IDENTIFICATION AND METABOLIC CHARACTERIZATION OF HOST-SPECIFIC ENTEROCOCCI FOR USE IN SOURCE-TRACKING FAECAL CONTAMINATION

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Abstract

Metabolic profiles were used to evaluate *Enterococcus* as an indicator of faecal pollution. Enterococci were isolated using m-Enterococcus agar and speciated using conventional biochemical tests. Forty percent of the isolates were identified and metabolically characterized by the automated Biolog system. The biochemical test scheme recognized 16 enterococcal species, while Biolog recognized nine. Both methods identified *E. faecalis* at the greatest frequency. Overall species frequencies varied between the two methods. Biolog was unable to identify 31% of the isolates; 7% of the isolates were unidentified by the biochemical test scheme. Of the identified isolates, metabolic profiling with Biolog achieved speciation with 60 substrates. Unique profiles were obtained for 89% of the isolates. Isolates also demonstrated inter-trial differential metabolism of substrates. This and the large number of unidentified isolates suggest great diversity among enterococci. Diversity and inter-trial metabolic inconsistencies will complicate use of enterococcal metabolic profiles as a source-tracking tool.

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ARA	antibiotic resistance analysis
B	blood
BE	bile esculin
BEA	bile esculin azide
BHI	brain-heart infusion
BMP	better management practise
bp	base pair
BUG agar + B	Biolog universal growth agar plus 5% defibrinated sheep blood
CFU	colony forming unit
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Ef-Tu	elongation factor of the *tuf* gene
GI	gastrointestinal
GP2	Gram positive 2
GP-coc	Gram-positive coccus
HC	Health Canada
IF	inoculating fluid
ISR	isolation success rate
LAP	leucine amino peptidase
LB	Little Bow
mE	m-*Enterococcus*
MGP	methyl-α-D-glucopyranoside

MST	microbial source tracking
%P	percentage of positive replicates
pAD1	pheromone responsive plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
sp.	species
%T	percent transmittance
TSA	tryptic soy agar
TSB	tryptic soy broth
TTC	triphenyltetrazolium chloride
WCP	whole cell protein

Carbon Source Legend for Gram Positive 2 (GP2) MicroPlates™

Well	Substrate	Well	Substrate	Well	Substrate
A1	water	C9	α-methyl D-galactoside	F5	L-malic acid
A2	α-cyclodextrin	C10	β-methyl D-galactoside	F6	methyl pyruvate
A3	B-cyclodextrin	C11	3-methyl glucose	F7	methyl succinate
A4	dextrin	C12	α-methyl D-glucopyranoside	F8	propionic acid
A5	glycogen	D1	β-methyl D-glucoside	F9	pyruvate
A6	inulin	D2	α-methyl D-mannoside	F10	succinamic acid
A7	mannan	D3	palatinose	F11	succinic acid
A8	tween 40	D4	D-psicose	F12	N-acetyl L-glutamic acid
A9	tween 80	D5	D-raffinose	G1	alaninamide
A10	N-acetyl glucosamine	D6	L-rhamnose	G2	D-alanine
A11	N-acetyl manosamine	D7	D-ribose	G3	L-alanine
A12	amygdalin	D8	salicin	G4	L-alanyl-glycine
B1	L-arabinose	D9	sedoheptulosan	G5	L-asparagine
B2	D-arabitol	D10	D-sorbitol	G6	L-glutamic acid
B3	arbutin	D11	stachyose	G7	glycyl-L-glutamic acid
B4	cellobiose	D12	sucrose	G8	L-pyroglutamic acid
B5	D-fructose	E1	D-tagatose	G9	L-serine
B6	L-fucose	E2	D-trehalose	G10	putrescine
B7	D-galactose	E3	turanose	G11	2,3-butanediol
B8	D-galacturonic acid	E4	xylitol	G12	glycerol
B9	gentiobiose	E5	D-xylose	H1	adenosine
B10	D-gluconic acid	E6	acetic acid	H2	2'-deoxyadenosine
B11	α-D-glucose	E7	α-hydroxybutyric acid	H3	inosine
B12	m-inositol	E8	β-hydroxybutyric acid	H4	thymidine
C1	α-D-lactose	E9	γ-hydroxybutyric acid	H5	uridine
C2	lactulose	E10	p-hydroxyphenyl acetic acid	H6	adenosine-5'-monophosphate
C3	maltose	E11	α-ketoglutaric acid	H7	thymidine-5'-monophosphate
C4	maltotriose	E12	α-ketovaleric acid	H8	uridine-5'-monophosphate
C5	D-mannitol	F1	lactamide	H9	fructose-6-phosphate
C6	D-mannose	F2	D-lactic acid methyl ester	H10	glucose-1-phosphate
C7	D-melezitose	F3	L-lactic acid	H11	glucose-6-phosphate
C8	D-melibiose	F4	D-malic acid	H12	D-L-α-glycerol phosphate

1.0 Introduction

Southern Alberta, Canada is home to intense agricultural practices. The Oldman River watershed is the region's principle water supply, used for maintaining feedlot operations, livestock watering, as well as recreational and residential purposes. During the past 15 years the livestock industry has expanded significantly within Alberta. From 1996 to 2001, the number of cattle in Alberta increased from ~5.9 to 6.6 million, numbers of swine increased from ~1.7 to over 2 million and poultry increased from ~19 to 25 million (Statistics Canada, 2001). Sheep numbers also increased from ~260 to 307 thousand during the same period (Statistics Canada, 2001).

Increasing livestock numbers may be having a negative impact on southern Alberta, raising concern over water quality within the basin. High levels of reportable enteric disease within the region caused by pathogens such as *Salmonella*, *Escherichia coli* O157:H7, and *Campylobacter* (Khakhria et al., 1996; Thomas et al., 2000; Edge et al., 2003) may be the result of environmental contamination. *Salmonella* isolated throughout the Oldman River basin during May to October (1999 and 2000) was ~2.5X more prevalent in 2000 than in 1999 (Johnson et al., 2003).

In order to remedy problems associated with faecal pollution, it is important to track main sources of contamination. While there is evidence that the agricultural industry has expanded in southern Alberta (Statistics Canada, 2001), the origin of contamination is not clear (Johnson et al., 2003). Microbial source tracking (MST) employs microbiological, genotypic, phenotypic and chemical techniques to elucidate sources of faecal contamination within a region accurately and efficiently. These techniques are used to identify and characterize specific microorganisms called indicator species, which are

found in close association with enteric pathogens. Characterization information may then be used to track host origin of these pathogens.

If sources of faecal pollution can be tracked, better management practices (BMP's) may be implemented to reduce contamination levels. For example, addition of in-pasture watering stations and fencing can restrict cattle access to stream water and significantly reduce numbers of fecal streptococci in the water (Hagedorn et al., 1999).

The current study examined the possibility of *Enterococcus* as an indicator organism based on host-specific metabolic characterization. Six hundred and seventy-three presumptive enterococci isolated from water and sewage, bovine, porcine, poultry, ovine, goose and human faecal matter were speciated using conventional morphological and biochemical tests. Tests included Gram stain, presence of pigment, tolerance to 6.5% NaCl and 45° C, esculin hydrolysis in the presence of bile, leucine amino peptidase (LAP) and catalase activity, and metabolism of mannitol, arabinose, pyruvate, sorbitol, raffinose, methyl-α-D-glucopyranoside (MGP), sucrose, trehalose and xylose. Following speciation, carbon metabolism profiles of ~40% of the isolates were generated using the Biolog MicroStation™ in conjunction with MicroLog™ 3 software and 96-well microtitre plates. Profiles reflected isolate ability to metabolize a collection of 95 carbon sources. Biolog software also generated species identification. Agreement of species identification and diversity results between the two methods employed was determined. Metabolic profiles of enterococci isolated from the faecal sources listed above were examined and compared to profiles of enterococci from water samples. The overall intent of the study was to: 1) isolate and identify presumptive enterococci; 2) determine enterococcal species abundance; 3) compare and evaluate identification results of Biolog

and a conventional biochemical test scheme; 4) determine metabolic capabilities of enterococcal isolates; and 4) examine the potential of Biolog as a MST tool / enterococci as indicator organisims.

2.0 Literature Review

2.1 Microbial Source Tracking (MST)

Concerns regarding faecal pollution are prevalent across North America (Hagedorn et al., 2003; Bernstein et al., 2002; Graves et al., 2002; Hartel, 2002b; Scott et al., 2002; Bahirathan et al., 1998). MST methods are currently studied and employed in Canada and the United States, although most techniques require further development and improvement. Tracking the source of faecal contamination is a task that begins in one particular watershed or region, with the examination of a limited number of samples from suspect sources of contamination by the chosen MST technique(s) (Bernstein et al., 2002). This is followed by development of comprehensive databases from MST generated results. While it increases reliability of MST tools, extensive sampling of different sources and the development of databases are extremely tedious. A universal MST tool that would allow researchers to exchange database information might decrease sampling and processing time, allowing quicker detection and treatment of enteric pathogens in water (Bernstein et al., 2002).

2.1.1 Indicator Organisms

MST methods rely on the presence of indicator microorganisms to predict the corresponding presence of potentially harmful microorganisms (Scott et al., 2002). Indicator organisms are identified and characterized using phenotypic and genotypic techniques that are able to capitalize on differences between microorganisms based on the host or environment from which they were originally derived. They are especially useful because they eliminate the need for screening every pathogen found in water sources (Scott et al., 2002).

Indicators must be strongly associated with the presence of pathogenic microorganisms. They should be rapidly isolated and characterized, and have survival characteristics similar to the pathogens of concern (Scott et al., 2002). An ideal indicator microorganism is also nonpathogenic, reducing the risk of human infection through repeated exposure. Microorganisms currently in the spotlight for indicator auditions include *Escherichia coli* (Pourcher et al., 1991), *Clostridium perfringens* and enterococci (Pourcher et al., 1991; Griffin et al., 2001). None of the proposed indicators are without shortcomings (Scott et al., 2002), however the development of new MST techniques could possibly overcome any limitations of a particular organism.

2.1.2 Enterococci as Indicator Organisms

Enterococci are hardy, opportunistic pathogens that are abundant in faeces (Facklam et al., 2002). They demonstrate long survival in the environment (Manero et al., 2002) and are easily isolated. Although research on host range is contradictory (Wheeler et al., 2002), enterococci are differentially distributed between hosts, making them attractive indicators of faecal contamination (Facklam et al., 2002).

Enterococci have been used successfully as reliable indicators of faecal pollution in marine environments and recreational waters (Cabelli et al., 1982). Of the indicators examined, enterococci demonstrated the best correlation to total gastrointestinal (GI) symptoms. This supports use of enterococci as faecal indicators because it suggests that this group of bacteria has a strong association with pathogenic microorganisms. Although enterococci are known to be associated with faecal contamination (Cabelli et al., 1982), it is not known whether enterococci could be used to indicate specific sources of contamination. To answer this question, water-borne enterococci should be profiled

(characterized by an MST technique) and compared to enterococcal profiles isolated from known sources of faecal contamination as in an MST regime.

One reported downfall to using enterococci as indicators is the existence of natural reservoirs (Aarestrup et al., 2002). Once enterococci are introduced into the environment, further growth may be possible (Desmarais et al., 2002). This indicates that the relationship of enterococci existence and numbers may no longer correspond to pathogen existence and numbers; it may degenerate over time. As long as enterococcal existence in natural reservoirs correlates with pathogen existence, further growth of enterococci may not be such a shortcoming if the primary goal is to track sources of contamination rather than estimate numbers of pathogens. It is also possible that pathogens are capable of further growth once released into the environment, as this phenomenon was not only noted with enterococci but also with faecal indicator *E. coli* (Desmarais et al., 2002). Desmarais et al. (2002) studied growth of indicator organisms in a tidally influenced, subtropical region, whereas Southern Alberta is an extremely dry region. Growth dynamics of indicator organisms are likely very different between the two region types; therefore there may not be one universally applicable indicator organism.

2.1.3 Host Range of *Enterococcus*

Since the genus *Enterococcus* includes more than thirty members, more than one species may be considered when selecting an indicator organism for MST. Host range becomes an important factor to the selection process, as it is pertinent to know whether or not contamination by a particular species could have originated from one or several sources.

Many of the enterococcal species identified to date have been isolated from a range of mammals and / or birds (Aarestrup et al., 2002). An indicator species should be well characterized and documented. Both are pertinent to reliable indication of faecal pollution. Thorough characterization aids in the differentiation between species and strains. As frequency of isolation for a species or strain increases, knowledge of host range increases. Table 2.1 provides an up-dated list of all enterococcal species isolated thus far (DSMZ, retrieved December, 2004). Many of the newly described species (Law-Brown and Meyers, 2003; Teixeira et al., 2001; Vancanneyt et al., 2001) described in this table are not yet well documented.

Some enterococci are present in a variety of animals, but are more frequently isolated from a particular animal species (Devriese et al., 1992a). Species that are isolated from a variety of animals may still be used as indicators of specific sources of faecal contamination as long as the enterococcal species from one host can be differentiated from the same species isolated from other possible hosts.

Wheeler et al. (2002) suggested that *E. faecalis* is a good candidate for tracking faecal contamination due to its host range limited to humans, dogs and chickens. Previous research on host range of this particular species is contradictory, expanding the range of *E. faecalis* to include cattle, horses, rabbits, rodents, sheep, swine and wild birds (Wheeler et al., 2002). Inter-research discrepancies concerning host range might be attributable to isolation method and identification media used (Wheeler et al., 2002; Hudson et al., 2003). This emphasizes the need for development of a regional MST standard operating procedure.

Table 2.1. Alphabetically sorted *Enterococcus* species isolated and characterized to date. Adapted from DSMZ, retrieved December, 2004. Detailed species information may be accessed at: http://www.dsmz.de/bactnom/nam1140.htm

Species	Year First Described
asini	1998
avium	1984
canis	2003
casseliflavus	1984
cecorum	1983
columbae	1993
dispar	1991
durans	1984
faecalis	1906
faecium	1919
flavescens	1992
gallinarum	1982
gilvus	2002
haemoperoxidus	2001
hermanniensis	2004
hirae	1985
italicus	2004
malodoratus	1984
moraviensis	2001
mundtii	1986
pallens	2002
phoeniculicola	2003
porcinus	2001
pseudoavium	1989
raffinosus	1989
ratti	2001
saccharolyticus	1985
saccharominimus	2004
seriolicida	1991
solitarius	1989
sulfureus	1991
villorum	2004

2.1.4 MST Methodology

MST utilizes techniques for identification and characterization of indicator organisms. Tools should have the potential to differentiate strains of the same species based on host origin. For example, restriction fragment length polymorphism of amplified 16S rDNA employed on six enterococcal species demonstrated 98-99% homology between some species (Facklam et al., 2002). Analysis of this particular genetic target does not adequately detect variation between species so it would likely be less successful at distinguishing between different strains of the same species. In contrast, results of *Vagococcus* speciation via pulsed field gel electrophoresis indicated some host specificity among profiles (Teixeira et al., 1997). Of the isolates examined, two were isolated from swine and four were isolated from humans. The two porcine isolates demonstrated the greatest amount of similarity in banding pattern. The four isolated from humans however, did not demonstrate this similarity. This might have been because the four human-origin strains were isolated from different sources in the body (blood, peritoneal fluid and from a bite wound), or the similarity between the porcine isolates may have been coincidental since only two isolates were examined.

Many methods of bacterial characterization exist (Facklam et al., 2002). Results such as the ones from the study mentioned above show promise for MST. Sections 2.1.4.1 and 2.1.4.2 briefly review some of the genotypic and phenotypic MST methods currently studied.

2.1.4.1 Genotypic Methods

Genotypic methods used for bacterial identification and characterization were primarily used in taxonomy labs, but have become popular methods of study in

microbiology labs during the past ten years (Facklam et al., 2002). Development of molecular tools attempts to identify enterococcal species more rapidly and accurately. These methods may also be useful in differentiating host-specific strains of *Enterococcus*.

Pulsed-field gel electrophoresis (PFGE) is a method of DNA fingerprinting where genomic bacterial DNA is treated with rare-cutting restriction endonucleases (Scott et al., 2002). PFGE is considered one of the most useful and reliable typing methods for epidemiological analyses of hospital-acquired enterococci infections (Facklam et al., 2002) and epidemiological relatedness in other bacteria (Barrett et al., 1994). PFGE was successfully used to match *E. coli* isolated from stream water to its host on one occasion, but on another occasion analysis of the *E. coli* failed to produce a relationship between the PFGE fingerprint and the isolate source (Scott et al., 2002). This may be due to the existence of numerous procedures used to perform PFGE analyses; there is a lack of standardized conditions for gel electrophoresis and criteria for interpreting banding patterns (Facklam et al., 2002).

Another potential tool used in MST is ribotyping. Highly conserved rRNA genes are identified with oligonucleotide probes after genomic DNA has been treated with restriction endonucleases (Scott et al., 2002). Although this method was useful in tracking human and non-human sources of faecal pollution using *E. coli* (Carson et al., 2001; Hartel et al., 2002a; Parveen et al., 1999) it is extremely time consuming. Discriminatory ability of ribotyping also tends to decrease when bacteria from different geographical regions are analyzed (Hartel et al., 2002a; Hartel et al., 2002b; Malakoff, 2002).

The polymerase chain reaction (PCR) is a method of DNA amplification that can be used to detect genetic differences and similarities based on DNA sequences. It has been used successfully to rapidly identify clinically important enterococci (Ke et al., 1999). Primers derived from conserved regions of the *tuf* gene, which encode elongation factor EF-Tu were designed in order to amplify an 803 bp region of four enterococcal species. Determination of complete nucleotide sequences revealed regions conserved among enterococci but distinct from other bacteria. Within the main sequence, some species-specific variations existed that could be used to detect clinically important species. There was no indication that this method of characterization had the potential to discriminate between host-specific enterococcal strains.

2.1.4.2 Phenotypic Methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a phenotypic method of speciation whereby whole cell protein (WCP) profiles are analyzed (Facklam et al., 2002). SDS is used to solubilize polypeptides of cell walls, which are separated by PAGE (Facklam et al., 2002). WCP profiles have been useful in establishing *Enterococcus* species (Merquior et al., 1994; Facklam et al., 2002) and distinguishing enterococci from closely related species of other genera. *Vagococcus fluvialis* and *Lactococcus* species that are commonly misidentified as enterococci have unique WCP profiles (Facklam et al., 2002). It is not known if WCP profiles of enterococci can be used to distinguish the origin of faecal contamination. It is unlikely since subspecies and atypical strains of *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* have the same WCP profiles as common strains (Facklam et al., 2002; Merquior et al., 1994; Niemi et al., 1993).

Antibiotic resistance analysis (ARA) relies on the idea that various animals are administered different types and concentrations of antibiotics at different frequencies; hence, the bacteria in their GI tract are also subjected to these antibiotics. Over time, selection for "survivor" bacteria occurs within specific animal groups and these bacteria will have host-reflective antibiotic resistance fingerprints. This technique of bacterial fingerprinting was successful when used in tracking *E. coli* and faecal streptococci to a host (Harwood et al., 2000; Wiggins, 1996; Wiggins et al., 1999). Because antibiotic use tends to vary between livestock and humans, this method may require a large database that incorporates results for many different host organisms in a large geographic area. Wiggins et al. (1999) discovered that classification success of isolates based on antibiotic resistance profiles increased as numbers and combinations of drugs tested increased. This could become a very expensive method of bacterial fingerprinting. Further, it was not determined whether ARA could accurately identify bacteria isolated from mixed samples (Wiggins et al., 1999). An MST tool must be able to identify species in water that originated from mixed samples. Recent research, however, indicated that ARA could be used to track the source of faecal contamination in a Virginia recreational watershed (Graves et al., 2002). Correct classification rates of 94.6% for 203 human isolates, 93.7% for 734 livestock isolates and 87.8% for 237 wildlife isolates were achieved. ARA could be used as a reliable MST method, but potentially high costs associated with this method and the temporal stability of antibiotic resistance profiles must be considered. Wiggins et al. (2003) found that antibiotic resistance profiles were stable for at least one year. MST library updates are expected as new profiles are

encountered, although it is important that novel profiles are not displacing well-established profiles at a high rate.

Carbon source utilization profiling, or biochemical fingerprinting, is an example of a phenotypic method that is potentially less time consuming than ARA. The ability of a bacterium to metabolize any given carbon source is dictated by genetic make-up and complementary biosynthetic pathways, which in turn have been influenced by long- and short-term changes driven by evolution. This suggests that bacterial metabolic capabilities reflect life history, resulting in differences between species of a genus and possibly differences between conspecifics isolated from different hosts. For example, a particular strain may have evolved to metabolize only those substrates frequently encountered in the niche(s) it inhabits. Souza et al. (1999) demonstrated that the metabolic capabilities of *E. coli* were associated with the taxonomic group of the host from which the strains were isolated. In general, animal species require specific diets unique from the diets of other species. A specific diet results because of unique physiological differences between hosts, but diet also contributes to creating a unique internal host environment. The bovine (ruminant) digestive system and diet is different from that of a human or chicken (non-ruminants). The GI flora would presumably reflect these differences. Floral differences between hosts may not only be due to physiological differences, but due to diet resulting from variation in host geographic location (Hagedorn et al., 2003; Hartel et al., 2003; Hartel et al., 2002a).

Not all researchers (Park et al., 1999; Ulrich and Muller, 1998; Teixeira et al., 1995; Devriese et al., 1993) agree that metabolic differences between strains of *Enterococcus* can be used to track faecal contamination to host. This may be due to the great

heterogeneity in phenotypic characteristics of enterococcal and other Gram-positive coccal species. *Streptococcus, Lactococcus, Vagococcus, Tetragenococcus* and *Pediococcus* contain species that are biochemically similar to enterococci (Facklam et al, 2002). Identification of enterococci via physiological tests alone is very difficult and may be erroneous regardless of the origin of the isolate (Facklam and Sahm, 1995; Devriese et al., 1993; Devriese et al., 1992a). Although this may be true, certain automated systems utilize standard operating procedures to help limit discrepancies. Recent research that used the Biolog commercial identification system (Hagedorn et al., 2003) demonstrated the potential of carbon utilization profiles for use in MST of faecal contamination. Manero et al. (2002) utilized the Phene-Plate System to phenotypically characterize enterococcal isolates from pig slurry, hospital sewage and urban sewage. Twenty-nine different profiles resulted; twenty profiles were unique to source of faeces, while seven profiles were detected more than once. Overlapping biochemical profiles between sources are expected when there is a possibility of contamination of one source by another. For example, animal faeces may contaminate sewage collection ponds, possibly explaining why there was profile overlap between enterococci isolated from animal faeces and urban sewage. Further research that examines profiles of enterococci isolated from fresh known-source faeces is required to test this.

2.1.5 The Commercial Biolog MicroStation™ and MicroLog™ 3 Software

The Biolog system uses carbon metabolism results for rapid identification to genus and species for over 1900 microorganisms (Biolog, 2004a). Although the system was developed primarily for identification of clinically important pathogens, the technology is potentially useful to all fields of microbiology such as clinical, veterinary,

pharmaceutical, quality control, agriculture, environmental, research, etc. (Holmes et al., 1994). Several examples exist where researchers have used the technology in their studies (Hale Boothe and Arnold, 2002; Choi and Dobbs, 1999; Gamo and Shoji, 1999; Truu et al, 1999; Lindstrom et al., 1998; Mergaert et al., 1996; Baillie et al., 1995).

MicroLog™ 3 software consists of a database or library of carbon metabolism results for bacterial and fungal genera (Biolog, 2004b). The software, in conjunction with the MicroStation™ plate-reader, identifies microorganisms based on metabolic patterns of an unknown organism compared to a standard set of organisms in the database. Biolog assumes a Gram stain has been performed on all isolates so that Gram reaction and cell shape are known.

A variety of microplates are available for use in species identification of microorganisms. This study utilized the 96-well GP2 (Gram-positive 2) MicroPlate™, enabling the characterization of enteric, non-enteric and fastidious Gram-positive bacteria (Biolog, 2001). Carbon sources corresponding to wells of the GP2 microplate are identified on p. xvi. Once organism type and Gram reaction are established, the appropriate Biolog plates are inoculated and incubated following a standard operating procedure. The MicroStation™ plate reader scans inoculated microtitre plates and the intensity of metabolic reaction in each well is assessed via spectroscopy. Spectrophotometer-generated results are transferred to Biolog identification software. Results are cross-referenced to the selected library or database where an identification of the isolate may be produced based on "goodness of match" to a profile in the database (Gadzinski, Biolog Inc.). Generated identifications are displayed with a probability value that the match is correct.

2.1.5.1 BIOLOG Characterization of Bacteria

The use of metabolic fingerprinting in MST is advantageous, as it eliminates ambiguity and judgment error that can often occur in other MST techniques. Biolog is quick, employs a standardized protocol and utilizes 95 different carbon sources. The more substrates available for testing, the greater the specificity a system will have. Biolog not only uses 95 different substrates, but the substrates chosen vary depending on whether the organism is a yeast, fungus, Gram-positive bacterium or Gram-negative bacterium (Biolog, 2004a).

Biolog has been examined previously for its specificity and repeatability capabilities (Odumeru et al., 1999). Genera examined included *Listeria* and *Salmonella*, as well as *E. coli* strains. Specificity was determined by testing Biolog with 40 isolates similar to the pathogen of interest and calculating the proportion of those isolates that were not identified as the pathogen of interest. Biolog demonstrated high specificity results for *Listeria* and *Salmonella* species, but not *E. coli.* Repeatability was examined for replicates examined by the system. Repeat identification was successful for *Listeria*, while repeatability decreased for *Salmonella* and *E. coli.* Specificity and repeatability depended upon the organism of interest. Based on these results, it seems reasonable to question the use of *E. coli* for MST when using Biolog as the MST tool.

Since Biolog has demonstrated discriminative capabilities and repeatability, it would be interesting to see if these capabilities extend to other faecal indicator bacteria, specifically the enterococci. Biolog has recently been evaluated as an MST tool (Hagedorn et al., 2003). Known-source enterococcal isolates were characterized by Biolog and compared to unknown-source isolates from various stream sites in Virginia

with at least one obvious source of faecal contamination. Biolog was able to distinguish between sources of contamination; however, the study only examined isolates from sources that were known to have contact with the streams in some way. Biolog would be an even more powerful MST tool if it were capable of discriminating between sources of faecal contamination without already knowing the most likely sources of contamination.

2.2 The Genus *Enterococcocus*

2.2.1 Taxonomy

The genus *Enterococcus* was first named by Thiercelin and Jouhaud in 1903 (Devriese et al., 1993). Despite this, recognition of enterococci apart from the genus *Streptococcus* is rather recent. Kalina (1970) proposed that the enterococci be recognized as a genus separate from *Streptococcus*, but no action was taken at this time. In 1984, Schleifer and Kilpper-Balz provided genetic evidence that *S. faecalis*, *S. faecium* and subspecies of the two taxa were significantly different from the other streptococcal species. Within ten years, *Enterococcus* was considered a valid genus (Facklam and Sahm, 1995). Since 1984, many new enterococcal species have been included in the genus (Facklam and Sahm, 1995; Gilmore et al., 2002). Because the genus *Enterococcus* is relatively young and presumptive members are not always easy to elucidate, species are still being added and removed (Facklam and Sahm, 1995). Species previously belonging to the genus *Streptococcus*, renamed as *Enterococcus avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. malodoratus*, were added to the new genus based on chemotaxonomic studies (Collins et al., 1984). Following this addition, *E. hirae*, *E. mundtii*, *E. cecorum*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus*, *E. columbae*, *E. dispar and E. sulfureus* were added to the genus *Enterococcus* (Farrow and Collins,

1985; Collins et al., 1986; Collins et al., 1989; Rodrigues and Collins, 1990; Devriese et al., 1990; Collins et al., 1991; Martinez-Murcia and Collins, 1991). More recently, *E. gilvus* and *E.* pallens were isolated from clinical specimens, bile and peritoneal dialysate respectively, and described by Tyrrell et al. (2002). Including *E. casseliflavus*, *E. mundtii* and *E. sulfureus*, *E. gilvus* and *E. pallens* are also yellow-pigmented enterococci. A few species listed in Table 2.1 (DSMZ, retrieved December, 2004) are no longer considered distinct species. Specifically, WCP profiling has indicated that *E. seriolicida* is actually a species belonging to *Lactococcus* (Teixeira et al., 1996) and *E. solitarius* belongs to *Tetragenococcus* (Facklam et al., 2002). *E. flavescens* and *E. casseliflavus*, and also *E. porcinus* (Teixeira et al., 2001) and *E. villorum* (Vancanneyt et al., 2001) have been recognized as the same species (Poyart et al., 2000; Facklam et al., 2002).

Currently accepted species of *Enterococcus* grouped based on reactions in mannitol, sorbose and arginine broths are shown in Table 2.2. Only those species with complete information regarding biochemical test results have been included in the table (Facklam et al., 2002).

2.2.2 Phenotypic Description of *Enterococcus*

The enterococci are ubiquitous, occurring widely in the environment. They may be isolated from food, soil, water and insects but predominantly inhabit the GI tract of humans and other animals (Giraffa, 2002; Facklam and Sahm, 1995; Holt et al., 1994). They are Gram-positive, coccus-shaped bacteria that occur singly, in pairs, or as short chains and may appear slightly elongated (coccobacillary) when Gram-stained from an agar culture (Facklam and Sahm, 1995). They do not form endospores and lack obvious

Table 2.2. Phenotypic tests that may be used to identify presumptive enterococci. Phenotypic test abbreviations: mannitol (man), sorbose (sor), arginine (arg), arabinose (ara), sorbitol (sbl), raffinose (raf), tellurite (tel), motility (mot), pigment (pig), sucrose (suc), pyruvate (pyu), methyl-α-D-glucopyranoside (mgp), litmus milk (lm), hippurate (hip), glycerol (gyl), trehalose (tre) and xylose (xyl). Enterococcal grouping (I to IV) is based on results in man, sor and arg broths. Utilization of 1% pyu, and production of acid in 1% man, sor, ara, sbl, raf, suc, mgp, gyl, tre and xyl is indicated by "p" for positive or "n" for negative. Hydrolysis of arg and hip is indicated by "p" or "n" . Growth in 0.04% tellurite, motile and pigmented strains are indicated by "p" and strains unable to grow in 0.04% tellurite, non-motile and non-pigmented strains are indicated by "n". The production of acid in lm is indicated by "a/", no acid production is indicated by "n/", a clot formed in lm is indicated by "/c" and no clot formation is indicated by "/n". A positive result ("p") indicates that greater than 90% of strains are positive and a negative result ("n") indicates that less than 10% of strains are positive. Occasional exceptions are indicated by "p*" or "n*" where less than 3% of strains show aberrant reactions. Strains of species that demonstrate variable reactions are indicated by "v". "n/a" indicates unavailable data. For group III species, "p" indicates that 85% or more of strains are positive, "n" indicates that 15% or less of strains are negative, "v" indicates that 16-84% of strains are positive. "p, n, or v" / "number" (e.g. p/82) indicates the overall interpretation is positive, negative or variable (whichever one is indicated), but "number" % of the strains are positive. For example, "p/82" indicates that 82% of strains are positive and "n/20" indicates that 20% of strains are positive. Adapted from Facklam et al. (2002).

Group	Species	Phenotypic Characteristic																
		man	sor	arg	ara	sbl	raf	tel	mot	pig	suc	pyu	mgp	lm	hip	gyl	tre	xyl
I	*avium*	p	p	n	p	p	n	n	n	n	p	p	v	n/a	n/a	n/a	n/a	n/a
	malodoratus	p	p	n	n	p	p	n	n	n	p	p	v	n/a	n/a	n/a	n/a	n/a
	raffinosus	p	p	n	p	p	p	n	n	n	p	p	v	n/a	n/a	n/a	n/a	n/a
	pseudoavium	p	p	n	n	p	n	n	n	n	p	p	p	n/a	n/a	n/a	n/a	n/a
	saccharolyticus	p	p	n	n	p	p	n	n	n	p	n	p	n/a	n/a	n/a	n/a	n/a
	pallens	p	p	n	n	p	p	n	n	p	p	n	p	n/a	n/a	n/a	n/a	n/a
	gilvus	p	p	n	n	p	p	n	n	p	p	p	n	n/a	n/a	n/a	n/a	n/a
II	*faecalis*	p	n	p	n	p	n	p	n	n	p*	p	n	n/a	n/a	n/a	n/a	n/a
	faecium	p	n	p	p	v	v	n	n	n	p*	n	n	n/a	n/a	n/a	n/a	n/a
	casseliflavus	p	n	p	p	v	p	n*	n*	p	p	v	p	n/a	n/a	n/a	n/a	n/a
	mundtii	p	n	p	p	v	p	n	n	p	p	n	n	n/a	n/a	n/a	n/a	n/a
	gallinarum	p	n	p	p	n	p	n	n*	n	p	n	p	n/a	n/a	n/a	n/a	n/a
III	*durans*	n	n	p	n	n	n	n	n	n	n	n	n	a/c	p/82	n	p	n
	porcinus	n	n	p	n	n	n	n	n	n	n	n	n	a/n	n	n	p	p
	ratti	n	n	p	n	n	n	n	n	n	n	n	n	n/n	v/60	n	n/20	n
	hirae	n	n	p	n	n	p	n	n	n	p	n	n	a/n	n/3	n/5	p	n
	dispar	n	n	p	n	n	p	n	n	n	p	p	p	a/n	p/82	p	p	n
	faecalis	n	n	p	n	n	n	p/88	n	n	n/12	p/76	n	a/c	n/13	n/12	n/12	n
	faecium	n	n	p	p	n	n/13	n/6	n	n	v/38	n	n	a/v	v/56	n/6	p/75	n
IV	*asini*	n	n	n	n	n	n	n	n	n	p	n	n	n/a	n/a	n/a	n/a	n/a
	sulfureus	n	n	n	n	n	p	n	n	p	p	n	p	n/a	n/a	n/a	n/a	n/a
	cecorum	n	n	n	n	p	p	n	n	n	p	p	n	n/a	n/a	n/a	n/a	n/a
V	*casseliflavus*	p	n	n	p	v	p	v	p	p	p	v	p	n/a	n/a	n/a	n/a	n/a
	gallinarum	p	n	n	p	n	p	n	p	n	p	n	p	n/a	n/a	n/a	n/a	n/a
	faecalis	p	n	n	n	p	n	p	n	n	p	p	n	n/a	n/a	n/a	n/a	n/a
	columbae	p	n	n	p	p	p	n	n	n	p	p	n	n/a	n/a	n/a	n/a	n/a

capsules (Holt et al., 1994). There are only two reported motile enterococci, *E. gallinarum* and *E. casseliflavus* (Facklam et al., 2002). Enterococci are facultative anaerobes with a growth range from 10 to 45°C and an optimal temperature of 35°C (Facklam and Sahm, 1995; Holt et al., 1994). *Enterococcus dispar* and *E. sulfureus* do not grow at 45°C (Martinez-Murcia and Collins, 1991) and *E. cecorum* and *E. columbae* are unable to grow at 10°C (Devriese et al., 1993). Although a carbon dioxide enriched environment is not required for growth, some strains grow better in atmospheres containing increased levels of CO_2 (Facklam and Sahm, 1995). All strains possess the Lancefield group D antigen (Schleifer and Kilpper-Balz, 1984). Despite this, several species fail to react with Lancefield group D antisera (Devriese et al. 1983, 1990; Farrow et al., 1984; Facklam and Collins, 1989; Collins et al. 1989). Most strains are capable of growth in broth containing 6.5% NaCl with the exception of *E. cecorum*, *E. columbae* and *E. avium*. These species grow slowly and poorly and sometimes not at all (Devriese et al., 1993). All strains can hydrolyze esculin in the presence of 40% bile salts (Facklam and Moody, 1970) and all strains produce leucine aminopeptidase (LAP) (Facklam and Sahm, 1995). All strains are catalase negative, as enterococci do not contain cytochrome enzymes. Occasionally a pseudocatalase is produced, resulting in a weak reaction during the catalase test (Facklam and Sahm, 1995). All strains, with the exception of *Enterococcus cecorum*, *E. columbae*, *E. pallens* and *E. saccharolyticus*, can hydrolyze pyrrolidonyl-β-naphthylamide (PYR) (Facklam et al., 1989; Facklam and Washington, 1991). Enterococci are chemoorganotrophs, capable of fermenting a wide range of carbohydrates with the production of mainly lactic acid but no gas (Facklam and Sahm, 1995). Nutritional requirements are complex (Devriese et al., 1992a).

2.3 Identification of *Enterococcus*

2.3.1 Presumptive Identification and Speciation

Because other Gram-positive cocci demonstrate similarities to enterococcal species, the identification of enterococcal strains via previously described standard tests is only presumptive. In the past (Teixeira et al., 1997), species from the genus *Vagococcus*, although phylogenetically distinct from the enterococci, have been wrongly classified as unidentified enterococci when phenotypic identification schemes have been employed. Results (Teixeira et al., 1997) indicated that *Vagococus fluvialis* was also positive for PYR and LAP production and could hydrolyze esculin in the presence of bile. Growth at 45 °C and in 6.5% NaCl was variable between strains. These characteristics are the same for the enterococci; some strains may not grow in high salt concentrations and high temperatures (Facklam and Sahm, 1995). Since unidentified enterococci are often members of *Vagococcus*, Teixeira et al. (1997) suggested the use of raffinose and arabinose broths to help distinguish vagococci from members of enterococci. Vagococci are unable to metabolize raffinose and arabinose (Facklam et al., 2002). At least six enterococcal species are also unable to metabolize both raffinose and arabinose. However, the incorporation of mannitol, MGP and pyruvate broths into the identification scheme can differentiate these species from vagococci (Table 2.2).

Due to practicality constraints, the following approach for presumptive identification of *Enterococcus* has been used: selection for Gram-positive, catalase-negative cocci, growth in 6.5% NaCl broth and strong growth on a medium selective for enterococci containing sodium azide (Devriese et al., 1992a; Devriese et al., 1993; Facklam and Sahm, 1995; Facklam et al., 2002). Since some strains of enterococci produce results

atypical of the genus, this identification approach is biased toward the isolation of classically defined (Devriese et al., 1993) enterococcal strains. Many novel enterococci may not grow on selective media, and if they do they may produce atypical test results leading to wrong identification (Devriese et al., 1993). Unless a medium that selects for all species of enterococci is developed, MST may only rely on typical species, otherwise the process would be too time consuming. For typical enterococci, identification to the species level must be employed in order to confirm presumptive genus identification. Table 2.2 identifies the tests that may be employed for species identification of suspect enterococcal isolates (Facklam et al., 2002).

2.4 Significance of *Enterococcus*

Enterococci are considered opportunistic pathogens, *i.e.*, they are classified as low-grade pathogens because they generally only infect immunosuppressed or intensive care patients (Levy, 2002). Although this is true, they are widely reported as causing nosocomial infections, and these reports continue to rise (Malani et al., 2002). The use of broad-spectrum antibiotics is increasing in hospitals and in feedlots as growth promoters, leading to an increase in antibiotic resistance by enterococci (Conly, 2002; Giraffa, 2002; Hancock and Gilmore, 2002; Levy, 2002; Butaye et al., 2001; McCormick, 1998). Animal husbandry practices provide reservoirs for genetic exchange of antibiotic resistance genes in enterococci via the food chain (Khan et al., 2002; Butaye et al., 2001). The transfer of resistance genes to truly pathogenic species such as *Staphylococcus aureus* further complicates the issue (Giraffa, 2002; Tejedor Junco et al., 2001).

Enterococci are responsible for many hospital-acquired infections such as urinary tract infections (Malani et al., 2002; Franz et al., 1999; Schaberg et al., 1991), intra-

abdominal or pelvic wound infections (Malani et al., 2002; Franz et al., 1999; Nichols and Muzik, 1992), bacteremia (Malani et al., 2002; Franz et al., 1999; Graninger and Ragette, 1992; Moellering, 1992; Schaberg et al., 1991; Gullberg et al., 1989; Maki and Agger, 1988), bacterial endocarditis (Malani et al., 2002; Poyart et al., 2002; Franz et al., 1999; Watanakunakorn and Burkert, 1993; Megran, 1992), endoophthalmitis (Hancock and Gilmore, 2002) and respiratory tract or central nervous system infections (Franz et al., 1999). Reports of the latter two infections are rare (Murray, 1990).

2.4.1 Virulence / Pathogenicity

Enterococci are generally only a concern in clinical settings, but the concern is a great one. Adaptation to detergents enables enterococci to persist in hospital settings where cleaning regimes may sometimes be inadequate (Hancock and Gilmore, 2002). Enterococci are typically transmitted from hands and medical instruments in hospitals (Hancock and Gilmore, 2002). Furthermore, enterococci not only possess virulence traits and natural resistance to antibiotics, but they are also capable of exchanging genetic material and acquiring novel antibiotic resistance genes (Haack et al., 1995).

2.4.1.1 Virulence Traits

Enterococci are normally found in the GI tract of individuals with healthy immune systems. Approximately 105-107 organisms / g exist in human faeces (Hancock and Gilmore, 2002). In order to infect an individual, enterococci must be able to colonize a mucosal surface, evade host clearance and induce pathological changes in the host (Hancock and Gilmore, 2002). The possession of many virulence traits facilitates the infection process by enterococci. These traits include adherence to host tissue, invasion and abscess formation, resistance to and inflection of host defense mechanisms, secretion

of toxins such as cytolysins and the production of plasmid-encoded pheromones (Franz et al., 1999; Jett et al., 1994; Eaton and Gasson, 2001). Virulence traits as well as antibiotic resistance can be transmissible via gene transfer mechanisms (Haack et al., 1995; Chow et al., 1994; Gilmore et al., 1994; Kreft et al., 1992; Wirth, 1994). Furthermore, the same plasmid may carry genes for a sex pheromone response and antibiotic resistance or haemolysin production genes (Franz et al., 1999; Wirth, 1994).

The sex pheromone response has been demonstrated to greatly facilitate the exchange of genetic material as well as elicit inflammation in the host (Ember and Hugli, 1989; Johnson, 1994; Sannomiya et al., 1990). The pheromone response plasmid, pAD1, codes for an aggregation substance and cytolysin (haemolysin) production. Both enhance vegetative growth in enterococcal endocarditis (adherence of enterococci to heart cells) (Hancock and Gilmore, 2002). Cytolysin may contribute to overall patient lethality while aggregation substance has led to an increase in enterococcal growth weight (Hancock and Gilmore, 2002). It has also been documented that enterococci with pheromone response plasmids (such as pAD1) were better able to bind to the cultured pig renal tubular cell line, LLC-PK, than enterococci without these plasmids (Hancock and Gilmore, 2002). Despite these findings, it appears that the binding of enterococci to epithelial tissue is complex and also involves protein and carbohydrate surface adhesins (Hancock and Gilmore, 2002). It should also be noted that the presence of plasmids is not a requirement for enterococcal infections. Plasmids may simply enhance enterococcal pathogenicity (Hancock and Gilmore, 2002).

2.4.1.2 Immune Invasion

The role of complement and anti-enterococcal antibodies in phagocytosis is important to host defense (Hancock and Gilmore, 2002). Healthy human individuals possess antibodies to aid in phagocytosis. In immune-compromised individuals, enterococcal strains can become resistant to phagocytosis (Hancock and Gilmore, 2002). Some enterococci evade phagocytosis by the possession of a protective material around their cell wall (proposed to be a carbohydrate), while other times enterococci can actually survive within a macrophage. Research in this area is still lacking.

2.4.1.3 Indirect-Tissue Damage

Induced pathological changes in the host, the last step in enterococcal infections, have been observed in studies of *E. faecalis* pathogenesis in the form of direct- and indirect-tissue damage (Hancock and Gilmore, 2002).

The group D streptococcal antigen (lipoteichoic acid) in enterococci has been implicated in immune response modulation, causing indirect tissue damage. Enterococcal lipoteichoic acid was documented to be just as strong an inflammatory agent as the lipopolysaccharide of Gram-negative bacteria (Hancock and Gilmore, 2002). The antigen is also important to mediation of adherence to host cells and the enhancement of exchange and dissemination of genetic determinants (Hancock and Gilmore, 2002).

2.4.1.4 Direct-Tissue Damage

Enterococcal cytolysin and gelatinase (zinc metalloprotease) are secreted toxins that play a significant role in direct tissue damage of human and animal hosts (Hancock and Gilmore, 2002). Cytolysin activity is generally encoded on pheromone-response

plasmids and possesses both toxin and bacteriocin properties, promoting growth and survival of enterococci in blood (Gilmore et al., 2002). Cytolysin has also been known to reduce retinal function as well as destroy retinal architecture (Hancock and Gilmore, 2002). The advantage of gelatinase to enterococci is not fully understood, but studies do indicate the potential for host immune response modulation (Hancock and Gilmore, 2002). Despite documentation of enterococcal virulence and pathogenicity, exact mechanisms and correlational effects of several factors require extensive research to be better understood (Hancock and Gilmore, 2002).

2.4.2 Antibiotic Resistance

Enterococci are generally resistant to cephalosporins, lincosamides, β-lactams and low levels of aminoglycosides (Morrison et al., 1997; Murray, 1990; Moellering, 1990; Leclercq, 1997; Hancock and Gilmore, 2002). They are not only intrinsically resistant to antibiotics, but they have acquired genetic determinants enabling resistance to all classes of antimicrobials (Khan et al., 2002). Genetic elements conferring antibiotic resistance can be transferred to other enterococci and more virulent pathogens by pheromone-mediated conjugative plasmids (Morrison et al., 1997). Few antibiotics are currently available to treat enterococcal infections, and acquired resistance to many of these, such as vancomycin (Manson et al., 2003; Conly, 2002; Georgopapadakou, 2002; Levy, 2002; McCormick, 1998), is on the rise.

Enterococcus faecalis and *E. faecium* are important where clinical epidemiology is concerned, as they are the two species associated with the majority of enterococcal infections (Georgopapadakou, 2002). Statistics indicate that *E. faecalis* is the most common enterococcal species isolated from human infections, although *E. faecium* may

be a greater concern in the way of antibiotic resistance (Jett et al., 1994; Huycke et al., 1998; Georgopapadakou, 2002). Both species harbour multiple determinants, *i.e.*, multiple virulence traits; however, molecular screening of *Enterococcus* virulence determinants indicated that *E. faecalis* strains harboured the most (Eaton and Gasson, 2001; Franz et al., 2001).

The prevalence of enterococci as nosocomial pathogens is increasing (Hancock and Gilmore, 2002), emphasizing the importance of continued enterococcal research to facilitate understanding of the genus. Reducing the number of infections by opportunistic pathogens through controlling faecal pollution would save millions of dollars in health care costs each year (Malani et al., 2002).

2.5 Specific Study Objectives

The overall intent of this study was to address the hypothesis that enterococcal strains of the same species can be differentiated based on the host of origin by using metabolic profiles as a method of phenotypic characterization. Enterococci were isolated and identified from several different environmental sources and metabolically characterized, and the potential of Biolog as an MST tool based on metabolic fingerprints was examined. More specific objectives were: 1) to isolate presumptive enterococci from water and sewage samples as well as bovine, porcine, human, goose, ovine and chicken faeces using mE (m-*Enterococcus*) agar as a selective medium; 2) to confirm presumptive enterococci; 3) to evaluate methods used to isolate and identify presumptive enterococci; 4) to speciate presumptive enterococci using a biochemical test scheme and the automated Biolog system and assess the ability of Biolog to identify environmental enterococci; 5) to evaluate usefulness and accuracy of the two identification methods; 6)

to evaluate species abundance and diversity; 7) to examine metabolic capabilities of enterococci and generate metabolic fingerprints of the Biolog-speciated isolates; 8) to examine anomalies of metabolic results compared to Biolog GP2 database results; 9) to determine which carbon sources are metabolized and not metabolized by all enterococci, conspecific enterococci and conspecific enterococci isolated from the same host; 10) to determine the number of different metabolic profiles encountered; 11) to match host-specific enterococcal profiles to profiles of water-borne isolates; and 12) to evaluate the possibility of Biolog as an MST tool.

3.0 Materials and Methods

3.1 Origin of Samples

Suspect enterococci were isolated from various livestock faeces including cattle, swine, ovine, chicken and geese, as well as human faeces, sewage effluent and several water sources. All samples were obtained in southern Alberta. Fresh faeces were obtained from livestock surrounding the Oldman River watershed located near Picture Butte, Claresholm, Coaldale and Lethbridge; porcine faeces were also obtained from Maple Leaf Processors. Fresh wild goose faeces were collected from Keho Lake boat launch, a pond in Lethbridge and a pond at the Birds of Prey Center in Coaldale. Fresh human faeces were obtained from volunteers living in the city of Lethbridge. Frozen glycerol stocks of suspect *Enterococcus* isolates were received from Health Canada (HC), Lethbridge, Alberta and originally isolated during the summer of 2002 from Coaldale, Coalhurst, Fort Macleod, Lethbridge and Picture Butte sewage treatment plants (plant in-flow) and from various points along the Oldman River watershed in southern Alberta. Suspect enterococci were also isolated from in-flow sewage effluent samples collected at the Lethbridge Sewage Treatment Plant during the summer of 2003.

American Type Culture Collection (ATCC) strains used in this study are listed in Table 3.1. All protocols to follow were also performed on each of the type strains for comparison and as examples of positive and negative controls.

Table 3.1. *Enterococcus* species, American Type Culture Collection (ATCC) type strains, cultured for phenotypic analysis and quality control.

Species	Strain
avium	ATCC 14025
casseliflavus	ATCC 700327
faecalis	ATCC 29212
gallinarum	ATCC 49573
hirae	ATCC 10541
saccharolyticus	ATCC 223062

3.2 Collection and Processing of Faecal Matter

Figure 3.1 outlines the steps followed for strain isolation from various samples. Isolates were selected from mE agar and used to generate parent cultures and glycerol stocks. All tests performed on suspect enterococci listed under sections 3.3 to 3.5 are also contained in Figure 3.1.

Faecal samples were collected from all non-human animals when defecation could be confirmed. This was to ensure freshness and origin of faeces. Human volunteers collected their own faecal sample.

3.2.1 Collection of Non-Human Animal Faeces

For each animal subject, approximately 5-30ml of faeces were collected using a sterile, cotton tipped applicator. Samples were placed into a sterile 50ml Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ) and stored on ice until they could be processed.

