Enumeration of probiotic strains from synbiotic samples produced by Myternak Trading.

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Abstract. Natural defense mechanisms of animals by using probiotics, prebiotics, and synbiotics have been seen as an alternative way to improve animal health and to overcome the food-borne diseases among the livestock. This is as the previous use of antibiotics as the growth promoter in the poultry industry has been banned by the European Union. Synbiotic is a combination of probiotics and prebiotics where probiotics are live microorganisms that give a gainful medical advantage on the host whereas prebiotics are nondigestible food ingredients that influence the host by stimulating the development and the action of a set number of microscopic organisms in the colon. This study was conducted to assess the viability of probiotic strains in the synbiotic powder samples from Myternak Trading as well as to see the correlation between the viability of probiotic strains with the amount of its selective prebiotics. Two different types of prebiotics; fructooligosaccharides (FOS) and inulin with seven different compositions of prebiotics had been used in this study. The viability of probiotic strains in synbiotic powder samples were assessed by enumeration of probiotic strains in two different selective media which are TOS and MRS agar. The highest average colony forming units (CFU) for \textit{Bifidobacterium sp.} can be found in Batch 7 samples $(4.10 \times 10^{11} \text{ CFU/ml})$ as the volume of FOS is the highest, which is 0.5 mL. While the highest CFU for \textit{Lactobacillus sp.} can be found in Batch 1 samples $(5.560 \times 10^{11} \text{ CFU/ml})$ as the weight of inulin is the highest, which is 5.0 g. These results proved the effects of prebiotic compositions in synbiotics towards the viability of the probiotic strains. The viable counts of each probiotic strains that contain in the synbiotic powder sample significantly decreases as the amount of selective prebiotic decreases. Therefore, the maximum CFU obtained as the composition of prebiotic increases. Hence it can be concluded that the produced synbiotic powder samples by MyTernak Trading could be used as an alternative feed additive in boosting the livestock health due to the viability of both probiotic strains which are \textit{Bifidobacterium sp.} and \textit{Lactobacillus sp.} in the synbiotic samples.

1. Introduction
Food-borne diseases especially originated from the livestock has been widely discussed and becoming more challenging as the use of antibiotics as growth promoters for the livestock has been banned in the European Union [1]. The development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota are the two major concerns for the use of antibiotics and hence it has been removed from the animal diets [2]. However, the food-borne diseases are vital concern to the poultry industry as it may cause loss of productivity, increment of mortality and the associated contamination of poultry products for human consumption [3]. A new feed additives need to
be used in order to enhance the natural defense mechanisms of the animals. Thus the use of probiotics, prebiotics and synbiotics has been seen as an alternative method to support the animal health [4]. Probiotics are live microorganisms which when controlled in sufficient amounts give a gainful medical advantage on the host. The most widely recognized probiotics are *Lactobacillus* and *Bifidobacterium*. Almost all probiotics are Gram positive, for the most part catalase-negative, roundly ended rods, and happen in sets, short, or long chains [5]. Meanwhile Gibson and Roberfroid [6] has characterized prebiotics as "a nondigestible food ingredients that helpfully influence the host by specifically stimulating the development as well as action of one or a set number of microscopic organisms in the colon". The most dominant prebiotic are from Fructooligosaccharide products such as oligofructose and inulin. The combination of prebiotics and probiotics is known as synbiotics. The use of probiotics, prebiotics and synbiotics as feed additives affects the animal health through the modulation of the gut microbiota. These feed additives have been approved to improve resistance toward pathogenic bacteria colonization and enhanced the host mucosa immunity which leads to an improvement of health status of the animals [7]. Study by Higgins [8] showed a significant recovery from Salmonella enteritidis by neonatal broiler chicks once by feeding the *Lactobacillus*–based probiotic. Vila [9] also reported a reduction of *Salmonella enteritidis* colonization in both broiler chicken and white leghorn chickens by continuous feeding of *Bifidobacterium* – based probiotics. In order to assess the viability of probiotic bacteria, selective enumeration of these probiotic bacteria need to be done. The most common method that has been used for enumeration is culture dependent techniques to enumerate *Lactobacillus* and *Bifidobacterium* strains [10]. Probiotics were portrayed by their phenotypic attributes, (e.g., colony morphology), microscopic details, (e.g., Gram stain response and cell morphology), and physiologic qualities, (e.g., fermentation designs and enzymatic actions) [11]. By using selective media, particular types of *Lactobacillus* are accessible by differentiating individuals from the variety. As for *Bifidobacterium*, it can also be distinguished, however no standard specific media are accessible to separate among *Bifidobacterium* species [12]. Myternak Trading is known as a leading company in supplied a high quality food products and supplement for animal feed. Synbiotic is one of an example for this company products which contain a combination of prebiotics (*Bifidobacterium* and *Lactobacillus* strains) and probiotics (FOS and inulin). Hence this research was aimed to assess the viability of the probiotic strains in the synbiotic samples provided by Myternak Trading by using the selective media and followed by enumeration of probiotic strains in the synbiotic samples. The effect of different prebiotic composition in the synbiotic powder samples towards the viability of the probiotic strains was also studied in this research.

