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ORIGINAL CONTRIBUTION

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Suitability of a batch *in vitro* fermentation model using human faecal microbiota for prediction of conversion of flaxseed lignans to enterolactone with reference to an *in vivo* rat model

Summary *Background In vitro* fermentation models have been used widely for studies of short-chain fatty acid (SCFA) formation from carbohydrates, whereas the suitability of these methods for enterolactone (ENL) formation has received less attention. *Aim* The aim was to study the suitability of an *in vitro* fermentation model for prediction of bioconversion of lignans to ENL, to compare the approach with that of an *in vivo* rat model and to study the SCFA formation in both models. *Methods* Predigested samples of rye bran (R), flaxseed meal (F) alone, or in combination with rye bran (R&F) and a faecal control were incubated in an *in vitro* fermentation model using human faecal microbiota. In the *in vivo* experiment rats consumed a non-fibre control diet (C) or diets supplemented either with rye bran (R), flaxseed meal (F) alone, or with their combination (R&F) for four weeks. Enterodiol (END), ENL and SCFA concentrations were measured from *in vitro* faecal fermentation samples and from the intestinal contents of rats. Plasma ENL concentrations from rats were also measured. *Results* The highest ENL production was found *in vitro* with the F supplement (areas under curve: 740 ± 4, 7500 ± 400, 2600 ± 500 and 1520 ± 70 nmol · h for the R, F, R&F supplements and faecal control, respectively). *In vivo*, the concentration of ENL in caecal digesta from flaxseed meal was significantly ($P < 0.05$) enhanced by the presence of rye bran (medians 261, 407 and 24 nmol/g in the F, R&F and C groups, respectively). No correlation was found between the models regarding ENL production, possibly due to different responses to the presence of rye bran matrix, differences in microbiota or application of a batch in the *in vitro* fermentation model. Rye bran supplementation enhanced butyrate production both *in vitro* and *in vivo*. *Conclusions In vitro* fermentation and the *in vivo* rat models responded differently to the presence of rye bran and no correlation with regard to the ENL formation from flaxseed lignans was observed.

Key words lignans – non-digestible carbohydrates – *in vitro* faecal fermentation – *in vivo* rat model

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Introduction

Lignans are non-nutrient, diphenolic compounds abundant in plants, e. g. in cereals, berries and fruit [1]. Lignan content is substantial in the outer layers of rye kernel, where the dietary fibre (DF) content is also highest [2]. Recently new lignans have been found in rye bran; in addition to secoisolariciresinol (SEC)[1] and matairesinol (MAT), syringaresinol (SYR), pinoresinol

1 List of abbreviations: *END* enterodiol; *ENL* enterolactone; *SCFA* short-chain fatty acid; *SEC* secoisolariciresinol; *MAT* matairesinol; *SYR* syringaresinol; *PIN* pinoresinol; *LAR* lariciresinol; *GC-MS* gas chromatography – mass spectrometry; *HPLC* high-performance liquid chromatography; *R* rye bran; *F* flaxseed; *R&F* rye bran and flaxseed; *C* non-fibre control diet

(PIN), lariciresinol (LAR) and isolariciresinol (iso-LAR) have been quantified in rye. The SYR content of rye bran is almost ten-fold compared to its SEC and MAT contents [3]. Flaxseed is the richest source of plant lignans, because it contains high amount of SEC, some MAT [4], and also PIN and iso-LAR have been identified in it [5].

Plant lignans are converted to enterodiol (END) and enterolactone (ENL) by colon microbes [3]. Several intermediary metabolites have been found as conversion products of plant lignans [6]. Furthermore, different pure lignans found in rye bran were shown to vary in their extent of conversion to ENL in fermentation with human faecal flora *in vitro* [3]. Radiolabeled lignins were also converted to mammalian lignans in rats [7]. Thus mammalian lignans have several possible precursors. High urinary or plasma concentrations of ENL have been associated with the consumptions of fruit, berries and cereals, particularly plant lignan-rich whole-grain products such as rye bread and flaxseed [8–10]. High plasma and urinary ENL concentrations have been associated with a lower risk of several diseases, e. g. acute coronary events and breast cancer [11, 12].

