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Synbiosis between *Enterococcus faecium* DSM 3530 and Fructan Compounds of Different Degree of Polymerization: a Preliminary *In vitro* Assay in a Condition Simulated Chicken Caecum

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ABSTRACT

This experiment was conducted to determine the potential synergistic relationship between *Enterococcus faecium* and fructans with different average Degree of Polymerization (DPav) including OligoFructose (OF, DPav 4), Standard-inulin (ST-inulin, DPav 10), Synergy1-Inulin (SYN1-inulin, DPav 15) and High-Polymer inulin (HP-inulin, DPav 25). A sterilized minimal MRS broth media was prepared by omission of glucose. The media pH was adjusted to a constant initial value of 5.8±0.1 and the temperature was maintained at 41°C. Sterilized fructans were added (1% wt/vol) to the broths, as experimental treatments with 3 replications each, and the medium with no added prebiotic was considered as the control. The same starting density of 10^8 *E. faecium* cells per ml was introduced to all media. The media pH, viable cells count, as well as growth of the latter were determined during 24 hours of incubation. The lowest pH and best growth rates were observed in the media enriched with OF and ST-inulin. Unlike aerobic, the anaerobic conditions produced no significant differences in growth of the bacteria among SYN1-inulin and HP-inulin treatments compared to the control. The viable cells count in the media containing OF was significantly higher than in the control and other treatments. The significant differences were also found among the control and treatments supplemented with ST-inulin and SYN1-inulin. In conclusion, the fructans with lower DP were preferentially metabolized by *E. faecium*, and hence it follows that a synbiotic blend of *E. faecium* and OF has the potential to be used in poultry nutrition.

Keywords: Degree of polymerization, *Enterococcus faecium*, *In vitro*, Inulin-type fructans, Synbiotic.

INTRODUCTION

It has been well defined that the avian gut microbiota has a vital role in bird health and performance (Zhu *et al.,* 2002; Xu *et al.,* 2003). This microbial community is a complicated complex of many different species of bacteria, differing from host to host (Stanley *et al.,* 2012). Since oral supplementation with antibiotics does not have growth-promoting effects in germ-free animals, the importance of the role of the gut microbiota is well understandable (Dibner and Richards, 2005; Brisbin *et al.,* 2008). Antibiotic growth promoters have been used extensively in the poultry industry to reduce pathogens and thereby increase animal performance (Gaskins *et al*., 2002; Jones and Ricke, 2003). However, with ban of antibiotics growth promoters in animal feed due to the continuous use of antimicrobials and consumer demand for high quality products, there is increasing interest in finding alternatives to antibiotics for poultry production to improve the animal performance (Windisch *et al*., 2008; Park *et al*., 2015). Administration of microbial dietary supplements like probiotics is one of the major

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tools for modulation of gut microbiota (Alloui *et al.,* 2013). Probiotics have been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). Many authors clearly confirmed the positive impact of dietary probiotics in broilers (Cavazzoni *et al*., 1998; Jin *et al*., 1998; Zulkifli *et al*., 2000; Mountzouris *et al*., 2007; Samli *et al*., 2007; Salarmoini and Fooladi, 2011; Khosravi *et al*., 2012), which creates growing interest in application of probiotics in poultry industry. However, as the efficacy of probiotics may be affected by different factors, considerable attention has recently been paid to find ways for amplifying their ability to serve as effective feed additives (Saminathan *et al*., 2011). A way of potentiating the efficacy of probiotics is co-administration with appropriate prebiotic as a synbiotic that beneficially affects the survival and implantation of dietary probiotic in the gastrointestinal tract (Awad *et al*., 2009; Rurangwa *et al*., 2009). Although several trials have been recently performed on synbiotics (Awad *et al*., 2009; Yitbarek *et al*., 2015), there is no introduced synbiotic for poultry based on in vitro studies with determination of the best prebiotic compound as a substrate.

Enterococcus faecium is a Gram-positive, catalase-negative, non-spore-forming and facultative anaerobic bacterium that can tolerate bile salts and grow in a wide range of pH, and temperature (Van den Berghe *et al*., 2006; Fisher and Phillips., 2009), therefore being able to colonize the gastrointestinal tract. There are some trials showing efficacy of *E. faecium* for increasing growth performance, villus height, as well as improving gut microbiota status in broilers (Samli *et al*., 2007; Samli *et al*., 2010; Cao *et al*., 2013). However, there is no information about the proper prebiotic as effective substrate for the given bacterial species. Therefore, the aim of the present in vitro appraisal was to evaluate optimum synergistic effects of *E. faecium* and co-added inulin-type fructan preparations with different DP for introduction of a synbiotic to be used in poultry nutrition.