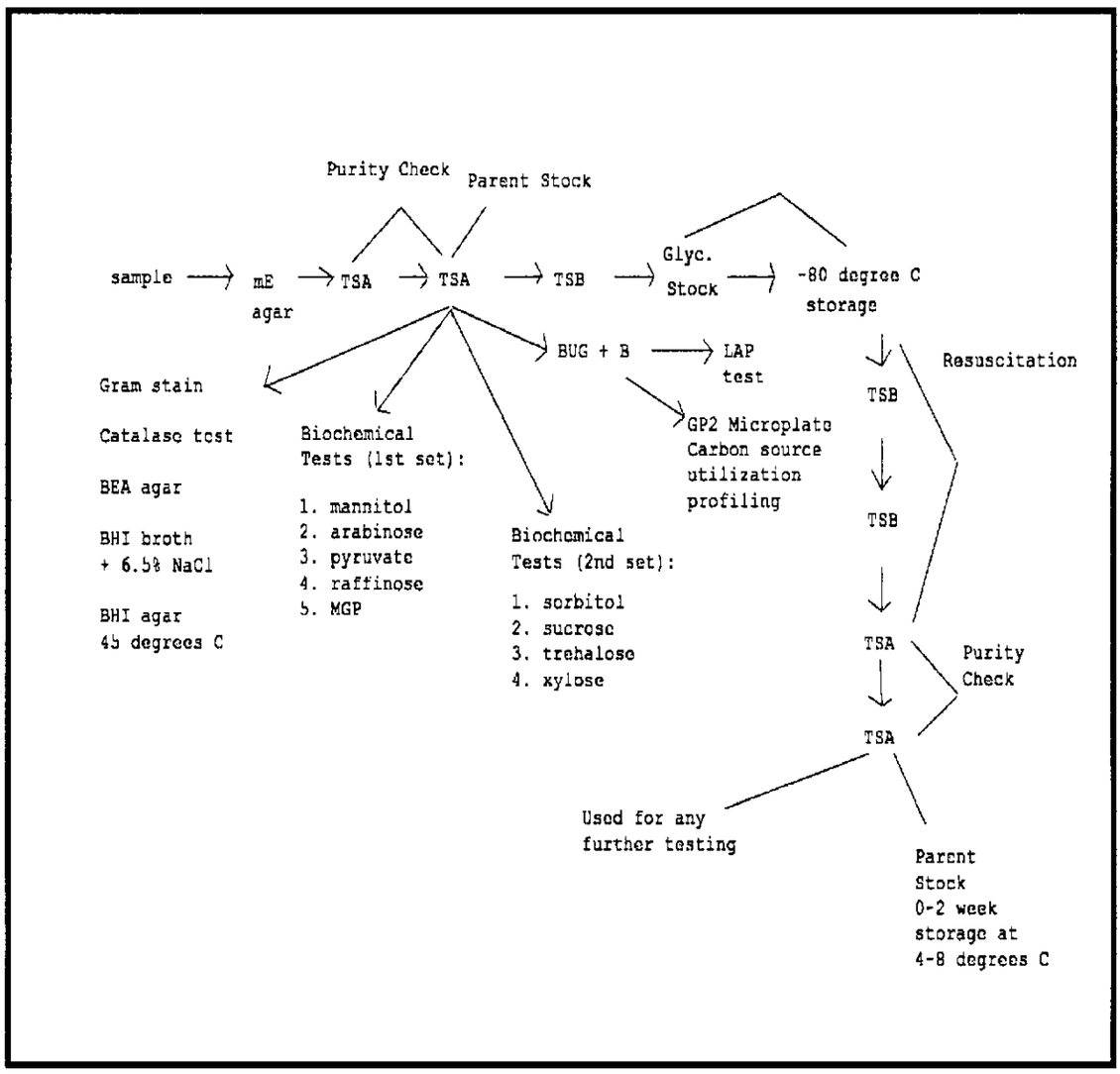
3.2.2 Collection of Human Faeces

Volunteers collected a dime-sized amount of faeces using a sterile Starplex collector vial with spork (Starplex Scientific Inc., Etobicoke, ON). Samples were refrigerated at 4-6°C or placed on ice until processing could take place.

3.2.3 Processing of Human and Animal Faeces

Phosphate buffered saline (PBS) was prepared by dissolving 8.0g NaCl, 0.2g of KCl, 1.44g Na_2HPO_4 and 0.24g of KH_2PO_4 in 800ml of distilled H_2O. Solution pH was adjusted to 7.4 with HCl and the volume brought to 1L with distilled H_2O. The buffer was autoclaved for 20min at 15lb / sq. in. on liquid cycle (Sambrook et al., 1989). Hard faeces that were difficult to spread were diluted 1:1 with PBS, shaken and vortexed until

Figure 3.1. The isolation scheme and tests performed on presumptive enterococci isolated from all sources of faecal matter and frozen glycerol stocks received from HC. Samples were isolated from water, sewage and bovine, porcine, human, goose, ovine and chicken faeces. Abbreviations: m-*Enterococcus* (mE), tryptic soy agar (TSA), tryptic soy broth (TSB), glycerol (glyc), bile esculin azide (BEA), brain-heart infusion (BHI), methyl-α-D-glucopyranoside (MGP), Biolog universal growth (BUG), 5% defibrinated sheep blood (B), leucine amino peptidase (LAP), GP2 (Gram-positive 2).


Purity Check
Parent Stock
sample
mE agar
TSA
TSA
TSB
Glyc. Stock
-80 degree C storage
Gram stain
Catalase test
BEA agar
BHI broth + 6.5% NaCl
BHI agar 45 degrees C
Biochemical Tests (1st set):
1. mannitol
2. arabinose
3. pyruvate
4. raffinose
5. MGP
BUG + B
LAP test
GP2 Microplate Carbon source utilization profiling
Biochemical Tests (2nd set):
1. sorbitol
2. sucrose
3. trehalose
4. xylose
Resuscitation
TSB
TSB
TSA
TSA
Purity Check
Used for any further testing
Parent Stock 0-2 week storage at 4-8 degrees C

a uniform mixture was achieved.

For each human or livestock faecal sample, a 10µl sterile loop (VWR International, Edmonton, AB) of faeces or PBS/faeces solution was streaked onto selective medium, m-*Enterococcus* (mE; Difco Laboratories, Sparks, MD) agar, for the growth of isolated colonies (Figure 3.1). mE agar was used for its ability to detect enterococcal species from water, food, faeces and other material (Slanetz and Bartley, 1957; Eaton et al., 1995; Manero and Blanch, 1999). The medium contained 0.04% sodium azide, for growth inhibition of Gram-negative bacteria, and 0.01% triphenyltetrazolium chloride (TTC), the agent responsible for the light to dark maroon colouring of suspect enterococcal colonies (Slanetz and Bartley, 1957). TTC is colourless, but is reduced to an insoluble red pigment (formazan) once incorporated into a growing bacterial cell. The red colouring formed only where suspect enterococci were growing (MacFaddin, 1985; Rose and Litsky, 1965). Plates were incubated at 35 ± 2°C for 48h. After incubation, any light and dark maroon colonies were considered suspect enterococci.

3.2.4 Long-term Storage of Faecal Matter

A 25% glycerol stock was prepared for long-term storage of faecal matter by mixing one part of glycerol with three parts of faeces or PBS/faeces solution. Glycerol stocks were labeled, vortexed and stored long-term in 15ml Falcon tubes at –80°C.

3.2.5 Collection of Sewage Effluent from Lethbridge Sewage Treatment Plant

Four 50ml samples of raw sewage, following mechanical removal of large debris and grit, were collected in sterile specimen containers. Samples were stored on ice until processing could take place.

3.2.6 Processing of Sewage Effluent from Lethbridge Sewage Treatment Plant

Two dilutions with PBS were performed (10^{-1} and 10^{-2}). A spread-plate using 150µl of effluent from each dilution was performed on mE agar and incubated for 48h at 35 ± 2°C.

3.2.7 Processing Colonies of Sewage or Animal Origin From mE Agar

Three to ten suspect enterococcal isolates were randomly selected from each mE agar plate and streaked on tryptic soy agar (TSA; Difco, Sparks, MD). Plates were incubated for 24-48h at 35 ± 2°C. After incubation, a colony from each plate was streaked to a new plate of TSA to ensure purity and incubated at 35 ± 2°C for 24h. These plates were labeled as parent stocks for each original isolate. Parent stocks were stored at 4-6°C and streaked to fresh TSA biweekly. Figure 3.1 illustrates the isolation of a pure colony for parent stock cultures.

3.2.8 Processing Frozen Glycerol Stocks of Suspect *Enterococcus* Water and Sewage Isolates Received From Health Canada, Lethbridge, Alberta

Tubes of tryptic soy broth (TSB; Difco, Sparks, MD) were inoculated with a loop of frozen culture stored at –80°C containing suspect *Enterococcus* isolates of water or sewage origin. Tubes were incubated at 35 ± 2°C for 4-10h. After incubation, 100µl of the TSB cultures were transferred to a second tube of TSB and incubated overnight at 35 ± 2°C. A 10µl loop of culture from each of the second tubes of TSB was streaked on TSA. These plates were incubated for 24h at 35 ± 2°C. Following incubation, a single isolated colony from each plate was streaked to fresh TSA and incubated for 24h at 35 ± 2°C. These TSA cultures were labeled as parent stocks for each original isolate. Parent stocks were stored at 4-6°C and streaked to fresh TSA biweekly. These steps ensured

quality control and maintained a uniform physiological state of the isolates (Reuter, 1992).

3.2.9 Preparation of Glycerol Stocks for Individual Suspect Enterococcal Isolates

From each of the parent stock cultures of water, sewage, bovine, porcine, human, goose, ovine and chicken suspect enterococcal strains, one single isolated colony was selected and inoculated to fresh TSB. Cultures were incubated overnight at 35 ± 2°C. After incubation, for each isolate three parts of liquid culture was mixed with one part of glycerol in labeled micro-centrifuge tubes. Tubes were shaken until uniform and stored long-term at –80°C. If parent cultures were not streaked to fresh TSA before two weeks, new parent stocks were created from freezer glycerol stocks by following the protocol under Section 3.2.8.

3.3 Confirmation of Presumptive *Enterococcus* Isolates From all Sources

Tests were chosen based on genus description by Facklam and Sahm (1995). Type strains and suspect *Enterococcus* colonies from parent cultures were streaked on TSA and incubated at 35 ± 2°C for 24h. All confirmation tests described below were performed on colonies from these plates. Figure 3.1 illustrates the scheme followed for confirmation tests.

Gram staining. A Gram stain was performed for each suspect enterococcal isolate and viewed under a light microscope using oil immersion. Gram reaction and cell shape were recorded. Any Gram-negative and non-coccoid bacteria were eliminated from the study.

Catalase activity (MacFaddin, 2000). For each isolate, one drop of 3% hydrogen peroxide (H_2O_2) was added to a slide smeared with a single colony. Catalase activity was

scored as positive if H_2O_2 was degraded and negative if H_2O_2 was not degraded. Bubbling after the addition of H_2O_2 to the smear indicated degradation. Positive results for this test in enterococci have been reported, however they are rare. Any isolates positive for catalase activity were removed from the study (Facklam and Sahm, 1995).

Esculin hydrolysis (MacFaddin, 2000). Following the isolation of colonies on mE agar, bile esculin azide (BEA) agar was used to further select for enterococci. Sodium azide and oxgall contained in the medium inhibit growth of Gram-negative bacteria and other Gram-positive bacteria respectively (MacFaddin, 2000). Plates containing BEA agar (Difco, Sparks, MD) plus 0.004% sodium azide were divided into ten squares and colonies were replica-plated to the appropriately labeled position. All plates were incubated at 35 ± 2°C for 24h. A browning of the agar indicated that esculin was hydrolyzed, a characteristic of enterococci (Facklam and Sahm, 1995). Any isolates unable to hydrolyze esculin were removed from the study.

Survival at 45°C. Plates containing brain heart infusion (BHI; Difco, Sparks, MD) agar were divided into 20 sections. Colonies were replica-plated to the corresponding labeled section and incubated at 45°C ± 0.5°C for 24-48h. No growth indicated a negative result, whereas growth indicated a positive result. Not all enterococci grow well at 45°C, therefore isolates negative for growth at 45°C were not eliminated from the study (Facklam and Sahm, 1995).

Tolerance to 6.5% NaCl. Tubes of brain heart infusion broth (BHIB; Difco, Sparks, MD) plus 6.5% NaCl were inoculated with each of the isolates and incubated at 35 ± 2°C for 48h. Turbid cultures indicated growth in 6.5% NaCl. Because some enterococci

grow weakly or not at all in 6.5% NaCl, those isolates negative for growth in the broth were not eliminated from the study (Facklam and Sahm, 1995).

LAP activity (MacFaddin, 2000). A single colony was streaked on Biolog universal growth agar (BUG; Difco, Sparks, MD) containing 5% defibrinated sheep blood (B). Plates were incubated at 35 ± 2°C for 24h. Following incubation, refrigerated LAP disks impregnated with 70μg / disk of leucine-β-naphthylamide hydrochloride (Dalynn Biologicals, Calgary, AB), were placed into labeled, sterile containers and warmed to room temperature. Leucine-β-naphthylamide can be hydrolyzed by the enzyme leucine aminopeptidase produced by some organisms, including enterococci. Disks were hydrated with a small drop of sterile water and inoculated with colonies from corresponding pure cultures. Following disk incubation for five minutes at room temperature, a drop of LAP reagent (ρ-dimethylaminocinnamaldehyde) was added to each disk. When leucine-β-naphthylamide was hydrolyzed by leucine aminopeptidase, β-naphthylamine was released and detected by ρ-imethylaminocinnamaldehyde. A red-coloured Schiff base was formed due to the coupling of β-naphthylamine and ρ-dimethylaminocinnamaldehyde (Dalynn Biologicals). Results were interpreted after one minute. Red or pink colouring on the disk indicated a positive result for the presence of leucine aminopeptidase. No change or yellow colouring indicated a negative result for LAP activity (MacFaddin, 2000; Dalynn Biologicals). All enterococci strains should produce leucine aminopeptidase, therefore, those strains that did not were eliminated from the study (Facklam and Sahm, 1995).

A running total of "failed" isolates (those isolates that did not pass the confirmation test scheme) was kept for suspect enterococci obtained from bovine faeces. These

isolates were considered not to belong to *Enterococcus* and were removed from the study. Selective ability of mE agar was evaluated from this information.

3.4 Species Identification via Conventional Biochemical Tests

Figure 3.1 illustrates the steps followed for speciation via conventional biochemical tests. Table 3.2 provides the names of the tests performed on all presumptive enterococci in this study, as well as known biochemical test results for typical strains of *Enterococcus*. This subset of tests (Table 3.2) that allowed speciation of the isolates obtained in the study were chosen from Table 2.2, adapted from Facklam et al. (2002).

All presumptive enterococci and type strains from less than two-week old parent cultures were streaked on TSA and incubated at 35 ± 2°C for 24h. The ability to utilize D-mannitol, L-arabinose, pyruvate, D-raffinose and methyl-α-D-glucopyranoside (MGP) as sole carbon sources was examined for all isolates (Facklam et al., 2002). A sterile swab was rolled over pure bacterial culture on TSA, picking up several colonies. The swab was rolled against the side of each carbohydrate tube just above the liquid level to release cells. The tube was slanted in order to pick up the inoculum and shaken gently. Tubes were incubated for a minimum of 24h to a maximum of two weeks at 35 ± 2°C (MacFaddin, 2000). Mannitol, arabinose and raffinose broths changed from red to yellow if a strain was able to metabolize the carbon source (MacFaddin, 2000). The pyruvate medium changed from dark blue to yellowish green (Nash and Krenz, 1991) and the MGP medium changed from purple to yellow (Lauderdale et al., 1999) in the event of pyruvate or MGP utilization by a bacterium. Strain pigmentation was also examined by

Table 3.2. Phenotypic tests performed on unknown, presumptive *Enterococcus* isolates. Expected results for typical species are listed. Results of this table were used in the speciation of experimental isolates. Phenotypic test abbreviations: mannitol (man), arabinose (ara), sorbitol (sbl), raffinose (raf), pigment (pig), sucrose (suc), pyruvate (pyu), methyl-α-D-glucopyranoside (mgp), trehalose (tre) and xylose (xyl). Enterococcal grouping (I to IV) is based on acid production in man and sor and hydrolysis of arg (Table 2.2). Utilization of 1% pyu, and production of acid in 1% man, ara, sbl, raf, suc, mgp, tre and xyl is indicated by "p" for positive or "n" for negative. Pigmented strains are indicated by "p" and non-pigmented strains are indicated by "n". A positive result (p) indicates that greater than 90% of strains are positive and a negative result (n) indicates that less than 10% of strains are positive. Occasional exceptions are indicated by "p*" or "n*" where less than 3% of strains show aberrant reactions. Strains of species that demonstrate variable reactions are indicated by "v". Where data was unavailable "n/a" is indicated. For group III species, "p" indicates that 85% or more of strains are positive, "n" indicates that 15% or less of strains are negative, "v" indicates that 16-84% of strains are positive. "p, n, or v" / "number" (e.g. p/82) indicates that the overall interpretation is positive, negative or variable (whichever one is indicated), but "number" % of the strains are positive. For example, "p/82" indicates that 82% of strains are positive and "n/20" indicates that 20% of strains are positive. Adapted from Facklam et al. (2002).

Group	Species	Phenotypic Characteristic									
		man	ara	sbl	raf	pig	suc	pyu	mgp	tre	xyl
I	*avium*	p	p	p	n	n	p	p	v	n/a	n/a
	malodoratus	p	n	p	p	n	p	p	v	n/a	n/a
	raffinosus	p	p	p	p	n	p	p	v	n/a	n/a
	pseudoavium	p	n	p	n	n	p	p	p	n/a	n/a
	saccharolyticus	p	n	p	p	n	p	n	p	n/a	n/a
	pallens	p	n	p	p	p	p	n	p	n/a	n/a
	gilvus	p	n	p	p	p	p	p	n	n/a	n/a
II	*faecalis*	p	n	p	n	n	p*	p	n	n/a	n/a
	faecium	p	p	v	v	n	p*	n	n	n/a	n/a
	casseliflavus	p	p	v	p	p	p	v	p	n/a	n/a
	mundtii	p	p	v	p	p	p	n	n	n/a	n/a
	gallinarum	p	p	n	p	n	p	n	p	n/a	n/a
III	*durans*	n	n	n	n	n	n	n	n	p	n
	porcinus	n	n	n	n	n	n	n	n	p	p
	ratti	n	n	n	n	n	n	n	n	n/20	n
	hirae	n	n	n	p	n	p	n	n	p	n
	dispar	n	n	n	p	n	p	p	p	p	n
	faecalis	n	n	n	n	n	n/12	p/76	n	n/12	n
	faecium	n	p	n	n/13	n	v/38	n	n	p/75	n
IV	*asini*	n	n	n	n	n	p	n	n	n/a	n/a
	sulfureus	n	n	n	p	p	p	n	p	n/a	n/a
	cecorum	n	n	p	p	n	p	p	n	n/a	n/a
V	*casseliflavus*	p	p	v	p	p	p	v	p	n/a	n/a
	gallinarum	p	p	n	p	n	p	n	p	n/a	n/a
	faecalis	p	n	p	n	n	p	p	n	n/a	n/a
	columbae	p	p	p	p	n	p	p	n	n/a	n/a

picking up a swab of colonies and looking for the absence or presence of pigment. A bright yellow colour indicated pigmentation, while light yellow, beige or clear colonies were considered non-pigmented (Facklam et al., 2002). Depending on the test results, species identification was recorded or further testing took place.

If further testing was required, the ability to metabolize sorbitol, sucrose, trehalose and xylose was examined. Tubes were inoculated in the same manner as described above and incubated for a minimum of 24h to a maximum of two weeks at 35 ± 2°C. Each broth changed from reddish-orange to yellow if a bacterium was able to metabolize the sole carbon source (MacFaddin, 2000). Following all tests, biochemical speciation results were recorded.

3.5 Species identification via BIOLOG MicroStation™

3.5.1 Bacterial Culturing

Approximately 40% of the enterococci isolated were examined using the Biolog MicroStation™ (Hayward, CA). Standard protocol as described by Biolog (2001) was followed. One colony from each parent culture of all type strains and various presumptive enterococci isolates was streaked on fresh TSA and incubated at 35 ± 2°C for 24h. Following incubation, several colonies from the overnight, pure culture were swabbed and spread evenly across a plate of BUG agar plus 5% defibrinated sheep blood (BUG agar + B). In some cases two plates of BUG agar + B were inoculated for the weaker growing strains. Blood plates allowed abundant growth of the isolates. Bacteria subjected to sub-optimal conditions may yield results that are not representative of a particular species or strain (Hughes and Andersson, 1997). BUG agar + B is the medium

Biolog database results were based on, so it was required to achieve accurate results for metabolic testing. All plates were incubated at 35 ± 2°C for 24h.

3.5.2 Establishing the Inoculum

Biolog GP2 microplates were removed from the refrigerator and placed into a 35 incubator for five minutes. The Biolog turbidimeter was calibrated before creating the inocula. Using an uninoculated tube of Biolog inoculating fluid (IF), the turbidimeter was set to 100% transmittance (%T). The bottoms of all tubes were cleaned with Kimwipes™ before insertion into the turbidimeter. The "Gram-positive coccus" (GP-coc) turbidity standard was placed into the turbidimeter and its percent transmittance (%T) recorded. All tubes were inoculated to a %T three units above or below the recorded GP-coc turbidity of 20%T.

Gram-positive coccal species require the addition of three drops of 5% sodium thioglycolate to the IF before the addition of bacteria. Sodium thioglycolate is an anti-capsule agent that partially or completely inhibits colour formation in the control well, A1, and other negative wells when bacteria metabolize their polysaccharide capsule in cases of starvation (Franco-Buff et al., 1998). Following the addition of sodium thioglycolate, a tube of IF was inserted into the turbidimeter and set to 100%T. This step was performed for each new tube, as the tubes were not optically uniform. Tubes were not rotated or moved once in the turbidimeter. A uniform suspension of bacteria and IF was prepared. A sterile, Biolog long-swab was pre-moistened with IF and gently twirled over the BUG agar + B culture sample. Nutrients were not removed along with the bacteria. The swab was twirled against the inside of the tube of IF just above the fluid surface to break up any clumps of culture and release cells. The inoculum was stirred in

a vertical motion so that aerosols and / or bubbles were not created. Bubbles can interfere with turbidity readings. More cells or IF were added until the desired turbidity value of 17-23%T was reached. Tubes were mixed well before a final %T reading was taken. Precise inoculum turbidity ensured necessary oxygen concentration for cells (Biolog, 2001).

Prior to the protocol under 3.5.2, a 23%T turbidity value for *E. faecalis* was established. Serial dilutions were prepared and vortexed before plating 100µL of dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} each on three separate petri plates of BUG agar + B. Cultures were incubated for 24h at 35 ± 2°C. Following incubation, colony forming units (CFU's) were counted on plates containing 30-300 colonies and converted to CFU's / ml. The average value at 23%T was $1.18 \times 10^{6} \pm 4.4 \times 10^{4}$ CFU / ml.

3.5.3 Inoculation of GP2 Microplates

Cell suspensions were poured into a multichannel pipet reservoir. An 8-channel repeating pipetor was set to dispense 150µl. Taking up the inoculum and dispensing it back into the reservoir primed the pipet tips. Following priming, 150µl of inoculum were dispensed into each set of eight wells until all 96 wells were inoculated. All plates ordered from Biolog were received ready for inoculation with bacteria. Each well, with the exception of the control or "blank" (well A1), was previously impregnated with tetrazolium dye and a different carbon source. Carbon sources were pre-selected based on previous and current research on Gram-positive bacteria metabolism. The control well was impregnated with distilled water and tetrazolium dye. All substances in the wells were dried. Tetrazolium violet allowed visualization of results because it changed from clear to purple after reduction due to substrate metabolism. To account for any

colour changes due to the natural oxidation of a substrate, a separate GP2 MicroPlate™ was inoculated with saline and 5% sodium thioglycolate minus bacteria. After inoculation, all microplates were incubated at 35 ± 2°C for 16-24h.

3.5.4 Reading the GP2 Microplates

Following incubation for 16-24h at 35°C ± 2°C, microplates were read using the Biolog MicroStation™ and MicroLog™ 3 software (GP2 Database, Release 6.01, 2001; Biolog, Hayward, CA). The microstation read each well of a plate at a dual wavelength of 590 nm and 750 nm. The final output value was the culture absorbance or turbidity at 590 nm minus the absorbance at 750 nm (Gadzinski, Biolog Inc.). Well A1 contained only tetrazolium dye plus water, which accounted for any colour change due to the accidental carry over of BUG agar + B nutrients. Since A1 was the reference well, any value in this well was subtracted from the values in all other wells. Output values were named "intensity of metabolic reaction". Based on reaction intensities for each well on a single microplate, the plate reader in conjunction with MicroLog™ 3 software calculated relative minimum and maximum threshold values unique to that plate. Any absorbance value recorded by the plate reader that fell below the minimum threshold value was considered to be a negative result, while any value above the maximum value was scored as a positive result. Intensity values that fell within the threshold range were considered borderline reactions. Negative reactions indicated the inability of a bacterium to metabolize a carbon source and positive reactions indicated a bacterium's ability to metabolize a carbon source. Borderline values indicated a weak positive result. In this study, borderline reactions were considered negative.

Following each plate reading, automated results were compared to results from manual readings for verification of consistency. After verification, results were entered into a spreadsheet. Isolates were examined for positive and negative similarities as well as species- and host-specific positive or negative reactions.

The entire protocol listed under section 3.8 was followed for each isolate tested, multiplied by the number of replicates performed.

3.6 Biolog Generated Profiles

3.6.1 Percentage of Positive Replicates

For each strain tested, replicate results were examined and a "percentage of times replicates were positive" (%P) was calculated for each well. For example, if six different microplates were inoculated with the same isolate, each well of each plate was examined for evidence of metabolism and the number of positive reactions or times metabolism took place were tallied. A rule regarding what was considered an overall positive result after consideration of replicate results was generated. The minimum number of replicates tested was four. To allow for error during the testing of at least one replicate, substrates where replicates demonstrated a %P greater than or equal to 75 were considered positive overall. A positive substrate was defined as a carbon source that was metabolized by 75% or more of the replicates for a single isolate. Substrates where replicates demonstrated a %P less than or equal to 25 were considered negative overall. A negative substrate was defined as a carbon source that was metabolized by 25% or less of the replicates for a single isolate. Any substrates that generated a %P between 25 and 75 were considered inconsistent. These carbon sources were still included in the profile;

however, they were not used to define profiles in the current study. To suit the definitions, all percentages involving replicates were rounded to whole numbers.

3.6.2 Genus Profiles

Profiles common to all enterococci in this study were generated by examining all of the %P values for each isolate tested, sorting the carbon sources based on the previously defined threshold and recording all of the shared positive and negative carbon sources.

3.6.3 Type Strain Profiles

Removing the carbon sources common to all enterococci in this study from Biolog-generated results created unique type strain profiles.

3.6.4 Species Profiles

Experimental isolates were sorted by species according to Biolog identification. Examination of all %P values was performed again, resulting in positive and negative carbon sources common to each species. These were considered conspecific common positive and negative carbon sources.

3.6.5 Host-Specific Species Profiles

Species were sorted according to source of isolation. %P values were examined, resulting in positive and negative carbon sources common to conspecifics from one source. These have been labeled "host-specific species commons". Unique host-specific species profiles were created by removing the carbon sources common to all enterococci and carbon sources common to the particular species in question regardless of source of isolation. Host-specific common carbon sources were not removed from host-specific species that were encountered at a frequency of one, as there were no other host-specific isolates of the same species for comparison. Species-specific common carbon sources

were not removed from the unique type strain profiles so that differences between type strains were more easily visualized.

3.6.6 Inconclusive Identifications

Only enterococcal commons were removed from profiles for any isolates that were unidentified or given two possible identifications. Unidentified isolates were still considered enterococci.

To summarize, the examination above resulted in: carbon sources common to all enterococci, distinct type strain profiles, carbon sources common to conspecific enterococci, carbon sources common to each host-specific species of *Enterococcus* and potentially unique profiles for all isolates examined. Host-specific profiles were compared to each other to identify profile overlap within species isolated from the same host. Host-specific profiles and carbon sources common to conspecifics were compared to profiles isolated from water-borne enterococci of the same species. This was done to identify sources of contamination in water.

4.0 Results and Discussion

4.1 Isolation and Presumptive Confirmation of *Enterococcus* from mE Agar

Specific objectives of Section 4.1 were: 1) to isolate presumptive enterococci from water and sewage samples as well as bovine, porcine, human, goose, ovine and chicken faeces using mE agar as a selective medium and a set of six presumptive confirmation tests; 2) to examine and evaluate the six presumptive confirmation test results; and 3) to evaluate the selective ability of mE agar.

Table 4.1 indicates the number of enterococci isolated from water and sewage, as well as bovine, porcine, human, goose, ovine and chicken faeces. A total of 673 isolates were classified as presumptive enterococci.

4.1.1 Confirmation of Presumptive *Enterococcus* Isolates from all Sources

All presumptive *Enterococcus* isolates used in the study were confirmed to be Gram-positive, catalase negative, positive for growth in 6.5% NaCl and at 45°C and positive for LAP activity and esculin hydrolysis, with the exception of the isolates listed in Table 4.2. Only three isolates were unable to survive at 45°C and four isolates demonstrated weak hydrolysis of esculin. Forty-five isolates were unable to tolerate 6.5% NaCl and 52 isolates demonstrated weak LAP activity. Of the isolates that could not tolerate 6.5% NaCl, greater than 50% were isolated from chicken and goose faeces. Of the isolates that showed weak LAP activity, more than 40% were isolated from chicken and goose faeces. The total proportion of goose and chicken strains initially isolated that could not grow in 6.5% NaCl was 37% and 32% respectively. The total proportion of goose and chicken strains initially isolated weakly positive for LAP activity was 16% and 32% respectively.

Table 4.1. Total number of enterococcal isolates following presumptive confirmation. mE agar was spread with sewage, bovine, porcine, human, goose, ovine and chicken faecal matter. Presumptive enterococcal isolates from water samples were obtained from Health Canada, Lethbridge, Alberta. Strains were labeled from 0 to 724 based on source of isolation (Identification). Confirmation tests were employed on isolates resulting in 673 enterococcal strains. Tests included: Gram stain, tolerance to 6.5% salt, survival at 45°C, hydrolysis of esculin, and presence of catalase and leucine amino peptidase activity. Total number of isolates identified on the Biolog automated system are also included.

Source	Lab Identification #	Total Isolates	Biolog Isolates
cow	10's	101	49
water	100's	98	50
sewage	200's	103	43
pig	300's	103	43
human	400's	100	40
goose	500's	43	17
sheep	600's	100	39
chicken	700's	25	10
	Grand Total	**673**	**291**

Table 4.2. Identification of isolates that produced aberrant results for confirmation tests. Isolate identifications (ID): 10's, bovine origin; 100's, water origin; 200's, sewage origin; 300's, porcine origin; 400's, human origin; 500's, goose origin; 600's, ovine origin; 700's, chicken origin. Isolates listed were negative for growth at 45°C and 6.5% NaCl tolerance and weakly positive for esculin hydrolysis and leucine amino peptidase (LAP) activity. Starred (*) isolates demonstrated aberrant results for more than one of the tests appearing in the table. Isolate totals based on source of isolation and test are identified.

Test	Growth at 45 °C	Tot.	Tolerance to 6.5% NaCl	Tot.	Esculin Hydrolysis	Tot.	LAP Activity	Tot.	Tot. by Source
Isolate ID	49*	1	5,19,44,48,49*,51,65,68 72,84,91,94,99A*,99B*	14	57,62,99A*	3	69,74,87,99B*	4	**22**
		0		0		0	113,116,118,119,127 128,132,155,177	9	**9**
		0	209	1		0	201,214,234,243,257 258,264,265,273,276 294,299C	12	**13**
		0		0		0	361	1	**1**
		0	401,402,414*,453,454	5		0	410,412,414*,417,431 434,439,464,465	9	**14**
	538*	1	520,521,522*,523*,524 528*,529,530,531,532,535* 538*,540*,541*,543,544	16		0	522*,523*,528*,535* 538*,540*,541*	7	**24**
		0	686	1		0	679,681	2	**3**
	711	1	700,701,702,706*,707,712 715*,720*	8	722	1	706*,708,709,710,713 715*,720*,723	8	**18**
Tot. by Test		**3**		**45**		**4**		**52**	**104**

All type strains were Gram-positive, catalase negative, positive for growth in 6.5% NaCl and at 45°C and positive for LAP activity and esculin hydrolysis. Isolates that were negative for growth in 6.5% NaCl and at 45°C may have been slower growing strains. These isolates were still considered enterococci, as slower growing strains often show no growth initially (Devriese et al., 1993; Facklam and Sahm, 1995). Any strains negative for esculin hydrolysis in the presence of bile and LAP production were removed from the study since all enterococci should be positive for these tests. Any isolates that demonstrated weak positive reactions (Table 4.2) were still considered to belong to the genus. This was to reduce elimination of any fastidious strains of *Enterococcus*. Based on aberrant results of the confirmation tests, more fastidious enterococcal strains were isolated from birds than from other sources.

4.1.2 Selective Ability of mE Agar

Selective ability of mE agar was examined for isolates of bovine faeces origin (Table 4.3) after confirmation test results were assessed. Differences in isolation success rate (ISR) were noted between faecal samples from the same farm and between farms. For example, samples A and B from a site in Picture Butte generated an ISR of 100%, while faecal samples C, D and E from the same farm generated ISR's of 31%, 0% and 43% respectively. Low overall ISR's were obtained from sites in Picture Butte and Little Bow (LB) 4.14, 54% and 53% respectively. High overall ISR's were achieved for sites in Claresholm and LB4, 92 and 100% respectively. When all sites and faecal samples were considered, an overall ISR of 73% was achieved.

Table 4.3. Selective ability of m-*Enterococcus* agar for the isolation of enterococci. Enterococci were isolated from bovine faeces, which were collected from different animals and different locations. Collection sites included LB 4.14, LB 1 and LB 4 along the Little Bow River (LB), a pasture just outside of Picture Butte and a livestock operation near Claresholm. Faeces were labeled A,B,C... or 1,2,3... The number of isolates from a faecal sample that did not meet confirmation requirements (*i.e.*, Gram-positive, hydrolysis of esculin, LAP activity) are indicated by "# fails". "# trys" indicates the total number of isolates that were examined from a faecal sample and "# confirmed" indicates the total number of enterococcal isolates positive for esculin hydrolysis and LAP activity from a faecal sample. "% selectivity" indicates the percentage of confirmed isolates out of the total number isolated from one faecal sample. Overall % selectivity is included for each individual collection site, and for all collection sites combined. "NA" under sites LB1 and Claresholm indicate that faeces information was not available.

Faeces Collection Site	Faeces ID	# Trys	# Fails	# Confirmed	% Selectivity	Overall % Selectivity
Picture Butte	A	10	0	10	100	
	B	5	0	5	100	
	C	13	9	4	31	
	D	6	6	0	0	
	E	7	4	3	43	
Totals		**41**	**19**	**22**		**54**
LB 4.14	1	1	0	1	100	
	2	4	1	3	75	
	3	6	3	3	50	
	4	2	0	2	100	
	5	7	4	3	43	
	6	10	6	4	40	
Totals		**30**	**14**	**16**		**53**
LB1	1	5	0	5	100	
	2	6	3	3	50	
	NA	1	0	1	100	
Totals		**12**	**3**	**9**		**75**
LB4	1	4	1	3	75	
	2	4	0	4	100	
	3	6	0	6	100	
	4	4	1	3	75	
	5	8	0	8	100	
Totals		**26**	**2**	**24**		**92**
Claresholm	2	1	0	1	100	
	3	5	0	5	100	
	4	1	0	1	100	
	5	3	0	3	100	
	7	9	0	9	100	
	8	9	0	9	100	
	NA	2	0	2	100	
Totals		**30**	**0**	**30**		**100**
Overall Totals	**NA**	**139**	**38**	**101**		**73**

The ability of mE agar to select for growth of enterococci was lower than originally reported (Slanetz and Bartley, 1957), yet higher than percent selectivity reported by Figueras et al. (1996). Slanetz and Bartley (1957) originally reported 100% selectivity for faecal streptococci or enterococci, while Figueras et al. (1996) reported a selective ability of 47% for faecal streptococci. mE selectivity increased to 60% when colonies from mE agar were confirmed with BEA agar (Figueras et al., 1996). Reuter (1992) did not report % selectivity of mE, but did indicate which Gram-positive bacteria grew on the medium. Including enterococci, members of *Streptococcocus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* were isolated from mE agar (Reuter, 1992). In this study, confirmation tests from section 3.3 were employed to account for the isolation of other Gram-positive cocci and reduce misidentification of isolates. New media are always being evaluated so that some confirmation tests may be eliminated in order to speed up isolation and identification of enterococci (Manafi, 1998).

Differences in % selectivity values between animals and farms may be due to frequency of bacterial types present within the animals studied. Although total colony counts from mE agar were not recorded, differences in total numbers of colonies between faecal samples did exist. This was especially true for bovine, ovine and human faeces. Some faecal samples were not used because there were no discernable colonies on the medium by 48h, while other samples generated growth of only a few colonies.

4.1.3 Summary

mE agar had an overall selective capability of 71% in the isolation of enterococci from bovine faeces. In this study, selective ability of mE agar varied between animals

and sampling location. Based on these results, selective ability of mE agar may also vary depending upon the source of faecal matter.

A total of 673 strains were isolated and confirmed to be enterococci. Confirmation tests are necessary when using mE agar to isolate enterococci, although care should be taken in the interpretation of these tests. Specifically, growth at 45°C and tolerance to 6.5% NaCl only increase the probability that an isolate belongs to *Enterococcus*. This is because some enterococci demonstrate contrary results to typically defined strains (Facklam and Sahm, 1995). Nonetheless, these tests are still useful. Growth and browning of agar on BEA agar and testing for LAP activity are useful in distinguishing enterococci from other Gram-positive cocci because all enterococcal strains should be positive for these tests. To be sure that a strain does belong to *Enterococcus*, identification to the species level should be made (Facklam and Sahm, 1995).

4.2 Identification of Type Strains

Specific objectives of Section 4.2 were: 1) to identify enterococcal type strains using a conventional biochemical test scheme and the automated Biolog system; and 2) to evaluate and compare identification results generated by the two methods.

4.2.1 Identification Using a Conventional Biochemical Test Scheme

Type strain results were as expected (Table 3.2) for pigmentation and production of gas in mannitol, arabinose, raffinose, sucrose and trehalose broths. There were no type strains able to metabolize sorbitol and / or xylose to compare to experimental strain results. There was often a colour change from green to bluish-green for strains positive for pyruvate utilization and a change from dark purple to lighter purple for strains positive for MGP utilization. According to protocol, these are negative results (Nash and

Krenz, 1991; Lauderdale et al., 1999). Despite these findings, type strains did at some point produce expected results in pyruvate and MGP broths.

Identification errors often occur in the differentiation of *E. gallinarum / E. casseliflavus* from other enterococci, particularly *E. faecium* (Devriese et al., 1996). *E. casseliflavus* is pigmented, making it easier to differentiate strains of this species from most other enterococci. Despite this, the occasional isolate was yellowish in the current study (Section 4.3), making it difficult to decide whether or not the strain was pigmented. According to Devriese et al. (1996), incorporation of methyl-α-D-glucopyranoside (MGP) into the identification scheme assists in the differentiation of *E. gallinarum* and *E. casseliflavus* from other enterococcal species. This is because just less than half of the species are positive for acid production in MGP broth allowing MGP utilization to further differentiate species where a collection of other tests may not be sufficient (Facklam et al., 2002).

In general, the metabolism of a carbohydrate in broth with a pH indicator should yield a distinct colour change. In the current study, *E. gallinarum* ATCC 49573 and *E. casseliflavus* ATCC 700327 incubated in MGP broth did not always demonstrate a strong colour change from purple to yellow. *E. faecalis, E. saccharolyticus* and *E. avium* also demonstrated aberrant results; often weakly positive in pyruvate broth. *E. saccharolyticus* should be negative for pyruvate utilization while *E. faecium* and *E. avium* should be able to utilize pyruvate. According to Nash and Krenz (1991), a positive reaction in pyruvate utilization medium is indicated when the broth changes from green to a distinct yellow colour. A yellow-green colour indicates a weak reaction and should be considered negative for pyruvate utilization. From personal experience, *Bacillus*

subtilis does not always produce a distinct colour change from purple to yellow in lactose broth and pH indicator bromcresol purple when it should be capable of fermenting lactose. The problem is how to determine whether a slight colour change is a weak reaction or simply a false negative reaction. Part of the solution may be in the method of culturing and ingredients making up the medium. Devriese et al. (1996) cultured test isolates on blood agar before inoculating MGP broth and used phenol red for a pH indicator. In the current study, isolates were cultured on TSA before inoculating any of the carbohydrate broths. Method of culturing has been known to impact results (Biolog, 2001; Hudson et al., 2003). Also in the current study, MGP and pyruvate media were prepared with bromcresol purple and bromthymol blue respectively, according to protocol (Lauderdale et al., 1999; Nash and Krenz, 1991). It is likely easier to detect a distinct colour change from a lighter pH indicator than from a darker pH indicator, supporting the use of standardized automated systems with built in spectrophotometers to eliminate judgment error. Both MGP and pyruvate media are very dark and minimally transparent, while broths prepared with phenol red are easy to see through.

4.2.2 Identification by Biolog MicroStation™ and MicroLog3™ Software

Type strains *E. faecalis* ATCC 29212, *E. casseliflavus* ATCC 700327, *E. gallinarum* ATCC 49573 and *E. saccharolyticus* ATCC 223062 were identified correctly according to Biolog. *E. avium* ATCC 14025 was identified correctly with a low probability (11%) on one occasion, but was unidentified on two other occasions. *E. hirae* was identified correctly on one occasion, unidentified on another occasion and identified as *E. mundtii* on three of the trials.

In general, Biolog demonstrated the ability to identify the type strains. There were only difficulties identifying *E. avium* ATCC 14025 and *E. hirae* ATCC 10541. *E. avium* tended to grow slowly and produced small coloniesd, possibly explaining why a 100% probability of identification did not come up the three times the strain was characterized. *E. hirae* ATCC 10541 was incorrectly identified or unidentified by Biolog 80% of the time. When Biolog results were compared to the expected *E. hirae* and *E. mundtii* results in the database, there wasn't a great difference between *E. hirae* ATCC 10541 and the database results. Metabolism of amygdalin (well A12; p. XXIII) was positive for *E. hirae* ATCC 10541 when it should have been negative 100% of the time according to Biolog. That same well was positive for 31% of the *E. mundtii* strains tested in the Biolog system. Metabolism of substrates in wells B3, B9, C1, D4 and H4 (arbutin, gentiobiose, α-D-lactose, β-methyl D-glucoside and thymidine) should be negative for 65% or greater of *E. hirae* strains according to the GP2 database, but in the case of *E. hirae* ATCC 10541 these carbon sources were metabolized. All other results matched the expected results in the Biolog database. According to the GP2 database, the wells listed above were positive for a greater number of *E. mundtii* strains than *E. hirae* strains. Because Biolog generates results based on probability, it was more probable that *E. hirae* ATCC 10541 be identified as *E. mundtii* according to the database. These two particular species may be similar and hard to distinguish for the Biolog system. For biochemical tests, the two species are easy to differentiate because *E. hirae* is negative and *E. mundtii* is positive for mannitol metabolism. According to the GP2 database, both species should be negative for mannitol metabolism. This is most likely because Biolog results are based on 24h of incubation. Biochemical test results can take up to a month before a

negative result is confirmed. Since Biolog is standardized, it must use results based on a defined time frame of 16-24h. Specific differences for type strain metabolism between test results and GP2 database results will be discussed in Section 4.4.

4.2.3 Summary

Expected results of type strains are known, but this is not true with unknown isolates. If an identification scheme is not reliable with type strains, it cannot be considered reliable with experimental strains. Both methods of speciation demonstrated imperfections in the ability to properly identify type strains from Table 3.1. Conventional biochemical tests used in this study (specifically MGP, pyruvate and pigmentation) to confirm type strain identification often produced ambiguous results, reducing confidence in identification. Testing should be repeated when intermediate results are encountered. Speciation results, however, were 100% reliable with Biolog in the identification of all type strains except *E. avium* and *E. hirae*. Difficulties identifying *E. avium* may have been due to poor growth of the strain. Any experimental isolates identified by Biolog as *E. mundtii* might actually be *E. hirae*. Comparison of results between *E. hirae* ATCC 10541 and *E. hirae* and *E. mundtii* from the GP2 database suggest that the database may require updating. Specific differences between test results and database results will be discussed in Section 4.4. Based on examination of type strain identification by the two methods employed, greater confidence was placed in Biolog-generated identifications.

4.3 Identification of Experimental Isolates

Specific objectives of Section 4.3 were: 1) to speciate presumptive enterococci using a biochemical test scheme; 2) to further evaluate usefulness and accuracy of traditional

biochemical tests based on identification results of experimental isolates and type strains from Section 4.2; 3) to speciate ~40% of the biochemical test-speciated isolates using the Biolog automated identification system; 4) to examine and evaluate overall species diversity according to biochemical test- and Biolog-generated results; 5) to compare overall species diversity results of the two identification methods employed; and 6) to examine biochemical test results of isolates unidentified according to the conventional biochemical test scheme.

4.3.1 Total Species Frequencies According to the Biochemical Test Scheme

All biochemical test speciation results can be viewed in appendix 8.1. Strains are numbered zero to 724 and are grouped according to source of origin (Table 4.1).

Table 4.4 ranks species by total proportion of all isolates. A total of 16 different species were recognized according to speciation by the biochemical test scheme employed. *E. faecalis* and *E. gallinarum* were most commonly isolated, followed by *E. hirae*, *E. mundtii*, and *E. faecium*. The top five species most commonly encountered made up nearly 80% of the total isolates. All other isolates identified as enterococci comprised less than 12% of the total. 2.3% of the isolates were identified as *Vagococcus* or *Lactococcus* sp., 2.4% of the isolates were narrowed down to two possible species and 4.6% of the isolates could not be identified according to the biochemical tests in Table 3.2. The biochemical test scheme employed resulted in the identification of 93% of the isolates, while 7% were unidentified.

Table 4.4. Total species frequencies and proportions (%) of 673 presumptive enterococci. Isolates were identified using conventional biochemical tests. "No id" indicates isolates that were not identified according to the scheme employed (Table 3.2). Isolates with two possible identifications are indicated by "double id".

	Identification	**Total**	**%**
1	*faecalis*	164	24.4
2	*gallinarum*	109	16.2
3	*hirae*	94	14.0
4	*mundtii*	90	13.4
5	*faecium*	74	11.0
6	no id	31	4.6
7	*avium*	24	3.6
8	*casseliflavus*	21	3.1
9	double id	16	2.4
10	*pseudoavium*	12	1.8
11	*Lactococcus sp.*	9	1.3
12	*Vagococcus sp.*	7	1.0
13	*maladoratus*	5	0.7
14	*asini*	4	0.6
15	*raffinosus*	3	0.4
16	*durans*	3	0.4
17	*sulfureus*	2	0.3
18	*saccharolyticus*	2	0.3
19	*ratti*	2	0.3
20	*pallens*	1	0.1
Total		**673**	**100.0**

4.3.2 Total Species Frequencies According to Biolog MicroStation™ and MicroLog3™ Software

Biolog-speciation results can be viewed in Appendix 8.2. A total of nine different species were identified by Biolog from all sources (Table 4.5). *E. faecalis* was identified most frequently at ~33%, followed by *E. mundtii* and *E. faecium*. The top three species comprised 57.9% of the total isolates. A significant proportion (22%) of the isolates could not be positively identified by Biolog or the identification was only narrowed down to two probable species (8.9%), totaling 30.9%. All other isolates accounted for ~11% of the total number of isolates. Biolog identified ~69% of the isolates, leaving ~31% with no identification or a questionable one (double id isolates).

4.3.3 Comparison of Total Species Frequencies According to Both Identification Methods

4.3.3.1 Identified Species

Overall species frequencies were similar for both identification methods (Tables 4.4 and 4.5), although the percentage of speciation agreement between the two methods was less than 41% (Table 4.7; to be discussed in Section 4.3.6). Further, ~43% of the total isolates were characterized by Biolog (Table 4.1), and these were randomly chosen; therefore they may not represent true proportions within the total population of isolates examined.

E. faecalis was identified most frequently and *E. faecium* was listed in the top five species encountered for both identification methods (Tables 4.4 and 4.5). This is typical since *E. faecalis* and *E. faecium* are more broadly distributed throughout the environment; *i.e.*, they are encountered more often (Aarestrup et al., 2002). *E. faecium*

Table 4.5. Total species frequencies and proportions of 291 presumptive enterococci. Isolates were identified by Biolog MicroStation™ and MicroLog3™ software. "Double id" indicates more than one possible "low probability" match according to results of the GP database. "No id" indicates that there is no probability that the isolate is a species from the Biolog database.

	Identification	**Total**	**%**
1	*faecalis*	97	33.3
2	no id	64	22.0
3	*mundtii*	48	16.5
4	double id	26	8.9
5	*faecium*	23	7.9
6	*hirae*	13	4.5
7	*gallinarum*	9	3.1
8	*casseliflavus*	8	2.7
9	*sulfureus*	1	0.3
10	*flavescens*	1	0.3
11	*durans*	1	0.3
Total		**291**	**100.0**

was in the top five, however it was unexpected that it would only be the fifth most frequent species isolated. Rather, due to its broad distribution and according to results from other studies, it was expected to be the second most frequently isolated species (Tejedor Junco et al., 2001; Pinto et al., 1999). *E. gallinarum* was the second most frequently isolated species according to the biochemical test scheme (Table 4.4), but it was only the seventh most encountered species according to Biolog (Table 4.5). The latter results are typical, as *E. gallinarum* is not frequently isolated from the intestines of farm animals. When *E. gallinarum* is isolated from humans, it is usually in a clinical setting and would have originated from a non-human animal (Tannock and Cook, 2002). All human volunteers in this study were healthy participants.

4.3.3.2 Isolates With Two Possible Identifications According to Biochemical Tests

Biochemical test results were not sufficient to produce only one species identification for several isolates. These isolates were assigned a "double identification". Eight '*faecium / asini*' isolates demonstrated what appeared to be intermediate results (weakly positive) for arabinose utilization, resulting in a double identification. These isolates may be an *E. faecium* strain that does not readily metabolize arabinose, or the isolates are a different species altogether. *E. asini* has so far only been isolated from donkeys (de Vaux et al., 1998; Aarestrup et al., 2002) reducing the likelihood that the isolates are indeed *E. asini*. One must be careful however, not to ignore the possibility of isolating *E. asini* from other animals as it has only recently been acknowledged as an enterococcal species (DSMV, retrieved Dec., 2004). *E. asini* should not be able to metabolize arabinose. Arginine hydrolysis could be used to differentiate these species (Table 2.2) as *E. faecalis* is positive for arginine hydrolysis and *E. avium* is negative for arginine hydrolysis.

