Sensitivity of pathogenic and commensal bacteria from the human colon to essential oils

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The microbiota of the intestinal tract plays an important role in colonic health, mediating many effects of dietary components on colonic health and during enteric infections. In the context of the increasing incidence of antibiotic resistance in gut bacteria, complementary therapies are required for the prevention and treatment of enteric infections. Here we report the potential application of essential oils (EO) and pure EO compounds to improve human gut health. Nerolidol, thymol, eugenol and geraniol inhibited growth of the pathogens Escherichia coli O157 : H7(VT−), Clostridium difficile DSM1296, Clostridium perfringens DSM11780, Salmonella typhimurium 3530 and Salmonella enteritidis S1400 at a half-maximal inhibitory concentration (IC50) varying from 50 to 500 p.p.m. Most EO showed greater toxicity to pathogens than to commensals. However, the beneficial commensal Faecalibacterium prausnitzii was sensitive to EO at similar or even lower concentrations than the pathogens. The EO showed dose-dependent effects on cell integrity, as measured using propidium iodide, of Gram-positive bacteria. These effects were not strongly correlated with growth inhibition, however, suggesting that cell membrane damage occurred but was not the primary cause of growth inhibition. Growth inhibition of Gram-negative bacteria, in contrast, occurred mostly without cell integrity loss. Principal component analysis showed clustering of responses according to bacterial species rather than to the identity of the EO, with the exception that responses to thymol and nerolidol clustered away from the other EO. In conclusion, the selective effects of some EO might have beneficial effects on gut health if chosen carefully for effectiveness against different species.

INTRODUCTION

Essential oils (EO) are steam-volatile or organic solvent extracts of plants, comprising mainly terpene, terpenoid and other aromatic and aliphatic constituents of fairly low molecular mass (Bakkali et al., 2008). Typically, EO contain a mixture of about 20–60 different compounds with two or three at high concentration (20–70 %). Throughout history, herbs and spices and their constituent EO have been used as antiseptics, to preserve food and to enhance flavour (Shelef, 1984). More recently, EO have been used in animal feeds to combat infection and improve productivity (Wallace et al., 2002, 2010; Newbold et al., 2004; Wallace, 2004; Franz et al., 2010). For example, EO and EO compounds, including thymol, carvacrol and eugenol, reduced the bacterial load in different parts of the gut and lowered the shedding of Clostridium perfringens in broiler chickens (Mitsch et al., 2004). EO also improved productivity in ruminants by altering the community of commensal bacteria in the rumen, altering protein degradation and reducing ammonia losses (Wallace et al., 2002; McIntosh et al., 2003; Wallace, 2004). In man, however, the roles of EO in microbial manipulation and gut fermentation have not been evaluated.

The main targets relating to gut health in man are enteric infection, inflammation and carcinogenesis. Enteric infections by gut pathogens, such as Escherichia coli, Salmonella spp., Campylobacter spp. and Clostridium spp., occur when host defences fail to prevent colonization and proliferation (Sekirov & Finlay, 2009). The complex community of commensal bacteria has a role to play in protecting against this type of infection. They also have a role in gastrointestinal homeostasis. Dysbiosis of intestinal microbiota occurs in ulcerative colitis, irritable bowel syndrome and colon cancer (Moore & Moore, 1995; Flint et al., 2007; Flint & Wallace, 2010; Noor et al., 2010; Sekirov et al., 2010). In a recent cohort study, the microbial community of patients with Crohn’s disease was found to be markedly different from healthy controls and their unaffected relatives (Joossens et al., 2011). Density gradient gel electrophoresis (DGGE) of ribosomal PCR amplicons indicated that numbers of Faecalibacterium prausnitzii and Bifidobacterium

Abbreviations: EO, essential oils; PCA, principal component analysis; PI, propidium iodide; RFU, relative fluorescence units.
Essential oils and gastrointestinal bacteria

(Bif) adolescents were lower and Ruminococcus gnavus higher than in healthy relatives. Thus, if EO are to be useful in promoting gut health in man, their effects on commensal as well as pathogenic bacteria must be determined.

