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### Accepted Manuscript

Aroma compound diacetyl suppresses glucagon-like peptide-1 production and secretion in STC-1 cells

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2	in STC-1 cells
3	
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18	Abbreviated running title: Diacetyl suppresses the satiety hormone GLP-1
19	
20	

#### Abstract 21

22

23	Diacetyl is a volatile flavour compound that has a characteristic buttery aroma and is widely
24	used in the flavour industry. The aroma of a food plays an important role in food palatability
25	and thus intake. This study investigates the effect of diacetyl on the satiety hormone,
26	glucagon-like peptide (GLP-1), using the enteroendocrine cell line, STC-1. Diacetyl
27	decreased proglucagon mRNA and total GLP-1 from glucose stimulated STC-1 cells. This
28	dampening effect on GLP-1 appears to be mediated by increasing intracellular cAMP levels,
29	increasing synthesis of the G protein coupled receptor, GPR120, and its recruitment to the
30	cell surface. Voltage gated Ca <sub>2+</sub> channels, $K^{+}_{ATP}$ channels and the $\alpha$ -gustducin taste pathway
31	do not appear to be involved. These findings demonstrate that components contributing to
32	food palatability suppress GLP-1. This ability of diacetyl to reduce satiety signals may
33	contribute to overconsumption of some palatable foods.
34	
35	Keywords
36	Glucagon-like pentide 1

#### Keywords 35

- Glucagon-like peptide 1 36
- Diacetyl 37
- Flavour compound 38
- Satiety 39
- **GPR120** 40

#### 41 **Chemical Compounds**

42 Diacetyl (PubChem CID:650); Nicardipine (PubChem CID:41114); Nitrendipine (PubChem

CID: 4507); Tolbutamide (PubChem CID 5505) 43

### **1. Introduction**

46	The gut hormone glucagon-like peptide 1 (GLP-1) has attracted considerable interest in
47	recent years due to its ability to enhance glucose-dependent insulin secretion, promote
48	pancreatic $\beta$ -cell proliferation and reduce food intake. It has also been reported that GLP-1
49	may reduce the rewarding and reinforcing properties of palatable foods (Dickson, Shirazi,
50	Hansson, Bergquist, Nissbrandt, & Skibicka, 2012). Secretion of this nutrient-responsive
51	hormone is impaired in obesity and type 2 diabetes (Toft-Nielsen, et al., 2001) and infusion
52	of GLP-1 has been shown to improve glycemia and reduce food intake in obese patients
53	(Nauck, et al., 1998).
54	GLP-1 is produced by L cells of the distal jejunum and ileum following tissue-specific
55	proteolytic processing of the <i>proglucagon</i> gene (Baggio & Drucker, 2007). The arrival of
56	carbohydrate, fat and protein in the gut lumen triggers GLP-1 release (Bruen, O'Halloran,
57	Cashman, & Giblin, 2012). Inhibition of GLP-1 by food has not been extensively studied.
58	Within the L cells, GLP-1 secretion occurs in response to an increase in intracellular levels of
59	cyclic adenosine monophosphate (cAMP) and $Ca^{2+}$ (Tolhurst, Reimann, & Gribble, 2009).
60	Changes in these mediators are brought about by nutrient uptake pathways, metabolic
61	closures of potassium channels and/or activation of nutrient-responsive G protein-coupled
62	receptors (GPCRs) (Reimann, et al., 2012). These GPCRs play various roles in GLP-1
63	secretion. The taste GPCRs, T1r3/T1r2, and the G protein $\alpha$ -gustducin are involved in the
64	secretion of GLP-1 in response to sugars (Jang, et al., 2007). On the other hand, free fatty
65	acids (FFA) can induce GLP-1 secretion via the GPCRs, GPR40 and GPR120 (Hirasawa, et
66	al., 2005; Reimann, et al., 2012). GPR120 is highly expressed in enteroendocrine L cells
67	(Anbazhagan, et al., 2016; Hirasawa, et al., 2005) and is activated in response to unsaturated
68	long-chain free fatty acids (FFAs) (Tanaka, Yano, Adachi, Koshimizu, Hirasawa, &
69	Tsujimoto, 2008). Recent evidence also suggests that GPR120 plays an important role in the

70	orosensory detection and preference for fats (Cartoni, et al., 2010). GPR120 is classified as a
71	$G_{q/11}$ -coupled receptor, capable of increasing GLP-1 secretion via phospholipase C $\beta$ and
72	intercellular Ca <sup>2</sup> signalling (Blad, Tang, & Offermanns, 2012) although there is also some
73	evidence of signalling promiscuity (Reimann, et al., 2012; Tsukahara, et al., 2015).
74	While the role of taste receptors and tastants in GLP-1 secretion has been widely investigated
75	in recent years, there is less information available on whether aromatic compounds may also
76	influence satiety signals. The aroma of a food plays an important role in food palatability and
77	intake (Massolt, van Haard, Rehfeld, Posthuma, van der Veer, & Schweitzer, 2010;
78	Ruijschop, Boelrijk, de Graaf, & Westerterp-Plantenga, 2009). While such effects may be
79	mediated through neural pathways, it is also possible that aroma compounds may influence
80	satiety signals. Indeed, it has been demonstrated that food-derived odorants present in the gut
81	lumen may stimulate serotonin release via olfactory receptors present in human
82	enterochromaffin cells (Braun, Voland, Kunz, Prinz, & Gratzl, 2007) and aroma intensity
83	certainly influences perceived satiation (Ruijschop, et al., 2009).
84	Diacetyl (2,3-butanedione) is a volatile favour compound that occurs naturally in several
85	foods, such as butter, milk, cheese, fruit and coffee and has a characteristic buttery aroma
86	(Bartowsky & Henschke, 2004). It is primarily produced by citrate fermenting lactic acid
87	bacteria during pyruvate metabolism. It is widely used in the flavouring industry. This
88	pleasant buttery aroma is perceived as a positive attribute by consumers and has been shown
89	to play a significant role in food preference and palatability (Liggett, Drake, & Delwiche,
90	2008).
91	The present study was undertaken to evaluate whether diacetyl alters GLP-1 production and
92	secretion, using the murine secretin tumor cell line (STC-1). STC-1 is a popular and reliable
93	enteroendocrine model to investigate gut hormone production and secretion. Similar to native

