access to the different organs throughout the body. Typhoid fever can be divided into four individual stages each of approximately one-week duration. During the first week, there is a slowly rising temperature with relative bradycardia (slowing of the heart rate), malaise, headache, cough and abdominal pain. In the second week, the patient lies prostrate with high fever (up to 40°C), delirium is common and rose spots may appear on the lower chest and abdomen. Diarrhoea can also occur during this stage although constipation is also common and the spleen and liver may also become enlarged. In the third week of typhoid fever, several complications can occur. These include haemorrhage of the intestine caused by bleeding in congested Peyer's patches which, although serious, is not usually fatal. However, intestinal perforation in the distal ileum is a serious complication, which is often fatal. Other complications include septicaemia, diffuse peritonitis, encephalitis, metastatic abscesses, cholecystitis, endocarditis and osteitis. In the fourth stage, towards the end of the third week and into the fourth week, the fever begins to subside and the surviving patient begins to recover.

Paratyphoid fever

Three *Salmonella enterica* serotypes that cause paratyphoid fever are Paratyphi A, Paratyphi B (previously *S. schotmulleri*) and Paratyphi C (previously *S. hirschfeldii*). Strains belonging to serotype Paratyphi B that do not ferment D-tartrate have been designated *S. enterica* ser. Java, although they are closely related and both cause similar disease, including gastroenteritis. These salmonellae are transmitted by contaminated water or food. Infections with *S. enterica* ser Paratyphi A are common in Africa, with a recent increase in the Far East and India. The disease is similar to typhoid but rose spots are more abundant and larger. In Europe Paratyphoid B is more common. This is characterised by typhoid-like illness, or as a severe gastroenteritis or illness with features of both. Although an uncommon infection, Paratyphoid C is still found in the Far East. Clinical features include septicaemia with metastatic abscesses and cholecystitis can sometimes occur.

5.2 *Yersinia enterocolitica*

Yersinia enterocolitica is widely distributed in nature and can be found in soil, water and animals, notably pigs in which the bacterium is a commensal. A large number of *Y. enterocolitica* biogroups and serogroups exist, but only a few of these are pathogenic to humans and these serogroups are frequently associated with a specific host (Bottone, 1997). Strains belonging to biogroup 1A are not commonly associated with human infections unlike serogroups within biogroup 1B (O:8; O:4; O:13a,13b; O:18; O:20 and O:21), biogroup 2 (O:9; O:5,27), biogroup 3 (O:1, 2, 3 and O:5, 27), biogroup 4 (O:3) and biogroup 5 (O:2, 3; O:3 and O:1, 2, 3). In Europe, *Yersinia enterocolitica* biogroup 4 (serogroup O:3) and biogroup 2 (serogroup O:9) are commonly associated with human disease (EFSA, 2007a). In the USA, where sporadic infections are less commonly reported, biogroup 1B is commonly associated with outbreaks. Pathogenicity is associated with carriage of a virulence (64-75 kb) plasmid designated pYV, which codes for a range of outer membrane proteins termed *Yersinia* outer proteins (YOPs) that are involved in pathogenicity along with chromosomally encoded invasion genes (e.g., *inv* and *ail*). The first stage of infection involves colonisation of the intestinal gut and the bacterium transverses the intestinal lumen, attaches to and penetrates the mucus barrier overlying the intestinal cell brush border membranes. The bacterium localises to the terminal ileum and proximal colon with the aid of a large outer membrane protein (YadA). Most of their pathological effects occur in this region. Invasion involves evading host cell defences and like other enteroinvasive bacterial species (e.g., *Salmonella* and *Shigella*), *Y. enterocolitica* can penetrate the M cells of the host with the aid of chromosomally encoded gene products and the phagocytic activity of the M cells. Pathogenic *Y. enterocolitica* strains also produce a heat-stable enterotoxin (Yst) that resembles STa of *E. coli*, although its role in diarrhoeal disease remains controversial.

Infection by *Y. enterocolitica* can invoke a wide range of gastrointestinal symptoms that are dependent on the strain, dose and susceptibility and age of the host. Children under 5 years of age are most susceptible and in children and adolescents gastroenteritis and inflammation of the lymph glands are the main symptoms. Pseudo-appendicitis is also associated with *Y. enterocolitica* infection, especially in young adults. In adults, diarrhoea and abdominal disorders are the commonest symptoms. Infection can occasionally result in complications including skin disorders and arthritis.

A variety of foods can become contaminated with *Y. enterocolitica*. Raw or undercooked pork and pork products are commonly associated with this pathogen, although unpasteurised milk and untreated water can also act as vehicles for infection. Most infections are sporadic, although in the USA outbreaks have been traced to contaminated pork and pork products.

5.3 *Yersinia pseudotuberculosis*

Yersinia pseudotuberculosis is the cause of chronic diarrhoea and mesenteric adenitis (inflammation of the mesenteric lymph nodes in the abdomen) in animals. In humans, symptoms are similar to those of infection with *Y. enterocolitica* (fever and right-sided abdominal pain), although diarrhoea is often absent. Consequently, the condition is difficult to diagnose and infections can mimic appendicitis, especially in children and younger adults. In rare cases the disease may cause skin complaints, reactive arthritis (joint stiffness and pain) or the bacteria can spread into the bloodstream (bacteraemia). Infection of humans by *Y. pseudotuberculosis* is now less common due to improvements in the hygiene of the water supply. However, contamination of fresh produce has been linked to this organism; for example, several cases of gastroenteritis in Finland in 2004 that were attributed to grated carrots, possibly contaminated on the farm during storage.

5.4 Pathogenic *Escherichia coli*

Escherichia coli is commonly found in the gastrointestinal tract of humans and a wide range of other animals. This bacterium can be shed, often in high numbers, via the faeces into the environment. Most strains are harmless commensals whereby the hosts remain as asymptomatic carriers of these bacteria. Some carriers can harbour pathogenic strains and consequently act as reservoirs for subsequent contamination of water, foods and the environment. The concept of pathotypes has long been used as a convenient way to describe *E. coli* strains carrying specific virulence-associated genes, the production of distinct toxins and the clinical characteristics associated with infection. This bacterium is genetically diverse and there are several traditional *E. coli* pathotypes recognised that are responsible for distinctive clinical diseases or syndromes (Nataro and Kaper, 1998; Baylis *et al.*, 2006). Owing to the genetic mobility of some virulence-associated genes (i.e., they can be passed between bacteria via mobile genetic elements such as plasmids), *E. coli* strains cannot simply be classified as belonging to a single pathotype, as the pathotype could change following the acquisition of new virulence-associated genes. Nevertheless, the different pathotypes described below still provide a convenient way to describe pathogenic *E. coli* strains.

The extraintestinal *E. coli*, include uropathogenic *E. coli* (UPEC), which are responsible for a large proportion of urinary tract infections, and the meningitis associated *E. coli* (MAEC), which cause meningitis in neonates. Additionally, *E. coli* has been implicated in infections of the blood: reports of bacteraemia associated with *E. coli* have increased recently. In comparison, the intestinal pathogenic *E. coli* (IPEC) is responsible for a range of diarrhoeal diseases and syndromes in man. Most of the pathotypes (such as enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC)) have a human reservoir and are predominantly diffused via the faecal-oral route and only occasionally transmitted via foods or water. It is one group in particular, the so called Enterohaemorrhagic *E. coli* (EHEC), that are known to also have a source in animals, especially ruminants, and this pathotype has often been associated with foodborne infections. The EHEC represents a sub-group of a much larger group of *E. coli* termed Verocytotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC).