DF is decomposed and fermented to short-chain fatty acids (SCFA) by gut microbiota [13]. SCFA formation is characteristic of the source of carbohydrate, as individual carbohydrates give rise to different relative proportions of acetate, propionate and butyrate [13]. Butyrate has been associated with local beneficial effects for colon health: improved cell proliferation and induction of apoptosis enhancing healthy tissue turnover [14].

The aim of this work was to evaluate the suitability of an *in vitro* fermentation model using human faecal microbiota for prediction of bioconversion of plant lignans with reference to an *in vivo* rat model. The approaches of the two models are compared and influence of particular carbohydrates in the fermentation is discussed.

Materials and methods

Materials

Rye bran was donated by Melia Ltd (Raisio, Finland) and de-oiled flaxseed meal by Elixi Oil Ltd (Somero, Finland). Powdered cellulose of pharmaceutical grade was purchased (Elcema G 250; Degussa AG, Frankfurt am Main, Germany).

In vitro fermentation model

Prior to the *in vitro* fermentation model the starch and protein contents of rye bran and flaxseed were reduced by *in vitro* enzymatic digestion mimicking the upper intestine according to Aura et al. [15], and the digestion products were removed by dialysis according to Karppinen et al. [16]. After the dialysis the retentates were freeze-dried. The supplements used as substrates in the *in vitro* fermentation model (Table 1) were constructed in the same way as the diets in the rat model. DF contents of rye bran (R), flaxseed (F) and the R&F supplements were standardized to 96.7 mg/200 mg by addition of pharmaceutical cellulose to the R and F supplements.

In vitro fermentation model was performed according to Aura et al. [17] with the following modifications: 200 mg of R, F or R&F supplements was placed in the bottles. Human faeces were collected from 4 healthy volunteers, who had not received antibiotics for at least 15 months and had not eaten flaxseed, rye or berries during the preceding 3 to 4 days. Freshly passed faeces were immediately taken into strictly anaerobic conditions and faecal suspension (16.7 %; w/v as final concentration) was prepared and triplicate samples were drawn as described previously [17]. Values were expressed as mean ± standard deviation (SD). Areas under the curve were calculated for lignan metabolites (END and ENL), for the total amount of SCFA production (a sum of acetate, propionate and butyrate) and for the butyrate production alone.

In vivo rat model

The animal protocol used was approved by the Laboratory Animal Ethics Committee of the University of Helsinki. The experimental groups received a modified AIN-93G [18] diet containing (g/kg) 236.2 casein, 478.9 dextrose, 148.9 butter, 13.3 sunflower-seed oil, 62.2 rapeseed oil, 41.6 AIN-93G mineral mixture, 11.8 AIN-93G vitamin mixture, 3.6 L-cystine, 3.6 choline cloride, 0.014 tertiary butylhydroxyquinone (control diet, C), or the same diet supplemented with 9 % rye bran (R), 1 % flaxseed (F), or 9 % rye bran + 1 % flaxseed (R&F). R, F and R&F diets were standardized to a dietary fiber content of 33.7 g/kg by the addition of pharmaceutical cellulose. The total lignan contents were 14, 153 and 168 μmol/kg diet for R, F, and R&F diets, respectively. All diets were similar with respect to protein (20 %), fat (40 %) and carbohydrates (40 %) on an energy basis (kJ). Male Wistar rats (Animal Center of Helsinki University, Helsinki, Finland) aged 9 weeks were divided on the basis of their body weights into four comparable experimental groups ($n = 7–8$, average weight 295 ± 15), and housed in plastic cages at controlled temperature and in a 12:12-hour light-dark cycle. The animals had free access to the diets and to tap water. Their body weights were recorded weekly. After four weeks feeding period, rats were killed using carbon dioxide asphyxation. Blood samples were collected from the abdominal aorta, centrifuged (6000g, 1 min), and the plasma was stored at –70 °C for analysis. The distal part of the small intestine,

Table 1 Compositions of rye bran (R), flaxseed (F) and rye bran and flaxseed (R & F) supplements used as substrates in the *in vitro* fermentation model using human faecal microbiota