- 94 L cells, STC-1 cells secrete GLP-1 in response to sugars, peptides, fatty acids, sweeteners,
  - 4

95	bitter tastants, food bioactives, hormones and bile (McCarthy, Green, Calderwood,
96	Gillespie, Cryan, & Giblin, 2015). However levels of response may differ to the native state
97	(Kuhre, et al., 2016). STC-1 cells also express GPR120, taste receptors (T1r1, T1r2, T1r3)
98	and α-gustducin (Hirasawa, et al., 2005; Wu, Rozengurt, Yang, Young, Sinnett-Smith, &
99	Rozengurt, 2002) and are recognized as a good model for taste signalling (Saitoh, Hirano, &
100	Nishimura, 2007).
101	
102	
103	2. Materials and methods
104	2.1. Chemicals
105	Diacetyl (2,3 butanedione), KREBS ringers bicarbonate buffer, nicardipine, nitrendipine,
106	tolbutamide, pertussis toxin, polyethylene glycol, DMSO, Hanks Balanced Salt Solution,
107	DMEM media, glucose, L-glutamine, Foetal Bovine Serum, penicillin, streptomycin, 3-
108	Isobutyl-1-methylxanthine (IBMX), forskolin, poly-1-lysine coated glass-slides,
109	paraformaldehyde, HEPES, NaCl, EDTA, ethylene glycol tetraacetic acid, Nonident P40,
110	dithiothreitol, Na <sub>3</sub> VO <sub>4</sub> , phenylmethysulphonyl fluoride, aprotinin, leupeptin, NaF, NaPPi,
111	Tris-HCL, SDS, glycerol, bromophenol blue, Tween-20, Phosphate Buffered Saline (PBS),
112	Tris buffered saline, Triton X-100, paraformaldehyde, Bovine Serum Albumin (BSA),
113	donkey serum and $\beta$ -actin horseradish peroxidase antibody were all sourced from Sigma
114	Aldrich, Ireland. Alpha-linolenic acid was sourced from Cayman Europe, Estonia. Glyoxyl
115	buffer was sourced from Ambion, USA. Rabbit anti-GPR120 antibody was purchased from
116	Life Span Biosciences, USA. Donkey anti-rabbit Alexa 488 antibody was purchased from
117	Santa Cruz Biotechnology, USA and horseradish peroxidase-conjugated anti-rabbit IgG was
118	purchased from Jackson Immunoresearch, USA. Alamar Blue, NuPAGE 4-12 % Bis-Tris
119	Gels and TOPO-TA cloning kit were purchased from Invitrogen, USA. QIAzol Lysis

120 Reagent Qiagen miRNeasy Mini Kit, QIAquick PCR Purification Kit and QuantiTect reverse 121 transcription kit were sourced from Qiagen, UK. LightCycler 480 SYBR Green I Master kit 122 was sourced from Roche Diagnostics, Germany. Halt Protease and Phosphatase Inhibitor were purchased from Thermo Fisher Scientific, USA. Total GLP-1 MSD<sup>®</sup> metabolic assay 123 124 was purchased from Meso Scale Discovery, USA. The cAMP HTRF kit was purchased from 125 Cisbio Bioassays, France. Pierce Biotechnology, USA supplied the BCA protein assay kit 126 and the chemiluminescence Western blotting Substrate Kit. Polyvinylidene difluoride 127 membranes were purchased from BIO-RAD, France. Non-fat dry milk and skim milk were 128 purchased from Marvel, Premier Foods Limited, UK. 129 Stock solution of nicardipine (5 mM) was prepared in polyethylene glycol. Stock solution of 130 nitrendipine (1 mM) was prepared in DMSO. Tolbutamide stock solution was prepared in DMSO at a concentration of 100 mM. Stock solution of Pertussis toxin (50 µg/ml) was 131 132 prepared in distilled H<sub>2</sub>O. All test compounds were prepared fresh, immediately before 133 experiments in KREBS Ringers bicarbonate buffer (0.0468 g/l MgCl<sub>2</sub>, 0.34 g/l KCl, 7 g/l NaCl, 0.1 g/l Na<sub>2</sub>HPO<sub>4</sub>, 0.18 g/l NaH<sub>2</sub>PO<sub>4</sub>, 1.8 g/l D-glucose, 1.26 g/l NaHCO<sub>3</sub>) unless stated 134 135 otherwise. Alpha-linolenic acid (100 µM) was prepared in pre-warmed KREBS and sonicated 136 to aid solubility.

