

TOPICAL REVIEW

Microbial volatile compounds in health and disease conditions

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Abstract

Microbial cultures and/or microbial associated diseases often have a characteristic smell. Volatile organic compounds (VOCs) are produced by all microorganisms as part of their normal metabolism. The types and classes of VOC produced is wide, including fatty acids and their derivatives (e.g. hydrocarbons, aliphatic alcohols and ketones), aromatic compounds, nitrogen containing compounds, and volatile sulfur compounds. A diversity of ecological niches exist in the human body which can support a polymicrobial community, with the exact VOC profile of a given anatomical site being dependent on that produced by both the host component and the microbial species present. The detection of VOCs is of interest to various disciplines, hence numerous analytical approaches have been developed to accurately characterize and measure VOCs in the laboratory, often from patient derived samples. Using these technological advancements it is evident that VOCs are indicative of both health and disease states. Many of these techniques are still largely confined to the research laboratory, but it is envisaged that in future bedside 'VOC profiling' will enable rapid characterization of microbial associated disease, providing vital information to healthcare practitioners.

Introduction

Volatile organic compounds (VOCs) are produced by all microorganisms as part of their normal metabolism. Some species of bacteria have been noted to have a characteristic smell *in vitro* when isolated on recovery media as pure cultures, and odiferous VOC production by anaerobes in tissue infections *in vivo* has been recognized for many years; for example the characteristic odour of gas gangrene by cytotoxic clostridia (Boland 1929). The types and classes of VOC produced is wide, including fatty acids and their derivatives (e.g. hydrocarbons, aliphatic alcohols and ketones), aromatic compounds, nitrogen containing compounds, and volatile sulfur compounds (Schulz and Dickschat 2007).

The specific composition of VOCs produced from a given bacterial community will depend on the diversity and

metabolic types/groups of species present, as well as the variety of principle carbon-energy sources available for the microbes to act upon. In the majority of cases, the largest amounts of carbon-energy are in the form of cell-impermeable polymers which require hydrolytic depolymerization before they can cross the cytoplasmic membrane, and enter into the cell (via specific transporter molecules). Figure 1 shows the main groups of macromolecules that are targeted within a host organism; polysaccharides (glycogen), proteins (e.g. collagen, albumin, globulin serum proteins, and haemoglobin), glycoproteins (mucins), lipids, phospholipids and nucleic acids. These compounds act as substrates for microbial growth via specific hydrolysis pathways (shown in figure 1) particularly during tissue infections, for example within wounds or periodontal disease (Harrington 1996). The breakdown of polymeric substrates is therefore dependent on the microflora having the right mix of hydrolytic (digesting) enzymes, and these are classified by the reactions they

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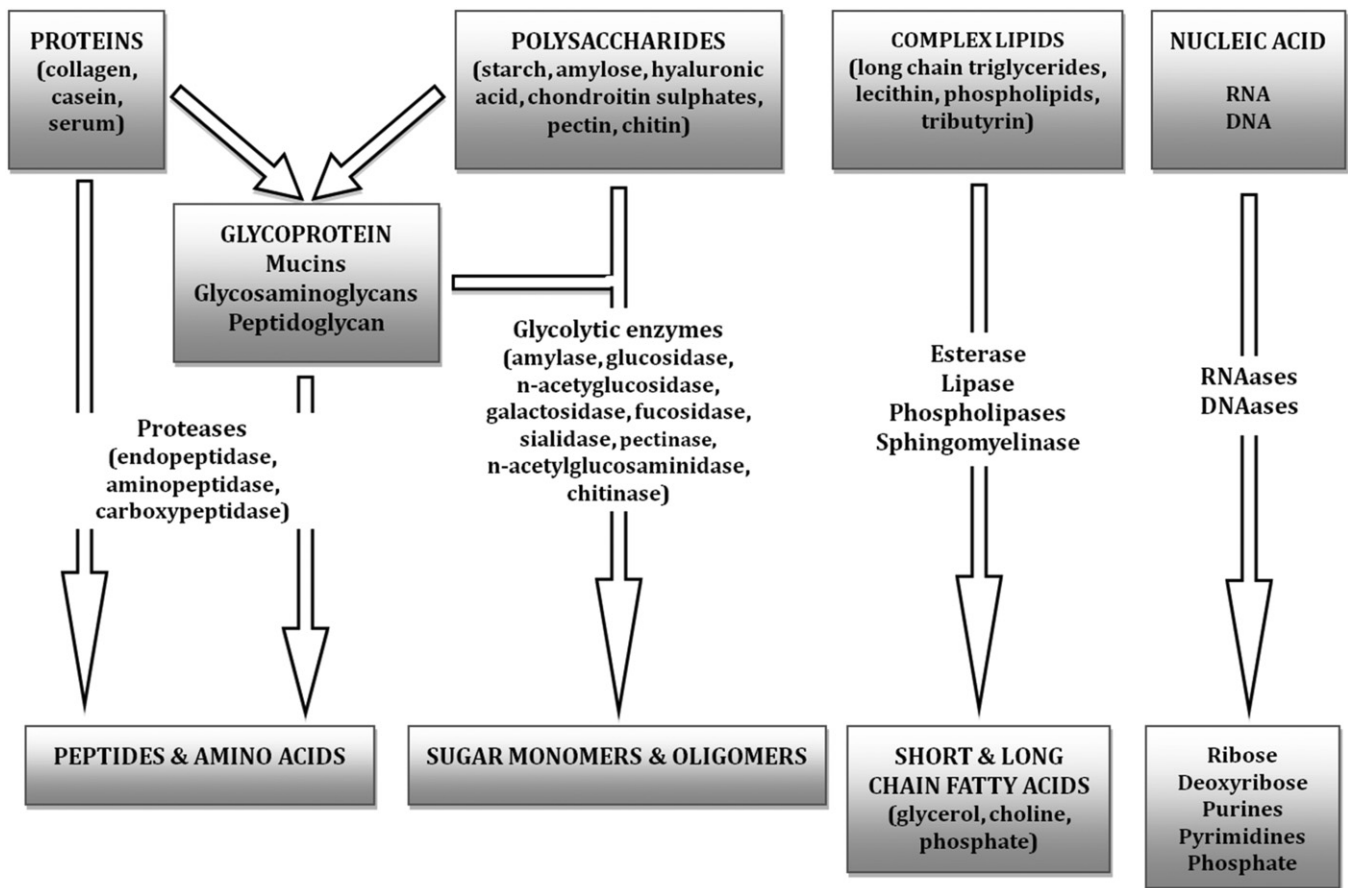


Figure 1. Composition and microbial hydrolysis pathways for complex feedstock. The majority of carbon-energy nutrient is in the form of soluble or insoluble polymers.

Table 1. Common microbial hydrolytic enzymes used to breakdown polymeric substances (depolymerization).

Microbial enzyme	EC number	Description
Esterase Lipase Phosphodiesterase Phosphatase Nucleases (DNAase/RNAase)	EC 3.1: Ester bonds	Common microbial enzymes associated with breakdown and metabolism of lipids.
DNA Glycosylases Glycoside hydrolase	EC 3.2: Sugars	Aid tissue breakdown. Glycoside hydrolases break off sugar chains from glycoproteins allowing them to be more easily ‘digested’ with proteases.
Proteases (Endoprotease/Exoprotease)	EC 3.4: Peptide bonds	Cleave proteins from the termini inwards (exoproteases) or within the polypeptide chain (endoproteases).
Hyaluronate lyase	EC 4.2.2.1: Carbon–oxygen bonds	Cleaves hyaluronate chains (not classed as a hydrolysis reaction).

catalyze (enzyme commission number (EC number); table 1, Webb 1992). The majority of the microbial hydrolytic enzymes are thought to be extracellular, hence their activity is likely to benefit all species present within a microcosm. An important group of molecules in tissues and wounds are the glycosaminoglycans (mucopolysaccharides) including hyaluronic acid, chondroitin-4 and chondroitin-6, heparin and dermatan-sulfate (Wadström and Ljungh 1999). The microbial enzymes that attack these substrates are classified

as hyaluronate lyases (EC 4.2.2.1; see table 1), since this is not a hydrolysis reaction, but instead involves breakage of carbon–oxygen bonds. A number of important microbial pathogens possess hyaluronate lyase (considered a ‘spreading factor’) including *Staphylococcus aureus* and *Streptococcus pyogenes* (Makris et al 2004). Collectively, the microbial enzymic degradation of available macromolecules is known to result in the production of a diversity of products, some of which are volatile.

Putrefaction

Undoubtedly the earliest detection of odorous volatiles being produced by microbes would have been from the process of putrefaction. Putrefaction is known to occur when heterotrophic microorganisms (bacteria, fungi and yeast) utilize organic material from once-living tissues (plants and animals, or their products, such as milk or sewage) as a source of carbon-energy. This 'feedstock' has high nutritional value and the presence of easily utilizable polymeric carbohydrates, lipids, nucleic acids, fats, and proteins. Microbes breakdown these compounds using various hydrolytic enzymes (see figure 1 and table 1) and resultant products are utilized by heterotrophic species as both a source of building blocks for the synthesis of their own structural polymers (anabolism), but most frequently by the main route of catabolism, as a source of biochemical energy (NADH and ATP) obtained either from aerobic respiration (O_2), anaerobic respiration (using end-terminal electron acceptors such as NO_3^- or SO_4^{2-} in place of oxygen), or fermentation. Depending on the nature of the microbes and the local physicochemical conditions (i.e. types of organic matter, pH, salt concentration and temperature) the putrefaction process will produce VOC gases, including many that are odiferous. This includes ammonia, volatile sulfur compounds (VSC), indole and certain volatile amines, (including putrescine and cadaverine; Statheropoulos *et al* 2005), all products of amino acid utilization for catabolism. The amino acids and small peptides are supplied from proteins following hydrolysis and digestion by proteolytic enzymes. Therefore, microbial processes will ultimately produce a complex mixture of VOCs and/or odour depending on both intrinsic and extrinsic factors, including the composition and pH of 'feedstock'. For example, carbohydrates do not result in major VOC odours whilst proteins and/or fats result in a variety of foul putrefactive odours (Statheropoulos *et al* 2005). In addition, a low pH (<6.0) is thought to allow yeasts and moulds to dominate, whilst a higher pH favours bacteria and the putrefaction processes, which will release foul-smelling VOCs (Mackie 1995), including volatile sulfide compounds (VSC), indoles and amines.

Microbial succession

In general, when complex organic compound mixtures (including those within host organisms) are exposed to complex, highly diverse mixed cultures of microorganisms, then a well described theoretical sequence of ecological shifts (succession) and corresponding metabolic 'phases' or stages occur. Aerobes and facultative anaerobes are the first colonizers, with high growth rate and population expansion, giving accelerated reduction of the oxygen concentration and redox. Mixed culture fermentation produces carboxylic acids that may lower the pH enabling aciduric and acidophilic species to gain a selective advantage (e.g. *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Propionibacterium* spp.; Wilson 2005). When all small molecular weight, easy to assimilate sugars and amino acids have been fully utilized, hydrolytic mechanisms become induced or relieved of catabolite repression (Magasanik 1961) and microbial

biotransformation of polymeric molecules (proteins and glycoproteins) becomes of more marked importance. The general phenomena of catabolite repression (Magasanik 1961) occurs when there is an abundance of small molecular weight (cell-transportable) nutrients (e.g. sugars or amino acids), and is a mechanism by which cells may conserve their energy (e.g. ATP) and amino-acid building blocks by repressing synthesis of enzymes involved in hydrolytic depolymerization of large molecules outside the cell. The enzymes are thus redundant under small molecule replete nutrient conditions. Even asaccharolytic species (e.g. *Porphyromonas* spp.) produce little proteolytic activity when grown in the presence of low molecular weight peptides and amino acids, compared to much higher amounts when grown in the presence of proteins (Minhas *et al* 1991).

Following proteolysis, decarboxylation reactions produce amines, and transamination and deamination reactions produce ammonium ion (Wilson 2005). The pH may then start to increase, as carbon dioxide is lost and amines and ammonia accumulate. Physicochemical conditions now favour anaerobic proteolytic species, especially strict anaerobes (e.g. *Clostridium* spp.). Volatile short chain fatty acids (SCFAs) will be produced through primary fermentation, producing mainly lactate, acetate, propionate and ethanol from the main carbohydrates, but is ultimately replaced by secondary fermentation, producing e.g. n-butyrate, propionate, propanol, n-butanol, and n-hexanol (Mackie 1995). The volatile SCFAs propionate and n-butyrate are produced by *Propionibacterium* spp. and *Butyribacterium* spp. respectively, as end-products of central metabolism of sugars, and additional amounts are produced by fermentation of amino acids by species such as *Clostridium* and *Fusobacterium* (MacFarlane and MacFarlane 1995). If methanogens are present (e.g. within the gastrointestinal tract) and the hydrogen partial pressure becomes sufficiently high, then acetate will be converted to methane (Jones *et al* 1987). Hence, the specific microbial succession (and associated microcosm metabolism) that occurs at a given anatomical location will impact on the volatiles produced.