Verocytotoxin-producing E. coli (VTEC)

The VTEC are characterised by their ability to produce potent toxins termed Verocytotoxins or Shiga toxins, so termed because of their structural similarity to Shiga toxin from *Shigella dysenteriae* I. Two antigenically distinct forms of VT (VT1 and VT2) were initially identified (Scotland *et al.*, 1985). At least 3 subtypes of VT1 (VT1, VT1c and VT1d) and seven subtypes of VT2 (VT2, VT2c, VT2d, VT2d3, VT2e, VT2f and VT2g) are recognised (Persson *et al.*, 2007). The toxin-encoding genes (*vtx/stx*) are generally elaborated by prophages of the lambda (λ) family and VTEC strains produce either VT1 or VT2 alone or both. Besides VT, some VTEC also possess additional virulence associated genes that are involved with colonisation and pathogenicity. Some VTEC, notably EHEC strains, cause attaching and effacing (A/E) lesions on epithelial cells using similar mechanisms to those found in EPEC. Transmitted by horizontal transfer, the pathogenicity island (termed the locus of enterocyte effacement (LEE) is an important cluster of genes in the bacterial chromosome involved in A/E lesions in the large intestine. The LEE encodes a type III secretion system (TTSS) and associated chaperones and effector proteins responsible for the characteristic re-arrangement of the actin in the cytoskeleton of the host cell beneath adherent organisms leading to the effacement of the microvilli and the formation of the actin-rich pedestal. The *eaeA* gene, located at the LEE, codes for an important outer membrane protein, intimin, which mediates direct binding of the bacterium to the host cell surface.

Infection with VTEC can result in a range of clinical manifestations. These include asymptomatic carriage through mild diarrhoea to life-threatening conditions such as haemorrhagic colitis (HC)

and the severe complications of haemolytic uraemic syndrome (HUS) causing kidney damage and renal failure. Thrombotic thrombocytopenic purpura (TTP), which involves severe neurological symptoms, may occur in a small proportion of the HUS cases, especially among elderly patients. Both HUS and TTP conditions can be fatal. The infectious dose can be as low as 10-100 cells. Severe human disease is commonly associated with strains carrying *eaeA* and producing VT2 and VT2c. Production of VT1 is often associated with milder disease and is often found in VTEC isolated from animals and food. EHEC strains, such as VTEC O157:H7 are often associated with severe disease and the carriage of additional virulence factors (e.g., enterohaemolysin) on large plasmids.

Apart from serogroup O157, other VTEC serogroups have also been associated with human disease, the five dominant non-O157 VTEC serogroups being O26, O111, O145, O103 and O91 (EFSA 2007b). However, further VTEC serotypes may also be involved in human infections; for example, the O104:H4 strain of EHEC isolated from cases in the EHEC infection outbreak in Germany in Spring 2011 was reported to be seen only rarely in humans and never before in an EHEC outbreak. Prior to the 2011 outbreak, only one case identified as *E. coli* O104:H4 had been documented in the literature. In Germany, with more than 3,600 people reported ill, over 850 of them suffering from HUS, including 40 fatalities, the implicated strain of *E. coli*, O104:H4, was unusual in many respects. It carried one of the Verocytotoxin (Shiga toxin) genes, namely the Verocytotoxin 2 gene (*vtx2a* variant) but contained neither the *eae* gene nor the enterohaemolysin gene. In this outbreak, it was not just the observation of a rare serotype (O104:H4), but also and importantly the combination of virulence traits, that occurred in the outbreak strain, that is to be noted. The outbreak strain possessed an unusual combination of virulence factors of STEC/VTEC and enteroaggregative *E. coli* (EAEC), which are described later. The strain was shown to share 93% of its genome sequence with a previously isolated O104:H4 EAEC strain. For this reason the outbreak strain was referred to as an Enteroaggregative Verocytotoxin-producing *E. coli* (EAggEC VTEC). EAggEC strains can belong to a wide range of different serogroups and EAEC VTEC have been found before. EAEC that produce VT2/Stx2, but do not harbour the genetic markers for classical EHEC, have been reported to be involved in HC and HUS in patients with HIV in Bangui, Central African Republic (Mossoro *et al.*, 2002). Furthermore, EAEC VTEC have also been reported to be involved in a European outbreak (Morabito *et al.*, 1998). EAEC and, according to research findings currently available, EAEC VTEC have a human reservoir, whereas the "classical" VTEC strains have a predominantly animal reservoir. It should be noted however, that VTEC strains (defined as *E. coli* strains that carry one or multiple VT genes) in sheep and cattle, which merely carry the VT genes, but do not possess the ability to adhere (as encoded by *eae*-gene in EHEC or *aggR*-gene in EAEC), are unlikely to be pathogenic to humans. The adhesion factor seems to be a prerequisite for the bacteria to cause severe disease in humans.

The German EAEC VTEC O104:H4 outbreak strain was also shown to produce extended-spectrum β -lactamase enzymes (also referred to as ESBL *E. coli*) that render the *E. coli* bacteria resistant to many different antibiotics. This illustrates the potential for horizontal gene transfer within the *E. coli* group. In addition, the complexity of this VTEC group of *E. coli* (including EAEC VTEC) with its various serotypes, possessing a variety of virulence factors and different modes of pathogenicity, is reflected in the subsequent difficulties in defining and characterising the *E. coli* group. In turn, this causes difficulties in using the group as targets for food monitoring and in the detection and identification of *E. coli* strains that can cause severe disease (diarrhoea, HUS, fatalities) in humans. Since 1982, when it was first recognised as a foodborne pathogen, *E. coli* O157:H7 has become one of the most important pathogens responsible for sporadic disease and large water and foodborne outbreaks. Raw meats, especially beef and undercooked burgers, were the first foods to be associated with this pathogen. Unpasteurised milk and dairy products and contaminated fresh produce (e.g., sprouted seeds, lettuce and spinach) are also known vehicles for *E. coli* O157:H7 as well as other VTEC. Cattle and sheep are common reservoirs for VTEC, including serotype O157:H7, which are also commonly found in the environment.

Enteropathogenic E. coli (EPEC)

The EPEC pathotype can be divided into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC). Both tEPEC and aEPEC have the ability to produce A/E lesions, a feature encoded and regulated by genes of the LEE, using a mechanism similar to that found in VTEC. A key characteristic of tEPEC is their adherence to the surface of HEp-2 cells in a localised adherence (LA) pattern. Adhesion of tEPEC is mediated by the bundle-forming pilus (BFP) encoded by the *bfpA* gene, which is located on a 50-70 MDa EPEC adherence factor (EAF)-encoding plasmid (pEAF). The presence of the pEAF and the associated plasmid encoded regulator (Per) in tEPEC and its absence in aEPEC differentiates these two types of EPEC. By comparison, aEPEC strains exhibit a variant LA pattern characterised by loose clusters of bacteria, termed LA Like (LAL), whereas other aEPEC can express a diffuse adherence pattern, aggregative or no detectable adherence.