137

### 138 2.2. STC-1 exposure to diacetyl and blockers

139 STC-1 was received as a kind gift from by Prof. B. Wice (Washington University, St. Louis,

140 USA) with permission from Prof. D. Hanahan (Swiss Institute for Experimental Cancer

141 Research, Switzerland). STC-1 cells were cultured in DMEM containing 4.5 g/l of glucose

and L-glutamine and supplemented with 17.5 % foetal bovine serum, 100 U/ml of penicillin

and 100 mg/l of streptomycin. Cells were passaged upon reaching 80-90 % confluence.

144 Experiments were performed with cells at passage numbers 15-30 (Gonzalez-Abuin,

145	Martinez-Micaelo, Blay, Green, Pinent, & Ardevol, 2014). STC-1 cells were seeded into 6-
146	well plates at a density of 1.5 x $10^6$ cells/well and incubated at 37 °C, 5 % CO <sub>2</sub> , for 18 h prior
147	to exposures. Cell monolayers in each well were washed with KREBS (1 ml) and then pre-
148	incubated for 1 h with KREBS (500 $\mu$ l). Following the pre-incubation period, KREBS was
149	aspirated off and replaced with 1 ml of diacetyl, freshly prepared in KREBS, at various
150	concentrations. Cells were exposed to diacetyl solution for 10 min, 30 min, 1 h, 2 h, 3 h and 4
151	h in sealed plates at 37 °C, 5 % CO <sub>2</sub> . STC-1 cells were incubated with the blocker of interest
152	for 15 min prior to exposure to 1000 ppb of diacetyl for a further 4 h. The final working
153	concentrations of the blockers, prepared in KREBS buffer, were as follows: 5 $\mu$ M nicardipine
154	and nitrendipine, 100 $\mu$ M tolbutamide and 10 $\mu$ g/ml pertussis toxin, as previously described
155	(Chen, Wu, Reeve, & Rozengurt, 2006; McLaughlin, Lomax, Hall, Dockray, Thompson, &
156	Warhurst, 1998; Ristoiu, Pluteanu, Flonta, & Reid, 2002).
157	
158	2.3. Cellular viability
159	Following the 1 h pre-incubation step in 10 µl of KREBS, 100 µl of various concentrations of

diacetyl (500 ppb, 1000 ppb and 2000 ppb) were added to  $1 \times 10^5$  STC-1 cells/well. Alamar

161 Blue (10  $\mu$ l) was added to each well and the plate was incubated for 4 h at 37 °C.

162 Fluorescence was measured at 570 nm. STC-1 cell viability in the presence of each blocker

163 was also tested. Data were expressed as % viability of STC-1 cells exposed to test

164 compounds compared to STC-1 cells exposed to KREBS alone.

165

166 *2.4. Real time-PCR* 

167 Following exposures, cell monolayers were washed with 1 ml of Hanks Balanced Salt

168 Solution. Cells were then lysed, using QIAzol Lysis Reagent and total RNA was isolated

169 from cell suspensions using the Qiagen miRNeasy Mini Kit, according to the manufacturer's

- instructions. Total RNA was quantified spectrophotometrically, using the Nanodrop 1000
- 171 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5 %
- 172 glyoxyl gel with 1 X glyoxyl buffer. Complementary DNA was prepared from 1 µg of RNA,
- using the Qiagen QuantiTect reverse transcription kit. Real time PCR was performed in a
- 174 LightCycler 480 instrument (Roche Diagnostics, Germany) to quantify proglucagon (of
- which exon 4 codes for GLP-1), GPR40 and GPR120 mRNA levels. All primers were
- 176 designed across intron/exon boundaries. Primers for murine proglucagon were designed,
- based on the GenBank sequence (accession Z46845): Forward primer 5'
- 178 AGGGACCTTTACCAGTGATGTGA- 3', Reverse primer 5'-
- 179 ACGAGATGTTGTGAAGATGGTTGT -3'. The annealing temperature for amplification
- 180 was 56 °C. Primers for murine *GPR40* were designed, based on the Genbank sequence
- 181 (accession AB095745): Forward Primer 5' –TGCCCGTCTCAGTTTCTCCATTC- 3',
- 182 Reverse primer 5' –TGTTCCCAAGTAGCCAGTGACCAG- 3'. Primers for murine *GPR120*
- 183 were designed, based on the Genbank Sequence (accession AY288424): Forward primer 5'

184 GGCCCAACCGCATAGGAGAAAT- 3', Reverse primer 5' –

185 TGAAGGCCACCACCAGAAGAAAA- 3'. The annealing temperature for amplification

186 for GPR40 and GPR120 primers was 50 °C. Plasmid standards for proglucagon were created

- 187 by cloning an amplified PCR product into the pCR4-TOPO vector, using the TOPO-TA
- 188 cloning system, according to the manufacturer's instructions. Standards for *GPR40* and
- 189 GPR120 were created from amplified PCR products, which were purified using a QIAquick
- 190 PCR Purification Kit, according to the manufacturer's instruction. The cloned amplicon and
- 191 PCR products were confirmed by sequencing (Beckman Coulter Genomics, UK). For RT-
- 192 PCR standards, linearized plasmid DNA or PCR products were quantified, using the
- 193 Nanodrop 1000 (Thermo Fischer Scientific, USA), and a series of dilutions from  $10^9$  to
- 194  $10^2$  copies were generated. For each 10 µl of Lightcycler reaction, 1 µl of test cDNA or