The generalized scheme of microbial succession described above assumes that all main metabolic steps are represented within the microcosm, whereby different species can replace one another in terms of the functional metabolic groups, enabling cross feeding and full diversity of reactions and pathways. However, the human microflora is exposed to a variety of physicochemical environments at different anatomical locations, resulting in diverse but often restricted microbial ecology. For example, the diversity of species found in the large intestine of man and mammals is a strongly selected group that can withstand bile secretion and other physicochemical conditions of the gut (Wilson 2005). Skin presents a hostile environment because of its low water activity, salt content, pH, and host defence molecules, but for those strains able to tolerate these conditions easily available nutrients are present, including sweat and sebum (Roth and James 1988). In reality, the predominant microbial flora at a given anatomical site is relatively specialized, and the authors postulate that the VOC profile is therefore likely to be more

Table 2. Microbial fermentation pathways. Adapted from Mackie (1995).

Fermentation pathway	Products	Example species
Alcoholic	Ethanol + CO ₂	<i>Saccharomyces</i> and other fungi <i>Zymomonas mobilis</i>
Lactic acid		
Homolactic	All pyruvate metabolized to lactate	<i>Lactobacillus</i>
Heterolactic	Pyruvate metabolized to lactic acid and other products (ethanol, CO ₂)	<i>Streptococcus</i> <i>Bacillus</i> <i>Leuconostoc</i> <i>Bifidobacterium bifidum</i>
Propionic acid	Propionate (lesser amounts of acetate, lactate, succinate and CO ₂)	<i>Propionibacterium pentosaceum</i> <i>Veillonella alcalescens</i> <i>Bacteroides ruminicola</i>
Butyric acid	butyrate, acetate + H ₂ + CO ₂ (some species produce butyl alcohol, acetone, 2-propionate ethanol or capronate)	<i>Clostridium butyricum</i> <i>Clostridium acetobutyricum</i> <i>Clostridium kluyveri</i>
Mixed acid	Ethanol + fatty acid mixture (acetic, lactic, formic and succinic)	<i>Escherichia coli</i>
Butanediol	2,3-butanediol + ethanol (and smaller amounts of organic acids)	<i>Serratia</i> <i>Enterobacter</i> <i>Erwinia</i>
Stickland	Carboxylic acids (acetate, propionate, butyrate, isobutyrate, isovalerate)	<i>Clostridium</i>

characteristic of the metabolic pathways favoured by the predominant groups of organisms present (which in turn is reliant on the substrate precursors available).

VOC generating bacterial metabolic pathways

Microbial biotransformations resulting in the production of VOCs within a host can be broadly grouped into three main categories of systems. Firstly, main carbon-energy metabolic pathways (e.g. SCFAs as end products of fermentation), and whose production rate is strongly linked to growth-rate. Secondly, enzyme associated reactions whose full expression is dependent on the physicochemical environment of the cell, or presence/absence of inducer molecules. For example the generation of hydrogen sulfide by cysteine desulfhydrase in the presence of thiol-containing molecules (Starkenmann *et al* 2008), since even if the enzyme in the cells is fully expressed, the VOC generation rate will be rate limited depending on the concentration of enzyme substrate present (cysteine, in our example). Finally, VOCs produced from enzymatic reactions that liberate the VOCs in question from a specific type of precursor molecule produced by the mammalian host. For example, 3-hydroxy-3-methylhexanoic and other related acids are generated from amino acid, peptide or protein conjugate precursors present in axillary secretions, via microbial aminoacylase activity (Natsch *et al* 2003).

Short chain fatty acid (SCFA) metabolism

Volatile SCFAs (fatty acids with <6 carbons in the aliphatic tail) are produced via numerous metabolic fermentation pathways within microbial cells, and these are summarized in table 2 along with example species which utilize each pathway. Following digestion/ depolymerization, carbon-energy monomers (e.g. sugars) are metabolized into pyruvate

Table 3. Microbial deamination of amino acids into fatty acids. Adapted from Macfarlane and Macfarlane (1995).

Amino acid	Deamination fatty acid product
Alanine, glycine and serine	Acetate
Glutamine, aspartate	Acetate, propionate and butyrate
Isoleucine	2-methylbutyrate
Leucine	Isovalerate
Phenylalanine	Phenylacetate
Tyrosine	<i>p</i> -hydroxyphenylacetate, phenylacetate and phenylpropionate
Threonine	Propionate
Tryptophan	Indoleacetate and 3-methylindole
Valine	Isobutyrate

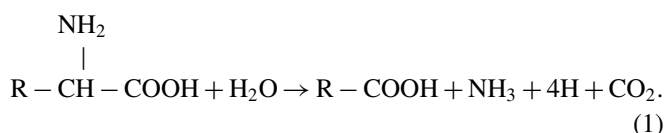
being an intermediate in several metabolic pathways. Acetate is produced via pyruvate metabolism by many species of microorganisms, as either major or minor components of other metabolic products. Butyrate is also produced as a major end-product of pyruvate fermentation in a small number of different species of obligate anaerobe (*Clostridium* spp., *Roseburia* spp. and *Eubacterium* spp.; Louis and Flint 2009), which are commonly isolated from the oral cavity and gastrointestinal tract of man and animals, as well as from infected wounds. Alanine can react with α -ketoglutarate to form pyruvate and glutamate, and is then again metabolized by a whole variety of routes depending on the species of microbe, and the availability of different types of end-terminal electron acceptors. Amino acids may be deaminated, decarboxylated or transaminated depending on type. The SCFAs produced include; acetate, butyrate, propionate, butylene glycol and occasionally acetone and butanol (Mackie 1995), many of which are volatile under physiological conditions.

SCFA products through deamination reactions are shown in table 3, and in particular the cyclic ring amino acids pheny-

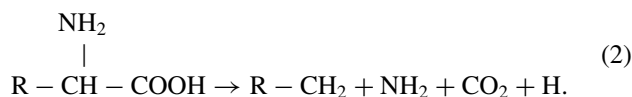
Table 4. Microbial decarboxylation products of amino acids. Adapted from Macfarlane and Macfarlane (1995).

Amino acid	Decarboxylase product
α -aminobutyrate	Propylamine
Alanine	Ethylamine
Arginine	Putrescine (some to pyrrolidine by ring closure) and agmatine
Cystine	Taurine
Glycine	Methylamine
Histidine	Histamine
Lysine	Cadaverine (some to piperidine by ring closure)
Norvaline	Butylamine
Ornithine	Putrescine (some to pyrrolidine by ring closure)
Phenylalanine	Phenylethylamine
Tyrosine	Tyramine
Tryptophan	Tryptamine

alanine, tyrosine and tryptophan produce interesting volatile products, including; indoleacetate, 3-methylindole (skatole), p-hydroxyphenylacetate, phenylacetate and phenylpropionate (Mackie 1995). The generalized deamination equation is



SCFAs are also produced through decarboxylase reactions commonly found in bacteria, (shown in table 4), and the generalized equation is



Coupled oxidation and reduction reactions of amino acids to produce SCFAs is known as the Stickland reaction (see table 2) (Nisman 1954). All amino acids with exception of histidine can act as either Stickland acceptors, Stickland donors, or both donor and acceptor. The electron donor amino acid is oxidized to a volatile carboxylic acid one-carbon atom shorter than the original amino acid, whilst the electron acceptor amino acid is reduced to a volatile carboxylic acid the same length as the original amino acid (Nisman 1954).

Microbial amine production and ammonia

Many amines are bioactive (in addition to their basic properties), particularly histamine and tryptamine which are both inflammatory mediators (Allaker *et al* 1987), as well as cadaverine and putrescine which possess a putrid odour (Goldberg *et al* 1994). Trimethylamine is produced by many species of bacteria from the breakdown of choline or trimethylamine oxide (TMAO) found in meat, eggs, fish and certain vegetables (Brassica) (Groninger 1959, Yamada 1967). In fish (post mortem), TMAO is reduced by bacteria (notably Gram-negative rods) to trimethylamine (TMA), which gives rise to the characteristic off-flavour and odour of marine fish in the early stage of decay (Beatty and Gibbons 1937, Tarr 1954). In humans, TMA is produced from TMAO (in the diet) or from

choline, by intestinal bacteria. From the gut, TMA is then absorbed into the bloodstream and is oxidized into odourless TMAO by the liver enzyme flavin mono-oxygenase 3 (FMO₃) and excreted into urine. Trimethylaminuria (TMAU; fish odour syndrome) is a rare condition caused as a consequence of an autosomal recessive mutation in FMO₃, where TMA is excreted into body fluids (including saliva and sweat) and is detected on breath giving rise to persistent or sporadic oral and body malodour (Mackay *et al* 2011, Whittle *et al* 2007). TMAO levels in the liver may vary for reasons other than TMAU mutation, including hepatic disease, anxiety and stress, diet and menstruation (Mitchell 2005, Shimizu *et al* 2007). A strict diet avoiding choline-rich and TMAO containing foods is advised in the management of these cases (Mitchell 2005).

Ammonium ion is a common product of many different types of reactions in microbial cells including; deamination reactions, arginine dihydrolase pathway, tryptophanase and methionine gamma lyase, and the breakdown of glutamine and glutamate. Ammonium ion production may be balanced by acid production, but if fermentable substrates are in short supply, the pH will tend to increase converting the ammonium ion present into ammonia gas. In strongly neutral buffered environments, the amount of ammonium ion production is probably not high enough to affect large pH changes. However, additional ammonium ion can be produced from the action of microbial urease enzymes on the substrate urea (found in blood, sweat, saliva and other body fluids in addition to urine), producing ammonium and bicarbonate ions (Mobley and Hausinger 1989). Bicarbonate is a weak acid and easily lost into the gas phase particularly when in the presence of carbonic anhydrase, an enzyme found in numerous microbial species, (particularly *Escherichia coli*; Guilloton *et al* 1992, Chirica *et al* 1997), as well as mammalian species. This allows the pH of these environments to increase and causes production of significant amounts of ammonia gas.

Volatile sulfur compounds

Although hydrogen sulfide is not organic (and so not strictly a VOC), volatile sulfur compounds (VSCs) are associated with desulfurization reactions of the sulfur containing amino acids cysteine, cystine and methionine, and plays a central role in the maintenance of a low redox potential (ideal for proteolytic anaerobes). There are two different mechanisms by which microbes produce hydrogen sulfide. One group of microbial species, collectively known as dissimilatory sulfate-reducing bacteria (SRB), reduce oxyanions of sulfur (SO₄, SO₃, S₂O₃²⁻) as terminal electron acceptors, using cytochromes to perform 'anaerobic respiration' (Postgate 1984). In the mammalian oral cavity SRB (e.g. *Desulfovibrio* spp.) have been detected, but are generally only a small fraction of the total population (Willis *et al* 1995), and their presence shows a negative correlation with VSC production and oral malodour in humans (Willis *et al* 1999). It is much more likely that the main microbial path to hydrogen sulfide within mammalian host organisms is via desulfurization of amino acids, and also by species that can utilize glutathione or other thiol containing peptides removing the sulfur in the form of hydrogen sulfide

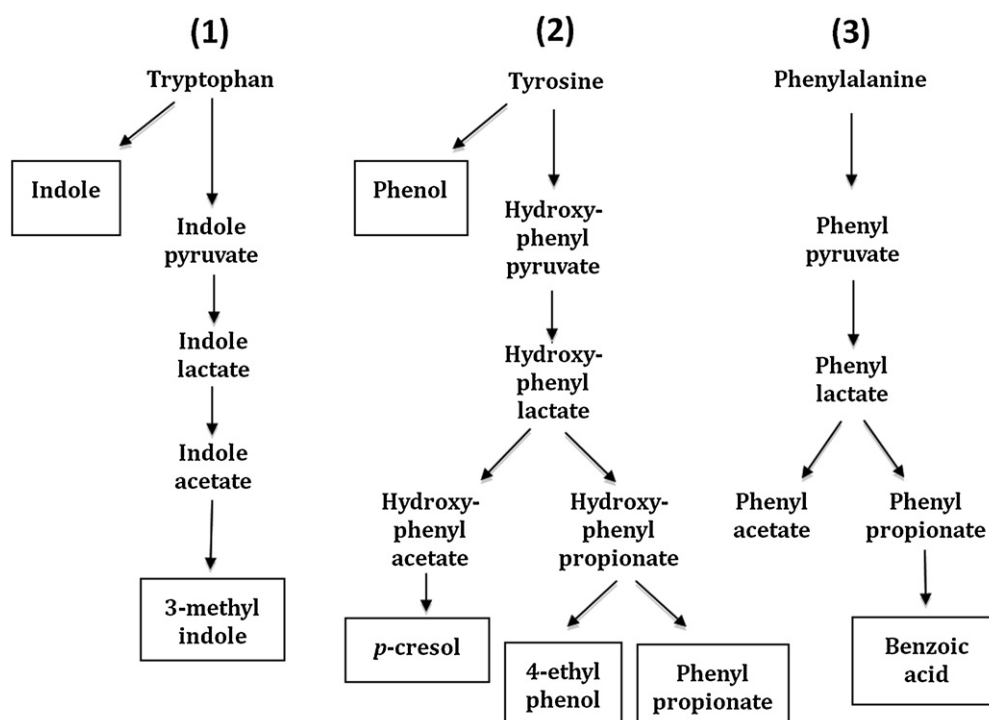


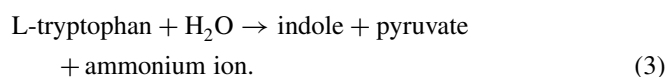
Figure 2. Formation of indolic (1) and phenolic (2 and 3) volatile compounds. (Adapted from Macfarlane and Macfarlane 1995.)