2. Materials and methods

2.1. Materials

All the reagents proteose peptone, beef extract, yeast extract, D (+) glucose, sodium acetate, ammonium citrate, dipotassium phosphate, manganese sulphate, magnesium sulphate, agar, L-cysteine, sodium propionate and dipotassium hydrogen phosphate were purchased from Sigma Aldrich.

2.2. Samples collection

The synbiotic powders (animal feed) as shown in Figure 1 were collected from Myternak Trading in Dengkil, Selangor. They were seven batches of synbiotic powder samples where each sample in the same batch has the same prebiotics composition, which are fructooligosaccharides and inulin. The only different between each samples in the same batch was the composition of soybean (the exact amount was not given by the provider as it is confidential). The compositions of the prebiotics in each synbiotic powder samples are given in Table 1.
Figure 1: The synbiotic powder samples.

Table 1: Compositions of prebiotics in each synbiotic powder samples

| Synbiotic Sample | Fructooligosaccharides, (mL) | Inulin, (g) |
|------------------|-----------------------------|------------|
| Batch 1 (Samples 1-2) | -                           | 5.0        |
| Batch 2 (Samples 3-6)  | 0.1                         | 4.0        |
| Batch 3 (Samples 7-8)  | 0.2                         | 3.0        |
| Batch 4 (Samples 9-10) | 0.3                         | 2.0        |
| Batch 5 (Samples 12-13) | 0.4                        | 1.0        |
| Batch 6 (Samples 11 & 14) | -                         | -          |
| Batch 7 (Samples 15-16) | 0.5                         | -          |

2.3 Sample preparation
For identification and enumeration of the probiotic strains, 0.2 g of each symbiotic powder samples was taken and diluted with 19.8 mL of distilled water. It was followed by a series of 10-fold dilution from $10^{-1}$ to $10^{-9}$.

2.4 Media preparation
Two different selective media were used for identification and enumeration of probiotic strains from synbiotic powder samples. MRS agar with pH 5.4 and 6.2 were used for *Lactobacillus* strain while TOS agar were used for *Bifidobacterium* strain. Each of the selective medium was prepared accordingly [13] with some modifications.

2.5 Enumeration of probiotic strains
For the enumeration purposes, 0.1 mL sample from the dilution $10^{-7}$ to $10^{-9}$ were spread onto a selective agar media and anaerobically incubated at 37°C for 3 days in anaerobic jars. This step was repeated for
the other 15 samples of the synbiotic powder. AnaeroPack® (Thermo Fisher Scientific, MA. USA) was used for the production of anaerobic environment in the anaerobic jars.

The total plate count (TPC) for each plate was determined by using Equation 1 and only plate with bacterial colony between 30 and 300 colonies was proceeded for TPC purposes.

$$\text{cfu/ml} = \frac{\text{number of colonies}}{(\text{volume of sample plated x dilution})}$$ (1)

3. Results and discussion

3.1 Colony morphology on selective agar media

The colonies of Bifidobacterium sp (for samples 1 and 5) in MRS agar pH 5.4 were of white-cream color, of circular form with entire margins. The same observations also found for the colonies of Bifidobacterium (for samples 2-5, 8 and 12-13) in MRS agar pH 6.2. While the colonies of Bifidobacterium (for samples 6 and 9) in MRS agar pH 6.2 were of white-cream color, of irregular form with undulate margins. On the other hand, the colonies of Lactobacillus sp (for samples 3-4, 6-7,10, 12-13 and 15-16) in TOS agar were of white-cream color, of circular form with entire margins.