195	serially-diluted standard was used. The LightCycler 480 SYBR Green I Master kit was used
196	for quantification, using 0.5 $\mu$ M concentrations of both the forward and reverse primers.
197	
198	2.5. Secretion studies of Total GLP-1
199	Following the incubation period, 10 $\mu$ l of 10 X Halt Protease and Phosphatase Inhibitor were
200	added to each well to inactivate endogenous dipeptidyl peptidase-4 activity. The cellular
201	supernatants were collected by aspiration and stored at -80 °C prior to analysis. Cellular
202	supernatant levels of total GLP-1 were assayed, using an MSD <sup>®</sup> metabolic assay, according
203	to the manufacturer's instructions. The MSD assay reports a minimum detection limit of 0.3
204	pM of total GLP-1. Total GLP-1 concentrations (pM) in the samples were quantified by
205	interpolating the intensity of emitted light from standard curves generated in the same assays.
206	
207	2.6. cAMP accumulation assay
208	Intracellular cAMP levels were measured, using a cAMP HTRF kit, according to
209	manufacturer's instructions. Briefly STC-1 cells were seeded into a 96-well plate at a density
210	of 6 x 10 <sup>5</sup> cells/ml and incubated overnight at 37 °C, 5 % CO <sub>2</sub> . Medium was aspirated off and
211	the cell monolayers were washed with 100 $\mu$ l of KREBS supplemented with 1 mM IBMX.
212	The cells were incubated for 30 min in 25 $\mu$ l of KREBS-IBMX solution. Cells were then
213	exposed to 25 $\mu$ l of diacetyl (1000 ppb) for a further 30 min. The positive control used was 1
214	$\mu M$ forskolin, a known activator of the enzyme adenylyl cyclase, which causes an increase in
215	intracellular cAMP. The negative control was KREBS-IBMX solution alone. After treatment,
216	the reactions were stopped by addition of lysis buffer containing homogeneous time-resolved
217	fluorescence reagents. Plates were then incubated for 60 min at room temperature, and time-
218	resolved fluorescence resonance energy transfer signals were measured, using a FLUOstar
219	Omega microplate reader (BMG Labtech, Germany).

220

### 221 2.7. Immunocytochemistry

222 For immunocytochemistry, STC-1 cells were seeded at a density of 8 x10<sup>5</sup> cells/ml in 24 well plates containing poly-1-lysine-coated glass-slides and incubated overnight at 37 °C, 5 % CO<sub>2</sub> 223 224 prior to exposure to diacetyl (1000 ppb) or  $\alpha$ -linolenic acid (100  $\mu$ M) for 30 min, 1 h and 4 225 h. As a control, cells were exposed to KREBS alone for the same time intervals. Following 226 exposure, cells were fixed with 4 % paraformaldehyde in PBS for 20min and permeabilized 227 in PBS supplemented with 0.1 % Triton X-100 for 5 min. Slides were than blocked with 10 % donkey serum diluted in PBS supplemented with 0.1 % Triton X-100. The slides were then 228 229 incubated overnight with rabbit anti GPR120 antibody (1:500 dilution), after which the slides 230 were washed and incubated for 2 h at room temperature with donkey anti-rabbit Alexa 488 antibody (1:1000). Primary and secondary antibodies were prepared in PBS supplemented 231 232 with 0.1 % Triton X-100 and 1 % donkey serum. Specificity of GPR120 antibody was 233 confirmed by the absence of fluorescent staining in HEK293T cells which do not express the 234 GPR120 receptor (Tanaka, Yano, Adachi, Koshimizu, Hirasawa, & Tsujimoto, 2008). 235 Specificity of the secondary antibody was confirmed by the absence of fluorescent staining 236 when the primary antibody was omitted (data not shown). Slides were analyzed, using an 237 inverted microscope set-up with a sensitive XM10 camera with an infrared cut filter, mercury 238 burner and fluorescence condenser (Olympus, Japan). Pictures were processed using Cell<sup>A</sup>F 239 Imaging Software (Olympus, Japan). For fluorescence data, intensity was determined to be 240 the mean pixel intensity for the entire cell using Image J software (National Institutes of 241 Health, USA). Staining was conducted in triplicate and, from an image of each staining; five 242 individual cells were selected for fluorescence intensity analysis.

243

#### 244 2.8. Western blotting

245	STC-1 cells were seeded into 12-well plates at a density of $1.5 \times 10^6$ cells/well and incubated
246	at 37 °C, 5 %CO <sub>2</sub> , for 18 h prior to exposures. Following a 30 min, 1 h or 4 h exposure to
247	KREBS alone, 1000 ppb of diacetyl or 100 mM $\alpha$ -linolenic acid, cells were lysed with lysis
248	buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid,
249	1 % Nonident P40, 0.5 mM dithiothreitol and 0.1 mM Na <sub>3</sub> VO <sub>4</sub> ) containing protease inhibitors
250	(0.1 mM phenylmethy sulphonyl fluoride, 2 $\mu$ g aprotinin/ml, 2 $\mu$ g of leupeptin/ml, 0.02 mM
251	NaF and 0.025 mM NaPPi) and centrifuged at 12,000 g for 10 min to remove insoluble
252	debris. Protein content of the lysates was quantified using a BCA kit. The cell lysate was
253	diluted in 4 X sample buffer (333 mM Tris-HCl, 3 % SDS, 26.7 % glycerol, 130 mM
254	dithiothreitol and 0.2 % bromophenol blue) and heated for 10 min at 95 °C. Samples were
255	loaded at 20 $\mu$ g of protein per lane onto precast NuPAGE 4–12 % Bis-Tris Gels. The
256	separated proteins were transferred onto polyvinylidene difluoride membranes by
257	electroblotting. The membrane was blocked for 1.5 h with Tris buffered saline solution
258	containing 0.1 % Tween-20 supplemented with 3 % non-fat dry milk and 2 % BSA.
259	Membranes were incubated overnight at 4 °C with rabbit anti GPR120 antibody (1:1,000
260	dilution) in 0.1% Tween-20 supplemented with 1 % skim milk and 1 % BSA. After three
261	washes in 0.1% Tween-20, the membranes were incubated for 1.5 h at room temperature with
262	horseradish peroxidase-conjugated anti-rabbit IgG (1:8,000). Visualisation was performed,
263	using the enhanced chemiluminescence Western blotting Substrate Kit and GelDoc Image
264	Reader (Las3000; Fujifilm, Japan). Sample loading of protein was corrected by staining with
265	a 1:15,000 dilution of a $\beta$ -actin horseradish peroxidase antibody.