(H₂S) or methyl mercaptan (CH₃SH) (Persson *et al* 1990). For example, *Fusobacterium nucleatum* is a Gram-negative, anaerobic species found in the human oral cavity with a high propensity for producing H₂S (Persson *et al* 1990). Three or four distinct VSC producing enzymes have been isolated and characterized, including: (1) α,β -elimination of L-cysteine to produce H₂S, pyruvate and ammonia; (2) β -elimination of L-cysteine to produce H₂S and L-serine; (3) β -replacement reaction, where two molecules of cysteine condense to produce H₂S and L-lanthione (Fukamachi *et al* 2002, Yoshida *et al* 2010, Yoshida *et al* 2011). A fourth mechanism has also been described involving the enzyme L-methionine γ -lyase (which normally catalyses methionine into methylmercaptan). This enzyme will produce H₂S when L-methionine is replaced by L-cysteine (Kyosuke *et al* 2011).

Indole

Indole production from tryptophan is a property of a number of different microbial species including; *E. coli* (Newton and Snell 1964), *Proteus vulgaris* (Kamath and Yanofsky 1992), *Haemophilus influenzae* (Martin *et al* 1998), *Porphyromonas gingivalis* (Yoshida *et al* 2009) and *F. nucleatum* (Imamura *et al* 2010). The enzyme responsible is tryptophanase (more correctly named as tryptophan indole-lyase; EC4.1.99.1) and it transforms tryptophan into indole, pyruvate and ammonium ion (figure 2, Newton and Snell 1964). Indole has a low volatility coupled with a very low odour threshold. The odour has been described as foul and repellent (Tso and Adler 1974), and due to its low volatility the smell 'lingers' remaining for hours or days (called stench). Indole acts as a cell-to-cell signalling molecule that mediates biofilm formation in various bacteria carrying the gene for tryptophanase (Newton

and Snell 1964, Martino *et al* 2003, Mueller *et al* 2009), as well as affecting the expression of several other genes, including those associated with amino acid metabolism (Wang *et al* 2001), multidrug exporters (Hirakawa *et al* 2005) and polysaccharide production (Mueller *et al* 2009). The enzyme consists of four identical monomers, each containing one molecule of pyridoxal 5'-phosphate (PLP). The PLP molecule forms an aldimine bond with a lysine residue (Ku *et al* 2006) and catalyzes the reversible hydrolytic cleavage of L-tryptophan to produce indole and ammonium pyruvate via an α,β -elimination mechanism (Snell 1975).



Methyl indole is produced from tryptophan via an indole pyruvate pathway by intestinal flora (figure 2, Yokoyama and Carlson 1974) and pathways for phenolic VOCs (phenol, p-cresol, 4-ethyl phenol, phenyl propionate, phenylacetate and benzoic acid) have also been described (figure 2, Macfarlane and Macfarlane 1995).

The effect of pH on the relative volatility of VOCs

For organic acids (including SCFAs) when the pH is above neutral they exist in the ionic dissociated (salt) form and are not volatile, whereas at decreasing pH, a greater proportion of the acid is in the associated (hydrophobic) state, hence more can partition between the liquid and gas phases. For amines, it is the other way round; when the pH is above neutral, amines exist more in their base-form, and it is only in this associated form that the compound can partition into the gas phase. Independent of the effects of

pH on VOC phase transitions, pH will also variably affect the production rate of microbial VOCs, e.g. pH inducible amino decarboxylases in bacteria (Viala *et al* 2011). Amine production and its role in pH homeostasis in *E. coli* and *Micrococcus lysodeikticus* was described nearly 70 years ago by Gale and Epps (1942). The mechanism occurred as a consequence of low pH inducible amino acid decarboxylase enzymes, which acted on amino acids (particularly glutamate, arginine, lysine and ornithine) producing carbon dioxide and free amine base. At low pH, carbon dioxide is lost from the system (especially in the presence of microbial carbonic anhydrase activity) and the overall balance is for the system to increase the pH from acid towards neutrality. More recently it has been shown how enteric bacteria survive exposure to acidic environments, by inducing genes that provide for acid resistance and pH homeostasis (Bearson *et al* 1997, Hall *et al* 1995). *E. coli* has three distinct systems for acid resistance: a glucose-catabolite-repressed system and two amino acid decarboxylase-dependent systems (Castanie-Cornet *et al* 1999). Glutamate decarboxylase activity was thought to be the most important pathway in *E. coli*, the product being gamma-aminobutyric acid (GABA; which is not volatile at ambient temperatures), but interestingly lysine decarboxylase (producing the volatile cadaverine) has been shown to be central to pH homeostasis in *Salmonella typhimurium* (Park *et al* 1996). These systems overlap in ways that protect both growing and non-growing cells from acid stress across a broad range of naturally occurring acidic environments, and can result in pH dependent volatile products.

Microbial VOC detection (then and now)

The detection of VOCs is of interest to various scientific disciplines, and numerous analytical approaches have been developed to accurately characterize and measure VOCs in the laboratory. Early research studies used the Duclaux method to analyse volatile fatty acids produced by bacteria. This was based on the differential volatility of fatty acids in steam (in essence preparative chromatography), whereby sequential distillate portions could be collected and titrated with alkali to determine the concentration (Richmond 1895). The method was used very effectively to assay for formic, acetic, propionic and butyric acid (Zoller and Clark 1920), and was later improved with more refined quantitative chemical analysis of fatty acid fractions (e.g. mercuric chloride method for detection of formic acids; McNair 1933). The detection of volatile compounds has been used for many years as part of bacterial identification systems, for example the imvic (indole, methyl red, Voges-Proskauer, and citrate) system which differentiates coliforms according to their production of indole, acetoin, and mixed acid fermentation products, in addition to their ability to metabolize citrate (MacFaddin 1980). This biochemical approach to the detection of microbial volatiles continues to the present day, for example functionalized nanoporous xerogels are currently being developed for detection of the presence of acetate and indole (Crunaire *et al* 2011).

In most modern analytic research laboratories, the detection of microbial volatiles is carried out using gas chromatography (the separation step), and subsequent mass spectrometry, electron capture or flame ionisation detection, which coupled with analytical software enables quantification and identification of VOCs (with high sensitivity and specificity) from a given microbial species or mixed consortia. Early studies on microbial volatiles used direct gas-liquid chromatography (Gorbach *et al* 1976, Bogaard *et al* 1986), whereby compounds were identified from their retention times, using a flame ionisation detector (Ettre 1993). This requires preparation of whole microbial cultures by chemical extraction, and modified methods were developed to enable direct injection of whole microbial cultures or faeces for detection of anaerobic bacteria (Bricknell and Finegold 1973). The development of 'head-space analysis' of microbial cultures using gas chromatography mass spectrometry (GC-MS) provided more information, was less laborious, and was capable of automation (Larsson *et al* 1978a, 1978b, Zechman *et al* 1986). In particular, the use of mass spectrometry (via electron ionisation) has enabled qualitative and quantitative detection of microbial volatiles by measuring the mass-charge ratio of charged sample particles. Subsequent software mining of mass spectral libraries (e.g. NIST or Massfinder) then enables identification of the volatiles present based on the sample mass chromatogram produced. However, this analysis should be performed with caution since many related compounds generate similar mass spectra (Schulz and Dickschat 2007), and it has been suggested that the structure of many microbial volatiles is yet to be 'unequivocally' identified (Kai *et al* 2009). The application of head-space GC-MS has been shown capable of identifying anaerobes (Julak *et al* 2000, Mardh *et al* 1981), facultative bacteria, (Zechman *et al* 1986), and fungi (Larsen and Frisvad 1995). The headspace gas sampling procedure initially involved simple purge trap approaches using various adsorbent materials (e.g. charcoal, tenax, super Q), but these are thought to preferentially bind certain compounds according to their chemical composition (Kai *et al* 2009). Hence, as GC-MS technology has advanced, so has the sampling procedure, and pre-concentration solid phase micro-extraction (SPME; Bojko *et al* 2011, Julak *et al* 2003) and closed-loop stripping apparatus (CLSA; Hassett and Rohwer 1999) have greatly improved the sampling efficiency. GC-MS does have some disadvantages being relatively expensive and time-consuming, requiring highly skilled operators, and is reliant on the use of internal standards to identify and quantify each VC component. Therefore, although it is largely considered the 'gold-standard' for microbial volatile detection, other analytical approaches have been explored.

A significant development in microbial volatile analysis is soft ionisation mass spectrometry techniques, enabling direct sampling in air. For example, ion mobility mass spectrometry separates and identifies ionized molecules (including VOCs) in the gas phase based on their mobility in a carrier buffer gas, and has enabled rapid determination of microbial volatiles in humid samples (milliseconds to minutes) (Eiceman 2002, Shnayderman *et al* 2005). This is a promising technology

with real-world applications, but suffers from being unable to differentiate all volatiles within complex samples due to insufficient sensitivity and mass resolution (Smith and Spanel 2005). To address this issue, a new approach to direct, online microbial headspace analysis has emerged in the form of proton transfer reaction mass spectrometry (PTR-MS) and selective ion flow tube mass spectrometry (SIFT-MS). These techniques are capable of determining absolute head-space concentrations of VOCs regardless of humidity, and without need for any sample preparation (Mayr *et al* 2003, Smith and Spanel 1996, Smith and Spanel 2005). PTR-MS uses protonated water (H_3O^+) as a chemical ionisation reagent enabling direct and real-time sampling and analysis of sample specimen headspace (Lindinger *et al* 1998). This has been used to quantify VOCs emanating from microbial cultures, enabling not only species specific volatile profile discrimination, but also temporal evolution of VOCs during bacterial growth (Bunge *et al* 2008, O'Hara and Mayhew 2009). In contrast, SIFT-MS uses multiple reagent ions (H_3O^+ , O_2^+ and NO^+) to perform soft ionisation. The use of three precursor ions enables internal verification of the VOCs detected, making identification more accurate, and enabling discrimination of isomeric and isobaric compounds (Hu and Storer 2008). The sample gas is delivered to the instrument at a constant rate where it is mixed with microwave generated reagent ions within a fast-flowing helium flow tube. The resultant reaction products are separated and counted downstream by quadrupole mass spectrometry, enabling quantitative measurements of complex volatile mixtures (Allardyce *et al* 2006a, Smith and Spanel 2005). Accurate identification and quantification of trace gaseous molecules is possible since the reaction time for molecular interactions is predictable within SIFT-MS instruments, being defined by predetermined rate co-efficients of the ion-molecule reactions (Smith and Spanel 1996). This technology has now been successfully applied to microbial headspace analysis (Allardyce *et al* 2006a, Allardyce *et al* 2006b), being capable of discrimination of bacterial species when coupled with multivariate analysis (Storer *et al* 2011, Thorn *et al* 2011).

Non-specific pattern recognition of microbial volatiles can be achieved through the use of electronic olfactory systems (e-nose). These comprise of non-specific, gas sensitive, chemical sensors connected to data-capture and analysis software, which generates an olfactory fingerprint of a specimen (Gibson *et al* 1997). Sample discrimination is then achieved through pattern recognition algorithms, principal component analysis (PCA), discriminant function analysis, cluster analysis or artificial neural networks (Spinelli *et al* 2010). These systems have been used successfully in detecting the presence of microbial activity (Spinelli *et al* 2010), and to differentiate microbial species by VOC profiling (Pavlou *et al* 2002a, 2002b). However, it should be noted that this is not a quantitative approach and cannot identify or provide absolute concentrations of VOCs, relying purely on comparative detection of patterns.

