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PII: S0308-8146(18)31255-X
DOI: https://doi.org/10.1016/j.foodchem.2018.07.110
Reference: FOCH 23225

To appear in: *Food Chemistry*

Received Date: 26 March 2018
Revised Date: 28 June 2018
Accepted Date: 17 July 2018

Please cite this article as: Pentikäinen, S., Koistinen, V., Kolehmainen, M., Poutanen, K., Hanhineva, K., Aura, A-M., Mastication-induced release of compounds from rye and wheat breads to saliva, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.07.110

Mastication-induced release of compounds from rye and wheat breads to saliva

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Abstract

Mastication initiates digestion, disintegrating food structure and mixing it with saliva. This study aimed to provide understanding about the first step of bread digestion by exploring release of compounds from bread matrix during mastication. Furthermore, the aim was to identify compound groups that differentiate rye and wheat breads.

Fifteen participants masticated whole-meal rye bread, endosperm rye bread, endosperm rye bread with added gluten and wheat bread. The masticated samples were studied with non-targeted LC-MS metabolic profiling.

A great number of compounds were released from bread matrices in mastication, and the identified compounds differed largely between bread types. Specifically, rye bread samples were characterized by a greater release of peptides and amino acids, whereas sugars and nucleosides were characteristic for wheat bread. These compounds could potentially act as signal molecules in the alimentary tract and may explain, at least partly, the postprandial physiological effects of the breads identified in earlier studies.

Keywords: bread; mastication; metabolomics; peptides; rye

1. Introduction

Breads are an important part of diets all over the world. Cereal flour is the main ingredient in bread baking, but due to the use of different cereal grains, as well as grinding and baking processes, breads constitute a wide range of food items with distinct nutritional profiles, structures and health effects. Rye bread and refined wheat bread represent very different structures, refined wheat bread having more cohesive and springy but less hard texture than rye bread, which is reflected in the mastication process (Pentikäinen et al., 2014). The differences between rye breads and wheat bread in the nutritional content, *in vitro* digestion of starch and protein, and postprandial metabolism have been studied extensively; however, the first step of digestion, namely mastication, has been thus far largely neglected (Bondia-Pons et al., 2011; Juntunen et al., 2003; Moazzami, Shrestha, Morrison, Poutanen, & Mykkänen, 2014). Mastication initiates digestion by disintegrating food to smaller particles and by lubricating the food mass with saliva. Ingested food is transformed into a food bolus structure that is swallowed and processed in further digestion (Bornhorst & Singh, 2012).

Disintegration of foods and the consequences for sensory perception have been studied using different foods and food models (Chen, 2015). However, food structure and mastication process do not determine only the sensory perception and the physical form (particle size, cohesion etc.), in which the bolus proceeds to further digestion, but they also determine, which compounds and to what extent they are dissolved from food matrix first to saliva and further to other digestive fluids. After mastication, the food bolus enters the stomach, where it is mixed with gastric juice and where protein digestion is initiated. The liquid phase with solute compounds generally passes through the stomach faster than the solid phase and may reach the gut in front (Siegel et al., 1988). The compounds that are released in mastication may act as flavour agents in the mouth, but may also act as signal molecules for further digestion, for example stimulating hormone excretion or activating vagal nerve receptors (Delzenne et al., 2010; Raybould, 2008).

For various types of rye breads, the low acute postprandial insulin response compared to wheat bread is a specific feature (Johansson, Lee, Risérus, Langton, & Landberg, 2015; Juntunen et al., 2003; Leinonen, Liukkonen, Poutanen, Uusitupa, & Mykkänen, 1999). Blood glucose concentration is the main trigger for insulin secretion and insulinogenic amino acids absorbed into the circulation augment insulin secretion (Nilsson, Stenberg, Frid, & Holst, 2004). In addition to the insulin-triggering effect of postprandial nutrient concentration in blood, the digestion of amino acids and glucose in the gut stimulates the secretion of incretin hormones (gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1)), which, in turn, augment glucose induced insulin secretion (Fu, Gilbert, & Liu, 2013). Thus, the flow of nutrients to gut lumen is one aspect determining insulin response.

Starch in breads begins to hydrolyse during mastication (Hoebler et al., 1998; Pentikäinen et al., 2014). Little is known about other compounds, which in addition to starch hydrolysis products could be released from the bread matrix to saliva during mastication. We hypothesize that the wheat and rye breads, which are known to have distinct postprandial metabolic responses differ already regarding the compounds that are released from the bread matrix and mix with saliva in mastication. We aimed to explore specifically those compounds that differentiate rye breads from wheat bread.