3.2 MRS and TOS agar as the selective medium for enumeration of probiotic strains

MRS medium is a selective medium that should permit the growth of Lactobacillus sp strains meanwhile TOS medium is selective medium that should stimulate the growth of Bifidobacterium sp strain. This can be seen from the results in Table 2 where both synbiotic powder samples 1 and 2 only showed a growth in MRS selective medium agar with $1.62 \times 10^{11}$ cfu/ml and $9.50 \times 10^{11}$ cfu/ml colony counts respectively with no colony growth in TOS selective agar medium. This results are accordingly with the fact that both synbiotic powder samples 1 and 2 only contained inulin as its prebiotic components and the inulin only promote the growth of Lactobacillus sp strains. While for both synbiotic powder samples 15 and 16, the results showed the growth in TOS selective agar medium only ($4.60 \times 10^{11}$ and $3.60 \times 10^{11}$ colony counts respectively) with no colony growth in MRS selective medium agar. This is as both synbiotic powder samples 15 and 16 only contained fructooligosaccharide as its prebiotic components and the fructooligosaccharides only promote the growth of Bifidobacterium sp strains.

Hence, from above results it can be proved that both MRS and TOS agar could act as selective medium for enumeration of probiotic strains.

3.3 Enumeration of probiotic strains in synbiotic powder samples

Table 2 shows the counted colony forming units for each synbiotic powder samples. All the visible colonies were calculated and represented as colony forming units (CFU). Two different selective media were used for identification and enumeration of probiotic strains from synbiotic powder samples. MRS agar with pH 5.4 and 6.2 were used for Lactobacillus sp. strain while TOS agar were used for Bifidobacterium sp. strain. From the results, it was showed that synbiotic powder sample 2 grown in MRS selective agar medium pH 6.2 has the highest CFU count which is $9.50 \times 10^{11}$ cfu/ml. This were then followed by both synbiotic powder samples 15 and 16 which were grown in TOS selective agar medium with the CFU count $4.60 \times 10^{11}$ and $3.60 \times 10^{11}$ cfu/ml respectively.

The highest number of CFU for synbiotic powder sample 2 is well corresponded with the highest amount of prebiotic contents (inulin, 5 g) used in the synbiotic powder samples. The same trend was also found for both synbiotic powder samples 15 and 16 where the highest number of CFU were well corresponded with the highest amount of prebiotic contents (fructooligosaccharides, 0.5 ml) used in the synbiotic powder samples.
From the above results, it was observed that the highest number of CFU count obtained for the synbiotic powder samples 2, 15 and 16 are the synbiotic powder samples with a single content of its prebiotic component. Synbiotic powder sample 2 only contained inulin as its prebiotic component while both synbiotic powder samples 15 and 16 which are the second and third highest CFU count only contained fructooligosaccharides as their prebiotic component. The highest number of CFU count for the above mentioned synbiotic powder samples might be due to no growth competition among the two probiotic strains; \textit{Bifidobacterium sp.} and \textit{Lactobacillus sp.} as each of the synbiotic powder samples only have single components of its prebiotic; either fructooligosaccharide or inulin. According to Süle [13], fructooligosachharides mainly stimulate the growth of \textit{Bifidobacterium sp} strains and retain its viability while inulin stimulates the growth and retains the viability of \textit{Lactobacillus sp} strains.