The reservoirs for tEPEC, which are almost exclusively associated with developing countries, are human as they have not been found in other animals. They are transmitted by inter-human contact and traditionally associated with infantile diarrhoea. The tEPEC cause either watery or bloody diarrhoea and during infections, EPEC bacteria adhere to intestinal epithelial cells and form actin-rich pedestals which is also a common feature of some EHEC strains. However, unlike *Shigella*, *Salmonella* and *Yersinia*, tEPEC strains are less invasive and unlike ETEC or EAEC, they cause an inflammatory response.

In contrast, aEPEC, which are found in developed as well as developing countries, are more closely related to VTEC and share similarities such as their association with a range of animal hosts and their subsequent transmission to humans via contaminated foods. Although any food exposed to faecal contamination can be associated with aEPEC, outbreaks have commonly been associated with raw meats but also with consumption of contaminated water. The aEPEC cause diarrhoea, although less is known about these strains than about tEPEC. In common with VTEC the aEPEC are recognised as emerging pathogens.

Enteroinvasive E. coli (EIEC)

Enteroinvasive *E. coli* (EIEC) closely resemble *Shigella* and are genetically, biochemically and pathogenically similar. The EIEC are atypical compared to the majority of *E. coli* strains. They are usually non-motile, do not decarboxylate lysine and approximately 70% of EIEC strains do not ferment lactose. These characteristics are similar to *Shigella*, although some strains of *S. sonnei* are able to ferment lactose, albeit slowly. Genetically *Shigella* is closely related to *E. coli*, in particular EIEC. Both cause an invasive, dysenteric form of diarrhoea in humans and infected humans appear to be the primary source as there are no known animal reservoirs for EIEC. Although uncommon in developed countries, EIEC have occasionally been implicated in food- and water-borne outbreaks. Outbreaks have been associated with contaminated soft cheese, potato salad and guacamole. Infection occurs via the faecal-oral route with contaminated food and water common vehicles. Food is usually contaminated by infected (carrier) food handlers. Person-to-person transmission has also been reported. The site of infection is predominantly the colon and the ability of EIEC to invade and destroy colonic tissue is associated with genes such as the invasion plasmid antigens IpaA to IpaH contained on a high (140 MDa) molecular weight plasmid (pINV). Common symptoms include watery diarrhoea that may precede dysenteric stools containing blood and mucus. Ulceration of the bowel can occur in severe cases.

Enterotoxigenic E. coli (ETEC)

Common in developing countries where sanitation is poor, ETEC continues to be a major cause of diarrhoea among infants and travellers. A characteristic feature of ETEC is their ability to express one or more heat-stable (ST) or heat-labile (LT) enterotoxins. One of the toxins (LTI) is plasmid encoded and resembles the cholera toxin produced by *Vibrio cholerae*. Another toxin (LTII) is chromosomally encoded yet is similar to LTI both in mode of action and in structure. Another toxin is

the plasmid-encoded heat-stable enterotoxin (ST) of which there are two groups (STa/STI and STb/STII). Infection by ETEC is characterised by watery diarrhoea with little or no fever. Consumption of contaminated foods, notably raw vegetables and soft cheeses, has been implicated in cases of infection and there have been sporadic waterborne outbreaks caused by ETEC.

Enteroaggregative *E. coli* (EAggEC) and Diffusely Adherent *E. coli* (DAEC)

Enteroaggregative *E. coli* (EAEC) are a major cause of chronic infantile diarrhoea in some developing countries. A characteristic phenotype of EAEC is the 'stacked-brick' pattern of adherence to the surface of host cells, which is associated with the carriage of a 60 MDa plasmid. The EAEC is a very heterogeneous group with strains producing a range of putative virulence factors that are associated with disease. These include plasmid-encoded aggregative adhesion fimbriae (AAF/I and AAF/II) regulated by transcriptional regulator AggR, which plays an important role in pathogenesis by EAEC. Enteroaggregative heat-stable toxin-1 (EAST-1), plasmid-encoded toxin (pet) and *Shigella* enterotoxin-1 (ShET1) are also produced by strains of EAEC. However, not all EAEC strains produce EAST-1 or Pet toxins and it is not known whether other toxins or virulence factors are involved.

Pathogenesis by EAEC involves adherence to the intestinal mucosa and colonisation. This results in damage to the hosts' epithelial cells, enhanced mucus production and diarrhoea, which is usually watery and protracted and often associated with abdominal pain. Some EAEC strains have been associated with production of VT2/Stx2 and the cause of severe disease, including HUS. This was highlighted by the EAEC O104:H4 outbreak strain in Germany in May/June 2011.

Diffusely adherent *E. coli* (DAEC) are characterised by a diffuse pattern of adherence to HEp-2 cells, presence of adhesions involved in diffuse adherence (AIDA) and the absence of virulence genes found in other *E. coli* pathotypes. Strains of DAEC may also produce EAST-1 and genes (*set*) encoding toxins found in *Shigella* spp. Unlike other *E. coli* pathotypes, little is known about the role of DAEC in disease. Clinical features include watery diarrhoea, usually without blood.

5.5 *Cronobacter* spp.

Cronobacter spp. are pathogens commonly associated with illness in neonates, especially low birth weight infants less than 2.5 kg and immuno-compromised infants. Before 1980 these pathogens were referred to as yellow pigmented *Enterobacter cloacae* because of the ability of strains to produce yellow-pigmented colonies, especially at 25°C on trypticase soy agar, blood agar and brain heart infusion agar after 48-72 h. In 1980, the bacterium was named *E. sakazakii* after the Japanese bacteriologist Riichi Sakazaki. More recently, polyphasic analysis revealed that *E. sakazakii* consisted of six different species, and it was therefore proposed that these species be moved to a novel genus "*Cronobacter*" (Iversen *et al.*, 2007; 2008). Unlike most *Enterobacter* spp, *Cronobacter* elaborate an α -glucosidase activity and this has been used as an important diagnostic marker to distinguish *Cronobacter* from *Enterobacter* spp. and other Enterobacteriaceae.

Cronobacter spp. (identified by their previous name, *E. sakazakii*) have been isolated from a variety of sources including the environment, clinical sources and a wide range of foods (Gurtler *et al.*, 2005; Lehner and Stephan, 2004; Friedemann, 2007). However, powdered infant formula (PIF) has been identified as one of the most epidemiologically important vehicles for transmission and there have been a number of documented outbreaks of neonatal *Cronobacter* spp. infections linked to this food product (Forsythe, 2005; Gurtler *et al.*, 2005). PIF is not sterile and poor treatment together with environmental contamination by *Cronobacter* spp. and subsequent improper storage of the reconstituted product, leading to growth of the bacteria, can result in the product being unsafe at the point of consumption. Despite its widespread distribution, the natural habitat of *Cronobacter* spp. remains unknown.

Neonatal meningitis is the most widely recognised clinical feature of *Cronobacter* spp. infection. This can result in further complications such as ventriculitis, brain abscess, cerebral infarction, cyst formation and development of hydrocephalus. A clinical manifestation associated with consumption of *Cronobacter*-contaminated PIF is the development of neonatal necrotising enterocolitis caused by bacterial colonisation of the intestinal tract. Infection can also result in bacteraemia. The virulence factors of *Cronobacter* spp. have yet to be fully elucidated, although strains are known to produce a variety of secretory factors that probably play a role in pathogenicity, e.g., endotoxins and a variety of proteases, including elastase and collagenase. Once the bacterium has translocated through the chordus plexus, the secretory factors increase the permeability of the blood-brain barrier and facilitate cellular invasion.

