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Irish Cheddar cheese increases glucagon-like peptide-1 secretion *in vitro* but bioactivity is lost during gut transit

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ABSTRACT

Appetite can be effectively reduced by targeting the production, secretion, circulation time or receptor of the enteric satiety hormone glucagon-like peptide-1 (GLP-1). The objective of this study was to investigate the potency of Irish Cheddar cheeses to modulate GLP-1 levels. Nine out of ten water-soluble extracts (WSEs) of representative Irish Cheddar cheeses, post 6 months ripening, significantly ($p < 0.05$) stimulated active GLP-1 secretion from the mouse enteroendocrine cell line STC-1. This secretion was associated with protein content and ripening time of cheese. C-57BL/6 mice ($n = 9/10$), who received the most potent sample, C2-WSE-8 M, had a significantly reduced cumulative food intake at 6 h compared to control ($p < 0.05$), but not overall treatment × time effect over a 7 h period. Simulated *in vitro* gastrointestinal digestion, that models the upper human gut, indicated loss of GLP-1 stimulating activity once C2-WSE-8M entered the intestinal phase, suggesting efficacy of C2-WSE-8M will depend on protection during gut transit.

1. Introduction

Cheese, originated as a method to preserve milk, is now an essential part of the cuisine of many countries. As a whole food, it contains high amounts of protein (10–30%) and calcium (0.1–1%) with a fat content that ranges from 4% in cottage cheese to 34% in Cheddar cheese. Cheddar cheese is produced by the addition of lactic acid bacteria (LAB) to milk, followed by curd formation of the casein fraction using rennet. Liquid whey is drained away and the curd is milled, dry salted, pressed and typically allowed to ripen for 2–24 months at 8–12 °C. Over this ripening period, casein proteins are hydrolyzed by the action of rennet (chymosin), indigenous milk proteinases and bacterial proteinases into smaller peptides and free amino acids (AAs) and subsequently metabolized into flavor compounds resulting in the distinctive Cheddar flavor (McSweeney, 2004).

In epidemiological studies, dairy food consumption, particularly low and reduced fat dairy, has been positively associated with appetite control and weight management (Dougkas, Reynolds, Givens, Elwood, & Minihane, 2011). However, weight management associations with cheese are limited with some data even linking cheese, and its high fat content, to increased BMI and the incidence of obesity (Beydoun, et al., 2008). There are some positive association studies linking cheese to subjective appetite ratings and energy intake, suggesting components within the cheese matrix may modulate appetite (Dougkas, Minihane, Givens, Reynolds, & Yaqoob, 2012; Lanou & Barnard, 2008). Potier et al. (2009) reported that consumption of 75 g cheese by 27 normal weight women 1 h before lunch during 9 days significantly increased feelings of fullness, reduced feelings of hunger and energy intake at lunch compared to controls. However, energy intake over the subsequent 24 h period remained unchanged (Potier et al., 2009). In another study, 49 g of mild Cheddar cheese effectively quenched feelings of hunger and reduced energy intake in an *ad libitum* lunch for 40 overweight men, however overall energy intake was not lowered (Dougkas, et al., 2012). Mozzarella cheese (63 g) consumed by 23 normal weight children aged 9–11 years old was shown to reduce food intake *ad libitum* compared to an isocaloric dairy control (milk), when subjective appetite ratings were unchanged (Chi-Yan Li, 2013). The solid state and texture of cheese compared to other dairy products,

Abbreviations: AA, amino acid; AEBSF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride; BCA, bicinchoninic acid; CCK, cholecystokinin; DMEM, Dulbecco's modified eagle medium; DPP-IV, dipeptidyl peptidase IV; GC, gas chromatography; GLP-1, glucagon-like peptide-1; I.p., intraperitoneal; LAB, lactic acid bacteria; MS, mass spectrometry; NSLAB, non-starter LAB; PYY, peptide YY; SGID, simulated gastrointestinal digestion; SPME, solid-phase microextraction; WSE, water soluble extract

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allows for longer oral processing and exposure times to the taste and smell which together with higher fat content could modulate appetite ratings. In addition to this, casein peptides released during cheese ripening as a result of hydrolysis, may influence satiety by targeting production or activity of satiety stimulating hormones.