Another isolate with the identification of '*columbae / raffinosus*' resulted because both strains share the same profile according to the biochemical test scheme. Testing for acid production in sorbose broth could be added to the isolation test scheme to help differentiate these species (Table 2.2). Without using sorbose to confirm, this isolate is more likely to be *E. raffinosus* because *E. columbae* has only been isolated from pigeons (Devriese et al., 1990; Aarestrup et al., 2002). Also, *E. columbae* requires CO_2 for growth and does not grow on selective media used to isolate other enterococci (Aarestrup et al., 2002). If researchers are using aerobic conditions and a medium that does not select for all enterococci, this could explain why *E. columbae* has not been isolated from other host faeces.

4.3.3.3 Isolates With Two Possible Identifications According to Biolog

Isolates with two possible identifications characterized by Biolog, resulted when metabolic properties closely matched those properties of two different species. On some occasions Biolog assigned low probabilities to two species identifications from one read on the MicroStation. On other occasions, the same isolate was identified with confidence as one species. This was unusual because it implied that certain strains may or may not metabolize a specific substrate on any given day. Researchers are generally aware that strains of the same species can demonstrate metabolic differences, however, the same strain demonstrating metabolic variability from day-to-day is not well documented. This will be discussed further in Section 4.4.12.

4.3.3.4 Unidentified Isolates of the Biochemical Test Scheme

Although the biochemical test scheme identified a greater proportion of isolates than Biolog, some percentage of error must be expected when conventional biochemical tests

are used. According to the definition of positive and negative results in Table 3.2 (adapted from Facklam et al., 2002), biochemical test results had an approximate 10% chance of error. For the tests listed in Table 3.2, a positive result indicated that > 90% of strains are positive for a test and a negative result indicated that < 10% of strains are positive for a test. This means that 10% of conspecific strains could produce aberrant results and be misidentified (Table 3.2, Facklam et al., 2002).

4.3.3.5 Unidentified Biolog Isolates

Biolog tests for utilization of 95 different substrates and has been used to examine several different strains on different occasions, so it should be more powerful than a conventional biochemical test scheme. With such a large number of tests, results are available in under 24h. With Biolog, a smaller number of substrates would not have a high differentiating capability, especially because some isolates do not begin metabolizing certain substrates until after 24h. Biolog uses characterization information of all previously examined strains and considers possibilities or probabilities each time a new strain is examined. Through repetition of testing during database development, Biolog assigns probability of identification to account for strain variability. In the current study, if any strains novel to Biolog reacted in such a way that the outcome resulted in a low probability match to a previously characterized strain, the new strain was left unidentified. This suggests that Biolog could be useful in screening for evolving strains.

It appears that the database may be limiting Biolog's identification capabilities for some of the isolates in this study. The GP2 database includes results for 18 different species, but it appears that no new species described after characterization of *E. asini* have been added to the database. *E. asini* was described in 1998 by De vaux et al. The

database was developed in 2001, suggesting that no database updates have been made since then. Unidentified isolates might be more recently described species not included in the GP2 database; or, with a relatively new genus it is possible that the unidentified species are novel and have not yet been characterized. Furthermore, if the database was developed primarily to identify important clinical pathogens, many metabolic profiles of environmental bacteria are likely novel to the GP2 database used in the study.

4.3.4 Unidentified Isolates from the Biochemical Test Scheme

According to the biochemical test scheme (Table 3.2; adapted from Facklam et al., 2002), 31 isolates were not identified. Two of these isolates (isolates 173 and 253) matched the profile of *E. hirae*, with the exception of acid production in xylose broth. Facklam et al. (2002) did not indicate what percentage of *E. hirae* strains could metablolize xylose. For study purposes, the identification of isolates 173 and 253 using biochemical tests could not be confirmed.

Of the remaining 29 unidentified isolates, there were five different phenotypes that did not match any of the species according to the biochemical tests employed. A description of these strains with regards to Table 3.2 is found in Table 4.6. There were two type-1 strains isolated from human faeces; seven, two and three type-2 strains isolated from human, goose and chicken faeces respectively; two and six type-3 strains isolated from goose and ovine faeces respectively; and four type-4 and three type-5 strains isolated from ovine faeces. Only strain types- 2 and 3 were evident among more than one host species and unidentified isolates were encountered only from ovine, human,

Table 4.6. Phenotypic description of isolates unidentified by a biochemical test scheme. Unidentified isolates are presumptive enterococci and have been grouped by type according to biochemical test results from Table 3.2 and according to source of isolation. Numerical identification of isolates are included below the source. Frequency of each type from a source is indicated as "# isolated". (man = mannitol, ara = arabinose, pig = pigmentation, pyu = pyruvate, raf = raffinose, mgp = methylglucopyranoside, sbl = sorbitol, suc = sucrose, tre = trehalose, xyl = xylose, "+" = positve for utilization, "-" = negative for utilization).

Type	Source(s)	# Isolated	Description
1	Human faeces 401,402	2	pig,tre + man,ara,pyu,raf,sbl,suc,xyl -
2	Human faeces 407, 458, 461, 476, 477, 478, 480 Chicken faeces 706, 720, 721 Goose faeces 529, 535	7 3 2	man,ara,mgp + pig,pyu,raf -
3	Sheep faeces 609, 610, 612, 614, 665, 669 Goose faeces 516, 518	6 2	man,ara,pig + pyu,mgp,raf -
4	Sheep faeces 615, 632, 637, 652	4	man,ara,pig,mgp + pyu,raf -
5	Sheep faeces 684, 691, 693	3	suc,tre + man,ara,pig,pyu,raf,sbl,xyl -
Total		**29**	

goose and chicken faeces. Ovine faeces contained the most unidentified isolates (13), followed by human faeces (9).

Table 4.7 lists the 13 isolates from Table 4.6 that were also examined by Biolog. Isolates are identified according to source and sorted by strain type according to biochemical test identification. Biolog identified six of the isolates as *E. mundtii*, one as *E. hirae* and one as either *E. mundtii* or *E. hirae*. The remaining five isolates were also unidentified by Biolog. All Biolog-examined type-2 strains were also not identified and all type- 4 and 5 strains were identified as *E. mundtii*. Biolog- and biochemical-generated identifications can be viewed in Appendices 8.2 and 8.3.

For all unidentified species, including the two suspect *E. hirae*, additional biochemical tests need to be employed and perhaps an alternative method of speciation. Because a limited number of biochemical tests were employed (Table 3.2), these isolates may or may not be novel species.

4.3.5 Summary

Since manual judgment of the biochemical test results is subjective, greater confidence might be placed in the Biolog identification approach. Type strains recognized by Biolog (Section 4.2) were generally identified with a high level of confidence; Biolog makes use of 95 biochemical tests under highly controlled and standardized conditions. Although conditions were controlled as much as possible for the conventional biochemical tests, only six to ten characteristics were examined for each strain (Table 3.2), depending on need to allow speciation of an unknown. While cost

Table 4.7. Biolog identification for isolates unidentified using biochemical tests. Numerical identification and origin of presumptive enterococci is indicated: 400's = human faeces; 500's = goose faeces; 600's = ovine faeces; 700's = chicken faeces. Isolates are sorted by strain type (described in Table 4.6). Identification according to Biolog is listed.

Isolate	Type	Biolog
401	1	*hirae*
402	1	no id
478	2	no id
706	2	no id
721	2	no id
516	3	no id
609	3	*hirae / mundtii*
610	3	*mundtii*
632	4	*mundtii*
637	4	*mundtii*
652	4	*mundtii*
684	5	*mundtii*
693	5	*mundtii*

effective, this approach will limit identification of potential discrepancies in the biochemical testing. Both identification methods recognized *E. faecalis* as the most encountered species, followed by *E. gallinarum*, *E. mundtii*, *E. hirae* and *E. faecium* in varying order (Tables 4.4 and 4.5). The overall rank of encounter frequency of *E. faecium* was unexpected (3rd and 5th most encountered species according to Biolog and biochemical tests respectively). This is discussed in Sections 4.3.8 to 4.3.10 when host-specific species frequencies are examined.

The conventional biochemical test scheme recognized a greater number of species than Biolog (18 vs. 9), and also identified a greater proportion of isolates than Biolog (93% vs. 69%). Despite this, Biolog is more sensitive in recognizing subtle differences between strains of the same species, because output values are in the form of reaction intensity, whereas traditional biochemical tests are scored as positive or negative; metabolic reaction intensity is not considered. Isolates unidentified by Biolog may be strain variants or they may be novel species. As the Biolog GP2 database expands to include profiles of more environmental isolates, numbers of unidentified isolates may decrease.

The 29 isolates unidentified by the biochemical test scheme used in this study need to be reexamined by an alternate method of speciation. Thirteen of the 29 isolates were examined by an alternative method; Biolog. Five of the isolates were confirmed to have no identification according to either method employed. Eight of the isolates were given a different identification by both methods. A third method of speciation would be useful in either identifying these isolates, or confirming the novel identification ("no identification") provided by the methods employed in this study.

All other isolates unidentified or with a "double identification" by either identification technique employed in this study also require further characterization in order to determine whether or not isolates are novel species. Where the biochemical tests are concerned, some of the newer species listed in Table 2.1were not included in Table 3.2 (*i.e.*, the table used to identify strains in this study). Unidentified isolates should be examined by alternate methods of speciation or by a biochemical test scheme that utilizes a greater number of tests.

4.3.6 Identification Accordance of Conventional Biochemical Tests and Biolog

Specific objectives for Section 4.3.6 were to: 1) determine percentage of accordance of speciation results for the two identification methods employed; 2) specifically examine percentage of accordance for the two identification methods employed for isolates identified as *E. faecalis*; and 3) discuss specific differences between the two methods of identification using *E. faecalis* as an example.

4.3.6.1 Overall Accordance

All Biolog and biochemical test speciation results for each strain can be viewed in Appendices 8.2 and 8.3. Species are listed alphabetically and sorted by source of isolation. Table 4.8 lists the percentage of accordance between Biolog and biochemical test speciation results. Approximately 40% of the isolates shared the same speciation result. Of this 40%, matches were greatest for isolates identified by Biolog as *E. faecalis* (77% accordance), followed by *E. faecium, E. mundtii, E. gallinarum* and *E.hirae*. 94%

Table 4.8. Percent accordance between Biolog and biochemical test speciation results. Any Biolog-generated species identifications that agreed with at least one isolate's identification according to biochemical test results are listed. Those isolates that were unidentified by Biolog are also listed. "Other" indicates the remaining Biolog-identified isolates. "Number of matches" refers to the number of isolates identified using biochemical tests that agreed with the Biolog identification. "Total possible" indicates the total number of isolates identified as a species by Biolog. "%" indicates the percentage of identifications for a species in accordance with both methods of speciation.

Species	# Matches	Total Possible	%
faecalis	74	97	76.3
faecium	10	23	43.5
mundtii	17	48	35.4
gallinarum	3	9	33.3
hirae	7	33	21.2
casseliflavus	1	8	12.5
no id	6	64	9.4
other	0	9	0.0
Total	**118**	**291**	**40.5**

of the matches involved these six species. The remaining isolates comprised 6% of the total matches.

E. faecalis was originally characterized in 1906 (DSMZ, retrieved Dec., 2004). The species has been further characterized since then and is considered "the type strain" of enterococci (Facklam et al., 2002). As one of the oldest enterococcal species (DSMZ, retrieved Dec., 2004), *E. faecalis* was also probably characterized thoroughly during the generation of the Biolog database. This explains why both methods identified *E. faecalis* with the highest accordance (76.3%; Table 4.8). A similar phenomenon was encountered in another study where API 20 STREP, an automated identification system, identified *E. faecalis* isolates with high success but demonstrated lower success with other species of *Enterococcus* (Devriese et al., 1995).

Hudson et al. (2003) came across an interesting phenomenon while identifying enterococci of veterinary origin using the BBL Crystal Identification Gram-Positive ID kit. They found that the sub-culture method and number of times sub-culturing was performed impacted enterococcal speciation. This phenomenon could possibly have occurred in the current study. Two different speciation techniques were used and both employed different sub-culture techniques. Hudson et al. (2003) also proposed that mixed cultures with colonies too small to see with the naked eye could have confounded the results. Single isolated colonies from mE agar were streaked to fresh medium a minimum of 2X to account for this.

4.3.6.2 Identification Accordance of *E. faecalis*

Accordance percentages can be determined for each source individually. Table 4.9 (generated from Appendix 8.2) uses *E. faecalis* as an example. The same analysis may

Table 4.9. Accordance % for *E. faecalis* identification by Biolog and biochemical tests. Identified isolates are sorted according to origin (100's = water, 200's = sewage, 300's = porcine faeces, 400's = human faeces, 500's = goose faeces, 700's = chicken faeces). Biolog and conventional biochemical test speciation results are indicated. Isolates whose identification is in agreement by both methods are bolded and percentage of isolates (for each source of origin) whose identification is in agreement by both methods (% accordance) is indicated.

ID	Biolog	Biochemical
101	*faecalis*	*avium*
102	***faecalis***	***faecalis***
110	***faecalis***	***faecalis***
111	***faecalis***	***faecalis***
117	***faecalis***	***faecalis***
121	***faecalis***	***faecalis***
124	***faecalis***	***faecalis***
125	***faecalis***	***faecalis***
126	*faecalis*	*avium*
154	***faecalis***	***faecalis***
156	***faecalis***	***faecalis***
158	***faecalis***	***faecalis***
160	***faecalis***	***faecalis***
165	***faecalis***	***faecalis***
166	***faecalis***	***faecalis***
168	***faecalis***	***faecalis***
169	***faecalis***	***faecalis***
171	***faecalis***	***faecalis***
172	***faecalis***	***faecalis***
175	***faecalis***	***faecalis***
Totals	***20***	***18***
% Accordance	***90***	

ID	Biolog	Biochemical
200	***faecalis***	***faecalis***
201	***faecalis***	***faecalis***
202	*faecalis*	*Lactococcus*
203	***faecalis***	***faecalis***
204	***faecalis***	***faecalis***
205	***faecalis***	***faecalis***
206	***faecalis***	***faecalis***
208	***faecalis***	***faecalis***
209	*faecalis*	*Lactococcus*
211	*faecalis*	*Lactococcus*
212	***faecalis***	***faecalis***
213	*faecalis*	*Lactococcus*
215	*faecalis*	*Lactococcus*
216	***faecalis***	***faecalis***
217	***faecalis***	***faecalis***
218	***faecalis***	***faecalis***
219	***faecalis***	***faecalis***
221	*faecalis*	*faecium*
223	***faecalis***	***faecalis***
224	***faecalis***	***faecalis***
227	***faecalis***	***faecalis***
228	***faecalis***	***faecalis***
229	***faecalis***	***faecalis***
230	***faecalis***	***faecalis***
231	***faecalis***	***faecalis***
234	***faecalis***	***faecalis***
273	***faecalis***	***faecalis***
275	***faecalis***	***faecalis***
276	***faecalis***	***faecalis***
295	***faecalis***	***faecalis***
296	***faecalis***	***faecalis***
297	***faecalis***	***faecalis***
298	***faecalis***	***faecalis***
299A	***faecalis***	***faecalis***
299B	***faecalis***	***faecalis***
299C	***faecalis***	***faecalis***
Totals	**36**	**30**
% Accordance	**83**	

ID	Biolog	Biochemical
300	***faecalis***	***faecalis***
301	*faecalis*	*Lactococcus*
302	*faecalis*	*avium*
303	***faecalis***	***faecalis***
305	*faecalis*	*avium*
306	*faecalis*	*avium*
307	***faecalis***	***faecalis***
328	***faecalis***	***faecalis***
329	***faecalis***	***faecalis***
330	***faecalis***	***faecalis***
331	***faecalis***	***faecalis***
332	***faecalis***	***faecalis***
333	***faecalis***	***faecalis***
334	***faecalis***	***faecalis***
335	***faecalis***	***faecalis***
336	***faecalis***	***faecalis***
Totals	**16**	**12**
% Accordance	**75**	
422	***faecalis***	***faecalis***
432	*faecalis*	*avium*
436	***faecalis***	***faecalis***
437	*faecalis*	*maladoratus*
441	*faecalis*	*pseudoavium*
442	*faecalis*	*pseudoavium*
445	***faecalis***	***faecalis***
446	***faecalis***	***faecalis***
451	***faecalis***	***faecalis***
452	***faecalis***	***faecalis***
457	***faecalis***	***faecalis***
466	***faecalis***	***faecalis***
471	*faecalis*	*pseudoavium*
472	*faecalis*	*pseudoavium*
486	*faecalis*	*avium*
487	*faecalis*	*avium*
490	*faecalis*	*avium*
491	*faecalis*	*pseudoavium*
Totals	**18**	**8**
% Accordance	**44**	

ID	Biolog	Biochemical
537	***faecalis***	***faecalis***
543	*faecalis*	*maladoratus*
Totals	**2**	**1**
% Accordance	**50**	
701	***faecalis***	***faecalis***
711	***faecalis***	***faecalis***
714	***faecalis***	***faecalis***
716	***faecalis***	***faecalis***
724	***faecalis***	***faecalis***
Totals	**5**	**5**
% Accordance	**100**	

be performed for the remaining species and isolates, however, only individual results of isolates identified by Biolog as *E. faecalis* versus biochemical test speciation results were examined in this study. According to Table 4.9, both methods of speciation identified the same five isolates from chicken faeces as *E. faecalis*, an accordance of 100%. This is 60% greater than the overall accordance for *E. faecalis*. Eighteen of the 20 water isolates were identified as *E. faecalis* by both methods of speciation, for an accordance of 90%, which is 14% greater than the overall agreement value for *E. faecalis* isolates. Thirty of the 36 Biolog-speciated *Enterococcus faecalis* sewage isolates were also given the same identification according to biochemical test results. The two methods were in accordance for 83% of these isolates. This is 7% higher than the overall agreement percentage for all of the *E. faecalis* Biolog-speciated isolates. The percentage of *E. faecalis* isolates from porcine, human and goose faeces whose Biolog and biochemical test identifications matched was 75%, 44% and 50% respectively.

Biolog identified a sewage isolate as *E. faecalis* and the biochemical test scheme identified the isolate as *E. faecium*. The only difference between these species according to Table 3.2 is the production of acid in arabinose and pyruvate. *E. faecalis* cannot utilize arabinose but can utilize pyruvate. The opposite is true for *E. faecium*. Where Biolog identified porcine, human or goose origin isolates as *E. faecalis* biochemical tests identified these isolates as *Lactococcus* sp., *E. avium*, *E. pseudoavium* and *E. malodoratus*. Based on the biochemical test scheme employed (Table 3.2), *E. faecalis* and the strains listed above are only differentiated by one carbon source. *E. faecalis* can metabolize pyruvate, while *Lactococcus* species cannot. *E. avium*, *E. pseudoavium* and

E. malodoratus can metabolize arabinose, raffinose and MGP respectively, whereas *E. faecalis* cannot metabolize any of these carbon sources.

4.3.7 Summary

Identification accordance of *E. faecalis* and *E. faecium* between methods of speciation was 76.3% and 43.5% respectively (Table 4.8). This is explained by the fact that these species are well characterized, most frequently isolated and most frequently studied (Facklam et al., 2002). When all isolate identifications were considered, overall accordance was 40.5%. Hudson et al. (2003) indicated that the sub-culturing method and number of times isolates are sub-cultured had the potential to impact speciation results. Both methods of speciation in this study used different sub-culturing methods.

Host-specific accordance values were different from overall percent accordance values for *E. faecalis*. This is likely true for other species isolated in this study. Differences could be due to different strains of the same species inhabiting different hosts. Strains of species inhabiting one host may be better characterized than strains of the same species inhabiting another host.

4.3.8 Biochemical Test-Generated Host-Specific Species Frequencies

Specific objectives of Sections 4.3.8 to 4.3.11 were: 1) to examine host-specific species frequencies according to biochemical test scheme results; 2) to examine host-specific species frequencies according to Biolog-examination; 3) to compare host-specific species diversity results of the two identification methods employed; and 4) to compare species diversity and frequency results from animal faeces to that of water samples.

Table 4.10 contains species frequencies from conventional biochemical test results and the total number of isolates examined per source. *E. faecalis* occurred at the greatest frequency in sewage, water and chicken and goose faeces. It was also common in human and porcine faeces. *E. faecium* was most common in bovine faeces, but was also isolated from sewage and water, as well as porcine, human, chicken and goose faeces. *E. hirae* was most common in porcine faeces and was the second most frequent species isolated from ovine faeces. *E. mundtii* was most frequently identified in ovine faeces, but was also isolated from bovine and goose faeces, as well as from water. *E. gallinarum* occurred at the greatest frequency in human faeces, and also occurred in water and sewage, as well as in bovine, ovine, porcine, goose and chicken faeces. *E. asini, E. casseliflavus, E. pseudoavium, E. saccharolyticus E. ratti, E. avium, E. malodoratus, E. raffinosus, E. pallens* and *E. durans* were also identified, but at a much lower frequency (less than 7%). The greatest percentage (13%) of unidentified isolates was isolated from ovine faeces.

Table 4.10 also identifies specifically which two species the "double id" isolates could possibly be. Most of the "double id" species occurred at a frequency of 2% or less, however '*faecium / asini*' appeared in the bovine isolates 8 times (8%). The '*faecium / asini*' isolates were weakly positive for arabinose utilization, and were positive for sucrose utilization, a characteristic of *E. asini* and 38% of *E. faecium* strains (Table 3.2). Possible explanations for the double identifications were previously discussed in Section 4.3.3.2.

Table 4.10. Species frequencies and proportions of 673 presumptive enterococci. Isolates were identified using conventional biochemical tests. Frequencies are sorted by source of isolation. "No id" indicates isolates that were not identified according to the scheme employed (Table 3.2). Isolates with two possible identifications are indicated by "species or genus / species or genus".

Source	Species	Frequency	%	Source	Species	Frequency	%
Cow	*faecium*	21	20.8	**Sheep**	*mundtii*	33	33.0
	gallinarum	21	20.8		*hirae*	28	28.0
	hirae	21	20.8		*casseliflavus*	19	19.0
	mundtii	16	15.8		no id	13	13.0
	faecium / asini	8	7.9		*faecium / mundtii*	2	2.0
	Vagococcus	7	6.9		*sulfureus*	2	2.0
	asini	2	2.0		*faecalis*	1	1.0
	Lactococcus	2	2.0		*mundtii / casseliflavus*	1	1.0
	casseliflavus	1	1.0		*pallens*	1	1.0
	pseudoavium	1	1.0				
	saccharolyticus	1	1.0				
	Total	101	100.0		Total	100	100.0
Water	*faecalis*	33	33.7	**Pig**	*hirae*	44	42.7
	mundtii	27	27.6		*faecalis*	27	26.2
	gallinarum	14	14.3		*gallinarum*	13	12.6
	faecium	13	13.3		*faecium*	6	5.8
	avium	7	7.1		*avium*	4	3.9
	casseliflavus	1	1.0		*asini*	2	1.9
	columbae / raffinosus	1	1.0		*Lactococcus*	2	1.9
	no id	1	1.0		*mundtii*	2	1.9
	pseudoavium	1	1.0		*maladoratus*	1	1.0
					raffinosus	1	1.0
					ratti	1	1.0
	Total	98	100.0		Total	103	100.0

Source	Species	Frequency	%	Source	Species	Frequency	%
Human	*gallinarum*	28	28.0	**Sewage**	*faecalis*	62	60.2
	faecalis	23	23.0		*gallinarum*	17	16.5
	pseudoavium	10	10.0		*faecium*	10	9.7
	faecium	9	9.0		*Lactococcus*	5	4.9
	no id	9	9.0		*avium*	3	2.9
	avium	9	9.0		*maladoratus*	2	1.9
	mundtii	6	6.0		no id	1	1.0
	gallinarum / faecium	2	2.0		*raffinosus*	1	1.0
	faecalis / Lactococcus	2	2.0		*ratti*	1	1.0
	hirae	1	1.0		*saccharolyticus*	1	1.0
	maladoratus	1	1.0				
	Total	100	100.0		Total	103	100.0
Chicken	*faecalis*	9	36.0	**Goose**	*faecalis*	9	20.9
	gallinarum	7	28.0		*faecium*	9	20.9
	faecium	6	24.0		*gallinarum*	9	20.9
	no id	3	12.0		*mundtii*	6	14.0
					no id	4	9.3
					durans	3	7.0
					maladoratus	1	2.3
					avium	1	2.3
					raffinosus	1	2.3
	Total	25	100.0		Total	43	100.0

4.3.9 Biolog-Generated Host-Specific Species Frequencies

Table 4.11 contains the frequency of enterococci isolated from each source for Biolog-identified strains. *E. faecalis* was identified in all sources except bovine and ovine faeces. This observation was the same as for the biochemical test results (Table 4.10), except that one isolate from ovine faeces was identified as *E. faecalis* instead of zero. *E. faecalis* dominated sewage isolates at a frequency of approximately 84%. Forty percent of the isolates from water, 37% from porcine faeces, 45% from human faeces and 50% from goose faeces were identified as *E. faecalis*. *E. mundtii* occurred most frequently in bovine and ovine faeces.

Eighteen percent of the strains isolated from goose faeces were identified as *E. faecium*. *E. faecium* and *E. hirae* also occurred frequently in bovine faeces. *E. casseliflavus* was identified at a frequency of 14% in water samples while another 14% of water isolates remained unidentified. Twenty-six percent of the strains isolated from porcine faeces, 33% from human faeces, 18% from ovine faeces, 30% from goose faeces and 59% from chicken faeces were not identified by the Biolog system One sewage isolate was not identified by Biolog.

4.3.10 Comparison of Host-Specific Species Frequencies

4.3.10.1 Species Isolated From Porcine Faeces

E. faecalis, E. hirae and *E. faecium* were the most prevalent species isolated from porcine faeces in previous studies (Devriese et al., 1994; Aarestrup et al., 2002). Kuhn et al. (2003) found *E. hirae* to be most frequent in swine from Sweden and Denmark, while *E. faecium* was most frequent in swine from Spain. In the current study, the same was

Table 4.11. Species frequencies and proportions of 291 presumptive enterococci. Isolates were identified by Biolog MicroStation™ and MicroLog3™ software. Frequencies are sorted by source of isolation. "No id" indicates isolates that were not identified according to Biolog. Isolates with two possible identifications are indicated by "species / species".

Source	Species	Frequency	%	Source	Species	Frequency	%
Cow	*mundtii*	14	28.0	**Sheep**	*mundtii*	28	71.8
	no id	12	24.0		no id	7	17.9
	faecium	8	16.0		*gallinarum / mundtii*	2	5.1
	hirae	7	14.0		*casseliflavus*	1	2.6
	mundtii / hirae	3	6.0		*hirae / mundtii*	1	2.6
	gallinarum	1	2.0				
	faecium / durans	1	2.0				
	mundtii / faecium	1	2.0				
	gallinarum / mundtii	1	2.0				
	hirae / gallinarum	1	2.0				
	Total	49	100.0		Total	39	100.0
Water	*faecalis*	20	40.0	**Pig**	*faecalis*	16	37.2
	no id	7	14.0		no id	11	25.6
	casseliflavus	7	14.0		*faecium*	4	9.3
	gallinarum	5	10.0		*mundtii*	4	9.3
	faecium	3	6.0		*hirae / mundtii*	3	7.0
	hirae / mundtii	2	4.0		*hirae*	2	4.7
	sulfureus	1	2.0		*gallinarum*	1	2.3
	mundtii / gallinarum	1	2.0		*gallinarum / faecalis*	1	2.3
	mundtii	1	2.0		*avium / faecium*	1	2.3
	hirae	1	2.0				
	flavescens	1	2.0				
	faecalis / casseliflavus	1	2.0				
	Total	50	100.0		Total	43	100.0

Source	Species	Frequency	%	Source	Species	Frequency	%
Human	*faecalis*	18	45.0	**Sewage**	*faecalis*	36	83.7
	no id	13	32.5		*gallinarum*	2	4.7
	faecium	4	10.0		*faecium*	1	2.3
	hirae / gallinarum	2	5.0		*hirae*	1	2.3
	durans	1	2.5		*gallinarum / faecalis*	1	2.3
	hirae	1	2.5		*hirae / faecium*	1	2.3
	gallinarum / casseliflavus	1	2.5		no id	1	2.3
	Total	40	100.0		Total	43	100.0
Chicken	*faecalis*	5	50.0	**Goose**	no id	10	58.8
	no id	3	30.0		*faecium*	3	17.6
	hirae	1	10.0		*faecalis*	2	11.8
	hirae / gallinarum	1	10.0		*gallinarum / mundtii*	1	5.9
					mundtii	1	5.9
	Total	10	100.0		Total	17	100.0

true except *E. gallinarum* replaced *E. faecium* for isolates speciated via traditional biochemical tests (Table 4.10) and *E. mundtii* replaced *E. hirae* for isolates speciated via Biolog (Table 4.11). Because Biolog had a tendency to identify *E. hirae* ATCC 10541 as *E. mundtii*, some concern over identification results of the two species must be expressed. Isolates identified as one of these species should be examined by an alternative method.

4.3.10.2 Species Isolated From Chicken Faeces

E. faecalis, E. gallinarum and *E. faecium* were isolated from chicken faeces in this order from greatest frequency to least according to the traditional biochemical tests (Table 4.10). Only ten isolates were examined by Biolog from chicken faeces. Fifty percent of these isolates were *E. faecalis* and 30% of the isolates were not identified. Kuhn et al. (2003) found that *E. faecalis* dominated the faeces of broiler chickens from Spain and Denmark. Aarestrup et al. (2002) found that prevalence of enterococcal species isolated from chickens was age-dependent. From one-day old chicks to 12-week old chicks, there was a high prevalence of *E. faecalis*, which was quickly replaced by *E. faecium*, *E. hirae* and *E. durans*, followed by a gradual replacement by *E. cecorum*. For the current study, age of the chickens sampled was not known. This is because several birds were housed per cage and the cages allowed faecal matter to fall into a collection canal filled with water. This made it difficult to be sure that faeces were selected from an identified chicken. If the chickens were 12 weeks old, one would expect to isolate *E. cecorum*. Unfortunately *E. cecorum* does not grow on mE agar and prefers the addition of CO_2 to its environment, making it a poor indicator species. According to Aarestrup et al. (2002), *E. gallinarum* is occasionally isolated from chickens. Biochemical test results indicated that seven out of 25 isolates obtained from chicken faeces were *E. gallinarum*

(Table 4.10). Because the sample size was small, it is difficult to draw any conclusions from the Biolog data (Table 4.11).

4.3.10.3 Species Isolated From Goose Faeces

Goose faecal samples came from both wild and domestic geese. According to the biochemical test scheme employed (Table 3.2), the same species of enterococci were isolated from goose as chicken faeces (Table 4.10). *E. mundtii*, *E. durans*, *E. malodoratus*, *E. avium* and *E. raffinosus* were also isolated from goose faeces, but not chicken faeces. *E. faecalis, E. faecium, E. gallinarum* and *E. mundtii* made up over 75% of the strains isolated from goose faeces according to the biochemical tests. According to Biolog results, *E. faecium, E. faecalis* and *E. mundtii* were isolated from goose faeces (Table 4.11). As well, nearly 60% of the isolates from goose faeces examined by Biolog were not identified, compared to 30% of the chicken isolates. The small sample sizes of both sources should be considered. Although documentation is limited, *E. faecium* (Thal et al., 1995; Wheeler et al., 2002), *E. faecalis, E. gallinarum* (Thal et al., 1995), *E. hirae* (Devriese et al., 2002), *E. durans* (Wheeler et al., 2002) and *E. mundtii* (Bahirathan et al., 1998; Thal et al., 1995) have been isolated from goose faeces.

4.3.10.4 Species Isolated From Bovine Faeces

Both methods of characterization identified *E. faecium*, *E. hirae* and *E. mundtii* in bovine faeces (Tables 4.10 and 4.11). Again, the tendency for Biolog to identify *E. hirae* ATCC 10541 as *E. mundtii* must be considered each time the species comes up; however, *E. mundtii* is a common inhabitant of plant material. Since cows eat plant matter, this might explain why *E. mundtii* was isolated from the faeces. Twenty-four percent of the isolates of bovine origin were unidentified by Biolog (Table 4.11), and *E. gallinarum* was

identified via traditional biochemical tests at a high frequency (Table 4.10). Devriese et al. (1992b) isolated several species of enterococci from non-ruminating calves, including *E. faecium* and *E. hirae*. Some enterococci were isolated from ruminating young cattle, mostly *E. faecalis* and *E. cecorum*, while very few enterococci (low numbers of *E. faecalis, E. hirae* and *E. casseliflavus*) were isolated from dairy cows. *Streptococcus bovis* was identified in the greatest proportion from ruminating young cattle and dairy cows. It was not the intent of this study to isolate *S. bovis*. Again, *E. cecorum* grows better in the presence of CO_2 and it does not grow with 0.3% sodium azide, an ingredient in mE agar and bile esculin azide agar. This explains why *E. cecorum* was never isolated in this study. Cattle sampled in this study were a mixture of steers, bulls, cows and pre-ruminating calves. *E. faecalis* has been isolated from cattle faeces of various age groups (Devriese et al., 1992b) including pre-ruminant calves (Aarestrup et al., 2002). In the current study, however, *E. faecalis* was not isolated from any bovine faecal samples (Tables 4.10 and 4.11). Wheeler et al. (2002) found *E. faecalis* to be absent from bovine faeces as well, however, they did not specify the age or sex of the cattle sampled. Consistent with Biolog results (Table 4.11), Wheeler et al. (2002) were unable to identify 31.9% of the isolates. In other studies *E. hirae* was found to be the most frequent in bovine faeces (Kuhn et al., 2003; Thal et al., 1995). Yanke et al. (2002) found the most frequently isolated enterococcal species from steers to be *E. durans, E. casseliflavus, E. hirae* and *E. gallinarum*. Species frequencies changed over time and depended upon antibiotics used in feed (Yanke et al., 2002). Despite the findings above, Devriese et al. (1992b) claimed that *E. durans* is rarely isolated from cattle. From the literature it appears that a variety of enterococcal species may be isolated from cattle, and factors

such as age, geographic distribution and antibiotics can have an impact on which species dominates.

4.3.10.5 Species Isolated From Ovine Faeces

Both methods of identification recognized *E. mundtii* in ovine faeces (Tables 4.10 and 4.11). *E. mundtii* was also abundant in cattle faeces. This could be attributed to diet, since *E. mundtii* is commonly found on plants. All of the sheep sampled were free grazing and half of the cattle were also free grazing. Biochemical tests identified many *E. hirae* and *E. casseliflavus* as well (Table 4.10). Like *E. mundtii, E. casseliflavus* is commonly found on plants. Both methods were unable to identify a significant number of isolates (Tables 4.10 and 4.11). *E. faecalis* was rare or absent from ovine faeces according to both methods of speciation. Some researchers have isolated *E. faecalis* from sheep, while others have not (Wheeler et al., 2002). Research regarding the isolation of enterococci from sheep was limited.

4.3.10.6 Species Isolated From Human Faeces

E. faecalis and *E. faecium* were the most frequent enterococcal species isolated from human faeces according to Biolog (Table 4.11). According to traditional biochemical test results (Table 4.10), *E. gallinarum* was the most frequently isolated species. As previously stated, this is unusual since *E. gallinarum* would more likely be isolated from clinical samples or from the gut of non-human animals. Hagedorn et al. found that 90% of all *E. gallinarum* identified from various sources were of non-human origin. *E. faecium* and *E. faecalis* are most commonly isolated from healthy adults (Devriese et al., 1992a; Tannock and Cook, 2002), although *E. durans* has been isolated less frequently (Tannock and Cook, 2002). Either *E. faecalis* or *E. faecium* may be most frequent in

human faecal matter, depending on geographic location (Devriese et al., 1992a). Kuhn et al. (2003) found *E. faecalis* to be most prevalent in healthy humans and in hospitalized patients, although the frequency of *E. faecalis* in healthy adults was slightly lower than that of patients. This was likely due to *E. faecalis* being an important hospital-acquired pathogen.

4.3.10.7 Species Isolated From Sewage

Dominant species isolated from sewage according to both methods of speciation (Tables 4.10 and 4.11) were *E. faecalis, E. gallinarum* and *E. faecium* in descending order. *E. faecium* and *E. faecalis* are the most common species of the human GI flora, but *E. gallinarum* is not, despite being identified in human faeces in the current study. *E. gallinarum* is occasionally present in the gut of other animals (Tannock and Cook, 2002). Other studies have also isolated *E. gallinarum* at a high frequency from sewage (Tejedor Junco et al., 2001). Manero et al., (2002) also isolated *E. faecalis, E. faecium* and *E. gallinarum*, as well as *E. hirae* from urban and hospital sewage. Biolog identified one *E. hirae* strain from sewage (Table 4.11). This study originally only examined sewage isolates, but this could not ensure the isolation of enterococci from humans only. People often flush pet faeces down toilets, plus faeces from feedlots or livestock processing plants can end up in sewage collection ponds as well. While there should be similarities between the composition of enterococci from sewage and human faeces, differences due to contamination of sewage by other sources of faecal matter are expected.

Although there is not a large concern regarding goose and ovine faeces contaminating food and water, enterococci were characterized from these animals for comparison to chicken and cows respectively (Tables 4.10 and 4.11). Chickens and geese may have a

similar composition of enterococci because they are both avians. Cattle and sheep are both ruminate mammals, making it interesting to compare the gut flora here as well.

4.3.10.8 Species Isolated From Water

As previous research has mentioned (Wheeler et al., 2002), *E. faecalis* and *E. faecium* are commonly isolated from warm-blooded animals, so it was expected that both identification methods would recognize these species in the water samples. *E. faecalis* comprised ~40% of the total isolates in water samples for both methods of speciation (Tables 4.10 and 4.11). Biochemical test results also identified a large proportion of *E. mundtii, E. gallinarum* and *E. faecium* isolates (Table 4.10), while Biolog-speciation results identified *E. casseliflavus, E. gallinarum* and a small proportion of *E. faecium* isolates (Table 4.11). One *E. flavescens* strain was identified by Biolog among the water isolates, however DNA reassociation studies and studies with the *sodA* gene encoding manganese-dependent superoxide dismutase have shown that *E. flavescens* and *E. casseliflavus* are a single species (Facklam et al., 2002; Poyart et al., 2000). *E. casseliflavus* and *E. mundtii* are plant-associated species (Devriese et al., 1992b), therefore they may have arrived in the water from the faeces of a herbivore or they may have inhabited the plants in or near the water. Pinto et al. (1999) isolated several different species from harbour sediments, brackish water and swimming pool water. Species included *E. faecium, E. faecalis, E. durans, E. hirae, E. casseliflavus, E. gallinarum* and *E. raffinosus*. Svec and Sedlacek (1999) isolated 523 enterococcal strains from various surface waters in Dobra, Frydek-Mistek of the Czech Republic. In their study, *E. faecium* and *E. faecalis* were most prevalent, comprising 48% of the isolates. *E. mundtii* was third most prevalent, followed by *E. hirae, E. casseliflavus* and *E.*

gallinarum, while 29% of the strains isolated were only identified to the genus level. Results appeared to overlap somewhat in this study and the studies mentioned above.

4.3.10.9 General Discussion of Species Isolated From all Sources

Although *E. faecalis* and *E. faecium* are expected to appear more frequently than other enterococcal species in most faeces from warm-blooded animals and water samples, inter-research differences in species percentages do arise. Factors such as diet, time and geographic location may be responsible for these differences. Liebana et al. (2002) demonstrated that *Salmonella enterica* serovar Dublin strains could have a diverse geographical and temporal distribution. Strain presence varied according to time and geographic location. Souza et al. (1999) found that genetic diversity of *Escherichia coli* strains correlated with geographic location and the taxonomic group of the host. Hartel et al. (2002a) found similar results to Souza et al. (1999), as different ribotype profiles were identified between *E. coli* from Idaho and Georgia isolated from cattle, horse, swine and chicken. Further, as geographic distance decreased, ribotype similarities increased between strains isolated from cattle and horse. This was not true of *E. coli* ribotypes from swine and chicken. Hartel et al. (2003) also demonstrated that diet impacted the number of *E. coli* ribotypes from penned and wild deer. Although research on the correlation of genetic make-up with geographic location, diet and time in enterococci is currently limited, these factors could potentially affect numbers and species of enterococci isolated from various sources.

One trend noted with speciation results from the biochemical test scheme (Table 3.2) was that *E. gallinarum* was isolated more than expected (Table 4.10). *E. gallinarum* strains by definition are able to metabolize mannitol, arabinose, raffinose and MGP.

They are unable to utilize pyruvate. *E. faecium* strains share the same characteristics except fewer strains can metabolize raffinose (between 10% and 90%) and less than 10% can utilize MGP (Table 3.2). Because *E. gallinarum* ATCC 49573 tended to produce intermediate results, similar results produced by unknown strains were considered positive as well. Ability to utilize MGP was used to differentiate *E. gallinarum* and *E. faecium* according to the biochemical identification scheme employed. If an *E. faecium* strain produced an intermediate result in MGP broth (but matched the rest of the *E. faecium* profile), the isolates would have been identified as *E. gallinarum* instead. Since *E. gallinarum* should be encountered less frequently than *E. faecium*, it is possible that the *E. gallinarum* strains were misidentified. The overall frequency of *E. gallinarum* was more than five percent greater than the frequency of *E. faecium* according to the biochemical test scheme (Table 3.2). This suggests that a significant proportion of *E. gallinarum* isolates identified via traditional biochemical tests may actually be *E. faecium* (Table 4.10). When this is considered, biochemical test results are consistent with the literature (Tanock and Cook, 2002).

4.3.11 Species Diversity and Frequency of Isolates From Water and Faecal Samples

The top four species isolated from water samples based on identification results of both methods employed are listed in Table 4.12. Both methods identified *E. faecalis*, *E. gallinarum* and *E. faecium* at the greatest frequency. *E. mundtii* was also one of the top four species encountered when biochemical tests were used, while *E. casseliflavus* was in the top four species according to Biolog. Table 4.12 also indicates the proportion of these species isolated from water, sewage and bovine, porcine, human, goose, ovine and

Table 4.12. The top four enterococcal species isolated from water samples. Species identifications are separated according to biochemical test results and Biolog results. The number of isolates from water samples identified as each of the four species and the number of isolates from faecal samples identified as each of the four species are identified for each method of speciation. Proportions of each species are with reference to the total number of isolates identified as any of the four species. This applies to isolates of water origin and isolates of faecal origin separately.

Biochemical Tests				
Species	**Water**	**Proportion (%)**	**Faecal Sources**	**Proportion (%)**
faecalis	33	37.9	131	37.4
mundtii	27	31.0	63	18.0
gallinarum	14	16.1	95	27.1
faecium	13	14.9	61	17.4
Total	**87**	100.0	**350**	100.0
Biolog				
Species	**Water**	**Proportion (%)**	**Faecal Sources**	**Proportion (%)**
faecalis	20	57.1	85	83.3
casseliflavus	7	20.0	1	1.0
gallinarum	5	14.3	4	3.9
faecium	3	8.6	12	11.8
Total	**35**	100.0	**102**	100.0

chicken faeces.

Similar proportions of *E. faecium* and *E. faecalis* were obtained from water and all faecal samples when biochemical tests were used for identification (Table 4.12). *E. mundtii* was more abundant from water than from faeces (31% vs. 18%) and *E. gallinarum* was less abundant from water than from faeces (16% vs. 27%). According to the identification results of Biolog (Table 4.12), proportions of the species were quite different between water and all faecal sources, with the exception of *E. faecium*. *E. faecium* was isolated at a frequency of 8.6% from water and 11.8% from faecal sources. *E. faecalis* comprised 83% of the total number of isolates identified as one of the four species isolated from animal faeces, while it was isolated at a frequency of 57% from water. Although the proportions are different, *E. faecalis* was isolated at the greatest frequency from both water and faecal material. The difference between *E. casseliflavus* proportions isolated from water and faeces was nearly 20%, and the difference between the proportion of *E. gallinarum* isolated from water and faeces was ~10%.

Numbers and types of species isolated from water sources may be influenced by factors such as presence of feedlot operations, slaughter houses and poultry-processing plants, abundance of wildlife and livestock in the area, heavy rainfall causing drainage from farmlands, sewage leakage, improper disposal of faecal matter from infants and pets, and differential survival of species. Water sampling and sampling of faecal matter for this study were performed in different years, and likely contributed to differences in species proportions between water and faecal samples. In the case of an MST regime, all sampling may need to be performed within the same time frame to properly assess sources of faecal pollution.

4.3.12 Summary

Frequency of species isolated in the current study varied between sources of origin, and varied compared to and between other studies (Devriese et al., 1992a; Devriese et al., 1992b; Devriese et al., 1994; Thal et al., 1995; Bahirathan et al., 1998; Pinto et al., 1999; Svec and Sedlacek, 1999; Tejedor Junco et al., 2001; Aarestrup et al., 2002; Devriese et al., 2002; Tanock and Cook, 2002; Wheeler et al., 2002; Yanke et al., 2002; Kuhn et al., 2003). Species frequencies isolated from animals depend upon factors such as selective medium used, geographic location of host, age of host, presence of antibiotics in host, health of host, and host diet. Differences in species proportions from water samples and faecal samples can also be attributed to many of these factors, as well as the proximity of potential sources of pollution to water.

Proportions of the top four species isolated from water samples (Table 4.12), according to biochemical tests, varied somewhat from species proportions isolated from faecal samples. Proportions of *E. faecalis* and *E. faecium* were similar, while proportions of *E. mundtii* and *E. gallinarum* varied by more than 10%. All proportions of the top four species isolated from water samples, according to Biolog, also varied by more than 10% from species proportions isolated from faecal samples, with the exception of *E. faecium*. Similar proportions of this species were isolated from both water and faecal samples. Both methods recognized the greatest proportion of isolates as *E. faecalis* from water and faecal samples.

4.4 Biolog-Generated Metabolic Profiles of Type strains and Experimental Isolates

Objectives of Sections 4.4.1 to 4.4.4 were: 1) to identify carbon sources commonly metabolized and commonly not metabolized by all type strains and experimental strains

examined in the study; 2) to identify differences in carbon metabolism results when threshold values defining positive and negative reactions are varied; 3) to identify unique carbon metabolism profiles of type strains; 4) to compare metabolic capabilities of type strains according to Biolog-generated results of the current study and the GP2 database results; 5) to identify carbon sources commonly metabolized and commonly not metabolized by conspecific enterococci; and 6) to identify carbon sources commonly metabolized and not metabolized by conspecific enterococci isolated from different hosts; 7) to compare isolate metabolic capability of substrates that overlapped between the Biolog GP2 MicroPlate and the conventional biochemical test scheme employed in the study.

4.4.1 Metabolism Results Common to all Biolog Identified Isolates and Type Strains

Isolate profiles and common carbon source profiles were generated based on strain ability to metabolize the various carbon sources (Carbon Source Legend, p. XXIII). Substrates are referred to by their well identification number and can be cross-referenced to the legend on p. XXIII to obtain the chemical name.

Table 4.13 identifies carbon sources that all type strains and all isolates characterized by Biolog were able and unable to metabolize. A total of 32 substrates were not metabolized by any of the isolates and only three substrates were metabolized by all of the isolates examined. None of the substrates examined using the conventional biochemical test scheme of this study (Table 3.2), were metabolized or not metabolized by all of the strains. These substrates are highlighted grey (Carbon Source Legend, p. XXIII).

Table 4.13. Carbon sources that were commonly metabolized (positive) and not metabolized (negative) by type strains and 291 isolates characterized via Biolog. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after incubation for 24h at 35°C (top half of the table). A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after incubation for 24h at 35°C (top half of the table). Carbon sources are listed next to the corresponding well of the Biolog GP2 MicroPlate™. The bottom half of the table indicates what the common carbon sources would have been if the definition of positive and negative carbon sources had been more stringent. For the bottom half of the table (*), a "positive carbon source" indicates that ≥ 80% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after incubation for 24h at 35°C, and a "negative carbon source" indicates that ≤ 20% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after incubation for 24h at 35°C. Wells affected by the change in threshold values for positive and negative carbon sources are highlighted grey in the top half of the table.

Postive Carbon Sources		Negative Carbon Sources		Negative Carbon Sources	
Well	**Carbon Source**	**Well**	**Carbon Source**	**Well**	**Carbon Source**
B5	D-fructose	A8	tween 40	F4	D-malic acid
B11	α-D-glucose	A9	tween 80	F7	methyl succinate
C6	D-mannose	B6	L-fucose	F8	propionic acid
		B8	D-galacturonic acid	F10	succinamic acid
		C11	3-methyl glucose	F11	succinic acid
		D6	L-rhamnose	F12	N-acetyl L-glutamic acid
		D9	sedoheptulosan	G2	D-alanine
		E4	xylitol	G3	L-alanine
		F6	acetic acid	G5	L-asparagine
		E7	α-hydroxybutyric acid	G6	L-glutamic acid
		E8	β-hydroxybutyric acid	G7	glycyl-L-glutamic acid
		E9	γ-hydroxybutyric acid	G8	L-pyroglutamic acid
		E10	p-hydroxyphenyl acetic acid	G10	putrescine
		E11	α-ketoglutaric acid	G11	2,3-butanediol
		F1	lactamide	H6	adenosine 5'-monophosphate
		F2	D-malic acid methyl ester	H12	D-L-α-glycerol phosphate
Total	**3**	**Total**	**16**		**16**

Well	Carbon Source*	Well	Carbon Source*	Well	Carbon Source*
B5	D-fructose	A8	tween 40	F7	methyl succinate
B11	α-D-glucose	B6	L-fucose	F8	propionic acid
		B8	D-galacturonic acid	F10	succinamic acid
		C11	3-methyl glucose	F11	succinic acid
		D6	L-rhamnose	F12	N-acetyl L-glutamic acid
		D9	sedoheptulosan	G2	D-alanine
		E4	xylitol	G3	L-alanine
		E7	α-hydroxybutyric acid	G5	L-asparagine
		E8	β-hydroxybutyric acid	G6	L-glutamic acid
		E9	γ-hydroxybutyric acid	G7	glycyl-L-glutamic acid
		E10	p-hydroxyphenyl acetic acid	G8	L-pyroglutamic acid
		E11	α-ketoglutaric acid	G10	putrescine
		F1	lactamide	G11	2,3-butanediol
		F4	D-malic acid	H12	D-L-α-glycerol phosphate
Total	**2**	**Total**	**14**		**14**

The Biolog GP2 database contains results for all of the species named in Table 3.2, except *E. pallens, E. gilvus, E. porcinus, E. ratti* and *E. asini*. Results were consistent with the Biolog GP2 database, with the exception of wells C11 (3-methyl glucose) and H6 (adenosine-5'-monophoshpate). According to Biolog, 32%, 29%, 42% and 70% of *E. sulfureus, E. cecorum, E. casseliflavus* and *E. saccharolyticus* strains respectively should be able to metabolize 3-methyl glucose. For well H6, 29%, 54%, 81% and 86% of *E. avium, E. cecorum, E. columbae* and *E. sulfureus* strains respectively should be able to metabolize adenosine-5'-monophoshpate. Isolates in the current study metabolized the two substrates less than 25% of the time. One *E. sulfureus* and eight *E. casseliflavus* strains were isolated in the study and zero *E. saccharolyticus, E. cecorum* and *E. columbae* strains were isolated. *E. saccharolyticus* type strain ATCC 223062 and *E. avium* type strain ATCC 14025 were the only strains of these species examined in the study. Low numbers or lack of these species isolated in the study most likely contributed to the inconsistent results. None of the database %P values are zero or 100, so it is reasonable to expect that some strains of these species would have varying metabolic capabilities.