5.6 *Shigella* spp.

From a taxonomical perspective *Shigella* should be regarded as a sub-group of *E. coli* along with EIEC, with which they share a number of characteristics. There are 4 species of *Shigella* (*dysenteriae*, *flexneri*, *boydii* and *sonnei*). Infection is normally limited to the distal ileum and colon. Although rare, intestinal perforation can occasionally occur. An important complication associated with *Shigella* infection is bacteraemia and seizures are common, especially with *S. dysenteriae* infection. Invasion of the host's epithelial cells occurs via the M cells and involves two loci (*ipa* and *mxi-spa*) encoded by a virulence plasmid. Invasion plasmid antigens (*IpaA*, *IpaB*, *IpaC* and *IpaD*) are encoded by the *ipa* operon, and the *mxi-spa* operon encodes components of a TTSS that delivers *Ipa* proteins from the bacterium to the host cell, triggering bacterial invasion. Uptake of the bacterium is followed by *IpaB*-, and possibly *IpaC*-, induced lysis of the vacuole and release of the bacteria into the epithelial cytoplasm. Bacterial cell replication follows inducing the host inflammatory response and the eventual lysis of the epithelial cells. Some *Shigella* produce enterotoxin and *S. dysenteriae* 1 also produce a potent toxin (Shiga toxin), which is structurally related to VT1 produced by strains of VTEC (see above), but unlike VTEC this toxin gene is chromosomally encoded. Shiga toxin can cause vascular damage and HUS in some patients.

Common symptoms associated with *Shigella* infection include diarrhoea, fever, nausea, vomiting, stomach cramps and flatulence. In cases of *Shigella*-associated dysentery, which results in the destruction of the epithelial cells of the intestinal mucosa in the caecum and rectum, the stool may contain blood, mucus, or pus. Seizures can occur in rare cases, especially in young children and *Shigella* have been implicated as one of the causes of reactive arthritis. *Shigella* are adapted to humans and the infective dose is very low (as few as 10 cells). Shigellosis is commonly contracted through person-to-person contact or via the faecal-oral route. In many European countries, foodborne shigellosis is uncommon. Foods implicated in outbreaks in the USA and S. Africa include fresh produce (salads), fresh juices and foods that are susceptible to contamination by infected food handlers.

5.7 Opportunistic and emerging pathogenic Enterobacteriaceae

Besides the well-known pathogens that belong to the Enterobacteriaceae family, some members such as *Providencia alcalifaciens*, *Klebsiella* spp., *Serratia* spp. and *Citrobacter* spp. have been implicated in human disease or can be the cause of opportunistic infections including bacteraemia, meningitis, urinary tract infections and wound infections, and are therefore important in clinical settings.

Providencia alcalifaciens

Providencia alcalifaciens are widely distributed in nature and can be isolated from both human and animal faeces. This bacterium was once assumed not to be a human pathogen although it has been implicated as a cause of diarrhoea (Hawkey, 2006). However, there is now good evidence

that this species can cause diarrhoeal illness, especially in children, as well as being a cause of traveller's diarrhoea and that food could be a vehicle. In a large outbreak of food poisoning in Japan, *P. alcalifaciens* was identified as the likely aetiological agent (Murata *et al.*, 2001). Although the sources and routes of transmission of *P. alcalifaciens* in diarrhoeal illness have not been fully investigated, excretion of this bacterium in the faeces of patients suffering from diarrhoea as well as asymptomatic carriage are potential risk factors. Transient hand carriage and faecal contamination of foods are therefore likely routes of transmission.

Citrobacter spp.

Citrobacter spp. are widely distributed in the environment and are commonly found in the intestinal tract of humans and other animals. *Citrobacter* spp. are opportunistic pathogens and *C. koseri* would appear to be an important neonatal pathogen responsible for meningitis. *Citrobacter* spp. have been shown to carry various virulence determinants found in other pathogens. The *eae* gene found in EPEC and EHEC has been found in *C. rodentium* as well as some strains of *C. freundii*. An outbreak of HUS in Germany was linked to the consumption of butter containing parsley contaminated with *C. freundii* carrying the *vtx2* gene (Tschäpe *et al.*, 1995).

Serratia spp.

Serratia spp. are also widely distributed in the environment, being commonly found in water, soil and plants as well as occasionally in the human intestinal tract. They are opportunistic and nosocomial pathogens, with the most important being *S. marcescens*. Contaminated soil and plants have been linked to community-acquired infections.

5.8 Extended-spectrum β -lactamase (ES β L)-producing Enterobacteriaceae

There is increasing concern that foods could act as potential vehicles for the dissemination of antibiotic-resistant bacteria among humans. Some Enterobacteriaceae, particularly *E. coli*, *Klebsiella* spp. and *Salmonella* spp. produce plasmid-mediated enzymes (class A β -lactamases) termed extended-spectrum β lactamases (ES β Ls). These enzymes can confer resistance by catalysing the hydrolysis of a variety of β -lactam antimicrobial compounds, including oxyimino- 2nd, 3rd and 4th generation cephalosporins (e.g., ceftazidime, cefotaxime, ceftriaxone and cefepime), monobactam (e.g., aztreonam) and penicillins (except temocillin), but not carbapenems or cephamycins. ES β Ls are inhibited by β -lactamase inhibitors, e.g., clavulanic acid, but many ES β L producing strains are resistant to commercially available penicillin-inhibitor combinations and are multiple resistant to other antibiotics including fluoroquinolones and aminoglycosides.

ES β L-producing strains have been associated with increased morbidity and mortality in patients with severe infections, particularly bacteraemias and nosocomial pneumonias. The presence of ES β L-producing Enterobacteriaceae and the resistance they cause complicates the treatment of many simpler infections resulting in hospitalisation of patients who would normally be treated with an oral antibiotic in the community. ES β Ls have been known since the 1980s and most early types were mutants of the long-established TEM and SHV plasmid mediated penicillinases. These were largely associated with nosocomial *Klebsiella* spp. and transmission was within hospitals via staff or contaminated equipment. However, since the turn of the century a new class of ES β Ls, the CTX-M types, has spread and are now more prevalent in *E. coli* than the earlier TEM and SHV variants. Five major sub-groups of CTX-M ES β Ls and over 90 minor variants are known. All appear to represent genetic escapes from the chromosomes of *Kluyvera* spp. The CTX-M ES β Ls have spread among *E. coli* and have become at least as frequent as TEM and SHV ES β Ls in hospital *Klebsiella* spp. There has also been a more limited spread among *Salmonella* spp. The VTEC EAEC O104:H4 responsible for the outbreak in Germany in 2011 was carrying ES β L CTX-M-15 and TEM-1 making it resistant to several antibiotics.

