Plant Archives Vol. 19, Supplement 2, 2019 pp.1824-1829 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

THE STUDY OF SOME CHEMICAL PROPERTIES OF *PLANTAGO OVATA* SEEDS AND ITS UTILIZATION AS SYNBIOTICS IN FERMENTED MILK

Ali Adnan Abbas AL-Ogaidi[1] and Amer Abdulrahman Alshaikh Daher[2]

[1]Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad, Iraq

[2] Iraqi General Secretarial of the Ministers Council, Iraq

Corresponding author: alirsoole2005@yahoo.com, dramer61@yahoo.com

Abstract

The present study aimed to identify some of the chemical properties of *Plantago ovata* (local and commercial powders) and its potential to utilization it for the first time in fermented milk as prebiotic for pure and co-culturing of probiotics, by adding the both powder (0.125% and 0.50%) individually. The results of the chemical analysis shown rising in the commercial psyllium content of total carbohydrate, protein and fat compared to local psyllium, and they were (56.21%, 15.74%, 16.22%) and (54.01%, 14.70%, 15.68%) respectively. The technic TLC for carbohydrate separation, shown an absence of simple sugars for water extract for both seeds powders, the exception was trace trisaccharides and the most of carbohydrates were polysacchrides. The peaks were obtained in the IR spectrum of commercial psyllium seed were same to local psyllium and matched with IR spectroscopy of arabinoxylan that presence in the psyllium husks. psyllium powder was added to MRS broth to study the possibility of utilization as prebiotic, the result shown that addition led increase the logarithmic count of *L. rhamnosus*, the increasing ratio were 11.48%. Co-culturing fermented milk, while increasing in *L. rhamnosus* plus *S. boulardii* logarithmic count compared to their pure culture, were 3.04% and 11.13%, respectively.

***Keywords*:** Probiotics, prebiotics, synbiotic, *p. ovata*, arabinoxylane, FT-IR, *S. boulardii*, *L. rhamnosus*, Co-culture.

Introduction

P. ovata is called Isabgol or psyllium that described as a short-stemmed annual herb, which grows between (30- 40 cm), many of flowering shoots arise from the base of the plant (Jat *et al.,* 2015). The utilization of *P. ovata* recorded in many countries around the world since ancient times, and its use in India back to 1500 BC as medication, also in Chinese used it as traditional medicine thousands years ago. Both Indians and Chinese used plant seeds to treat constipation, diarrhea, hemorrhoids, bladder problems and high blood pressure, in Europeans and North Americans began using it as cholesterol and diabetes treatment (Ashwini *et al*. 2014). Prebiotics are compounds in food that induce the growth or activity of beneficial microorganisms such as bacteria and fungi, the most common example is in the gastrointestinal tract, where prebiotics can alter the composition of organisms in the gut microbiome (Hutkins *et al.,* 2016). Furthermore, in order for carbohydrates to consider as prebiotics, they should not cause undesirable effects to the host, such as flatulence due to increased gas production or metabolized by pathogenic microbes (Gibson *et al*., 2017). Arabinoxylan, considered as the main carbohydrate in psyllium plant, its classified as a polysaccharides, with molecular weight 300-2000 KD, multiple branches chain, as well as it appear as peripheral branches with xylose (Guo *et al*., 2008). The main structural units of arabinoxylan is composed from arabinose and xylose with β (1-4) linked (Ficsher *et al.,* 2004), arabinoxylans containing 56.72% xylose, 21.96% arabinose, 1.5% rhamnose, 0.4% mannose, 3.7% galactose, and galacturonic acid 5-8% (Van-Creayveld *et al.,* 2009). Many kinds of food products that fortifying by fibers has been developed, include baked foods, milk, juice and fermented foods, The unique properties of psyllium husk have captured the attention of food producers for production functional foods with nutritional characteristics in the world (Vega-Lopez *et al.,* 2003). The US Food and Drug Administration (USFDA) has authorized the consumption of foods containing psyllium (Leeds, 2009). Pandey *et al.* (2016), shows the role of arabinoxylan mucilage after extract it, to gave high viability to *Lactobacillus case* in the ice cream product, as well to the role of the psyllium husks in increasing counts number of *Lactobacillus case*, which was due to the presence of xylose and arabinose as prebiotic. And its ability to maintain the survival of *S. boulardii* (Elmer *et al.,* 1999). In spite of the use many prebiotics plant-derived in the manufacture of fermented dairy products, but the powder and psyllium seeds did not used till now in fermented products. Probiotic defined as "live microbial organism, when ingested in sufficient amounts, award a health benefits to the consumer, (WHO/FAO) with a minor grammatical changes (Hill *et al.,* 2014). Ozcan *et al.* (Ozcan *et al*., 2016), defined as living organisms (yeasts or bacteria). Probiotics defined as live microorganisms intended to provide health benefits when consumed, generally by improving or restoring the gut flora (Goldenberg *et al*., 2015). Despite the use of prokaryotic probiotic frequently (Suvarna and Boby 2005), however scientific studies approved on the presence of eukaryotic microorganisms which have favorable advantages in enhancing the health and defenses of the host (More and Swidsinski 2015), and the most important yeast is *Saccharomyces boulardii*, that is the only species of *Saccharomyces* genus that is certified for human consumption (Buchl 2010), as its registered in the General Recognized As Safe (GRAS) by FDA in 1996. Probiotics recently gets significant importance on the medical and commercial as a new global approach has begun to be used as an alternative treatment of antibiotics against many diseases (Akin and Tozun 2014). In addition, probiotics have a therapeutic and functional role in concentration that should not less than 10^7 CFU/mL or gm in order to have the therapeutic and health benefits and to strengthen the immune system (Floch, 2014).