266

267 2.9. Statistical analysis

STC-1 cell exposures to vehicle control (KREBS), diacetyl and blockers were performed in
duplicate and on two separate occasions. For cellular viability assays, at least 2 wells on two

270	separate occasions were examined per test compound. RT-PCR assays were performed in
271	triplicate. Total GLP-1 assays were performed in duplicate with both technical and
272	experimental repeats.
273	Data were analysed by one-way analysis of variance (ANOVA), followed by, where
274	appropriate, Fishers least significant difference (LSD) multiple comparison <i>posthoc</i> test.
275	Means without a common letter differ significantly from each other (P<0.05).
276	
278 279	3. Results
280	3.1. Diacetyl reduces proglucagon mRNA levels and total GLP-1 secretion in the presence of
281	10mM glucose
282	STC-1 cells were exposed to physiologically relevant concentrations of the volatile flavour
283	compound, diacetyl. As in other studies (Zhou & Pestka, 2015), exposures were performed in
284	the presence of the known stimulator, glucose. In our study, experiments were performed in
285	KREBS Ringers bicarbonate buffer, which contains 10 mM glucose. Exposure of STC-1 cells
286	to diacetyl resulted in a significant ( $P < 0.05$ ) dose-dependent decrease in <i>proglucagon</i>
287	mRNA levels compared to the vehicle control at all timepoints (Figure 1A). Increasing the
288	exposure time from 10 min to 4 h led to an overall reduction in proglucagon mRNA levels
289	(Figure 1A). This reduction in <i>proglucagon</i> mRNA coincided with a significant ( $P < 0.05$ )
290	decrease in secreted levels of total GLP-1 compared with the control (Figure 1B). In STC-1
291	cells, the presence of diacetyl reduces proglucagon transcription and total GLP-1 secretion in
292	response to glucose. In order to address potential cytotoxicity of diacetyl to STC-1 cells, the
293	Alamar Blue assay was employed which is based on the ability of viable cells to reduce
294	resazurin to resorufin. There was no significant difference in the ability of cells to reduce

295 resazurin to resorufin in the presence of various concentrations of diacetyl compared to 296 vehicle control (Figure 2). These results indicated that diacetyl suppression of GLP-1 297 production and secretion is not due to cytotoxicity. GC/MS analysis of cellular supernatant 298 after 4 h exposure to diacetyl revealed that this volatile compound was still present in the 299 supernatant, albeit at a reduced level (data not shown). 300 3.2. Is diacetyl suppression of total GLP-1 mediated via  $K^+$ ,  $Ca^{2+}$  channels, taste pathways or 301 302 *intracellular cAMP?* To investigate whether the reduction of transcript levels of *proglucagon* mRNA by diacetyl 303 was mediated through Ca<sup>2+</sup> channels, glucose stimulated STC-1 cells were preincubated with 304 L-type voltage-dependent Ca<sup>2+</sup> channel blockers, nicardipine and nitrendipine, prior to 305 306 exposure to diacetyl. Both failed to restore proglucagon mRNA levels to those observed with 307 KREBS (Figure 3A). Indeed in both cases, there was a further small yet significant reduction 308 (P < 0.05) in *proglucagon* mRNA levels compared with exposure to diacetyl alone. Preincubation with the K<sup>+</sup> channel blocker, tolbutamide, followed by exposure to diacetyl, 309 310 failed to restore *proglucagon* mRNA levels to vehicle control (Figure 3A). To examine if the 311 effects of diacetyl were mediated through taste signalling pathways, glucose stimulated STC-312 1 cells were incubated in the presence of pertussis toxin, a known blocker of the  $\alpha$ -subunit of 313 gustducin prior to exposure to diacetyl (Figure 3A). Blocking α-gustducin resulted in a 58-314 fold decrease in proglucagon mRNA levels in glucose stimulated STC-1 cells compared with 315 the vehicle control and 17-fold decrease compared with levels observed with diacetyl (Figure 316 3A). These observed decreases were not as a result of decreases in cellular viability in the 317 presence of the various blockers (data not shown). 318 Secreted levels of total GLP-1 were measured in STC-1 cells stimulated with glucose in the

319 presence of the various blockers (data not shown). Nicardipine increased levels of secreted