Table 2: The Colony Forming Unit (CFU) of each synbiotic samples

| Samples       | MRS Agar 5.4 37°C, 72h, anaerobic | MRS Agar 6.2 37°C, 72h, anaerobic | TOS Agar 37°C, 72h, anaerobic |
|---------------|-----------------------------------|-----------------------------------|-------------------------------|
| Batch 1       |                                   |                                   |                               |
| Sample 1      | 1.62×10^{11}                      | -                                 | -                             |
| Sample 2      | -                                 | 9.50×10^{11}                      | -                             |
| Batch 2       |                                   |                                   |                               |
| Sample 3      | -                                 | 7.20×10^{10}                      | 3.00×10^{9}                   |
| Sample 4      | -                                 | 5.70×10^{10}                      | 3.20×10^{9}                   |
| Sample 5      | 4.70×10^{10}                      | -                                 | -                             |
| Sample 6      | -                                 | 4.30×10^{10}                      | 1.60×10^{9}                   |
| Batch 3       |                                   |                                   |                               |
| Sample 7      | -                                 | -                                 | 4.50×10^{9}                   |
| Sample 8      | -                                 | -                                 | 4.50×10^{9}                   |
| Batch 4       |                                   |                                   |                               |
| Sample 9      | -                                 | 4.00×10^{9}                       | -                             |
| Sample 10     | -                                 | -                                 | 9.80×10^{9}                   |
| Batch 5       |                                   |                                   |                               |
| Sample 12     | -                                 | 3.20×10^{9}                       | 8.90×10^{10}                  |
| Sample 13     | -                                 | 3.00×10^{9}                       | 3.50×10^{10}                  |
| Batch 6       |                                   |                                   |                               |
| Sample 11     | -                                 | -                                 | -                             |
| Sample 14     | -                                 | -                                 | -                             |
| Batch 7       |                                   |                                   |                               |
| Sample 15     | -                                 | -                                 | 4.60×10^{11}                  |
| Sample 16     | -                                 | -                                 | 3.60×10^{11}                  |

3.4 Effect of prebiotics amount towards the viability of probiotic strains in synbiotic powder samples.

As mentioned earlier in part 2.2, there are seven batches of synbiotic powder samples where each sample in the same batch has a same amount of its prebiotics component. From the respective batches, the CFU number in each sample was added and divided by number of samples in corresponding batch, in order obtain the average colony forming units for each batch as shown in Table 3.

The results in Table 3 showed that as the amount of fructooligosaccharides increased in the synbiotic powder samples, the average colony forming units (CFU) in TOS agar also significantly increased. This is as fructooligosaccharides helps to promote the growth of \textit{Bifidobacterium sp}. The same trend also
observed for the MRS agar where as the amount of inulin increased in the synbiotic powder samples, the average colony forming units (CFU) in MRS agar also increased. This is as inulin helps to promote the growth of *Lactobacillus* sp. Both fructooligosaccharides and inulin supply the nutrients and trace elements to their specific probiotics strain that presence in the synbiotic samples and it helps the probiotic to be viable.

**Table 3**: Correlation between the average colony forming units (CFU) in each batch of synbiotic powder samples with the composition of prebiotics in synbiotic powder samples

| Batch | Average CFU in TOS agar | Fructooligosaccharides (mL) | Average CFU in MRS agar | Inulin (g) |
|-------|-------------------------|-----------------------------|------------------------|------------|
| 1     | -                       | -                           | 5.60 × 10^11           | 5.0        |
| 2     | 5.475 × 10^10           | 5.20 × 10^9                 | 5.475 × 10^10          | 4.0        |
| 3     | 4.50 × 10^9             | 0.1                         | 4.50 × 10^9            | 3.0        |
| 4     | 4.0 × 10^9              | 0.2                         | 4.0 × 10^9             |            |
| 5     | 3.10 × 10^9             | 0.4                         | 3.10 × 10^9            | 1.0        |
| 6     |                         | -                           | -                      |            |
| 7     | 4.10 × 10^11            | 0.5                         |                        | -          |

4. Conclusion

Different selective media promote the growth of different probiotic strains. From the results it can be concluded that the viability of probiotic strains are depends on the composition of prebiotics in the synbiotic powder samples. As the composition of prebiotics increase, the number of probiotics strains and its viability also increased as the prebiotics helps in providing an optimum amount of nutrients for the probiotic strains to grow and replicate. Furthermore, it can be concluded that the produced synbiotic powder samples by MyTernak Trading could be used as an alternative feed additive in boosting the livestock health due to the viability of both probiotic strains which are *Bifidobacterium* sp. and *Lactobacillus* sp in the synbiotic samples.

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