Person-to-person dissemination within the community is recognised as an important source of *E. coli* with CTX-M ES β Ls in the community (Mesa *et al.*, 2006; Lo *et al.*, 2010). Food contamination could contribute to the wider spread of producer strains, (Lavilla *et al.*, 2008). There are several reports of ES β Ls being isolated from foods, especially those of animal origin (Machado *et al.*, 2008; Jouini *et al.*, 2007; Rodriguez *et al.*, 2009; Kolar *et al.*, 2010; Dhanji *et al.*, 2010).

6. ENTEROBACTERIACEAE IN FOOD SPOILAGE

Enterobacteriaceae may be present as the natural microflora of certain foods or can be introduced as a result of (post-)process contamination. *Proteus*, *Escherichia* and *Salmonella* can enter the food chain via faecal contamination and these may be associated with particular foods such as meat and poultry. *Citrobacter*, *Klebsiella*, *Serratia* and *Pantoea* are commonly found in the environment (soil, water and plants). *Erwinia*, *Brenneria* and *Pectobacterium* spp. are associated with plants, so they can often be found in foods of vegetable or plant origin. Other bacteria, such as *Enterobacter*, *Hafnia* and *Yersinia*, are also widely distributed and are therefore commonly encountered in foods. Enterobacteriaceae can cause food spoilage in a broad range of food products, namely milk, dairy products, meats, poultry, fish, seafoods, fruit, vegetables and other foods. The growth and metabolic activity of Enterobacteriaceae in food can result in off-flavours, odours, colour defects and other organoleptic deviations. These changes may arise from enzymatic breakdown of proteins or lipids, production of volatile components and gas production.

In milk and other dairy products, some strains of Enterobacteriaceae can impart bitterness by producing enzymes that break down casein. The thermostability of some lipases and proteinases suggests that high temperature-short time (HTST) and ultra-heat (UHT) treatments will not inactivate these enzymes. Besides flavour defects, some Enterobacteriaceae, notably *Serratia* spp., can produce a reddish pigment, whilst other bacteria may cause other colour defects (ICMSF, 1998). During the early ripening stage of fresh cheese production Enterobacteriaceae can also cause a spoilage termed "early blowing" which can result in holes within the product, or blown packs, caused by the production of gas from the fermentation of lactose (ICMSF, 1998). *Proteus*, *Escherichia* and *Enterobacter* are associated with defects including a condition termed "slimy curd" in cottage cheese (Cousin, 1982; Jay, 2000). *Serratia* spp. and other members of the Enterobacteriaceae are associated with spoilage of fresh cream desserts, characterised by clotting of the cream following acid and gas production (Sutherland et al., 1986). Growth of Enterobacteriaceae in meat and poultry is favoured by storage at 5°C and above. Enterobacteriaceae in fresh meats can utilise glucose and other simple carbohydrates and when these become exhausted they can then make use of free amino acids and related simple nitrogenous compounds. When this occurs, noticeable odours and spoilage become apparent. Foul odours in meats are generally associated with the breakdown of amino acids to yield hydrogen sulphide (H₂S), which is a product of sulphur-containing amino acids, ammonia (NH₃) from many amino acids and indole from tryptophan (Jay, 2000).

Fresh fish stored on ice is particularly susceptible to bacterial spoilage, although the role of Enterobacteriaceae is less significant compared to other spoilage bacteria, notably *Pseudomonas* spp. and *Shewanella putrefaciens*. Two important characteristics of fish spoilage bacteria is their ability to produce H₂S and to reduce trimethylamine oxide (TMAO) to trimethylamine (TMA), which has a strong "fishy" smell even at low concentrations. Most species of Enterobacteriaceae, with the exception of *Erwinia* and some *Shigella* spp., can reduce TMAO to TMA (Barrett and Kwan, 1985) leading to spoilage. More important than their potential to cause deterioration in the organoleptic properties of fish is the histidine decarboxylase activity of some members of the Enterobacteriaceae leading to production of histamine in fish belonging to the Scombridae family (e.g., tuna, mackerel), which in turn can result in scombroid poisoning (Baylis 2006).

Soft rots are a common type of spoilage of vegetables and fruits caused by Enterobacteriaceae, notably *Erwinia* spp., and especially *Pectobacterium* (*Erwinia*) *carotovorum*. This results from pectic enzymes breaking down pectins resulting in a characteristic mushy appearance, which is sometimes accompanied by a bad odour and water-soaked appearance.

Obesumbacterium proteus is associated with beer spoilage and various members of the Enterobacteriaceae have been associated with spoilage of maple syrup. Bacterial rots in shell eggs have been attributed to Enterobacteriaceae with black rots caused by *Escherichia* and *Proteus* and red rots caused by *Enterobacter* spp. (ICMSF, 1998).

7. GROWTH, INACTIVATION AND SURVIVAL

Growth and survival characteristics of Enterobacteriaceae will determine if these bacteria will cause spoilage or adversely affect the organoleptic quality of a particular food, or both. Enterobacteriaceae are more susceptible to adverse conditions than Gram-positive bacteria. Their capacity to grow and cause food spoilage is mostly limited to perishable foods (i.e., those with a shelf-life < 10 days) especially foods kept under refrigerated storage. However, the precise conditions that enable growth and survival or cause inactivation of a particular strain of Enterobacteriaceae can differ significantly depending upon the genus and species, the inherent chemical and physical characteristics of the food (intrinsic factors), food processing (any action that substantially alters the initial product) and the storage conditions (extrinsic factors), and interactions with other microbial populations within the food (implicit conditions). As with other large bacterial families, these organisms show a large physiological diversity; thus, it is difficult to make generalised statements regarding growth and survival.

Intrinsic and extrinsic factors such as reduced temperature and pH, modified atmosphere and appropriate water activities (a_w) are often used in combination to ensure product stability and safety. The main objective of this so-called hurdle technology is to provide for an extended shelf-life, even for so-called shelf-stable products, and to prevent the growth or survival of pathogens in the food. Some traditional products may also contain intrinsically inhibitory substances that may also contribute to stability. Examples include essential oils and their active compounds such as carvacrol, eugenol and others, which are known to exert an antimicrobial activity towards many Enterobacteriaceae although their effects are not always reproducible in foods. The remainder of this section provides information on some key intrinsic and extrinsic factors, and how these can affect the Enterobacteriaceae in foods.

7.1 Temperature

The Enterobacteriaceae include psychrotrophic, mesophilic and thermotolerant bacteria. The majority are mesophilic, including the foodborne pathogens *Salmonella* and EHEC, with growth temperatures ranging between 15 and 40°C. Rapid cooling to the normal refrigeration temperatures of 0-8°C for storage facilitates growth inhibition (but not inactivation) of mesophilic Enterobacteriaceae, therefore they only multiply in perishable products when subjected to temperature abuse. An optimum growth temperature of 37°C is typical for Enterobacteriaceae of faecal origin. Psychrotrophic strains (defined as those bacteria capable of growing at temperatures down to 0°C and with an optimum growth temperature of 22-30°C) include strains of *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Proteus* and *Serratia* species (ICMSF, 1978) as well as some pathogenic *Yersinia enterocolitica*. The thermotolerant Enterobacteriaceae, which include strains of *E. coli* and *Klebsiella oxytoca*, comprise a sub-group of mesophiles capable of growth up to 44°C and having a minimum temperature for growth of > 7-8°C. Growth of micro-organisms, including Enterobacteriaceae, ceases at temperatures below -8°C. Along with most Gram-negative bacteria, Enterobacteriaceae are particularly susceptible to damage by freezing, which causes sub-lethal-injury along with a further gradual inactivation during prolonged storage. However, some cells can survive and may remain viable for long periods, so freezing is not a reliable control measure or inactivation process during food manufacture.