Table 4.13 also lists what the common carbon sources would be if the positive and negative threshold values were changed from 25% to 20% and 75% to 80%. This translates to "number of positive replicates at a frequency of 80% or greater" and "number of negative replicates at a frequency of 20% or less" respectively. With a more stringent threshold value, 30 substrates would be commonly metabolized or not metabolized (bottom of Table 4.13), rather than 35 (top of Table 4.13). Specifically,

wells A9, E6, F2 and H6 would not be negative and well C6 would not be positive for all enterococcal strains tested. They would fall under the "inconsistently metabolized" category (number of positive replicates at a frequency between the defined threshold values) and remain part of an isolate's unique profile. A unique profile is one where all substrates common to enterococci, conspecific enterococci and host-specific enterococci of the same species have been removed from the profile. Conspecific and host-specific enterococci are defined in Sections 4.4.3 and 4.4.4.

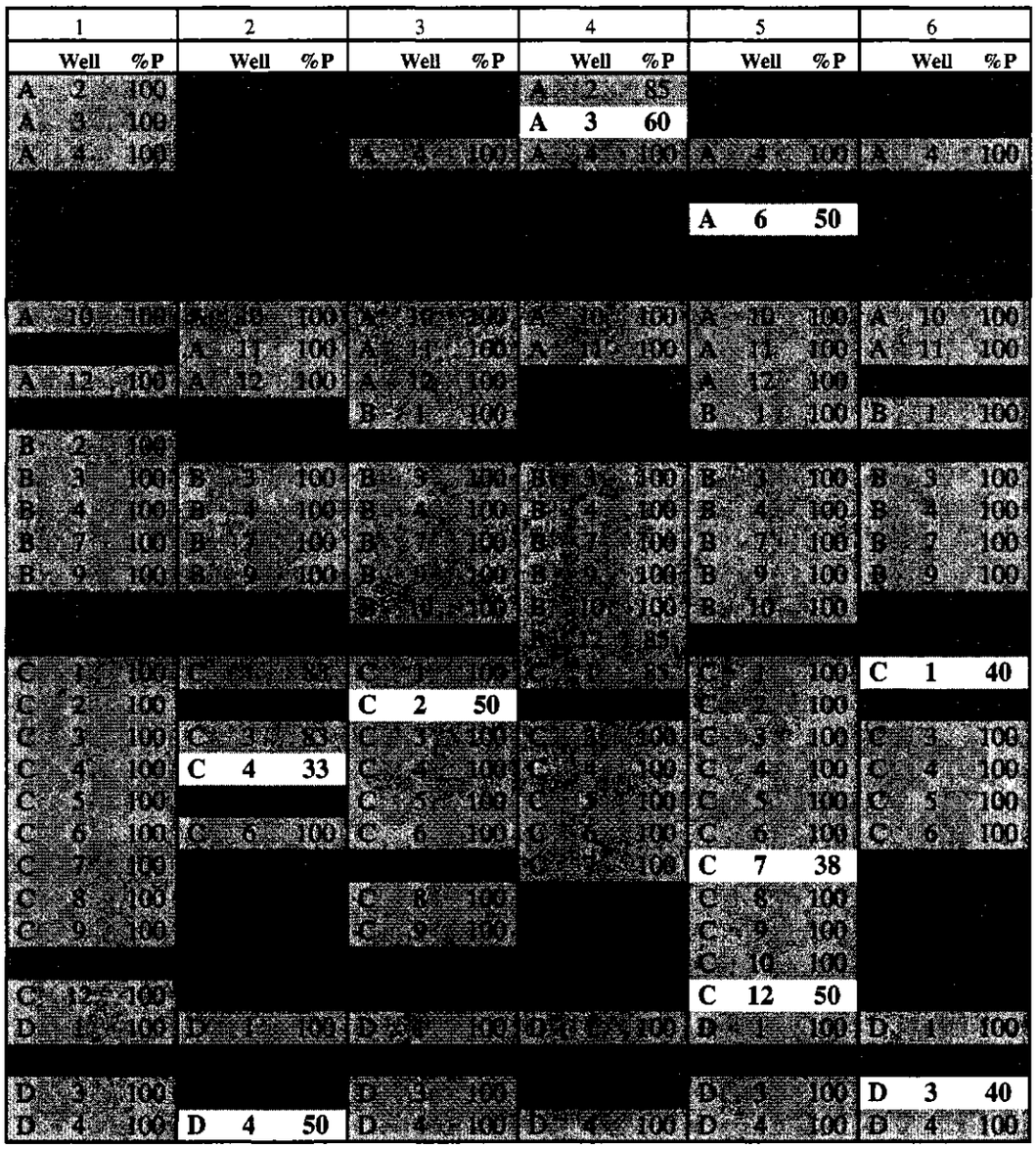
By subtracting all substrates that were metabolized and not metabolized by all strains characterized by Biolog, preliminary results indicated that more commonly isolated species of *Enterococcus* could be differentiated between with as few as 60 substrates. In a study that used a similar sample size to the current study (105 human isolates and 195 non-human isolates), Biolog results of known-source enterococci were used to build a library to test against unknown-source isolates (Hagedorn et al., 2003). Results indicated that 30 of the 95 carbon sources were not only sufficient, but also most effective at discriminating between species of enterococci. Only two of the 30 substrates (α-D-glucose, well B11 and D-mannose, well C6) were carbon sources metabolized by all of the strains tested in the current study (Table 4.13). Hagedorn et al. (2003) found that 97.6% of human and 99% of non-human isolates could metabolize substrate B11, while 78% of human and 100% of non-human isolates could metabolize substrate C6. Based on their results, Hagedorn et al. (2003) suggested pipetting the 30 carbon sources and two controls mixed with tetrazolium dye in triplicate into a generic microplate. With the correct software, the Biolog MicroStation also serves as a regular plate reader. Reducing the number of carbon sources would also decrease cost and waste generated from the

unused wells. The current study supports this suggestion when results of the uninformative wells are considered. Hagedorn et al. (2003) found that 65 of the carbon sources were not necessary to identify enterococci isolated in their study. The current study found that 35 of the carbon sources were not required in order to identify more commonly isolated enterococci. Thirty-three of these substrates matched with the results of Hagedorn et al. (2003), with the exception of wells B11 and C6. If a plate specifically used for the isolation of enterococci were to be developed, a selective medium and the six confirmation tests used in this study would be required to initially identify presumptive enterococci. With the current GP2 plate, only a Gram stain needs to be performed.

4.4.2 Type Strain Profiles

Metabolic profiles of type strains after the removal of common substrates from Table 4.13, are displayed in Table 4.14. For all profile tables to follow, dark grey shading indicates the inability to metabolize a carbon source each time testing is replicated (0-25% P), light grey shading indicates the ability to metabolize a substrate each time testing is replicated (75-100% P) and no shading indicates inconsistent metabolism of a carbon source within replicates (26-74% P). Patterns in Table 4.14 demonstrate distinct metabolic profiles between the six type strains examined. Wells where study results and Biolog GP2 database results (%P*) differed are identified in Table 4.15. %P* values indicate the percentage of times strains of a species (a combination of different strains and replicates of the same strain) demonstrated metabolism of a carbon source after incubation for 24h at 35°C.

Table 4.14. Enterococcal type strain profiles after the removal of commonly metabolized and not metabolized carbon sources by all Biolog-characterized isolates.
Wells are indicated by position on the GP2 MicroPlate™. Both test results (%P) and Biolog database results (%P*) are displayed. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. ["% P" = percentage of positive replicates; 1 = *E. saccharolyticus* ATCC 223062, 4 replicates; 2 = *E. hirae* ATCC 10541, 6 replicates; 3 = *E. gallinarum* ATCC 49573, 4 replicates; 4 = *E. faecalis* ATCC 29112, 20 replicates; 5 = *E. casseliflavus* ATCC 700327, 8 replicates; 6 = *E. avium* ATCC 14025, 5 replicates].


1
2
3
4
5
6
Well %P
A 2 100
A 3 100
A 4 100
A 10 100
A 12 100
B 2 100
B 3 100
B 4 100
B 7 100
B 9 100
C 1 100
C 2 100
C 3 100
C 4 100
C 5 100
C 6 100
C 8 100
C 9 100
C 12 100
D 1 100
D 3 100
D 4 100
A 2 85
A 3 60
A 6 50
C 1 40
C 2 50
C 4 33
C 7 38
C 12 50
D 3 40
D 4 50

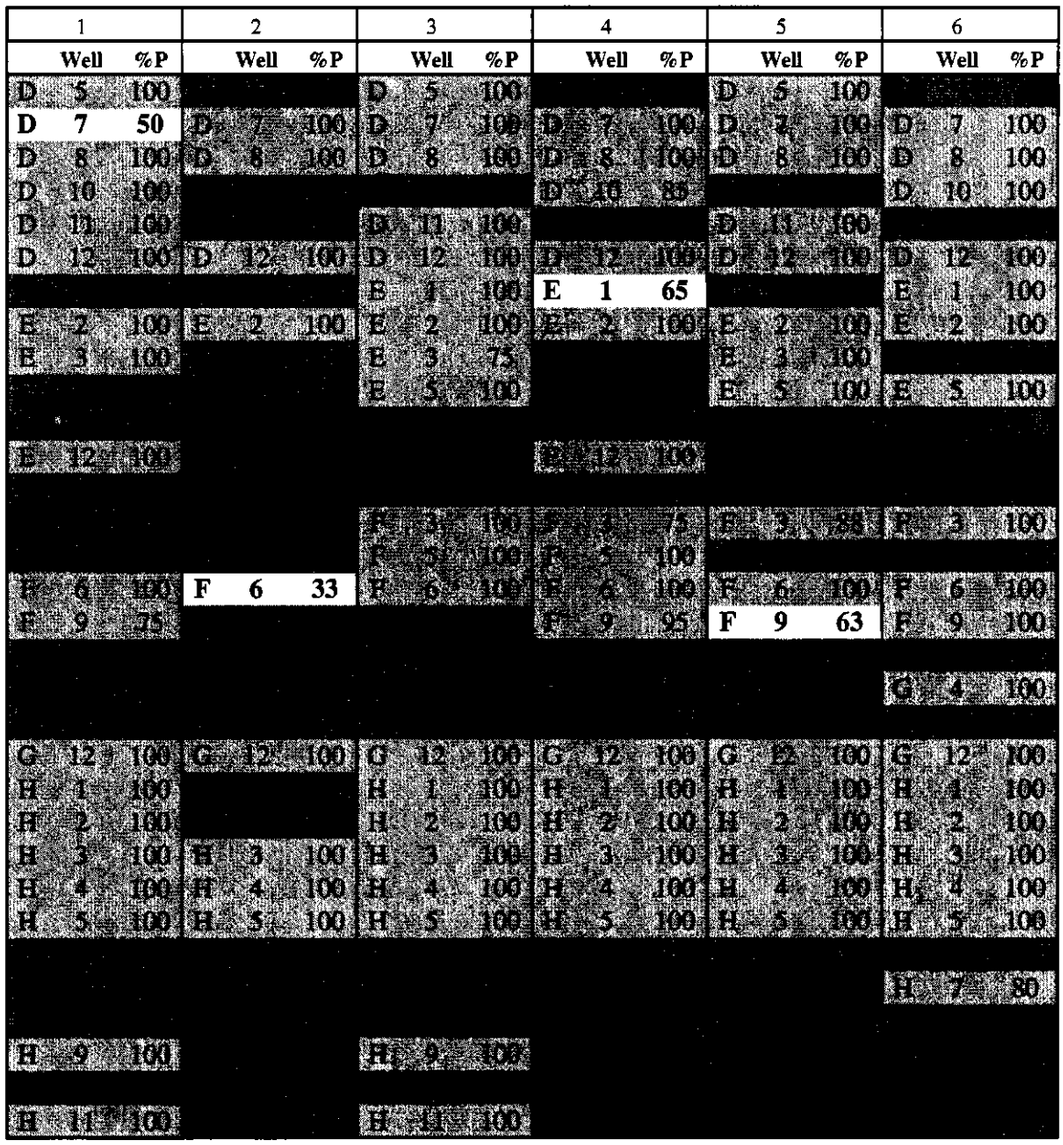

1
Well %P
D 5 100
D 7 50
D 8 100
D 10 100
D 11 100
D 12 100
E 2 100
E 3 100
E 12 100
F 6 100
F 9 75
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
H 9 100
H 11 100
2
Well %P
D 7 100
D 8 100
D 12 100
E 2 100
F 6 33
G 12 100
H 3 100
H 4 100
H 5 100
3
Well %P
D 5 100
D 7 100
D 8 100
D 11 100
D 12 100
E 1 100
E 2 100
E 3 75
E 5 100
F 3 100
F 5 100
F 6 100
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
H 9 100
H 11 100
4
Well %P
D 7 100
D 8 100
D 10 85
D 12 100
E 1 65
E 2 100
E 12 100
F 3 75
F 5 100
F 6 100
F 9 95
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
5
Well %P
D 5 100
D 7 100
D 8 100
D 11 100
D 12 100
E 2 100
E 3 100
E 5 100
F 3 88
F 6 100
F 9 63
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
6
Well %P
D 7 100
D 8 100
D 10 100
D 12 100
E 1 100
E 2 100
E 5 100
F 3 100
F 6 100
F 9 100
G 4 100
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
H 7 80

Table 4.15. Wells of the GP2 MicroPlate™ where metabolic fingerprints of enterococcal type strains differ according to Biolog test results (%P) and GP2 database results (%P*). A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. ["% P" = percentage of positive replicates (study results); "%P*" = percentage of times well is positive at end-point (GP2 database information); 1 = *E. saccharolyticus* ATCC 223062, 4 replicates; 2 = *E. hirae* ATCC 10541, 6 replicates; 3 = *E. gallinarum* ATCC 49573, 4 replicates; 4 = *E. faecalis* ATCC 29112, 20 replictes; 5 = *E. casseliflavus* ATCC 700327, 8 replicates; 6 = *E. avium* ATCC 14025, 5 replicates].

Well	%P	%P*	Well	%P	%P*	Well	%P	%P*
1			**3**			**5**		
		33	A 4 100		61	A 6 50		
C 9 100		43	B 12 100		64	B 10 100		60
		47	B 10 100			C 7 38		
E 12 100		47	C 5 100		58	C 9 100		58
		47	C 9 100			D 7 100		
H 9 100		67			42			65
H 11 100		60	D 3 100					78
2			D 7 100		28	E 5 100		42
		33	E 1 100		42	F 6 100		62
		27	E 3 75			F 9 63		
A 12 100			E 5 100			**6**		
B 3 100		35	F 3 100		39	A 4 100		40
B 4 100		50	F 5 100		53	B 1 100		
B 9 100			F 6 100		56	B 3 100		57
C 12 83		27	H 2 100		56	B 9 100		57
		27	H 9 100			C 4 100		69
C 3 83		54	H 11 100			D 1 100		29
		27	**4**			D 3 40		
D 1 100		58	A 2 85		55	D 8 100		74
D 4 50					26	D 10 100		69
		27	B 10 100		30	D 12 100		26
D 7 100		65	C 1 85		45	E 1 100		74
		42	C 5 100		69	E 5 100		
F 6 33			D 1 100		54	F 6 100		55
H 3 100		50	D 10 85		40	F 9 100		
H 4 100			E 12 100		59			55
			F 3 75			H 2 100		67
			F 5 100		48			29
			F 9 95		73	H 7 80		
			H 4 100		68			

Table 4.16 indicates the number of wells that differed for each species between test and database results. It should be noted that Biolog study results refer to the percentage of positive replicates for one strain, while the Biolog database results refer to a combination of the percentage of positive strains and strain-replicates of a given species. This indicates that results may vary from test to test, unless database values are zero or 100. Numbers of strains and numbers of replicates tested during the generation of the GP2 database could not be provided (Lisa Schibler of Biolog, telephone conversation, March 4, 2005).

4.4.2.1 *E. saccharolyticus* ATCC 223062

The least number of differences (7) between study results and database results occurred with *E. saccharolyticus* (Table 4.16). For these wells, differences were all insignificant; *i.e.*, none of the database results indicated that 0% or 100% of *E. saccharolyticus* strains should metabolize the substrate. Differences do not contradict profile results of *E. saccharolyticus* according to the Biolog database.

4.4.2.2 *E. hirae* ATCC 10541 and *E. avium* ATCC 14025

The greatest number of differences (18) occurred in the examination of *E. hirae* and *E. avium* (Table 4.16). Neither one of the strains was accurately identified by Biolog (Section 4.2.2). For *E. hirae*, all of the differences involved intermediate results, except for well A12 (amygdalin) (Table 4.15). According to the database results, all strains of *E. hirae* should be negative for metabolism of amygdalin, while *E. hirae* ATCC 10541 metabolized amygdalin each time it was tested. Devriese et al. (1993) also found that *E. hirae* could metabolize amygdalin. This suggests that the Biolog GP2 database requires updating.

Table 4.16. Number of wells that differed between study results and GP2 database results for enterococcal type strains examined via Biolog MicroStation™ and MicroLog3™. Type strains were obtained from the American Type Culture Collection (ATCC). "Code" refers to the identification given to each species on Tables 4.14 and 4.15 and "# Replicates" indicates how many different times type strains were examined with the Biolog system.

Code	Species	ATCC ID	# Replicates	# Differences
1	*saccharolyticus*	223062	4	7
2	*hirae*	10541	6	18
3	*gallinarum*	49573	4	17
4	*faecalis*	29112	20	12
5	*casseliflavus*	700327	8	10
6	*avium*	14025	5	18

Wells H7 (thymidine-5'-monophosphate) and E5 (D-xylose) differed significantly between test results and database results for *E. avium* (Table 4.15). *E. avium* ATCC 14025 was able to metabolize D-xylose 100% of the time when tested and was able to metabolize thymidine-5'-monophosphate 80% of the time when tested. According to database results, no strains of *E. avium* should be able to metabolize either of these substrates. Tyrrell et al. (2002) also found that *E. avium* could not metabolize D-xylose. Devriese et al. (1993) reported that some *E. avium* strains could metabolize D-xylose, although it was rare.

The high %P for *E. avium* involving metabolism of D-xylose (well E5) and thymidine-5'-monophosphate (well H7) in the current study (Table 4.15) suggests that the values were not due to error. Type strains have been selected because of their thorough characterization and physiological stability. *E. avium* grows best with the addition of 5% CO_2, which was not added in the current study. Despite this, *E. avium* reliably metabolized both substrates. Tyrrell et al. (2002) also omitted CO_2 and used the same *E. avium* type strain, ATCC 14025. The metabolism of D-xylose (well E5), however, was tested using conventional biochemical tests rather than with an automated system (Tyrrel et al., 2002). *E. avium* was also streaked on TSA + B (Tyrrell et al., 2002), whereas all strains were grown on BUG + B in the current study. This could account for differences in metabolic capability (Hudson et al., 2003).

4.4.2.3 *E. gallinarum* ATCC 49573

There were 17 differences between test results and database results for *E. gallinarum* (Table 4.16). None of these differences were significant. They did not contradict *E. gallinarum* profile results according to the Biolog database.

4.4.2.4 *E. faecalis* ATCC 29112

There were 11 insignificant differences and one significant difference between study and database results for *E. faecalis* (Table 4.16). L-lactic acid (well F3) should not be metabolized by any *E. faecalis* strains, however, *E. faecalis* ATCC 29112 was able to metabolize this substrate 75% of the time it was examined in this study (Table 4.15).

4.4.2.5 *E. casseliflavus* ATCC 700327

There were ten differences between study and database results for *E. casseliflavus* (Table 4.16). Only one of these differences was significant. According to the GP2 database (Table 4.15), no *E. casseliflavus* strain should metabolize D- melezitose (well C7). *E. casseliflavus* ATCC 700327 demonstrated the ability to metabolize this substrate 38% of the times it was examined by Biolog. Although the type strain was able to metabolize melezitose on occasion, 38% does not indicate a strong ability to metabolize the substrate.

4.4.3 Metabolism Results Common to Conspecifics

Where two or more isolates were identified by Biolog as the same species, all carbon sources commonly metabolized or not metabolized by strains of the same species (conspecific carbon sources) were identified and removed from the unique profile (Section 4.4.6). These conspecific carbon sources of the more commonly isolated enterococcal species are displayed in Table 4.17. Isolates identified as *E. faecalis* had the least number of carbon sources in common and the greatest number of isolates identified. Isolates identified as *E. casseliflavus* had the second greatest number of carbon sources in

Table 4.17. Biolog-generated carbon sources commonly metabolized (positives) and not metabolized (negatives) by enterococcal strains of the same species.

Species most commonly isolated (Table 4.5) from water, sewage and bovine, porcine, human, chicken, goose and ovine faeces are identified. Number of isolates identified per species is indicated below the species name. "Conspecific" carbon sources are indicated by well identification. "Positives" indicate that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. "Negatives" indicate that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C.

Species	Positives	Negatives		Species	Positives	Negatives	
casseliflavus	A- 4,10,11,12	A- 5,7		*faecalis*	A- 10	A- 6	
8	B- 1,3,4,7,9	B- 12		97	B- 3,4	D- 2	
	C- 1-5,8	E-12			C- 3	G- 1,4,9	
	D- 1,4,5,7,8,11,12	G- 4,9			D- 7,8	H- 8	
	E- 2,3	H- 8-10			E- 2		
	G- 12				F-6		
	H- 1-5				G- 12		
					H- 1,3,5		
Total	**30**	**9**	**39**	**Total**	**12**	**6**	**18**
gallinarum	A- 10	A- 3,5,6,7		*faecium*	G- 12	A- 3,5,6,7	
9	B- 3,4,7	B- 12		23	H- 5	B- 10,12	
	D- 8,12	C- 12				C- 5,7,9,12	
	G- 12	D- 2,10				D- 2,3,5,10	
		G- 1,4,9				E- 1,3,5	
		H- 8,10				F- 3,5,9	
						G- 1,4,9	
						H- 2,7,8,10,11	
Total	**7**	**13**	**20**	**Total**	**12**	**28**	**40**
hirae	A- 10	A- 6,7		*mundtii*	A- 10	A- 2,3,5,6,7	
13	B- 3,4	B- 12		48	B- 3,4	B- 10,12	
	D- 8	D- 2			D- 1,7,8	C- 7,12	
	G- 12	E- 1,12			G- 12	D- 2,3,5,10	
		F- 5,9			H- 5	E- 1,3,5,12	
		G- 1,4,9				F- 3,5,9	
		H- 8-11				G- 1,4,9	
						H- 7-11	
Total	**5**	**15**	**20**	**Total**	**8**	**28**	**36**

common and the least number of isolates identified. *E. faecium* had the greatest number of carbon sources in common and the third most number of isolates identified. With the exception of *E. hirae* and *E. gallinarum,* numbers of common carbon sources tended to decrease as species frequency increased (Figure 4.1). This suggests that sample sizes could be larger in order to get a more accurate representation of conspecific carbon sources.

Where few isolates were examined, there appeared to be a lower chance of intra-species differences arising (Table 4.17). Despite this, numbers of strains of the same species should be representative of what exists in the environment when ~300 isolates are selected. If a particular species, *E. casseliflavus* for example, is encountered infrequently in the environment, it likely would not be a good candidate to indicate sources of faecal pollution. A good faecal indicator should be rapidly isolated and strongly associated with pathogenic organisms (Scott et al., 2002). Overall numbers and combinations of carbon sources commonly metabolized or not metabolized by conspecific enterococci (Table 4.17) varied significantly. Differences appeared to be attributed more to number of carbon sources than actual carbon sources. That is, the same substrates tended to appear as positive and negative carbon sources for all species. A commonly positive substrate for one species was generally not a commonly negative substrate for another species. Only wells C5 (D-mannitol), D5 (D-raffinose), E3 (turanose) and H2 (2'-deoxyadenosine) were positive for one species and negative for another. *E. faecium* strains tested were negative for metabolism of these substrates and *E. mundtii* was negative for the metabolism of D-raffinose. The remaining species were either positive

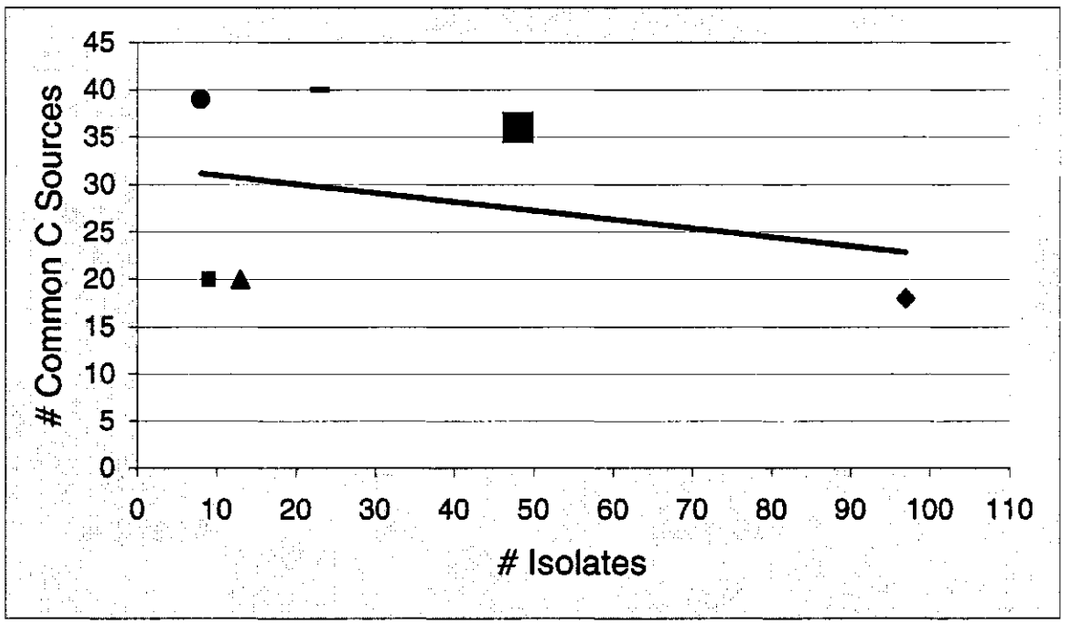


Figure 4.1. Relationship between the number of commonly metabolized and not metabolized carbon sources and numbers of con-specific enterococci isolated from water and sewage, as well as bovine, ovine, chicken, goose and human faeces.

" " = *E. casseliflavus*; " " = *E. mundtii;* " " = *E. faecium*; " " = *E. gallinarum*; " " = *E. hirae*; " " = *E. faecalis*;

for metabolism of these four substrates, or they demonstrated inconsistent metabolism.

4.4.4 Metabolism Results Common to Host-specific Species

Where two or more host-specific strains of the same species were encountered, all carbon sources commonly metabolized or not metabolized by host-specific strains of the same species (host-specific carbon sources) were removed from the unique profile. The most common host-specific species identified by Biolog (isolated from faecal matter) are displayed in Table 4.18. Substrates that were commonly metabolized or not metabolized are listed in the table and sorted according to source of isolation. Host-specific commons were not included for water isolates, as it could not be assumed that the water-borne isolates originated from one source.

Each group of host-specific *E. faecium* strains (Table 4.18) isolated from bovine, goose, human and porcine faeces demonstrated different metabolic capabilities. *E. faecium* isolated from porcine and bovine faeces contained the same number of common positive and negative carbon sources (9), but a different collection of them. This was also true for *E. faecium* isolated from human and goose faeces. Isolates from each of these sources had 20 common positive and negative carbon sources.

Numbers and combinations of negative and positive carbon sources common to all host-specific species varied from source to source (Table 4.18). For example, *E. faecalis* isolated from goose faeces had the most common carbon sources (33), followed by isolates from chicken faeces (32), porcine (22), human (20) and sewage (7). With the exception of *E. faecium* isolated from bovine and porcine faeces, there was a negative correlation between the number of strains isolated from each source and number of

Table 4.18. Biolog-generated carbon sources commonly metabolized (positives) and not metabolized (negatives) by faecal-borne enterococci of the same species.

Species most commonly isolated from sewage and bovine, porcine, human, chicken, goose and ovine faeces are identified. Number of host-specific species identified per source is indicated below the species name. "Host-specific" carbon sources are indicated by well identification. Total number of positive and negative commons are summed for each host-specific species (highlighted numbers are the combined positive and negative totals). "Positives indicate that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. "Negatives" indicate that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C.

Source	Species	Positives	Negatives	Source	Species	Positives	Negatives
Cow	*faecium*	B- 4	B- 1	Goose	*faecium*	A- 10,11	A- 2,4,12
	8	D- 8	C- 2,8,10		3	B- 3,4,7	C- 1,2,8,10
			D- 11			D- 8,12	D- 1,11
			E- 12			H- 3,4	E- 12
			H- 9				H- 9
Total	**9**	**2**	**7**	**Total**	**20**	**9**	**11**
Human	*faecium*	A- 10	A- 2,4,12	Pig	*faecium*	A- 10,11	A- 2
	4	D- 12	B- 1,6,9		4	B- 4	B- 1
		G- 12	C- 1-4,8,10			D- 8	E- 12
		H- 4	D- 1,11			H- 4	H- 1
			H- 1,9				
Total	**20**	**4**	**16**	**Total**	**9**	**5**	**4**
Chicken	*faecalis*	A- 2,4,11	A- 3,5,7,12	Goose	*faecalis*	A- 4,11	A- 3,5,7,12
	5	B- 7,9,10	B- 1		2	B- 7,9,10,12	B- 1
		D- 4,10,12	C-1,2,8-10,12			C- 4,5,7	C- 10,12
		E- 12	D- 3,5,11			D- 1,4,10,12	D- 3,5,11
		H- 2,4,7	E- 3,5			E- 12	E- 3,5
			F- 2			F- 9	F- 3
			H- 10,11			H- 2,4	H- 9,10,11
Total	**32**	**13**	**19**	**Total**	**33**	**17**	**16**

Source	Species	Positives	Negatives	Source	Species	Positives	Negatives
Pig	*faecalis*	A- 4,11	A- 5,7	Human	*faecalis*	A- 4	A- 3,5,7,12
	16	B- 7	B- 1		18	B- 7	C-8,9,10,12
		C- 4	C- 9,10,12			C- 4	D- 3,5,11
		D- 4,12	D- 3,11			D- 4	E- 3,5
		E- 12	E- 3,5			E- 12	F- 3
		H- 2,4	F- 3				H- 10
Total	**22**	**11**	**11**	**Total**	**28**	**5**	**15**
Sewage	*faecalis*	B- 7	C- 9	Cow	*mundtii*	B- 7,9	B- 1
	36	C- 4	H- 10		14	C- 6	C- 5,9
		D- 4,12				E- 2	H- 2
		H- 4				H- 3,4	
Total	**7**	**5**	**2**	**Total**	**10**	**6**	**4**
Sheep	*mundtii*	C- 6	A- 4	Pig	*mundtii*	A- 10	A- 4
	28	D- 12	C- 5,7,12		4	B- 7,9	B- 1
						D- 12	C- 5
						E- 2	E- 12
						H- 3,4	H- 2
Total	**6**	**2**	**4**	**Total**	**12**	**7**	**5**
Cow	*hirae*	A- 11	A- 5	Pig	*hirae*	A- 10	A- 3,5
	7	B- 9	B- 10		2	B- 7	B- 1,10
		C- 3,4	C- 2,5,7,9,10,12			C- 8	C- 2,5,7,10,12
		D- 1,7,12	D- 3,10			D- 1,7,12	D- 3,10
		E- 2	E- 3			H- 3-5	E- 3,5
			F- 3				F- 3,9
			H- 1,2,7				H- 1,2,7
Total	**23**	**8**	**15**	**Total**	**27**	**9**	**18**
Sewage	*gallinarum*	C- 8	A- 2,4,12				
	2	D- 5,11	B- 1,9,10				
			C- 1,2,5,7,9,10				
			D- 1,3,4				
			E- 1,3,5,12				
			F- 3,5,6,9				
			H- 1-3,7,9,11				
Total	**32**	**3**	**29**				

common carbon sources. Only two *E. faecalis* strains were isolated from goose faeces (33 common sources), while 36 strains were isolated from sewage (7 common sources).

Again, changing the threshold value for positive and negative reactions to ≥ 80% and ≤ 20% respectively would have an impact on the carbon sources listed in Tables 4.17 and 4.18. Using *E. faecalis* as an example, carbon sources in wells F6, H1, H3 and H5 would not be considered common positives and carbon sources in wells A6 and H6 would not be considered common negatives between all *E. faecalis* identified by Biolog; a reduction of 18 commons to 12. Increasing the maximum threshold value appears to decrease the number of carbon sources common between groups of enterococci.

Overall number and collection of common positive and negative carbon sources varied between conspecifics (Table 4.17) and host-specific strains of the same species (Table 4.18); however, a commonly positive substrate for a host-specific strain of the same species was not a commonly negative substrate for the same species isolated from a different host. This poses a problem if common carbon sources are to be used to track a bacterium to its source of origin. Substrates that were metabolized at a percentage between chosen threshold values (25% and 75%) were not helpful in defining the metabolic capabilities of an isolate. The collection of host-specific carbon sources did indeed vary between conspecifics isolated from different hosts, but they did not thoroughly define host-specific enterococci. This is because all host-specific strains of the same species needed to metabolize or not metabolize a carbon source most of the time in order for it to be considered common. Some of the strains demonstrated the ability to metabolize a source, while other conspecific strains isolated from the same host did not. To further complicate the matter, strains demonstrated metabolic inconsistencies. That is,

the ability to metabolize a particular substrate varied from day to day, decreasing the %P value, but this did not mean the strain could never metabolize the substrate.

Metabolic results of conspecific and host-specific enterococci suggest that using more lenient threshold values could better define metabolic capabilities of enterococci. If threshold values were adjusted to eliminate inconsistent metabolic results ($\geq$ 50% = P and $<$ 50% = N), this would account for all carbon sources. Carbon sources would be defined as positive, negative or variable. Variable carbon sources would be those substrates that some conspecific enterococci from the same host could metabolize while other conspecific strains isolated from the same host could not.

Threshold values (25% and 75%) were chosen based on the lowest number of replicates. A minimum of four Biolog trials were performed for each isolate. The chosen threshold values allowed at least one error. If one out of four replicates were negative for a carbon source, this would be attributed to error. It was not anticipated that metabolic ability would be so variable from day to day with some of the substrates; otherwise a threshold value of 50% may have been chosen initially.

4.4.5 Metabolic Results of Subtrates Common to Both Identification Methods

The substrates used in both the identification of enterococci with the Biolog system and the conventional biochemical test scheme are highlighted grey in the "Carbon Source Legend" (p. XXIII) and are listed in Table 4.19. Expected type strain and *Enterococcus* sp. results for conventional biochemical tests are included in Table 4.19. Metabolic capability, according to the Biolog system, for type strains and Biolog-identified isolates that were encountered more frequently (Table 4.5) were compared to literature results (Facklam et al., 2002; Table 3.2). A total of 54 differences were possible for type and

Table 4.19. Literature (L; Facklam et al., 2002) and study (S) results for substrate metabolism by enterococcal type strains and environmental strains (Table 4.5). Type strains are highlighted grey. Subtrates included in the Biolog GP2 MicroPlate and the biochemical test scheme used in this study have been listed by abbreviation and well position on the GP2 MicroPlate (ara = arabinose, B1; man = mannitol, C5; mgp = α-methyl-D-glucopyranoside, C12; raf = raffinose, D5; sbl = sorbitol, D10; suc = sucrose, D12; tre = trehalose, E2; xyl = xylose, E5; pyu = pyruvate, F9). Literature results and definitions were adapted from Facklam et al. (2002). A "p"or "n" under column "L" indicates that > 90% and < 10% of strains respectively, are able to metabolize the substrate when a conventional biochemical protocol is followed. A "v" under column "L" indicates variable reactions among strains of that species. "na" indicates that data was unavailable and "na*" indicates that data was unavailable for group II and V of *E. faecalis*. A "p" or "n" respectively, under column "S" indicates that all isolates of the species were able to metabolize the substrate ≥ 75% of the times tested and all isolates of the species were able to metabolize the substrate ≤ 25% of the times tested. A "v" under column "S" indicates that the ability to metabolize the substrate varied between environmental isolates of that species; a "v" under column "S" for highlighted type strains indicates that the ability to metabolize the substrate varied between replicates. Where metabolic capability varied between literature and study results, reactions are highlighted grey.

Well Substrate Literature / Study	B1 ara		C5 man		C12 mgp		D5 raf		D10 sbl		D12 suc		E2 tre		E5 xyl		F9 pyu	
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
casseliflavus II + V	p	p	p	p	p	v	p	p	v	v	p	p	na	p	na	v	v	v
casseliflavus (ATCC 700327)	p	p	p	p	p	v	p	p	v	n	p	p	na	p	na	p	v	v
gallinarum II + V	p	p	p	v	p	n	n	n	n	n	p	p	na	v	na	v	n	v
gallinarum (ATCC 49573)	p	p	p	p	p	n	p	p	n	n	p	p	na	p	na	p	n	n
hirae	n	v	n	v	n	v	n	v	n	n	p	v	p	v	n	v	n	n
hirae (ATCC 10541)	n	n	n	n	n	n	n	n	n	n	p	p	p	p	n	n	n	n
faecalis II, III, V	n	n	v	v	n	v	n	n	v	v	v	v	na*,p	p	na*,n	n	p	v
faecalis III (ATCC 29212)	n	n	v	p	n	n	n	n	v	p	v	p	na*,p	p	na*,n	n	p	p
faecium II + III	n	v	n	n	n	n	v	n	v	n	p	v	na	v	na	n	n	n
mundtii	n	v	n	v	n	n	n	n	v	n	p	v	na	v	na	n	n	n
avium (ATCC 14025)	p	p	p	p	v	n	n	n	p	p	p	p	na	p	na	p	p	p
saccharolyticus (ATCC 23463)	n	n	p	p	p	p	p	p	p	p	p	p	na	p	na	n	p	p

environmental strains (six species multiplied by nine different carbon sources). Literature and study results agreed except for where shaded (Table 4.19). There were fewer differences involving type strains (5/54) than environmental strains (27/54). This was expected since type strains are selected for stability and are often used for quality control in the lab. Environmental strains are faced with different selection pressures than type strains, and therefore may not always match type strain profiles.

4.4.5.1 Type Strains

According to the literature (Facklam et al., 2002), *E. casseliflavus* ATCC 700327 should be able to metabolize MGP. In the current study, *E. casseliflavus* ATCC 700327 was able to metabolize MGP 50% of the times tested on the Biolog system. *E. gallinarum* ATCC 49573 and *E. hirae* ATCC 10541 should be able to metabolize MGP and raffinose respectively (Facklam et al., 2002), but neither of the type strains metabolized the carbon sources when examined by the Biolog system. Where type strain results differed from the literature (Facklam et al., 2002) slow metabolism of these substrates may be indicated. According to the GP2 database results, *E. casseliflavus* strains only demonstrate metabolism of MGP 35% of the time. Biolog results for *E. gallinarum* ATCC 49573 and *E. hirae* ATCC 10541 matched the expected results of the GP2 database.

E. saccharolyticus ATCC 223062 should not be able to metabolize pyruvate (Facklam et al., 2002), but did so in the current study when examined using Biolog. This result is consistent with the GP2 database, and indicates that current understanding of metabolism of this substrate needs to be reassessed.

Three different strains of *E. faecalis* have been characterized (Facklam et al., 2002). Results for the metabolism of trehalose were not available for *E. faecalis* belonging to group II and V. *E. faecalis* belonging to group III should not metabolize trehalose (Facklam et al., 2002); however, *E. faecalis* ATCC 29212 could metabolize trehalose when tested using the Biolog system. Again, study results agreed with Biolog GP2 database results. Although group III *E. faecalis* strains do not metabolize trehalose (Facklam et al., 2002), other strains of *E. faecalis* can metabolize trehalose (Andersson et al., 2001).

4.4.5.2 Environmental Isolates

Twenty-three of the 27 differences between literature results and Biolog-generated metabolic results of environmental isolates involved a positive or negative literature result matched with a variable study result. A variable result indicates that experimental strains of a species demonstrated a mixture of metabolic capabilities (*i.e.* some isolates of a species metabolized the substrate in question, while others did not). Inherent strain variability does exist among isolates of the same enterococcal species (Facklam et al., 2002). This might explain why some isolates of the same species could metabolize the substrate in question, while other isolates of the same species could not. Table 4.20 contains the percentage of isolates that demonstrated inherent strain variability with regards to metabolic capability involving specific substrates. The greatest percentage of isolates that produced the same result generally matched literature results. From 63-97% of the environmental isolates reacted in a manner similar to the type strain. A positive value indicates that > 90% of the isolates should metabolize the substrate in question (Facklam et al., 2002); however, positive values have been previously defined as > 85%

Table 4.20. Substrates where conspecific enterococci did not demonstrate the same metabolic capability ("v"; Table 4.19). "L" and "S" indicate expected (Facklam et al., 2002) and study results for type and environmental isolates respectively (Table 4.5). Subtrates included in the Biolog GP2 MicroPlate and the biochemical test scheme used in this study have been listed by abbreviation and well position on the GP2 MicroPlate (ara = arabinose, B1; man = mannitol, C5; mgp = α-methyl-D-glucopyranoside, C12; raf = raffinose, D5; sbl = sorbitol, D10; suc = sucrose, D12; tre = trehalose, E2; xyl = xylose, E5; pyu = pyruvate, F9). Literature results and definitions were adapted from Facklam et al. (2002). A "p"or "n" under column "L" indicates that > 90% and < 10% of strains respectively, are able to metabolize the substrate when a conventional biochemical protocol is followed. "na*" under column "L"indicates that data was unavailable for group II and V of *E. faecalis*. "#p" or "#n" respectively, under column "S" indicates the number of isolates of the species that were able to metabolize the substrate ≥ 75% of the times tested and the number of isolates of the species that were able to metabolize the substrate ≤ 25% of the times tested. "#v" under column "S" indicates the number of isolates that demonsrated variable metabolism between replicate trials (metabolic capability between 25% and 75% of the times tested). Expected results of type strains (L) and number and percentage of isolates matching literature results for each substrate are bolded.

Well		B1			C5			C12			D5			D10		
Substrate		ara			man			mgp			raf			sbl		
Literature / Study	# Isolates	L	S	%	L	S	%	L	S	%	L	S	%	L	S	%
casseliflavus II + V	8							**p**	1n	12						
									2v	25						
									5p	**63**						
gallinarum II + V	9	**p**	6n	67	**p**	6n	67				**p**	1n	11			
			1v	11		2v	22					1v	11			
			2p	**22**		**1p**	**11**					**7p**	**78**			
hirae	13	**n**	**11n**	**85**	**n**	**12n**	**92**	**n**	**12n**	**92**	**p**	9n	70	**n**	**10n**	**77**
			2v	15		1v	8		1v	8		2v	15		1v	8
			0p	0		0p	0		0p	0		**2p**	**15**		2p	15
faecalis II, III, V	97	**n**	**92n**	**95**				**n**	**94n**	**97**	**n**	**90n**	**93**			
			3v	3					2v	2		5v	5			
			2p	2					1p	1		2p	2			
faecium II + III	23	**p**	22n	96												
			1v	4												
			0p	**0**												
mundtii	48	**p**	41n	85	**p**	47n	98									
			5v	11		1v	2									
			2p	**4**		**0p**	**0**									
Well		**D12**			**E2**			**E5**			**F9**					
Substrate		**suc**			**tre**			**xyl**			**pyu**					
Literature / Study	**# Isolates**	**L**	**S**	**%**	**L**	**S**	**%**	**L**	**S**	**%**	**L**	**S**	**%**			
casseliflavus II + V	8															
gallinarum II + V	9										**n**	**7n**	**78**			
												2v	22			
												0p	0			
hirae	13	**p**	1n	8	**p**	1n	8	**n**	**11n**	**85**						
			0v	0		0v	0		2v	15						
			12p	**92**		**12p**	**92**		0p	0						
faecalis II, III, V	97							**na*,n**	**91n**	**94**	**p**	1n	1			
									2v	2		13v	13			
									4p	4		**83p**	**86**			
faecium II + III	23	**p**	8n	35												
			0v	0												
			15p	**65**												
mundtii	48	**p**	1n	2												
			1v	2												
			46p	**96**												

of isolates metabolizing a substrate (Facklam et al., 2002; Holt et al., 1994). It is possible that as research expands to include profiling of more environmental isolates, inherent strain variability may increase. Literature may currently be underestimating this variability. For those substrates where the majority of study results did not match literature results (arabinose for *E. gallinarum, E. faecium* and *E. mundtii*; mannitol for *E. gallinarum* and *E. mundtii*; raffinose for *E. hirae*), it may be that most isolates of these species take longer than 20-22h to begin metabolism of these substrates. With manual biochemical tests, it has been suggested to wait for one month before confirming a negative metabolic result (MacFaddin, 2000).

The remaining four metabolic differences between environmental isolates and literature results involved the metabolism of MGP by *E. casseliflavus*, mannitol by *E. faecium*, raffinose by *E. mundtii* and trehalose by *E. faecalis*. All tested environmental isolates of these species demonstrated metabolic capabilities opposite to what was expected. Because type strains for two of these substrates (*E. gallinarum*, MGP; *E. faecalis*, trehalose) also demonstrated metabolic capabilities opposite to what was expected, metabolism of these substrates may not be evident after 20-22h. If a type strain requires more time to metabolize a substrate, an environmental strain may also require more time. The Biolog GP2 database is based on results of isolate profiles generated from GP2 microplates. Because 95 different carbon sources are examined, isolate identification can be generated within 20-22h of incubation. This does not mean, however, that negative Biolog-generated metabolic results indicate that an isolate can never metabolize the substrate. The isolate may simply require more time to do so.

4.4.6 Summary

According to results of the current study, Biolog should be able to assign a species identification for enterococci based on metabolic reactions in as few as 60 wells. The remaining 35 wells were common to all Biolog-examined isolates in the study. Metabolic results of these 35 wells were consistent with the GP2 database results for 16 *Enterococcus* sp., with the exception of 3-methyl glucose and adenosine-5'-monophoshpate. Results differed for rarely isolated species such as *E. columbae* and *E. cecorum* (Devriese et al., 1993). These two substrates may be added to the 60 carbon sources identified if less common enterococcal species are to be studied.

The six type strain profiles were distinct from one another and were generally consistent with the GP2 database (Tables 4.14 and 4.15). A few inconsistencies were noted. Some studies (Devriese et al., 1993) supported the results of this study, whereas others (Tyrrell et al., 2002) supported results included in the GP2 database. Differences could be attributed to variations in protocol such as culturing media and method of species identification (Hudson et al., 2003). Support from this study and research by Devriese et al. (1993) suggests that the Biolog GP2 database used in this study requires updating.

In general, as sample size increased for conspecifics or host-specific species, the number of common carbon sources decreased (Tables 4.17 and 4.18). Variability may increase as sample size increases, but only to a point until the number of different phenotypes plateaus. The downfall to increasing variability is a decrease in the ability to distinguish between conspecifics isolated from different hosts. This emphasizes the importance of choosing threshold values to be used for interpretation of metabolic ability.

A 5% change in threshold values reduced the number of carbon sources commonly metabolized and not metabolized by all enterococci from 36 to 30. Conspecific commons for *E. faecalis* were reduced from 18 to 12 when the threshold values were changed. Threshold values that result in only either positive or negative substrates might define profiles of conspecific enterococci and conspecific enterococci from different hosts more clearly.

Biolog-generated metabolic results for type strain and environmental strains did not appear to contradict expected manual biochemical test results according to the literature (Facklam et al., 2002); however, inherent strain variation may be underestimated in the literature. The length of incubation of inoculated GP2 plates can explain most of the differences. Biolog generates isolate identification, but does not indicate whether or not an isolate could metabolize a Biolog-generated negative substrate if incubated for up to one month, as the literature suggests (MacFaddin, 2000).

4.4.7 Metabolic Profiles of Host-Specific Species

Objectives of Sections 4.4.6 to 4.4.10 were: 1) to identify metabolic profiles for enterococci in the study by removing all carbon sources common to enterococci and removing carbon sources common to conspecifics; 2) to organize profiles based on source of isolation and to remove carbon sources common to conspecific enterococci isolated from the same host; 3) to determine the number of unique profiles encountered among isolates in the study; 4) to match profiles of faecal-borne enterococci to profiles of water-borne enterococci; 5) to briefly examine geographic location of strains isolated from bovine faeces that shared metabolic profiles; and 6) to evaluate Biolog as an MST tool.

The first two objectives involved elucidating unique metabolic profiles of enterococci; therefore, carbon sources that were commonly metabolized or not metabolized by all isolates examined via Biolog (Table 4.13) were removed from profiles. All carbon sources that were commonly negative or positive between conspecifics isolated from all hosts (conspecific commons, Table 4.17) and all carbon sources that were commonly negative or positive between conspecifics isolated from the same host (host-specific commons, Table 4.18) were also removed from profiles. Common carbon sources identified differences between species and conspecifics isolated from different hosts and also identified similarities between enterococci (Table 4.13), conspecific enterococci (Table 4.17) and host-specific enterococci of the same species. The purpose of creating unique profiles was to examine differences and similarities between individual strains (rather than a collective group) sorted by species and source of isolation. Host-specific groups of enterococci have been defined (Table 4.18); but how many different profiles exist? Are there several profiles among host-specific enterococci that separate these strains further?

Tables 4.21 to 4.23 and Appendices 8.4 to 8.8 identify unique metabolic profiles of enterococci (sorted alphabetically) isolated from sewage, bovine, human, porcine, goose, ovine and chicken faeces. Carbon sources from Tables 4.17 and 4.18 were only removed from a profile if more than one isolate was identified as the particular species or host-specific species in question, because "conspecific commons" and "host-specific commons" imply that a comparison between isolates has been made.

