Footprint International: Probio Efficacy Stage 2

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Aims of work:

Refer to scope document (R+D Scope Probio Cleaner 21.12.2009).

1. Experimental Methodology

A) Bacterial Strains, Culture Preparation and Incubation Temperatures

The strain used is from a commercial bacterial culture collection and is fully traceable.

1. *Escherichia coli (E. coli)*: ATCC 25922
2. *E. coli* was cultured for 18h in tryptone soya broth (TSB) at 44°C prior to use in the experiments.

B) Membrane Preparation

1. Probio Cleaner was diluted 1:5 in sterile water. 10ml was filtered through a cellulose nitrate membrane allowing concentration of the bacterial spores present in the product on the surface of the membrane.
2. Filtration was repeated on separate membranes with 10ml of sterile water (control).
3. The membranes were then placed on the surface of tryptone soya agar (TSA) - a standard nutrient rich bacterial growth medium - plates and incubated for 3h at 22°C.

C) Bacterial Exposure Methodology

1. Stationary phase *E. coli* cultures were diluted in maximum recovery diluent (MRD), an isotonic protective medium, to a final concentration of approximately 1000 cells per ml of diluent. 1ml of diluent was then added directly to the surface of the membrane (giving a final concentration of approximately 1000 cells per membrane). Membranes were incubated at 22°C for 0, 2, 4, 6 and 8 days. The time course was performed

using membranes exposed to each of the experimental conditions i.e: sterile water (control) and Probio cleaner, to enable accurate comparison of the growth of *E. coli* in relation to each treatment.

2. After the appropriate incubation time the membranes were removed into 10ml MRD. After vortexing for 10 seconds to release the bacteria from the membrane into solution, 1ml of the MRD / bacteria suspension was taken and added to a sterile petri dish. 15ml of molten Tryptone Bile X-Glucuronide (TBX) Agar was subsequently added to the sample and mixed thoroughly. The plates were allowed to set and then incubated at 44°C for 18h.

   NOTE: TBX medium contains bile salts which inhibit the growth of gram positive bacteria other than E. coli. The medium also contains X-β-D-glucuronide which detects glucuronidase activity, an enzyme specific to E. coli, causing E. coli colonies to turn blue/green. This enables easy identification of E. coli colonies.

3. After 18h the number of viable *E. coli* cells on each plate was determined by colony count – each colony is taken to represent 1 bacterium from the initial suspension.

4. All experiments were performed in duplicate.

2. Results

Results are displayed as follows:

1. Graph 1: a graphical representation of the number of *E. coli* cells expressed as a percentage of water treated membrane (control).

   NOTE: Error bars are calculated as the standard deviation of the mean. Results are the averages of the duplicate experiments.

2. Table 1: the overall results for each treatment and time point indicating the effectiveness of the probiotic as an inhibitor of *E.coli* growth of over time.