2. Material and methods

2.1. Test breads

The test breads were baked at the VTT Technical Research Centre of Finland Ltd. The test breads included three sourdough-baked rye breads, which were baked using wholemeal rye flour (wholemeal rye bread, WRB), endosperm rye flour (endosperm rye bread, ERB) and endosperm rye flour and wheat gluten (endosperm rye bread with added wheat gluten, ERBG) and a yeast leavened wheat bread (WB) which was baked using endosperm wheat flour. The test breads were chosen to represent different bread structures. WRB had the most dense and hard structure and WB the least

dense and hard structure. ERB and ERBG had a less dense and hard structure than WRB but a denser and harder structure than WB. Table 1 shows the macronutrient composition of the test breads by dry weight basis. The recipes, baking processes, and structural properties of the test breads are described in detail in our previous paper (Pentikäinen et al., 2014).

Table 1. Macronutrient composition of the test breads. Values are percentage of dry weight.

	Wholemeal rye bread	Endosperm rye bread	Endosperm rye bread with gluten	Refined wheat bread
Starch	59.1 ± 0.1	76.2 ± 0.1	74.0 ± 0.2	66.0 ± 0.2
Total dietary fibre	19.1	9.4	9.9	4.6
Insoluble dietary fibre	13.0 ± 0.1	5.9 ± 0.0	6.6 ± 0.0	3.3 ± 0.0
Soluble dietary fibre	6.0 ± 0.3	3.5 ± 0.2	3.2 ± 0.0	1.4 ± 0.2
Protein	10.3 ± 0.0	6.8 ± 0.1	9.1 ± 0.1	12.3 ± 0.0
Fat	1.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	8.9 ± 0.0

2.2. Mastication trial

A mastication trial with 15 females aged 20–40 years was conducted at the end of the year 2013. Only female participants were included to outline some of the inter-individual variation in mastication. Exclusion criteria were smoking, missing teeth (except 3rd molars) and diagnosed functional mastication problems. The study was conducted according to the ethical principles of good research and clinical practice described in the declaration of Helsinki. Research Ethics Committee, Hospital District of Northern Savo gave ethical approval to the study (record 87/2013). Written informed consents were collected from the participants prior to the study.

The participants attended the study visit in the morning between 8–11 a.m. They were instructed to eat their habitual breakfast 1–1.5 hours prior the study visit. The breads were served in random order as three 2 × 2 × 2 cm cubes, which were masticated one after the other. Each bread cube was masticated until the subjective swallowing point. At that point, the bolus was expectorated to a plastic container, which was kept on ice. There was a break of two minutes between the bread types. Mouth was rinsed with water during the break. Bolus samples were stored at −70 ºC.

2.3. Metabolite profiling

2.3.1. Bolus sample preparation

200 mg bolus sample by dry weight basis was weighed in 2 ml plastic tubes and 610 µl of water was added. The tubes were centrifuged and the supernatant was collected. The metabolites were extracted and proteins precipitated by adding 200 µl methanol to 100 µl of sample. The tubes were mixed, stood on ice for 30 minutes and centrifuged. The supernatant was collected and stored at −70 ºC.

2.3.2. Non-targeted LC-MS metabolite profiling analysis

The samples were filtered (0.2 µm PTFE membrane; PALL corporation) prior to analysis by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-qTOF-MS). The samples were analysed in a random order using hydrophilic interaction (HILIC) chromatography. The quality control samples were injected after every nine samples.

The liquid chromatography was performed on a 1290 Infinity Binary UPLC system (Agilent Technologies, Santa Clara, CA, USA). For the separation, an Aqcuity UPLC BEH amide column (Waters, Milford, MA, USA; dimensions 2.1 × 100 mm, particle size 1.7 µm) was used. The column temperature was +50 °C, flow rate 0.5 ml/min, injection volume 2 µl, and sample tray temperature +4 °C. The gradient elution consisted of HPLC grade water (solution A) and HPLC

grade methanol (solution B), both containing formic acid (0.1 % v/v). A following gradient was used: 0–10 min: 2 % → 100 % of solution B; 10–14.5 min: 100 % of solution B; 14.5–14.51 min: 100 % → 2 % of solution B; and 14.51–16.5 min: 2 % of solution B. The mass spectrometric analysis was performed on a 6540 UHD Accurate-Mass Q-TOF (Agilent Technologies). The ionization was carried out using jet stream electrospray ionization (ESI) in the positive mode. The collision energies for the MS/MS analysis were chosen as 10, 20 and 40 V, for compatibility with spectral databases.

2.3.3. Metabolomics data analysis

The data were collected by using the vendor software (MassHunter Qualitative Analysis B.05.00; Agilent Technologies), and the output was transferred in compound exchange format (.cef) into the Mass Profiler Professional software (MPP 2.2; Agilent Technologies) for data pre-processing (Koistinen, Katina, Nordlund, Poutanen, & Hanhineva, 2016). Only features that were found in at least 80 % of replicates, in at least one of the sample types (four masticated breads) were included in the analyses. The features were normalized row-wise and clustered, based on peak areas, into 15 clusters by k-means clustering using Multiple Experiment Viewer software (version 4.9). Clustering was conducted in order to categorize features occurring in a similar manner within certain sample types into distinct groups.