4.4.7.1 *E. casseliflavus* (Table 4.21) and *E. durans* (Table 4.22) Profiles

Table 4.21 contains the profile of the only *E. casseliflavus* identified from ovine faeces and Table 4.22 contains the profile of the only *E. durans* isolated from human faeces. Since only one isolate out of 291 was identified as *E. durans*, a larger profile resulted as only the enterococcal commons were removed. Comparisons between unique profiles of *E. casseliflavus* and *E. durans* strains cannot be made, as only one of each of these species was isolated from a host organism.

For all profile tables to follow, the species at the top of the table is the isolate identification according to Biolog and the species below that is the identification according to the biochemical test scheme. Isolate lab identification numbers are also included in all tables to follow.

4.4.7.2 *E. faecalis* Profiles (Table 4.23)

Table 4.23 contains unique profiles of *E. faecalis* isolates identified from sewage. Appendix 8.4 identifies profiles of *E. faecalis* isolated from human, porcine, chicken and goose faeces.

Although the isolates were from only one source, there were many different profiles for this species isolated from sewage and human faeces. There were only two *E. faecalis* profiles from sewage that appeared twice (isolates 295 and 296) and one profile that appeared three times (isolates 204, 299A and 299C), resulting in 32 distinct profiles among the *E. faecalis* strains isolated from sewage. Only one profile appeared twice among the human isolates (isolates 472 and 491), leaving 17 distinct profiles. There were several profiles, however, that differed by only one to two carbon sources. For example,

Table 4.21. Biolog-generated metabolic profile of *E. casseliflavus* from ovine faeces. Carbon sources that generated common positive and negative metabolic reactions between all 291 strains and all Biolog-speciated *E. casseliflavus* strains have been removed from the profile. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

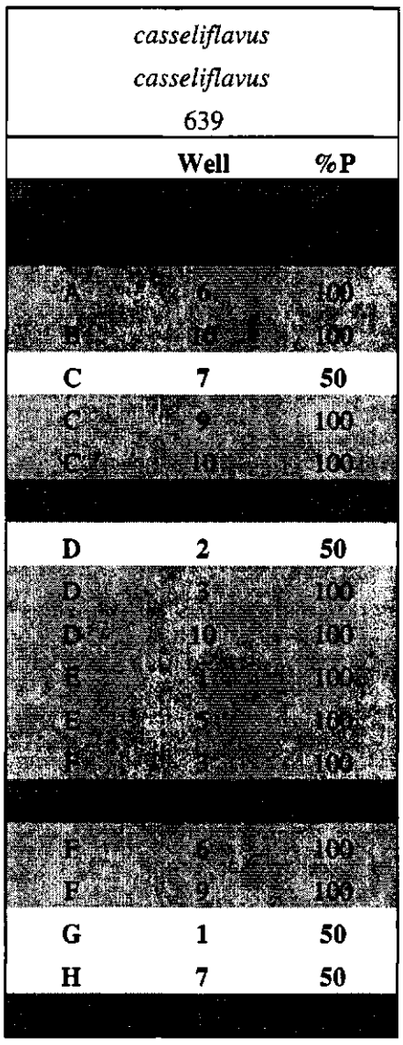

casseliflavus
casseliflavus
639
Well
%P
A
6
100
B
10
100
C
7
50
C
9
100
C
10
100
D
2
50
D
3
100
D
10
100
E
1
100
E
5
100
F
6
100
F
9
100
G
1
50
H
7
50

Table 4.22. Biolog-generated metabolic profile for *E. durans* from human faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profile. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

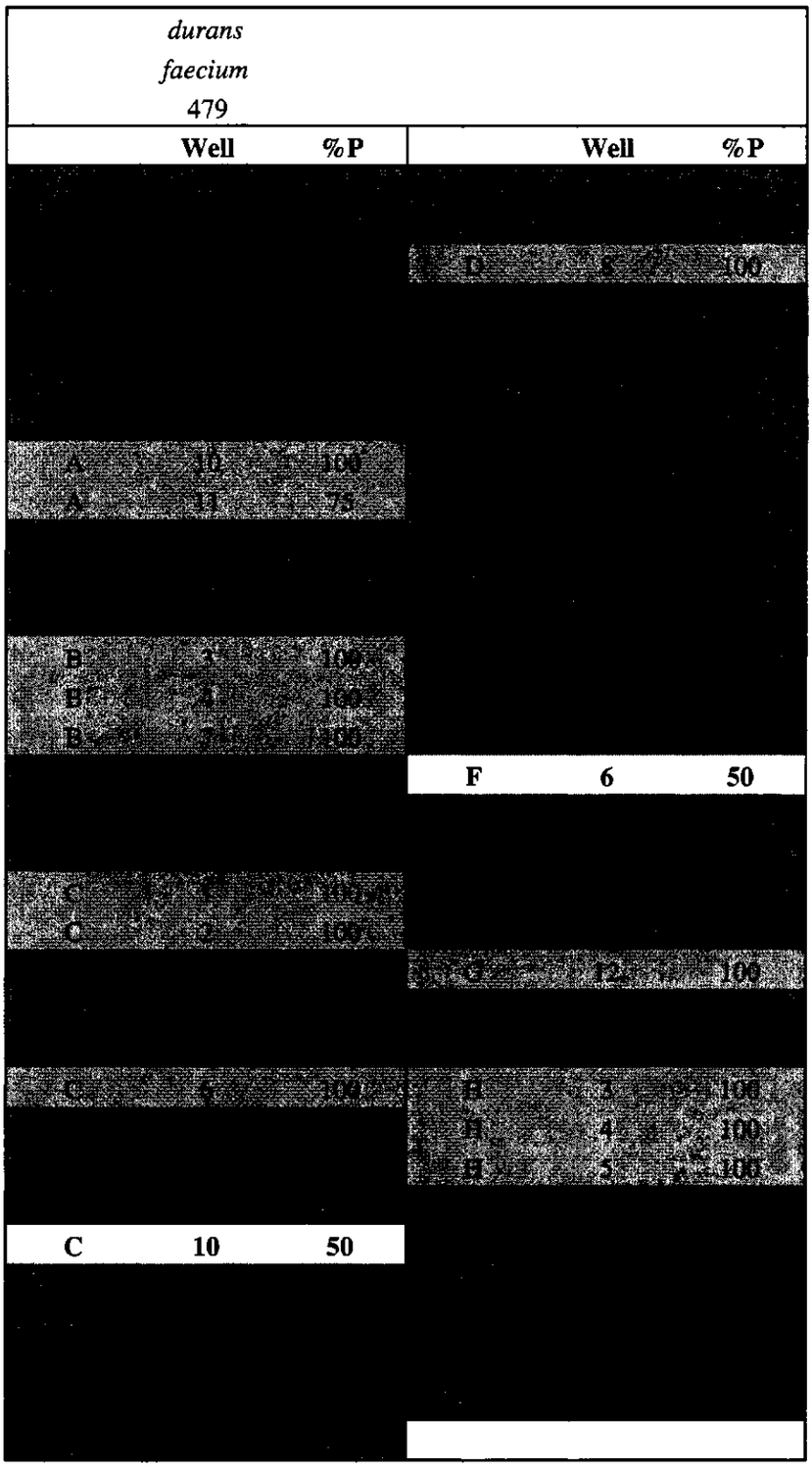

durans
faecium
479
Well
%P
Well
%P
C
10
50
F
6
50

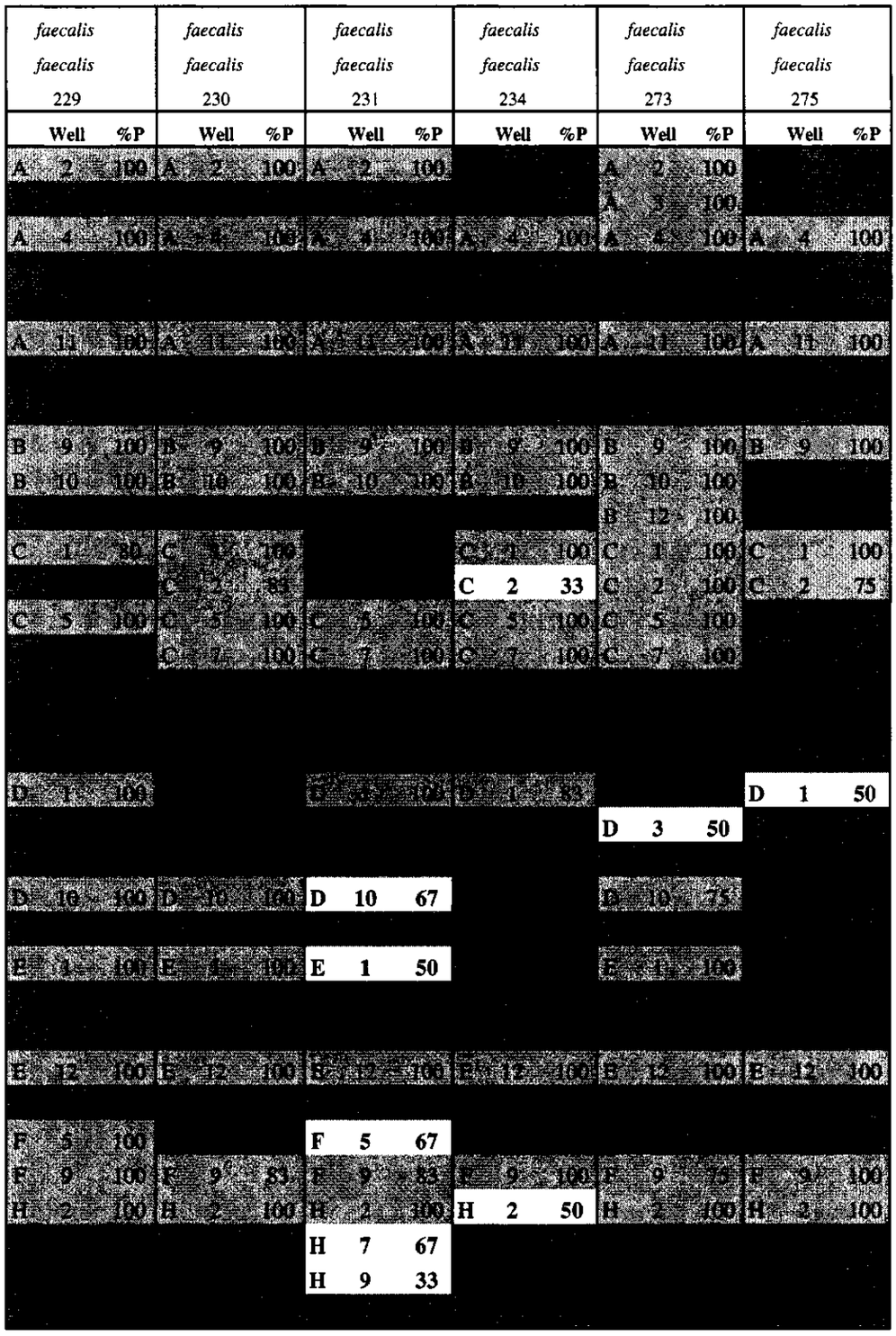
Table 4.23. Biolog-generated metabolic profiles of *E. faecalis* isolated from sewage. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. faecalis*-specific and *E. faecalis* host-specifc common carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

faecalis *faecalis* 200			*faecalis* *faecalis* 201			*faecalis* *Lactococcus* 202			*faecalis* *faecalis* 203			*faecalis* *faecalis* 204			*faecalis* *faecalis* 205		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	50	A	2	100				A	2	100	A	2	50	A	2	33
			A	3	100				A	3	100						
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100			
															A	5	50
															A	7	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
															A	12	100
															B	1	100
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
B	12	100							B	12	100						
						C	1	100				C	1	100	C	1	100
						C	2	50				C	2	100	C	2	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100							C	7	100	C	7	100	C	7	100
															C	8	100
															C	10	100
															C	12	100
D	1	67	D	1	67				D	1	100	D	1	100	D	1	100
															D	3	100
															D	5	100
D	10	100										D	10	33	D	10	33
															D	11	100
E	1	100				E	1	50				E	1	33	E	1	33
			E	5	100										E	3	100
															E	5	83
E	12	83	E	12	100	E	12	100	E	12	100	E	12	100			
F	3	100							F	3	100				F	3	100
F	5	100				F	5	100	F	5	100	F	5	100			
F	9	100	F	9	100	F	9	100	F	9	50	F	9	100	F	9	83
H	2	100	H	2	100	H	2	50	H	2	100	H	2	100	H	2	100
H	7	67							H	7	100				H	7	33
H	9	100	H	9	100				H	9	100						
H	11	100	H	11	100				H	11	100						

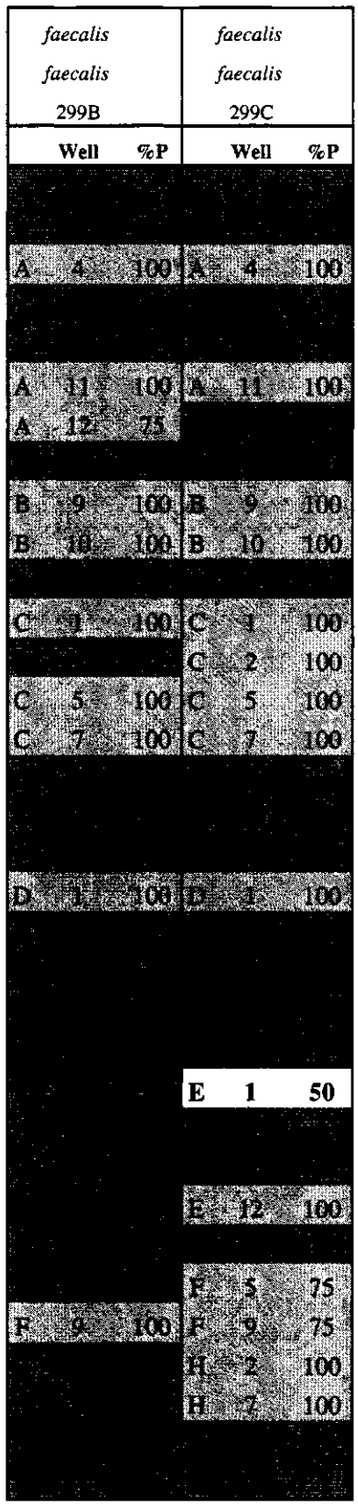
faecalis *faecalis* 206			*faecalis* *faecalis* 208			*faecalis* *Lactococcus* 209			*faecalis* *Lactococcus* 211			*faecalis* *faecalis* 212		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	33				A	2	100
			A	3	100							A	3	33
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100				A	11	100	A	11	100	A	11	100
A	12	50				A	12	33				A	12	33
B	1	50												
B	9	100				B	9	100	B	9	100	B	9	100
B	10	100				B	10	100	B	10	100	B	10	100
			B	12	100	B	12	100				B	12	67
C	1	100				C	1	100	C	1	100	C	1	100
C	2	100				C	2	100	C	2	33	C	2	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
C	8	50												
C	10	50												
C	12	50												
D	1	50	D	1	33	D	1	100	D	1	100	D	1	33
D	3	50	D	3	100									
D	5	50												
D	10	50				D	10	33				D	10	67
D	11	50												
E	1	67				E	1	33				E	1	83
E	3	50												
E	5	50												
			E	12	100	E	12	100	E	12	67	E	12	100
F	3	50	F	3	100									
F	5	33	F	5	67	F	5	100	F	5	67	F	5	100
F	9	50	F	9	50	F	9	100	F	9	67	F	9	83
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
			H	7	33	H	7	100	H	7	50	H	7	33
H	9	50	H	9	100	H	9	33				H	9	67
H	11	50	H	11	100	H	11	33				H	11	50

faecalis *Lactococcus* 213			*faecalis* *Lactococcus* 215			*faecalis* *faecalis* 216			*faecalis* *faecalis* 217			*faecalis* *faecalis* 218		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	83				A	2	100	A	2	50	A	2	100
						A	3	100						
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
									A	12	100			
									B	1	83			
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
												B	12	100
C	1	100	C	1	100				C	1	100			
C	2	100	C	2	100				C	2	83			
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
						C	8	40	C	8	83			
									C	10	83			
									C	12	67			
D	1	100				D	1	100	D	1	100	D	1	100
									D	3	83			
						D	5	40	D	5	83			
D	10	33	D	10	33	D	10	60	D	10	67	D	10	83
									D	11	83			
E	1	33				E	1	40	E	1	50	E	1	100
									E	3	83			
									E	5	67			
E	12	100	E	12	100	E	12	80				E	12	100
									E	3	83			
F	5	100	F	5	100	F	5	100				F	5	100
F	9	83	F	9	100	F	9	100	F	9	100	F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
H	7	100	H	7	33				H	7	50	H	7	50
						H	9	100				H	9	100
						H	11	100				H	11	100

faecalis *faecalis* 219			*faecalis* *faecium* 221			*faecalis* *faecalis* 223			*faecalis* *faecalis* 224			*faecalis* *faecalis* 227			*faecalis* *faecalis* 228		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100				A	2	100	A	2	100
A	3	50				A	3	100									
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
			A	12	40				A	12	50						
B	1	33															
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
B	12	100				B	12	100	B	12	100						
			C	1	40	C	1	100	C	1	100	C	1	100	C	1	100
						C	2	100	C	2	100	C	2	100	C	2	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100						
D	1	67	D	1	100	D	1	33	D	1	100	D	1	100	D	1	100
						D	5	100									
D	10	67	D	10	80	D	10	100	D	10	67						
E	1	83	E	1	100	E	1	100	E	1	100				E	1	67
E	5	33															
E	12	100	E	12	100	E	12	100	E	12	100						
F	5	100	F	5	100	F	5	83	F	5	100	F	5	100	F	5	100
F	9	83	F	9	100	F	9	67	F	9	83	F	9	100	F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
									H	7	83						
H	9	100	H	9	100				H	9	50						
H	11	100	H	11	100												


faecalis
faecalis
229
Well %P
A 2 100
A 4 100
A 11 100
B 9 100
B 10 100
C 1 20
C 5 100
D 1 100
D 10 100
E 1 100
E 12 100
F 5 100
F 9 100
H 2 100
faecalis
faecalis
230
Well %P
A 2 100
A 4 100
A 11 100
B 9 100
B 10 100
C 1 100
C 2 83
C 5 100
C 7 100
D 10 100
E 1 100
E 12 100
F 9 83
H 2 100
faecalis
faecalis
231
Well %P
A 2 100
A 4 100
A 11 100
B 9 100
B 10 100
C 5 100
C 7 100
D 10 67
E 1 50
E 12 100
F 5 67
F 9 83
H 2 100
H 7 67
H 9 33
faecalis
faecalis
234
Well %P
A 4 100
A 11 100
B 9 100
B 10 100
C 1 100
C 2 33
C 5 100
C 7 100
D 1 33
E 12 100
F 9 100
H 2 50
faecalis
faecalis
273
Well %P
A 2 100
A 3 100
A 4 100
A 11 100
B 9 100
B 10 100
B 12 100
C 1 100
C 2 100
C 5 100
C 7 100
D 3 50
D 10 75
E 1 100
E 12 100
F 9 75
H 2 100
faecalis
faecalis
275
Well %P
A 4 100
A 11 100
B 9 100
C 1 100
C 2 75
D 1 50
E 12 100
F 9 100
H 2 100

faecalis *faecalis* 276			*faecalis* *faecalis* 295			*faecalis* *faecalis* 296			*faecalis* *faecalis* 297			*faecalis* *faecalis* 298			*faecalis* *faecalis* 299A		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	75	A	2	75									
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
A	**12**	**50**															
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
B	12	100															
			C	1	100	C	1	100	C	1	100	C	1	75	C	1	100
			C	2	100	C	2	100	C	2	100	C	2	75	C	2	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
			C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
D	1	75	D	1	100	D	1	100	D	1	100	D	1	100	D	1	100
			D	10	75	D	10	75									
			E	1	75	E	1	75	E	1	75						
			E	12	100	E	12	100	E	12	75	E	12	100	E	12	100
			F	5	100	F	5	100	**F**	**5**	**50**	F	5	75	F	5	75
F	9	100	F	9	100	F	9	75	F	9	100	F	9	75	F	9	75
H	2	100	H	2	100	H	2	100	H	2	75	H	2	100	H	2	100
			H	7	100	H	7	100	H	7	75						



faecalis *faecalis* 299B			*faecalis* *faecalis* 299C		
	Well	%P		Well	%P
A	4	100	A	4	100
A	11	100	A	11	100
A	12	75			
B	9	100	B	9	100
B	10	100	B	10	100
C	1	100	C	1	100
			C	2	100
C	5	100	C	5	100
C	7	100	C	7	100
D	1	100	D	1	100
			E	1	50
			E	12	100
			F	5	75
F	9	100	F	9	75
			H	2	100
			H	7	100

only results in well A2 (α-cyclodextrin) differed between isolates 437 and 441. Isolate 437 demonstrated inconsistent metabolism of the substrate, while isolate 441 was negative for metabolism of the substrate. One *E. faecalis* profile from porcine faeces appeared twice (isolates 331 and 332) and 12 profiles differed from another profile by only one carbon source, but not necessarily the same carbon source (isolates 300, 301, 303, 305, 307, 329, 330, 331, 332, 333, 335 and 336). Out of the 5 strains identified as *E. faecalis* from chicken faeces, there were three isolates with different profiles. Isolates 711 and 724 shared the same profile. There were two isolates identified as *E. faecalis* from goose faeces. These strains were also distinct according to Biolog-generated profile results. Based on Tables 4.17 and 4.18 and individual strain profiles, it does not appear that any profiles overlapped between *E. faecalis* strains isolated from all hosts; the majority of *E. faecalis* strains in this study have their own unique metabolic profile, with the exception of the indicated isolates.

All remaining profiles of Biolog-speciated isolates are identified in Appendices 8.5 to 8.8. Appendix 8.5 identifies profiles of *E. faecium* strains isolated from sewage, bovine, human, porcine and goose faeces, Appendix 8.6 identifies profiles of *E. gallinarum* strains isolated from sewage, bovine and porcine faeces, Appendix 8.7 identifies profiles of *E. hirae* strains isolated from sewage, porcine, bovine, human and chicken faeces and Appendix 8.8 identifies profiles of *E. mundtii* strains isolated from bovine, ovine, porcine and goose faeces.

4.4.7.3 *E. faecium* Profiles (Appendix 8.5)

Two different *E. faecium* profiles each appeared twice (75, 98 and 99, 97) in isolates from bovine faeces. This is a total of six unique profiles out of eight possible. Only one *E. faecium* profile was encountered in sewage. This profile was different from any of the other *E. faecium* profiles. Only four *E. faecium* isolates were identified from porcine faeces. These four profiles were also very different from each other. The profile of isolate 313 contained only one positive carbon source (sucrose, well D12), while another profile (isolate 363) contained 16 carbon sources that were metabolized. A similar trend was noted for the profiles encountered in strains isolated from human faeces. Four different profiles resulted, one with zero carbon sources metabolized (isolate 404) and another with eight sources metabolized (isolate 424). Three unique profiles were also identified among the four *E. faecium* strains isolated from goose faeces.

4.4.7.4 *E. gallinarum* Profiles (Appendix 8.6)

Four unique *E. gallinarum* profiles were identified; one isolated from bovine faeces, one isolated from porcine faeces and two isolated from sewage matter. Because only one *E. gallinarum* strain was identified from bovine and porcine faeces, host-specific common carbon sources were not removed from each of the profiles. The bovine isolate was able to metabolize 14 carbon sources and the porcine isolate was able to metabolize six carbon sources. When the same carbon sources are considered, that is, without removing the host-specific commons (Table 4.18; the 32 positive and negative carbon sources common to sewage *E. gallinarum* isolates) from the sewage *E. gallinarum* profiles, the two profiles are different from the *E. gallinarum* bovine and porcine profiles.

From the unique profiles, one of the sewage isolates could metabolize four carbon sources and the other metabolized eight.

4.4.7.5 *E. hirae* Profiles (Appendix 8.7)

Two different *E. hirae* profiles were identified in strains isolated from porcine faeces. Six different *E. hirae* profiles were identified in strains isolated from bovine faeces. One profile appeared twice (isolates 10 and 95), resulting in six different profiles out of seven possible. Only one *E. hirae* isolate was identified from sewage, human and chicken faeces. None of the corresponding profiles were identical to each other, nor did they appear among the other *E. hirae* profiles.

4.4.7.6 *E. mundtii* Profiles (Appendix 8.8)

E. mundtii profiles were identified for strains isolated from bovine, porcine, ovine and goose faeces. Eight (out of 14 possible) different *E. mundtii* profiles were identified among the bovine isolates. One profile appeared twice (isolates 18 and 33), while another appeared six times (isolates 7, 8, 15, 35, 36 and 50). Four distinct *E. mundtii* profiles were encountered among strains isolated from porcine faeces. One isolate (315) was negative for metabolism of all the carbon sources, while two of the isolates (316 and 365) metabolized seven carbon sources. These two profiles (isolates 316 and 365) differed slightly in wells C2 (lactulose), D4 (D-psicose) and F6 (methyl pyruvate). The difference between replicate ability (for 316 and 365) to metabolize these substrates ranged from only 16% to 50%. Twenty-four out of 28 possible different *E. mundtii* profiles were identified among strains isolated from ovine faeces. Isolate pairs 625 and 632, 611 and 628, 605 and 633 and 619 and 629 each shared a profile. Isolates 600, 610 and 616 differed from isolates 611, 625, 628 and 632 by only one to two carbon sources.

Isolates 637 and 659 only differed by 25%P between wells C4 and H1, 648 and 649 differed by 25%P in well H4 and 602 and 623 differed by 50%P between wells B9 and D11 only. The one *E. mundtii* profile isolated from goose faeces was similar to the profile of isolate 600 from ovine faeces. The only differences between these two profiles concerned wells D4 (D-psicose) and F6 (methyl pyruvate). Both carbon sources were metabolized 75% of the time by isolate 600 while D-psicose and methyl pyruvate were metabolized by isolate 517 50% and 25% of the time respectively.

4.4.8 Unique Metabolic Profiles of Water-Borne Enterococci

All enterococcal species isolated from water are found in Appendix 8.9: *casseliflavus, faecalis, faecium, mundtii, gallinarum, hirae, flavescens* and *sulfureus*. Because water-borne enterococcal profiles did not have host-specific carbon sources removed, their profiles needed to match unique profiles of faecal-borne isolates and the appropriate host-specific common carbon sources.

None of the seven *E. casseliflavus* profiles were encountered more than once among the water isolates. Only one *E. casseliflavus* profile was identified from all of the faecal samples and this profile did not match any of the *E. casseliflavus* water profiles.

Of the *E. faecalis* strains isolated from water, profiles for pairs 156 and 158 and 102 and 154 matched each other. No other profiles appeared more than once among the *E. faecalis* water isolates, resulting in 18 different profiles. Profiles for isolates 117 and 166 matched the profiles of strains 330 (porcine origin) and 334 (porcine origin) respectively, while the profile shared by isolates 102 and 154 matched the profile of isolate 216 (sewage origin).

The three water-borne *E. faecium* strains had different profiles from each other and none of them matched profiles of strains isolated from sewage nor bovine, porcine and human faeces. The profile of isolate 122 did however match the profile of isolate 533 from goose faeces.

None of the five *E. gallinarum* profiles encountered from water samples matched one another, nor did they match the bovine, porcine or sewage *E. gallinarum* profiles.

Only one profile each for *E. hirae* and *E. mundtii* profile was identified among the water isolates. The *E. hirae* profile did not match *E. hirae* profiles encountered in sewage or porcine, bovine, human and chicken faeces. The *E. mundtii* profile did not match any of the profiles from bovine, porcine, ovine or goose faeces.

Profiles for *E. flavescens* and *E. sulfureus* were identified only in water, and not isolated from any other source.

When only faecal-borne isolates assigned a species identification by Biolog were considered, 142 unique profiles resulted out of 162 possible. A total of 37 unique profiles were identified from the 39 possible water-borne enterococci. Isolates from goose, human and porcine faeces and water demonstrated the greatest percentage of unique profiles, while isolate profiles of bovine origin demonstrated more overlap (Table 4.24). Overlap refers to identical profiles. *E. mundtii* strains demonstrated the greatest proportion of profile overlap, while *E. casseliflavus* and *E. gallinarum* demonstrated the greatest proportion of unique profiles. Only one of each *E. durans, E. flavescens* and *E. sulfureus* were isolated, allowing no possibility of intra-species profile overlap.

Table 4.24. Number of unique profiles for faecal- and water-borne enterococci. Strains were isolated from water, sewage and bovine, ovine, goose, chicken, porcine and human faeces. Profiles are based on Biolog-generated metabolism results. Only those profiles of isolates assigned a species identity were considered. "Total" indicates number of profiles for each source and species, "total possible" identifies the total number of strains isolated from one source and the total number of strains identified as each species, " % unique" identifies the proportion of unique profiles for each source and species.

Species / Source	bovine	ovine	goose	chicken	porcine	human	sewage	water	**Total**	**Total Possible**	**% Unique**
casseliflavus	0	1	0	0	0	0	0	7	8	8	100
durans	0	0	0	0	0	1	0	0	1	1	100
faecalis	0	0	2	4	15	17	32	18	88	97	91
faecium	6	0	3	0	4	4	1	3	21	23	91
flavescens	0	0	0	0	0	0	0	1	1	1	100
gallinarum	1	0	0	0	1	0	2	5	9	9	100
hirae	6	0	0	1	2	1	1	1	12	13	92
mundtii	8	24	1	0	4	0	0	1	38	48	79
sulfureus	0	0	0	0	0	0	0	1	1	1	100
Total	**21**	**25**	**6**	**5**	**26**	**23**	**36**	**37**	**179**		
Total Possible	**30**	**29**	**6**	**6**	**27**	**24**	**40**	**39**	**201**		
% Unique	**70**	**86**	**100**	**83**	**96**	**96**	**90**	**95**	**89**		

4.4.9 Unique Metabolic Profiles of Unidentified Isolates

4.4.9.1 Isolates With two Possible Identifications (Appendix 8.10)

Isolates that Biolog narrowed down to two species were encountered in water, sewage and bovine, human, chicken, goose and ovine faeces. Isolates 34 and 45 from Appendix 8.10 shared the same profile. No other isolates from Appendix 8.10 shared a profile.

4.4.9.2 Isolates With no Identification (Appendix 8.11)

Unidentified isolates were encountered in water, sewage, bovine, porcine, human, goose, chicken and ovine faeces. Profiles are identified in Appendix 8.11. None of the profiles appeared more than once, but there were many profiles with subtle differences between them. For example, isolates 463 and 464 from human faeces shared identical profiles with the exception of well C3 (maltose). This was also true of isolates 481 and 482 with the exception of well B1 (L-arabinose). Isolate 464 could metabolize maltose 100% of the time it was tested, while isolate 463 metabolized maltose 50% of the time it was tested. Isolate 481 could not metabolize L-arabinose, while isolate 482 metabolized L-arabinose 50% of the time it was tested.

4.4.10 Shared Profiles

Each pair of ovine-origin isolates that shared the same profile came from different animals on the same farm. The two sewage profiles that appeared more than once belonged to isolates that originated from the Coalhurst sewage treatment plant. The two isolates of human origin with the same profile were isolated from different volunteers. Because the porcine isolates that shared the same profile were isolated from Maple Leaf Processors, details regarding geographic location or animal are not known. Chicken

isolates that shared the same profile were isolated from different animals on the same farm. Of the six profiles isolated from bovine faeces that appeared more than once, two profiles were encountered in the same animals, two were identified from different animals and different locations, one was encountered from different animals on the same farm and the last profile was identified in four different animals and two different locations. None of the enterococcal profiles detected from one host species were identified in a different host species.

4.4.11 Discussion of Metabolic Profiles

4.4.11.1 Biolog as an MST Tool

Two objectives of this study were to elucidate metabolic profiles for enterococcal species and match those profiles generated from water-borne isolates to profiles of faecal-borne isolates. In the current study, 291 metabolic profiles were elucidated. Of the 201 possible profiles for speciated isolates, there were 179 unique profiles; metabolic profiles of 89% of the identified strains were distinct. This suggests there is great diversity among enterococci, regardless of origin. The analysis used to distinguish identical profiles capitalized on distinct differences in metabolic capabilities between enterococci. Matching of water enterococcal profiles to profiles from faecal samples did not allow origin-identification of most water isolates. This does not mean, however, that an analysis capitalizing on subtle differences could not. Many of the profiles were similar in the current study, differing only between one to three carbon sources. Furthermore, profiles identified from water-borne isolates were identical to a profile identified among sewage isolates and two profiles identified among porcine faeces. These are promising results, especially because water samples were collected in different years.

Analyses that consider other factors such as metabolic reaction intensity may increase the discriminative ability of Biolog. Other possible methods of analysis include: 1) using the Biolog-generated metabolism results from this study to build a host-specific database for enterococci; and 2) performing a phylogenetic analysis on isolates based on positive, negative and inconsistent metabolism results. These methods of analysis will be discussed further under Section 6.0 (Future Directions).

4.4.11.2 Identical Profiles

For profiles that were identical, some belonged to: 1) isolates from animals of the same species on different farms; 2) isolates from the same animal on the same farm; 3) isolates from different animals of the same species on the same farm; 4) isolates from the same sewage plant; and 5) isolates from different human volunteers. Defining geographic location is important to accurate MST. Hagedorn et al. (2003) examined enterococci collected from areas they defined as different geographical regions. Researchers noticed that classification success increased when non-human sources of faecal pollution were separated from human sources of faecal pollution, and when species-specific or geographic location-specific libraries were tested. This suggests a number of things: 1) geographic location must be clearly defined in order to launch an effective MST regime; 2) databases should be frequently updated by adding new test results or by sharing results between researchers. This is especially true if areas with extensive faecal contamination span different geographic regions; 3) region-specific databases may be developed to increase the accuracy of MST; 4) species-specific databases may be developed to increase the accuracy of MST if isolates are speciated prior to elucidating source of contamination; 5) there is a chance that errors in tracking

the source of contamination will be greater in areas suspected of human and non-human faecal contamination.

The current study was not specifically designed to examine the impact of geographic location on carbon source utilization profiles, although some insight was shed upon the matter. Faecal samples were collected within an approximate 75km radius. Geographic boundaries for MST likely differ between regions, but profile overlap of enterococci isolated between farms suggests that the areas sampled constitute one geographical region. To reiterate the importance of defining geographic location and developing region-specific databases, it would be interesting to test the known-host isolates from the current study against the known-host library in the study mentioned above (Hagedorn et al., 2003).

4.4.12 Summary

Eighty-nine percent of the Biolog-generated profiles for identified isolates were unique, indicating great diversity in metabolic capabilities of enterococci. The greatest proportion of identical profiles was identified among bovine isolates and *E. mundtii*. *E. mundtii* was encountered most frequently in bovine and ovine faeces. Among the unique profiles, there were often minimal differences (%P values just above or below the defined threshold) involving one to three carbon sources. This suggests that more lenient threshold values could reduce the number of profiles encountered.

A few profiles of water-borne isolates matched one profile identified from sewage and two profiles identified among porcine faeces. Although Biolog did not identify sources of faecal contamination in most water samples, alternative analyses that utilize Biolog-generated results from this study may be performed. Analyses should consider

either intensity of metabolic reactions or positive, negative and inconsistent profile results collectively.

4.4.13 Inter-Trial Variability

The purpose of this section is to address isolate variability in metabolic capabilities between trials. Isolate metabolic ability tended to vary between days. Whether or not an isolate could metabolize a carbon source on any given trial was the information used to calculate %P values. Values were interpreted based on the threshold values (25% and 75%) described in Sections 3.6 and 4.4.4. %P values above the maximum and below the minimum threshold values defined positive and negative results respectively. %P values that fell between the minimum and maximum values defined inconsistently metabolized substrates. Inconsistently metabolized substrates are identified in isolate profiles from Tables 4.21 to 4.23 and Appendices 8.4 to 8.11. These are the substrates with which strains demonstrated significant inter-trial variability. The ability of an isolate to metabolize a substrate on any given trial was identified by Biolog based on metabolic reaction intensity explained in Section 3.5.4. Differences based on reaction intensity results were not anticipated, and raise questions as to why they occurred.

The inter-trial differences were likely not attributed to technical error. Biolog is very precise and strict regarding how protocol should be carried out. Inoculating fluid containing bacteria was measured carefully and fell within 17% and 23%T, sterilized equipment and gloves were used at a clean workstation, plates were incubated for 21 ± 1h and BUG + B agar was used to culture the isolates.

One explanation for the inter-trial differences in metabolic capability involves gene regulation. If genes controlling metabolic pathways involving questionable substrates

were switched on during one trial and switched off during another trial, an isolate would be able to metabolize the substrate on one occasion, yet not on the other (Caldwell, 1995). This theory requires testing.

The hypothesis of genes "turning on and off" raises the question: What is causing this differential gene regulation? One explanation may be the loss of metabolic function by some strains that are more sensitive to colder temperatures. Hudson et al. (2003) entertained this explanation in their study when different sub-culturing methods produced different speciation results. They found where isolate identification disagreed, there was not enough evidence to attribute the differences to freezing. In the current study, some isolates were characterized on the Biolog system from refrigerated cultures while others were characterized from frozen cultures. To account for this, resuscitation that included culturing frozen isolates two times before testing was performed. This step was meant to bring cultures to the same physiological state; therefore freezing should not have impacted isolate metabolic capability between trials.

If inter-trial differences occurred so that an isolate could not metabolize a substrate on one trial but could on the next, these differences could be attributed to prior exposure of an isolate to the substrate. Some isolates may have required priming by adding the sole carbon source of interest to the resuscitation medium before testing.

Biolog is generally used once to identify an isolate. Inter-trial differences suggest that replicates should be considered when examining specific metabolic characteristics of a bacterium.

4.4.14 Summary

Variation in isolate metabolic ability occurred between trials for inconsistently metabolized substrates. This variability is probably not due to experimental error, but may be attributed to gene regulation. Factors capable of influencing gene regulation, such as sub-culturing, are important to source-tracking methods that employ carbon source utilization profiles. Inter-trial strain variability should be examined more thoroughly and replicates should be considered when using the Biolog system to assess metabolic capabilities of bacteria.

4.4.15 Barriers to Source-Tracking

The purpose of this section is to address individual factors that may impact metabolic profiles of bacteria, and therefore MST regimes that utilize carbon metabolism profiles.

It was mentioned earlier that factors such as host diet (Hartel et al., 2003), age of host (Devriese et al., 1992b; Aarestrup et al., 2002), use of antibiotics, time (Yanke et al., 2002) and host geographic location (Hartel et al., 2002a; Liebana et al., 2002) can impact the diversity of enterococcal species isolated from faecal samples. Some of these same factors might also impact metabolic profiles of enterococci.

The importance of geographic location to accurate MST was also discussed in section 4.4.10. Bacterial classification success increased when region-specific databases were used (Hagedorn et al., 2003). Souza et al. (1999) noted that geographic location had an effect on profiles of *E. coli* isolated from wild hosts on different continents. Also, as distance decreased between cattle and horses sampled for *E. coli*, the sharing of bacterial ribotypes within animals of the same species decreased (Hartel et al., 2002a). Each study

indicates that differences between strains of the same species increase as distance increases.

Depending upon the method of characterization, the size of the region in which MST is done and the breadth of the database used, it may not be possible to accurately track isolates to the source of contamination. MST has its limitations, geography being one of them. This limitation can be overcome by generating location-specific databases (Hagedorn et al., 2003).

Just as diet influences the types of enterococcal species isolated from an organism, diet may also be responsible for differences seen within strains of the same species (Hartel et al., 2003). Thirty-five ribotypes were identified in *E. coli* from wild deer, while 11 ribotypes were identified in *E. coli* from penned deer. Penned deer were fed a standard diet while wild deer ate plant matter from their surrounding environment. Hagedorn et al. (2003) attributed this diversity in ribotypes to diet. Diet is more difficult to control than geographic location, especially where wildlife is concerned.

If enterococcal populations change with host age (Aarestrup et al., 2002; Devriese et al., 1992b; Devriese et al., 1991), age and host diet likely interact and select for different strains of enterococci. For example, non-ruminating calves are drinking milk and the GI flora will reflect that. Although species existing in the guts of pre-ruminant calves overlap with species in the guts of ruminant calves (Aarestrup et al., 2002), these bacteria may have different metabolic capabilities as strains of the same species often do (Facklam et al., 2002). This may impact Biolog-generated metabolic profiles of age-dependent strains. Research concerning age-attributed metabolic differences between strains of the same species is lacking.

The health of an organism has been demonstrated to affect the presence of certain strain types of the same bacterial species in chickens (Nolan et al., 1991). Metabolic capabilities of *Salmonella* species isolated from healthy and ill chickens were examined. Results showed that conspecifics isolated from both healthy and ill chickens demonstrated differences in metabolic capabilities. Host health was not a controlled variable in the current study and could easily have contributed to profile differences.

4.4.16 Summary

Barriers to MST such as host geographic location, diet, health and age can reduce the effectiveness and applicability of MST. Those factors that can be controlled should be considered when constructing libraries.

5.0 Summary of General Conclusions

5.1 Isolation and Confirmation of *Enterococcus* from mE Agar

Using mE agar as the selective medium, a total of 673 presumptive enterococci were isolated from water, sewage and bovine, porcine, human, goose, ovine and chicken faeces. Isolates were confirmed to belong to *Enterococcus* according to results of a confirmation test scheme: 1) Gram stain; 2) catalase activity; 3) tolerance to 6.5% NaCl; 4) growth at 45°C for 24h; 5) esculin hydrolysis in the presence of bile; and 6) LAP activity. Each test was useful to the confirmation of enterococci, although some of the isolates were negative for 6.5% NaCl tolerance and growth at 45°C for 24h, when most enterococci are positive for these tests. Despite this, the tests should not be removed from the confirmation scheme because they add confidence to identification results when reactions are positive. Results are further strengthened when isolates are identified to the species level.

Selective ability of mE agar was examined for the isolation of enterococci from bovine faeces. From a total of 139 isolates, 101 (73%) were confirmed to belong to *Enterococcus* according to the tests listed above. When animals and geographic location were considered, the selective ability of mE agar varied. Selective ability of mE agar for individual cattle ranged from 40% to 100%, and from 53% to 100% for sampling locations.

5.2 Identification of Type Strains

Type strains were identified using a conventional biochemical test scheme and an automated identification system (Biolog MicroStation™ and MicroLog3™ software). This was to test the validity and reliability of both identification techniques employed and

maintain quality control. Intermediate reactions were produced in MGP and pyruvate broths used to identify enterococcal type strains. Some metabolic reactions that should have been positive appeared only weakly positive. According to Nash and Krenz (1991) and Lauderdale et al. (1999), weakly positive results are considered negative.

Biolog accurately identified all type strains with the exception of *E. hirae* and *E. avium*. *E. hirae* was identified as *E. mundtii* for 80% of the trials and *E. avium* was unidentified for 66% of the trials. Any isolates identified as *E. mundtii* should be flagged as possible *E. hirae* strains. Weak growth of *E. avium* ATCC 14025 may have contributed to misidentification of the strain. The GP2 database may need to be updated to include results of *E. hirae* ATCC 10541.

Type strain results suggest that Biolog is a more accurate method of identification when identifying enterococcal species such as *E. saccharolyticus, E. faecalis, E. gallinarum* and *E. casseliflavus*. It would be useful to examine more enterococcal type strains with the Biolog system so that this conclusion can be extended to other species of *Enterococcus*. Difficulties interpreting conventional biochemical tests will likely be a problem regardless of species.

5.3 Identification of Experimental Isolates

A greater number of species were recognized according to the conventional biochemical test scheme (16 sp.) compared to the number of species recognized by Biolog (9 sp.). Both methods of identification recognized *E. faecalis* as the most encountered species overall. This is in agreement with the literature (Facklam et al., 2002).

Species frequencies varied depending upon method of speciation and source of isolation. This is also consistent with the literature (Devriese et al., 1992a; Devriese et al., 1992b; Devriese et al., 1994; Thal et al., 1995; Bahirathan et al., 1998; Pinto et al., 1999; Svec and Sedlacek, 1999; Tejedor Junco et al., 2001; Aarestrup et al., 2002; Devriese et al., 2002; Tanock and Cook, 2002; Wheeler et al., 2002; Yanke et al., 2002; Kuhn et al., 2003). Because species prevalence may depend upon a number of factors (Sections 4.3.10 to 4.3.11), inter-research differences regarding species frequencies are to be expected. These factors were not accounted for in the current study, but help explain inter-research discrepancies.

Less than 50% of the identifications of Biolog-examined isolates matched the conventional biochemical test scheme-generated identifications. Media and sub-culturing techniques varied between the two methods of identification, possibly impacting speciation results. On the other hand, the conventional biochemical test scheme identified a greater proportion of isolates than Biolog, but difficulties interpreting these test results were experienced. Tests were inefficient because of the long incubation period and results were not always accurate. When this and the number of tests employed by both methods were considered, it is more likely that some isolates were misidentified where the conventional biochemical tests were used. Although Biolog was unable to identify a significant proportion of isolates, overall identifications were likely more accurate than those according to the biochemical test scheme. Biolog was accurate in identifying four of the six type strains examined. The system is also standardized and follows a rigid, yet quick and efficient protocol to decrease chances of experimental error.

The large proportion of isolates unidentified by Biolog may be attributed to the great diversity of enterococci identified by host-specific metabolic profiles from Section 4.4.6. It may also indicate evolving enterococcal strains. Unidentified isolates may be mutated strains of recognized species or they may be novel species. Unidentified strains should be analyzed by an alternate method of speciation. Although the GP2 database appears to require updating for this study region, it does not mean the database would be more efficient if used in another region. Inter-research disparity in species diversity and evidence of diverse metabolic capabilities of enterococci from this study indicates the need for regional databases. This would be true when using Biolog as an identification tool or a MST tool.

5.4 Biolog-Generated Metabolic Profiles of Type strains and Experimental Isolates

Differences in metabolic capability of tested type strains for individual carbon sources compared to the GP2 database suggest the database requires updating. That is, more type strains and environmental strains of the same species should be examined and included in the database. Any species identified to date not included in the database (for e.g., *E. asini*) should also be examined and included. Although diversity of enterococci limits the discriminative ability of a database, type strains should be regularly added to the database. Although type strains have been selected for stability, it is possible that even these strains may evolve over time; or variability among more obscure characteristics, such as the metabolism of a rare carbon source, are being noted.

Biolog was able to differentiate enterococcal species with as few as 60 substrates since 35 of the substrates were common to all enterococci in the study. However, if larger study samples are examined and more characterized and novel enterococcal

species are added to the GP2 database, 60 substrates may not be sufficient. Results of this study do suggest that fewer carbon sources may be used in the development of an "*Enterococcus*" Biolog plate. If the system is to be used as a source-tracking tool, it is recommended that a plate be developed specifically for enterococci in order to reduce cost and waste.

Biolog demonstrated great specificity with regards to metabolic profiles of each isolate. Very few duplicate profiles resulted in the current study; however, some profiles were minimally different from others. Three profiles from porcine faeces and sewage matched three of the water profiles. Profile results indicate that threshold values need to be adjusted. If differences between enterococci are to be established according to overall reactions in wells (positive, negative and inconsistent), varying threshold values need to be developed, tested and compared. In doing so, values that separate enterococci according to host may be established.

Biolog was unable to identify sources of contamination in the water samples according to the threshold values and the analysis performed in the current study. Despite this, valuable metabolic information has been collected for 292 enterococcal isolates. By using these results and a different method of analysis, host-specific differences between enterococci may be established. Such methods may take advantage of reaction intensity or reactions may be grouped according to %P replicates and a phylogenetic analysis employed.

It was also noted that carbon metabolism capabilities varied between trials for some substrates. This phenomenon may be linked to gene regulation and further research on the topic should be pursued.

To summarize the major findings, species diversity of enterococci varies greatly between sources of isolation and between research findings. Metabolic capabilities of enterococcal strains of the same species are also very diverse. This complicates the use of enterococci for MST. It emphasizes the need for a central database where researchers may bank characterization results and download data collected by other researchers. From this study, a great amount of useful data has been collected with reference to metabolic ability of enterococci and their possible role in source tracking; however, based on metabolic profiles, conspecifics isolated from one host organism were not clearly differentiated from strains of the same species isolated from a different host.

6.0 Future Directions

Examination of enterococci using the automated Biolog identification system has produced a large amount of useful information and potentially useful data regarding metabolomics. The system itself identified subtle differences between strains; however, the method of analysis was not able to capitalize on these differences. Differences tended to involve the overall metabolism of a substrate, the intensity of the reaction and the number of replicates producing the reaction. Each of these components may be required in the analysis of metabolic profiles to be used in MST. Future studies utilizing Biolog should initially focus on a discriminative method of analysis before more isolates are examined.

Of the isolates in this study, infrequently encountered species such as *E. sulfureus*, *E. casseliflavus* and *E. avium* would not be suitable indicator organisms. An alternative analysis should consider those species that are frequently isolated because they would be better correlated with potential enteric pathogens. *E. faecalis* was encountered at the greatest frequency in the current study. This species was isolated from all sources except bovine and ovine faeces; *E. mundtii* was isolated at the greatest frequency from the faeces of these host organisms. For efficiency, alternative analyses should focus on examining metabolic capabilities of *E. faecalis* and *E. mundtii*.

Perhaps one of the best ways to analyze the results would be to enter the known source isolate profiles into the Biolog database and test the water isolate profiles against them. By doing this, actual intensities of the metabolic reactions would be taken into account. Biolog allows researchers to develop their own databases, so a new system would not need to be developed. The GP2 database and a second method of speciation

such as WCP profiling should be used to confirm species identification of the isolates. Following this, profiles of known-host isolates would be entered into the host-specific enterococcal database. Water-borne isolates would be examined by Biolog and matched to isolates used to generate the host-specific enterococcal database. The database could be updated at any time and used in the future for the identification of host-specific enterococci isolated from water samples.

Another possible way of analyzing the data would be to perform a phylogenetic analysis. A phylogenetic analysis would group organisms by relatedness based on metabolic capability. If strains of *E. faecalis* isolated from one host are more related than *E. faecalis* strains isolated from a different host, they should be clustered together. Substrates used in metabolic testing would be categorized based on replicate metabolic ability. Using the same definitions for overall positive and negative reactions as in the current study, possible categories might be –1 (negative; $\%P \leq 25$), 0 (intermediate; %P between 25 and 75) and 1 (positive; $\%P \geq 75$). Types and numbers of categories would be based on chosen threshold values for what constitutes negative, intermediate and positive reactions, although they need not be limited to those numbers of categories or threshold values chosen for the current study. The current study utilized three categories, while it may be more insightful to sort results further. For example, five different categories may be chosen and defined. Categories could be based on the number of isolates that generated replicate values of 100%P, 61-99%P, 40-60%P, 1-39%P and 0%P, making up five different categories. This method of data analysis may not be as accurate as developing a host-specific enterococcal database using Biolog since it does not

consider reaction intensities. Reaction intensities are important because they may indicate how readily a particular strain utilizes a carbon source.