The antimicrobial properties of EO have been demonstrated against a wide range of food micro-organisms, including bacteria, protozoa and fungi (Burt, 2004). Several studies also found that the most pathogenic gut bacteria, E. coli O157 : H7 (Burt & Reinders, 2003; Delaquis et al., 2002), Salmonella typhimurium (Anderson et al., 2002), Campylobacter jejuni (Anderson et al., 2009) and Helicobacter pylori (Bergonzelli et al., 2003) are inhibited by EO in vitro. The aim of the present study was to compare the effects of a range of EO and EO compounds on human pathogenic and commensal intestinal bacteria. Further studies were undertaken to explain the nature of the selectivity of EO against different bacterial species.

METHODS

Chemicals and reagents. The pure oils of clove, coriander and curcuma, a commercial blend of EO (‘Agolin’), analytical grade eugenol, geraniol, geranlyacetate, linalool, methylisoeugenol, nerol and thymol, and chestnut extract were provided by Agolin SA, Bière, Switzerland. The test materials were selected on the basis of traditional and potential commercial usefulness, on their published effects on pathogens and their safety. The EO and EO compounds were >98% pure, while the chestnut extract contained >75% tannins. Stock solutions (100 mg ml⁻¹ in methanol) were stored in air-tight capped bottles at 4 °C in the dark. Propidium iodide (PI) was purchased from Sigma-Aldrich, UK. All other reagents were of analytical grade.

Bacteria. Five species of recognized pathogens were investigated, along with 11 species of commensal bacteria. Clostridium difficile DSM 1296, C. perfringens DSM 11780, Propionibacterium shermanii DSM 4902, Propionibacterium freudenreichii DSM 20271 and Bacteroides (Bac.) thetaiotaomicron 5482 (DSM 2679) were obtained from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Bifidobacterium breve NCFB 2258 and Bif. adolescents NCFB 2204 were from the National Collection of Food Bacteria (Reading, UK). Lactobacillus plantarum NCFB 7220 was from the National Collection of Industrial, Food and Marine Bacteria (Aberdeen, UK). Salmonella typhimurium 3530 and Salmonella enteritidis S1400 were kindly provided by George Grant (University of Aberdeen). E. coli O157:H7 NCTC 12900, a verotoxin-deleted strain, is a human isolate. An aerotrophes acaec L-1-92 (DSM 14667), Eubacterium (Eu.) hallii L-2-7 (DSM 17630), Roseburia inulinivorans A2-194 (DSM 16841), Roseburia hominis A2-181 and F. prausnitzii L-2-6 were isolated from human faeces (Bercella et al., 2000) and are maintained at the Rowett Institute of Nutrition and Health. Salmonella spp. and E. coli were grown in LB medium aerobically, and all others were grown in the liquid form of medium 2 (Hobson, 1969) under CO₂.

Influence of EO on bacterial growth. The influence of EO on the growth of E. coli, S. typhimurium and S. enteritidis was tested by a broth dilution method on 96-well plates. A range of concentrations of EO in methanol was prepared on a dilution plate from the stock solution (100 mg ml⁻¹) and 10 μl was transferred to a culture plate to give final concentrations of 50, 100, 200, 300, 500, 750 and 1000 p.p.m. A 200 μl volume of LB medium, containing 5% overnight culture as inoculum, was transferred to corresponding wells in triplicate. Positive control wells contained only 10 μl methanol. Plate control and negative control wells were set up using sterile water and uninoculated medium, respectively. Plates were sealed with plastic adhesive tape (Fasson S695, catalogue no. SH 236269, Nunc) and growth at 37 °C was measured spectrophotometrically (SpectraMax spectrophotometer, Molecular Devices Corporation) as OD₆₅₀ for 24 h at 30 min intervals. All other bacteria were inoculated as a 5% inoculum in the liquid form of medium 2 (Hobson, 1969) in Hungate-type tubes containing different concentrations of EO in methanol. Incubation was carried out at 37 °C and growth was measured spectrophotometrically (Novaspec II spectrophotometer, Amersham Biosciences) as OD₆₅₀ at different incubation times. The methanol concentration was 5%, which had no effect on the growth of the tested bacteria. The calculation of percentage growth and percentage inhibition by EO treatment was based on the growth of positive controls (Sultanbawa et al., 2009):

Percentage growth= (OD₆₅₀/OD₆₅₀) × 100

Percentage inhibition= 100 – percentage growth

where OD₆₅₀ is the OD₆₅₀ of test samples at incubation time t, OD₆₅₀ is the positive control at time zero (0), OD₆₅₀ is the positive control at time t and OD₆₅₀ is the positive control at time zero (0).