320	total GLP-1 fror	n glucose	stimulated STC	-1 cells b	y 2-fold	(P <	< 0.05).	GLP-1	levels
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- remained unchanged with nitrendipine compared with vehicle control. STC-1 cells secreted
- significantly more GLP-1 in the presence of tolbutamide (P < 0.05) compared with glucose
- alone (vehicle control). Pertussis toxin had no effect on GLP-1 levels. To determine if the
- various blockers could block the inhibition of diacetyl on secreted GLP-1 levels, STC-1 cells
- 325 were preincubated with each individual blocker prior to diacetyl exposure. None of the
- 326 blockers were capable of altering levels of secreted GLP-1 from glucose-stimulated STC-1
- 327 cells exposed to diacetyl (Figure 3B).
- 328 The failure to respond to tolbutamide in a diacetyl background may indicate that diacetyl
- exposure causes cAMP levels to rise in STC-1 cells, closing K<sub>ATP</sub>-channels (Reimann, 2010).
- 330 Intracellular cAMP was therefore measured in a competitive immunoassay. Glucose-

stimulated STC-1 cells exposed to 1000 ppb of diacetyl had a 5-fold increase in intracellular

cAMP compared with cells incubated with glucose alone (Figure 4).

333

#### 334 3.3. Diacetyl increases GPR120 mRNA levels and cell surface expression

335 As diacetyl is a methyl ketone and is structurally similar to methyl ketones produced during 336 oxidation of free fatty acids, we investigated if GPR40 and GPR120 responded to diacetyl 337 presence. Diacetyl had no significant effect on mRNA levels of the medium- to long-chain 338 fatty acid receptor, GPR40, in glucose-stimulated STC-1 cells (Figure 5A). However, mRNA 339 levels of the long chain fatty acid receptor, GPR120, were significantly (P < 0.05) increased 340 in glucose-stimulated STC-1 cells exposed to diacetyl compared with KREBS (Figure 5B). 341 To explore whether GPR120 proteins levels were also affected by diacetyl, time-course 342 experiments was performed by immunofluorescent staining and Western blotting. Exposure 343 of glucose-stimulated STC-1 cells, over a 4 h period, to diacetyl significantly (P < 0.05) and 344 progressively increased GPR120 levels, both at protein and cell surface level, compared with

vehicle control (Figure 6). These increases with 1000 ppb of diacetyl (which equates to 11.7

 $\mu$ M) were also significantly higher than those observed with the GPR120 agonist,  $\alpha$ -linolenic

acid (100  $\mu$ M), indicating that diacetyl is more potent.

- 348
- 349

#### 350 4. Discussion

The widely used flavour ingredient diacetyl inhibits production and secretion of GLP-1 by

352 intestinal endocrine cells *in vitro*. This damping effect on GLP-1 appears to be mediated by

recruiting GPR120 to the cell surface, increasing intercellular cAMP levels and increasing

354 GPR120 synthesis. The mechanism of GLP-1 reduction by diacetyl appears to be

electroneutral, as evidenced by independence from  $K^{+}_{ATP}$  channels and voltage-gated Ca<sup>2+</sup>

 $\alpha$ -gustducin taste pathway also does not appear to be involved in response to

this flavour compound. Whether diacetyl enters the cell is unknown.

358 Diacetyl is structurally similar to methyl ketones produced during oxidation of free fatty

acids. Ketones produced in this manner are mainly derived from fatty acids with 6 to 12

360 carbons. This may thus explain the ability of diacetyl to interact with GPR120. GPR120 is

361 classified as a  $G_q$  protein-coupled free fatty acid (Blad, et al., 2012) that is internalized in the

362 presence of long chain free fatty acids with concomitant increase in cytosolic  $Ca^{2+}$  but with

no effect on cAMP levels (Hirasawa, et al., 2005). However, with the knowledge that

364 GPR120 also plays roles in apoptosis (Katsuma, et al., 2005) and inflammatory response in

365 vintestinal L cells (Anbazhagan, et al., 2016; Tsukahara, et al., 2015), there is growing

sevidence that GPR120 can couple to alternative downstream pathways (Iakoubov, Izzo,

367 Yeung, Whiteside, & Brubaker, 2007; Tsukahara, et al., 2015). Tsukahara et al. (2015) have

recently demonstrated that activation of GPR120 can inhibit *proglucagon* production via G<sub>s</sub>

369 cAMP pathway in inflamed rat L cells and GLUTag cells (Tsukahara, et al., 2015). Treatment

370 of L-cells with 10 ng/ml of TNF- $\alpha$ , a pro-inflammatory cytokine, resulted in a 50 % decrease 371 in *proglucagon* mRNA transcript levels. This effect was mediated by upregulating GPR120 372 and increasing cellular levels of cAMP. Similar to diacetyl, 30 ng/ml of TNF had no effect on 373 the GPR40 mRNA transcript whilst increasing GPR120 mRNA by 250 % (Tsukahara, et al. 2015). Although the inflammatory status of STC-1 cells exposed to diacetyl, a GRAS food 374 375 additive, was not monitored, cell viability was not altered. 376 In our study, STC-1 cells incubated with 1000 ppb of diacetyl resulted in 518 % increase in 377 cAMP levels with a 70 % inhibition of proglucagon transcription and 93 % reduction in 378 GLP-1 exocytosis compared with KREBS alone. Elevation of cAMP levels in STC-1 cells usually enhances transcription of proglucagon (Gevrey, et al., 2004; Islam, et al., 2009; Lotfi, 379 380 et al., 2006). The proglucagon promoter contains a cAMP response element (CRE) at -291 bp 381 to -298 bp. Increase in cAMP levels, leads to activation of the enzyme protein kinase A 382 (PKA) which phosphorylates CRE-binding protein (CREB). Phosphorylated CREB then 383 binds to other transcription factors (e.g. Pax-6, Isl-1) and usually recruits them to the 384 promoter in order to bridge the pre-initiation complex (Gevrey, et al., 2004). In STC-1 cells, 385 site-directed mutagenesis of the CRE site causes a 50 % reduction in the ability of forskolin 386 to increase proglucagon promoter activity (Gevrey, et al., 2004). In GLUTag cells, cAMP 387 rise by 10 µM; forskolin/IBMX stimulates GLP-1 exocytosis (Simpson, et al., 2007) but 388 surprisingly does not increase GLP-1 exocytosis in STC-1 cells (Kuhre, et al., 2016). In 389 GLUTag cells, this GLP-1 exocytosis is mediated through PKA phosphorylation of Kir6.2, 390 the pore-forming subunit of K<sup>+</sup> channels (Reimann, 2010). This results in channel closure and an increase in intracellular Ca<sup>2+</sup>, either *via* influx into the cell through voltage-gated Ca<sup>2+</sup> 391 channels or by mobilisation of intracellular Ca<sup>2+</sup> stores (Reimann, 2010). In our study, neither 392 the L-type voltage-dependent Ca<sup>2+</sup> channel blockers, nicardipine and nitrendipine, nor the K<sup>+</sup> 393 394 channel blocker, tolbutamide could influence the inhibitory effect of diacetyl on GLP-1

