

## **Author's response to reviews**

**Title:** Four-week short chain fructo-oligosaccharides ingestion leads to an increase in fecal bifidobacteria and cholesterol excretion in healthy elderly volunteers

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**Author's response to reviews:** see over

**The Editor,  
Nutrition Journal**

November 20, 2007

Dear Editor,

Please find enclosed our revised manuscript entitled "*Four-week short chain fructo-oligosaccharides ingestion leads to an increase in fecal bifidobacteria and cholesterol excretion in healthy elderly volunteers*" (MS: 1315258621152779).

Changes in the revised manuscript are underlined (yellow). We have addressed all the questions raised by the reviewers in this letter.

We hope that the manuscript is now suitable for publication in your journal. Should the manuscript be accepted for publication, all authors would provide a completed copy of the author's form.

Whatever your final decision is, I would like to thank the editorial board and the reviewers for their pertinent remarks, which have helped us to improve the manuscript.

Yours sincerely,

Damien Paineau

## Answers to reviewer comments

### Reviewer #1 (Kevin Whelan):

#### General:

This is an interesting and carefully conducted investigation of the impact of scFOS on faecal microbiota, pH and sterol concentrations. It appears to be a well conducted study with appropriately measured outcomes. Although not entirely novel in terms of the impact of scFOS on the faecal bifidobacteria, this study is novel in terms of its age group (older people), impact upon sterol metabolism and impact upon other microbiota. The authors should be congratulated on some of their extremely novel findings relating to impact upon faecal clostridia.

#### Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached):

##### **Question 1:**

Results and discussion – the major improvement required from this manuscript is extensive discussion of the remarkable effects on faecal clostridia. The results show that scFOS supplementation increases clostridia and withdrawal of scFOS then increases them even further – the authors are truly the first group to have demonstrated such effects. Although these changes are mentioned in the results they are not mentioned in the discussion. These are major observations and warrant extensive discussion, particular due to the age related changes in microbiota and the increased risk of *C. difficile* colonisation in older people. Perhaps supplementation may be negative in older people?? Older people may be more frequent antibiotic users – and certainly in vitro studies (MacFarlane) have shown that FOS supplementation in the presence of antibiotics actually increases clostridia. In view of the effect of scFOS on clostridia there should be significant discussion of this finding and the title of the manuscript should also probably reflect this.

Answer 1: The reviewer is right, and we appreciate the opportunity to be clearer. In this study, we found significant increase in *Clostridium* spp. after sc-FOS ingestion discontinuation. We decided not to highlight this finding in the manuscript first version, since we were concerned about potential misinterpretations. *Clostridium* spp. is indeed a major component of normal anaerobic microflora and it can not be considered as a deleterious or beneficial genus. Even if

there is an abundant literature regarding the risk of pseudo membranous colitis and/or *Clostridium difficile* infection in elderly, global increase of this genus without any characterisation of the species and toxinogenic properties prevents from drawing any conclusions. It would have been interesting to measure the effect of prebiotic consumption on toxinogenic *Clostridium difficile* subspecies, which are the only pathogenic *Clostridium difficile* strains. However, such measurements require special methods (cellular cultures, biomolecular analysis), that were out of our work scope.

Considering that this remark was provided as a major compulsory revision, we mentioned *Clostridium* spp. increase in the comment section of the revised manuscript:

“We also found an increase in *Clostridium* spp. after sc-FOS ingestion discontinuation. *Clostridium* spp. is a major component of normal anaerobic microflora and it can not be considered as a deleterious or beneficial genus. Since some toxinogenic subspecies of *Clostridium difficile* are related to an increased risk of pseudo membranous colitis and/or infection in older people, it would have been interesting to measure sc-FOS effects on these subspecies. However, we did not perform those analyses, which were out of our study scope. Further studies may investigate this point, using adequate measurement methods of species concentrations and toxinogenic properties (cellular cultures, biomolecular analysis)”.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

**Question 2:**

Introduction - you should make reference to the studies already done of prebiotics in older people in the introduction – although you quote Bartosch there are others (e.g. Bunout, JPEN, 2002 – although didn't measure microbiota).