Measurement of cell integrity. The influence of EO on the cell integrity of bacteria was determined by a PI uptake method based on Amor et al. (2002) and later modified by Maia et al. (2007). Briefly, 1 ml of overnight culture was inoculated into 9 ml M2 medium and incubated at 37 °C until it reached mid-exponential phase (OD₆₅₀ approx. 0.6). The bacterial culture was centrifuged at 3000 g for 10 min at 4 °C. The pellet was washed twice with anaerobic potassium phosphate buffer (100 mM, pH 7.0) containing 1 mM DTT. Cells were resuspended to OD₆₅₀ 0.4 in the same buffer for assay. Dilution series of EO were prepared in methanol in separate 96-well plates and 10 μl was added to 200 μl of cell suspension. Control cultures contained the same volume of methanol, which did not affect measurements. The suspension was incubated at 37 °C for 30 min. Cell suspensions without EO and sonicated cells (10 μm amplitude, 3 min; MFE Soniprep 150) served as controls. A working solution of PI (1.5 mM) was prepared in distilled water and stored at 4 °C in the dark. Fifty microlitres of each sample was added to 149 μl of PI working solution. Fluorimetry measurements were done using a Gemini XPS Microplate Reader (Molecular Devices Corporation) at λex=530 nm and λem=620 nm. Calculation of percentage cell integrity loss was based on the relative fluorescence units (RFU) of the positive control (sonicated damaged cells):

Percentage cell integrity loss=(RFU_treatment/RFU_positive control) × 100

Principal component analysis (PCA). PCA (Jolliffe, 2002) of the data was carried out in order to look for patterns of similarity and clustering in the organisms and compounds. For the PCA, each of the
99 combinations of organisms and compounds was considered as an observation. The growth inhibition and cell membrane damage at each of the seven doses were considered as variables, leading to 14 variables in total, and so a 99 by 14 data matrix. Scores plots of the first two components then show a map of the similarities and differences among the organism and compound combinations.

Statistical analysis. Growth inhibition data are presented as mean ± SD of triplicate observations. Data on membrane integrity loss were transformed to log values and analysed using one-way ANOVA, and means for the treatments were separated by Bonferroni post-hoc multiple comparisons in SPSS 19 software with significance set at $P < 0.05$. The PCA was performed with R (R Foundation for Statistical Computing, ISBN 3-900051-07-0; http://www.R-project.org).

**RESULTS**

**Differential effects of EO on bacterial growth**

The growth of human gut bacteria in the presence of a range of EO or EO compounds at concentrations ranging from 50 to 1000 p.p.m. was investigated. Most pathogenic strains of *E. coli* O157 : H7, *Salmonella* spp., *Clostridia* spp. and the abundant commensal Firmicutes and Bacteroidetes were affected to different extents by different EO in a dose-dependent manner. Fig. 1 illustrates the type of growth data that were obtained. The measurement of percentage growth compares the optical density of bacteria in EO-containing medium with that of a parallel non-amended culture at the entry into stationary phase. Thymol showed the strongest effect on the growth of all bacteria, with most of the bacteria unable to grow at 300 p.p.m. (Fig. 1a). In contrast, clove oil was less toxic to all the bacteria at similar concentrations (Fig. 1b). Eugenol, the major EO compound present in clove oil, had similar effects on the growth of *E. coli*, *S. typhimurium* and *C. difficile*, while the commensal species *L. plantarum*, *Eu. hallii* and *Bif. adolescentis* were less affected (Fig. 1c).