It was indicated in Section 4.4.4 that the threshold values chosen for the current study could be altered so that only positive or negative reactions are possible. For example, any %P values ≥ 50% would be positive and any %P values < 50% would be negative. Profiles would need to be adjusted according to the alternate threshold values and then compared. The advantage to having only two reactions is that all carbon sources would be accounted for, as inconsistently metabolized substrates were not necessarily inconsistent for every host-specific strain. This made profile analysis and interpretation more complicated.

To confirm speciation results of Biolog and / or the conventional biochemical tests, it would be useful to employ an alternative method of identification for isolates in this study. WCP profiles were discussed in Section 2.1.4.1. Although they are not suggested for use in MST, WCP profiles analyzed by SDS-PAGE have high discriminative capabilities when distinguishing one enterococcal species from another (Facklam et al., 2002). Even species such as *E. casseliflavus* and *E. gallinarum* that are closely related according to 16S rRNA gene sequences have distinct WCP profiles. WCP profiles may also be able to identify isolates that were given no identification or a double identification by one of the two identification methods employed in the study.

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8.0 Appendices

Appendix 8.1. Identification of 673 confirmed enterococci using biochemical tests. Enterococci were isolated from water (100's), sewage (200's), and bovine (10's), porcine (300's), human (400's), goose (500's), ovine (600's) and chicken (700's) faeces. Numbers indicate an isolate's lab identification (ID). Speciation results are based on a conventional biochemical test scheme (Table 3.3). Species-identifications are displayed next to the numerically sorted isolates. Isolates with two possible identifications are included; identifications are separated with a slash (/).

ID	Species	ID	Species
0	*mundtii*	31	*hirae*
1	*faecium*	32	*hirae*
2	*mundtii*	33	*Vagococcus*
3	*mundtii*	34	*hirae*
4	*mundtii*	35	*hirae*
5	*faecium*	36	*Vagococcus*
6	*faecium*	37	*mundtii*
7	*Vagococcus*	38	*mundtii*
8	*faecium*	39	*mundtii*
9	*gallinarum*	40	*mundtii*
10	*Lactococcus*	41	*mundtii*
11	*Vagococcus*	42	*mundtii*
12	*hirae*	43	*mundtii*
13	*faecium / asini*	44	*faecium*
14	*faecium / asini*	45	*Vagococcus*
15	*faecium / asini*	46	*gallinarum*
16	*faecium / asini*	47	*gallinarum*
17	*faecium / asini*	48	*faecium*
18	*hirae*	49	*faecium*
19	*faecium*	50	*hirae*
20	*hirae*	51	*faecium*
21	*hirae*	52	*gallinarum*
22	*hirae*	53	*faecium / asini*
23	*hirae*	54	*Vagococcus*
24	*faecium / asini*	55	*faecium / asini*
25	*hirae*	56	*asini*
26	*hirae*	57	*asini*
27	*hirae*	58	*Vagococcus*
28	*hirae*	59	*mundtii*
29	*hirae*	60	*gallinarum*
30	*gallinarum*	61	*faecium*
62	*mundtii*	93	*gallinarum*
63	*hirae*	94	*Lactococcus*
64	*faecium*	95	*hirae*
65	*gallinarum*	96	*faecium*
66	*faecium*	97	*gallinarum*
67	*mundtii*	98	*faecium*

ID	Species	ID	Species
68	*faecium*	99	*gallinarum*
69	*casseliflavus*	99A	*gallinarum*
70	*mundtii*	99B	*faecium*
71	*gallinarum*	100	*mundtii*
72	*faecium*	101	*avium*
73	*gallinarum*	102	*faecalis*
74	*faecium*	103	*columbae / raffinosus*
75	*gallinarum*	104	*mundtii*
76	*gallinarum*	105	*faecium*
77	*gallinarum*	106	*faecium*
78	*gallinarum*	107	*mundtii*
79	*gallinarum*	108	*mundtii*
80	*faecium*	109	*gallinarum*
81	*mundtii*	110	*faecalis*
82	*hirae*	111	*faecalis*
83	*gallinarum*	112	*casseliflavus*
84	*faecium*	113	*mundtii*
85	*hirae*	114	*mundtii*
86	*faecium*	115	*faecium*
87	*gallinarum*	116	*gallinarum*
88	*gallinarum*	117	*faecalis*
89	*hirae*	118	*mundtii*
90	*pseudoavium*	119	*mundtii*
91	*saccharolyticus*	120	*mundtii*
92	*faecium / Lactococcus*	121	*faecalis*
122	*faecium*	153	*faecalis*
123	*faecium*	154	*faecalis*
124	*faecalis*	155	*mundtii*
125	*faecalis*	156	*faecalis*
126	*avium*	157	*mundtii*
127	*gallinarum*	158	*faecalis*
128	*faecium*	159	*mundtii*
129	*avium*	160	*faecalis*
130	*avium*	161	*mundtii*
131	*faecium*	162	*gallinarum*
132	*gallinarum*	163	*mundtii*
133	*avium*	164	*gallinarum*

ID	Species	ID	Species
134	*faecalis*	165	*faecalis*
135	*mundtii*	166	*faecalis*
136	*avium*	167	*gallinarum*
137	*mundtii*	168	*faecalis*
138	*mundtii*	169	*faecalis*
139	*faecium*	170	*gallinarum*
140	*mundtii*	171	*faecalis*
141	*mundtii*	172	*faecalis*
142	*mundtii*	173	no id
143	*mundtii*	174	*faecalis*
144	*mundtii*	175	*faecalis*
145	*faecalis*	176	*gallinarum*
146	*faecalis*	177	*faecium*
147	*faecalis*	178	*mundtii*
148	*faecium*	179	*gallinarum*
149	*faecalis*	180	*mundtii*
150	*faecalis*	181	*faecium*
151	*faecalis*	182	*mundtii*
152	*gallinarum*	183	*mundtii*
184	*faecalis*	217	*faecalis*
185	*faecalis*	218	*faecalis*
186	*faecium*	219	*faecalis*
187	*faecium*	220	*faecium*
188	*gallinarum*	221	*faecium*
189	*gallinarum*	222	*faecalis*
190	*gallinarum*	223	*faecalis*
191	*faecalis*	224	*faecalis*
192	*faecalis*	225	*faecalis*
193	*mundtii*	226	*faecalis*
194	*pseudoavium*	227	*faecalis*
195	*faecalis*	228	*faecalis*
196	*avium*	229	*faecalis*
197	*faecalis*	230	*faecalis*
200	*faecalis*	231	*faecalis*
201	*faecalis*	232	*saccharolyticus*
202	*Lactococcus*	233	*gallinarum*
203	*faecalis*	234	*faecalis*

ID	Species	ID	Species
204	*faecalis*	235	*gallinarum*
205	*faecalis*	236	*faecium*
206	*faecalis*	237	*faecalis*
207	*faecium*	238	*faecalis*
208	*faecalis*	239	*gallinarum*
209	*Lactococcus*	240	*gallinarum*
210	*gallinarum*	241	*gallinarum*
211	*Lactococcus*	242	*faecium*
212	*faecalis*	243	*gallinarum*
213	*Lactococcus*	244	*gallinarum*
214	*faecium*	245	*faecalis*
215	*Lactococcus*	246	*faecium*
216	*faecalis*	247	*gallinarum*
248	*gallinarum*	279	*avium*
249	*faecalis*	280	*faecalis*
250	*faecalis*	281	*faecalis*
251	*faecalis*	282	*faecalis*
252	*gallinarum*	283	*faecalis*
253	no id	284	*avium*
254	*faecalis*	285	*faecalis*
255	*gallinarum*	286	*faecalis*
256	*gallinarum*	287	*faecalis*
257	*faecium*	288	*raffinosus*
258	*gallinarum*	289	*faecalis*
259	*faecalis*	290	*gallinarum*
260	*maladoratus*	291	*gallinarum*
261	*ratti*	292	*faecalis*
262	*gallinarum*	293	*faecalis*
263	*faecium*	294	*faecalis*
264	*faecalis*	295	*faecalis*
265	*faecalis*	296	*faecalis*
266	*faecium*	297	*faecalis*
267	*faecalis*	298	*faecalis*
268	*maladoratus*	299	*faecalis*
269	*avium*	299A	*faecalis*
270	*faecalis*	299B	*faecalis*
271	*faecalis*	299C	*faecalis*

ID	Species	ID	Species
272	*faecalis*	300	*faecalis*
273	*faecalis*	301	*Lactococcus*
274	*faecalis*	302	*avium*
275	*faecalis*	303	*faecalis*
276	*faecalis*	304	*avium*
277	*faecalis*	305	*avium*
278	*faecalis*	306	*avium*
307	*faecalis*	338	*faecalis*
308	*raffinosus*	339	*faecalis*
309	*faecium*	340	*faecalis*
310	*faecium*	341	*faecalis*
311	*faecium*	342	*faecalis*
312	*gallinarum*	343	*faecalis*
313	*gallinarum*	344	*faecalis*
314	*gallinarum*	345	*faecalis*
315	*hirae*	346	*faecalis*
316	*hirae*	347	*maladoratus*
317	*ratti*	348	*hirae*
318	*hirae*	349	*hirae*
319	*hirae*	350	*hirae*
320	*hirae*	351	*hirae*
321	*gallinarum*	352	*hirae*
322	*hirae*	353	*hirae*
323	*hirae*	354	*hirae*
324	*hirae*	355	*hirae*
325	*hirae*	356	*hirae*
326	*hirae*	357	*hirae*
327	*hirae*	358	*mundtii*
328	*faecalis*	359	*hirae*
329	*faecalis*	360	*Lactococcus*
330	*faecalis*	361	*hirae*
331	*faecalis*	362	*hirae*
332	*faecalis*	363	*hirae*
333	*faecalis*	364	*hirae*
334	*faecalis*	365	*hirae*
335	*faecalis*	366	*hirae*

ID	Species	ID	Species
336	*faecalis*	367	*hirae*
337	*faecalis*	368	*faecium*
369	*gallinarum*	399A	*hirae*
370	*gallinarum*	399B	*hirae*
371	*gallinarum*	399C	*mundtii*
372	*gallinarum*	400	*faecium*
373	*gallinarum*	401	no id
374	*gallinarum*	402	no id
375	*gallinarum*	403	*faecium*
376	*faecalis*	404	*faecium*
377	*faecalis*	405	*faecium*
378	*faecalis*	406	*faecalis*
379	*faecalis*	407	no id
380	*gallinarum*	408	*faecalis*
381	*faecium*	409	*faecalis*
382	*asini*	410	*gallinarum*
383	*hirae*	411	*mundtii*
384	*hirae*	412	*mundtii*
385	*gallinarum*	413	*mundtii*
386	*hirae*	414	*mundtii*
387	*hirae*	415	*mundtii*
388	*hirae*	416	*gallinarum*
389	*asini*	417	*gallinarum*
390	*hirae*	418	*gallinarum*
391	*hirae*	419	*gallinarum*
392	*hirae*	420	*faecium*
393	*faecium*	421	*gallinarum*
394	*hirae*	422	*faecalis*
395	*hirae*	423	*gallinarum*
396	*hirae*	424	*gallinarum*
397	*faecalis*	425	*faecium*
398	*hirae*	426	*faecalis*
399	*hirae*	427	*faecalis*
428	*faecium*	459	*faecalis*
429	*gallinarum / faecium*	460	*gallinarum*
430	*gallinarum / faecium*	461	no id
431	*gallinarum*	462	*gallinarum*
432	*avium*	463	*gallinarum*

ID	Species	ID	Species
433	*gallinarum*	464	*gallinarum*
434	*gallinarum*	465	*gallinarum*
435	*faecalis*	466	*faecalis*
436	*faecalis*	467	*faecalis / Lactococcus*
437	*maladoratus*	468	*faecalis / Lactococcus*
438	*gallinarum*	469	*gallinarum*
439	*mundtii*	470	*faecalis*
440	*hirae*	471	*pseudoavium*
441	*pseudoavium*	472	*pseudoavium*
442	*pseudoavium*	473	*pseudoavium*
443	*pseudoavium*	474	*pseudoavium*
444	*pseudoavium*	475	*pseudoavium*
445	*faecalis*	476	no id
446	*faecalis*	477	no id
447	*gallinarum*	478	no id
448	*faecalis*	479	*faecium*
449	*faecalis*	480	no id
450	*faecalis*	481	*gallinarum*
451	*faecalis*	482	*gallinarum*
452	*faecalis*	483	*gallinarum*
453	*avium*	484	*gallinarum*
454	*avium*	485	*gallinarum*
455	*avium*	486	*avium*
456	*gallinarum*	487	*avium*
457	*faecalis*	488	*avium*
458	no id	489	*avium*
490	*avium*	517	*mundtii*
491	*pseudoavium*	518	no id
492	*faecalis*	519	*mundtii*
493	*faecalis*	520	*gallinarum*
494	*faecalis*	521	*gallinarum*
495	*gallinarum*	522	*faecium*
496	*gallinarum*	523	*faecium*
497	*gallinarum*	524	*faecalis*
498	*faecalis*	525	*raffinosus*
499	*faecium*	526	*faecium*
500	*durans*	527	*gallinarum*

ID	Species	ID	Species
501	*durans*	528	*faecium*
502	*faecium*	529	no id
503	*durans*	530	*gallinarum*
504	*faecium*	531	*gallinarum*
505	*faecalis*	532	*gallinarum*
506	*faecalis*	533	*faecium*
507	*faecalis*	534	*gallinarum*
508	*faecalis*	535	no id
509	*faecalis*	536	*gallinarum*
510	*mundtii*	537	*faecalis*
513	*mundtii*	538	*faecium*
514	*mundtii*	539	*faecalis*
515	*mundtii*	540	*faecium*
516	no id	541	*avium*
542	*faecalis*	628	*mundtii*
543	*maladoratus*	629	*mundtii*
544	*gallinarum*	630	*hirae*
600	*mundtii*	631	*casseliflavus*
601	*mundtii*	632	no id
602	*mundtii*	633	*mundtii*
603	*mundtii*	634	*mundtii*
604	*sulfureus*	635	*casseliflavus*
605	*mundtii*	636	*casseliflavus*
606	*hirae*	637	no id
607	*mundtii*	638	*faecium / mundtii*
608	*hirae*	639	*casseliflavus*
609	no id	640	*pallens*
610	no id	641	*casseliflavus*
611	*mundtii*	642	*mundtii*
612	no id	643	*casseliflavus*
613	*hirae*	644	*casseliflavus*
614	no id	645	*hirae*
615	no id	646	*casseliflavus*
616	*mundtii*	647	*hirae*
617	*mundtii*	648	*casseliflavus*
618	*hirae*	649	*casseliflavus*
619	*mundtii / casseliflavus*	650	*hirae*

ID	Species	ID	Species
620	*mundtii*	651	*mundtii*
621	*hirae*	652	no id
622	*hirae*	653	*mundtii*
623	*hirae*	654	*mundtii*
624	*mundtii*	655	*casseliflavus*
625	*casseliflavus*	656	*mundtii*
626	*hirae*	657	*mundtii*
627	*casseliflavus*	658	*mundtii*
659	*mundtii*	692	*hirae*
660	*mundtii*	693	no id
661	*mundtii*	694	*hirae*
662	*mundtii*	695	*hirae*
663	*mundtii*	696	*hirae*
664	*hirae*	697	*hirae*
665	no id	698	*hirae*
666	*casseliflavus*	699	*hirae*
667	*casseliflavus*	700	*faecium*
668	*hirae*	701	*faecalis*
669	no id	702	*faecium*
670	*casseliflavus*	703	*faecium*
671	*casseliflavus*	704	*gallinarum*
672	*casseliflavus*	705	*faecium*
673	*mundtii*	706	no id
674	*casseliflavus*	707	*faecium*
675	*mundtii*	708	*gallinarum*
676	*mundtii*	709	*faecium*
677	*mundtii*	710	*gallinarum*
678	*sulfureus*	711	*faecalis*
679	*mundtii*	712	*faecalis*
680	*hirae*	713	*gallinarum*
681	*mundtii*	714	*faecalis*
682	*hirae*	715	*gallinarum*
683	*faecium / mundtii*	716	*faecalis*
684	no id	717	*faecalis*
685	*hirae*	718	*faecalis*
686	*faecalis*	719	*gallinarum*
687	*hirae*	720	no id
688	*hirae*	721	no id
689	*hirae*	722	*faecalis*
690	*hirae*	723	*gallinarum*
691	no id	724	*faecalis*

Appendix 8.2. Speciation results of 291 confirmed enterococci. Identifications are based on Biolog examination and conventional biochemical test scheme results (Table 3.3). Isolates are organized alphabetically by Biolog-generated identification and numerically by source of origin. Numbers indicate a strain's lab identification (ID). (10's = bovine faeces, 100's =water, 200's = sewage, 300's = porcine faeces, 400's = human faeces, 500's = goose faeces, 600's = ovine faeces, 700's = chicken faeces). Isolates with the same identification according to Biolog and the biochemical test scheme are highlighted grey. Isolates with two possible identifications are indicated with a slash (/) between identifications and isolates with no identification (no id) are listed at the end of the table.

ID	Biolog	Biochemical
100	*casseliflavus*	*mundtii*
104	*casseliflavus*	*mundtii*
108	*casseliflavus*	*mundtii*
113	*casseliflavus*	*mundtii*
114	*casseliflavus*	*mundtii*
159	*casseliflavus*	*mundtii*
163	*casseliflavus*	*mundtii*
639	*casseliflavus*	*casseliflavus*
479	*durans*	*faecium*
101	*faecalis*	*avium*
102	*faecalis*	*faecalis*
110	*faecalis*	*faecalis*
111	*faecalis*	*faecalis*
117	*faecalis*	*faecalis*
121	*faecalis*	*faecalis*
124	*faecalis*	*faecalis*
125	*faecalis*	*faecalis*
126	*faecalis*	*avium*
154	*faecalis*	*faecalis*
156	*faecalis*	*faecalis*
158	*faecalis*	*faecalis*
160	*faecalis*	*faecalis*
165	*faecalis*	*faecalis*
166	*faecalis*	*faecalis*
168	*faecalis*	*faecalis*
169	*faecalis*	*faecalis*
171	*faecalis*	*faecalis*
172	*faecalis*	*faecalis*
175	*faecalis*	*faecalis*
200	*faecalis*	*faecalis*
201	*faecalis*	*faecalis*
202	*faecalis*	*Lactococcus*
203	*faecalis*	*faecalis*
204	*faecalis*	*faecalis*
205	*faecalis*	*faecalis*
206	*faecalis*	*faecalis*
208	*faecalis*	*faecalis*
209	*faecalis*	*Lactococcus*
211	*faecalis*	*Lactococcus*
212	*faecalis*	*faecalis*
213	*faecalis*	*Lactococcus*

ID	Biolog	Biochemical
215	*faecalis*	*Lactococcus*
216	*faecalis*	*faecalis*
217	*faecalis*	*faecalis*
218	*faecalis*	*faecalis*
219	*faecalis*	*faecalis*
221	*faecalis*	*faecium*
223	*faecalis*	*faecalis*
224	*faecalis*	*faecalis*
227	*faecalis*	*faecalis*
228	*faecalis*	*faecalis*
229	*faecalis*	*faecalis*
230	*faecalis*	*faecalis*
231	*faecalis*	*faecalis*
234	*faecalis*	*faecalis*
273	*faecalis*	*faecalis*
275	*faecalis*	*faecalis*
276	*faecalis*	*faecalis*
295	*faecalis*	*faecalis*
296	*faecalis*	*faecalis*
297	*faecalis*	*faecalis*
298	*faecalis*	*faecalis*
299A	*faecalis*	*faecalis*
299B	*faecalis*	*faecalis*
299C	*faecalis*	*faecalis*
300	*faecalis*	*faecalis*
301	*faecalis*	*Lactococcus*
302	*faecalis*	*avium*
303	*faecalis*	*faecalis*
305	*faecalis*	*avium*
306	*faecalis*	*avium*
307	*faecalis*	*faecalis*
328	*faecalis*	*faecalis*
329	*faecalis*	*faecalis*
330	*faecalis*	*faecalis*
331	*faecalis*	*faecalis*
332	*faecalis*	*faecalis*
333	*faecalis*	*faecalis*
334	*faecalis*	*faecalis*
335	*faecalis*	*faecalis*
336	*faecalis*	*faecalis*
432	*faecalis*	*avium*

ID	Biolog	Biochemical
436	*faecalis*	*faecalis*
437	*faecalis*	*maladoratus*
441	*faecalis*	*pseudoavium*
442	*faecalis*	*pseudoavium*
445	*faecalis*	*faecalis*
446	*faecalis*	*faecalis*
451	*faecalis*	*faecalis*
452	*faecalis*	*faecalis*
457	*faecalis*	*faecalis*
466	*faecalis*	*faecalis*
471	*faecalis*	*pseudoavium*
472	*faecalis*	*pseudoavium*
486	*faecalis*	*avium*
487	*faecalis*	*avium*
490	*faecalis*	*avium*
491	*faecalis*	*pseudoavium*
537	*faecalis*	*faecalis*
543	*faecalis*	*maladoratus*
701	*faecalis*	*faecalis*
711	*faecalis*	*faecalis*
714	*faecalis*	*faecalis*
716	*faecalis*	*faecalis*
724	*faecalis*	*faecalis*
17	*faecium*	*faecium / asini*
47	*faecium*	*gallinarum*
75	*faecium*	*gallinarum*
77	*faecium*	*gallinarum*
78	*faecium*	*gallinarum*
97	*faecium*	*gallinarum*
98	*faecium*	*faecium*
99	*faecium*	*gallinarum*
105	*faecium*	*faecium*
122	*faecium*	*faecium*
167	*faecium*	*gallinarum*
214	*faecium*	*faecium*
309	*faecium*	*faecium*
313	*faecium*	*gallinarum*
368	*faecium*	*faecium*
375	*faecium*	*gallinarum*
400	*faecium*	*faecium*
404	*faecium*	*faecium*

ID	Biolog	Biochemical
424	*faecium*	*gallinarum*
434	*faecium*	*gallinarum*
520	*faecium*	*gallinarum*
533	*faecium*	*faecium*
540	*faecium*	*faecium*
107	*flavescens*	*mundtii*
26	*gallinarum*	*hirae*
115	*gallinarum*	*faecium*
116	*gallinarum*	*gallinarum*
120	*gallinarum*	*mundtii*
123	*gallinarum*	*faecium*
128	*gallinarum*	*faecium*
232	*gallinarum*	*saccharolyticus*
233	*gallinarum*	*gallinarum*
314	*gallinarum*	*gallinarum*
10	*hirae*	*Lactococcus*
29	*hirae*	*hirae*
31	*hirae*	*hirae*
32	*hirae*	*hirae*
82	*hirae*	*hirae*
95	*hirae*	*hirae*
106	*hirae*	*faecium*
207	*hirae*	*faecium*
312	*hirae*	*gallinarum*
349	*hirae*	*mundtii*
401	*hirae*	no id
719	*hirae*	*gallinarum*
1	*mundtii*	*faecium*
7	*mundtii*	*Vagococcus*
8	*mundtii*	*faecium*
14	*mundtii*	*faecium / asini*
15	*mundtii*	*faecium / asini*
18	*mundtii*	*hirae*
25	*mundtii*	*hirae*
33	*mundtii*	*Vagococcus*
35	*mundtii*	*hirae*
36	*mundtii*	*Vagococcus*
42	*mundtii*	*mundtii*
50	*mundtii*	*hirae*
62	*mundtii*	*mundtii*
63	*mundtii*	*hirae*

ID	Biolog	Biochemical
92	*mundtii*	*faecium / Lactococcus*
118	*mundtii*	*mundtii*
315	*mundtii*	*hirae*
316	*mundtii*	*hirae*
356	*mundtii*	*hirae*
365	*mundtii*	*hirae*
517	*mundtii*	*mundtii*
600	*mundtii*	*mundtii*
602	*mundtii*	*mundtii*
603	*mundtii*	*mundtii*
605	*mundtii*	*mundtii*
608	*mundtii*	*hirae*
610	*mundtii*	no id
611	*mundtii*	*mundtii*
616	*mundtii*	*mundtii*
617	*mundtii*	*mundtii*
619	*mundtii*	*mundtii / casseliflavus*
623	*mundtii*	*hirae*
625	*mundtii*	*casseliflavus*
628	*mundtii*	*mundtii*
629	*mundtii*	*mundtii*
631	*mundtii*	*casseliflavus*
632	*mundtii*	no id
633	*mundtii*	*mundtii*
636	*mundtii*	*casseliflavus*
637	*mundtii*	no id
648	*mundtii*	*casseliflavus*
649	*mundtii*	*casseliflavus*
652	*mundtii*	no id
653	*mundtii*	*mundtii*
659	*mundtii*	*mundtii*
660	*mundtii*	*mundtii*
684	*mundtii*	no id
689	*mundtii*	*hirae*
693	*mundtii*	no id
174	*sulfureus*	*faecalis*
11	*mundtii / hirae*	*Vagococcus*
13	*mundtii / hirae*	*faecium / asini*
30	*gallinarum / hirae*	*gallinarum*
34	*faecium / mundtii*	*hirae / dispar*
38	*gallinarum / mundtii*	*mundtii*

ID	Biolog	Biochemical
45	*mundtii / hirae*	*Vagococcus*
83	*faecium / durans*	*gallinarum*
103	*faecalis / casseliflavus*	*columbae / raffinosus*
119	*mundtii / hirae*	*mundtii*
155	*gallinarum / mundtii*	*mundtii*
161	*mundtii / hirae*	*mundtii*
210	*gallinarum / faecalis*	*gallinarum*
220	*faecium / hirae*	*faecium*
308	*gallinarum / faecalis*	*raffinosus*
311	*avium / faecium*	*faecium*
318	*mundtii / hirae*	*hirae*
322	*mundtii / hirae*	*hirae*
348	*hirae / mundtii*	*hirae*
411	*gallinarum / casseliflavus*	*mundtii*
456	*gallinarum / hirae*	*gallinarum*
497	*gallinarum / hirae*	*gallinarum*
510	*gallinarum / mundtii*	*mundtii*
601	*gallinarum / mundtii*	*mundtii*
604	*gallinarum / mundtii*	*sulfureus*
609	*hirae / mundtii*	no id
708	*gallinarum / hirae*	*gallinarum*
6	no id	*faecium*
16	no id	*faecium / asini*
20	no id	*hirae*
21	no id	*hirae*
22	no id	*hirae*
24	no id	*faecium / asini*
27	no id	*hirae*
39	no id	*mundtii*
66	no id	*faecium*
76	no id	*gallinarum*
79	no id	*gallinarum*
88	no id	*gallinarum*
93	no id	*gallinarum*
109	no id	*gallinarum*
112	no id	*casseliflavus*
127	no id	*gallinarum*
157	no id	*mundtii*
162	no id	*gallinarum*
164	no id	*gallinarum*
173	no id	no id

ID	Biolog	Biochemical
299	no id	*faecalis*
304	no id	*avium*
310	no id	*faecium*
320	no id	*hirae*
337	no id	*faecalis*
355	no id	*hirae*
364	no id	*hirae*
367	no id	*hirae*
385	no id	*gallinarum*
392	no id	*hirae*
399	no id	*hirae*
399C	no id	*mundtii*
402	no id	no id
412	no id	*mundtii*
419	no id	*gallinarum*
420	no id	*faecium*
426	no id	*faecalis*
427	no id	*faecalis*
463	no id	*gallinarum*
464	no id	*gallinarum*
467	no id	*faecalis / Lactococcus*
478	no id	no id
481	no id	*gallinarum*
482	no id	*gallinarum*
496	no id	*gallinarum*
500	no id	*durans*
502	no id	*faecium*
503	no id	*durans*
515	no id	*mundtii*
516	no id	no id
522	no id	*faecium*
525	no id	*raffinosus*
526	no id	*faecium*
538	no id	*faecium*
544	no id	*gallinarum*
622	no id	*hirae*
626	no id	*hirae*
635	no id	*casseliflavus*
690	no id	*hirae*
694	no id	*hirae*
698	no id	*hirae*
699	no id	*hirae*
704	no id	*gallinarum*
706	no id	no id
721	no id	no id

Appendix 8.3. Speciation results of 291 confirmed enterococci. Identifications are based on Biolog examination and conventional biochemical test scheme results (Table 3.3). Isolates are organized alphabetically by identification according to the biochemical test scheme and numerically (ID) by source of origin. (10's = bovine faeces, 100's =water, 200's = sewage, 300's = porcine faeces, 400's = human faeces, 500's = goose faeces, 600's = ovine faeces, 700's = chicken faeces). Isolates with the same identification according to Biolog and the biochemical test scheme are highlighted grey. Isolates with no identification (no id) and isolates with two possible identifications are included. The two possible identifications are separated by a slash (/).

ID	Biolog	Biochemical
101	*faecalis*	*avium*
126	*faecalis*	*avium*
302	*faecalis*	*avium*
305	*faecalis*	*avium*
306	*faecalis*	*avium*
432	*faecalis*	*avium*
486	*faecalis*	*avium*
487	*faecalis*	*avium*
490	*faecalis*	*avium*
304	no id	*avium*
639	*casseliflavus*	*casseliflavus*
625	*mundtii*	*casseliflavus*
631	*mundtii*	*casseliflavus*
636	*mundtii*	*casseliflavus*
648	*mundtii*	*casseliflavus*
649	*mundtii*	*casseliflavus*
112	no id	*casseliflavus*
635	no id	*casseliflavus*
103	*faecalis / casseliflavus*	*columbae / raffinosus*
500	no id	*durans*
503	no id	*durans*
102	*faecalis*	*faecalis*
110	*faecalis*	*faecalis*
111	*faecalis*	*faecalis*
117	*faecalis*	*faecalis*
121	*faecalis*	*faecalis*
124	*faecalis*	*faecalis*
125	*faecalis*	*faecalis*
154	*faecalis*	*faecalis*
156	*faecalis*	*faecalis*
158	*faecalis*	*faecalis*
160	*faecalis*	*faecalis*
165	*faecalis*	*faecalis*
166	*faecalis*	*faecalis*
168	*faecalis*	*faecalis*
169	*faecalis*	*faecalis*
171	*faecalis*	*faecalis*
172	*faecalis*	*faecalis*
175	*faecalis*	*faecalis*
200	*faecalis*	*faecalis*

ID	Biolog	Biochemical
201	*faecalis*	*faecalis*
203	*faecalis*	*faecalis*
204	*faecalis*	*faecalis*
205	*faecalis*	*faecalis*
206	*faecalis*	*faecalis*
208	*faecalis*	*faecalis*
212	*faecalis*	*faecalis*
216	*faecalis*	*faecalis*
217	*faecalis*	*faecalis*
218	*faecalis*	*faecalis*
219	*faecalis*	*faecalis*
223	*faecalis*	*faecalis*
224	*faecalis*	*faecalis*
227	*faecalis*	*faecalis*
228	*faecalis*	*faecalis*
229	*faecalis*	*faecalis*
230	*faecalis*	*faecalis*
231	*faecalis*	*faecalis*
234	*faecalis*	*faecalis*
273	*faecalis*	*faecalis*
275	*faecalis*	*faecalis*
276	*faecalis*	*faecalis*
295	*faecalis*	*faecalis*
296	*faecalis*	*faecalis*
297	*faecalis*	*faecalis*
298	*faecalis*	*faecalis*
299A	*faecalis*	*faecalis*
299B	*faecalis*	*faecalis*
299C	*faecalis*	*faecalis*
300	*faecalis*	*faecalis*
303	*faecalis*	*faecalis*
307	*faecalis*	*faecalis*
328	*faecalis*	*faecalis*
329	*faecalis*	*faecalis*
330	*faecalis*	*faecalis*
331	*faecalis*	*faecalis*
332	*faecalis*	*faecalis*
333	*faecalis*	*faecalis*
334	*faecalis*	*faecalis*
335	*faecalis*	*faecalis*
336	*faecalis*	*faecalis*

ID	Biolog	Biochemical
422	*faecalis*	*faecalis*
436	*faecalis*	*faecalis*
445	*faecalis*	*faecalis*
446	*faecalis*	*faecalis*
451	*faecalis*	*faecalis*
452	*faecalis*	*faecalis*
457	*faecalis*	*faecalis*
466	*faecalis*	*faecalis*
537	*faecalis*	*faecalis*
701	*faecalis*	*faecalis*
711	*faecalis*	*faecalis*
714	*faecalis*	*faecalis*
716	*faecalis*	*faecalis*
724	*faecalis*	*faecalis*
174	*sulfureus*	*faecalis*
299	no id	*faecalis*
337	no id	*faecalis*
426	no id	*faecalis*
427	no id	*faecalis*
467	no id	*faecalis / Lactococcus*
479	*durans*	*faecium*
221	*faecalis*	*faecium*
98	*faecium*	*faecium*
105	*faecium*	*faecium*
122	*faecium*	*faecium*
214	*faecium*	*faecium*
309	*faecium*	*faecium*
368	*faecium*	*faecium*
400	*faecium*	*faecium*
404	*faecium*	*faecium*
533	*faecium*	*faecium*
540	*faecium*	*faecium*
115	*gallinarum*	*faecium*
123	*gallinarum*	*faecium*
128	*gallinarum*	*faecium*
106	*hirae*	*faecium*
207	*hirae*	*faecium*
1	*mundtii*	*faecium*
8	*mundtii*	*faecium*
220	*faecium / hirae*	*faecium*
311	*avium / faecium*	*faecium*

ID	Biolog	Biochemical
6	no id	*faecium*
66	no id	*faecium*
310	no id	*faecium*
420	no id	*faecium*
502	no id	*faecium*
522	no id	*faecium*
526	no id	*faecium*
538	no id	*faecium*
17	*faecium*	*faecium / asini*
14	*mundtii*	*faecium / asini*
15	*mundtii*	*faecium / asini*
13	*mundtii / hirae*	*faecium / asini*
16	no id	*faecium / asini*
24	no id	*faecium / asini*
92	*mundtii*	*faecium / Lactococcus*
47	*faecium*	*gallinarum*
75	*faecium*	*gallinarum*
77	*faecium*	*gallinarum*
78	*faecium*	*gallinarum*
97	*faecium*	*gallinarum*
99	*faecium*	*gallinarum*
167	*faecium*	*gallinarum*
313	*faecium*	*gallinarum*
375	*faecium*	*gallinarum*
424	*faecium*	*gallinarum*
434	*faecium*	*gallinarum*
520	*faecium*	*gallinarum*
116	*gallinarum*	*gallinarum*
233	*gallinarum*	*gallinarum*
314	*gallinarum*	*gallinarum*
312	*hirae*	*gallinarum*
719	*hirae*	*gallinarum*
30	*gallinarum / hirae*	*gallinarum*
83	*faecium / durans*	*gallinarum*
210	*gallinarum / faecalis*	*gallinarum*
456	*gallinarum / hirae*	*gallinarum*
497	*gallinarum / hirae*	*gallinarum*
708	*gallinarum / hirae*	*gallinarum*
76	no id	*gallinarum*
79	no id	*gallinarum*
88	no id	*gallinarum*

ID	Biolog	Biochemical
93	no id	*gallinarum*
109	no id	*gallinarum*
127	no id	*gallinarum*
162	no id	*gallinarum*
164	no id	*gallinarum*
385	no id	*gallinarum*
419	no id	*gallinarum*
463	no id	*gallinarum*
464	no id	*gallinarum*
481	no id	*gallinarum*
482	no id	*gallinarum*
496	no id	*gallinarum*
544	no id	*gallinarum*
704	no id	*gallinarum*
26	*gallinarum*	*hirae*
29	*hirae*	*hirae*
31	*hirae*	*hirae*
32	*hirae*	*hirae*
82	*hirae*	*hirae*
95	*hirae*	*hirae*
18	*mundtii*	*hirae*
25	*mundtii*	*hirae*
35	*mundtii*	*hirae*
50	*mundtii*	*hirae*
63	*mundtii*	*hirae*
315	*mundtii*	*hirae*
316	*mundtii*	*hirae*
356	*mundtii*	*hirae*
365	*mundtii*	*hirae*
608	*mundtii*	*hirae*
623	*mundtii*	*hirae*
689	*mundtii*	*hirae*
318	*mundtii / hirae*	*hirae*
322	*mundtii / hirae*	*hirae*
348	*hirae / mundtii*	*hirae*
20	no id	*hirae*
21	no id	*hirae*
22	no id	*hirae*
27	no id	*hirae*
320	no id	*hirae*
355	no id	*hirae*

ID	Biolog	Biochemical
364	no id	*hirae*
367	no id	*hirae*
392	no id	*hirae*
399	no id	*hirae*
622	no id	*hirae*
626	no id	*hirae*
690	no id	*hirae*
694	no id	*hirae*
698	no id	*hirae*
699	no id	*hirae*
34	*faecium / mundtii*	*hirae / dispar*
202	*faecalis*	*Lactococcus*
209	*faecalis*	*Lactococcus*
211	*faecalis*	*Lactococcus*
213	*faecalis*	*Lactococcus*
215	*faecalis*	*Lactococcus*
301	*faecalis*	*Lactococcus*
10	*hirae*	*Lactococcus*
437	*faecalis*	*maladoratus*
543	*faecalis*	*maladoratus*
100	*casseliflavus*	*mundtii*
104	*casseliflavus*	*mundtii*
108	*casseliflavus*	*mundtii*
113	*casseliflavus*	*mundtii*
114	*casseliflavus*	*mundtii*
159	*casseliflavus*	*mundtii*
163	*casseliflavus*	*mundtii*
107	*flavescens*	*mundtii*
120	*gallinarum*	*mundtii*
349	*hirae*	*mundtii*
42	*mundtii*	*mundtii*
62	*mundtii*	*mundtii*
118	*mundtii*	*mundtii*
517	*mundtii*	*mundtii*
600	*mundtii*	*mundtii*
602	*mundtii*	*mundtii*
603	*mundtii*	*mundtii*
605	*mundtii*	*mundtii*
611	*mundtii*	*mundtii*
616	*mundtii*	*mundtii*
617	*mundtii*	*mundtii*

ID	Biolog	Biochemical
628	*mundtii*	*mundtii*
629	*mundtii*	*mundtii*
633	*mundtii*	*mundtii*
653	*mundtii*	*mundtii*
659	*mundtii*	*mundtii*
660	*mundtii*	*mundtii*
38	*gallinarum / mundtii*	*mundtii*
119	*mundtii / hirae*	*mundtii*
155	*gallinarum / mundtii*	*mundtii*
161	*mundtii / hirae*	*mundtii*
411	*gallinarum / casseliflavus*	*mundtii*
510	*gallinarum / mundtii*	*mundtii*
601	*gallinarum / mundtii*	*mundtii*
39	no id	*mundtii*
157	no id	*mundtii*
399C	no id	*mundtii*
412	no id	*mundtii*
515	no id	*mundtii*
619	*mundtii*	*mundtii / casseliflavus*
173	no id	no id
401	*hirae*	no id
610	*mundtii*	no id
632	*mundtii*	no id
637	*mundtii*	no id
652	*mundtii*	no id
684	*mundtii*	no id
693	*mundtii*	no id
609	*hirae / mundtii*	no id
402	no id	no id
478	no id	no id
516	no id	no id
706	no id	no id
721	no id	no id
441	*faecalis*	*pseudoavium*
442	*faecalis*	*pseudoavium*
471	*faecalis*	*pseudoavium*
472	*faecalis*	*pseudoavium*
491	*faecalis*	*pseudoavium*
308	*gallinarum / faecalis*	*raffinosus*
525	no id	*raffinosus*
232	*gallinarum*	*saccharolyticus*
604	*gallinarum / mundtii*	*sulfureus*
7	*mundtii*	*Vagococcus*
33	*mundtii*	*Vagococcus*
36	*mundtii*	*Vagococcus*
11	*mundtii / hirae*	*Vagococcus*
45	*mundtii / hirae*	*Vagococcus*

Appendix 8.4. Biolog-generated metabolic profiles of *Enterococcus faecalis* strains isolated from human (400's), porcine (300's), chicken (700's) and goose faeces (500's). Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. faecalis*-specific and *E. faecalis* host-specifc carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

faecalis *faecalis* 422			*faecalis* *avium* 432			*faecalis* *faecalis* 436			*faecalis* *maladoratus* 437			*faecalis* *pseudoavium* 441		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
			A	2	100	A	2	50	A	2	50			
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
B	9	100	B	9	100	B	9	75	B	9	75	B	9	75
B	10	100												
B	12	100												
C	1	100	C	1	100	C	1	100	C	1	100	C	1	100
						C	2	50	C	2	50	C	2	50
			C	5	100	C	5	100	C	5	100	C	5	100
C	7	100												
D	1	100	D	1	50	D	1	50	D	1	50	D	1	50
D	10	75				D	10	50						
D	12	100				D	12	100	D	12	75	D	12	75
E	1	100	E	1	75	E	1	50	E	1	75	E	1	75
F	5	100	F	5	50									
F	9	75	F	9	100	F	9	50	F	9	75	F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
			H	7	100									

faecalis *pseudoavium* 442			*faecalis* *faecalis* 445			*faecalis* *faecalis* 446			*faecalis* *faecalis* 451			*faecalis* *faecalis* 452		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	50	A	2	75	A	2	50	A	2	50	A	2	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
B	9	100	B	9	100	B	9	50	B	9	100	B	9	100
												B	12	100
C	1	100	C	1	100	C	1	100	C	1	100			
C	2	75	C	2	75	C	2	50	C	2	50			
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
												C	7	100
D	1	75	D	1	100	D	1	75	D	1	100	D	1	75
D	10	50	D	10	75	D	10	50	D	10	50			
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
E	1	100	E	1	100	E	1	50	E	1	100			
F	9	100	F	9	75	F	9	100	F	9	100	F	9	50
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
												H	7	50

faecalis *faecalis* 457			*faecalis* *faecalis* 466			*faecalis* *pseudoavium* 471			*faecalis* *pseudoavium* 472			*faecalis* *avium* 486		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	50	A	2	50							A	2	50
A	11	100	A	11	100							A	11	100
B	9	100	B	9	50	B	9	100	B	9	100	B	9	100
			B	10	100	B	10	100	B	10	100	B	10	100
												B	12	100
C	1	100	C	1	100	C	1	100	C	1	100	C	1	100
C	2	50							C	2	50	C	2	100
C	5	100	C	5	100	C	5	50	C	5	100	C	5	100
			C	7	100	C	7	100	C	7	100	C	7	100
D	1	75										D	1	100
D	10	50	D	10	50							D	10	75
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
E	1	50	E	1	50				E	1	50	F	1	100
												F	5	75
F	9	75	F	9	75	F	9	100	F	9	100	F	9	100
H	2	100	H	2	50	H	2	50	H	2	100	H	2	100
H	4	100	H	4	50	H	4	100	H	4	100	H	4	100
						H	7	50	H	7	75	H	7	50
												H	9	75
												H	11	75

faecalis *avium* 487			*faecalis* *avium* 490			*faecalis* *pseudoavium* 491			*faecalis* *faecalis* 300			*faecalis* *Lactococcus* 301		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
			A	2	50				A	2	100	A	2	100
A	11	100	A	11	100							A	3	29
									A	12	33	A	12	29
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
B	12	100	B	12	100									
C	1	100	C	1	100	C	1	100	C	1	67	C	1	43
C	2	50	C	2	75	C	2	50						
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
D	1	100	D	1	100									
			D	10	75				D	1	100	D	12	86
D	12	100	D	12	100	D	12	100						
E	1	100	E	1	100				D	10	67	D	10	29
F	5	50	F	5	75				D	12	100	D	12	100
F	9	100	F	9	75	F	9	100	E	1	67	E	1	29
H	2	100	H	2	100	H	2	100	F	5	83	F	5	57
H	4	100	H	4	100	H	4	100	F	9	100	F	9	57
			H	7	50	H	7	100	H	7	33			
			H	9	50				H	9	100	H	9	86
			H	11	50									
									H	11	83	H	11	86

faecalis *avium* 302			*faecalis* *faecalis* 303			*faecalis* *avium* 305			*faecalis* *avium* 306			*faecalis* *faecalis* 307		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100	A	2	67	A	2	100
			A	3	50	A	3	50						
A	12	33	A	12	33	A	12	33				A	12	33
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	67	B	10	100	B	10	100	B	10	100	B	10	100
C	1	67	C	1	33	C	1	33	C	1	100	C	1	33
									C	2	100			
C	5	67	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
						C	8	33				C	8	33
D	1	100	D	1	100	D	1	100	D	1	100	D	1	100
						D	5	33				D	5	33
D	10	50	D	10	67	D	10	83				D	10	100
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
			E	1	67	E	1	33	E	1	50	E	1	50
F	5	50	F	5	67	F	5	50	F	5	50	F	5	50
F	9	100	F	9	83	F	9	100	F	9	100	F	9	100
						H	7	33	H	7	67			
H	9	67	H	9	100	H	9	100				H	9	83
H	11	67	H	11	83	H	11	100				H	11	67

faecalis *faecalis* 328			*faecalis* *faecalis* 329			*faecalis* *faecalis* 330			*faecalis* *faecalis* 331			*faecalis* *faecalis* 332		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100	A	2	100	A	2	100
A	3	50	A	3	83	A	3	50						
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
									D	12	83	B	12	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100	C	7	100	C	7	100	C	7	100
D	1	83	D	1	100	D	1	83	D	1	33	D	1	50
			D	10	67							D	10	33
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
			E	1	50									
F	5	50	F	5	100	F	5	83	F	5	100	F	5	100
F	9	67	F	9	83	F	9	100	F	9	100	F	9	100
									H	7	100	H	7	100
H	9	100	H	9	100	H	9	100	H	9	100	H	9	100
H	11	100	H	11	100	H	11	100	H	11	100	H	11	100

faecalis *faecalis* 333			*faecalis* *faecalis* 334			*faecalis* *faecalis* 335			*faecalis* *faecalis* 336		
	Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100	A	2	100
			A	3	83	A	3	50	A	3	50
B	9	100	B	9	100	B	9	83	B	9	50
B	10	100	B	10	100	B	10	100	B	10	100
B	12	67	B	12	100	B	12	67	B	12	50
C	1	50	C	1	100						
C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100						
D	1	50	D	1	83	D	1	67	D	1	33
			D	10	50	D	10	33			
D	12	100	D	12	100						
			E	1	33						
F	5	100	F	5	100	F	5	33	F	5	33
F	9	100	F	9	100	F	9	100	F	9	100
H	7	100	H	7	100	H	7	67			
H	9	100	H	9	100	H	9	100	H	9	100
			H	10	50						
H	11	100	H	11	100	H	11	100	H	11	100

faecalis *faecalis* 543			*faecalis* *faecalis* 537			*faecalis* *faecalis* 701			*faecalis* *faecalis* 711		
	Well	%P		Well	%P		Well	%P		Well	%P
			A	2	75	B	12	100	B	12	100
C	1	100				C	7	100			
C	2	100				D	1	100			
C	8	100				E	1	100			
C	9	100				F	5	100	F	5	50
			E	1	75	F	9	75	F	9	75
F	5	50	F	5	75	H	9	100			
			H	7	75						

faecalis *faecalis* 714			*faecalis* *faecalis* 716			*faecalis* *faecalis* 724		
	Well	%P		Well	%P		Well	%P
			B	12	100	B	12	100
C	7	100						
D	1	100						
E	1	100						
F	5	100	F	5	100	F	5	50
F	9	75	F	9	50	F	9	100

Appendix 8.5. Biolog-generated metabolic profiles of *Enterococcus faecium.* Strains were isolated from sewage (200's), bovine (10's), human (400's), porcine (300's), and goose (500's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. faecium*-specific and *E. faecium* host-specifc carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1^{st} species) and conventional biochemical test scheme identifications (2^{nd} species) are displayed above the numerical identity of an isolate.

faecium *gallinarum* 99			*faecium* *asini* 17			*faecium* *gallinarum* 47			*faecium* *gallinarum* 75		
	Well	%P		Well	%P		Well	%P		Well	%P
			A	2	40						
			A	4	40						
A	10	100	A	10	100	A	10	67	A	10	100
A	11	100	A	11	40	A	11	83	A	11	100
			A	12	100						
B	3	100	B	3	100	B	3	33	B	3	83
B	7	100				B	7	83	B	7	100
			B	10	100						
			C	2	100	C	1	50			
			C	3	100						
			C	4	100						
			C	11	100						
D	4	50	D	4	60						
D	7	33	D	7	100	D	7	33			
			D	12	100	D	12	100			
E	2	83				E	2	33			
F	6	33									
			F	1	100						
H	3	100	H	3	100	H	3	67	H	3	83
H	4	100	H	4	100	H	4	100	H	4	83

faecium *gallinarum* 77			*faecium* *gallinarum* 78			*faecium* *gallinarum* 97			*faecium* *faecium* 98		
	Well	%P		Well	%P		Well	%P		Well	%P
A	10	100	A	10	100	A	10	100	A	10	100
A	11	100	A	11	100	A	11	100	A	11	100
B	3	67	B	3	100	B	3	83	B	3	100
B	7	67				B	7	100	B	7	100
									D	4	33
						D	7	33	D	7	50
									E	2	33
F	6	67							F	6	33
H	3	67	H	3	100	H	3	100	H	3	100
H	4	67	H	4	100	H	4	83	H	4	100

faecium *faecium* 309			*faecium* *gallinarum* 313			*faecium* *faecium* 368			*faecium* *gallinarum* 375			*faecium* *faecium* 214		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
						A	4	50						
B	3	100	B	3	50							A	10	100
B	7	50	B	7	50							A	11	100
												B	3	100
C	3	100	C	3	50							B	8	100
			C	4	50							B	7	100
D	1	33												
D	4	33	D	4	50	D	4	67				C	3	100
												C	4	100
E	12	100	E	12	100									
E	2	100	E	2	50	E	2	100	E	2	100			
			F	6	33	F	6	33						
H	3	100	H	3	50	H	3	100	H	3	100			
			H	9	50							E	3	100
faecium *faecium* 400			*faecium* *faecium* 404			*faecium* *gallinarum* 424			*faecium* *gallinarum* 434					
	Well	%P		Well	%P		Well	%P		Well	%P			
												D	12	100
D	12	100												
									E	2	100			
												F	1	100
												F	3	100
												F	4	100
F	6	50												
H	3	50	H	3	50	H	3	100						
faecium *gallinarum* 520			*faecium* *faecium* 533			*faecium* *faecium* 540								
	Well	%P		Well	%P		Well	%P						
B	1	50												
B	9	50				B	9	50						
			C	3	75	C	3	100						
						C	4	100						
D	4	50	D	4	50	D	4	100						
D	7	100	D	7	50	D	7	75						
E	2	100	E	2	100									
F	6	50	F	6	50	F	6	50						
			H	1	50	H	1	50						