MATERIALS AND METHODS

Prebiotic Substrates

Four fructan preparations with different average Degrees of Polymerization (DPav) including Standard-inulin (ST-inulin), OligoFructose (OF), High-Polymer inulin (HP-inulin), and Synergy1-inulin (SYN1-inulin), were obtained from Beneo-Orafti (Tienen, Belgium). ST-inulin (DP between 3 and 65, DPav 10) is isolated from chicory roots by water extraction, followed by refining and spray-drying. OF (DP between 2 and 8, DPav 4) is a mixture of short-chain oligosaccharides consisting of glucose linked to fructose units through β-(2–1) bonds (GFn), which is obtained by partial enzymatic hydrolysis of ST-inulin. HP-inulin (DP between 10 and 65, DPav 25) is produced by physically removing the lower-DP units from ST-inulin. SYN1-inulin (DPav 15) is a 1/1 blend of OF and HP-inulin (Coudray *et al*., 2003).

Probiotic Bacteria

The probiotic strain was *E. faecium* DSM 3530, which belongs to the clade of Lactic Acid Bacteria (LAB). The strain was cultured in MRS (Merck, Germany) broth medium for 48 hours at 37°C. The fresh colonies were obtained after re-culturing on MRS agar (Merck, Germany) for 24 h at 37 °C.

Experimental Setup and Data Collection Procedures

To assess the growth of *E. faecium* on different fructans as prebiotic substrates, a modified minimal MRS (mMRS) broth media was prepared according to De Man *et al*. (1960) by omission of glucose. They contained the following ingredients: 1.0% peptone, 0.8% meat extract, 0.4% yeast extract, 0.5% sodium acetate trihydrate, 0.1 % polysorbate 80 (Tween 80), 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate and 0.005% manganese sulfate tetrahydrate. The media were autoclaved at 121°C for 15 minutes and their pH was adjusted to a constant value of 5.8±0.1 with 1N NaOH and 1N HCl. During the culturing period, temperature of all the media was maintained at 41°C, simulating the normal physicochemical conditions in the caecal lumen of chicken (Van Der Wielen *et al*., 2001). After sterilization, ST-inulin, OF, HP-inulin, and SYN1-inulin as the experimental treatments, with 3 replications each, were dissolved at the dose of 1% wt/vol in the media before inoculation. A treatment without any prebiotic substrate was also included in the experiment as the control. All the broths were

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inoculated with the same starting density of 10^8 ml^{-1} of *E. faecium*, and then incubated aerobically in a shaking incubator (Iran Khodsaz; Iran) for 24 h. The growth of *E. faecium* was monitored at 3-hour sampling intervals with measurement of optical density at 600 nm wavelength (OD_{600}) using a spectrophotometer (model BT 600; Brite Technology, Canada). In all media the pH was determined with 3-hour intervals using a digital pH meter (Hanna; Romania). The first sample, from a series taken for pH determination, was used for OD measurements. The number of live *E. faecium* cells per milliliter of each medium was determined at 12 hours after inoculation, corresponding for most bacteria to the exponential growth phase and the start of the stationary growth phase, by the method described by Dastar *et al.* (2016) for measuring the caecal LAB population.

The growth rates of *E. faecium* in media containing individual fructans selected for this experiment was also determined under anaerobic conditions. An experimental broth was prepared as described previously. All general procedures were the same as the previously described aerobic conditions, with the exception that, to create anaerobic conditions, the inoculated media were incubated under a seal of sterile paraffin (Elliot and Dole, 1947), and a non-shaking incubator (Iran Khodsaz; Iran) was used.

Statistical Analysis

The data were analysed in a completely randomized design using the GLM procedure of SAS software (SAS, 2001). Significant differences among means were determined using Duncan's multiple range test at the level of $P< 0.05$.