In order to condense large quantities of growth data to values that enable comparison between species and their response to different EO, IC$_{50}$ values of EO were calculated by linear interpolation. Most of the EO showed an IC$_{50}$ below 500 p.p.m. for pathogenic bacteria, while there was a wide range of effectiveness with different EO. Nerolidol and thymol were the most active EO, with IC$_{50}$ values of 41 and 108 p.p.m. for *C. difficile* and 156 and 111 p.p.m. for *E. coli* O157 : H7, respectively (Table 1). Clove oil, coriander oil, curcuma oil and Agolin blend were effective at higher concentration ranges of 234 and up to 1000 p.p.m. Similar effects were observed with pure compounds of EO such as eugenol, geraniol, linalool and methylisoeugenol, which are major constituents of a number of EO, particularly clove oil (eugenol) and geranium oil (geraniol). Nerolidol also had strong growth-inhibitory effects against most of the commensals, with IC$_{50}$ values of 33–244 p.p.m. depending upon the bacterial species. Thymol had similar effects against pathogens, and most commensals were affected by low concentrations. *F. prausnitzii* L2-6, in particular, was highly sensitive to all EO relative to all other commensals and the pathogens. However, most other commensals were insensitive to many EO at the concentrations that were effective against pathogens.

![Fig. 1. Differential inhibitory effect of EO on human intestinal pathogenic and commensal bacteria; (a) thymol, (b) clove oil and (c) eugenol. Percentage growth due to treatment was determined by taking growth in medium containing no EO as 100% at stationary phase. Results are mean ± SD of triplicate observations.](image-url)
Table 1. IC\textsubscript{50} values of EO on pure cultures of human gut bacteria

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Eugenol</th>
<th>Geraniol</th>
<th>Geranylacetate</th>
<th>Linalool</th>
<th>Methylisoeugenol</th>
<th>Nerolidol</th>
<th>Thymol</th>
<th>Clove oil</th>
<th>Coriander oil</th>
<th>Curcuma oil</th>
<th>Agolin blend</th>
<th>Chestnut extract</th>
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<tr>
<td>Variability (SEM as percentage of mean)*</td>
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<td>6.9</td>
<td>5.7</td>
<td>15.0</td>
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*Variability of means was not considered for doses >1000 p.p.m.
Influence of EO on cell integrity

The influence of EO on the cell integrity of different species of pathogenic and commensal bacteria was investigated using PI, which fluoresces when it reacts with DNA. The relative fluorescence was compared between undamaged cells, sonicated cells and cells exposed to EO. Typical results are illustrated in Fig. 2, where thymol or nerolidol were added to suspensions of *E. coli* O157 : H7 at 50, 100, 200 and 300 p.p.m. for 30 min. Thymol caused a dose-dependent loss of cell integrity in *E. coli* (Fig. 2a). In contrast, nerolidol had no effect at any concentration (Fig. 2b), despite having an IC\textsubscript{50} of 156 p.p.m. for growth (Table 1).

When the full range of bacteria, including both pathogens and commensals, was compared for cell integrity loss and growth inhibition by EO, a clear differential effect was observed (Fig. 3). In general, Gram-negative bacteria clustered along the y axis (Fig. 3a), indicating that cell integrity loss was not high and that growth inhibition occurred without loss of cell integrity. Gram-positive bacteria, on the other hand, tended to cluster along the x-axis, with only a few clusters at the top right of the graph (Fig. 3b). Thus, Gram-positive bacteria were much more susceptible than Gram-negative species to cell envelope disruption by EO, although this in itself was insufficient to cause growth inhibition.

When the results were analysed by PCA (Fig. 4), cell integrity loss explained 76% of the variability in growth inhibition. Patterns linking organisms and EO were identified by ellipses drawn on the PCA, based on our biological understanding of the species and compounds, rather than a statistically based cluster analysis, which would lack the biological interpretation. The observed effects could be explained with five clusters: cluster 1 revealed high growth inhibition of *Bac. thetaiotaomicron* relative to cell integrity loss; clusters 2 and 3 were mainly due to *C. perfringens* and *R. inulinivorans*, respectively, showing growth inhibition mainly due to high cell integrity. Cluster 4 was mainly due to *F. prausnitzii*, and cluster 5 revealed a subset of most other clusters and mainly contained the effects of thymol, nerolidol and geraniol on most of the bacteria. This plot also visualized the distinction between Gram-positive and Gram-negative species, as they clustered far from each other.