Ingredients	R supplement	F supplement	R&F supplement
Predigested rye bran (mg)	180	0	180
Predigested flaxseed meal (mg)	0	20	20
Cellulose (mg)	7.74	88.92	0
Dietary fibre*, total (mg)	96.66	96.66	96.66
Composition	**mg**	**mg**	**mg**
Moisture	14.22	1.68	15.90
Protein	25.38	3.48	28.86
Fat	19.44	5.12	24.56
Ash	7.38	0.96	8.34
Carbohydrates	113.58	8.76	122.34
Total starch	6.66	0.06	6.72
Fructan	5.40	0.06	5.46
β-Glucan	9.36	0.01	9.37
Pentosan, (ara + xyl)*0.88	38.97	1.48	40.45
Cellulose, 0.9′glu	29.36	61.58	25.78
Plant lignans	**nmol**	**nmol**	**nmol**
Secoisolariciresinol	0.71	383.60	384.31
Matairesinol	0.87	0.49	1.36
Lariciresinol	0.49	0.40	0.89
Pinoresinol	1.14	0.64	1.78
Isolariciresinol	3.29	9.92	13.21
Syringaresinol	15.39	0.05	15.44
Total plant lignans	**21.89**	**395.11**	**416.99**

caecum and colon were removed. The contents of these areas were collected and stored at –70 °C for further analysis.

Methods of analyses

Total starch, fructan and β-glucan contents were determined with specific enzymatic kits (Megazyme, Ireland). Total DF content was measured according to Asp et al. [19], the content of pentosan according to Douglas [20], protein content by the Kjeldahl method (6.25 × nitrogen) [21] and ash content by incineration overnight at 550 °C using a temperature-programmed muffle oven. The results are mean values from two replicate determinations. Accepted variation of the measurements was ± 0.1–0.5 % [15].

Plant lignans from native or predigested rye bran or flaxseed meal samples and END and ENL from the colon contents of rats were determined by a gas chromatographic-mass spectrometric (GC-MS) method according to Mazur et al. [22] with modifications [17]. Mammalian lignans in the fermentation samples were analysed using HPLC with coulometric electrode array detection (CEAD) as described by Heinonen et al. [3]. Time-resolved fluoroimmunoassay (TR-FIA) was used to analyse the ENL from plasma [23, 24] including fat removal by n-hexane extraction after hydrolysis. SCFA were extracted with diethyl ether and analysed with gas chromatography [16]. Determination of neutral sugars was carried out according to Karppinen et al. [16] except that a 50 mg sample was used for the acid hydrolysis and subsequent analysis was performed by DIONEX-HPLC.

Statistics

All the statistical analyses were performed with the SPSS software (SPSS Inc., Chicago, IL). Data of the rat experiment were analysed by the non-parametric Kruskall-Wallis and the Mann-Whitney test with Bonferroni adjustment. Differences of $P < 0.05$ were considered to be significant. All the correlations between variables were analysed with the non-parametric Spearman's correlation test. Values were expressed as median (minimum, maximum) or mean ± SD.

Results

In vitro fermentation model

When F and R&F supplements were fermented with human faecal microbiota *in vitro*, maxima of END produc-

tion occurred in 12 h (224 nmol) for the R&F supplement and from 4 to 8 hours (124 nmol) for the F supplement (Fig. 1A). ENL conversion increased steadily and reached its maximum in 36–48 hours, 96 nmol and 230 nmol for R&F and F supplements, respectively (Fig. 1B). Both END and ENL production remained below that of faecal control for R supplement. The highest ENL production was found *in vitro* with the F supplement (areas under curve: 740 ± 4, 7500 ± 400, 2600 ± 500 and 1520 ± 70 nmol · h with the R, F, R&F supplements and faecal control, respectively). Areas under the curves of the mammalian lignans (END and ENL) were summed and the relative proportion of ENL was calculated for each supplement. The proportion of ENL of total mammalian lignans was 32 % for the R&F supplement and 82 % for the F supplement. Therefore, when the correlation between production of the two mammalian lignans was investigated, ENL formation correlated weakly with END ($r = 0.783$; $P = 0.003$; $n = 12$).

The presence of rye bran enhanced the total SCFA formation (Fig. 1C). Also, relative butyrate proportion was highest with samples containing rye bran (Fig. 2). Therefore a good correlation ($r = 0.972$; $P = 0.000$; $n = 12$) was found between areas under the curves of SCFA and butyrate productions (results not shown). The rate of pH decrease was in accordance with the rate of SCFA formation (results not shown).

