Author's response to reviews

Title: Dosage effect on uropathogenic Escherichia coli anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study

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Version: 4 **Date:** 22 February 2010

Author's response to reviews: see over

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Dear Editor,

Please find attached the revised version of our manuscript entitled 'Dosage effect on uropathogenic *Escherichia coli* anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicenter study' by AB Howell, et al.

This manuscript has been substantially amended according to the comments of the reviewers.

We thank the two reviewers for their useful comments and discussion and you, as the Editor, for giving us the opportunity to resubmit our manuscript.

We hope the revised version will be suitable for being published BMC Infectious Diseases.

Your sincerely.

Jean-Philippe Lavigne, MD, PhD
On the behalf of the co-authors

Replies to Reviewer's comments: (9132281163257164)

REFEREE: 1

There are some specific aspects that need to be clarified:

- *the volunteers received the tablets at 8.00, the first urine period to be collected however started at 9.00 to 14.00. What happened to the 8.00 to 9.00 urine period* : Howell et al. have previously demonstrated the bacterial anti-adhesion activity of PAC. This activity continuously increased in a regular progression, peaking at 4–6 h post-consumption and persisting in the urine for at least 8 h, suggesting potential protection against bacterial attachment in the uroepithelium during this period. This study have demonstrated the low level of PAC in urines between 0 and 1h after PAC absorption. We added a comment (P. 14, Ln 3 ; new manuscript).

In addition the authors describe their findings sometimes with peak activity. It should be noted that there were only 2 collection periods, from 9.00 to 14.00 and from 14.00 to 8.00. Therefore it should rather be described that the activity was higher in one or the other collection periods. : No the second collections were made exclusively the following morning (8 :00AM) as mentioned P. 8, Ln 11, new manuscript.

Similarly in the tables it should be clarified that the results are from collection periods and not from a single collection time point at 24h, such as in blood kinetics for example. : No the second collections were made exclusively the following morning (8:00AM).

- t*he authors describe that urines with leucocytes were excluded. How many urines or volunteers were those and why were they excluded*. : Urines with leucocytes was an exclusion criteria. If a volunteer presented at inclusion leucocytes, he was excluded. This case does not happen.

- the C. elegans model is not clear. *How was this performed*. : We explain the technique in Methods part (P. 10-11, new manuscript)

An avirulent E. coli was used as control, but there was still a lethal time of 6 described in the table. What does this mean? The nematodes have a short (2-weeks) life span that facilitates host-bacteria interaction analysis.

Is this hours or days.? 'days'. we added this data in Table 3 (new manuscript)

Apparently an avirulent E. coli is also lethal to C. elegans. No the nematodes have a short (2-weeks) life span. OP50 is an avirulent strain and serves as control (P. 11, Ln 18, new manuscript).

In the methods the authors describe that LT 50 is the time to kill 50% respectively 100% of C. elegans. In table 3 only LT 50 is reflected. Is there also a LT 100? In the nematodes experiments, the most interesting statistical analysis corresponded to LT50. However we added the LT100 data in a new table 3 (new manuscript).

The validity of this assay should also be discussed. : This model have been previously published and validated in the study of host-bacteria interactions (1 146 references in PubMed). This model has been validated by different teams with *E. coli* (Ref 12, 24,25,26 in our manuscript) and we validated this model in this assay in a previous study (Ref 12). This point was discussed in Discussion section (P. 16-17 ; new manuscript).

- table 1 MRHA assay: There is 0% AAA at 0 h, except for the Japanese volunteers. *What does 0 h mean*? *Is this urine prior to ingestion of cranberry tablets, or is this the urine between 8.00 to 9.00*? 0 hr is the pre-cranberry consumption collection, so it is the background sample. We added the precision P. 8, Ln 12 and in the Table 1; new

manuscript.
Apparently the evaluation is semiquantiative, using steps of AAA of 100%, 50% and 0%. What was the reason to use a semiquantitative approach. : The assay is semi-quantitative based on the percentage of agglutination. Since, the results are red microscopically and not mechanically, the results must be reduced to differences that can be discernable by the human eye. Research has shown that humans can detect differences between 25 and 50%, but not between 50 and 60%, etc. This type of method gives us semi-quantitative data for more precision in the results, rather than just a plus or minus for activity.
The analysis of this assay is not clear. The japanese volunteers receiving placebo apparently showed a 50% AAA at 0h in one of the four times the assays were used. Was this a reproducable finding? Yes all the experiments were provided in triplicate (P. 9, Ln 19, new manuscript).
This was however not found at 1-6 h and 24 h. Is there an explanation for this? The explanation (or hypothesis) was done in Discussion section (P. 15-16, new manuscript). Occasionally we get a positive 0-time or background sample in a volunteer, as there is a certain percentage of the population that produces endogenous adhesion inhibitors, such as Tamm-Horsfall glycoprotein. These inhibitors can be induced by a number of factors, dietary and environmental.
- Page 5: *please explain DMAC* : The proanthocyanidin content has been determined using an updated dimethylaminocinnamaldehyde method (DMAC) taking advantage of the selective colorimetric reaction between PACs and DMAC after open column gel chromatography on Sephadex® LH-20. We added informations P. 7, Ln 23 ; new manuscript.