RESULTS

The growth of *E. faecium* on different prebiotics under aerobic and anaerobic conditions is presented in Figure 1. The results revealed sharp

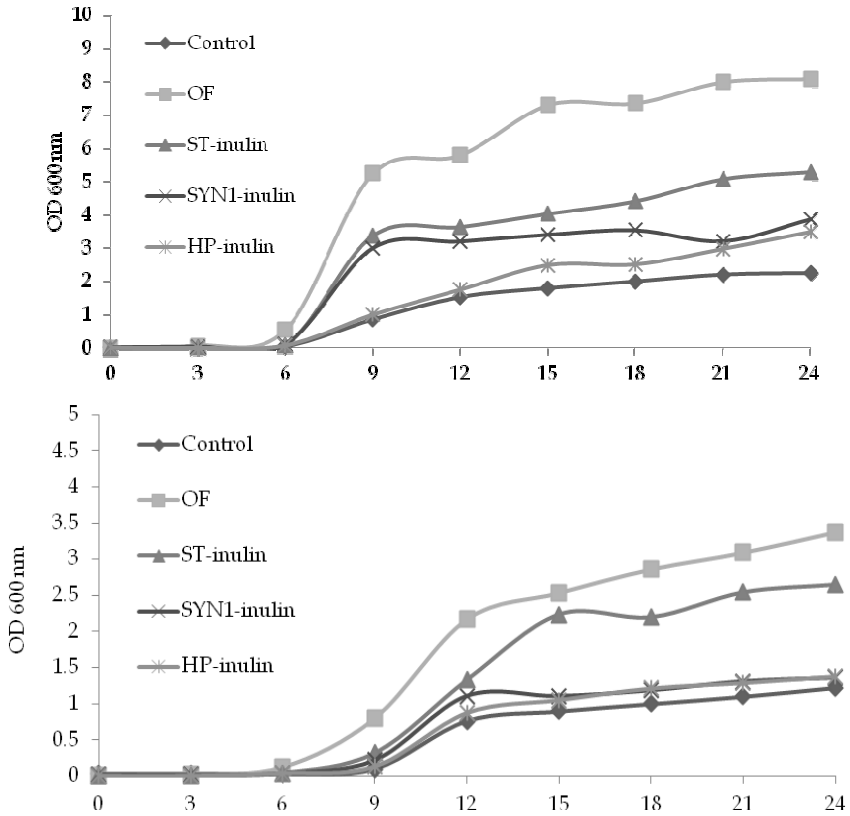

Figure 1. Aerobic (upper graph) and anaerobic (lower graph) growth curves of *Enterococcus faecium* in mMRS media supplemented with different fructan prebiotics obtained by 24-hours monitoring of the Optical Density of suspension (OD600). Values are means of three replications.

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increase in the growth of this probiotic strain in the media containing OF under both conditions. To a lower level, similar trends were observed in the ST-inulin, SYN1-inulin and HP-inulin treatments. As shown in Figure 2, there were significant differences among all treatments containing prebiotics when compared to control treatment under aerobic condition (P< 0.05). In addition, there were significant differences among prebiotics with different DP, except SYN1 and HP-inulin. Although the optical density was lower under anaerobic condition, the highest growth occurred in the treatments with OF and ST-inulin, respectively. Based on the optical densities obtained, the OF treatment showed the best growth of *E. faecium* which was significantly higher than all other treatments (P< 0.05). Moreover, there were significant differences between the growth of *E. faecium* on ST-inulin medium compared to the control, SYN1-inulin, and HP-inulin treatment media. Unlike the aerobic condition, when incubated anaerobically no significant differences were found in OD_{600} among the control and both the SYN1-inulin and HP-inulin treatment media, (Figure 2). The final pH of the media containing prebiotics was also measured after 24 hours aerobic and anaerobic incubations for determination of acidic product during fermentation (Figure 3). Under both conditions, the pH of the media was significantly lower in synbiotic treatment of *E. faecium* plus OF compared to the control and other synbiotic treatments (P< 0.05). Furthermore, pH of media containing ST-inulin was also significantly lower than control under both conditions (P< 0.05). Moreover, there was a significant difference in the final pH between the media containing ST-inulin and the media containing SYN1-inulin and HP-inulin under anaerobic conditions (P< 0.05).

The number of viable cells of *E. faecium* obtained from treatments under aerobic and anaerobic conditions is demonstrated in Figure 4. The results showed that there were significant differences among treatments supplemented with fructans of different DP. The number of cells in the media containing OF was significantly higher than in the control and other treatments under aerobic and anaerobic conditions (P< 0.05). For this criterion the significant differences were also detected among control and treatments supplemented with ST-inulin and SYN1-inulin, but not between the HP-inulin and control ones under both conditions.