A new emerging approach to gas analysis is laser (absorption) and photoacoustic spectroscopy. Laser (absorption) spectroscopy, based on the attenuation of an

electromagnetic signal to produce a characteristic adsorption profile, has been used for accurate detection and quantification of volatile compounds. An extensive review already exists on the use of this technology for detection of volatile sulfur compounds, whereby it was concluded that the detection limits (down to ppb) are sufficient for medical applications and could result in the future development of portable and affordable gas sensors (Ciaffoni *et al* 2011). Photoacoustic spectroscopy is the acoustic detection of the interaction of absorbed electromagnetic energy (light) with matter. This is based on a modulating excitation source which excites light absorbing gas molecules, subsequent transfer of kinetic energy to surrounding molecules, and generation of a sound wave which is detected by a specially designed microphone and amplifier (Chivukula *et al* 2007). This has been successfully used to measure complex VOCs using a spectrally tunable monochromatic radiation source, in combination with a sophisticated data analysis algorithm (Wolff *et al* 2011), and represents a highly sensitive and novel approach that could equally be applied to microbial volatile detection.

Relationship between VOCs and diseases or health conditions

It is widely known that microbes play an essential role in causation and prevention of disease in the human biosphere, but what is less well known is the potential role of microbial VOCs in disease processes. Many microbial VOCs have biogenic actions on mammalian cells (including cytotoxic and inflammatory effects), whereby the VOCs can be described as virulence factors or tissue aggressins. For example, experiments using Vero cells have shown carboxylic acid salts to be cytotoxic even down to concentrations of 1–2 mM, with the order of VOC cytotoxicity being: butyrate > propionate > valerate > isovalerate > isobutyrate, with human fibroblast cells being shown to be even more sensitive to the cytotoxic effects (Allaker *et al* 1985). Butyrate, histamine and tryptamine all produced detectible inflammatory effects on smooth muscle preparations *in vitro*, with 10 mM or less dosing still giving a 50% tissue response (ED_{50} values) particularly when added as mixtures (Greenman *et al* 1988). Moreover, crevicular fluid from patients with periodontal disease has been shown to contain propionate and butyrate at concentrations around 10 mM and 2.6 mM, respectively (Niederman *et al* 1997). Propionate and butyrate have also been shown to produce gingival inflammation in Beagle dogs when applied to the periodontium at concentrations down to 5 mM (Singer and Buckner 1981). Volatile sulfur compounds (VSCs) are a family of gases that are highly toxic to tissues even at quite low concentrations (Ng and Tonzetich 1984, Johnson *et al* 1992). Hydrogen sulfide (H_2S) has been reported to be cytotoxic to a wide range of mammalian cells, including those found in the periodontal regions of humans (Calenic *et al* 2010a, 2010b, Fujimura *et al* 2010, Yaegaki *et al* 2008). It is unknown to what extent a complex mixture of microbes produces a complex mixture of cytotoxic, inflammatory or biologically active compounds (e.g. VOCs) and whether such

compounds may have a synergistic effect when produced as mixtures.

The idea that complex metabolite profiles could be used to diagnose diseases in medicine was put forward by Linus Pauling and co-workers (Pauling *et al* 1971). They suggested the analysis of vapour and breath using gas chromatography (GC), in order to detect metabolic changes in indicative diseases, and identified over 200 different VOCs on breath condensate (Pauling *et al* 1971). However, the diagnosis of microbial disease through the analysis of breath samples is complicated by the diversity of VOCs present, as well as inter-individual VOC variation (Phillips *et al* 1999). VOC analysis for diagnosis of microbial associated disease is an emerging field in numerous areas of medical research, and will almost certainly play an important role in informing effective and appropriate treatment for individual patients.

Oral disease and malodour

Bad breath, halitosis or oral malodour, are all interchangeable terms used to describe the general condition of odiferous breath (Scully and Greenman 2012). Bad breath may rank only behind dental caries and periodontal disease as the cause of a patient's visits to the dentist, although the perception of halitosis is different in culturally diverse populations (Rayman and Almas 2008). The true prevalence of halitosis is unknown, particularly since it is not an all or nothing phenomena, and the degree to which it is severe enough to be considered socially unacceptable (also known as pathologic halitosis) will vary depending on how it is judged.

Human breath will contain VOCs that have originated from more than one source within the respiratory system. Three main VOC sources can be identified: (1) exogenous sources; VOC's inspired from background ambient gases and then expired again, (2) Extra-oral; from microbes further down the respiratory tract (oropharynx, bronchioles), or else blood-circulatory VOC's that enter the lungs and, through gaseous exchange, are removed from the body, and (3) Intra-oral; microbial VOCs from microbial biofilms that are encountered in the oral cavity. Exogenous sources depend on the ambient gases around the time and place of the sample streams, and may include industrial-synthetic sources or else VOCs emitted from plants, animals or microbes in the vicinity of the sample. The other two sources (extra-oral and intra-oral) can be differentiated by comparing lung gas samples (e.g. collected via nose) with samples obtained from the mouth, following a period of 'holding' the gas within the mouth (by breathing through the nose) to allow for phase equilibration with the aqueous phase (typically one or two minutes) and finally sampling the mouth gas after this period. The majority of human subjects with halitosis (80 to 90%) have oral malodour because of microbial activity from biofilms within the oral cavity (Miyazaki *et al* 1995). For extra-oral halitosis, (about 5–10% of all cases of halitosis), then the majority of patients have blood-borne halitosis (Tangerman and Winkel 2010). Blood-borne halitosis is frequently caused by odorous VSCs, in particular dimethyl sulfide (CH_3SCH_3). Extra-oral halitosis can potentially be a manifestation of a more serious disease, for

which treatment is much more complicated than for intra-oral halitosis. Potential sources of (non-microbial) halitosis include liver and kidney diseases, metabolic disorders, medications and certain foods (Greenman and Saad 2009), and most of these VOCs are therefore produced by the mammalian system (van den Veld *et al* 2007a). It is therefore of utmost importance to differentiate between intra-oral and extra-oral halitosis. For malodour to be perceived as noticeable and unpleasant, the VOCs in question must be well above the threshold concentration for smell detection by the perceiver. The relative 'smell' of a pure compound can be determined by accurate dilution of the VOC in question to the point where a panel of judges can just detect it. The degree of dilution necessary to reach this point is a measure of the olfactory power (p_{ol} value) of the compound in question (Greenman *et al* 2004). The main unit of measurement (p_{ol} by volume) is defined as the negative log of the threshold concentration expressed in volumic or molar fractions, for example, 1 ppm = p_{ol} 6, whilst 1 ppb = p_{ol} 9 (Devos *et al* 1990). Approximate averaged standardized thresholds have been published for a number of compounds (Devos *et al* 1990).

In the population as a whole, available epidemiological data suggests that halitosis is common with estimates between 30% (Liu *et al* 2006, Sanz *et al* 2001, Ueno *et al* 2007) and 50% (Tessier and Kulkarni 1991), and can affect people of all ages with a tendency to increase in age (Miyazaki *et al* 1995). Most reports now agree that the most frequent sources of halitosis exists within the oral cavity. This includes bacterial reservoirs such as the dorsum of the tongue, saliva and periodontal pockets, where anaerobic bacteria degrade sulfur-containing amino acids to produce the foul smelling volatile sulfur compounds (VSCs); especially hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulfide (CH_3SCH_3) (Tonzetich 1971). Figure 3 illustrates the main microbial mechanisms in generation of oral malodour gases. A 'generalized' bacterial cell is shown since no single species can produce all the enzymes, or possesses all the pathways, for changing primary substrates into corresponding volatile compounds. However, collectively there is sufficient diversity of species within the oral cavity such that all pathways usually operate to some degree, the same being true for the diversity of hydrolytic enzymes that change secondary into primary substrates.

The tongue biofilm coating is considered to be the most important source of VSCs, simply because it harbours the highest population of microbes (Greenman *et al* 2005). To date, the exact number of different species resident within the oral cavity is unknown, although several attempts have estimated numbers ranging from; 500 species (Moore *et al* 1985, Paster *et al* 2001), to 600 species (Kazor *et al* 2003), and up to 700 species (Aas *et al* 2005). Although the actual exact number will probably remain unknown due to the high diversity of resident oral flora, most estimates propose that over 50% of the microorganisms within the oral cavity are uncultivable, and their presence has only been identified by molecular rDNA testing (Aas *et al* 2005, Paster *et al* 2001). Hartley *et al* (1996) counted and identified microbes from tongue biofilm samples from 50 volunteers

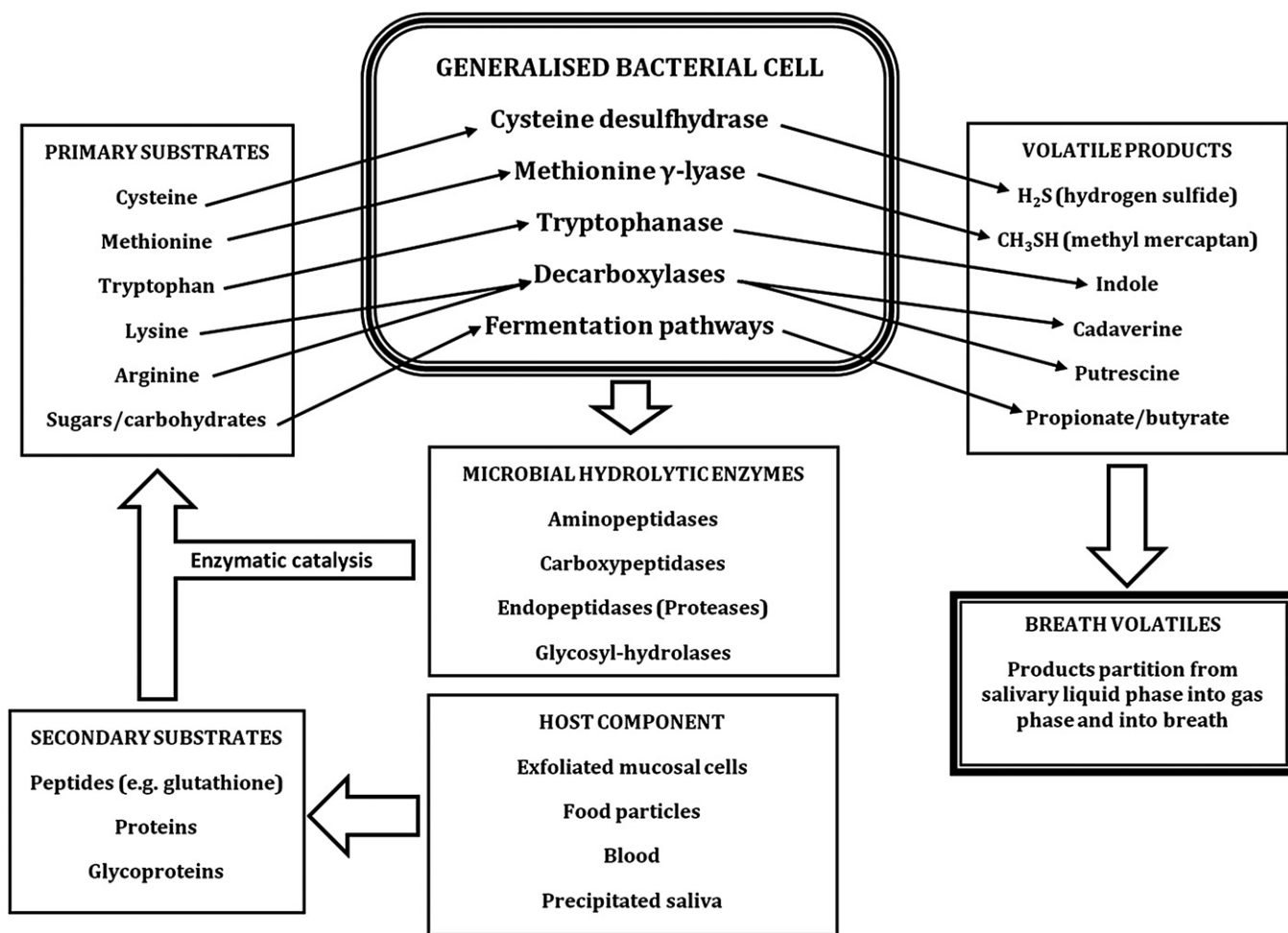


Figure 3. Microbial mechanisms in the generation of oral malodour gases from a ‘generalized’ bacterial cell.

and showed a strong relationship between microbial numbers isolated (cfu cm^{-2}) and the oral malodour status of human volunteers. For subjects grouped into low or high odour producers, significant increases in the total bacterial load and key bacterial groups, namely gram-negative anaerobes, were related to high odour, with two phenotypic characteristics (sulfide production and proteolytic activity) also strongly associated with malodour (Hartley *et al* 1996). Further research (Hartley *et al* 1999) analysed the tongue microbiota before and after use of a metronidazole mouthwash, a compound that is only effective against strict anaerobes. A reduction in oral malodour correlated closely with the reduction of strict anaerobes (predominantly Gram-negative anaerobes) whilst population levels of other species (facultative and aerobic species) remained constant throughout. Furthermore, malodour return (after 48 h) correlated with return of the strict anaerobes back to their starting numbers (Hartley *et al* 1999).