Answer 2: We thank the reviewer for this reference. As suggested, we have added several references in the revised version of our manuscript:

T. Mitsuoka, H. Hidaka, T. Eida (1987). Effect of fructo-oligosaccharides on intestinal microflora. *Nahrung*; 31 (5-6): 427-36.

J. M. Hamilton-Miller (2004). Probiotics and prebiotics in the elderly. *Postgrad Med J*; 80 (946): 447-51.

D. Bunout, S. Hirsch, M. Pia de la Maza, C. Munoz, F. Haschke, P. Steenhout, P. Klassen, G. Barrera, V. Gattas, M. Petermann (2002). Effects of prebiotics on the immune response to vaccination in the elderly. *JPEN J Parenter Enteral Nutr*, 26 (6): 372-6.

**Question 3:**

Methods – how (and where from) were subjects recruited.

Answer 3: Subjects were recruited through announcement near the investigation site. Volunteers received around 500 dollars for participating in the study, which was accepted by the local Ethics Committee.

**Question 4:**

Methods – please justify why scFOS was chosen rather than inulin (for example)

Answer 4: We decided to test sc-FOS, which have already been studied for their bifidogenic properties in most populations, and we extended these studies to look for potential effects in elderly. These prebiotics have been in our field of interest for many years.

**Question 5:**

Methods – it should be made even clearer that OFTT was measured at the end of each of the feeding periods.

Answer 5: The reviewer is right. We have modified the manuscript as suggested:

“Stools had been collected for three consecutive days before the end of weeks 2 (basal period), 6 (ingestion period), and 10 (follow-up period), that is to say at the end of each feeding period”.

**Question 6:**

Methods – please provide brief details of how the pellets were measured in faeces (presumably X-ray).

Answer 6: Detailed methodology is provided in the following reference, which is indicated in the manuscript method section: Cummings JH, Wiggins HS: Transit through the gut measured by analysis of a single stool. *Gut* 1976, 17:219-223.

**Question 7:**

Methods – please clarify – were stools frozen prior to culturing or were they cultured fresh? If they were cultured following freezing then the limitations of this should be acknowledged and the interpretation of the results should be amended accordingly.

Answer 7: This is an important point. In our study, stools were cultured fresh within 2 hours after emission. To make this point clearer, we have added the word “fresh” in the revised manuscript: “Fresh faecal samples (1 g) were introduced in the first pre-weighed tube of the dilution series and thoroughly mixed”.

**Question 8:**

Methods – page 7 – “outnumber” – is this the correct word?

Answer 8: We have substituted ‘outnumber’ by “distinguish” in the revised manuscript.

**Question 9:**

Methods – were samples diluted in buffer prior to pH measurement? If so details of the buffer should be provided.

Answer 9: Yes, samples were diluted in pH 7 buffer prior to pH measurement.

**Question 10:**

Data analysis – details of data analysis of bile acids etc should also be provided (2 way ANOVA presumably).

Answer 10: Faecal metabolites were analysed using 2 way ANOVA, as the other parameters.

We mentioned this point in the revised version of the manuscript:

“Two-way ANOVA, with time and treatment as factors, was used to compare bacterial concentrations, pH and faecal metabolites between the three periods”.

**Question 11:**

Results - first line – refers to “control” – please be consistent with terminology e.g. ‘baseline’ or ‘follow-up’.

Answer 11: We modified the manuscript as suggested.

**Question 12:**

Discussion - the discussion on page 13 includes the following sentences which seem to be contradictory: “microbial transformation of cholesterol into coprostanol was not influenced by scFOS in our study” “ingestion of low dose of scFOS .....prevented microbial conversion of cholesterol to.....coprostanol.....”