DISCUSSION

The aim of this in vitro trial was to investigate the potential synergistic effect of *E. faecium* in conjunction with inulin-type fructans differing in

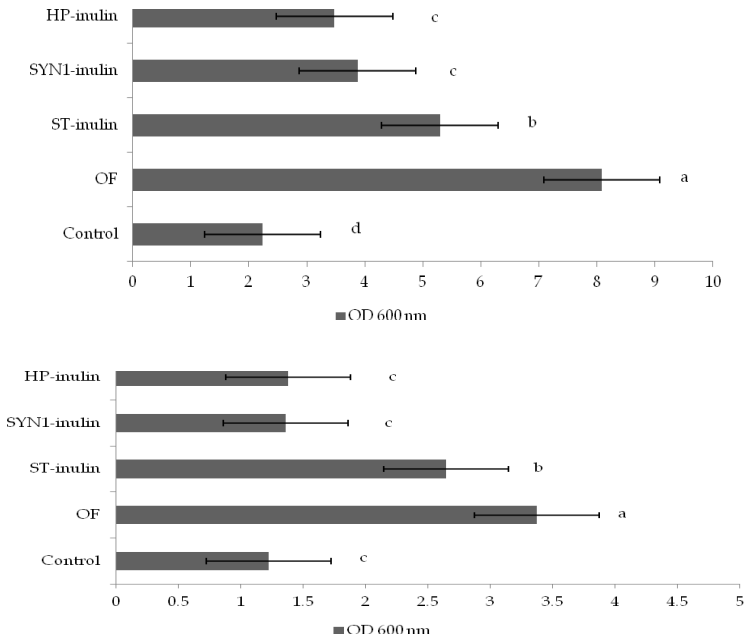

Figure 2. Optical Density (OD_{600}) reached by *Enterococcus faecium* grown in mMRS media supplemented with different fructan prebiotics under aerobic (upper graph) and anaerobic (lower graph) conditions after 24 hours of incubation at 41°C. Means with different letters differ (P< 0.05). Values are means of three replications.

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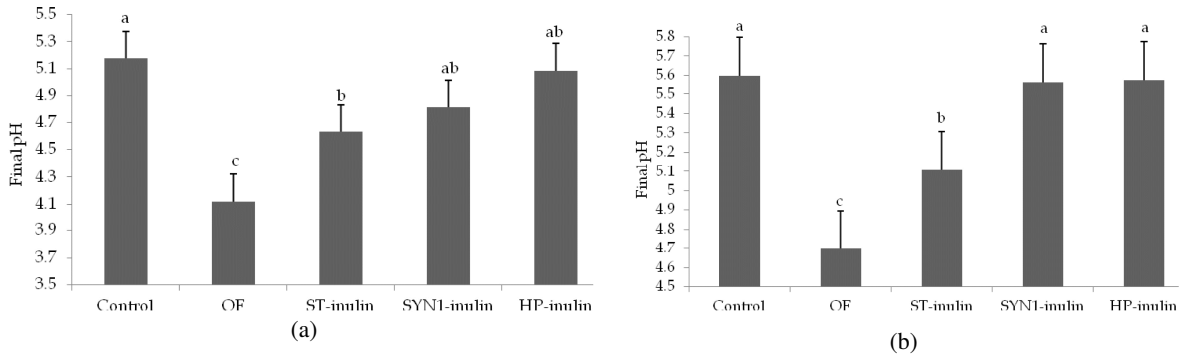

Figure 3. The final pH of mMRS media supplemented with *Enterococcus faecium* and fructan different prebiotics after 24 hours incubation at 41°C under aerobic (a) and anaerobic (b) conditions. Means with different letters differ (P< 0.05). Values are means of three replications.