Appendix 8.6. Biolog-generated metabolic profiles of *Enterococcus gallinarum.* Strains were isolated from sewage (200's), bovine (10's) and porcine (300's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. gallinarum*-specific and *E. gallinarum* host-specifc carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

gallinarum hirae/dispar 26			*gallinarum gallinarum* 314		
	Well	%P		Well	%P
A	4	33			
D	4	67			
D	5	67			
H	4	50	H	4	50
H	5	50	H	5	50

gallinarum saccharolyticus 232			*gallinarum gallinarum* 233		
	Well	%P		Well	%P
D	7	33			

Appendix 8.7. Biolog-generated metabolic profiles of *Enterococcus hirae.*

Strains were isolated from sewage (200's), bovine (10's), porcine (300's), human (400's) and chicken (700's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. hirae*-specific and *E. hirae* host-specifc carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

hirae *faecium* 207			*hirae* no id 401			*hirae* *gallinarum* 719			*hirae* *Lactococcus* 10			*hirae* *hirae/dispar* 28		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	50	A	2	100							A	2	71
			A	3	100							A	3	71
A	4	100	A	4	100				A	4	67	A	4	71
									A	12	67	A	12	71
A	11	100				B	4	100				B	1	29
A	12	100										B	7	100
B	1	67							C	8	50	C	8	57
B	7	100	B	7	100	B	7	100	D	4	50	D	4	71
B	9	67	B	9	100									
B	10	67												
C	1	67	C	1	100							E	5	29
C	2	67	C	2	100				F	6	33			
C	3	100	C	3	100	C	3	100	F	9	33			
C	4	100	C	4	100	C	4	100	H	3	67	H	3	57
C	5	67							H	4	83	H	4	57
C	7	67							H	5	100	H	5	100
C	8	67				C	8	67	*hirae* *hirae/dispar* 29			*hirae* *hirae/dispar* 31		
						C	9	83						
C	10	67								Well	%P		Well	%P
C	12	67							A	2	57	A	2	57
D	1	67	D	1	100				A	3	57	A	3	57
D	3	67							A	4	71	A	4	100
D	4	100	D	4	50	D	4	33	A	12	57	A	12	100
D	5	67				D	5	100						
D	7	100	D	7	100	D	7	100						
D	10	50							B	2	100	B	7	100
D	11	67				D	11	100	C	8	86	C	8	100
D	12	100				D	12	100	D	4	71	D	4	100
E	2	100	E	2	100							D	5	43
E	3	67										D	11	43
E	5	67												
F	3	67							F	6	57	F	6	57
F	6	100												
F	9	67							H	3	100	H	3	100
H	1	67							H	4	100	H	4	100
H	2	67							H	5	100	H	5	100
H	3	100	H	3	100	H	3	83						
H	4	100	H	4	100	H	4	67						
H	5	100	H	5	100	H	5	100						
H	7	33												

hirae *hirae/dispar* 32			*hirae* *hirae/dispar* 82			*hirae* *hirae* 95			*hirae* *gallinarum* 312			*hirae* *mundtii* 349		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	57							A	2	57			
A	3	57							A	4	57			
A	4	71	A	4	71	A	4	67				A	12	100
A	12	71	A	12	100	A	12	33	B	9	57	B	9	100
												C	1	33
B	7	100	B	7	100	B	7	33	C	3	100	C	3	50
C	8	71							C	4	100			
D	4	71										C	9	100
									D	4	57	D	4	67
									D	5	100			
									D	11	100			
F	6	29							F	6	100			
H	3	57	H	3	43	H	3	33						
H	4	71	H	4	100	H	4	100						
H	5	71	H	5	100	H	5	100						

Appendix 8.8. Biolog-generated metabolic profiles of *Enterococcus mundtii.* Strains isolated from bovine (10's), porcine (300's), ovine (600's) and goose (500's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. *E. hirae*-specific and *E. hirae* host-specifc carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

mundtii *faecium* 1			*mundtii* *Vagococcus* 7			*mundtii* *faecium* 8			*mundtii* *asini* 14			*mundtii* *asini* 15		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
									A	4	40			
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
A	12	50	A	12	100	A	12	100	A	12	60	A	12	100
C	1	100							C	1	80			
C	3	83							C	3	60			
									C	4	60			
D	4	83							D	4	80			
			D	12	100	D	12	100	D	12	100	D	12	100
F	6	89							F	6	40			
H	1	67												
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100

mundtii *Vagococcus* 33			*mundtii* *hirae/dispar* 35			*mundtii* *Vagococcus* 36			*mundtii* *mundtii* 42			*mundtii* *hirae/dispar* 50		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
												A	4	33
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
A	12	50	A	12	100	A	12	100				A	12	100
									C	1	67			
									C	2	33			
									C	3	100	C	3	33
									C	4	67	C	4	33
									C	8	33			
									C	10	33			
									D	4	100	D	4	33
									D	11	100			
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
									F	6	67			
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100

mundtii *hirae/dispar* 18			*mundtii* *hirae/dispar* 25			*mundtii* *mundtii* 62			*mundtii* *hirae/dispar* 63		
	Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	100				A	11	100
A	12	50	A	12	100				A	12	56
						C	1	67			
			C	3	50						
						C	8	67			
D	4	33	D	4	50	D	4	89			
						D	11	100			
D	12	100	D	12	100	D	12	100	D	12	100
H	4	100	H	4	67	H	4	100	H	4	100

mundtii *mundtii* 600			*mundtii* *mundtii* 602			*mundtii* *mundtii* 603			*mundtii* *mundtii* 605			*mundtii* *hirae* 608		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
												A	12	50
B	1	75												
B	7	100	B	7	100	B	7	100	B	7	100	B	7	100
B	9	100	B	9	50	B	9	75	B	9	50	B	9	100
C	1	100				C	1	100	C	1	100	C	1	50
						C	2	50						
C	3	100							C	3	50			
						C	8	100				C	8	50
									C	10	50			
D	4	75				D	4	73	D	4	100	D	4	50
D	11	100	D	11	100	D	11	100	D	11	100	D	11	50
E	2	100	E	2	100	E	2	100	E	2	100	E	2	100
F	6	75				F	6	50	F	6	100	F	6	50
H	3	100	H	3	100				H	3	50	H	3	50
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100

mundtii no id 610			*mundtii* *mundtii* 611			*mundtii* *mundtii* 616			*mundtii* *mundtii* 617		
	Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	100	A	11	100	A	11	100
			A	*12*	*50*						
B	1	50							B	1	50
B	7	100	B	7	100	B	7	100	B	7	100
B	9	75	B	9	100	B	9	100	B	9	100
C	1	100	C	1	100	C	1	100	C	1	50
C	3	100	C	3	100	C	3	100	C	3	50
			C	*8*	*50*						
D	4	50	D	4	100	*D*	*4*	*50*	D	4	100
D	11	100	D	11	100	D	11	100	D	11	75
E	2	100	E	2	100	E	2	100	E	2	100
F	6	75	F	6	75	*F*	*6*	*50*	F	6	75
H	3	100	H	3	100	H	3	100	H	3	50
H	4	100	H	4	100	H	4	100	H	4	100

mundtii *hirae* 623			*mundtii* *casseliflavus* 625			*mundtii* *mundtii* 628			*mundtii* *mundtii* 629		
	Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	100	A	11	100	A	11	100
A	12	50							A	12	50
B	7	100	B	7	100	B	7	100	B	7	100
B	9	100	B	9	100	B	9	100	B	9	50
C	1	50	C	1	100	C	1	100	C	1	50
						C	2	100	C	3	50
									C	4	50
C	8	50	C	8	50				C	8	50
									C	10	50
D	4	50	D	4	75	D	4	75	D	4	100
D	11	50	D	11	100	D	11	100	D	11	100
E	2	100	E	2	100	E	2	100	E	2	100
			F	6	75	F	6	100	F	6	75
									H	1	50
H	3	100	H	3	100	H	3	75	H	3	50
H	4	100	H	4	100	H	4	100	H	4	100

mundtii *casseliflavus / mundtii* 619			*mundtii* no id 632			*mundtii* *casseliflavus* 633			*mundtii* *casseliflavus* 636		
	Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	100	A	11	100	A	11	100
A	12	50	A	12	50	A	12	50	A	12	100
									B	1	50
B	7	100	B	7	100	B	7	100	B	7	100
B	9	50	B	9	100	B	9	50	B	9	100
C	1	50	C	1	100	C	1	100	C	1	100
C	2	50	C	2	50						
C	3	50	C	3	50	C	3	50	C	3	100
C	4	50									
C	8	50							C	8	50
C	10	50	C	10	50	C	10	50	C	10	100
D	4	100	D	4	100	D	4	75	D	4	100
D	11	100	D	11	100	D	11	100	D	11	100
E	2	100	E	2	100	E	2	100	E	2	100
F	6	100	F	6	100	F	6	100	F	6	75
H	1	50	H	1	50				H	1	50
H	3	50	H	3	100	H	3	50	H	3	100
H	4	75	H	4	100	H	4	100	H	4	100

mundtii *casseliflavus* 631			*mundtii* *casseliflavus* 649			*mundtii* no id 652			*mundtii* *mundtii* 653		
	Well	%P		Well	%P		Well	%P		Well	%P
A	11	100	A	11	50	A	11	100	A	11	100
A	12	100	A	12	50	A	12	100	A	12	100
			B	1	50						
B	7	100	B	7	100	B	7	100	B	7	100
B	9	100	B	9	100	B	9	100	B	9	100
C	1	100	C	1	100	C	1	100	C	1	100
									C	2	100
C	3	100	C	3	50	C	3	100	C	3	100
						C	4	50	C	4	50
C	8	50				C	8	50	C	8	100
C	10	75				C	10	100	C	10	100
D	4	100	D	4	100	D	4	100	D	4	100
D	11	100	D	11	100	D	11	100	D	11	100
E	2	100	E	2	100	E	2	100	E	2	100
F	6	100	F	6	100	F	6	100	F	6	100
						H	1	50	H	1	50
H	3	100	H	3	100	H	3	75	H	3	100
H	4	100	H	4	75	H	4	100	H	4	100

mundtii no id 637			*mundtii casseliflavus* 648			*mundtii asini* 684			*mundtii hirae* 689			*mundtii asini* 693			*mundtii mundtii* 517		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
			A	11	50	A	11	100	A	11	100	A	11	100			
A	12	100				A	12	100				A	12	100	A	11	100
B	7	100	B	7	100	B	7	100	B	7	50	B	7	100	B	1	100
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100	B	7	100
C	1	100	C	1	100										B	9	100
C	2	50													C	1	100
C	3	100	C	3	50							C	3	75			
C	4	50													C	3	100
C	8	100															
C	10	100															
D	4	100	D	4	100										C	6	100
D	11	100	D	11	100												
E	2	100	E	2	100	E	2	100				E	2	100			
E	6	75	F	6	100												
H	1	75													D	4	50
															D	11	100
H	3	100	H	3	100	H	3	100	H	3	75	H	3	100	D	12	100
H	4	100	H	4	50	H	4	100	H	4	100	H	4	100	E	2	100
mundtii mundtii 659			*mundtii mundtii* 660														
	Well	%P		Well	%P										H	3	100
A	11	50	A	11	100										H	4	100
A	12	100	A	12	100	*mundtii hirae* 315			*mundtii hirae* 316			*mundtii hirae* 356			*mundtii hirae* 365		
B	1	50															
B	7	100	B	7	100		Well	%P		Well	%P		Well	%P		Well	%P
B	9	100	B	9	100	C	1	67	C	1	100				C	1	100
C	1	100	C	1	100				C	2	50				C	2	100
			C	2	100										C	3	100
C	3	100	C	3	100	C	3	67	C	3	100				C	4	100
C	4	75							C	4	100				C	8	100
C	8	100	C	8	100	C	8	50	C	8	100	C	8	100			
C	10	100	C	10	100	C	9	50									
D	4	100	D	4	100				C	10	100				C	10	100
D	11	100	D	11	100				D	4	83	D	4	83	D	4	67
E	2	100	E	2	100				D	11	100				D	11	100
F	6	100	F	6	100				F	6	33						
H	1	50	H	1	50	H	1	50									
H	3	100	H	3	100												
H	4	100	H	4	100												

Appendix 8.9. Biolog-generated metabolic profiles of enterococci isolated from water. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. Species-specific and host-specific (for each species) carbon sources have also been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1st species) and conventional biochemical test scheme identifications (2nd species) are displayed above the numerical identity of an isolate.

casseliflavus mundtii 100			*casseliflavus mundtii* 104			*casseliflavus mundtii* 108			*casseliflavus mundtii* 113		
	Well	%P		Well	%P		Well	%P		Well	%P
A	6	50	A	6	100	A	6	100	A	6	100
B	10	100	B	10	100	B	10	100	B	10	100
C	7	33	C	7	100				C	7	100
C	9	100	C	9	100				C	9	100
C	10	100	C	10	100	C	10	100			
C	12	100	C	12	88	C	12	100	C	12	50
D	3	100	D	3	100	D	3	100	D	3	100
D	10	100	D	10	63	D	10	100	D	10	100
E	1	100				E	1	29	E	1	100
E	5	100	E	5	100	E	5	29	E	5	100
F	3	100	F	3	100				F	3	100
F	5	100	F	5	100						
F	6	100	F	6	100	F	6	100	F	6	100
F	9	100	F	9	100				F	9	100
			H	7	100				H	7	50

casseliflavus mundtii 114			*casseliflavus mundtii* 159			*casseliflavus mundtii* 163		
	Well	%P		Well	%P		Well	%P
						A	2	67
						A	3	50
A	6	50						
B	10	100	B	10	100	B	10	100
			C	7	67	C	7	100
			C	9	33	C	9	100
C	10	100	C	10	100	C	10	100
C	12	50	C	12	100	C	12	100
			D	3	100	D	3	100
D	10	100	D	10	67	D	10	100
E	1	100	E	1	50	E	1	100
E	5	100	E	5	67	E	5	100
F	3	50	F	3	100	F	3	100
F	5	50						
F	6	50	F	6	100	F	6	100
F	9	50	F	9	67	F	9	83
			H	7	33	H	7	83
						H	11	33

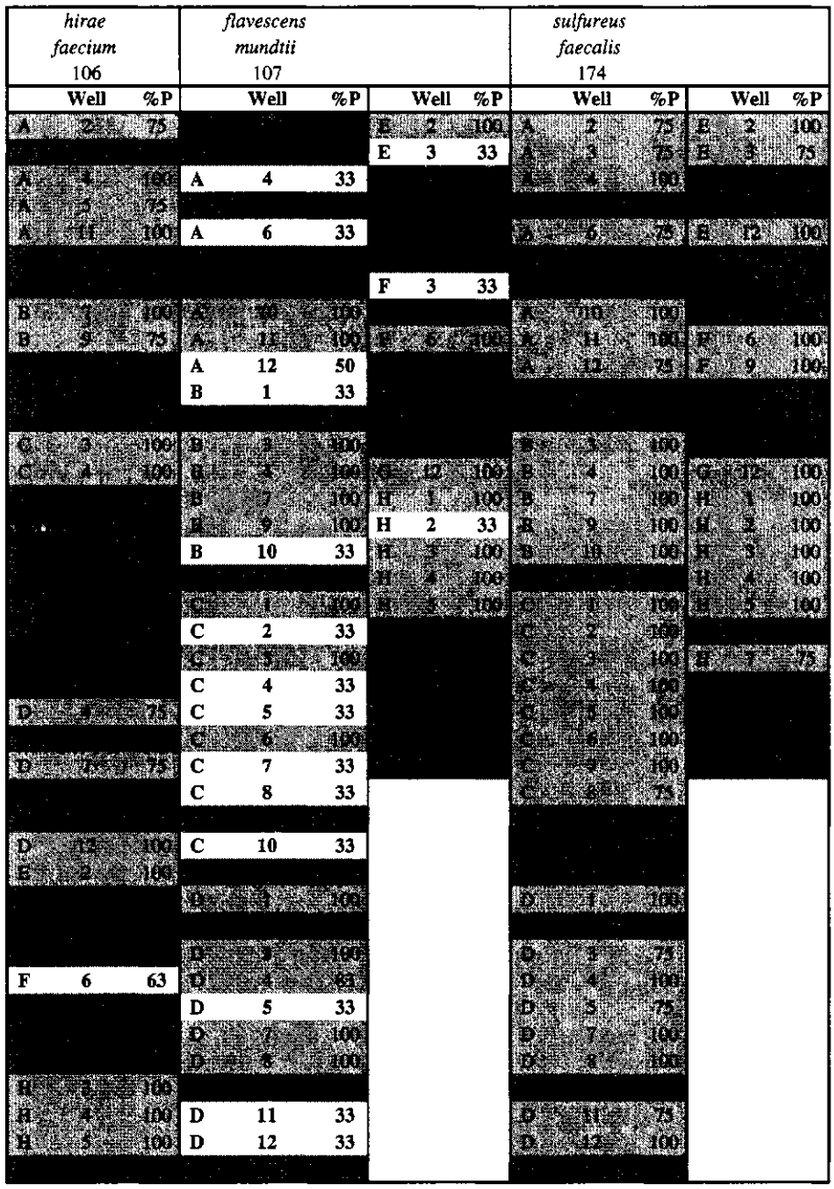
faecalis *avium* 101			*faecalis* *faecalis* 102			*faecalis* *faecalis* 121			*faecalis* *faecalis* 124			*faecalis* *faecalis* 125			*faecalis* *faecalis* 110		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100				A	2	100	A	2	100	A	2	100
A	3	50	A	3	100				A	3	100	A	3	100	A	3	67
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
B	7	100	B	7	100				B	7	100	B	7	100	B	7	100
			B	9	100				B	9	100	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100			
B	12	100	B	12	100				B	12	83	B	12	100	B	12	33
															C	1	67
C	4	100	C	4	100	C	4	100	C	4	100	C	4	100	C	4	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100				C	7	100	C	7	100	C	7	100
D	4	100	D	4	100	D	4	100	D	4	100	D	4	100	D	4	100
D	4	100	D	4	100	D	4	100	D	4	100	D	4	100	D	4	100
															D	10	33
															D	11	33
D	12	100	D	12	100				D	12	100	D	12	100	D	12	100
E	1	33													E	1	33
E	12	100	E	12	100	E	12	100	E	12	100	E	12	100	E	12	100
F	5	100	F	5	100										F	5	100
F	9	100	F	9	100	F	9	71	F	9	100	F	9	100	F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
			H	7	50	H	7	29				H	7	50			
			H	9	100	H	9	57	H	9	33	H	9	100	H	9	83
			H	11	100	H	11	57	H	11	33	H	11	100	H	11	67

faecalis *faecalis* 111			*faecalis* *faecalis* 117			*faecalis* *faecalis* 158			*faecalis* *faecalis* 160			*faecalis* *faecalis* 165			*faecalis* *avium* 126		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100	A	2	100	A	2	100	A	2	100
A	3	67	A	3	60	A	3	100	A	3	100	A	3	100	A	3	100
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100				A	11	100
A	12	50				A	12	100	A	12	100	A	12	50			
B	7	100	B	7	100	B	7	100	B	7	100	B	7	100	B	7	100
B	9	67	B	9	100	B	9	50	B	9	50	B	9	100	B	9	100
B	10	100	B	10	100	B	10	100	B	10	100	B	10	100	B	10	100
												B	12	100	B	12	100
															C	1	83
C	4	100	C	4	100	C	4	100	C	4	100	C	4	100	C	4	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	100	C	5	100
C	7	100	C	7	100							C	7	100	C	7	100
			C	8	40												
D	1	67	D	1	100	D	1	100	D	1	100	D	1	100	D	1	100
D	4	100	D	4	80	D	4	100	D	4	100	D	4	100	D	4	100
			D	5	40												
			D	10	80										D	10	100
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
			E	1	40												
E	12	100	E	12	80	E	12	100	E	12	100	E	12	100	E	12	100
F	5	67	E	5	100							E	5	100	E	5	100
F	9	100	F	9	100	F	9	50	F	9	67	F	9	100	F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	100	H	2	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
						H	7	50	H	7	33	H	7	67	H	7	83
H	9	67	H	9	100	H	9	83	H	9	100				H	9	100
H	11	67	H	11	100	H	11	83	H	11	67				H	11	100

faecalis *faecalis* 154			*faecalis* *faecalis* 156			*faecalis* *faecalis* 171			*faecalis* *faecalis* 172			*faecalis* *faecalis* 175			*faecalis* *faecalis* 166		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	2	100	A	2	100	A	2	100	A	2	100	A	2	29	A	2	100
A	3	100	A	3	100	A	3	100				A	3	29	A	3	100
A	4	100	A	4	100	A	4	100	A	4	100	A	4	100	A	4	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
			A	12	100	A	12	50				A	12	43	A	12	50
B	7	100	B	7	100	B	7	100				B	7	100	B	7	100
B	9	100	B	9	50	B	9	100				B	9	100	B	9	100
B	10	100	B	10	100	B	10	50	B	10	100	B	10	29	B	10	100
B	12	100													B	12	100
						C	1	50				C	1	71	C	1	100
															C	2	50
C	4	100	C	4	100	C	4	100	C	4	100	C	4	71	C	4	100
C	5	100	C	5	100	C	5	100	C	5	100	C	5	29	C	5	100
C	7	100				C	7	100							C	7	100
												C	10	29			
D	1	100	D	1	100	D	1	100	D	1	100	D	1	100	D	1	100
D	4	100	D	4	100	D	4	100	D	4	100	D	4	86	D	4	100
D	10	33															
D	12	100	D	12	100	D	12	100							D	12	100
															E	1	50
E	12	100	E	12	100	E	12	50	E	12	100				E	12	100
F	5	100				F	5	100				F	5	29	F	5	100
F	9	100	F	9	100	F	9	100	F	9	86				F	9	100
H	2	100	H	2	100	H	2	100	H	2	100	H	2	43	H	2	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
H	7	50	H	7	50	H	7	50	H	7	33				H	7	83
H	9	100	H	9	100	H	9	100				H	9	29	H	9	100
H	11	100	H	11	100	H	11	100				H	11	29	H	11	100

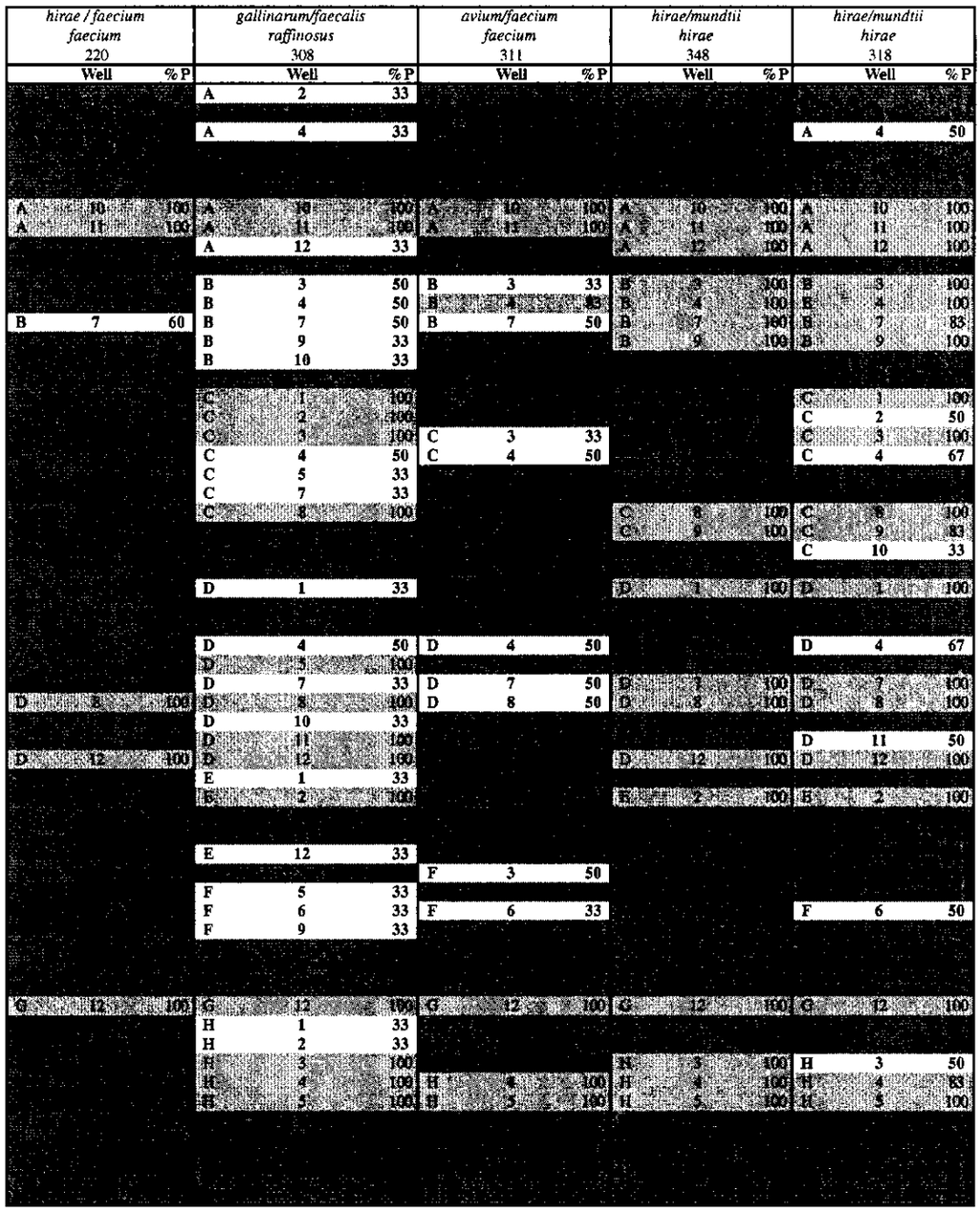
faecalis *faecalis* 168			*faecalis* *faecalis* 169			*faecium* *faecium* 105			*faecium* *faecium* 122			*faecium* *gallinarum* 167			*mundtii* *mundtii* 118		
Well		%P	Well		%P	Well		%P	Well		%P	Well		%P	Well		%P
A	2	100	A	2	100												
A	3	100	A	3	100										A	11	100
A	4	100	A	4	100	A	10	100	A	10	100	A	10	100			
						A	11	100	A	11	100	A	11	100			
															B	7	100
A	11	100	A	11	100										B	9	33
A	12	100	A	12	50	B	4	100	B	3	100	B	3	100	C	1	100
						B	4	100	B	4	100	B	4	100			
B	7	100	B	7	100	B	7	67	B	7	100	B	7	100	C	3	50
			B	9	100				B	9	29				C	4	50
B	10	100	B	10	100										C	5	33
			B	12	100										C	6	100
			C	1	100	C	3	100	C	3	86						
			C	2	100	C	4	67	C	4	71						
C	4	100	C	4	100												
C	5	100	C	5	100												
			C	7	100										D	11	100
						D	3	33							D	12	33
															E	2	83
						D	4	100	D	4	100	D	4	100			
D	1	100	D	1	100	D	12	100	D	12	100						
						E	2	50	E	2	100						
D	4	100	D	4	100										H	4	100
						F	6	67	F	6	57						
D	12	100	D	12	100	H	3	67	H	3	100	H	3	67			
			E	1	67	H	4	100	H	4	100	H	4	67			
E	12	100	E	12	100												
			F	3	100												
F	9	100	F	9	100												
H	2	100	H	2	100												
H	4	100	H	4	100												
			H	7	83												
H	9	67	H	9	100												
H	11	50	H	11	100												

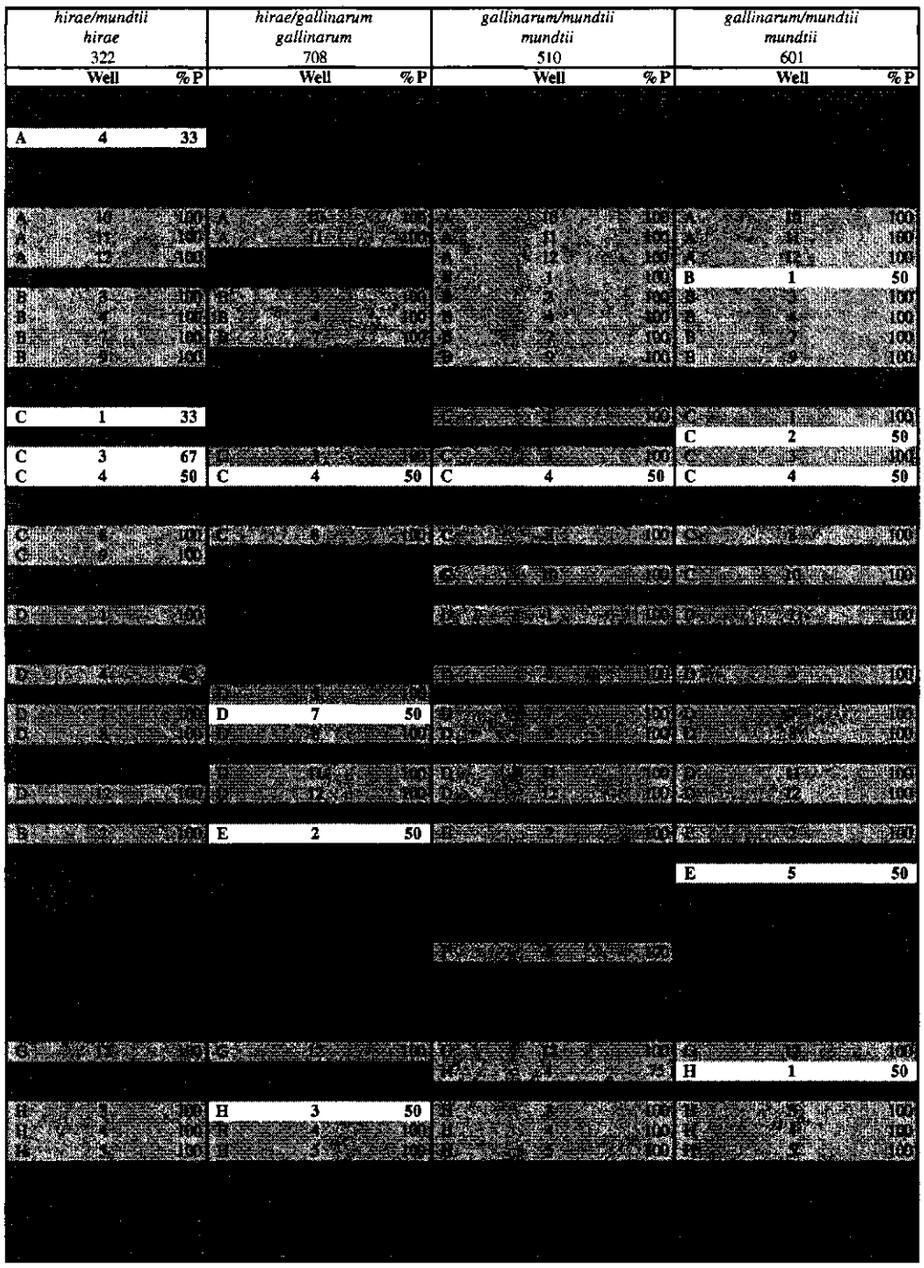
gallinarum gallinarum 116			*gallinarum mundtii* 120			*gallinarum faecium* 123			*gallinarum faecium* 128			*gallinarum faecium* 115		
Well		%P	Well		%P	Well		%P	Well		%P	Well		%P
						A	2	50				A	2	100
			A	4	33	A	3	100				A	4	100
A	11	100	A	11	100	A	11	50	A	11	100	A	11	100
			A	12	33	A	12	100				A	12	100
			B	1	33	B	1	100				B	1	100
			B	9	50	B	9	100				B	9	100
						B	10	50				B	10	100
			C	1	33	C	1	100				C	1	100
			C	2	33	C	2	50				C	2	100
C	3	100	C	3	100	C	3	100	C	3	100	C	3	100
C	4	100	C	4	50	C	4	100	C	4	100	C	4	100
			C	5	33	C	5	100				C	5	50
			C	7	33									
C	8	100	C	8	33	C	8	100	C	8	100	C	8	100
						C	9	100						
			C	10	33							C	10	100
			D	1	100	D	1	100				D	1	100
			D	3	33	D	3	100				D	3	50
						D	4	100				D	4	100
D	5	100				D	5	100	D	5	100	D	5	100
			D	7	100	D	7	100				D	7	100
D	11	100	D	11	33	D	11	100	D	11	100	D	11	100
						E	1	100				E	1	100
E	2	50	E	2	100	E	2	100	E	2	67	E	2	100
						E	4	100				E	5	50
			E	12	33									
						F	3	67				F	3	75
						F	5	100				F	5	100
F	6	100	F	6	33	F	6	100				F	6	100
			F	9	33							F	9	50
			H	1	33	H	1	100				H	1	100
			H	2	33	H	2	100				H	2	100
H	3	50	H	3	100	H	3	100	H	3	100	H	3	100
H	4	50	H	4	33	H	4	100	H	4	100	H	4	100
H	5	100	H	5	100	H	5	100	H	5	100	H	5	100

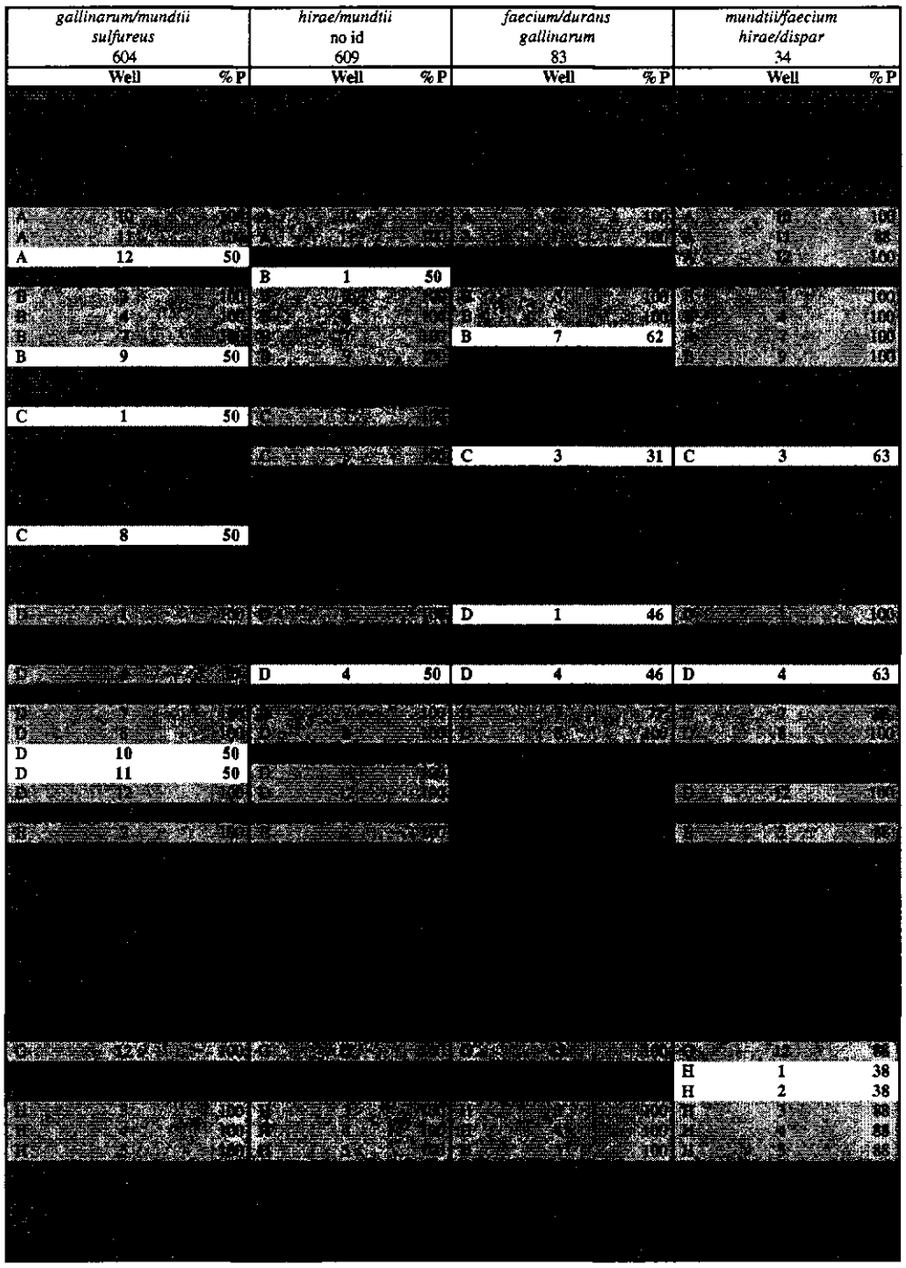

hirae
faecium
106
Well %P
A 2 75
A 4 100
A 7 75
A 11 100
B 7 100
B 9 75
C 3 100
C 4 100
D 9 75
D 75 75
D 12 100
E 2 100
F 6 63
H 3 100
H 4 100
H 5 100
flavescens
mundtii
107
Well %P
A 4 33
A 6 33
A 12 50
B 1 33
B 2 100
B 5 100
B 7 100
B 9 100
B 10 33
C 1 100
C 2 33
C 5 100
C 4 33
C 5 33
C 6 100
C 7 33
C 8 33
C 10 33
D 3 100
D 4 83
D 5 33
D 7 100
D 8 100
D 11 33
D 12 33
E 2 100
E 3 33
F 3 33
F 6 100
G 12 100
H 1 100
H 2 33
H 3 100
H 4 100
H 5 100
sulfureus
faecalis
174
Well %P
A 2 75
A 3 75
A 4 100
A 6 75
A 10 100
A 11 100
A 12 75
B 3 100
B 4 100
B 7 100
B 9 100
B 10 100
C 1 100
C 2 100
C 3 100
C 4 100
C 5 100
C 6 100
C 7 100
C 8 75
D 1 100
D 2 75
D 4 100
D 5 75
D 7 100
D 8 100
D 11 75
D 12 100
E 2 100
E 3 75
E 12 100
F 6 100
F 9 100
G 12 100
H 1 100
H 2 100
H 3 100
H 4 100
H 5 100
E 7 75

Appendix 8.10. Biolog-generated metabolic profiles of enterococci with two possible dentifcations. Strains were isolated from water (100's), sewage (200's) , bovine (10's), porcine (300's), human (400's), goose (500's), ovine (600's) and chicken (700's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1^{st} species) and conventional biochemical test scheme identifications (2^{nd} species) are displayed above the numerical identity of an isolate.

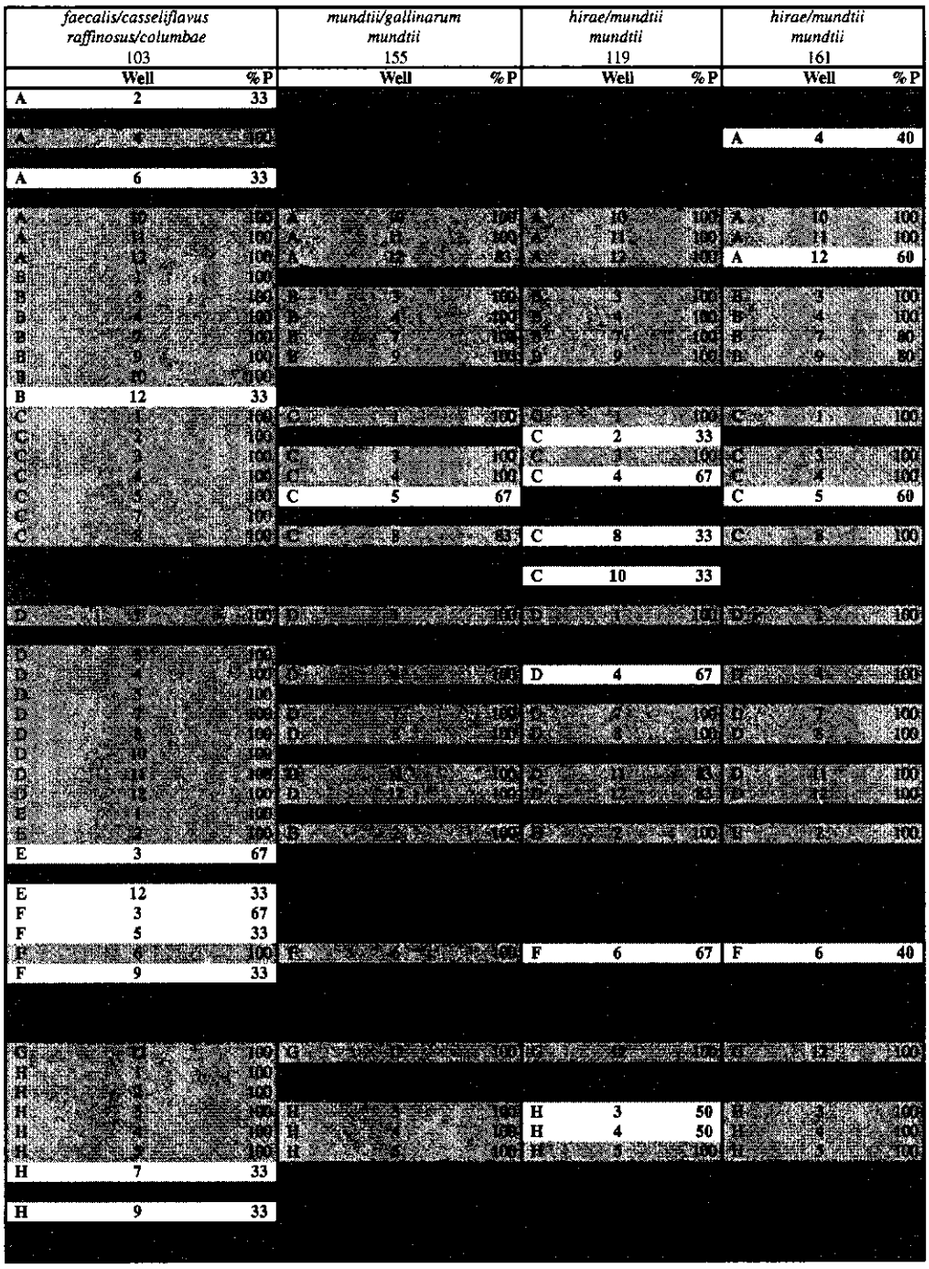
hirae/gallinarum gallinarum 456			*hirae/gallinarum gallinarum* 497			*gallinarum/casseliflavus mundtii* 411			*gallinarum / faecalis gallinarum* 210		
	Well	%P		Well	%P		Well	%P		Well	%P
						A	5	100	A	4	33
						A	6	50			
A	10	100	A	10	50	A	10	100	A	10	100
A	11	100				A	11	100	A	11	100
						A	12	100	A	12	33
B	1	50				B	1	100			
B	3	100	B	3	100	B	3	100	B	3	33
B	4	100	B	4	100	B	4	100	B	4	33
B	7	100	B	7	100	B	7	100	B	7	100
						B	9	100	B	9	33
						B	10	100	B	10	33
C	1	100				C	1	100	C	1	100
						C	2	100	C	2	100
C	3	100	C	3	100	C	3	100	C	3	100
C	4	100	C	4	50	C	4	100	C	4	100
						C	5	100	C	5	33
						C	7	50	C	7	33
C	8	100	C	8	100	C	8	100	C	8	100
						C	9	100			
						C	10	100			
						D	1	100	D	1	33
						D	2	100			
D	4	50				D	4	100	D	4	33
D	5	100	D	5	100	D	5	100	D	5	100
D	7	100	D	7	100	D	7	100	D	7	33
D	8	100	D	8	100	D	8	100	D	8	100
						D	10	100			
D	11	100	D	11	100	D	11	100	D	11	100
D	12	100	D	12	100	D	12	100	D	12	100
						E	1	100			
E	2	100	E	2	100	E	2	100	E	2	100
						E	3	100			
						E	5	100			
									E	12	33
						F	2	100			
						F	3	100			
			F	6	50	F	6	100	F	6	33
						F	9	100	F	9	33
G	12	100	G	12	100	G	12	100	G	12	100
						H	1	100	H	1	33
						H	2	100	H	2	33
H	3	100	H	3	50	H	3	100	H	3	100
H	4	100	H	4	100	H	4	100	H	4	67
H	5	100	H	5	100	H	5	100	H	5	100


hirae / faecium
faecium
220
Well % P
A 10 100
A 11 100
B 7 60
D 8 100
D 12 100
G 12 100
gallinarum/faecalis
raffinosus
308
Well % P
A 2 33
A 4 33
A 10 100
A 11 100
A 12 33
B 3 50
B 4 50
B 7 50
B 9 33
B 10 33
C 1 100
C 2 100
C 3 100
C 4 50
C 5 33
C 7 33
C 8 100
D 1 33
D 4 50
D 5 100
D 7 33
D 8 100
D 10 33
D 11 100
D 12 100
E 1 33
E 2 100
E 12 33
F 5 33
F 6 33
F 9 33
G 12 100
H 1 33
H 2 33
H 3 100
H 4 100
H 5 100
avium/faecium
faecium
311
Well % P
A 10 100
A 11 100
B 3 33
B 4 83
B 7 50
C 3 33
C 4 50
D 4 50
D 7 50
D 8 50
F 3 50
F 6 33
G 12 100
H 4 100
H 5 100
hirae/mundtii
hirae
348
Well % P
A 10 100
A 11 100
A 12 100
B 3 100
B 4 100
B 7 100
B 9 100
C 8 100
C 9 100
D 1 100
D 12 100
E 2 100
G 12 100
H 3 100
H 4 100
H 5 100
hirae/mundtii
hirae
318
Well % P
A 4 50
A 10 100
A 11 100
A 12 100
B 3 100
B 4 100
B 7 83
B 9 100
C 1 100
C 2 50
C 3 100
C 4 67
C 8 100
C 9 83
C 10 33
D 1 100
D 4 67
D 7 100
D 8 100
D 11 50
D 12 100
E 2 100
F 6 50
G 12 100
H 3 50
H 4 83
H 5 100


hirae/mundtii
hirae
322
Well %P
A 4 33
C 1 33
C 3 67
C 4 50
hirae/gallinarum
gallinarum
708
Well %P
D 7 50
E 2 50
H 3 50
gallinarum/mundtii
mundtii
510
Well %P
gallinarum/mundtii
mundtii
601
Well %P
B 1 50
C 1 100
C 2 50
C 4 50
E 5 50
H 1 50

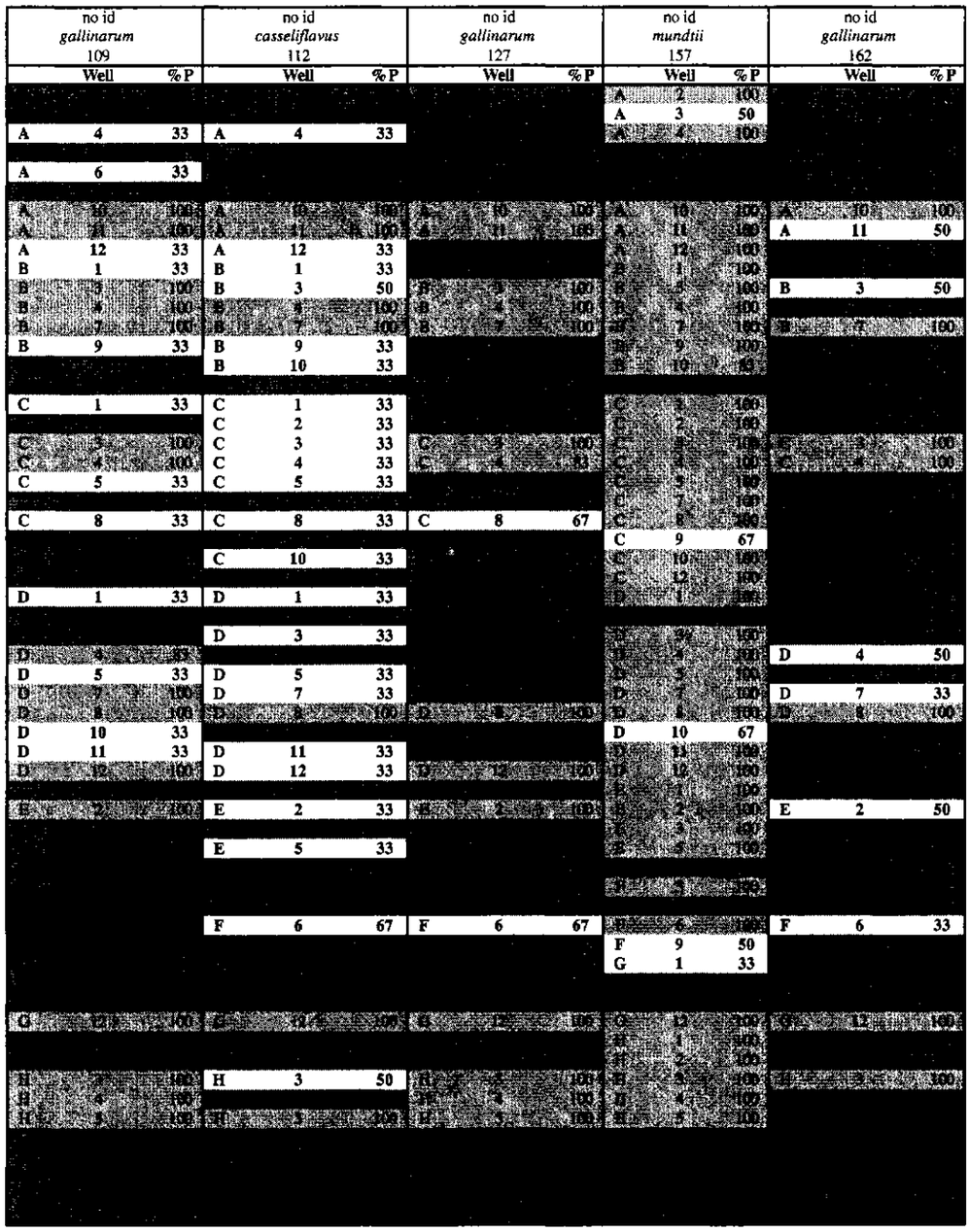

gallinarum/mundtii
sulfureus
604
Well %P
A 12 50
B 9 50
C 1 50
C 8 50
D 10 50
D 11 50
hirae/mundtii
no id
609
Well %P
B 1 50
D 4 50
faecium/durans
gallinarum
83
Well %P
B 7 62
C 3 31
D 1 46
D 4 46
mundtii/faecium
hirae/dispar
34
Well %P
C 3 63
D 4 63
H 1 38
H 2 38

gallinarum/mundtii *mundtii* 38			*mundtii/hirae* *Vagococcus* 11			*mundtii/hirae* *asini* 13			*mundtii/hirae* *Vagococcus* 45			*hirae/gallinarum* *gallinarum* 30		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
			A	2	50	A	2	50				A	2	50
			A	3	50	A	3	50				A	3	50
A	4	67	A	4	50	A	4	50				A	4	67
A	10	100	A	10	100	A	10	100	A	10	100	A	10	100
A	11	100	A	11	100	A	11	100	A	11	100	A	11	100
A	12	67	A	12	50	A	12	50	A	12	100	A	12	100
B	1	67				B	1	50						
B	3	100	B	3	100	B	3	100	B	3	100	B	3	100
B	4	100	B	4	100	B	4	100	B	4	100	B	4	100
B	7	100	B	7	100	B	7	100	B	7	100	B	7	100
B	9	100	B	9	100	B	9	100	B	9	100	B	9	100
C	1	100				C	1	33				C	1	83
C	2	33												
C	3	100	C	3	50	C	3	67	C	3	50	C	3	100
C	4	67	C	4	50	C	4	67	C	4	50	C	4	100
C	8	100										C	8	100
C	10	50												
D	1	100	D	1	100	D	1	100	D	1	100	D	1	100
D	2	100	D	4	67	D	2	83				D	2	100
												D	5	50
D	7	100	D	7	100	D	7	100	D	7	100	D	7	100
D	8	100	D	8	100	D	8	100	D	8	100	D	8	100
D	11	100										D	11	50
D	12	100	D	12	100	D	12	100	D	12	100	D	12	100
E	2	100	E	2	100	E	2	100	E	2	100	E	2	100
F	6	83				F	6	50				F	6	67
G	12	100	G	12	100	G	12	100	G	12	100	G	12	100
H	1	83												
H	2	67												
H	3	100	H	3	100	H	3	100	H	3	100	H	3	100
H	4	100	H	4	100	H	4	100	H	4	100	H	4	100
H	5	100	H	5	100	H	5	100	H	5	100	H	5	100