395	secretion, indicating differences in response to intracellular cAMP levels between GLUTag
396	and STC-1 cells.

397	The significant reduction by diacetyl of GLP-1 production in STC-1 cells in the presence of
398	glucose appears to be specific, as neither the butterscotch fruity aroma, 2-butanone, nor the
399	pineapple fruity odour, ethyl butyrate, altered proglucagon mRNA levels (unpublished data).
400	However Jang et al. (2007) demonstrated that the food additive, lactisole, also significantly
401	reduced GLP-1 secretion in sucrose-stimulated NCI-H716 cells (Jang, et al., 2007). Lactisole
402	(2.5 mM) reduced GLP-1 secretion 1-fold in these human enteroendocrine cells incubated
403	with 1-5mM sucrose compared to cells with sucrose alone (Jang, et al., 2007). Lactisole
404	mediates its GLP-1 attenuation via the taste receptor, T1r3 (Jiang, et al., 2005) and is
405	therefore added to food to suppress the perception of sweetness.
406	Gonzalez-Abuin et al. (2014) reported that 50 mg/l of grape seed procyanidin extract (GSPE)
407	significantly reduced secreted levels of active GLP-1 from STC-1 cells stimulated with either
408	20 mM glucose or 5 mM proline plus 2.5 mM glucose (Gonzalez-Abuin, et al., 2014).
409	Interestingly this flavonoid extract had no effect on GLP-1 secretion from STC-1 cells
410	stimulated with 30 $\mu$ M linoleic acid plus 10mm glucose (Gonzalez-Abuin, et al. 2014). GSPE
411	appears to mediate its effect on STC-1 cells by the hyperpolarization of cellular and
412	mitochondrial membranes, indicating that Na <sup>+</sup> chelation at the cation channel, TRPM5,
413	maybe involved (Gonzalez-Abuin, et al. 2014). GSPE is likely to be perceived as bitter with a
414	negative sensory attribute. The neuropeptide, galanin (100 nM) can also inhibit GLP-1
415	secretion by 75 % in primary duodenal cultures that are stimulated with 10 mM glucose and
416	100 µM IBMX (Psichas, Glass, Sharp, Reimann, & Gribble, 2016). This 29 amino acid
417	peptide appears to mediate its inhibitory effect on stimulated enterendocrine cells by
418	activating its $GAL_1$ receptor, employing $G_i$ coupling pathways, reducing intercellular cAMP
419	levels but not involving potassium channels (Psichas, et al., 2016). The nuclear receptor

420	Farnesoid X Receptor (FXR) is expressed in L-cells and activation of FXR in GLUTag cells
421	resulted in a 50 $\%$ inhibition of glucose (5.6 mM)-induced proglucagon transcription
422	(Trabelsi, et al., 2015). FXR physically interacts with the carbohydrate response element
423	binding protein (ChREBP), implying that it interferes with ChREBP docking on the
424	proglucagon promoter (Trabelsi, et al., 2015). Trabelsi et al. (2015) also observed that active
425	FXR decreases glucose-induced GLP-1 secretion by inhibiting glycolysis and lowering
426	intracellular ATP levels but not impeding membrane depolarization (Trabelsi, et al., 2015).
427	Given the important role of GLP-1 in food intake and glucose homeostasis, it is surprising
428	that the inhibition and fine-tuning of production and secretion of GLP-1 by foods has
429	received little attention. To our knowledge, this is the first study which shows an inhibitory
430	effect of a volatile aroma compound on satiety hormone production and secretion, albeit in
431	vitro. Although there is extensive information available on (1) the mechanisms of neural
432	responses to both orthonasal and retronasal aroma (Shirasu, et al., 2014), and (2) links
433	between aroma, sensory exposure and satiation (Ruijschop, et al., 2009), there appears to be
434	little information available on aroma directly modulating satiety signals. Massolt et al. (2010)
435	did observe that subjects who smelled dark chocolate before eating it reported significantly
436	higher levels of satiation, a result which correlated inversely with serum levels of the hunger
437	hormone, ghrelin, but had no effect on serum GLP-1 (Massolt, et al., 2010). If the aroma of a
438	food can dampen the gastrointestinal GLP-1 response to that food, the amount of that food
439	consumed may increase. Overconsumption of palatable food is considered a major factor
440	contributing to the global surge in obesity (Kenny, 2011).
441	In conclusion, the results of our study demonstrate that diacetyl reduces levels of
442	proglucagon mRNA transcripts and total GLP-1 secretion, with an associated increase in
443	cAMP levels and recruitment of GPR120 to the cell surface. It is feasible that inhibition of
444	GLP-1 by palatable food components can contribute to overconsumption.