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**Fig. 2.** Relative fluorescence of PI as a measure of cell integrity loss of *E. coli* O157 : H7 treated with (a) thymol (T 50, T 100, T 200 and T 300: 50, 100, 200 and 300 p.p.m.) and (b) nerolidol (N 50, N 100, N 200 and N 300: 50, 100, 200 and 300 p.p.m.), at 30 min exposure (n=4). Different letters differ significantly (P<0.05).

**Fig. 3.** Correlation plots of the effect of EO on growth inhibition and on cell integrity loss. Plots were obtained from mean observations of 11 EO at seven doses in duplicate. (a) Gram-negative bacteria (*E. coli*, *S. typhimurium*, *S. enteritidis*, *Bac. thetaiotaomicron*), (b) Gram-positive bacteria (*C. perfringens*, *Bif. adolescentis*, *Bif. breve*, *R. inulinivorans* and *F. prausnitzii*).
**DISCUSSION**

EO and their component compounds have been used by mankind for many centuries, and their antibacterial effects have been determined on various microbial species during recent years. The present work reports the results of an investigation of an unusually wide range of EO and bacterial species not investigated previously, in a depth that enabled PCA analysis to reveal correlations between different EO and different types of bacteria. This is also the first report of the influence of EO on commensal gut bacteria, which, if EO are to be used therapeutically or for prophylaxis, is an essential element of background information. The results form the first phase of possible development of EO as health-enhancing food additives/supplements in man.

The potency of the different EO depended on their chemical structure. The categories of EO compounds included (A) phenol derivatives (e.g. thymol), (B) alcohol derivatives [e.g. geraniol (Bg), linalool (Bl)] and (C) ester derivatives (e.g. geranylacetate) of monoterpenes; (D) alcohol derivatives (e.g. nerolidol) of sesquiterpenes; (E) phenol derivatives (e.g. eugenol) and (F) methoxy derivatives (e.g. methylisoeugenol) of aromatic compounds. The IC50 values and thus the overall antibacterial effects of EO compounds against C. difficile were in the order D>A>Bg>Bf>C>E>Bh, for C. perfringens D>A>F>Bg>C=E>Bh, for E. coli A>D>Bg>F>E>Bf>C, for S. typhimurium A>Bg>E>D>G>Bf>C, for S. enteritidis A>Bg>E>Bf>F>C. These relative potencies were quite similar when tested against most of the commensals (Table 1). Thus, sesquiterpenes and phenolic monoterpenes were the most active class of antibacterial agents followed by other phenolic aromatic compounds. A twofold lower activity of geranylacetate, compared with geraniol, which is an ester derivative of geraniol, indicates the importance of the free -OH group for this activity. Similar observations were made before, whereby the inhibitory effects of carvacrol against Bacillus cereus (Ultee et al., 2002), E. coli (Veldhuizen et al., 2006) and Staphylococcus aureus (Veldhuizen et al., 2006) were completely abolished in its methyl ester derivative. In those studies, the substituted groups and delocalized electrons in the benzene ring were postulated to be responsible for this difference in activity. It is important to note, however, that EO mixtures and natural EO may have activities different from those that might be expected based on the potency of pure EO compounds. Here we have established that such interactions depend on the bacterial species. For E. coli, C. difficile and L. plantarum, the results for eugenol and clove oil were very similar, while for S. typhimurium eugenol alone was clearly more effective. On the other hand, the sensitivity of Bif. adolescentis and Eu. hallii to clove oil was greater than that to eugenol, suggesting that a minor component of clove oil may act synergistically with eugenol. These observations have implications for the use of natural EO, in which changes in the chemical composition can occur by seasonal variation, growth period and geographical region (Mabrouk et al., 2011). Thus, careful quality control would be necessary when applying natural EO therapeutically or prophylactically.

The possible application of EO in manipulating the human gut microbiota to treat or prevent bowel diseases and enteric infections will depend on understanding the inhibitory...
profile of both pathogenic micro-organisms and the more abundant normal flora of the gut. Here, different EO showed different inhibitory effects on different species of bacteria. However, in general, EO were more inhibitory towards pathogens than commensals, a finding that confirms a similar conclusion made for porcine intestinal bacteria (Si et al., 2006). A very significant exception was, however, the greater sensitivity of F. prausnitzii to virtually all EO and EO compounds than the pathogens. F. prausnitzii plays an important anti-inflammatory role in the gut (Sokol et al., 2008), and lower numbers of F. prausnitzii are associated with Crohn’s disease (Marteau et al., 2001). Thus, the use of EO in man would have to guard against the suppression of F. prausnitzii as well as proving efficacy against pathogens.