Features in specific clusters were identified. Exact masses of the positive ions and MS/MS fragmentation data were compared to entries in METLIN, other publicly available spectral databases, and in our in-house standard library. MS-DIAL software version 2.64 (Tsugawa et al., 2015) was used in the identification process.

Fold changes were calculated as the ratio (B/A) of the average peak area of identified compounds in rye bread boluses (B) against the corresponding average peak areas in wheat bread boluses (A). In the cases where fold change was below 1 the negative inverse was calculated. T-tests with

Benjamini-Hochberg FDR correction were conducted to examine whether the fold changes were statistically significant. A *P*-value of 0.01 was set as a limit for statistical significance.

3. Results

3.1. Overview of the features released from breads to saliva in mastication

Altogether 1807 features were included in the data matrix collected from the non-targeted metabolite profiling analysis of saliva and bread bolus samples. Figure 1 provides an overview on how the features clustered in k-means cluster analysis across different sample types based on their peak areas. Approximately 57 % of the features were located in clusters 9, 10, 14 and 15, which contained those features specifically pronounced in rye bread boluses. Approximately 8 % of the features were located in cluster 11 representing compounds more pronounced in wheat bread boluses when compared to rye bread boluses. These five specific clusters, which most clearly differentiated rye bread bolus samples from wheat bread bolus samples, were further examined. Details about the identified compounds are presented in a supplementary table.

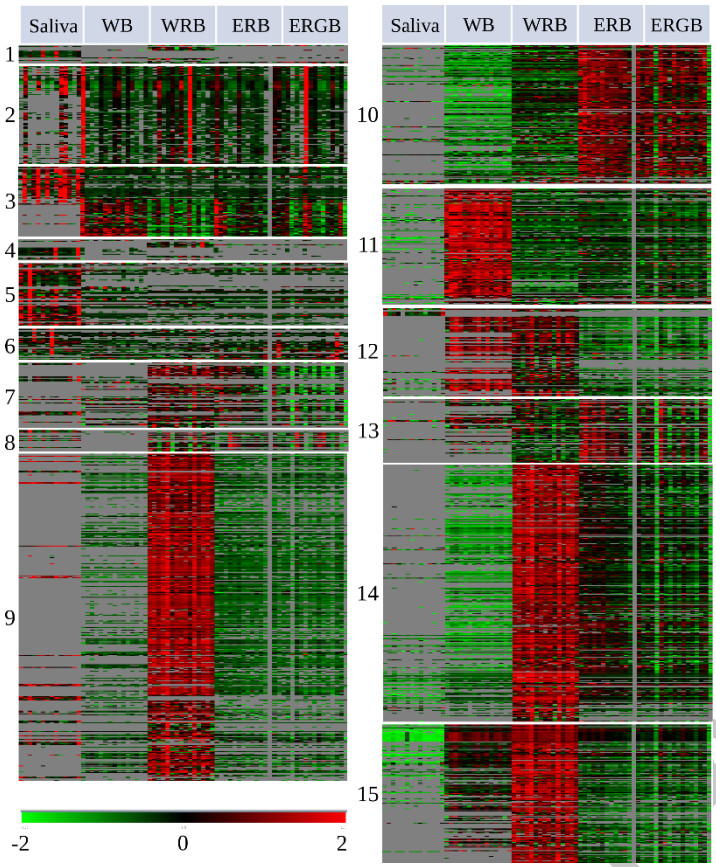

Figure 1. k-Means cluster analysis of metabolic features (n = 1807) in the dataset.

3.2. Differential compounds released from masticated rye versus wheat breads

3.2.1. Clusters of features more pronounced in masticated rye breads compared to wheat bread

Cluster 9 included 388 features of which 22 were identified (Table 2). The majority of the identified compounds were peptides. In addition, two amino acids, ribitol and pyridoxine were among the identified compounds. The fold changes were mainly positive, meaning that these compounds were mainly present in rye bread boluses and to a lesser extent in wheat bread boluses. All the identified peptides were more pronounced in wholemeal rye bread boluses than in wheat bread boluses, and generally, this was also the case with endosperm rye bread boluses yet with smaller fold changes and with less consistence. Both amino acids (L-asparagine and L-histidine) were more pronounced

in wholemeal rye bread boluses and L-asparagine was more pronounced in endosperm rye bread boluses, when compared to the wheat bread boluses.

Cluster 14 contained 305 features, of which 25 were identified. The identified compounds included several amino acids and peptides, betaines, nucleosides, one polyamine and thiamine. Among compound groups, peptides had the most identifications, but this cluster was not as dominated by peptides as cluster 9. All the identified compounds were mainly found in the three types of rye bread boluses but had relatively low levels in wheat bread boluses. Fold changes were generally higher for wholemeal rye bread boluses compared to endosperm rye bread boluses.