In vivo rat model

In the *in vivo* rat model, when both rye bran and flaxseed were added to the diet (R&F diet), the presence of rye bran increased caecal ENL concentration in rats compared with the caecal ENL concentration of rats consuming the F diet (Table 2). This was observed as the highest medians, minima and maxima in distal ileal and caecal contents of rats consuming the R&F diet. Differences in ENL concentrations in caecal contents were significant ($P < 0.05$) between all the diets (R, F, and R&F) and the control (C). In the distal ileum, ENL concentrations of rats consuming diets containing flaxseed (R&F and F) did not differ significantly from each other, although they differred significantly ($P < 0.05$) from the R and C diet groups. END concentration (Table 2) was significantly ($P < 0.05$) higher in the ileal and caecal contents of rats consuming the F diet.

Plasma ENL concentrations of the R&F and F groups was significantly higher ($P < 0.05$) than in the C group (Table 2). Plasma ENL concentration in the group consuming rye bran was between the groups consuming flaxseed and the control group, and it did not differ significantly from the other dietary groups.

Diets containing rye bran favoured significantly ($P < 0.05$) caecal butyrate production in rats (Fig. 2). The relative proportion (%) of butyrate in the caecal con-

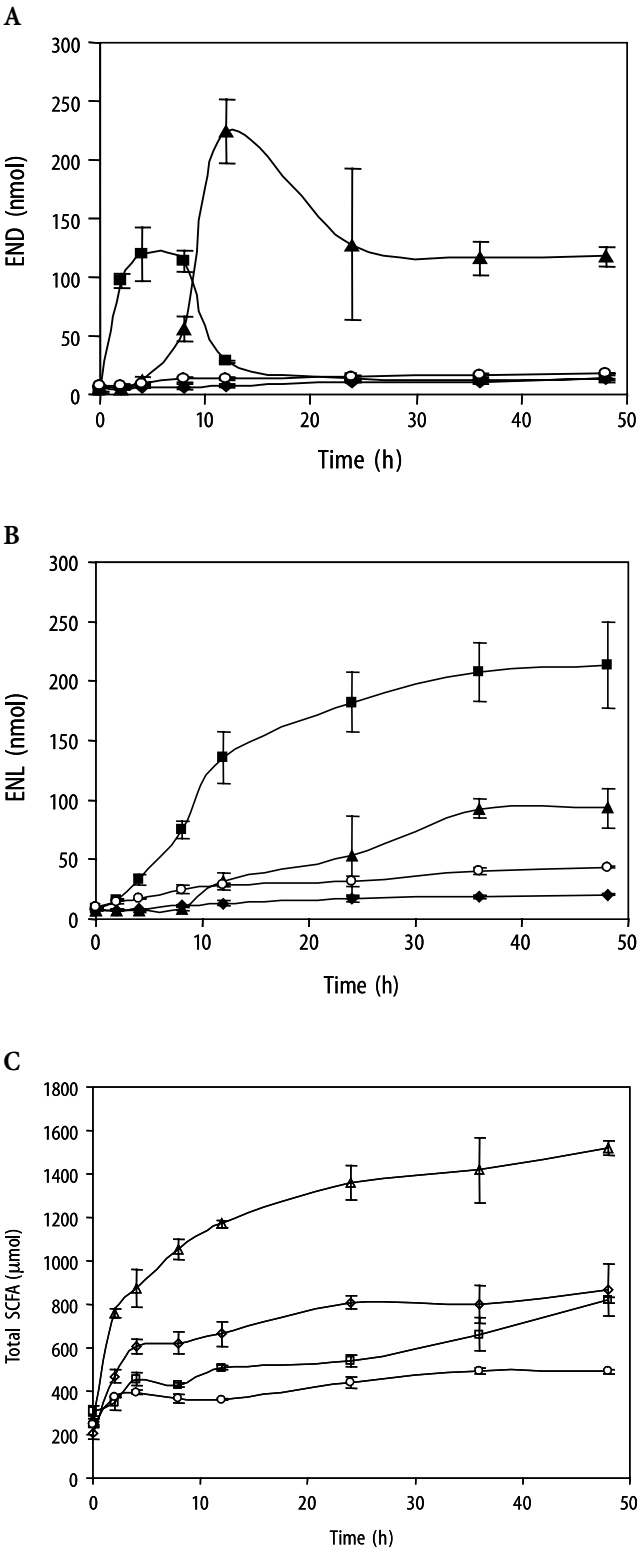

Fig. 1 The time course of enterodiol (**A:** END), enterolactone (**B:** ENL) and total short-chain fatty acid (**C:** SCFA; a sum of acetate, propionate and butyrate) formations from rye bran (R; ◆), flaxseed (F; ■) and rye bran and flaxseed (R&F; ▲) supplements and from faecal background (○) in the *in vitro* fermentation model using human faecal microbiota