A 10-minute treatment at 55°C would normally destroy most psychrotrophic bacteria and heat-sensitive mesophiles, whereas the more heat-resistant non-spore forming mesophiles, including some enterococci and lactobacilli, would be destroyed at 65°C. Heat resistance of micro-organisms however, depends on the history of the strain and the characteristics of the food, e.g., fat content and water activity. Heat tolerance at lower a_w is increased, particularly at temperatures $\geq 70^\circ\text{C}$. Moreover, the extent of protection afforded by reduced a_w varies with solute type (Mattick *et al.*, 2001).

Minimal time/temperature combination for pasteurisation used in the dairy industry, e.g., 30 min at 63°C or equivalent, will destroy all Enterobacteriaceae present. The heat resistance displayed by *Cronobacter* spp. in reconstituted powdered infant formula is reported to be diverse with decimal reduction time (D-values) at 58°C ranging from 0.39 to 0.60 min (Breeuwer *et al.*, 2003) and some with an average D-value at 58°C of 9.9 min (Edelson-Mammel and Buchanan, 2004). However, even the most heat-resistant *C. sakazakii* strain will be reduced by more than 8 log₁₀ units by standard pasteurisation practices (15 s at 72°C) and a 5 log₁₀ reduction by 68°C for 16 s (Nazarowec-White *et al.*, 1999). Inactivation of *Cronobacter* spp. will occur quickly at temperatures above 70°C as recommended by the World Health Organization for reconstitution of powdered formula.

Cooking burgers to 70°C for 2 min or equivalent has been upheld as a recommended heat treatment by the ACMSF *Ad Hoc* Group on Safe Cooking of Burgers (ACMSF, 2007). This treatment should destroy vegetative pathogenic bacteria including pathogenic Enterobacteriaceae.

7.2 Water activity (a_w)

Most Enterobacteriaceae grow best at above a_w 0.95, with a minimum a_w limit close to 0.94. Therefore, foods with a water activity providing optimal conditions for growth, especially those with a_w values of 0.98 or above, such as fresh meat and fish, fresh fruits and vegetables and milk are most commonly associated with spoilage and food safety issues involving Enterobacteriaceae.

Lowering the a_w will substantially reduce the growth rate of some Enterobacteriaceae. For example, some salmonellae can divide at only one half of their maximal rate at a_w 0.97 and at about one-quarter at a_w 0.96 (ICMSF, 1980). Moreover, in foods with a_w below 0.98 Enterobacteriaceae are often at a greater disadvantage compared to other bacteria (particularly Gram-positive) and they may be out-competed by the natural microflora.

The pathogens *Salmonella*, *Cronobacter* spp. and pathogenic *E. coli*, however, may survive for prolonged periods (several months) in low a_w foods. Examples include foods containing high levels of sucrose, e.g., chocolate, cake, biscuits and confectionery, and those containing salt, e.g., dry and semi-dry fermented meat products such as salami and dried foods such as milk powder, flour and powdered infant formula.

The length of survival in these foods may vary depending on the species. For example, *Salmonella enterica* ser. Typhimurium have been shown to survive for longer periods of time than *E. coli* in fine granulated sugar (Samaraweera *et al.*, 2001). Survival at low a_w also depends on the temperature, with better survival at refrigeration temperatures than at ambient temperature. The chemical composition of the matrix also plays a role: in foods with a high sugar content survival is better compared to survival in salted foods (with equal low a_w).

7.3 pH

The minimum pH for growth of Enterobacteriaceae is 3.8 with an upper limit of approximately pH 9.0. However, pH tolerance is often influenced by the nature of the acidulant, with organic acids (e.g. lactic acid) being more inhibitory than mineral acids (e.g. hydrochloric acid). Enterobacteriaceae are normally only responsible for deterioration of foods with neutral or slightly acidic pH. *Shigella* spp. and VTEC are sometimes referred to as particularly suited to survive at low pH. It is not possible precisely to define either the pH tolerance of a species, e.g., *Shigella* spp., or their survival under conditions below the minimum pH for growth, because of the variability of the strains of even a single species of bacterium. Furthermore, food isolates may exhibit increased resistance to acid compared to culture collection strains, including type strains as was illustrated with some *Shigella sonnei* and *S. flexneri* strains (Bagamboula *et al.*, 2002), which could be due to multiple sub-culturing and long-term storage

of these bacterial collections. Perhaps the most notable Enterobacteriaceae associated with acid resistance are strains of *E. coli* O157:H7, which are reported to survive at pH levels below pH 3.8. These pose a food safety risk for certain foods e.g. fermented meats. Survival under pH conditions that are sub-optimal for growth is also influenced by temperature, with ambient temperature enhancing inactivation (Zhang *et al.*, 2010). The same effect of temperature with faster inactivation at ambient temperature was noted for *E. coli* O157 kept under conditions representative of fermented sausage (Uyttendaele *et al.*, 2001a).

It is recognised that changes in the food chain and food preservation methods can exert a selective pressure on microbes enabling them to adapt and grow under challenging environmental conditions, which would normally be inhibitory to growth. The emergence of acid-resistant *E. coli* O157 strains, that can survive in contaminated fermented meat products, is an example of strains adapting to survive under conditions (e.g. low pH) that are otherwise considered inhibitory. It is also the case that some non-pathogenic *E. coli* strains can survive for prolonged periods at low pH. This increased potential for survival under acidic conditions combined with the low infectious dose has enabled *E. coli* O157 to emerge as a pathogen in acidic food products such as yoghurt, juices and salami that were assumed to be safe owing to their inherently low pH. For some strains of pathogenic bacteria, including *Salmonella* and *E. coli* O157, acid resistance is an intrinsic characteristic; for others it can be classified as an adaptive response and the previous history of the strain (i.e., prior exposure to sub-optimal pH) may enhance its subsequent survival under harsh acidic conditions (Buchanan and Edelson, 1996; Uyttendaele *et al.*, 2001b; Tassou *et al.*, 2009).

Acidity of foods can be increased either by fermentation or by addition of weak acids (e.g., lactic acid, acetic acid, citric acid). In fermented foods, growth inhibition and inactivation of Enterobacteriaceae can be accomplished by the rapid production of acid by lactic acid bacteria, or other bacteria used in some fermentation processes. These micro-organisms can be naturally occurring or added as starter cultures. In fermented meats, the fermentation process initially induces rapid sub-lethal injury of Enterobacteriaceae followed by inactivation. During commercial manufacture of fermented meat products, a significant inactivation (5 log₁₀ reduction in the USA) is required to ensure production of a safe product. This is essential due to the potential risk of survival of low infectious dose pathogens such as *E. coli* O157:H7.