Twenty-three of the 167 features in cluster 15 were identified The compound groups included amino acids, peptides, betaines and nucleobases. In addition, phenylethanolamine and glucose 6-phosphate were identified. All the identified compounds were found in particular in wholemeal rye bread boluses; phenylethanolamine and some of the peptides were completely missing from the other boluses. Regarding the endosperm rye bread boluses, the fold changes for some amino acids, betaines, peptides, nucleobase and glucose 6-phosphate were negative, indicating that these compounds were more pronounced in wheat bread boluses than in endosperm rye bread boluses. Thus, the identified compounds in this cluster seem to be specific to whole-meal rye bread.

Table 2. Identified compounds in clusters 9, 14 and 15. The fold changes (FC) are listed for the rye breads with comparison to wheat bread. The statistical significance of the fold change is marked with asterisks: $p < 0.01$*, $p < 0.001$** and $p < 0.0001$***. WB: Wheat bread, WRB: Wholemeal rye bread, ERB: Endosperm rye bread, ERGB: Endosperm rye bread with gluten.

Compound group	Identification	FC, WRB/WB	*p*	FC, ERB/WB	*p*	FC, ERGB/WB	*p*
			Cluster 9				
peptide	Thr-Val-Leu	∞***	0	∞***	0	∞***	0
peptide	Leu-Leu-Ala	∞***	0	∞***	0	∞***	0
peptide	Ile-Val-Lys	∞***	0	∞***	0	∞***	0
peptide	Ile-Ile-Arg	∞***	0	∞***	0	∞***	0
peptide	Leu-Cys-Arg	∞***	0	∞***	0	∞***	0
peptide	Ile-Val-Glu	∞***	0	∞***	0	∞***	0
peptide	Ala-Pro-Leu	∞***	0	∞***	0	∞***	0
peptide	Val-Val-Leu	21.4***	1.2×10^{-26}	3.6***	8.1×10^{-16}	2.5***	1.2×10^{-6}
peptide	Leu-Val-Ile	13.7***	4.1×10^{-24}	2.4***	3.3×10^{-11}	1.7*	6.2×10^{-3}
amino acid	L-asparagine	12.3***	3.7×10^{-30}	3.8***	7.7×10^{-23}	3.4***	1.2×10^{-16}
peptide	Val-Leu	11.7***	1.2×10^{-26}	2.1***	5.7×10^{-12}	1.7**	1.0×10^{-4}
peptide	diprotin B	8.4***	8.6×10^{-27}	1.7***	1.0×10^{-8}	1.6***	9.1×10^{-7}
vitamin	pyridoxine (vitamin B6)	6.7***	2.4×10^{-28}	2.3***	4.7×10^{-12}	2.4***	8.6×10^{-10}
peptide	Ile-Thr-Leu	6.2***	3.0×10^{-27}	2.0***	1.7×10^{-9}	1.6***	7.0×10^{-8}
peptide	Ala-Val-Leu	6.0***	1.9×10^{-18}	-1.0	0.84	-1.2	0.22
peptide	Leu-Thr-Lys	4.8***	1.5×10^{-18}	1.4***	7.9×10^{-5}	1.3**	2.7×10^{-4}
peptide	Val-Arg	4.7***	2.2×10^{-30}	1.1*	7.2×10^{-3}	-1.2	0.21
peptide	Ala-Val-Arg	4.0***	4.8×10^{-16}	1.4***	2.0×10^{-5}	-1.0	0.86
peptide	Ala-Ile-Lys	3.2***	2.2×10^{-20}	-1.1	0.52	-1.1	0.082
peptide	Val-Ser	2.2***	5.5×10^{-08}	-1.5***	2.6×10^{-7}	-1.5***	2.7×10^{-6}
sugar alcohol	ribitol	1.7***	1.2×10^{-13}	1.3***	5.8×10^{-5}	1.1	0.59
amino acid	L-histidine	1.5***	1.1×10^{-22}	-1.6	0.062	-1.2***	3.1×10^{-7}
			Cluster 14				
betaine	acetylcholine	204.5***	6.5×10^{-17}	120.4***	1.3×10^{-15}	107. 1***	7.8×10^{-15}
peptide	Pro-Leu	31.8***	9.1×10^{-30}	7.4***	1.1×10^{-20}	5.4***	2.2×10^{-11}
peptide	(contains Leu / Ile)	28.5***	3.5×10^{-36}	10.3***	6.1×10^{-28}	8.3***	2.0×10^{-16}
amino acid	L-leucine	26.9***	2.8×10^{-32}	3.7***	4.0×10^{-20}	3.2***	7.7×10^{-11}
amino acid	L-phenylalanine	25.4***	4.5×10^{-34}	5.0***	3.1×10^{-24}	4.4***	2.2×10^{-17}
peptide	Leu-Leu-Leu	24.8***	2.4×10^{-29}	8.6***	7.0×10^{-22}	6.2***	3.0×10^{-13}
peptide	Leu/Ile-Arg	18.0***	1.4×10^{-19}	2.4***	5.2×10^{-9}	2.1***	2.5×10^{-5}
polyamine	N8-acetylspermidine	15.7***	2.9×10^{-27}	8.7***	1.4×10^{-23}	7.8***	2.1×10^{-17}