Although there are hundreds of different VOCs reported on breath (Van den Velde *et al* 2007b), the most important contributors to oral malodour are thought to be volatile sulfur compounds (VSC). The microbial degradation of cysteine residues produces H_2S , and the sulfhydryl anion (HS^-) is a strong reducing agent that drops the redox potential (Eh) within the tongue biofilm (Kleinberg and Codipilly 2008), favouring the growth of anaerobes (McNamara *et al* 1972),

and activating thiol-dependent enzymes including trypsin-like protease (important in the putrefaction process; Smalley *et al* 1994) and cysteine desulphydrase (the very same enzymes responsible for $\text{H}_2\text{S}/\text{HS}^-$ production in the first place). The detection of these compounds is complicated by the high humidity within patient breath samples, but is made possible using pre-concentration steps for GC-MS (Ochiai *et al* 2001), or using analytical approaches able to handle high humidity samples without need for sample preparation (e.g. SIFT-MS or PTR-MS; Smith and Spanel 2005). Most of the non-sulfur VOCs that may contribute to halitosis appear to be produced endogenously and/or in the mouth, and include acetone, 2-butanone, 2-pentanone and 1-propanol, appearing in both alveolar and mouth air, as well as indole and dimethyl selenide found mainly in alveolar air (van den Velde *et al* 2007a). In recent years halitosis associated sulfide detectors have been developed for this market, including; the HalimeterTM (Rosenberg *et al* 1991), zinc-oxide and tin-oxide semiconductor sensors (Shimura *et al* 1996, Maita 1996) such as the BreathronTM (Tanda *et al* 2007), as well as the GC-based OralchromaTM (Tangerman and Winkel 2008). Being compact, affordable, easy to use and capable of producing rapid results, they have been widely utilized in research and dental clinics, but lack the same level of sensitivity and specificity obtained using GC-MS or soft ionisation MS. However, the emergence

of laser (absorption) spectroscopy for detection of volatile compounds may address this issue in future, being shown to have high sensitivity and specificity while potentially being scalable to bedside or chairside use (Ciaffoni *et al* 2011).

Periodontal disease

There are several categories of periodontal disease. The most common category (in terms of numbers of cases) is called gingivitis (inflammation of the gum tissue). This is also described as non-destructive periodontal disease since the tissue damage is limited to gum tissue without any involvement of bone or deeper tissue structures (Armitage 1999). The condition is characterized by its reversibility to normal tissue if treated appropriately, i.e. by removal or inhibition of the aetiological agents, considered to be gingival plaque as a whole (Armitage 2004). The most common form of gingivitis is termed plaque-induced gingivitis, since its magnitude relates to the amount or 'load' of microbes present in the gingival plaque around the gingival margin and crevice (Armitage 1999). In the absence of treatment, gingivitis may progress to periodontitis, which is a destructive form of periodontal disease. In contrast to gingivitis, there are at least six different types of termed *destructive* periodontal disease because they are essentially irreversible; these include; chronic periodontitis, aggressive periodontitis, periodontitis as a manifestation of systemic disease, necrotizing gingivitis/periodontitis, abscesses of the periodontium and combined periodontic-endodontic lesions (Armitage 2004). Whilst the destructive form involves specific species of periodontopathogens that have penetrated the gingival tissue, the non-destructive gingivitis might be simply the result of diffusion and penetration into gum tissue of small molecular weight biologically active molecules (e.g. VOCs butyrate, hydrogen sulfide, and/or histamine) produced by the plaque biomass 'as a whole' into tissue and known to be cytotoxic or inflammatory as pure compounds (Greenman *et al* 1988). While in some sites or individuals, gingivitis never progresses to periodontitis (Ammons *et al* 1972), data indicates that periodontitis is nearly always preceded by gingivitis. A group of mainly anaerobic species have been classed as 'periodontopathogens' and are commonly associated with chronic periodontitis. These include the proteolytic *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, and the asaccharolytic *Prevotella intermedia* and *Eubacterium* (Loesche and Grossman 2001). Microaerophilic species associated with chronic periodontitis include *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, and *Eikenella corrodens* (Loesche and Grossman 2001).

VOCs arising from saliva samples taken from periodontal disease patients (analysed immediately upon collection or upon subsequent incubation at 37 °C) have been compared for changes in VOC profile in relationship to different types of periodontal disease condition (Kostelc *et al* 1980, 1981). The diverse VOC profiles detected suggested that the generation rates of VOCs vary, and that increases in some VOCs related well with collagen breakdown (Kostelc *et al* 1980, 1981).

Hydrogen sulfide is usually the most predominant VSC in chronic malodour in otherwise healthy individuals. Patients with gingivitis are thought to produce higher levels of methyl mercaptan, but not as high as those generally found in cases of periodontitis (Yaegaki and Senada 1992). Indeed, it is thought that the ratio between H₂S to methyl mercaptan can be reliably used to differentiate gingivitis from periodontitis, and significant correlations have been shown between the H₂S to methyl mercaptan ratio and periodontal parameters (i.e. severity) (Takeuchi *et al* 2010). Likely reasons for the observed differences are thought to be due to the different exposure to sulfur (S)-containing substrates. In gingivitis, the microbes are outside of the tissues and the main sources of S-containing substrates are obtained from the salivary domain. In periodontal disease, microbes are in more intimate contact with connective tissue and this is described as a serum-tissue domain, due to the presence of gingival crevicular fluid where free methionine or methionine containing peptides are at higher concentrations than in saliva.

Respiratory tract and oropharynx

The respiratory tract is the most common site of infection by pathogens and the majority are viral in nature including; adenovirus and rhinovirus (>50% of all infectious cases), influenza virus, EBV, herpes simplex, measles virus, coronavirus, respiratory syncytial virus and parainfluenza virus. Many can also cause significant infection of the throat, ear, and lungs. It is unknown to what extent virus infections would cause target mammalian cells (or migrating white blood cells) to produce a profile of VOC that would be different to normal healthy cells. In adults, respiratory infections such as tonsillitis, bronchitis, or bronchiectasis, or neoplasms, and/or the presence of tonsilloliths may be responsible for causing malodour (Castellani 1930, Rio *et al* 2008). VOCs from the sinuses (nasal cavity) may be detected in breath and could play a role in clinical diagnosis. In fact electronic nose technology has already been applied to this problem, and preliminary studies have shown its potential in identifying bacterial species within nose and ear swabs (Shykhon *et al* 2004). Interestingly, one study has shown that GC-MS headspace analysis using SPME of the VOCs from above both pure sinus-infection associated bacterial cultures and sinus mucus itself differed significantly, and that identification of bacteria-specific volatiles was possible in some cases (Preti *et al* 2009).

The most common bacterial infection of the throat is probably *Strep. pyogenes* (Group A beta-haemolytic streptococcus), but less common throat infections include *Corynebacterium diphtheriae*, *Neisseria gonorrhoeae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Arcanobacterium haemolyticus*. Strict anaerobes such as *Fusobacterium* may also be associated with peritonsillar abscess formation, and some cases of pharyngitis are caused by fungal infection such as *Candida albicans* causing oral thrush (Choby 2009). A few other causes are rare, but possibly fatal, and include parapharyngeal space infections; peritonsillar abscess ('quinsy') submandibular space infection (Ludwig's angina) and epiglottitis, the latter

most often associated with the presence of *Haemophilus influenzae* type b (Johnstone and Lawy 1967). Bronchitis is characterized by inflammation of the mucous membranes of the bronchi that carry air and respiratory gases into or out from the lungs. About 90% of cases of acute bronchitis are caused by viruses, including rhinoviruses, adenoviruses, and influenza, whereas bacteria, including *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, and *Bordetella pertussis*, account for about 10% of cases (Cohen and Powderly 2004). In patients with cystic fibrosis, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* are the three most common organisms causing lung infections, whereas in later life 80% of patients harbour *P. aeruginosa* and 3.5% *Burkholderia cepacia* (Rowe *et al* 2005, Mitchell *et al* 2007). These respiratory disorders can result in odorous gases being expelled in air via the oral cavity and the nose (Castellani 1930, Sanz *et al* 2001, Yaegaki and Coil 2000). It therefore seems likely that all the main bacterial pathogens would indicate their presence in significant population numbers by emitting characteristic VOC profiles, which could then be detected in breath.

Diagnosis of respiratory tract infection through breath analysis may play a future role in identifying the various etiological agents of disease. For example, 2-aminoacetophenone has been detected (by GC-MS) in the breath of patients with cystic fibrosis, as a marker of colonisation of the lung by *P. aeruginosa* (Scott-Thomas *et al* 2010). Interestingly the volatile sample collection device significantly affected the results; when glass sampling bulbs and Tedlar[®] gas sampling bags were compared, this biomarker was found only to be stable within the glass sampling bulbs (Scott-Thomas *et al* 2010), and a more recent study has also highlighted the importance of the sampling procedure (Scott-Thomas *et al* 2011). *Aspergillus fumigates* colonisation of the respiratory tract of at-risk patients (chronic lung disease) has been associated with the presence of the volatile biomarker 2-pentylfuran in breath through GC-MS analysis (Chambers *et al* 2009), and this is now being developed as a novel diagnostic breath test (Chambers *et al* 2011). This volatile is produced by other medically important fungi including *Aspergillus flavus*, *Aspergillus niger*, *Scedosporium apiospermum* and *Fusarium* species as well as *Strep. pneumoniae*, and hence may have applications in other disease states. Electronic nose technology has also been applied to the diagnosis of respiratory tract infection, being shown capable of discriminating between common respiratory pathogens *in vitro*, including *S. aureus*, *Strep. pneumoniae*, *H. influenzae*, and *P. aeruginosa* (Lai *et al* 2002). Moreover, it has shown good correlation with clinical pneumonia score when used to monitor breath samples from mechanically ventilated surgical intensive care patients (Hanson and Thaler 2005).

Rapid, sensitive, specific, inexpensive and non-invasive diagnostic tools are needed in order to fight tuberculosis (TB; Syhre *et al* 2009), and hence the use of VOC analysis technologies coupled with *Mycobacterium tuberculosis* specific biomarkers is being explored. This area of research has been spurred on by the observation that rats are capable

of smelling the presence of TB in sputum samples, being as least as sensitive a detection method as smear microscopy (Weetjens *et al* 2009). Hence, non-specific e-nose studies have been shown capable of discriminating TB from non-TB cultures *in vitro* (Pavlou *et al* 2004, Fend *et al* 2006). Specific biomarkers of the growth of *M. tuberculosis* and *Mycobacterium bovis* cultures *in vitro* have also been identified (methyl phenylacetate, methyl p-anisate, methyl nicotinate and o-phenylanisole) using SPME and GC-MS (Syhre and Chambers 2008), and methyl nicotinate has shown promise as an *in vivo* marker of disease in the breath of patients (Syhre *et al* 2009). In another study the application of GC-MS consistently identified 130 volatile compounds in the headspace of *Mycobacterium tuberculosis*, and by using pattern recognition and fuzzy logic analysis of breath VOCs, enabled discrimination between hospitalized patients and healthy controls (Philips *et al* 2007). This approach has been further developed into a breath test of active pulmonary tuberculosis, by screening for the presence of both putative oxidative stress products (alkanes and alkane derivatives) and volatile metabolites of *M. tuberculosis* (using GC-MS) (Philips *et al* 2010). However, it should be noted that there is criticism of this studies' suggestion that alkanes and alkenes are biomarkers of oxidative stress (Kwak and Preti 2011). Moreover, breath studies in general often fail to translate into diagnostic aids, largely due to the fact that many diagnostic volatiles are present in high levels in the environment, that there are high levels of inter patient variability, and often a general lack of understanding of the biosynthetic pathways involved (Kwak and Preti 2009).