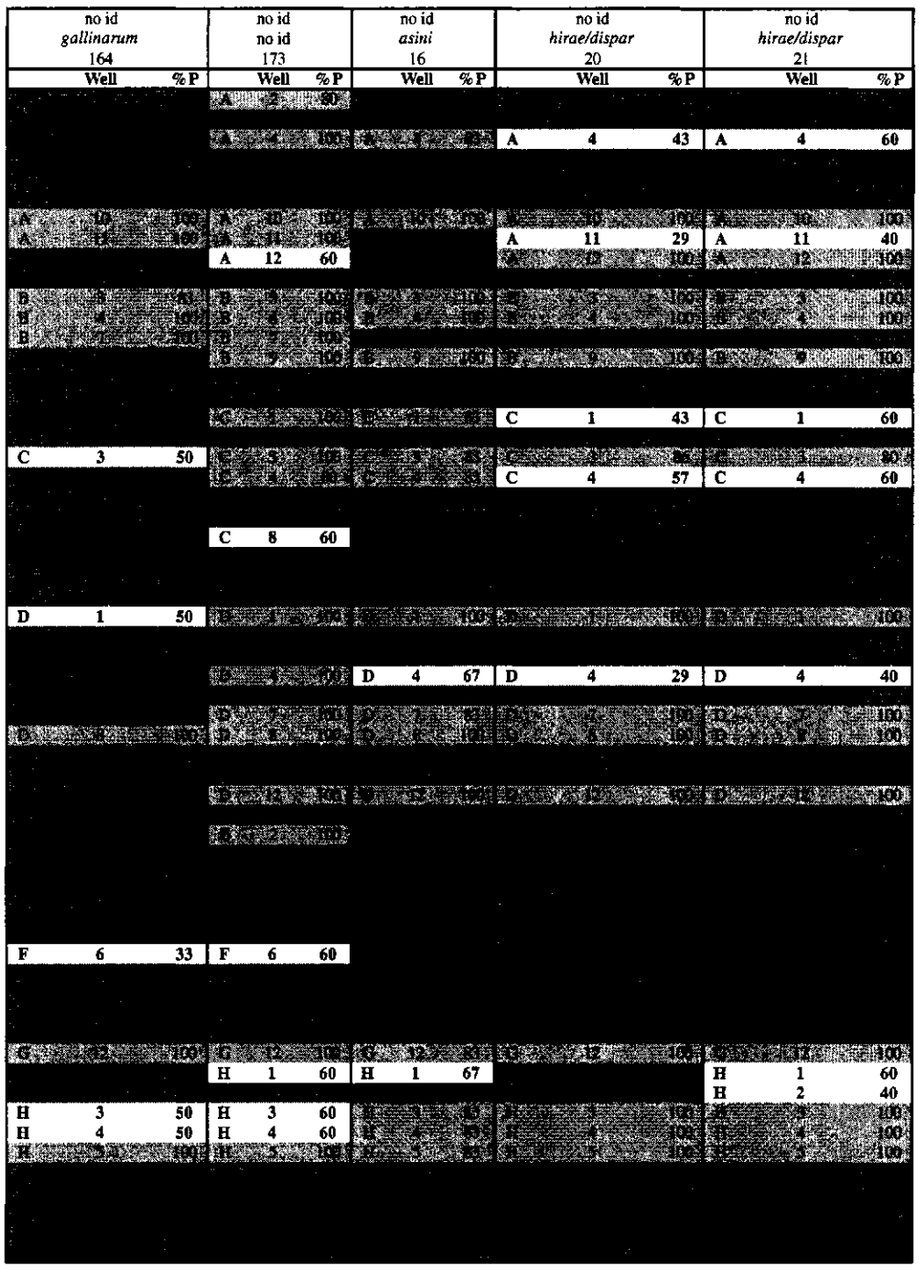


faecalis/casseliflavus raffinosus/columbae 103			*mundtii/gallinarum mundtii* 155			*hirae/mundtii mundtii* 119			*hirae/mundtii mundtii* 161		
	Well	%P		Well	%P		Well	%P		Well	%P
A	2	33									
A	4	100							A	4	40
A	6	33									
A	10	100	A	10	100	A	10	100	A	10	100
A	11	100	A	11	100	A	11	100	A	11	100
A	12	100	A	12	83	A	12	100	A	12	60
B	1	100									
B	3	100	B	3	100	B	3	100	B	3	100
B	4	100	B	4	100	B	4	100	B	4	100
B	7	100	B	7	100	B	7	100	B	7	80
B	9	100	B	9	100	B	9	100	B	9	80
B	10	100									
B	12	33									
C	1	100	C	1	100	C	1	100	C	1	100
C	2	100				C	2	33			
C	3	100	C	3	100	C	3	100	C	3	100
C	4	100	C	4	100	C	4	67	C	4	100
C	5	100	C	5	67				C	5	60
C	7	100									
C	8	100	C	8	83	C	8	33	C	8	100
						C	10	33			
D	1	100	D	1	100	D	1	100	D	1	100
D	3	100									
D	4	100	D	4	100	D	4	67	D	4	100
D	7	100									
D	8	100	D	8	100	D	8	100	D	8	100
D	10	100									
D	11	100	D	11	100	D	11	83	D	11	100
D	12	100	D	12	100	D	12	67	D	12	100
E	1	100									
E	2	100	E	2	100	E	2	100	E	2	100
E	3	67									
E	12	33									
F	3	67									
F	5	33									
F	6	100	F	6	100	F	6	67	F	6	40
F	9	33									
G	12	100	G	12	100	G	12	100	G	12	100
H	1	100									
H	3	100	H	3	100	H	3	50	H	3	100
H	4	100	H	4	100	H	4	50	H	4	100
H	5	100	H	5	100	H	5	100	H	5	100
H	7	33									
H	9	33									

Appendix 8.11. Biolog-generated metabolic profiles of unidentified enterococci. Strains were isolated from water (100's), sewage (200's) and bovine (10's), porcine (300's), human (400's), goose (500's), ovine (600's) and chicken (700's) faeces. Carbon sources that generated common positive and negative metabolic reactions between all Biolog-speciated isolates have been removed from the profiles. Wells are indicated by position on the GP2 MicroPlate™. Test results (%P) are displayed. "%P" indicates the percentage of replicates that were able to metabolize a carbon source. A "positive carbon source" indicates that ≥ 75% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Wells that are positive are shaded light grey. A "negative carbon source" indicates that ≤ 25% of the replicates for a given isolate demonstrated ability to metabolize the carbon source after 24h of incubation at 35°C. Negative wells are shaded dark grey. An "inconsistent carbon source" indicates that the number of replicates for a given isolate able to metabolize the carbon source fell between 25% and 75% after 24h of incubation at 35°C. Inconsistent wells are bolded and not shaded. Biolog-generated identifications (1^{st} species) and conventional biochemical test scheme identifications (2^{nd} species) are displayed above the numerical identity of an isolate.

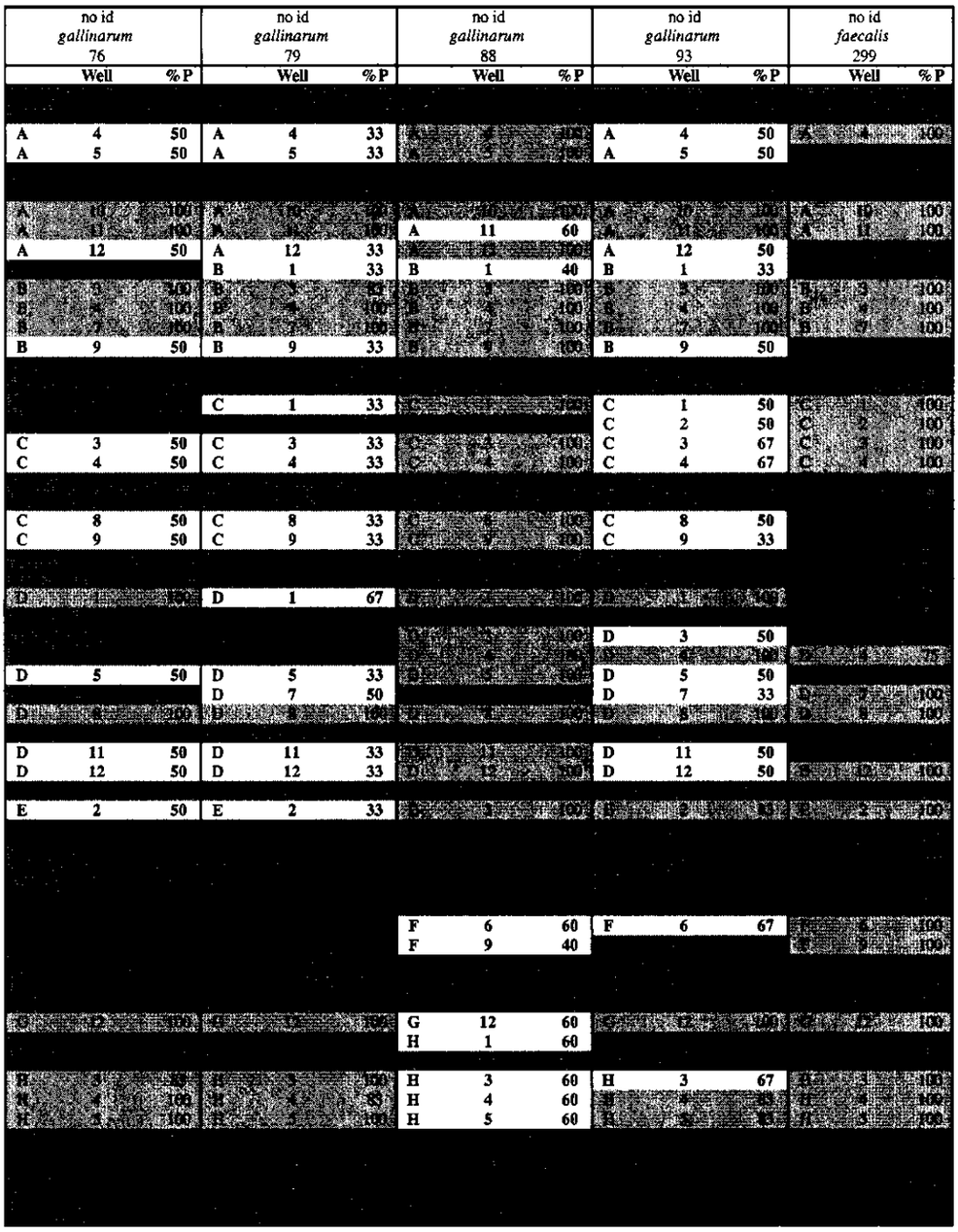


no id *gallinarum* 109			no id *casseliflavus* 112			no id *gallinarum* 127			no id *mundtii* 157			no id *gallinarum* 162		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	4	33	A	4	33				A	2	100			
									A	3	50			
A	6	33							A	4	100			
												A	11	50
A	12	33	A	12	33									
B	1	33	B	1	33									
			B	3	50							B	3	50
B	9	33	B	9	33									
			B	10	33									
C	1	33	C	1	33									
			C	2	33									
			C	3	33									
			C	4	33									
C	5	33	C	5	33									
C	8	33	C	8	33	C	8	67						
									C	9	67			
			C	10	33									
D	1	33	D	1	33									
			D	3	33									
												D	4	50
D	5	33	D	5	33									
			D	7	33							D	7	33
D	10	33							D	10	67			
D	11	33	D	11	33									
			D	12	33									
			E	2	33							E	2	50
			E	5	33									
			F	6	67	F	6	67				F	6	33
									F	9	50			
									G	1	33			
			H	3	50									

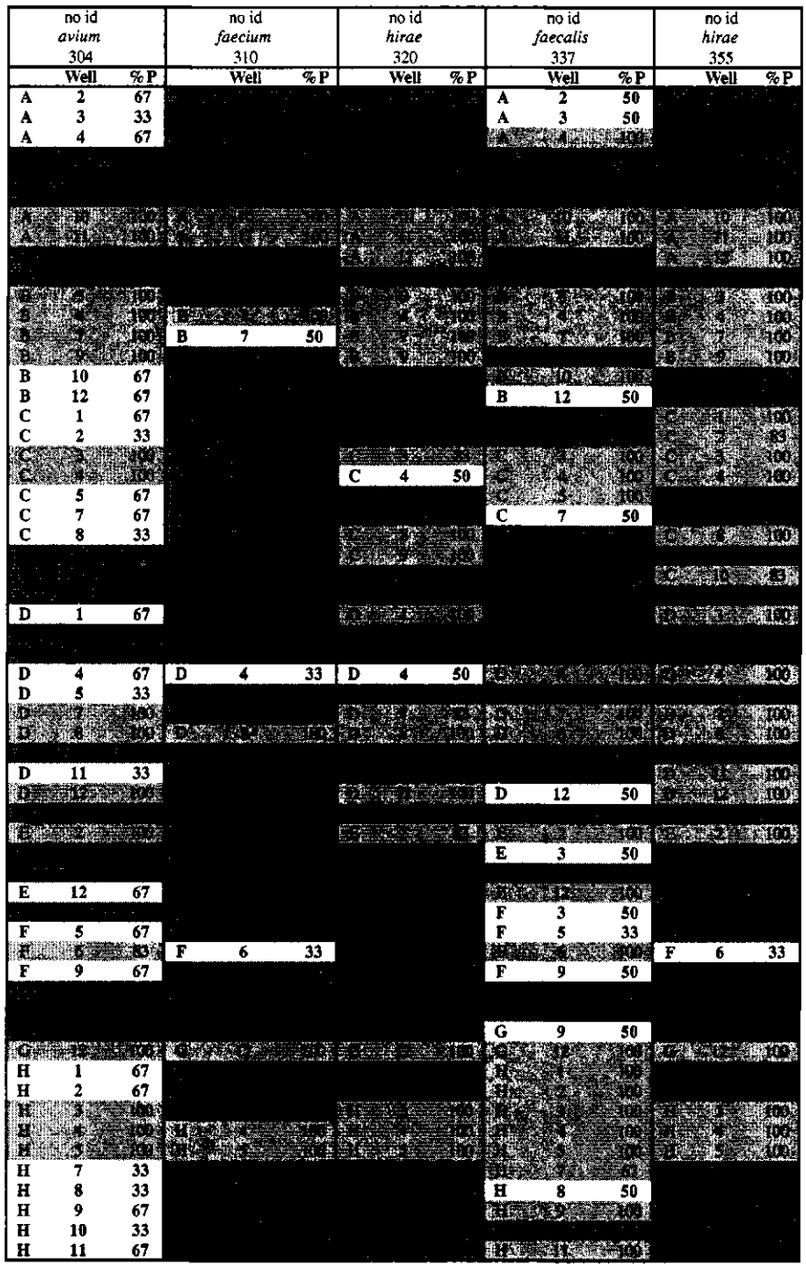


no id *gallinarum* 164			no id no id 173			no id *asini* 16			no id *hirae/dispar* 20			no id *hirae/dispar* 21		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
			A	2	50									
			C	4	100				A	4	43	A	4	60
			A	10	100									
			A	11	100				A	11	29	A	11	40
			A	12	60									
			C	1	100				C	1	43	C	1	60
C	3	50												
									C	4	57	C	4	60
			C	8	60									
D	1	50												
						D	4	67	D	4	29	D	4	40
			F	6	60									
			H	1	60	H	1	67				H	1	60
												H	2	40
H	3	50	H	3	60									
H	4	50	H	4	60									

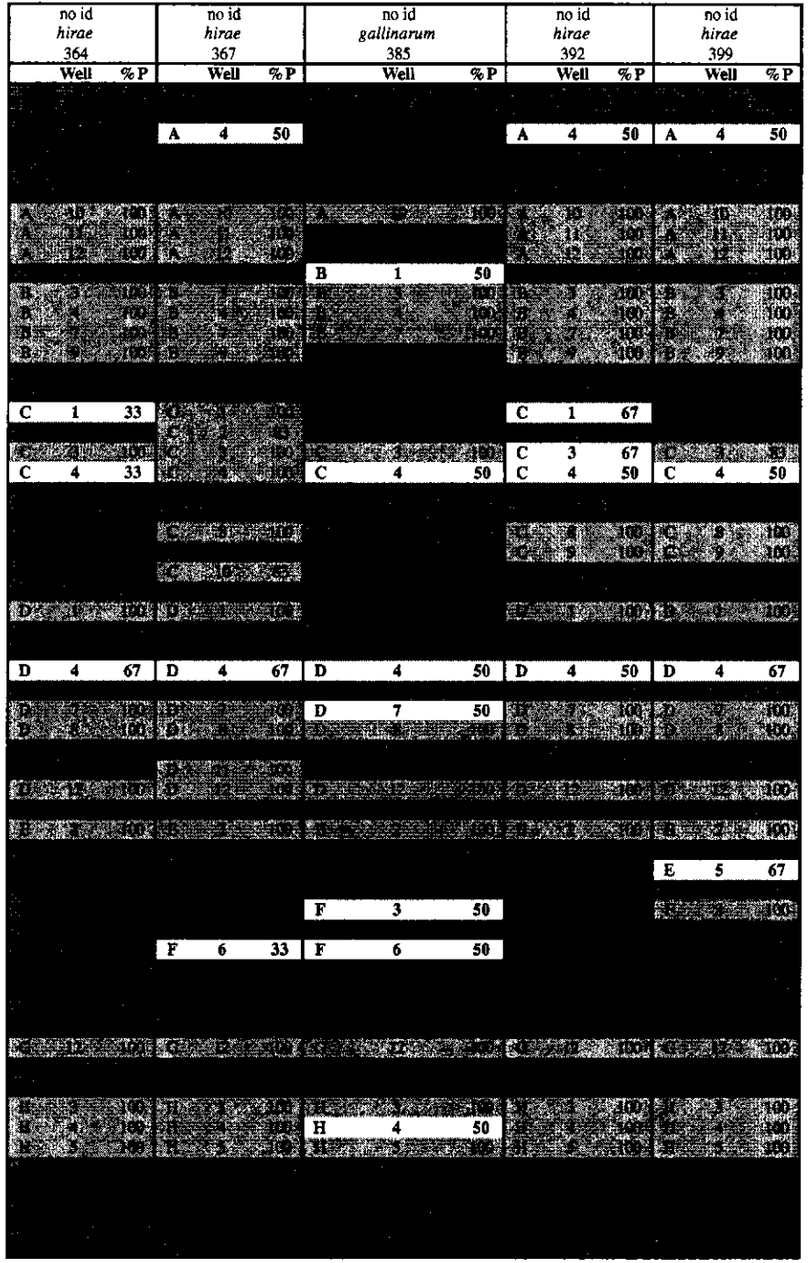

no id
hirae/dispar
22
Well %P
A 2 33
A 4 33
A 11 33
C 1 50
C 4 67
D 4 33
F 6 33
H 1 67
no id
asini
24
Well %P
A 11 50
no id
hirae/dispar
27
Well %P
A 4 57
C 1 29
D 4 57
H 1 57
no id
mundtii
39
Well %P
B 1 33
C 8 67
C 10 33
no id
faecium
66
Well %P
B 10 57
C 2 57
C 5 57
D 4 71
D 7 71
E 3 57
H 1 71
H 2 29



no id *gallinarum* 76			no id *gallinarum* 79			no id *gallinarum* 88			no id *gallinarum* 93			no id *faecalis* 299		
	Well	%P		Well	%P		Well	%P		Well	%P		Well	%P
A	4	50	A	4	33	A	11	60	A	4	50	A	4	100
A	5	50	A	5	33	A	12	60	A	5	50			
A	12	50	A	12	33	B	1	40	A	12	50			
			B	1	33				B	1	33			
B	9	50	B	9	33				B	9	50			
			C	1	33				C	1	50	C	1	100
									C	2	50	C	2	100
C	3	50	C	3	33				C	3	67	C	3	100
C	4	50	C	4	33				C	4	67	C	4	100
C	8	50	C	8	33				C	8	50			
C	9	50	C	9	33				C	9	33			
			D	1	67									
									D	3	50			
D	5	50	D	5	33				D	5	50			
			D	7	50				D	7	33			
D	11	50	D	11	33				D	11	50			
D	12	50	D	12	33				D	12	50			
E	2	50	E	2	33									
						F	6	60	F	6	67			
						F	9	40						
						G	12	60						
						H	1	60						
						H	3	60	H	3	67			
						H	4	60						
						H	5	60						



no id *avium* 304			no id *faecium* 310			no id *hirae* 320			no id *faecalis* 337			no id *hirae* 355		
	Well	% P		Well	% P		Well	% P		Well	% P		Well	% P
A	2	67							A	2	50			
A	3	33							A	3	50			
A	4	67												
B	7	50	B	7	50									
B	10	67												
B	12	67							B	12	50			
C	1	67												
C	2	33												
C	4	50				C	4	50						
C	5	67												
C	7	67							C	7	50			
C	8	33												
D	1	67												
D	4	67	D	4	33	D	4	50						
D	5	33												
D	11	33												
									D	12	50			
									E	3	50			
E	12	67												
F	3	50							F	3	50			
F	5	67							F	5	33			
F	6	33	F	6	33							F	6	33
F	9	67							F	9	50			
									G	9	50			
H	1	67												
H	2	67												
H	7	33												
H	8	33							H	8	50			
H	9	67												
H	10	33												
H	11	67												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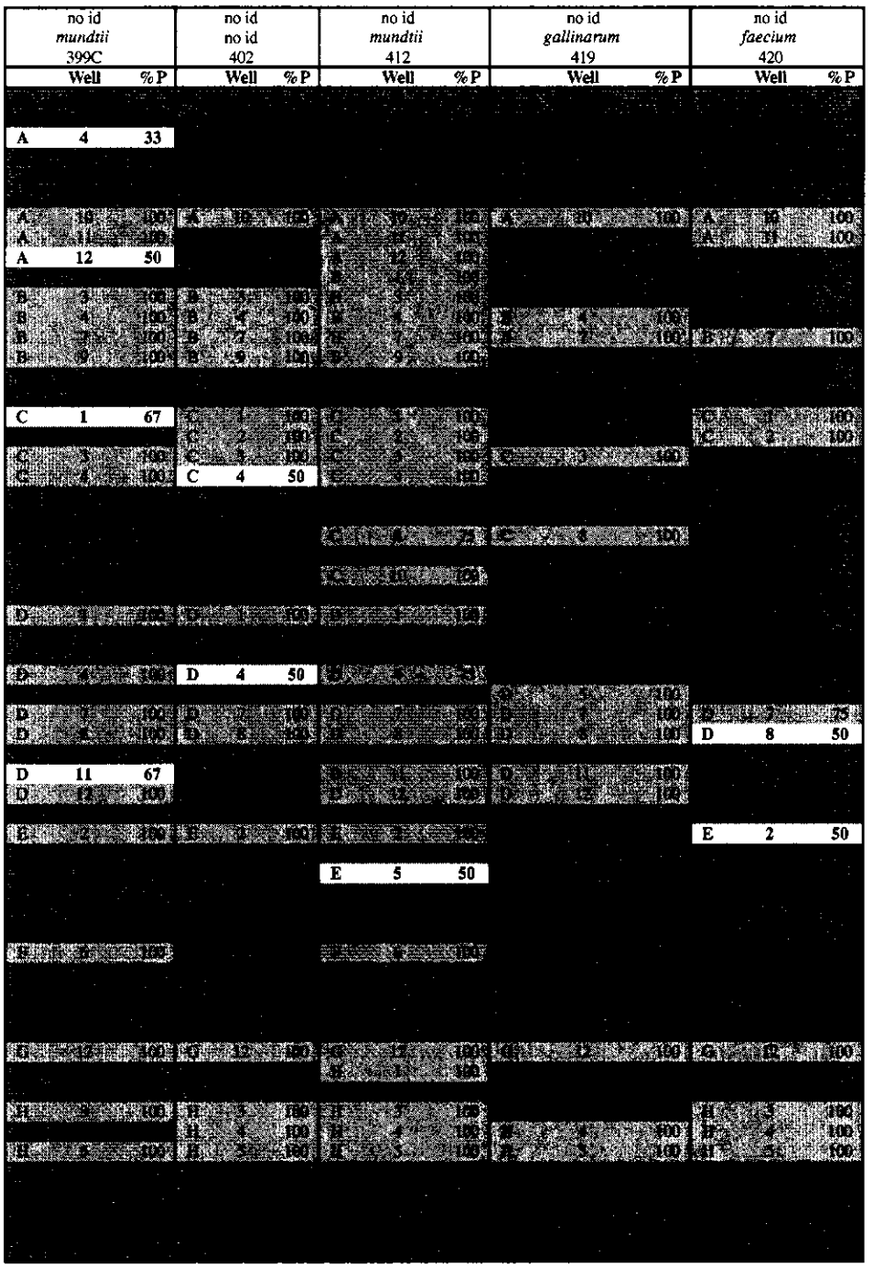
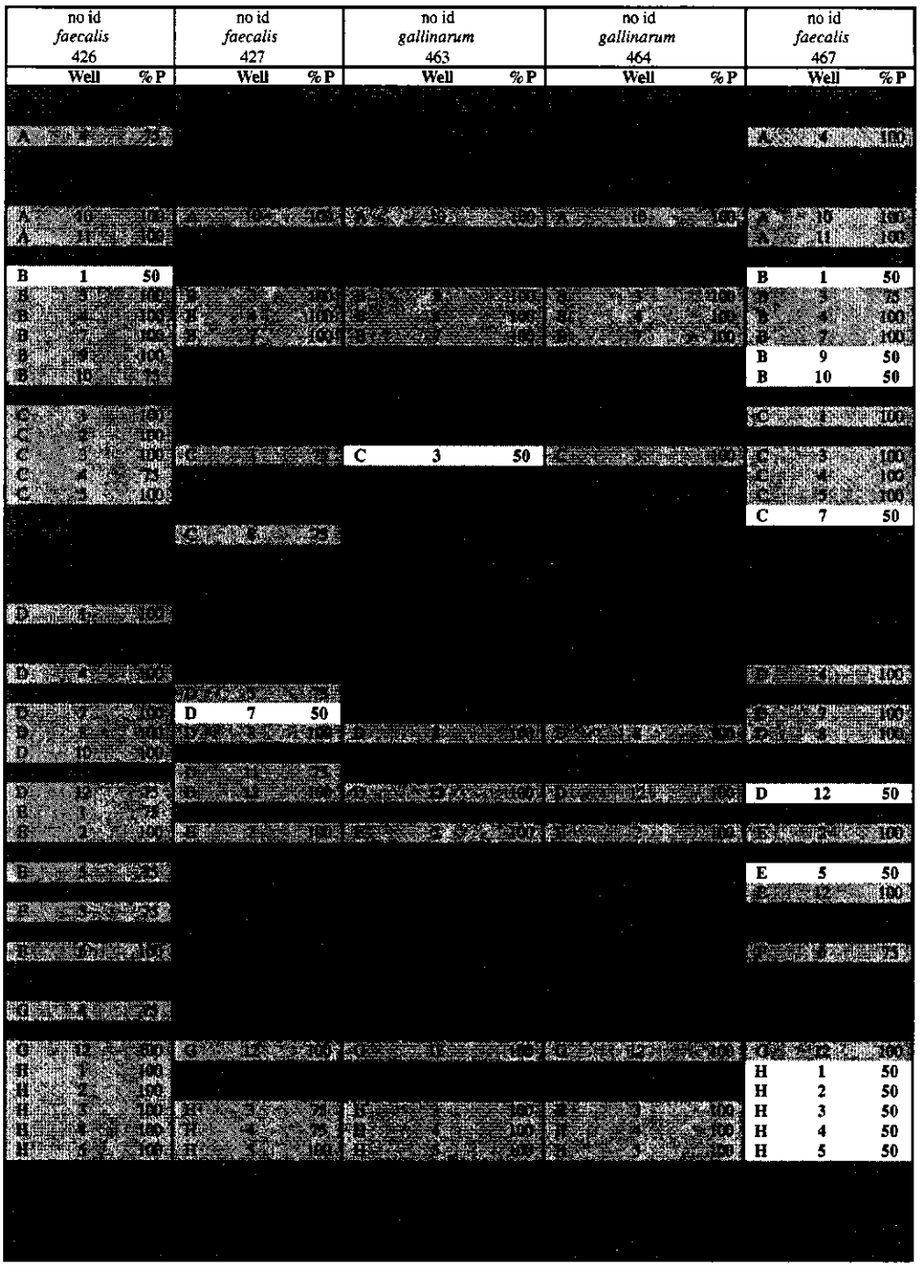
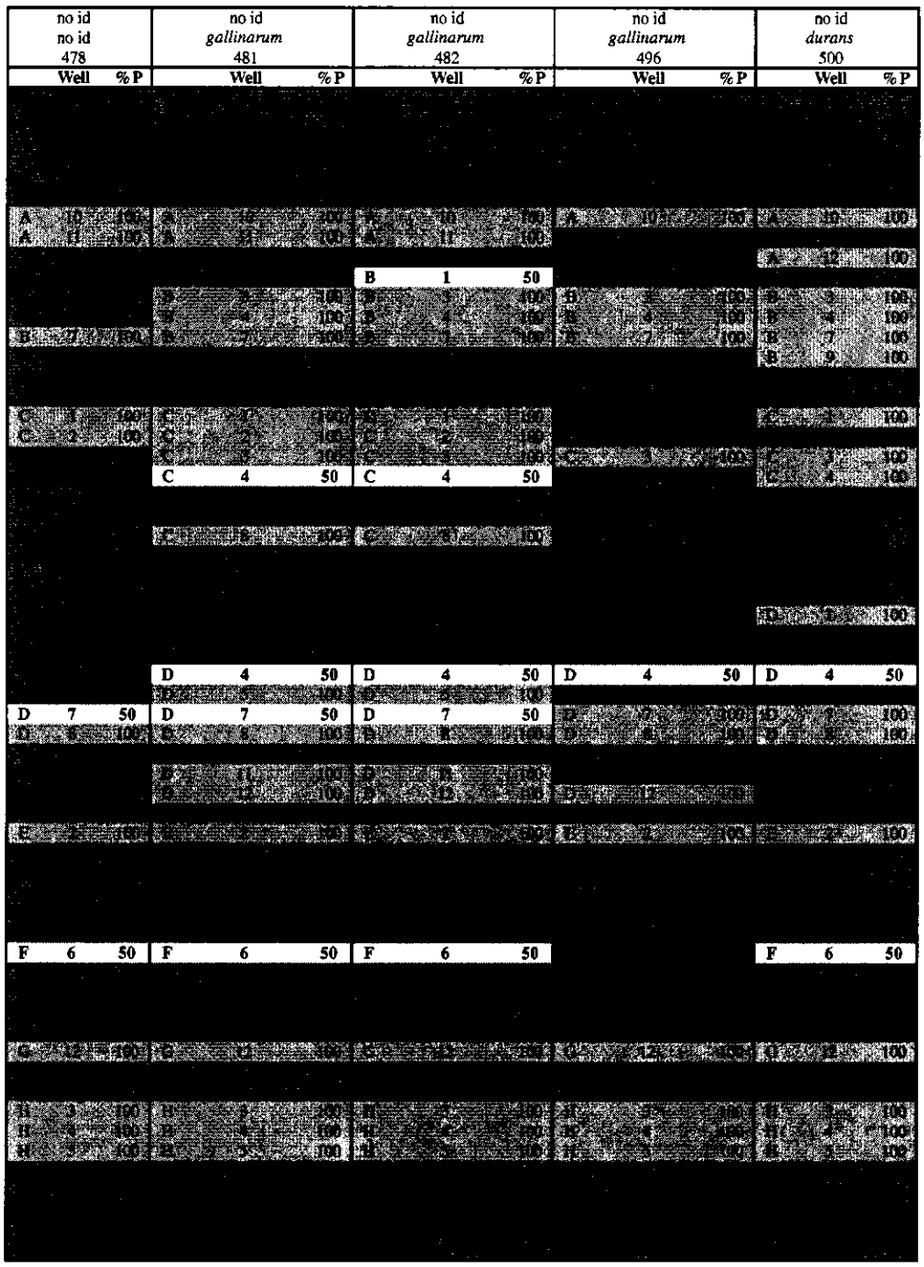
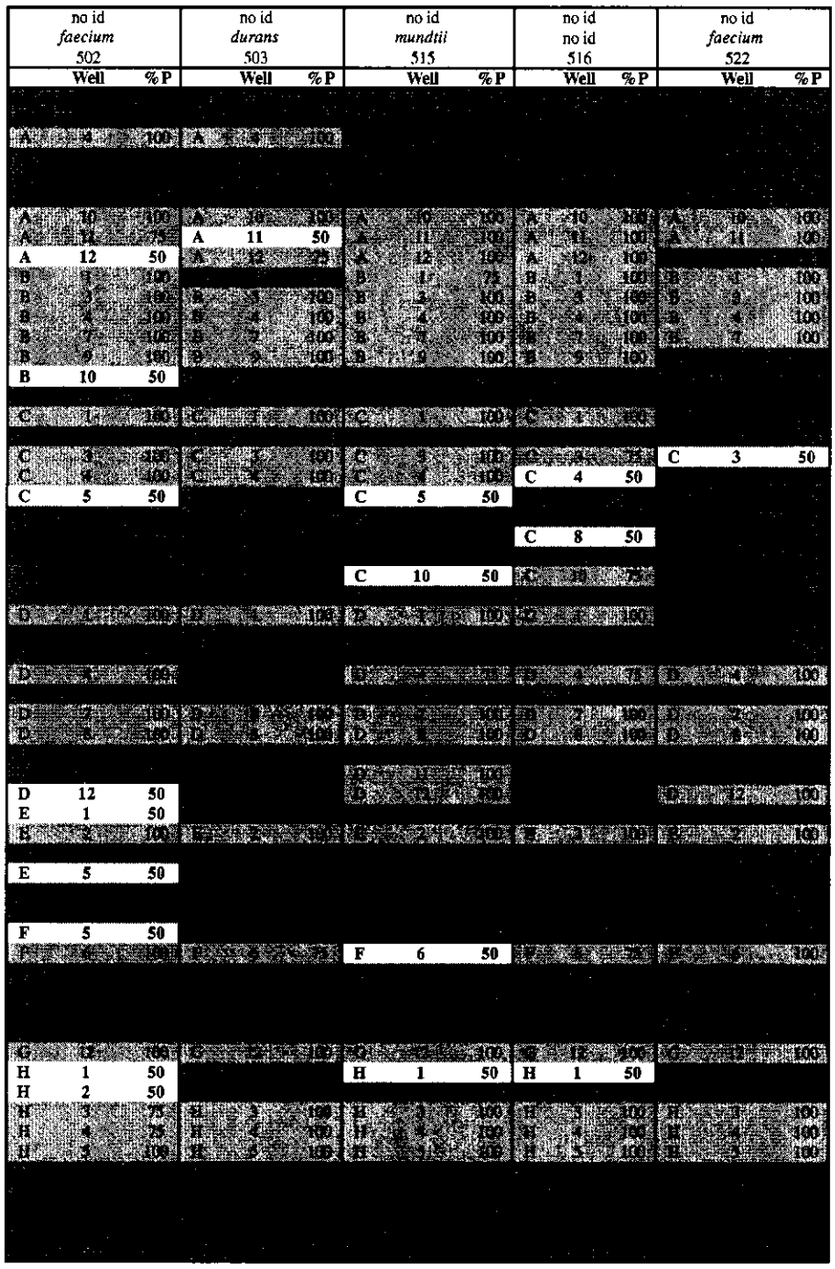
hirae
364
Well %P
A 4 50
C 1 33
C 4 33
D 4 67
F 6 33
no id
hirae
367
Well %P
A 4 50
C 1 33
C 4 33
D 4 67
F 6 33
no id
gallinarum
385
Well %P
B 1 50
C 4 50
D 4 50
D 7 50
F 3 50
F 6 50
H 4 50
no id
hirae
392
Well %P
A 4 50
C 1 67
C 3 67
C 4 50
D 4 50
no id
hirae
399
Well %P
A 4 50
C 4 50
D 4 67
E 5 67

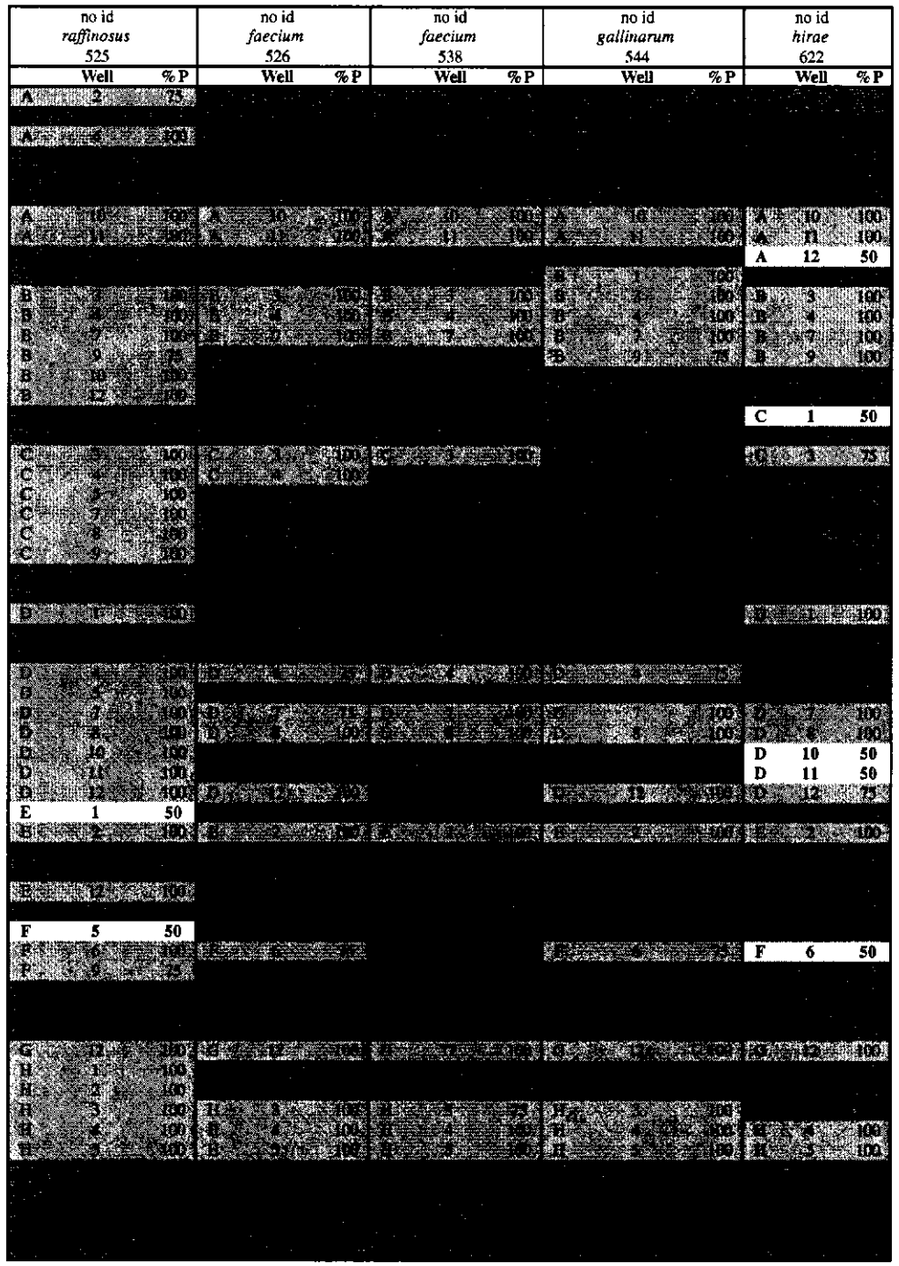

no id
mundtii
399C
Well %P
A 4 33
A 12 50
C 1 67
C 4 50
D 11 67
no id
no id
402
Well %P
D 4 50
no id
mundtii
412
Well %P
E 5 50
no id
gallinarum
419
Well %P
no id
faecium
420
Well %P
D 8 50
E 2 50

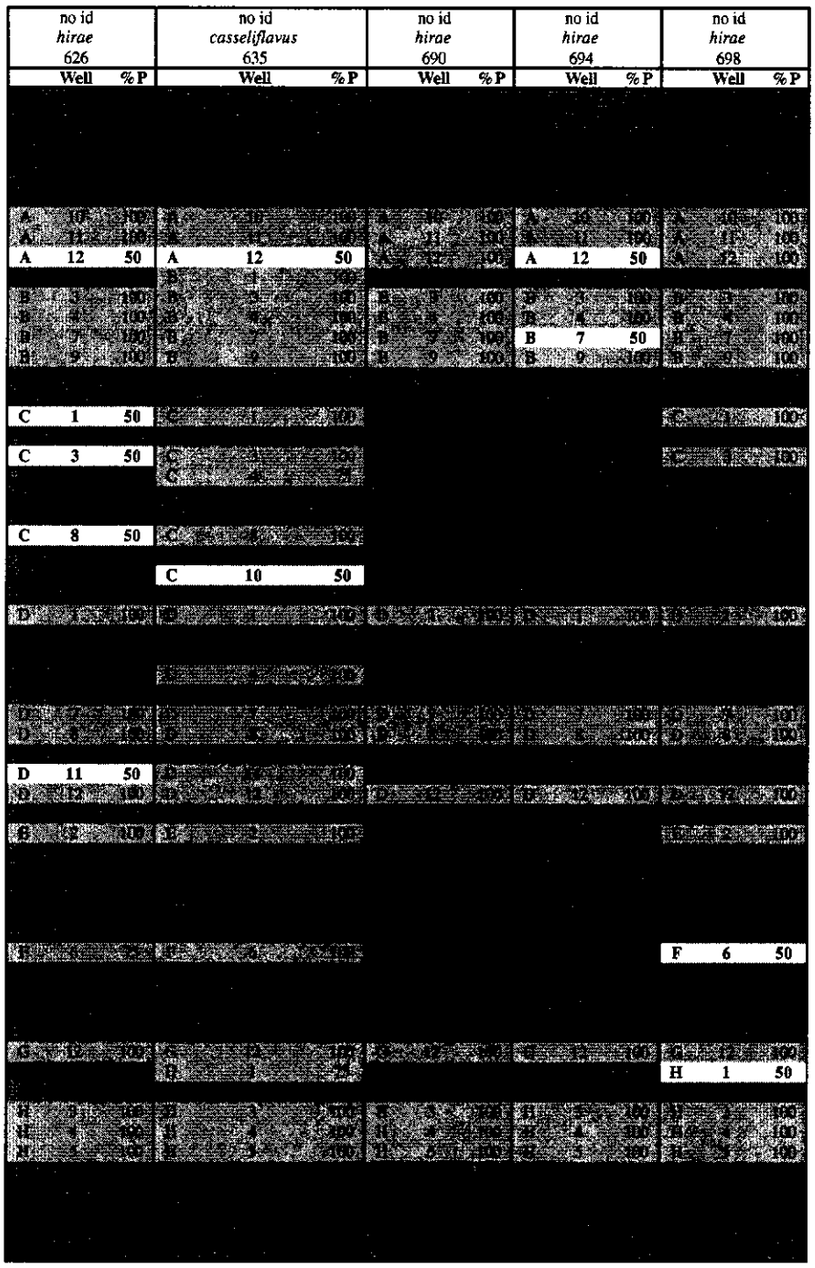


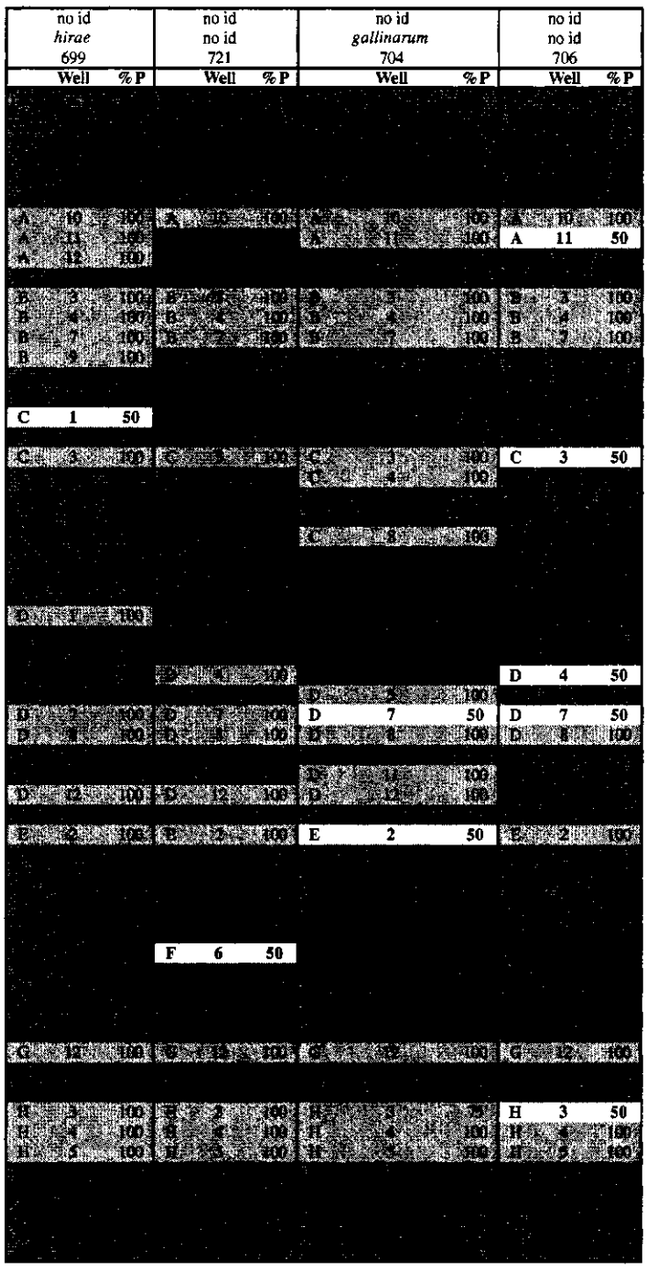
no id *faecalis* 426			no id *faecalis* 427			no id *gallinarum* 463			no id *gallinarum* 464			no id *faecalis* 467		
Well		%P	Well		%P	Well		%P	Well		%P	Well		%P
B	1	50										B	1	50
												B	9	50
												B	10	50
						C	3	50				C	7	50
			D	7	50									
												D	12	50
												E	5	50
												H	1	50
												H	2	50
												H	3	50
												H	4	50
												H	5	50


no id
no id
478
Well %P
no id
gallinarum
481
Well %P
no id
gallinarum
482
Well %P
no id
gallinarum
496
Well %P
no id
durans
500
Well %P
B 1 50
C 4 50
D 4 50
D 7 50
F 6 50


no id
faecium
502
Well %P
no id
durans
503
Well %P
no id
mundtii
515
Well %P
no id
no id
516
Well %P
no id
faecium
522
Well %P


no id
raffinosus
525
Well % P
no id
faecium
526
Well % P
no id
faecium
538
Well % P
no id
gallinarum
544
Well % P
no id
hirae
622
Well % P
A 12 50
C 1 50
D 10 50
D 11 50
E 1 50
F 5 50
F 6 50


no id
hirae
626
Well %P
A 12 50
B 7 50
C 1 50
C 3 50
C 8 50
D 11 50
F 6 50
H 1 50
no id
casseliflavus
635
Well %P
A 12 50
C 10 50
no id
hirae
690
Well %P
no id
hirae
694
Well %P
A 12 50
B 7 50
no id
hirae
698
Well %P


no id
hirae
699
Well %P
A 10 100
A 11 100
A 12 100
B 3 100
B 4 100
B 7 100
B 9 100
C 1 50
C 3 100
D 7 100
D 8 100
D 12 100
E 2 100
F 6 50
G 12 100
H 2 100
H 4 100
H 5 100
no id
no id
721
Well %P
A 10 100
B 4 100
B 7 100
C 3 100
D 7 100
D 8 100
D 12 100
E 2 100
G 12 100
H 2 100
H 4 100
H 5 100
no id
gallinarum
704
Well %P
A 10 100
A 11 100
B 3 100
B 4 100
B 7 100
C 3 100
C 4 100
C 5 100
D 4 100
D 7 50
D 8 100
D 11 100
D 12 100
E 2 50
H 3 75
H 4 100
H 5 100
no id
no id
706
Well %P
A 10 100
A 11 50
B 3 100
B 4 100
B 7 100
C 3 50
D 4 50
D 7 50
D 8 100
E 2 100
H 3 50
H 4 100
H 5 100