#### 445

446

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### 453

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581	
582	

# 583 Figure 1. GLP-1 production and secretion in STC-1 cells exposed to diacetyl. (A) STC-1 cells (1.5 x 10<sup>6</sup> cells/well) were exposed to various concentrations of diacetyl in KREBS, 584 585 which contains 10 mM glucose, from 10 min to 4 h. Different superscript letters (a-d) indicate significant proglucagon mRNA differences within the same exposure time (P<0.05) 586 587 \* indicates significant differences (P < 0.05) due to 500 ppb diacetyl after 10 min exposure compared with 4 h exposure. $\ddagger$ indicates significant differences (P < 0.05) due to 1000 ppb 588 589 diacetyl after 10 min compared with 4 h. § indicates significant differences (P < 0.05) due to 2000 ppb diacetyl after 10 min compared with 4 h. (B) STC-1 cells ( $1.5 \ge 10^6$ cells/well) 590 591 were exposed to 1000 ppb of diacetyl in KREBS for 4 h. Levels of total GLP-1 were 592 quantified in cell supernatant. Different superscript letters indicate significant differences in 593 total GLP-1 (P < 0.05). Both technical and experimental repeats were performed in duplicate 594 on two separate occasions. 595

#### Figure 2. STC-1 cellular viability in the presence of various concentrations of diacetyl. 597

- Viability of STC-1 cells (1 x 10<sup>5</sup> cells/well) was determined, by Alamar Blue, after 598
- 599 incubation for 4 h with diacetyl. KREBS alone was the vehicle control and represented 100 %
- cellular viability. Superscripts with a common letter are similar to each other (P > 0.05). Both 600
- technical and experimental repeats were performed in duplicate on two separate occasions. 601

602

#### Figure 3. GLP-1 production and secretion in STC-1 cells exposed to various blockers. 603

- (A) proglucagon mRNA levels: STC-1 cells ( $1.5 \times 10^6$  cells/well) were incubated with 604
- various blockers (Ca<sup>2+</sup> channel blockers (5 µM nicardipine or 5 µM nitrendipine), K<sup>+</sup>channel 605
- blocker (100  $\mu$ M tolbutamide) or the  $\alpha$ -gustducin inhibitor (10  $\mu$ g/ml pertussis toxin)) for 15 606
- min. Diacetyl (1000 ppb) was then added and cells incubated for a further 4 h. KREBS alone 607
- is the vehicle control. Superscripts with a different letter indicate a significant difference (P < P608
- rca 609 0.05). (B) Secreted levels of total GLP-1. Different superscripts indicate significant
- 610 differences (P < 0.05).

612

#### 613 Figure 4. Intracellular cAMP levels in STC-1 cells exposed to 1000 ppb of diacetyl for 4

- Acceleration 614 h. Each value represents the mean ± SEM. Vehicle control was KREBS alone. Means

617

#### Figure 5. (A) GPR40 mRNA levels and (B) GPR120 mRNA levels in STC-1 cells STC-1 618

- uti cells (1.5 x 10<sup>6</sup> cells/well) were exposed to 1000 ppb of diacetyl for 4 h. Means without a 619

624	Figure 6. (A) GPR120 surface expression and (B) GPR120 protein levels in STC-1 cells.
625	(A) For immunocytochemistry, seeded STC-1 cells (8 x $10^5$ cells/ml) were exposed, for 30
626	min, 1 h or 4 h, to either KREBS alone or 1000 ppb of diacetyl or 100 $\mu$ M $\alpha$ - linolenic acid.
627	Rabbit anti GPR120 (1:500 dilution) antibody was applied to fixed cells. Staining was
628	conducted in triplicate and from an image of each staining, five individual cells were selected
629	for fluorescence intensity analysis. Data at each time point were analysed, using a one-way
630	ANOVA with a Fisher's LSD comparison. Means without a common roman numeral, within
631	the same time interval, differ significantly from each other ( $P < 0.05$ ). (B) STC-1 cells were
632	seeded into 12-well plates at a density of $1.5 \times 10^6$ cells/well and exposed for 30 min, 1 h or
633	4h to KREBS alone, 1000 ppb of diacetyl or 100 mM $\alpha$ -linolenic acid. Separated proteins
634	were incubated with rabbit anti GPR120 antibody (1:1,000 dilution). Sample loading of
635	protein was corrected by staining with a 1:15,000 dilution $\beta$ -actin horseradish peroxidase
636	antibody. Both technical and experimental repeats were performed in duplicate on two
637	separate occasions.
638	
639	
P	





STC-1 exposure time to diacetyl







1000 ppb diacetyl

Figure 4



Figure 5

A



B

Figure 6



640	Highlights:
641	• A volatile favour compound with a pleasant aroma supresses GLP-1, a satiety
642	hormone
643	• Aromatic diacetyl recruits GPR120 to the cell surface and increases cAMP levels
644	• $K^{+}_{ATP}$ channels, Ca <sup>2+</sup> channels and the $\alpha$ -gustducin taste pathway are not involved
645 646	
5	