In a 12-week randomized double-blinded intervention study with 52 obese participants, on a daily intake of 60 g casein, the postprandial levels of the satiety hormone, glucagon-like peptide-1 (GLP-1), were 878 pmol/L × 360 min higher ($p < 0.01$) compared to consumption of the same quantities of whey (Bohl et al., 2015). Bendtsen et al. (2014) observed no overall differences in plasma GLP-1 levels post-consumption of 30 g intact casein, hydrolysed casein or intact whey in 24 overweight adults (Bendtsen et al., 2014). However at some time points the casein samples significantly increased GLP-1 secretion compared to whey.

GLP-1 is one of several satiety hormones, which is secreted by L cells in the distal gut (duodenum to colon) in response to food consumption and post-translationally modified to its biologically active form, GLP-1 (7-37) (Baggio & Drucker, 2007; Bruen, O'Halloran, Cashman, & Giblin, 2012). It reaches target receptors in the intestine, brain, heart, pancreas and stomach to promote feeling of fullness, insulin release and to slow gastric movements. It is then, rapidly degraded by dipeptidyl peptidase IV (DPP-IV) (Baggio & Drucker, 2007). To date stable GLP-1 analogs are the most effective drugs for weight management (Astrup et al., 2012) and for the treatment of type 2 diabetes mellitus (Meier, 2012; Ryan et al., 2017).

GLUTag, STC-1 and NCI-H716 cell lines, are routinely used as enteroendocrine L cell models to screen for food components capable of increasing production or secretion of GLP-1 (Kuhre et al., 2016). *In vitro*, intact casein, especially α- and β-caseins, and casein hydrolysates stimulate proliferation of enteroendocrine cells and improve their ability to secrete GLP-1 (Gillespie & Green, 2016; O'Halloran et al., 2018).

Other components of cheese that may play a role in satiety hormone modulation include individual fatty acids (Hirasawa et al., 2005) and flavor compounds (McCarthy, et al., 2017). The aim of this study was to focus on water soluble extracts (WSEs) of Irish Cheddar cheese, to investigate GLP-1 bioactivity over a 10 month ripening period using an *in vitro* cellular assay. A murine trial was then performed to compare food intake levels, over a 7 h period, in mice who consumed the most potent and the least potent WSE.

2. Material and methods

2.1. Material

STC-1 cell line was sourced from the American Tissue Culture Collection (ATCC code SD5482, supplied by LGC Standards, Teddington, UK). CellTiter One solution reagent was purchased from Promega (MyBIO, Ireland). 100× Halt Protease and Phosphatase Inhibitor was from Thermo Fisher Scientific (MSC, Ireland). Mouse Metabolic Magnetic Bead Panel for the active GLP-1 (#MMHMAG-44K) and Millex-HV Syringe Filter Units (0.45 μm) were sourced from Millipore (Ireland). RNeasy RNA extraction kit and On-column DNase digestion kits were purchased from Qiagen (UK). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Belgium). Tetro cDNA synthesis kit and BCA (bicinchoninic acid) protein assay kit were from Bioline (MSC, Ireland). 10× glyoxyl buffer was from Ambion (Applied Biosystems, USA). LightCycler 480 SYBR Green I Master mix for tPCR was from Roche (Roche Products Ireland Limited). Commercial cheese samples were sourced from 4 Irish cheese manufacturers. Filter-tubes with threshold 10 kDa were purchased from Satorius (Ireland). All other reagents were from Sigma Aldrich (Ireland).