Answer 12: We thank the reviewer for this comment. Although these sentences were actually not contradictory, we modified the revised manuscript to be clearer:

- 1) We have moved “in our study” at the beginning of the first sentence to highlight the fact that the results apply to the present experiment: “In our study, the microbial transformation of cholesterol into coprostanol was not influenced by scFOS ingestion”.
- 2) We have added “According to these studies” at the beginning of the second sentence: “According to these studies, ingestion of low scFOS dose by humans, which prevented microbial conversion of cholesterol to cytotoxic molecule, coprostanol, potentially carcinogenic, could be interesting for human”.

It is now clearer that the first sentence refers to our results, whereas the second one refers to previous experiments. Apparent contradiction between the two sentences may result from insufficient prebiotic dose in our study, as discussed in the comment section (see also answer to question 13).

**Question 13:**

Discussion - the discussion regarding increases in faecal cholesterol are reportedly due to the impact of the microbiota in blocking cholesterol conversion to coprostanol. However, wouldn't this result in a reduction in coprostanol concentrations (which didn't occur)? Is it at all possible that the increase in cholesterol concentrations may be due in part to the scFOS affecting cholesterol absorption in the gut?

Answer 13: We do not think that sc-FOS may affect cholesterol absorption in the gut, but it may probably act on cholesterol bacterial metabolism as discussed. The fact we did not find significant results regarding cholesterol bacterial metabolism in our study may be related to insufficient prebiotic doses, as discussed.

**Question 14:**

General – the manuscript is generally well written but would benefit from review by a scientist with English as a first language.

Answer 14: The manuscript has been reviewed by a scientist with English as a first language.

Discretionary Revisions (which the author can choose to ignore):

**Question 15:**

Methods – you ask subjects to exclude FOS containing foods from their diet. There are number of issues with this:

-firstly the largest source of FOS in the diet is from wheat – was this excluded – if so then, as wheat is so ubiquitous in Western diet this would have made a significant change to the overall dietary and nutrient intake.

-Although asparagus, artichoke etc contain high levels of FOS they are consumed in such low quantities to contribute negligible amounts of FOS to the diet (see Moshfegh) – perhaps their exclusion was unnecessary.

Answer 15: We agree with those comments. However, we mainly aimed at avoiding large discrepancies between volunteers regarding sc-FOS intake, which means we tried to detect abnormal consumers (subjects consuming, in addition to wheat, high sc-FOS sources, such as asparagus, artichoke...).

**Question 16:**

Methods – the limitations of culture-based enumeration of the microbiota should be acknowledged (perhaps in the discussion).

Answer 16: We have mentioned these limitations in the paragraph dealing with *Clostridium* spp. increase interpretation.

“Culture-based enumeration of the microbiota does not usually allow for measurement of bacterial species, but mainly bacterial genus”.

**Question 17:**

Methods – you say that subjects recorded frequency and consistency of stools and a cut-off for diarrhoea was defined. These results are not reported in the text. Also, consistency is notoriously subjective for people to record. How was stool consistency rating improved (e.g. stool charts??).

Answer 17: Stool consistency was assessed by subjects according to three levels: “normal / liquid / hard”. There was no diarrhoea during the study.

**Reviewer #1 (Randal Buddington):**

General:

There is a well established, and accepted, relationship between supplementing the diet with prebiotics and increasing the proportion of the GI bacteria represented by species considered to provide a diversity of health benefits. The senior author (Y Bouhnik) has been an author for several papers that describe and contribute to our understanding of how the prebiotic scFOS increases fecal bifidobacteria in human subjects. The present contribution describes a study that examined if and how supplementing the diet of 12 elderly subjects changes fecal bacterial and chemical composition and stool characteristics and patterns. Since elderly subjects exhibit declines in health promoting bacteria and are at greater risk of GI disturbances, the contribution addresses an issue of relevance to health, particularly with the increasing percentage of the population represented by the elderly.

