To
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Ghent, 12th June 2006

Report on microbial tests towards dust mite

In the framework of Chrisal’s R&D strategy for the Probiotics In Progress (PIP) product line, LabMET performed a number of microbial tests to verify the repellent activity of sporulating bacteria towards dust mite. Three types of experiments were performed in triplicate and are presented below.

1) Repellent action of PIP product towards dust mite

Sealed and aerated plastic containers, provided with a textile bottom, were inoculated with dust mite and incubated at 28°C at a humidity of 70%. No additional feed was added. Treatment of the containers with Chrisal’s PIP product was performed on the right half of the textile surface, with the other half being treated with an equal volume of sterile water. Treatment protocols were as follows:

- Container 1: Control (left and right side treated with water)
- Container 2: Daily treatment of the right side with PIP product (during 8 days)
- Container 3: 72 hours treatment of the right side with PIP product (during 2 weeks)
- Container 4: Weekly treatment of the right side with PIP product (during 3 weeks)

Results:

Container 1: Data are averaged over three replicate experiments. No significant differences were obtained between the dust mite counts on both halves of the surface. This indicates that with no treatment, the dust mites migrate randomly across the surface. The slight rise in absolute dust mite numbers also indicates that the lack of supplemented food is not detrimental to the population.

Fig 1 presents the results on dust mite counts of container 1.
Container 2: Daily treatment with the PIP product led to a significant drop in the numbers of dust mite on the treated half of the surface. Again, a slight rise on the untreated side was noticed, however, the total number of dust mites on the overall surface was lower compared to the control, indicating a possible stress on dust mite replication resulting from the PIP product. Results are presented in Fig 2.
Container 3: Treatment every 3 days showed a lower level of dust mite on the treated half of the surface after 2 weeks; however statistical data based on three experiments indicate that these results are not significant. Results are presented in Fig. 3.

**Fig 3: 72h treatment with PIP product**

- Water treated
- PIP treated

Container 4: The weekly treatment did not result in any significant result on the dust mite distribution as presented in Fig 4.

**Fig 4: Weekly treatment with PIP product**

- Water treated
- PIP treated
Conclusion: From the above results it can be concluded that the PIP product is effective in repelling dust mite when applied on a daily basis. Lowering of the treatment frequency after an initial intensive dose may result in a suppressed dust mite population.

2) Fecal deterioration:

A second experiment verified whether the spore forming bacteria present in the PIP product are able to grow on a faecal extract from dust mite. These extracts were obtained by removing dust mites from a 1-week populated container without additional food and suspending the remaining fractions in the container. This suspension was homogenised and filter sterilised, followed by its addition as a nutrient source to a watery suspension of the spore forming bacteria from the PIP product. Determination of the number of bacteria after 72 hours was performed by means of plate counts on Nutrient Agar at 37°C. Comparison was made to the control where sterile physiological solution instead of faecal extract was added to the bacterial suspension.

Results: As presented in Fig. 5, the PIP bacteria are able to grow on the faecal extract of dust mite. Even a 10-fold diluted extract resulted in a significant growth of the bacteria.

Conclusion: The above results indicate that the bacteria present in the PIP product are able to grow and hence consume faecal extract from dust mite.
3) Biocidal activity

Using Live/dead staining on the flow cytometry, it was determined whether the filtrate of a 48h old bacterial suspension of the PIP product was able to kill Staphylococcus aureus and Streptococcus faecalis.

Results: Live/dead counts on S. faecalis and S. aureus are presented in table 1.

Table 1: Viability counts on Streptococcus faecalis and Staphylococcus aureus to determine a possible biocidal action of the PIP product.

<table>
<thead>
<tr>
<th></th>
<th>Live</th>
<th>Dead</th>
<th>Total</th>
<th>Live (%)</th>
<th>Dead (%)</th>
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<tbody>
<tr>
<td>Control: Streptococcus faecalis</td>
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<td>18</td>
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<td>95</td>
<td>9433</td>
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<td>Control: Staphylococcus aureus</td>
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<td>Staphylococcus aureus + bacterial filtrate</td>
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<td>9717</td>
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</table>

Conclusion: No biocidal activity of the PIP product towards the tested bacteria was witnessed.

Overall conclusion: With a sufficiently high frequency of application, the PIP product was able to reduce the number of dust mite on the treated surface. Results further indicate that dust mite faeces can act as a nutrient source to the bacteria in the product. This may result in the suppression of dust mite allergy symptoms by application of the PIP product. No biocidal action of the PIP product towards Streptococcus and Staphylococcus was recorded.

Sincerely yours,

Dr. Robin Temmerman
Project manager