Gastrointestinal system

The anaerobic microbial communities of the gut are exceedingly complex (Hold *et al* 2001), being characterized by high cell densities and strain diversity, and a significant degree of interspecies cross-feeding and microbial interaction (Dolfing and Gottschal 1997). The main products of microbial fermentation in the large intestine can vary significantly in their relative concentrations and production rates, depending on diet and site of production, but typically high concentrations of acetate, propionate and butyrate are produced (see figure 4), at a ratio of roughly 3:1:1 (acetate: propionate: butyrate; Cummings 1995, Cummings *et al* 1987). Highly-fermentable fibre residues, such as pectin, bran and highly branched starch are transformed by colonic species of bacteria into SCFAs, including significant amounts of butyrate, whereas less fermentable fibres (such as celluloses) produce fewer SCFAs (Cummings *et al* 2001). The production of SCFA from fibres in ruminant animals such as cattle is responsible for the high butyrate content of blood-plasma, milk and butter (Cummings *et al* 2001).

The Embden–Meyerhof–Parnas (EMP) pathway is the major human colonic pathway for the catabolism of glucose, and the formation of significant amounts of acetate by the Wood–Ljungdahl pathway (reductive acetyl co-enzymeA (CoA) Pathway). The overall fermentation in the bovine rumen also proceeds mainly via the EMP pathway, with reductive

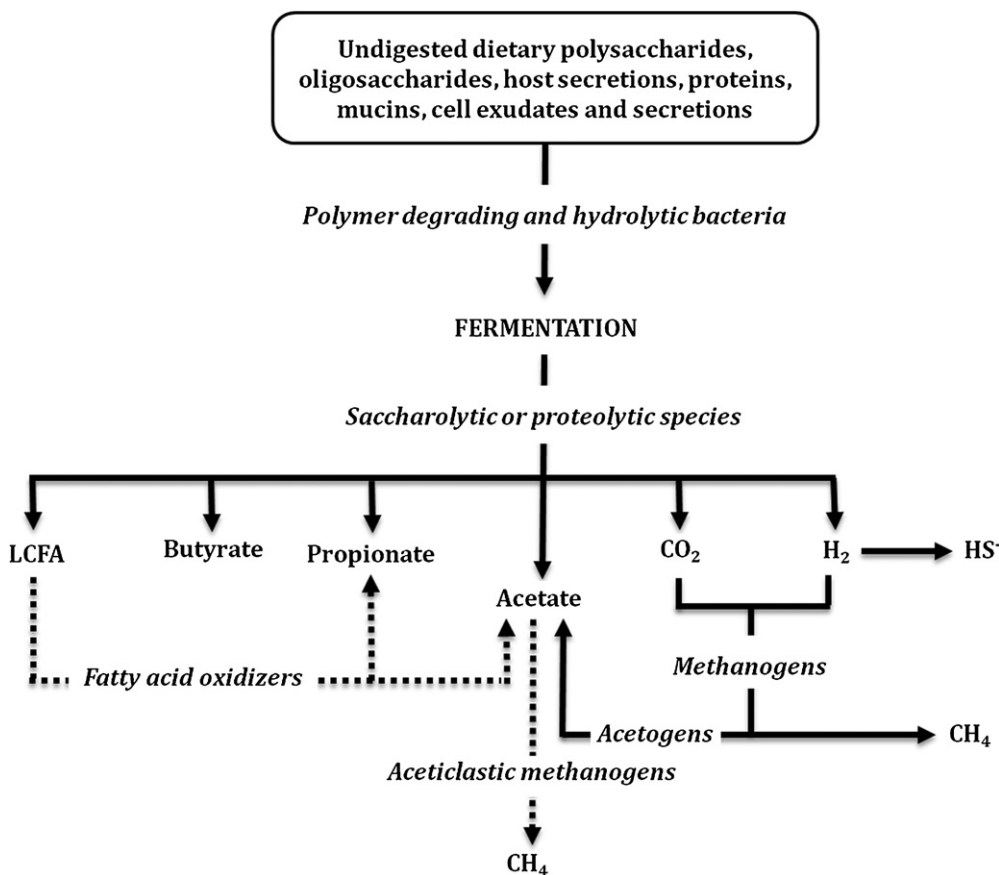


Figure 4. Main VOCs from fermentation in the gastrointestinal tract (large colon).

acetyl co-enzymeA (CoA) pathway accounting for up to one-third of the total acetate, probably associated with the utilization of free H₂ formed as an intermediate fermentation product and the utilization of electrons by homoacetogenic, glucose-fermenting species (Miller and Wolin 1996). The other major sources of acetate are produced from the glucose carbon skeleton, probably by a combination of the homoacetate and propionate fermentations. Propionate is formed by CO₂ fixation pathways, as in the Propionibacteria, and butyrate is formed from two molecules of acetyl CoA, yielding acetoacetyl-CoA, which is then converted to butyryl-CoA (Diez-Gonzalez 1999). Thereafter, butyryl-CoA may yield butyrate by two different mechanisms, either via butyrate kinase (as in some strains of ruminal *Butyrivibrio fibrisolvens*), or via butyryl-CoA:acetate-CoA transferase. The overall scheme is shown in figure 4. Colonic epithelium cells use butyrate (in preference to glucose) as their main energy source (Cummings *et al* 1987) and it has been proposed that butyrate could provide protection from colon cancer and colitis (Archer *et al* 1998, Christl *et al* 1996). Microbial species known to produce butyrate include: *Fusobacterium*, *Faecalibacterium*, *Coprococcus*, *Butyrivibrio* and *Roseburia* (Barcenilla *et al* 2000).

Although direct VOC analysis of the GI tract would prove problematic, research into VOC analysis of the microbial populations within human stool and urine samples has been performed. Direct gas chromatography coupled with a flame ionisation detector was capable of discriminating

between urinary tract infection caused by *E. coli* and *Klebsiella* spp., by analysing spent growth medium from urine samples (Manja 1983). This approach has been further developed using an e-nose system coupled to an automated headspace analyser, which is capable of screening for bacteriuria within clinical urine samples, with the same level of sensitivity as conventional culture (Aathithan *et al* 2001, Pavlou *et al* 2002b). Human faecal matter has a large microbial component and due to colonic fermentation processes gives rise to a diversity of VOCs, which are largely fatty acids and a smaller VSC component (Sato *et al* 2001). GC-MS analysis of ‘stool’ headspace has demonstrated that different gastrointestinal diseases produce distinct VOC profiles (Probert *et al* 2004, 2009). Using multivariate discriminate analysis, significant VOC profile differences have been observed between asymptomatic volunteers and patients with *Campylobacter jejuni*, ulcerative colitis, or *Clostridium difficile* infections (Garner *et al* 2007). Moreover, this approach has been used to characterize the VOC profile of fecal matter from premature neonates (De Lacy Costello *et al* 2008), and with the monitoring of certain VOC profile ‘shifts’ has potential for use as an early indicator of neonate necrotising enterocolitis (Garner *et al* 2009).

Vaginal infection and odour

Bacterial vaginosis is the most common cause of vaginal discharge characterized by an overgrowth of genital tract

organisms and a depletion of lactobacilli (Sobel 2000). Vulvovaginal candidiasis is the next most common caused by *Candida albicans* (90% of cases), *C. glabrata* or *Saccharomyces cerevisiae*. Trichomoniasis is a sexually transmitted disease caused by *Trichomonas vaginalis*, a protozoa. On occasion, vaginal discharge may be seen in cervicitis caused by *Neisseria gonorrhoeae* or *Chlamydia trachomatis* (Buimer *et al* 1996).

Bacterial vaginosis is characterized by a complex change in the vaginal flora. The healthy vaginal flora is dominated by *Lactobacillus* species that produce hydrogen peroxide and produce organic acids (mainly lactic acid) from glycogen in the vaginal epithelium; sufficient to maintain the pH to less than pH 4.7 (Vásquez *et al* 2002). They may also synthesize bacteriocins and other inhibitory compounds. In vaginosis, these lactobacilli are replaced by an increased concentration of other organisms, especially anaerobes such as *Gardnerella vaginalis*, *Mycoplasma hominis*, *Prevotella* spp., *Porphyromonas* spp., *Bacteroides* spp., anaerobic *Peptostreptococcus* spp., *Fusobacterium* spp., or *Atopobium vaginae* (Fredricks *et al* 2005). The change in the microflora at the advent of bacterial vaginosis can also be observed microscopically using Gram stain of smears from discharge samples. Gram-positive, long bacilli are predominant in a healthy microflora whilst Gram variable cocci and coccobacilli are dominant in disease. The symptoms of vaginosis can vary from asymptomatic, to one or more of the following: abnormal vaginal discharge (with an unpleasant odour), itching, burning sensation during urination and discomfort during intercourse. *Gardnerella vaginalis* produces proteolytic carboxylases that decompose vaginal peptides, releasing a variety of amines with fishy odour including putrescine, cadaverine and trimethylamine (Kwak and Preti 2011). These amines volatilize increasingly with rising pH, so patients often note a worsening of this symptom as the vaginal alkalinity is enhanced. Even women considered as clinically normal fall into two types based on their odorant production (Huggins and Preti (1983). These odour differences may be linked to differences in the population of bacterial flora harboured by individual females.

Skin and skin conditions

There is a large resident population of microbial species that reside and live harmlessly on the skin. The cutaneous flora consist of aerobic and facultative cocci of the micrococcaceae family (particularly *Staphylococcus epidermidis*), aerobic diphtheroids of the genera *Corynebacterium* and *Brevibacterium*, the anaerobic diphtheroids (*Propionibacterium acnes*, *P. granulosum*, and *P. avidum*), and yeasts from the genus *Pityrosporum* (Roth and James 1988). Significant variations in both the total number of bacteria and the compositions of bacterial flora exist for different body regions, and these variations reflect differences in the amount of water and nutrients available to support bacterial growth (Roth and James 1988). For example, eccrine sweat glands, which provide water, electrolytes, and minerals; sebaceous glands, which produce a mixture of lipids; apocrine sweat glands, which along with specialized sebaceous glands secrete a substance rich in protein and lipid, all

contribute to the ecology of the skin (Marples 1969). In addition, sweat contains amino acids, and minerals such as copper, iron, magnesium, zinc and calcium (Leyden *et al* 1987), which are important for bacterial growth and metabolism, as well as toxin production by pathogenic bacteria, for example iron dependent toxin production by *Corynebacterium* spp. (Wong and Groman 1984). The outermost region of the skin, the stratum corneum, is a compartment that is constantly being shed and provides a rich source of amino acids necessary for bacterial growth and metabolism. In areas of partial occlusion due to body surface-to-surface contact, such as the axilla, perineum, and toe web space, increase in CO₂ tension and moisture may also be an important factor promoting growth of bacteria.

There are various skin conditions with microbial aetiological agents, including *S. aureus* (Feingold and Elias 1988, Hay and Addriaans 1998), *Strep. pyogenes* (Bisno and Stevens 1996, Hay and Addriaans 1998), *S. epidermidis* (Roth and James 1988, O'Brien and Collin 1992, Hay and Addriaans 1998), in addition to yeasts e.g. *Candida albicans*. Erythrasma is a mild, chronic, localized superficial infection of the skin caused by a group of closely related aerobic coryneform bacteria, particularly *Corynebacterium minutissimum* (Sarkany *et al* 1961, Somerville 1970). Acne vulgaris is another extremely common skin disorder that affects almost all individuals at some time during their life (Brown and Shalita 1998, Cunliffe and Simpson 1998). *P. acnes* is strongly associated with the condition acne vulgaris (Holland *et al* 1981, Brown and Shalita 1998, Holland *et al* 1998), and it has been hypothesized that microenvironmental changes within a pilosebaceous follicle may be important in determining the physiology of the bacterial inhabitants, with consequential effects on the follicle due to the activities of the organisms (Holland *et al* 1978). A number of different types of biologically active compounds are produced by *P. acnes*, including exocellular enzymes (Holland *et al* 1981), complement activating compounds (Massey *et al* 1978), chemotactic substances (Russell *et al* 1976, Gould and Cunliffe 1978), cytotoxic agents (Allaker *et al* 1985) and compounds that are directly inflammatory (such as the VOCs histamine and propionate) (Greenman and Holland 1985, Allaker *et al* 1986). In addition cell components will act as antigenic compounds. It would be expected that these differences in microbial metabolic processes would be reflected in skin VOC profiles. Some studies have now looked at volatiles emanating from skin (Riazanskaia *et al* 2008, Martínez-Lozano and Mora 2009), including one study which sampled, identified, and postulated on the source of a range of compounds as part of a full skin VOC profile (Gallagher *et al* 2008). However, few have looked directly for volatile metabolic indicators of microbial processes.