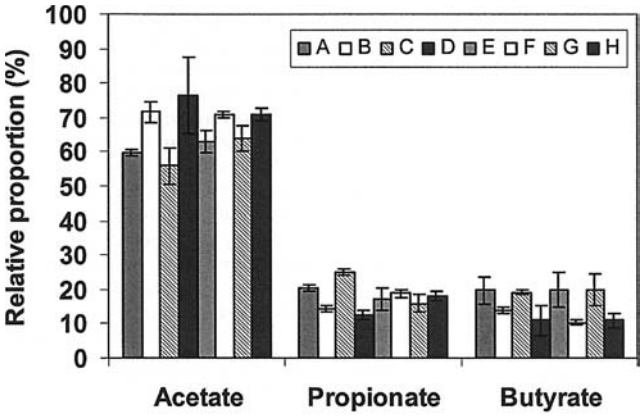

Fig. 2 Relative proportions (%) of acetate, propionate and butyrate of total SCFA. Symbols: A: rye bran (R: grey); B: flaxseed (F: white); C: rye bran and flaxseed (R&F: light grey) supplements and D: faecal background (black) after 24 h incubation with human faecal microbiota in vitro. The same response in caecal contents of rats consuming E: R diet (grey); F: F diet (white); G: R&F diet (light grey) and H: a control diet (black). Error bars correspond to standard deviations of the samples

tents was 20 % in the R and R&F groups, whereas it was only 10–11 % in the F and C groups.

Discussion

In order to evaluate the suitability of the *in vitro* fermentation model for prediction of ENL formation from plant lignans, substrates containing rye bran and flaxseed were fermented with human faecal microbiota *in vitro* and introduced to an *in vivo* rat model. The results showed that there were differences in the influence of rye bran on mammalian lignan production between the *in vitro* and *in vivo* models. On the contrary, SCFA productions were similar in both experimental models.

Rye bran (R supplement) showed formations of END and ENL, which were below those of the faecal background. Lignan content of rye bran was low. By contrast, rye bran provided a source of carbohydrates, which were readily fermented to SCFA and pH decreased more rapidly than in the samples containing F supplement. The rapid decrease in pH, lack of carbohydrate nutrients and accumulation of products at late timepoints may have caused external stress for the microbiota shown as a delay in END production and as suppression of ENL production after 24 h in *in vitro* samples containing R&F supplement. Batch cultures are static and conditions may not be physiologically relevant at late timepoints [25]. Thus the application of a semi-continuous model would mimic the conditions in the colon better than a batch model in the case of incubation times longer than 24 h. It is also possible that, when readily fermentable carbohydrates are present, the metabolism of the faecal microbiota is directed to the fermentation of readily available substrates rather than to bioconversion of phenolic compounds. Slowly fermented powdered cellulose, abundant in F supplement, provided a carbohydrate source for the late timepoints and pH decreased slowly during its fermentation, which may have favoured ENL production.

In contrast to the *in vitro* findings, in the *in vivo* rat model the conversion of ENL from plant lignans in flaxseed was enhanced in the presence of rye bran, when measured from the caecal contents of rats consuming the R&F diet. There was a high inter-individual variation in ENL concentrations in the *in vivo* rat model, which is in accordance with previous observations with both human subjects and animals [26, 27]. Complete conversion to ENL *in vivo* resulted in low concentrations of END in the caecal contents of rats. Thus the *in vivo* rat model did not correlate with the *in vitro* faecal fermentation model in the influence of rye bran carbohydrates on ENL conversion from flaxseed plant lignans.