7.4 Gaseous atmosphere

Although *Pseudomonas* are some of the most important spoilage organisms of proteinaceous foods, particularly meat and fish held at refrigeration temperature in air, psychrotrophic Enterobacteriaceae may also cause spoilage of these foods. Being facultative anaerobes, Enterobacteriaceae can grow on the surface and in the interior of foods. They are not usually inhibited by the growth of strict aerobes, whereas growth of Enterobacteriaceae can inhibit the growth of aerobic spoilage organisms. Packing meat and poultry under vacuum is effective when used in combination with refrigerated storage, although vacuum-packed meats are prone to spoilage by Enterobacteriaceae. Packaging foods under modified atmospheric packaging (MAP) is an effective way of extending a product's shelf-life. Common gas mixtures used include 60-80% oxygen and 20-40% carbon dioxide. In general, Gram-negative microorganisms are susceptible to CO₂. In fresh meat and fish in MAP, lactic acid bacteria will predominate and suppress growth of Enterobacteriaceae. However, upon temperature abuse, the concentration of dissolved CO₂ in the pack (and thus also its antimicrobial activity) will decrease, enabling growth of Enterobacteriaceae (Devlieghere *et al.*, 2002).

Cutting fresh produce affects the physiological processes of the plant tissue, e.g., respiration and ripening activities. To prolong shelf-life it is important to maintain physiological activity of the living plant tissue. Fresh produce is normally packed under low O₂ (1–5%) and high CO₂ (5–10%) concentrations (Jacxsens *et al.*, 1999). Maintaining the physiological activity of the plant tissue whilst retarding plant tissue die-off will retain some of the natural defences of the plant tissue against infection by different bacteria, including Enterobacteriaceae. Modifying the packaging atmosphere alone has little or no effect on growth of Enterobacteriaceae in packaged fresh produce, whereas temperature is an important factor that affects growth.

The nutritional composition of the fresh produce will also determine the type of bacteria that dominate during prolonged storage of pre-packed fresh produce. Gram-negative flora including Enterobacteriaceae will spoil the less-acidic produce e.g. leafy vegetables. Spoilage of sugar-rich vegetables such as carrots and bell peppers is commonly attributed to lactic acid bacteria that will suppress the Enterobacteriaceae. Intrinsic acidic properties of minimally processed fruits favour the growth of yeasts and moulds and in some cases lactic acid bacteria.

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GLOSSARY OF TERMS

Attaching and effacing (A/E) lesions: A/E lesions are characterised by the intimate adherence of certain bacteria (e.g., EPEC, *E. coli* O157 and other EHEC) to the epithelial cells of the gut cell wall, resulting in localised destruction of brush-border microvilli and rearrangement of host cell cytoskeletal proteins (pedestal formation) beneath the adherent bacterium. They are essential in the pathogenicity of the EPEC and EHEC infections. The genes involved in the A/E lesion formation are encoded by the LEE pathogenicity island.

Bacteraemia: Presence of bacteria in the blood (in contrast: septicemia refers to bloodstream infection which is clinically more significant).

Bacteriophage: Virus that infects a bacterium.

Cholecystitis: Inflammation of the gallbladder's wall.

Collagenase: Proteolytic enzyme that catalyzes the degradation of collagen.

Collagen: A protein consisting of large proportions of the amino acids glycine and proline and characterised by the presence of hydroxyproline and hydroxylysine.

Commensal: Living in a relationship in which one organism derives food or other benefits from another organism without adversely affecting or helping it.

Complement: Group of proteins normally found in normal blood plasma that combine with antibodies to destroy pathogenic bacteria and other foreign cells. It is part of the immune system (innate immune system) that is not adaptable and does not change over the course of an individual's lifetime. It can be recruited and brought into action by the adaptive immune system.

D value: The time required (usually expressed in minutes) at a given temperature to reduce the number of viable cells or spores of a given microorganism to 10% of the initial population.

eaeA: Gene associated with the production of the protein intimin, which mediates binding of the bacterium to the host cell surface.

Elastases: An enzyme that catalyzes the hydrolysis of elastin.

Elastin: A protein that coils and recoils like a spring within the elastic fibres of connective tissue. Provides elasticity of structures such the skin, blood vessels, intestines, tendons and ligaments.

Endotoxin: A toxin that forms an integral part of the cell wall (lipopolysaccharide complex) of certain bacteria and is released upon destruction of the bacterial cell.

Encephalitis: Inflammation of the brain.

Endocarditis: Inflammation or infection of the endocardium (inner lining of the heart muscle). Commonly involves the heart valves.

EspA: A protein secreted by various strains of pathogenic *E. coli*, required by the bacterium as part of the process of attachment to host cells surfaces.

Extrinsic factors: External factors (i.e. storage conditions) applied to a food e.g. temperature, humidity, gaseous atmosphere.

Facultative anaerobe: A microorganism that normally grows aerobically, but can grow equally well under anaerobic conditions.

Fimbriae: Thin proteinaceous filaments extending from the surface of certain bacteria including *E. coli*. Smaller than flagella but with a similar structure, fimbriae are associated with bacterial adhesion.

Flagella: Organelles of motility found in motile bacteria. Certain bacteria express only one flagellum while others express numerous flagella over the entire cell surface.

Gene: The basic unit of heredity.

Haemorrhagic colitis (HC): An acute disease characterised by abdominal cramps and bloody diarrhoea, usually without fever. Attributed to a self-limited infection by VTEC.

Haemolytic Uraemic Syndrome (HUS): A clinical condition, which sometimes arises from VTEC infection and is characterised by anaemia and kidney failure.

HEp-2 Cells: A human epithelial cell line of intestinal origin useful in the study of bacterial attachment and invasion.

Implicit conditions: Conditions that are the result of mutual interactions between mixed populations of microorganisms. These result in competition between microorganisms, i.e. for nutrients and associations between different microbial populations, that may result in the stimulation and inhibition of growth of certain species or microbial populations.

Intimin: A protein required by the bacterium to mediate intimate attachment to the host cell surface. Intimin cannot produce attaching and effacing lesions by itself.

Intrinsic factors: Factors (chemical and physical) that are inherent within a food, e.g., pH, water activity, salt and sugar content, preservatives.

Kilobase(s) (kb): The abbreviation for kilobase pairs or a measure of DNA chain length. 1 kb is 1000 base pairs.

KiloDaltons (kDa): The abbreviation for 1000 Daltons. A Dalton is the unit of atomic or molecular mass. A molecule of 1 kDa has a molecular mass of 1000.

The Locus of Enterocyte Effacement (LEE): Pathogenicity island found in the chromosome of *Citrobacter rodentium*, EPEC, and EHEC strains (including *E. coli* O157) as well as other VTEC. The LEE is characterised by a cluster of genes encoding a Type III secretion system and associated chaperones and effector proteins responsible for attaching and effacing (A/E) lesions in the large intestine.

M cells (microfold cells): Special epithelial cells associated with Peyer's patches and lymphoid follicles that actively take up particulate matter from the intestinal contents. Responsible for transporting organisms and particles from the gut lumen to immune cells across the epithelial barrier. They play an important role in stimulating mucosal immunity.

MDa: MegaDaltons or 1,000,000 Daltons.

Macrophage(s): Type of white blood cell (denoted as a leucocyte) that ingests foreign material (in a process known as phagocytosis). Macrophages become active when stimulated by inflammation. They require the presence of activated T cells, or their components for activation. Macrophages play an important role in the immune response to foreign invaders such as infectious microorganisms. They also release substances that stimulate other cells of the immune system and play a role in antigen presentation to T and B lymphocytes.