(i) *Body odour from axilla.* The precursors to axillary odour are produced by the apocrine glands (Shehadeh and Kligman 1963a, 1963b, Zeng *et al* 1992, Gower *et al* 1994), and the characteristic axillary odour of humans is formed by the interaction of odourless, precursor molecules found in apocrine secretions with cutaneous, axillary microorganisms (Shehadeh and Kligman 1963a, Leyden *et al* 1981). It is

generally considered that freshly secreted sweat is sterile and that body malodour (e.g. under arm odour; UAO) is the result of biotransformation of sweat components by micro-organisms living on the surface of the skin around the apocrine glands (in the proximity of the axilla) to produce VOCs that are odoriferous. *In vitro* and *in vivo* studies of the relationship between UAO and the cutaneous microflora have shown the presence of three dominant genera (aerobic coryneforms, *Micrococcaceae* and propionibacteria) with no competition for habitat between these groups (Preti and Leyden 2010 and references cited therein). A relationship has also been established between severity of cases and the dominance of aerobic coryneforms within the axillary microflora populations, with *Corynebacterium xerosis* identified as key (Rennie *et al* 1990). A statistically significant association between population density of aerobic coryneform bacteria and UAO intensity was observed, whilst no such associations could be found between UAO and population densities of staphylococci, micrococci or propionibacteria (Rennie *et al* 1990). With regard to the production of UAO then it had been shown that *Corynebacterium* species are unusual in possessing 'rare' enzymes or metabolic pathways to biotransform 16-androstene steroids into odiferous products e.g. 5 α -androstene, 5 α -androstene, 3 α (β)-androstene, 4,16-androstadien-3-one as well as keto-substituted compounds. All these example compounds were found to be more odiferous than the 16-androstene steroids used as precursors, and found naturally in fresh apocrine sweat secretions (Rennie *et al* 1991). Thus the causal links to the production of high UAO were thought to be clear. However, more recent studies have shown that other pathways to odiferous VOC may be much more important regarding UAO. For example, 3-hydroxy-3-methylhexanoic acid and 3-methylhex-2-enoic acid (and other related acids) are generated via microbial aminoacylase activity from precursors naturally present in axillary secretions (Natsch *et al* 2003, Natsch *et al* 2006). The enzyme is a zinc-dependent N α -acyl-glutamine aminoacylase (N-AGA) produced from *Corynebacterium xerosis*, *Corynebacterium striatum* and other species of *Corynebacterium*, but not in *Staphylococcus* or *Propionibacterium* genus. Other precursors may include the N-terminal amino acid of apolipoprotein D that is putatively covalently bound to 3-hydroxy-3-methyl hexanoic acid, and other key odour acids in axillary sweat (Zeng *et al* 1996, Akiba *et al* 2011). However, these alternate theories regarding biogenesis still correspond with the strong correlation between a high population of corynebacteria and strong axillary odour. These compounds are highly odiferous and their pattern of production may even be associated with MHC-allele-dependent mate selection phenomenon in rodents (Yamazaki *et al* 1976) and there are strong genetic influences in the patterns of VOCs in humans (Preti and Leyden 2010).

Another important class of VOC, sulfanylalkanols (3-sulfanylhexasan-1-ol, 2-methyl-3-sulfanylbutan-1-ol, 3-sulfanyl-pentan-1-ol and 3-methyl-3-sulfanylhexasan-1-ol), possess strong onion-sulfide smell and have been detected in axillary odour. These VOCs are produced from C-S lyase activity of malodour-forming corynebacteria (Natsch

et al 2004) and have broad substrate specificity for cysteine conjugates. The specific enzyme involved is probably the cystathionine- β -lyase enzyme (metC gene product) associated with corynebacteria but not staphylococcus (Natsch *et al* 2004). In addition, *Staphylococcus* spp. and *Propionibacterium* spp. are known to produce a wide range of volatile carboxylic acids, including; isovaleric, isobutyric and propionic acids, as main end-products of the primary fermentation pattern of the species, and may contribute to UAO.

(ii) *Foot odour*. Marshall *et al* (1987, 1988) examined the microflora found on normal feet and found that foot odour was associated with high population densities of staphylococci and aerobic coryneform. Feet with strong odours had significantly higher population densities of microorganisms that produced hydrolytic exoenzymes (lipase, protease, and callous degrading enzymes). Kanda *et al* (1990) found that isomers of SCFAs were the primary components of foot odour, and Kobayashi (1990) found that *Staph. epidermidis*, which is a normal resident of the skin, plays a major role in the development of foot odour. Athlete's foot (tinea pedis) is an infection of the foot caused by fungi called dermatophytes which invade the 'dead' outer layers of the skin (Weitzman and Summerbell 1995). The fungi thrive in warm, moist, and dark environments and the disease was thought to be fairly uncommon before human beings started wearing shoes. Pitted keratolysis occurs worldwide, seen in both tropical and temperate environments, and it can be related to occupation or sport activity. Microbial overgrowth of the normal flora occurs, with species including; *Kytococcus sedentarius*, *Dermatophilus congolensis* or species of *Corynebacterium*, *Actinomyces* or *Streptomyces* (Nordstrom *et al* 1987, Woodgyer *et al* 1985, Longshaw *et al* 2002). Under appropriate conditions such as prolonged occlusion, hyperhidrosis and increased skin surface pH, these species proliferate and produce proteinases that destroy the stratum corneum, creating pits (Holland *et al* 1992). *Dermatophilus congolensis* liberates keratinases in appropriate substrate (Hanel *et al* 1991), and *K. sedentarius* has been found to produce two keratin-degrading enzymes (Longshaw *et al* 2002). The malodour associated with pitted keratolysis is presumed to be the production of sulfur-compound by-products, such as thiols, sulfides, and thioesters (Nordstrom *et al* 1986). Strong foot odour (but without pitted skin changes) has also been described due to overgrowth of *Staph. epidermidis* and production of isovaleric acid from leucine (found in sweat; Ara *et al* 2006). Overgrowth of *Bacillus subtilis* and presence of *Brevibacterium* have also been associated with strong foot odour. The latter produces methyl mercaptan from methionine; a product of enzyme-hydrolysed skin components (Ara *et al* 2006).

(iii) *Wound infection*. When a wound is formed the integrity of the host defence mechanisms is compromised, giving rise to a new ecological niche favourable to microbial growth, which can potentially lead to an adverse effect on the process

of wound healing. It is generally accepted that all acute, traumatic, surgical and chronic wounds become contaminated with microorganisms (Bowler *et al* 2001). This does not necessarily provide a barrier to wound healing, and it is only if a wound progresses towards infection, that there will be a failure to move through the phases of healing in a timely manner (Edwards and Harding 2004). Chronic wounds, including diabetic foot ulcers, leg ulcers and pressure ulcers, are known to be regularly colonized by a large number and diverse species of microorganisms including anaerobic organisms (Howell-Jones *et al* 2005), even when no clinical sign of infection is evident, possibly correlating with critical colonisation (Panuncialman and Falanga 2009). The classic sign of wound infection is inflammation (localized heat, pain, oedema and erythema), but further criteria include abscess, discharge (often purulent), discolouration (within and around wound margins), bleeding (friable) granulation tissue and wound malodour, i.e. VOCs that are odiferous (Edwards and Harding 2004).

There have been few studies of volatiles directly emanating from wounds, or wound associated clinical material, as a method of wound classification or diagnosis of infection. An early research study provided evidence for the use of direct gas-liquid chromatography of clinical wound samples (pus and serous fluid) for rapid presumptive testing of anaerobic, Gram-negative bacilli (Gorbach *et al* 1976), whereby isobutyric, butyric and succinic acid were determined to be signature compounds. This was corroborated by a study analysing wound pus from a variety of superficial and deep infections using the same technique, concluding that it 'provides a rapid and reliable means for the presumptive differentiation of anaerobic from aerobic infection' (Philips *et al* 1976), to the extent that this technique was integrated as a standard protocol in at least one public health laboratory. Moreover, the technique was extended to the analysis of wound swabs, and a similar diagnostic potential was observed (Reed and Sanderson 1979).

Analysis of volatiles directly emanating from fungating breast and head-and-neck cancer wounds (known to have strong odour properties), has been performed by gas chromatography-mass spectrometry-olfactometry (GC-MS-O) to separate and identify the volatile compounds present, and associate these with a corresponding human sensory perception of odour (Shirasu *et al* 2009). Various compounds were detected (e.g. acetic, butyric and isobutyric acid), but the main fungating wound odorant was identified as dimethyl trisulfide ($C_2H_6S_3$), and although no attempt was made to identify its source, it was postulated that this may be the product of bacteria present within the wound bed (Shirasu *et al* 2009). Arrays of conducting polymer sensors (e-nose) have now been developed for the purpose of wound monitoring (Bailey *et al* 2008). One such instrument (the 'wound-monitor') analyses swab material, by first using a solid phase micro extraction (SPME) pre-concentration step, and then together with a pattern recognition system is hoped to assist early and rapid diagnosis of patient wounds (Persaud 2008). Early results have shown good differentiation between *P. aeruginosa* and *S. aureus* cultures (Persaud 2008). The

instrument has also been shown capable of differentiating between infected and uninfected burns patients by pre-concentrating volatiles from swabs and wound dressing material, using principle component analysis (PCA) to analyse the resultant volatile spectrum (Byun *et al* 2010). If any such devices are to be used for direct 'wound sniffing' then one problem to be overcome (for all wound types), is the large diversity of human biological volatiles emanating from skin/wounds unrelated to the microbial community present. These must be further identified and characterized, so that signature microbial metabolic processes or volatile profiles can then be detected for use as a diagnostic tool. This would enable the development of a true 'bedside' clinical tool for rapid and non-invasive wound assessment.

***In vitro* microbial VOC analysis and identification: models for studying VOC production**

The use of VOCs as a tool for microbial identification has been widely applied. As previously discussed, numerous existing biochemical tests are reliant on the detection of microbial VOCs in liquid phase to discriminate at the species level, for example production of indole (*E. coli*), hydrogen sulfide (*Proteus* spp. and *Citrobacter* spp.), and trimethylamine (*E. coli* and *Enterobacter* spp.). Modern VOC analysis tends to focus on one of two approaches (1) identifying VOC biomarkers only produced by particular species or (2) searching for a range of different VOCs, none of which are unique in themselves but together forming a unique pattern. For example, the first approach has been used in the identification of *P. aeruginosa*, using the grape-like odour 2-aminoacetophenone, detected by GC-MS (Cox and Parker 1979, Labows *et al* 1980). However, as with many other 'species-specific' biomarkers, there are reports of the non-specificity of this compound (Allardyce *et al* 2006a), and meticulous design of diagnostic approaches is required if they are to have utility in healthcare facilities.

A more recent approach has been in analysing microbial 'volatile profiles', since this negates the need for large numbers of species-specific VOCs, and instead is reliant on commonly produced VOCs. Different species will differ in their central metabolic pathways of carbon energy sources into fermentation products (summarized in table 2). Distinct pathways include acetone-butanol fermentation (a characteristic of some *Clostridium* species: products include acetone, butanol and ethanol), butanediol fermentation (*Aerobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Serratia*: products include acetate, 2,3-butanediol, butylene glycol, ethanol, carbon dioxide and hydrogen), butyric-butylic fermentation (*Clostridium* species: products include acetate, butyrate, ethanol, isopropanol, carbon dioxide and hydrogen), homolactic fermentation (*Bacillus*, *Lactobacillus*, *Streptococcus*: products include lactate and small amounts of acetate), heterolactic fermentation (*Bacillus*, *Lactobacillus*, *Streptococcus*: products include lactate with significant amounts of acetate and ethanol), mixed acid fermentation (*Escherichia*, *Proteus*, *Providencia*, *Salmonella*, *Shigella*, *Yersinia*: products include acetate, ethanol, formate,