Compound group	Identification	FC, WRB/WB	*p*	FC, ERB/WB	*p*	FC, ERGB/WB	*p*
peptide	Leu-Leu	13.8***	5.7×10^{-26}	1.8***	7.2×10^{-5}	1.5**	1.9×10^{-4}
nucleobase	adenine	10.9***	3.7×10^{-24}	3.6***	5.6×10^{-16}	3.4***	4.2×10^{-14}
amino acid	L-isoleucine	10.2***	2.7×10^{-29}	2.0***	6.0×10^{-14}	1.8***	5.6×10^{-6}
polyamine	1,2-diamino-2-methylpropane	9.1***	5.7×10^{-29}	2.6***	2.5×10^{-18}	2.2***	3.1×10^{-8}
amino acid	L-saccharopine	5.6***	4.8×10^{-27}	5.3***	2.3×10^{-26}	4.4***	4.1×10^{-16}
amino acid	tyrosine	3.8***	9.5×10^{-20}	1.3*	1.2×10^{-3}	1.2	0.089
betaine	trigonelline	3.7***	4.4×10^{-27}	2.0***	$1.7 \times ^{-}10^{18}$	1.7***	2.6×10^{-10}
amino acid	*N*-methyllysine	3.6***	2.2×10^{-25}	3.3***	5.3×10^{-22}	2.7***	8.8×10^{-11}
nucleoside	N6-methyladenine	3.5***	1.9×10^{-26}	1.7***	6.6×10^{-14}	1.5**	2.8×10^{-4}
vitamin	thiamine	3.4***	9.0×10^{-25}	2.0***	9.4×10^{-16}	1.8***	4.6×10^{-6}
amino acid	L-citrulline	3.3***	8.4×10^{-16}	1.7***	1.3×10^{-7}	1.7***	3.1×10^{-5}
peptide	Ile/Leu-Glu-Arg	3.3***	5.9×10^{-13}	-∞***	0	-∞***	0
amino acid	aspartic acid	3.1***	2.1×10^{-21}	2.8***	1.6×10^{-19}	2.6***	$1.1 \times ^{-}10^{12}$
betaine	L-carnitine	2.6***	8.5×10^{-18}	1.8***	2.0×10^{-12}	1.5**	6.6×10^{-4}
nucleoside	5'-methylthio-adenosine	2.4***	1.5×10^{-14}	2.1***	4.6×10^{-12}	1.9***	4.4×10^{-7}
nucleoside	4-guanidino-butanoate	2.1***	1.7×10^{-22}	1.8***	4.0×10^{-18}	1.7***	1.1×10^{-12}
			Cluster 15				
amine	phenylethanolamine	∞***	0	∞***	0	∞***	0
peptide	Met-Leu-Phe	∞***	0	∞***	0	∞***	0
peptide	Ile-Pro-Ile	∞***	0	–	–	–	–
peptide	Ala-Ile-Arg	∞***	0	∞***	0	∞***	0
peptide	Leu-Leu-Ala	∞***	0	∞***	0	∞***	0
peptide	Phe-Ile	∞***	0	∞***	0	∞***	0
betaine	valine betaine	7.2***	5.7×10^{-29}	-3.0***	3.2×10^{-21}	-2.7***	2.6×10^{-10}
amino acid	L-threonine	3.9***	3.9×10^{-14}	-1.2	0.25	-1.0	0.80
nucleobase	guanine	3.5***	2.2×10^{-18}	-1.1	0.13	-1.1	0.41
amino acid	L-arginine	3.2***	3.5×10^{-19}	-1.0	0.65	-1.2	0.18
peptide	Leu-Ala-Lys	2. 3***	2.4×10^{-10}	-1.5***	1.6×10^{-6}	-1.5***	2.6×10^{-5}
betaine	choline	1.8***	5.2×10^{-13}	-2.3***	8.9×10^{-14}	-2.4***	2.1×10^{-6}
peptide	Ile-Pro	1.7*	3.3×10^{-3}	1.0	0.89	1.0	0.95
amino acid	L-lysine	1.7***	5.4×10^{-18}	-1.8***	4.5×10^{-17}	-1.9***	3.4×10^{-9}
sugar	glucose 6-phosphate	1.7***	2.5×10^{-7}	-1.6	0.058	-1.3	0.013
phosphocholine	glycerophosphocholine	1.6***	2.4×10^{-16}	-1.0	0.92	1.3**	1.4×10^{-4}
nucleobase	5-methylcytosine	1.5*	1.5×10^{-3}	-2.4***	1.0×10^{-12}	-2.1***	1.1×10^{-7}
amino acid	L-arginine	1.5***	3.1×10^{-20}	-1.4	0.39	-1.1	0.11
amino acid	L-pipecolic acid	1.4***	1.6×10^{-7}	-1.3***	2.1×10^{-5}	-1.7**	1.8×10^{-4}
betaine	glycine betaine	1.3***	5.4×10^{-21}	-1.4	0.49	1.1**	9.9×10^{-4}
amino acid	*N*,*N*-dimethyl-L-arginine	1.3*	6.1×10^{-3}	-1.2	0.057	-1.3	0.023
peptide	Arg-Ala	1.2	0.079	-∞***	0	-4.1***	5.1×10^{-8}