- Page 7: *please explain cleared through IRB* : IRB (Institutional Review Board) corresponds to the ethical commitee. This committee reviews research studies in order to protect participants. We replaced the term IRB by the ethics committee (P. 7, Ln 6; new manuscript).

REFEREE: 2
Abstract: Conclusion: The last statement should be removed as the so-called universal effect is doubtful with the small sample size. Eight volunteers from 4 countries (Europe 3, Asia 1) is not representative of universality. There are no volunteers for North and South America, and Africa. : We have deleted the sentence.

Methods
1. *The first line should read…..even if cranberry is a supplement…..* : This sentence has been corrected (P. 6, Ln 20, new manuscript).
2. *Read would like to know the specific ethical committees in these study sites*. : In each country, the study has been submitted and approved by the corresponding ethical committees (sud Mediterrannée, Budapest, Barcelona and Kyushu). We added this information (P. 6, Ln 21; new manuscript)
3. *Authors chose 32 females from Japan, Hungary, Spain and France, i.e 8/country. Readers would like to know the rationale for choosing 32 females from these countries. Does this number represent universality as claimed by the authors*. : Statistical analysis was based on our first study (Lavigne et al, Clinical Microbiology and Infection, 2008) with a sample of French patients (N=8). From this study, it is expected that the mean value of the patients with placebo is around 20 with a standard deviation of 5.5 and the mean value of the patients with treatment and a dose 36mg is around 8.2 with a standard deviation of 7.3. Due to the cross-over design of the study, the standard deviation of the difference between placebo and dose 36mg is equal to (5.5*5.5+7.3*7.3)^0.5=9.1. Assuming a risk a of 0.05 bilateral and a statistical power of 90%, the minimal sample size is N=8 patients.

So we enrolled 8 patients in each country. (Ref. Matched Pairs - Machin D, Campbell M, Fayers, P, Pinol A (1997) Sample Size Tables for Clinical Studies. Second Ed. Blackwell Science IBSN 0-86542-870-0 p. 73-74). Moreover the power of the test was suffisant because the differences observed in each test were very important.
4. *Readers would like to know the form of consent given by the volunteers* : This consent was added in supplementary data.
5. *Urine with an abundance of leukocytes and nitrites were excluded. Readers would like to know at what point during the participation period this was done*. : The detection of leukocytes and nitrites was measured for each urines and each regimen. If urines with an abundance of leukocytes were detected, these urines were excluded and the participation stopped. This case does not happen. We modified our sentence (P. 8, Ln 14; new manuscript).
6. *Volunteers in Japan and Hungary received 0, 36 and 72mg of PAC, while volunteers from France and Spain received 0, 18 and 36mg of PAC. The authors should explain the reason for the disparity in dose regimen*. : The optimal dose of PAC recommanded previously was 36 mg. All the participants followed this regimen. In the aim to evaluate the real optimal dose, the half of patients consumed 18 mg of PAC and the other half 72 mg of PAC. This repartition was performed by a randomized assay organized by the statistician.
7. …*Is universal within the population..This statement need to be changed. The sample size from these countries is not from the same population*. : We have modified the sentence in the abstract. In Introduction, this sentence could be maintained because it is a hypothesis.

Results
1.*The authors should explain the Non-significance of the dose between 36 and 72, this negates the authors statement that the effect of cranberry is dose dependent*. : No, the effect is clearly dose dependant. The non significance corresponded exclusively in urines collected at 1-6h but a significant difference was noted in urines collected at 24h. This dose dependence effect was noted whatever the method used.
2. *Page 12, readers would like to know the meaning of "geographic effect" for the volunteers from Japan*. : We changed the sentence (P. 13, Ln 22 ; new manuscript)
3. Page 13, line 6…. Should read ….was increased to 32%... : The sentence has been changed (P. 14, Ln. 18; new manuscript)

Discussion
1. *Page 14. Last paragraph, …due to the production of endogenous adhesion inhibitors that are produced by some people…..The authors should provide reference. One wonders if the adhesion inhibition is known to be present in only Japanese women* : There is no reference. It's just an hypothesis to explain this surprinsingly results. The regimen followed by European and Asian populations are different. Perhaps the alimentation could explain the results observed in this population.
2. *The authors should provide reference for difference in the metabolism between the Asian and European lifestyles.* : We deleted this sentence but it's exactely the same answer to the following question.
3. *Readers would also like to know if men with UTI could also benefit from cranberry consumption*. : Yes of course but currently the problematic of UTI is a women' disease. Over 11 million women a year, in the United States alone, report having had a urinary tract infection (UTI), accounting for direct costs of over $1.6 billion [Fihn, S. D., Acute uncomplicated urinary tract infection in women, N. Engl. J. Med. 2003, 349, 259–266]. Most women experience another UTI during their lifetime and 25% suffer recurrent infections [Hooton, T. M., Recurrent urinary tract infection in women, Int. J. Antimicrob. Agents 2001, 17, 259–268].

4. *The authors should in addition explain the nyctohemeral production briefly*. : It's not a production but a protection. With 72 mg, an important protection (detected by the different experiments) was present 24h after cranberry consumption. We added a comment (P. 16, Ln 9; new manuscript).