The manuscript will require editing to correct grammar and spelling. The following comments are provided to assist the authors in addressing issues that some readers will find uncertain or controversial.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

**Question 1:**

The most pressing concern is that the study is not as novel as the authors contend. There have been several previous studies that have examined if and how prebiotics, including scFOS, influence the fecal bacteria of elderly humans. For example, Mitsuoka T, Hidaka H, Eida T. Effect of fructo-oligosaccharides on intestinal microflora. *Nahrung*. 1987;31(5-6):427-36. And there is a review available (Hamilton-Miller JM. Probiotics and prebiotics in the elderly. *Postgrad Med J*. 2004 Aug;80(946):447-51). Additional papers have described similar responses in elderly dogs (Grieshop et al., Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. *Arch Anim Nutr*. 2004 Dec;58(6):483-93). The authors will need to expand their review of the literature as others have previously examined if prebiotics influence the fecal bacteria in elderly subjects. Some readers will be aware of these references. The authors will also need to inform readers how the present contribution expands the literature by providing new information.

Answer 1: We thank the reviewer for these interesting comments. We have added the reference by Mitsuoka *et al.* in the introduction, and the one by Hamilton *et al.* We read with interest the paper describing similar effects in elderly dogs, but we did not add this reference to our manuscript as we consider important to focus our comments in humans. We have also added other references (see answer to question 2, first reviewer). To take into account those previous studies, we have modified the revised manuscript by tempering our comment regarding this study originality:

“The bifidogenic effect of sc-FOS has been extensively demonstrated in adults [6, 26-28], but rarely in elderly [19, 21, 22]”.

Regarding the contribution of this study to the literature, we would like to stress the following points:

- We extensively studied faecal metabolites of neutral sterols and bile acids in humans and found that sc-FOS prebiotics may change cholesterol metabolism in elderly, which is clearly associated with colon cancer risk.
- We measured oro-faecal transit time, which is a major problem in elderly population. We failed to show any effects of prebiotics.

We underlined those original results in our conclusion:

“Under our experimental conditions, i.e. 8 g/d for 12 days, we failed to show any sc-FOS effects on OFTT, which is commonly increased in elderly living in industrialised countries. We found significant change in cholesterol metabolism, which could potentially exert protective action against colon cancer; however, this finding warrants further studies”.

## **Questions 2:**

Methods: Some of the methods are not adequately described.

### **Question 2a:**

a. Were the radio-opaque pellets that were fed a mixture of different shapes, or was a different shape fed each day? Is there any evidence that the pellets traverse the GI tract at the same rate as digesta?

Answer 2a: See reference 20. Radio-opaque pellets were of three different shapes. This method has been validated in healthy volunteers by comparing it with mean transit time measured by a continuous marker method. Both methods were very closely correlated ( $p < 0.001$ ). We believe radio-opaque pellets present the same transit time as insoluble fibre.

**Question 2b:**

b. How were the bifidobacteria identified on the plates? Because the Beeren's medium used is a semi-selective agar, other bacterial groups will grow. Unless the authors confirmed the identities of representative colonies growing on the plates (e.g., by the fructose-6-phosphate phosphoketolase activity), the reported counts are not necessarily bifidobacteria and may be misleading, or at the least inaccurate.

Answer 2b: We checked the accurate bifidobacteria measurement by sampling typical and non-typical colonies and analysing them for bacterial shapes under optic microscopy. According to the results, we calculated the bifidobacteria concentration using the ratio method.

**Question 2c:**

c. Were the stools diluted before measuring pH, or was the pH probe placed into the solid stool?

Answer 2c: We measured the faecal pH in diluted stools.

**Question 2d:**

d. Were the methods used for bile acids and neutral sterols validated? Was the percent recovery determined to ensure readers the method is appropriate? Since a modification of a previous method was used, readers (and reviewers) will want to know if this improved the analysis, and if so how.

Answer 2d: Yes, methods for bile acids and neutral sterols measurement have been validated (see reference by Stempfel and Sidbury). After the first description of the method, some adaptations were made and the results observed with the adapted method were regularly published:

- Bouhnik Y, Neut C, Raskine L, Michel C, Riottot M, Andrieux C, Guillemot F, Dyard F, Flourie B. Prospective, randomized, parallel-group trial to evaluate the effects of lactulose and polyethylene glycol-4000 on colonic flora in chronic idiopathic constipation. *Aliment Pharmacol Ther.* 2004 Apr 15;19(8):889-99.
- Bouhnik Y, Flourie B, Riottot M, Bisetti N, Gailing MF, Guibert A, Bornet F, Rambaud JC. Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutr Cancer.* 1996;26(1):21-9.