3. Graph 2: a graph of the effectiveness of the probio over time.

Graph 1

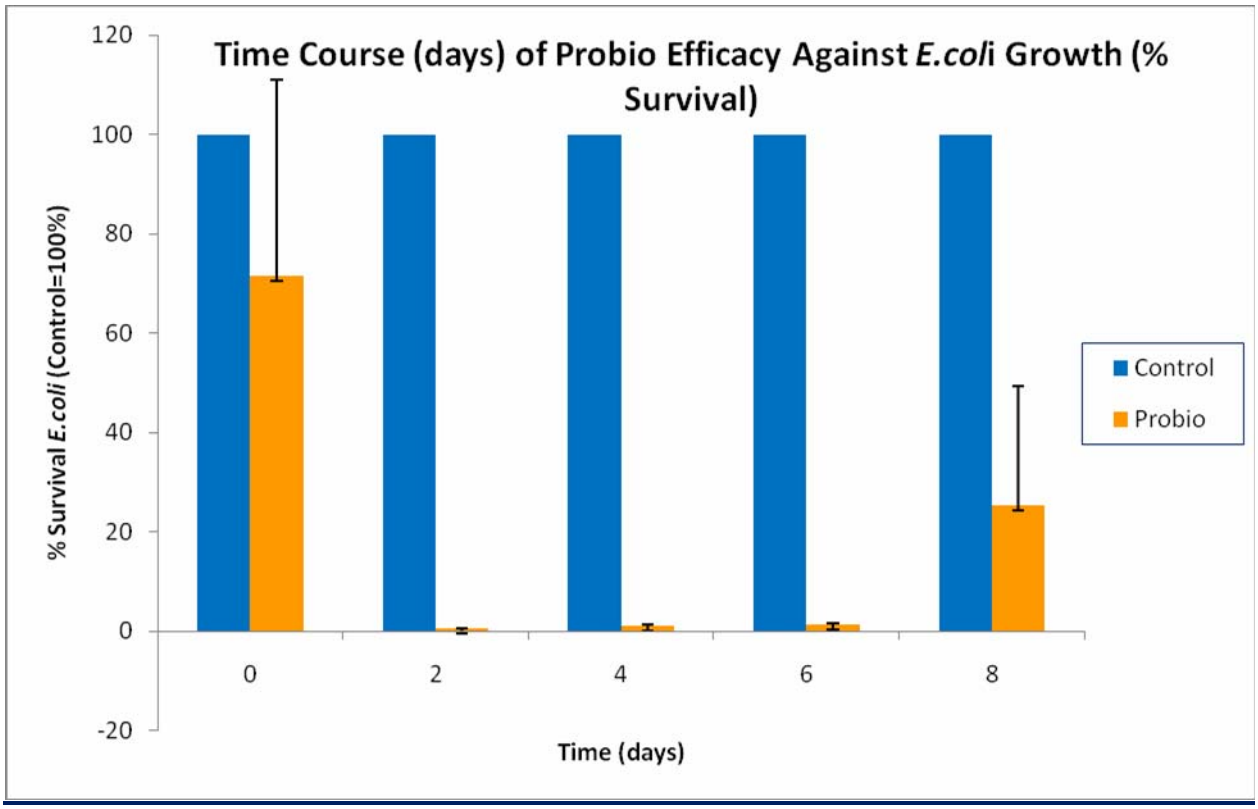
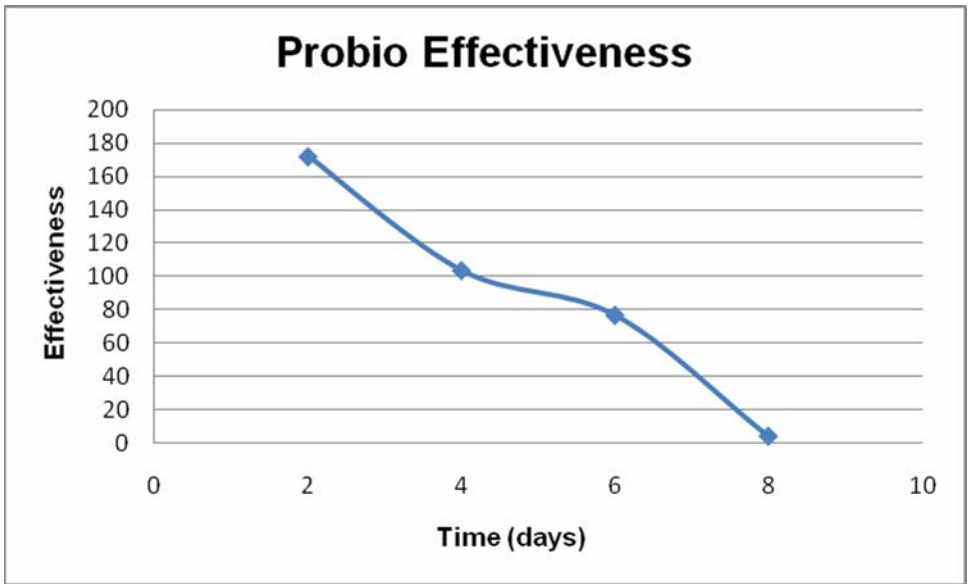


Table 1

Time (day)	Control	Probio	Effectiveness*
0	$8.25x10^4$	$5.9x10^4$	N/A
2	$8x10^9$	$4.65x10^7$	172.04
4	$1.45x10^9$	$1.4x10^7$	103.57
6	$1.15x10^9$	$1.5x10^7$	76.67
8	$1.3x10^8$	$3.3x10^7$	3.94

* The effectiveness is measured as a ratio between the control result and the probio result – the higher the value the more effective the probio product.

Graph 2



3. Conclusions

- In the control membranes treated with water, the number of *E. coli* cells continue to multiply significantly over time (increasing in number about 1 million times), due to the nutrient rich environment supplied by the tryptone soya agar.

- **Although *E. coli* cells do grow on membranes treated with probiotic, growth is significantly restricted. This effect is evident over the entire 8 day time-course, but becomes less pronounced over time.** This may be due to microorganisms in the probiotic entering a different growth phase (i.e. stationary/sporulation phase during which they will not be continuing to multiply) which enables the *E. coli* to access nutrients which were not previously available due to competition with the probiotic organisms.