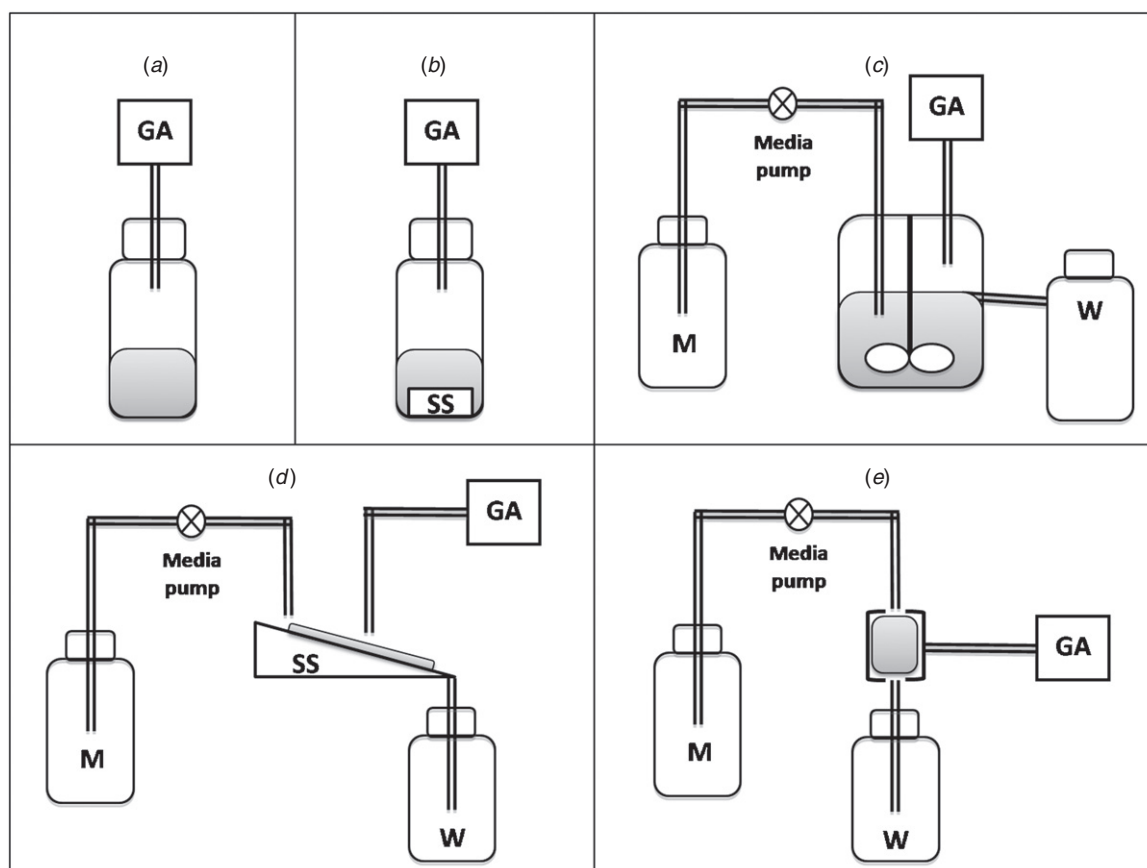


Figure 5. Classes of physical *in vitro* model for measuring VOC production rates from microbes by gas analysis (GA). (a) Planktonic batch culture; microbial headspace can be sampled from closed accumulative models. (b) Batch culture biofilm; a supporting substratum (SS) is provided to enable surface microbial growth. (c) Planktonic continuous culture; enabling steady state growth conditions, as nutrients (growth medium, M) are continuously added and waste products (W) taken away. (d) Continuous flow biofilms using impermeable substratum; nutrients (growth medium, M) are continuously added and waste products (W) taken away, enabling dynamic quasi-steady state microbial biofilms to form. (e) Continuous flow biofilms using highly permeable matrix perfusion substratum; all cells are close to nutrient micro-channels and are served with growth-limiting nutrient at approximately the same rate. More homogeneous environment with all cells growing at nutrient supply rate (i.e. μ proportional to flow rate) forming near true steady state biofilms under controlled conditions.

lactate, succinate, carbon dioxide, hydrogen) and propionic fermentation (*Propionibacterium*: products include acetate, propionate and carbon dioxide). Therefore different species can be differentiated by reference to their principle metabolic products, some of which will be volatile, and this has enabled differentiation and identification of potentially pathogenic organisms *in vitro* (Pavlou *et al* 2002a, Thorn *et al* 2011).

Many different physical *in vitro* models have been used to study growth, metabolism and VOC production in microorganisms, and these are summarized in figure 5. All models break down into two main systems: closed (figures 5(a) and (b)) and open (figures 5(c), (d) and (e)). Open systems have nutrient flow, which brings new nutrients and removes products. Closed systems usually refer to a batch culture where the microorganisms of interest are co-incubated with media and substrates with no additional inputs (i.e. nutrients, media, substrates) or removal of outputs (except those required for analysis). A further distinction can be made for planktonic (i.e. cells in suspension; figures 5(a) and (c)) or biofilm model (figures 5(b), (d) and (e)). Analysis of VOC profiles for a wide number of bacterial species growing planktonically in batch culture mode has now been described

(Bunge *et al* 2008, Thorn *et al* 2011). Although these models can reveal the presence of a wide range of characteristic VOCs for different species, the amounts produced will vary considerably over the period of incubation. In batch culture (figures 5(a) and (b)), growth of the cells goes through different phases (lag, exponential, stationary and decline) and all main physicochemical parameters (substrate concentration, product concentrations, growth rate, growth yield) are never constant. In general, this model is of limited use when trying to extrapolate to how microbes may be behaving *in vivo* (in response to set changes). In contrast, open systems refer to planktonic culture (figure 5(c)) or biofilm models (figures 5(d) and (e)) that have an element of continuous culture, whereby the nutrient and substrate requirements of the culture are constantly replenished and fed into the system, thus supporting continuous growth. Additionally, outputs from the biofilm (i.e. metabolites, VOCs, cells from microbial growth) are able to leave the system and are available for analysis if required, and dynamic steady states are possible (McBain 2009).

For the vast majority of cases of microbial infections (whether skin, oral cavity, lungs or wound) the microbes attach to a local substratum or matrix to form biofilms (Wilson 2001).

Depending on the particular physicochemical conditions, the microbes utilize local nutrient substrates and multiply. The most immediate source of supply of substrates depends upon the composition of the perfusing fluids found in the nutrient domain of the biofilm. For example, supragingival dental plaque is served by nutrients derived from saliva, whilst subgingival plaque is served with crevicular fluid (which is a serum-derived exudate) (Marsh and Martin 1999). Typically, a small amount of growth is sustained by immediately available small molecular weight compounds (sugars, amino acids) dissolved in the fluidic domain of the biofilm. This would be blood and serum for infected wounds, bronchoalveolar lavage fluid for biofilms in the bronchus/lung, or urine and gastric fluids for urinary tract and gastrointestinal infections respectively. For modelling purposes biofilms are grown under closed (figure 5(b)) or open (figures 5(d) and (e)) conditions. For example, caries-like biofilms can be grown by inoculating dental plaque samples onto slices of hydroxyapatite, glass, or plastic surfaces and saliva periodically added or allowed to flow continuously across the biofilm. For a solid impermeable substratum (such as bone and tooth), the bacterial biofilms are only supplying nutrients from above. They form inner layers, intermediate layers and outer layers, such that inner cells are starved of nutrients by the intermediate and outer layers that are more directly exposed to salivary nutrients. A chemical gradient of growth rate limiting nutrients is formed such that the growth rate of the biofilm cells on the inside are close to zero, whilst close to the maximum dictated by local physicochemical conditions on the outside. As a result, there is a wide distribution of both growth rates and molecule residence times within the system as a whole, and hence the biofilm is heterogeneous. This is not the situation on substrata that are freely permeable. For example, biofilms formed on cellulosic or carbon-veil substrata are served with nutrients by local perfusion via micro-channels (figure 5(e)); cells are more equally supplied with nutrients and all grow at similar rates (Spencer *et al* 2007, Greenman *et al* 2008a).

In bad breath research, early experimental studies into oral malodour used a planktonic batch culture model (Tonzetich 1971, McNamara *et al* 1972, Tonzetich and McBride 1981). Kleinberg and Codipilly (1995) examined a range of gram-positive ($n = 13$) and gram-negative ($n = 12$) pure species supplemented with a range of 18 separate amino acids, and showed that gram-negative organisms produced significantly higher malodorous compounds compared to other groups. Although only batch culture, these models nevertheless helped to confirm the importance of S-metabolism in oral malodour. In dental research, a defined consortium of microorganisms is often used to investigate ecological phenomena such as species interaction, communication (quorum sensing) and competition, which would otherwise be difficult to measure for reasons of complexity. Defined consortiums make use of simplified combinations of organisms that are able to grow together when supplied with same media, and are readily identifiable by selective culture techniques. An example of a defined consortium would be the 'Marsh consortium' developed by Philip Marsh (CAMR, Porton down, Salisbury, UK) for use in chemostats. This consortium comprises 10

bacterial strains (Bradshaw *et al* 1996), representing many of the important physiological and ecological groups that exist in plaque. Although, perhaps surprisingly VSC or microbial VOCs have been little studied using planktonic continuous culture models.

Model microcosms are stable mixed cultures of species that can grow together, being derived from real inocula from the particular ecosystem/habitat of interest. They are what remain through survival of the transfer process, from *in vivo* to model. The diversity of a microcosm is usually less than the original inoculum, although it is nevertheless more diverse than a defined consortium. Microcosms have the advantage of maintaining much of the complexity of the original sample, enabling the *in vivo* microbial community dynamics to be replicated within the biofilm model. For example, a constant depth film fermentor (CDFF) was used to develop plaque microcosm biofilms from inoculation with real plaque samples, and subsequently removed and analysed for VSC generation *ex situ* using a portable sulfide analyser (Pratten *et al* 2003). The model is an example of a continuous culture biofilm, but with biofilm formation over an impermeable substratum surface (figure 5(d)). In contrast, the 'Sorbarod' system is an example of a continuous flow biofilm using a highly permeable matrix (the Sorbarod; figure 5(e); Hodgson *et al* 1995). This system has been shown to be capable of maintaining a complex microcosm of tongue-scrape derived inoculums, and this has provided insight into the microbial ecology, physiology and VSC production of oral malodour production (Spencer *et al* 2007, Greenman *et al* 2008b, Taylor and Greenman 2010).

Future research directions

Research methods that elucidate the specific VOCs being produced by microbes or emanate from microbial associated disease processes need to be applied (e.g. GC-MS), before developing more 'VOC focused' or non-specific sensors (e.g. e-nose), as this information will inform and improve their development. Consequently, more research is required to further identify microbial specific VOCs, but also to 'unequivocally' identify the structure of these microbial associated VOCs (Kai *et al* 2009) so as to better enable their future detection. This situation is further complicated by the fact that the specific VOCs produced by a given microbial community *in vivo* will often differ from that *in vitro* due to varying growth substrates, presence of other (often uncultivable) competing microorganisms, and the impact of host processes. It is important to adequately define the 'research question' being investigated, and hence for *in vitro* identification systems substrates should be provided that best discriminate microbial metabolic processes as monitored through VOC analysis. However, when developing *in vivo* detection systems then any *in vitro* characterisation must attempt to best replicate the real-world environment, including provision of appropriate growth substrates, and potentially actual clinical material as has been suggested by one author investigating sinus infection (Preti *et al* 2009). In future it is likely that headspace gas sample VOC profiles

from real conditions (*in situ* from a human) will be compared to an analogous model in the laboratory (see section on *in vitro* modelling). The differences can then be used to identify VOCs arising from the mammalian system alone, against those produced in the model (microbial alone).

Microbial infection is the root cause and/or the complicating factor of many human diseases, and traditional bacterial identification methods (e.g., culture, serological, and genetic methods) are time-consuming (from hours to days), and in many cases it may be difficult or painful to get specimens or samples (e.g. lung disease, infected wound). The ultimate goal of diagnostic VOC analysis would be online sampling of patient breath and/or body odour, enabling bedside or chairside diagnosis of their status *in vivo*. The rapid advancement of online VOC analysis technologies (e.g. SIFT-MS, PTR-MS) goes some way to realizing this goal, but future developments must be focused on miniaturizing technology without losing analytical or clinical sensitivity or specificity. Perhaps one of the most under-researched areas in the application of microbial VOC analysis in the clinic is time-course VOC analysis (of patient or patient samples), to assess treatment success or failure. For example, preliminary investigations have shown e-nose technology capable of following progression towards healing during wound care treatment (Edwards-Jones and Greenwood 2003). However, for this to be more widely used, VOC sampling and analysis will need to be cost effective in terms of both time and money to enable repeat patient measurements to be made.

Conclusion

It is clear that a huge array of microbial metabolic processes give rise to VOCs, and that these processes are dependent on local physicochemical conditions (e.g. anatomical location). VOC analysis as a method to monitor the progression of microbial disease (and response to treatment) has obvious advantages since it would enable timely and effective treatment decisions, reducing the need for broad spectrum antibiotics and potentially reducing the spread of microbial resistance. Moreover, being non-invasive it would have high patient acceptability. Although more research is needed to truly elucidate microbial VOC generating processes, it is envisaged that in future 'VOC profiling' will enable rapid characterisation of microbial associated disease, providing vital information to healthcare practitioners.

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