- Sacquet E, Parquet M, Riottot M, Raizman A, Jarrige P, Huguet C, Infante R. Intestinal absorption, excretion, and biotransformation of hyodeoxycholic acid in man. J Lipid Res. 1983 May;24(5):604-13.
- Bento OP, Martins JM, Lanca MJ, de Abreu MC, Viegas-Crespo AM, Freire JP, Almeida JA, Riottot M. Effects of ileo-rectal anastomosis on cholesterol metabolism in pigs fed either casein or extruded soya beans. Br J Nutr. 2004 May;91(5):689-98.

**Question 2e:**

e. Log bacterial counts are generally transformed prior to analysis. The authors may want to consult a statistician about the most appropriate method to analyze their data.

Answer 2e: We agree with this important point. Log bacterial counts were transformed prior to analysis and converted again in log for the results.

**Questions 3:**

3. Results

**Question 3a:**

a. In light of the above concern of bifidobacteria identification, this reviewer has concerns about whether the data are 'real'

Answer 3a: These data are "real" (and not virtual!) as explained above.

**Question 3b:**

b. The authors may want to consider another approach for analysis of the data.

Specifically, since each subject effectively serves as their own control, the authors may want to consider expressing fecal characteristics as a percentage or ratio of the value measured during the initial period before the scFOS supplement. This accounts for individual variation and may reveal differences that are obscured because of individual variation in absolute values. It is possible that differences will be detected where none are now evident.

Answer 3b: Statistics were applied for all patients in the same period, as usually performed. However, taking into account all individual results, we did not find any major individual deviation. Such deviation is also evaluated by using SEM (Standard Error Mean), which is mentioned in the tables.

**Question 3c:**

c. Similarly, the authors could prepare ratios of apparent 'bifidobacteria' relative to clostridia and enterics, or the percentage of anaerobic bacteria represented by the different bacterial groups.

Answer 3c: We did not calculate ratios between bacteria genders since it results in comparing measures obtained with methods presenting sensitivity difference, thus potentially leading to abnormal results.

**Question 3d:**

d. Figure 1 should be redone such that each of the four measured parameters are presented as 3 bars basal, scFOS, follow-up. This will make it easier for readers to immediately see if there is an influence.

Answer 3d: We modified the figure as suggested.

**Questions 4:**

4. Discussion

**Question 4a:**

a. Can the authors speculate on the mechanism responsible for the increase in fecal cholesterol?

Answer 4a: See page 13: increasing faecal cholesterol was probably related to decreasing cholesterol conversion to other sterols (coprostanol, cholestanol, ketones). We have underlined this possible mechanism in the revised version of the manuscript:

“The mechanism of such increase could be related to decreasing cholesterol bacterial transformation, although we failed to find any significant sc-FOS effect on cholesterol bacterial metabolism”.

**Question 4b:**

b. Is it possible that the bacterial assemblages in the elderly respond slower than those of younger individuals? If so, the 4 week period might not have been long enough to alter both the species composition and metabolic activities.

Answer 4b: We thank the reviewer for this interesting comment. It would be interesting to perform further studies in order to compare the kinetics of the sc-FOS effects on microflora and bacterial metabolites in young people versus elderly people. To our knowledge, no such study has been performed yet.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Discretionary Revisions (which the author can choose to ignore)

**Question 5:**

1. The authors may want to discuss how various doses of scFOS have been used and the dose-response relations. Is it possible that a larger dose is needed for elderly subjects? The authors do raise this question, but leave readers wondering. Also, the authors mention a dose-response relationship noted among animals models for poorly digested carbohydrates.

Answer 5: We have discussed the fact according to which a higher dose of prebiotics may have result in significant results, especially regarding bacterial metabolites. That is why we mentioned a study in animals, which uses much higher doses of prebiotics compared to studies in humans (compared to body weight).