A study of the relationship between effects of EO on growth and their effects on cell integrity was undertaken in order to understand better the mechanisms by which different EO interact with and inhibit the growth of different bacterial species. While some of the data were difficult to explain, such as the difference between thymol and nerolidol in their effects on E. coli (Fig. 2), a general pattern emerged that the growth of Gram-negative bacteria was inhibited generally without a loss of cell integrity, while the opposite was true for Gram-positive bacteria. PI, which fluoresces when it interacts with DNA, was the indicator of cell integrity damage, as it has been in many other studies (Gill & Holley, 2006a). These results appear to contrast with observations reported in E. coli (Gill & Holley, 2006a), in which E. coli treated with 10 mM eugenol results in 100% staining of the cell and 100% cell death; however, this concentration corresponds to 1640 p.p.m., much higher than the concentrations used here. The same authors concluded that the primary action of EO in E. coli was against membrane-bound ATPases (Gill & Holley, 2006b). Our observations would also support the hypothesis that growth inhibition of gut bacteria by EO is not solely the result of membrane damage. The cellular membrane has a selective and low permeability for polar and charged particles. Lipophilic compounds such as cyclic hydrocarbons, including EO, can easily penetrate the membrane and increase the loss of ATP and intracellular metabolites, but more specific metabolic inhibition may occur with specific EO compounds (Di Pasqua et al., 2007).

PCA was used to analyse in greater depth the relationship between growth inhibition and cell integrity loss by EO in different species. The intention of PCA is to take a high-dimensional set of observations (in this case the 14 values that specify the dose–responses in growth inhibition and membrane damage) and reduce the dimensionality by calculating summaries of them which aim to capture as much as possible of the total variability. By plotting the scores of each observation (organism/compound combination) from the first few components, a map was produced of the similarities and differences among the organism/compound combinations (Fig. 4). Thus, observations close in the score plot share similar dose–response characteristics. The orientation of the axes is arbitrary: only proximity or distance is relevant. The PCA scores plot confirmed that the antimicrobial effects due to loss in cell integrity were specific to bacterial species, as shown by clusters 1, 2, 3 and 4 (Fig. 4), while Gram-negative bacteria (clusters 1 and 4) and Gram-positive bacteria (clusters 2 and 3) clustered more tightly. Some EO, such as thymol, nerolidol and geraniol, showed generalized effects and were more strongly associated with cell integrity loss and growth inhibition than other EO and EO compounds (cluster 5). These differences in clusters due to both micro-organisms and the EO illustrate the importance of the chemical nature of EO and the type of bacterium to an understanding of specific inhibitory effects.

In conclusion, although pathogenic species were generally more sensitive to EO than most commensal bacteria, EO may compromise F. prausnitzii, one of the most beneficial of the commensal microbiota. The possible usefulness of EO therefore depends on many factors. If the site of inhibition of pathogens precedes the large intestine, i.e. the stomach or small intestine, EO might be selected that are absorbed before reaching the terminal ileum or are metabolized by the intestinal microbiota to avoid toxicity to F. prausnitzii. Alternatively, combinations of EO compounds may be sought that increase the selectivity towards pathogens. Further information is thus required on differential effects of EO in mixed cultures of gut microbiota and in vivo to formulate different dietary regimes for specific inhibition of pathogens during enteric infections, and to treat or prevent colonic dysbiosis (Kaefer & Milner, 2008; Moore & Moore, 1995).

ACKNOWLEDGEMENTS

The authors thank Nest McKain for her expert technical assistance, and Graham Horgan of Biomathematics and Statistics Scotland for help with data analysis. D. T. received an Industrial Studentship from the University of Aberdeen and Agolin SA, Switzerland, supplemented by the Scottish Overseas Research Student Awards Scheme (SORSAS). The Rowett Institute of Nutrition and Health is funded by the Research Executive for Rural and Environment Research and Analysis Directorate of the Scottish Government (RERAD).

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