Malaise: A general feeling of lack of well-being discomfort, illness, often associated with a disease state.

Mesophilic: A microorganism with an optimum growth temperature between 20°C and 45°C.

Metastatic abscesses: Secondary abscess that develops at a point distant from an original site of infection, resulting from transportation of infectious particles to other locations via the bloodstream.

Mobile genetic element : A genetic unit that can insert into a chromosome, exit, and relocate. Examples include transposons, plasmids, insertion sequences and bacteriophage elements.

Neutrophil: An abundant type of granular white blood cell that is highly destructive of microorganisms. They play an important role in the early stages of many forms of acute inflammation.

Nosocomial infection: An infection acquired in a hospital or other health care unit. Infections first appearing 48 hours or more after hospital admission or within 30 days after discharge are considered nosocomial.

O-Antigen: This is also termed lipopolysaccharide or somatic antigen and has an unusual origin. Reaction of an anti-flagella antibody with a strain of *Escherichia coli* caused an agglutination resembling condensation on glass, termed "hauch" in German, hence the term H-antigen for bacterial flagella antigens. Where no hauch was detected the term "Ohnehauch" (without hauch) was used and became O-antigen.

Osteitis: Inflammation of bone or bony tissue.

Pasteurisation: A form of heat treatment that kills vegetative pathogens and spoilage microorganisms in milk and other foods, e.g. for milk, the legal requirement for the pasteurisation process in the European Union is at least 71.7°C for 15 s.

Pathogen: Any microorganism that causes disease in humans or animals by direct interaction with (infection of) the host.

Pathogenic: Pertaining to behaviour as a pathogen.

Pathogenicity: The ability of a bacterium to cause disease. A measure of pathogenicity is termed "virulence".

Peritonitis : Inflammation of the peritoneum (serous membrane which lines part of the abdominal cavity and viscera).

Peyer's patches: Aggregations of lymphoid tissue that are usually found in the lowest portion of the small intestine (ileum in humans). Covered by a special epithelium that contains specialised cells called microfold cells (M cells), which sample antigen directly from the lumen. Peyer's patches play an important role in the immune response.

Phylogenetic studies: The study of evolutionary relatedness among various groups of organisms through molecular and morphological data matrices.

Phagocytosis: The process of engulfing and ingesting particles by the cell or a phagocyte (e.g., macrophage) to form a phagosome (or food vacuole). These fuse with lysosomes to become phagolysosomes. The engulfed material is eventually degraded or digested and either released extracellularly via exocytosis, or released intracellularly to undergo further processing.

Phenotype: The observable characteristics of an organism, including biotype, serotype, phage type and bacteriocin-type.

Plasmid: Extrachromosomal DNA that replicates independently of the host cell chromosome. Plasmids (and the genetic characteristics they carry) may be transferable between cells.

Polymerase Chain Reaction (PCR): A widely used technique to amplify multiple copies of a target DNA sequence.

Prophage: A bacteriophage genome covalently integrated as a linear part of the bacterial chromosome.

Proteases: Group of enzymes that catalyze the hydrolytic breakdown of proteins into peptides or amino acids.

Psychrotrophic: A microorganism that can survive and grow at low temperatures e.g. 0-5°C, but grows optimally between 15°C and 20°C.

Pseudoappendicitis: A condition with symptoms like those of appendicitis but which do not result from inflammation of the appendix.

rRNA: Ribosomal RNA (rRNA) comprises the major part of RNA in the cell. It provides a mechanism for decoding messenger RNA (mRNA) into amino acids and interacts with transfer RNAs (tRNAs) during translation by providing peptidyl transferase activity.

Serotyping: A method of distinguishing among bacteria on the basis of their antigenic properties (reaction to known antisera). The O-antigen defines the serogroup of a strain and in combination with the H-antigen defines the serotype of the strain.

Species: A group of plants, animals or micro-organisms that have a high degree of similarity and generally can interbreed only amongst themselves to produce fertile offspring, so that they maintain their 'separateness' from other such groups.

Strain: An isolate or group of isolates that can be distinguished from other isolates of the same genus and species by either phenotypic and/or genotypic characteristics.

Thrombocytopenia: Low numbers of platelets circulating in the blood stream.

Type III secretion system: A specialised secretion system found in many Gram-negative bacterial pathogens, which is utilised to deliver virulence effector proteins directly into host cells.

Water activity (a_w): Amount of 'free' or 'available' water in a given substrate (e.g. food). Can be defined as 1/100th the relative humidity (RH%) of the air in equilibrium with the substrate. E.g., an RH of 95% corresponds to an a_w of 0.95.

ABBREVIATIONS

| | |
|--------|--|
| AAF | aggregative adherence fimbriae |
| A/E | attaching and effacing |
| ACMSF | Advisory Committee on the Microbiological Safety of Food |
| AEEC | attaching effacing <i>Escherichia coli</i> |
| BFP | bundle-forming pili |
| BCIG | 5-bromo-4-chloro-3-indolyl- β -glucuronide (chromogenic substrate) |
| bp | base pair(s) |
| BPW | Buffered Peptone Water |
| BGBLB | Brilliant Green Bile Lactose Broth |
| cfu | colony forming units |
| CFs | colonisation factors |
| CFAs | colonisation factor antigens |
| Da | Dalton(s) |
| DAEC | diffusely adherent <i>Escherichia coli</i> |
| DNA | deoxyribonucleic acid |
| eae | <i>Escherichia coli</i> attaching and effacing gene |
| EAF | EPEC adherence factor |
| EAST | enteroaggregative heat-stable toxin |
| EHEC | Enterohaemorrhagic <i>Escherichia coli</i> |
| EPEC | enteropathogenic <i>Escherichia coli</i> |
| EIEC | enteroinvasive <i>Escherichia coli</i> |
| ETEC | enterotoxigenic <i>Escherichia coli</i> |
| EAggEC | enteroaggregative <i>Escherichia coli</i> |
| Esp | <i>E. coli</i> secreted protein |
| HACCP | Hazard Analysis Critical Control Points |
| HC | haemorrhagic colitis |
| HUS | haemolytic uraemic syndrome |
| ICMSF | International Commission on Microbiological Specifications for Foods |
| kb | kilobase(s) |
| kDa | kilodalton(s) |
| LPS | lipopolysaccharide |
| LEE | locus of enterocyte effacement |
| LT | heat labile enterotoxin |
| LSTB | Lauryl Sulphate Tryptose Broth |
| MPN | most probable number |
| PCR | Polymerase Chain Reaction |
| REFPED | Refrigerated Processed Foods of Extended Durability |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| ST | heat-stable enterotoxin |
| STEC | Shiga-like toxin-producing <i>E. coli</i> |
| Stx | Shiga toxin |
| stx | Shiga toxin-encoding gene |
| TBX | Tryptone bile X-glucuronide agar |
| TMA | trimethylamine |

| | |
|-------|-----------------------------------|
| TMAO | trimethylamine oxide |
| TTSS | type III protein secretion system |
| VRBGA | Violet Red Bile Glucose Agar |
| VRBLA | Violet Red Bile Lactose Agar |
| VT | Verocytotoxin |
| vtx | Verocytotoxin-encoding gene |

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