2.2. Cheese compositional analysis, WSE preparation, total protein and free AA characterization

Ten commercial Irish Cheddar cheeses (C1–C10) were sourced from different Irish producers, stored at 8 °C and sampled after 2, 4, 6, 8 and 10 months of ripening (2 M–10 M). The following methods were used to determine the basic composition of cheese samples: moisture by standard drying method at 102 ± 2 °C (FIL-IDF 4A:1982); fat (FIL-IDF 5B:1986); salt (FIL-IDF 88A:1988) and total protein using Kjeldahl method (FIL-IDF 20B:1993). Cheese was macerated in distilled water (50 °C) for 5 min, incubated in a water bath at 55^0C for 1 h and then centrifuged. The sample was filtered through glass wool and fat was removed to produce WSE (C1-WSE–C10-WSE), using the modified method described by Kuchroo and Fox (Kuchroo & Fox, 1982). WSEs were lyophilized by freeze drying (FreeZone Stoppering Tray Dryer, Labconco, USA). Freeze dried powders were re-suspended in dH_2O at a concentration 50 mg/mL and stirred for 1 h. The pH of the dissolved sample was measured and adjusted to pH 7.0–7.4 with 1 M NaOH. Samples were left overnight at 4 °C to hydrate, then filter-sterilized with 0.45 μm syringe filter units and aliquoted for single use. Samples were stored at −20 °C before cell exposure. To determine AA concentrations in WSE, protein was removed by adding an equal volume of 24% (w/v) trichloroacetic acid. The mixture was allowed to stand for 10 min and then centrifuged at 14,000 rpm (Eppendorf centrifuge 5417 C) for 10 min. AAs were quantified from the supernatant using a Jeol JLC-500/V AA analyzer fitted with a Jeol Na^+ high performance cation exchange column. Total protein was measured using BCA protein assay according to manufacturer's instructions.

2.3. Cell culture

STC-1 cells (murine intestinal enteroendocrine tumor cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 4.5 g/L of glucose and L-glutamine, supplemented with 10% Foetal Bovine Serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown in a humidified incubator at 37 °C and 5% CO_2 and passaged every 2–3 days upon reaching 80–90% confluence. Cell passages 17–33 were used.

2.4. STC-1 cell exposure to WSEs and analysis of GLP-1 (active) secretion

STC-1 cell exposure to cheese WSEs was performed as described previously (Kondrashina et al., 2018). In short, cells were seeded at a density of 1.5×10^6 cells/well in 6 well plates and 1×10^5 cells/well in 96 well plates. Cells were exposed for 4 h to 10 mg/mL WSEs, prepared in Krebs-Ringer buffer. All non-sterile solutions were filter-sterilized (0.45 μm) prior to cell exposure. After incubation, 10 μL 100× Halt Protease and Phosphatase Inhibitor were added to inactivate any endogenous DPP-IV. Cellular debris was removed by centrifugation of cell supernatants in 1.5 mL tubes at 900 × *g* and 4 °C for 5 min. Supernatants were stored at −80 °C prior to analysis. This was performed with Milliplex Map Kit (Mouse Metabolic Magnetic Bead Panel) and MagPix fluorescent detection system (Luminex, The Netherlands) according to the manufacturer's instructions. Milliplex assay detects active GLP-1 in the concentration range 41–30,000 pg/mL.

2.5. RNA extraction and real-time PCR

After 4 h incubation with WSE samples, cell monolayers were pelleted, lysed with lysis buffer (Qiagen, UK) and frozen at -80 °C. RNA extraction and real-time PCR were performed as described previously (Kondrashina et al., 2018). RNeasy Mini Kit was used for the RNA extraction following the manufacturer's instructions. Total RNA was quantified spectrophotometrically with Nanodrop 1000 (Thermo Fisher Scientific, USA) and RNA integrity was assessed by electrophoresis (BioRad, USA). Real time PCR was performed with cDNA, prepared