Compound group	Identification	FC, WRB/WB	*p*	FC, ERB/WB	*p*	FC, ERGB/WB	*p*
betaine	proline betaine	1.1	0.10	-1.0	0.94	-1.2	0.044

Cluster 10 contained 165 features of which seven were identified including tri- and tetrasaccharides, 4-aminobutylguanidine (nuclebase derivative), 5'-*S*-methyl-5'-thioadenosine (nucleoside), and spermidine (polyamine) (Table 3). Sugars were more pronounced in the endosperm rye bread boluses than in wheat bread boluses, whereas they were less pronounced in wholemeal rye *vs.* wheat bread boluses. This was the case also for spermidine, whereas 4-aminobutylguanidine and 5'-*S*-methyl-5'-thioadenosine were mainly characteristic of all rye bread boluses.

Table 3. Identified compounds in cluster 10. The fold changes (FC) are listed for the rye breads with comparison to wheat bread. The statistical significance of the fold change is marked with asterisks: p < 0.01*, p < 0.001** and p < 0.0001***. WB: Wheat bread, WRB: Wholemeal rye bread, ERB: Endosperm rye bread, ERGB: Endosperm rye bread with gluten.

Compound group	Identification	FC, WRB/WB	*p*	FC, ERB/WB	*p*	FC, ERGB/WB	*p*
nucleobase derivative	4-aminobutylguanidine	6. 1***	6.3×10^{-30}	6.4***	2.6×10^{-28}	5.8***	9.2×10^{-21}
nucleoside	5'-*S*-methyl-5'-thioadenosine	1.3***	1.5×10^{-13}	1.4***	7.5×10^{-12}	1.3***	3.4×10^{-6}
sugar	tetrasaccharide	-1.1*	2.3×10^{-3}	1.3***	1.4×10^{-12}	1.2***	1.4×10^{-6}
sugar	trisaccharide	-1.3*	0.011	1.5***	9.8×10^{-9}	1.3***	1.5×10^{-5}
sugar	trisaccharide	-1.3***	5.4×10^{-5}	3.7*	3.5×10^{-3}	3.6*	4.1×10^{-3}
polyamine	spermidine	-1.5***	2.3×10^{-5}	2.4***	1.2×10^{-9}	2.2***	2.6×10^{-11}
sugar	trisaccharide	-2.0***	2.7×10^{-13}	3.3***	2.6×10^{-10}	2.9***	4.3×10^{-7}

3.2.2. Cluster of features more pronounced in masticated wheat bread compared to rye breads

Cluster 11 contained 138 features, of which 14 were identified (Table 4). The identified compounds included unidentified di-, tri- and tetrasaccharides, phosphocholines, nucleosides, pantothenic acid, and one peptide. The fold changes between the rye and wheat bread boluses were negative for most of the identified compounds, indicating that these compounds were found in particular in wheat bread boluses. All identified nucleosides and pantothenic acid were statistically significantly more pronounced in wheat bread than in rye bread boluses. The majority of the identified sugar compounds were also more pronounced in wheat bread boluses than in the three different rye bread boluses. Phosphocholines were more abundant in wheat bread boluses than in endosperm rye bread boluses, whereas there were no statistically significant differences between wheat bread boluses and wholemeal rye bread boluses.

Table 4. Identified compounds in cluster 11. The fold changes (FC) are listed for the rye breads with comparison to wheat bread. The statistical significance of the fold change is marked with asterisks: $p < 0.01$*, $p < 0.001$** and $p < 0.0001$***. WB: Wheat bread, WRB: Wholemeal rye bread, ERB: Endosperm rye bread, ERGB: Endosperm rye bread with gluten.