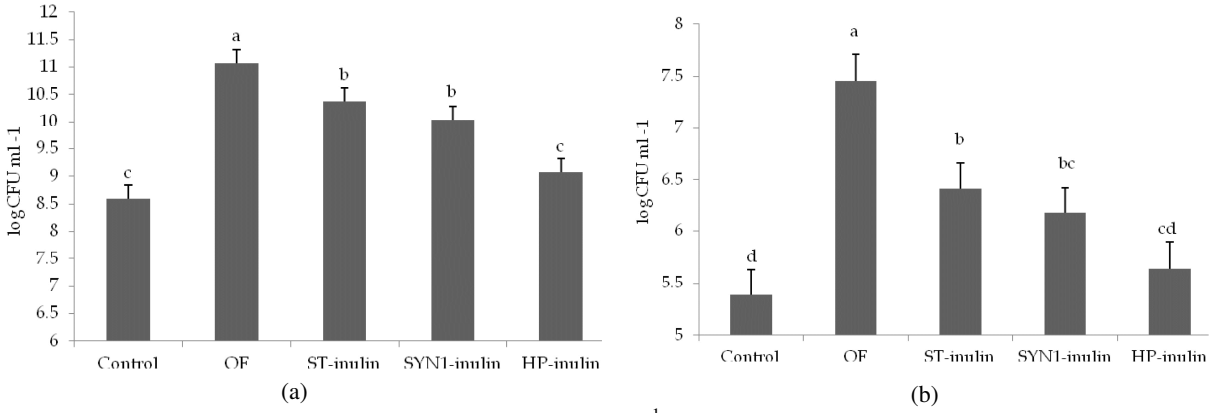

Figure 4. Viable *Enterococcus faecium* (log CFU ml $^{-1}$) at exponential growth phase in mMRS media supplemented with fructan different prebiotics after 24 hours incubation at 41°C under aerobic (a) and anaerobic (b) conditions. Means with different letters differ (P< 0.05). Values are means of three replications.

the polymer chain length. The maximum growth of the strain, under both the aerobic and anaerobic conditions, was observed when OF and ST-inulin were supplemented to the media. To the best of our knowledge, there is no study available on in vitro growth of *E. faecium* in combination with fructan compounds of different DP as substrates. However, Audisio *et al*. (2001) studied the growth of *E. faecium* CRL1385 on the range of common carbohydrates and found that the strain can grow in the presence of brown commercial sugars and molasses. In addition, their study showed that these synbiotics inhibited growth of *Salmonella pullorum*. The present results revealed lower growth of *E. faecium* when SYN1-inulin and HP-inulin were used as substrates and the difference between treatments containing these substrates and the control was not significant under anaerobic conditions. Fermentability of prebiotic compounds may be directly affected by DP; higher DP could result in their lower fermentability (Kolida *et al.,* 2002). Therefore, as it was shown in our experiment, *E.faecium* is not able to metabolize the fructans with DP> 10 in an easy way. Likewise, other experiments revealed that *Carnobacterium piscicolawas* (Khouiti and Simon, 1997) and *Pediococcus acidilactici* (Hoseinifar *et al.,* 2015) were unable to ferment prebiotics with high DP. DP= 10 is a critical physicochemical barrier and fructans with DP<11 have high solubility in water (up to 85%), making them very rapidly fermentable. On the other hand, the compounds with DP>10 are hardly soluble in water (up to 5%) and have been shown to be 5 times slower in fermentablity than OF by fecal slurry microbiota (Coudray *et al*., 2003).

The obtained results also showed the significant differences between fructans with DPav lower than 10, i.e. OF and ST-inulin. Regardless of the gap in DP, the significant difference found

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between these treatments could be related to their chemical composition. Although *E. faecium* has a complete enzymatic machinery allowing it to use complex carbohydrates (Barnes, 1964), the composition content of the carbohydrates present in the fructan compounds has a crucial effect on the growth of different bacteria. It is well evidenced that glucose is the main carbon source used by all microorganisms because of its size, rapid uptake, utilization and cellular energy conversion (Audisio *et al*., 2001). The carbohydrates content in all preparations used in this in vitro appraisal were glucose and fructose but each with a various number of molecules. As each branch of fructans is terminated by one glucose moiety, OF has the highest percentage of glucose moiety among the compounds studied, making it easy to use by *E.faecium.* Similar to our results, Audisio *et al*. (2001) found that composition of the prebiotic is a key factor in the antagonistic activity of *E. faecium* against some poultry pathogens because lactic acid and bacteriocin production were carbohydrate nature dependent.