The lack of correlation of the lignan results between the *in vitro* and *in vivo* models is likely to be due to differences between rat and human colonic microbiota [28]. However, there are differences also in the approaches of the two models in respect to the lignan conversion. In the *in vitro* fermentation model the inocu-

Table 2 The concentrations of mammalian lignans in the distal small intestinal and caecal contents and plasma enterolactone concentrations of rats fed with 9 % rye bran (R), 1 % flaxseed meal (F), 9 % rye bran and 1 % flaxseed meal (R&F) supplemented diets or the control (C) diet for four weeks[1]

Intestinal part	R (n = 8)	F (n = 8)	R&F (n = 8)	C (n = 7)
	nmol/g			
Distal small intestine				
Enterodiol	0.6 (0.4, 1.6) *a*	75.1 (7.3, 206.3) *c*	6.5 (2.6, 39.0) *b*	0.5 (0.1, 1.3) *a*
Enterolactone	116.5 (58.3, 155.5) *b*	298.0 (210.0, 998.6) *c*	621.7 (405.1, 1465.5) *c*	32.4 (25.6, 94.5) *a*
Caecum				
Enterodiol	0.5 (0.3, 1.1) *a*	50.0 (5.9, 182.8) *c*	3.8 (2.1, 20.5) *b*	0.5 (0.0, 1.2) *a*
Enterolactone	72.4 (30.6, 141.0) *b*	260.6 (137.0, 335.4) *c*	407.1 (229.8, 532.7) *d*	23.8 (12.3, 56.0) *a*
	nmol/l			
Plasma				
Enterolactone	23.9 (9.5, 72.2) *ab*	56.9 (22.9, 172.7) *b*	59.4 (27.5, 126.3) *b*	8.8 (6.6, 18.0) *a*

1 Values are median (minimum, maximum), n = 7–8. Medians in a row without a common letter differ, $P < 0.05$ (Mann-Whitney test with Bonferroni adjustment)

lum is a dense suspension maintaining a stable microbiota during the fermentation [29]. In the *in vivo* rat model the animals were fed with supplemented diets for four weeks, which is enough for adaptation of the colonic microbiota. It has been shown previously that a pre-adaptation period with ferulic acid enhanced the excretion of ENL in rats consuming wheat bran and ferulic acid [30]. Rye bran is a good source of ferulic acids [31], and it is possible that rye bran components, e. g. ferulic acid, enhanced the ENL formation in the *in vivo* rat model and suppressed its formation in the *in vitro* fermentation model. Further investigations are needed to reveal the components in rye matrix, which suppress ENL conversion from plant lignans.

In the present study the ileal appearance of END and ENL, well established bacterial metabolites of plant lignans [3, 28], was an indication of enterohepatic circulation. Enterohepatic circulation of mammalian lignans resulted in a low diurnal variation in plasma concentration of ENL in cannulated pigs [32]. It should be emphasized that in the present work conjugated hepatic metabolites of END or ENL [33] were not included in the method.

The fermentation of carbohydrates in rye bran showed enhanced total SCFA and butyrate productions in both *in vitro* and *in vivo* models. The molar proportions of butyrate were increased in colon contents of rats consuming rye bran [34]. High faecal butyrate concentration has also been associated with wholemeal rye bread in human subjects [35]. These results also suggest that the *in vitro* batch model suits better for metabolite formation occurring at earlier timepoints than for the slow ENL formation.

The *in vitro* fermentation model does not include absorption, hepatic metabolism or enterohepatic circulation, which are present in the animal models and in the human body. It mimics only the caecal conversion of dietary constituents and reveals the metabolite formation during the experiment. Presence of rye bran matrix suppressed conversion of rye lignans in the *in vitro* fermentation model [17]. Identification, order of appearance and rate and extent of metabolite formation *in vitro* have been demonstrated for pure plant lignans, quercetin derivatives, and for condensed and free catechins [3, 36, 37]. These findings elucidate the role of microbiota in the structural transformation reactions of dietary phenolics. In particular, influence of plant matrix, order of appearance and dynamics of metabolite formation are difficult to show in the *in vivo* experiments, because of high inter-individual variation [26, 27] and occurrence of many simultaneous biological events. Despite the limitations of the *in vitro* batch fermentation model it has a position in studies of release and bioconversion of phenolic compounds.

Conclusions

ENL formation from flaxseed plant lignans was a slow process in the *in vitro* fermentation model using human microbiota. The *in vitro* fermentation and the *in vivo* rat models responded differently to the presence of rye bran in ENL formation from flaxseed lignans and no correlation was found between the models. This may also be due to differences in the colonic microbiota or application of a batch *in vitro* fermentation model. However, SCFA patterns were similar in the two models.

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