Compound group	Identification	FC, WRB/WB	*p*	FC, ERB/WB	*p*	FC, ERGB/WB	*p*
nucleoside	2'-deoxyadenosine	-8.3***	2.8×10^{-24}	-3.7***	2.8×10^{-14}	-3.4***	7.7×10^{-15}
nucleoside	cytidine	-6.5***	8.9×10^{-25}	-7.3***	2.0×10^{-22}	-8.2***	8.3×10^{-16}
nucleobase	cytosine	-4.2***	1.7×10^{-24}	-4.7***	4.4×10^{-21}	-5.3***	2.3×10^{-14}
sugar	trisaccharide	-3.2***	8.9×10^{-16}	-1.1	0.071	-1.2	0.017
nucleoside	adenosine	-3.0***	2.2×10^{-29}	-3.0***	1.0×10^{-23}	-2.9	2.9×10^{-27}
nucleoside	1-methyladenosine	-2.9***	2.7×10^{-25}	-2.6***	2.1×10^{-20}	-2.5***	3.6×10^{-11}

sugar	disaccharide	-2.0***	8.3×10^{-7}	-1.5**	2.4×10^{-4}	-1.6**	1.8×10^{-4}
vitamin	pantothenic acid (vitamin B5)	-1.9***	4.1×10^{-11}	-2.6***	7.2×10^{-14}	-2.9***	3.0×10^{-10}
peptide	glutathione	-1.8***	5.4×10^{-6}	-2.1***	6.2×10^{-5}	-3.7***	3.6×10^{-8}
amino acid	glutamine	-1.7***	2.6×10^{-14}	-1.8***	5.6×10^{-14}	-2.1***	1.4×10^{-7}
sugar	trisaccharide	-1.3**	9.4×10^{-4}	-1.7	0.059	-1.5	0.036
sugar	tetrasaccharide	-1.2	0.13	-1.3	0.39	-1.1	0.18
phosphocholine	LysoPC(16:0)	-1.2	0.082	-1.6*	2.1×10^{-3}	-2.2**	3.5×10^{-4}
phosphocholine	unknown phosphocholine	-1.1	0.30	-1.5*	4.0×10^{-3}	-2.2**	5.0×10^{-4}
peptide	Leu-Leu-Arg	∞	0	–	–	–	–

4. Discussion

This is the first study demonstrating the wealth of compounds released from bread matrices and mixed with saliva in mastication. Intriguingly, the k-means clustering revealed clusters of compounds that clearly differentiated the masticated breads. The identified compound groups and the relative amounts of those compounds differed between wheat bread and the three types of rye breads, as well as between the different types of rye breads. The most evident differences between masticated rye and wheat breads was the greater release of peptides and amino acids from rye breads and release of sugar compounds from wheat bread.

Postprandial gastric inhibitory peptide (GIP) response has been found to be lower for rye breads than for wheat bread suggesting that there could be differences in the nutrient flow in the gut (Juntunen et al., 2003). We expected that protein hydrolysis products with incretin release stimulating activity could partly explain the higher insulin response after wheat bread *vs.* rye bread consumption, which has been observed in previous studies (Johansson et al., 2015; Juntunen et al., 2003; Leinonen et al., 1999). However, as opposed to what we expected, the release of peptides and

amino acids was greater from masticated rye than wheat breads. For example, leucine, isoleucine and phenylalanine, the concentration of which in blood has been previously connected with insulin response (Bondia-Pons et al., 2011; Moazzami et al., 2014), were more abundant in rye bread boluses than in wheat bread boluses.

The greater release of peptides and amino acids from rye breads might be explained by differences in the bread baking processes. Sourdough fermentation is typically applied in rye bread baking, as was the case also in the current study. During fermentation, endogenous rye proteases hydrolyze proteins and produce peptides and amino acids (Poutanen, Flander, & Katina, 2009; Tuukkanen, Loponen, Mikola, Sontag-strohm, & Salovaara, 2005). However, the main sites for protein digestion are in the stomach and small intestine. Thus, even though amino acids and peptides were released from rye breads in mastication to a greater extent than from wheat bread, the following steps of digestion might turn the situation around. The study of Bondia-Pons et al. showed that *in vitro* protein hydrolysis was slower from sourdough endosperm rye bread than from wheat bread (Bondia-Pons et al., 2011). However, in this study, the relative content of soluble proteins and smaller molecular weight peptides was higher in rye bread compared to wheat bread both in the beginning and in the end of the *in vitro* hydrolysis. As observed by Nordlund et al., sourdough rye breads were less disintegrated than wheat breads after chewing and gastric digestion *in vitro* (Nordlund, Katina, Mykkänen, & Poutanen, 2016). It could be interpreted that even if there is a pool of readily available peptides and amino acids in rye breads, the main protein pool remains intact for some time and hydrolyses more slowly than the protein pool of wheat bread. Compounds released from the food matrix could also have relevance for postprandial satiety responses, which are enhanced for rye products compared to refined wheat products (Isaksson, Fredriksson, Andersson, Olsson, & Aman, 2009; Rosén, Östman, Shewry, et al., 2011; Rosén, Östman, & Björck, 2011) but differ among rye products with varying structures (Isaksson et al., 2011; Pentikäinen et al., 2017). Protein hydrolysates in digestive tract increase cholecystokinin release

(Raybould, 2008). Cholecystokinin is an appetite suppressing hormone that is released shortly after the beginning of an eating episode (Delzenne et al., 2010). Therefore, protein hydrolysates in digestive tract could offer one explanation for the satiety-promoting effects of rye bread. The concentration of ribitol in plasma has been observed to increase after rye bread intake, in acute and 8-week interventions (Bondia-Pons et al., 2011; Lankinen et al., 2011) and it has been suggested to mediate the satiety response. This current study found that ribitol was released from whole-meal rye bread and endosperm rye bread and it was mixed with saliva supporting the potential role of ribitol for enhanced satiety responses.