The present results also indicated significant difference in the final pH of the media when different substrate fructans were used. The pH of the media is directly influenced by growth of the bacteria, as a general biochemical process. The major metabolic end products of LAB fermentation are acetate and lactate, responsible for lowering the pH of the media (Fooks and Gibson, 2002). Therefore, considering the higher growth of *E. faecium* in treatments supplemented with OF and ST-inulin and presumably production of the short chain fatty acids, the media pH values were significantly lower than in the control and other treatments. The results showed that there was a direct correlation between growth of the probiotic strain and the number of viable bacteria at the exponential growth phase as it was expected. Accordingly, significant difference found among the treatment containing OF in comparison with the control and other treatments for the number of viable bacteria was directly related to higher growth of the bacteria on this medium.

In addition, a significant difference in the number of viable bacteria was also observed between HP-inulin and the control treatments and SYN1-inulin treatment. SYN1-inulin is a product consisting of 1/1 mixture of OF and HP-inulin. Since OF is rapidly available to be used by the bacteria, as the present results showed, it seems that the significant difference between these treatments is related to higher growth of *E. faecium* consuming OF present in the SYN1-inulin medium. On the other hand, there was no significant difference between the control medium and the medium containing HP-inulin for the number of viable *E. faecium*. Similar to the control medium (not supplemented with prebiotic), it seems that nutrients required for growth of the bacteria in the medium containing HP-inulin could be mainly derived from peptone, yeast and/or meat extract present in the medium.

In conclusion, the results showed that inulin-type fructans with lower DP are preferentially metabolized by *E. faecium*. This finding obtained in vitro provides a preliminary observation that a synbiotic blend of *E. faecium* DSM 3530 and oligofructose with DPav = 4 has the potential to be used as a suitable feed additive in poultry nutrition.

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اثرات سینبیوتیکی *انتروکوکوس فاسیوم* DSM 3530 و فروکتان‌های با درجه پلیمریزاسیون متفاوت: یک آزمایش برون حیوانی مقدماتی تحت شرایط شبیه سازی شده سکوم جوجه‌های گوشتی

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چکیده

این آزمایش برون حیوانی به منظور بررسی اثرات سینبیوتیکی *انتروکوکوس فاسیوم* و فروکتان‌های با میانگین درجه پلیمریزاسیون (DPav) متفاوت شامل الیگوفروکتوز (۴ DPav)، اینولین طبیعی (۱۰ DPav)، اینولین سینرژی ۱ (۱۵ DPav) و اینولین پلیمر بالا (۲۵ DPav) انجام گردید. یک محیط مینیمال MRS بدون افزودن گلوکز تهیه گردید. pH و دمای محیط به ترتیب به ۱ ± ۵/۸ و ۴۱ درجه سانتی‌گراد رسانده شد. پربیوتیک‌های مختلف، به عنوان تیمار (با ۳ تکرار)، پس از استریل شدن به محیط افزوده شدند و یک محیط بدون پربیوتیک به عنوان تیمار شاهد در نظر گرفته شد. معادل 10^8 واحد کلنی‌ساز *انتروکوکوس فاسیوم* به تمامی محیط‌ها تلقیح گردید. pH محیط، تعداد باکتری‌های زنده و میزان رشد باکتری در طی انکوباسیون ۲۴ ساعته تحت شرایط هوازی و بی‌هوازی اندازه گیری شدند. کمترین pH و بهترین میزان رشد باکتری در تیمارهای حاوی الیگوفروکتوز و اینولین طبیعی مشاهده گردید. برخلاف شرایط هوازی، تفاوت معنی‌داری از لحاظ میزان رشد باکتری بین تیمارهای اینولین سینرژی ۱ و پلیمر بالا در مقایسه با تیمار شاهد در شرایط بی‌هوازی مشاهده نشد. تعداد باکتری-های زنده در محیط حاوی الیگوفروکتوز به طور معنی‌داری بیشتر از تیمار شاهد و سایر تیمارها بود. علاوه بر این، تفاوت معنی‌داری بین تیمار شاهد و تیمارهای حاوی اینولین طبیعی و سینرژی ۱ مشاهده شد. در مجموع، نتایج نشان داد که فروکتان‌های با درجه پلیمریزاسیون پایین‌تر در اولویت استفاده توسط *انتروکوکوس فاسیوم* هستند و این امر نشان می‌دهد که ترکیب سینبیوتیکی *انتروکوکوس فاسیوم* و الیگوفروکتوز پتانسیل این را دارد تا در تغذیه طیور مورد استفاده قرار گیرد.

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