Tri-, tetra- and monosaccharides were a distinct group of compounds released from masticated wheat bread to greater extent than from masticated rye breads. This result is in line with our earlier study related to glucose release, where we found a trend for faster salivary alpha-amylase induced starch hydrolysis in wheat bread compared to rye breads (Pentikäinen et al., 2014) and with a study where starch of wheat bread was hydrolysed faster than starch of rye breads (Juntunen et al., 2003). On the contrary, Bondia-Pons et al. found the starch hydrolysis rate from endosperm rye bread to be faster than that from wheat bread (Bondia-Pons et al., 2011). It seems that some part of the wheat bread starch starts to hydrolyze in the very beginning of digestion process. Faster starch hydrolysis, which stimulates the release of incretin hormones, could explain at least to some extent the higher postprandial insulin response to wheat bread very soon after ingestion. However, the compound identification did not reveal, of which sugar moieties the mono-, tri-, and tetrasaccharides were comprised. It will remain uncertain if these compounds were comprised of glucose units or of some other monosaccharides, and if those compounds could influence incretin and insulin secretions. The sugar compounds could be studied in depth in future studies.

In addition to peptides, amino acids and sugars, a great variety of other compound groups, such as vitamins, amines and betaines, were identified from bread boluses. Betaines were found in particular in masticated rye breads. Previously, betaine concentration in plasma has been linked to

whole grain consumption in humans (Ross et al., 2011). In mice urine, concentrations of betaines were increased after a rye containing diet (Pekkinen et al., 2015). This is the first study to show that some betaines are released from the bread matrix in mastication and mixed with saliva.

Whole grain consumption has been associated with many health benefits (G.-C. Chen et al., 2016; Schwingshackl, Schwedhelm, Hoffmann, Lampousi, & Knu, 2017). About one third of the features in the current data were related to wholemeal rye bread. These compounds will be an interesting field for future studies when aiming to understand the mechanisms for the benefits of whole grain consumption.

The features were divided to 15 clusters by k-means clustering. The five clusters of interest contained 1163 features of which we were able to identify 83 compounds (7 %) meaning that the majority of the metabolic features in the clusters remained unidentified. Food bolus samples represent a new sample type and there are no references regarding this specific sample type yet. The small proportion of identifications is a limitation of this study. We suggest that compounds that are released from food bolus in different stages of digestion could act as signal molecules for endocrine and neural responses. However, at this stage it is uncertain which compounds could be relevant and how large fold changes could be expected to deliver some difference in further physiological responses.

5. Conclusions

The current study revealed that, a diverse array of compounds was released from masticated bread samples and mixed with saliva. The study also outlined the magnitude of differences between different bread types. Peptides, amino acids and sugars were the most evident compound groups that differentiated rye and wheat breads. We expect these results and the metabolomics approach to inspire further research inspecting the actions of the released compounds in the gut lumen.

Authors' contributions

SP, MK, KP, KH, and A-MA designed the study. SP conducted the mastication trial. KH and SP were responsible for LC-MS analytics. KH, VK and SP were responsible for the data analyses and compound identification. SP drafted the manuscript and all authors contributed to the interpretation of the data and processing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Mrs. Miia Reponen for skilful technical assistance with the LC-MS analysis. Biocenter Finland, Academy of Finland and Lantmännen Research Foundation are thanked for financial support. This work was supported by Biocenter Finland, Academy of Finland and Lantmännen Research Foundation. The funding bodies were not involved in designing the study, collection, analysis, or interpretation of data, writing the manuscript or in the decision to submit the article for publication.

Conflicts of interest: none

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Figure caption

(title):

Figure 1. *k*-Means cluster analysis of metabolic features (n = 1807) in the dataset.

(description of the illustration):

Each row represents one molecular feature. The columns Saliva, WB, WRB, ERB and ERGB contain replicates obtained from bolus samples of 15 individual participants. Red color indicates upregulation (large relative peak area) whereas green color indicates downregulation (small relative peak area). WB: Wheat bread, WRB: Wholemeal rye bread, ERB: Endosperm rye bread, ERGB: Endosperm rye bread with gluten.

Supplementary files

title:

Supplementary file 1. Compound identification table

(description of the supplementary file):

RT: retention time; CID: collision induced dissociation, M: mass.

Highlights

- Mastication as part of bread digestion has been largely neglected.
- The release of compounds from the food matrix starts in mastication.
- The early released compounds may act as signal molecules in further digestion.
- A diverse array of compounds was released from masticated bread samples.
- Peptides, amino acids and sugars differentiated rye and wheat breads.