Synbiotic is defined as a relationship that combines prebiotics with probiotics, in order to maximize the therapeutic benefit, by prebiotics that providing of support and stimulation for probiotics, through increased its growth rates and its viability (Grimoud *et al*., 2010). Synbiotics refer to food ingredients or dietary supplements combining

probiotics and prebiotics in a form of synergism, hence synbiotic concept was first introduced as "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare" (Pandey *et al.*, 2015). Fortifying foods by prebiotics are widespread now, so to achieve those benefits, the therapeutic and health role of probiotics products is achieved by containing high counts of live cells of probiotic to ensure their entering through the gut and its resistance to extreme conditions and finally colonized in the colon (Oluwakemi *et al*., 2014).

Materials and Methods

This study conducted from 01-03-2018 until 05-07-2018 at the Baghdad University / Baghdad/ Iraq and the Ferdowsi University / Mashhad / Iran.

Probiotic strains Sources

Commercial probiotic strain of *S. Boulardii* (CS) was provided by Sarrow Fomalas Company, Los Angeles-USA, and Commercial probiotic strain of *Lactobacillus rhamnosus* (GG) was supplied by (NOVA, USA) *well-recognized probiotics.*

Preparation of eukaryotic and prokaryotic Inoculums and estimate their logarithmic numbers

In order to activated both of the probiotic yeast and bacteria, one capsule from each transferred a under sterile conditions individually, the Yeast activation by Peptone Dextrose broth (Cardoso *et al*., 2015) and then MRS broth medium (Robinson, 1990), respectively, and incubating at 37° C / two days (repeat three times), then 20 ml of skim milk (Regiliat comp. France) 12% (w/v) plus 2% of table sugar for yeast and without sugar adding for bacteria was prepared and inoculated active fresh liquid culture of each (Hanan *et al*., 2018). In addition, counting the viable logarithmic number of each probiotics was done as described using serial dilution method by (Bashar and Amer 2017).

Chemicals and reagents

Concentrated hydrochloric acid and concentrated sulfuric acid (BDH - England), Glucose, fructose, sucrose, mannose, xylose, raffinose and arabinose were obtained from (Sigma-Aldrich, St. Louis, MO, USA) and (Merck Germany), Standard chicory inulin (Sigma, Berlin, Germany). Ethanol, were obtained from (Alpha Chemicals-India). Chromatographic silica gel 60 (20 cm×20 cm) pre-coated glass plates were obtained from (Merck, Germany).

Preparation of local and commercial psyllium seed powder

Local psyllium seed purchased from local market (Baghdad-Iraq), the preparation steps were included, cleaning, milling by electric coffee miller then sieved by using the sieve with pore size was (mesh 60/250 µm) and kept in a glass bottle and store in refrigerator. The commercial powder was prepared by the commercial company Herbal secrets / USA, which is provided in capsule form

Study of some chemical properties of psyllium

Determination of chemical composition

The chemical composition of moisture, ash, fat and protein content of the local and commercial psyllium seeds was estimated as mentioned (A.O.A.C. 1980). The % total, reducing and non-reducing sugars was estimated by 60% Ethanol solvent extraction, as follows:

Determination of total carbohydrates

The phenol sulfuric acid method was used to estimate total carbohydrate (Dubois *et al.,* 1956), and D-fructose sugar was used to prepare the standard curve.

Determination of reducing and non - reducing sugars

The method of 3,5-Dinitrosalicylic acid (DNSA) was used to estimate the reducing sugars using D-fructose to prepare the standard curve (Miller, 1959). The non-reducing sugars were estimated by calculate the difference between total sugars and reducing sugars.

Sugars Separation and diagnosis by Thin-layer chromatography TLC

The qualitative identification of monosacchara-ides, oligosaccharides and polysaccharides in psyllium seed extract achieved by using TLC technique, in order to determine the carbohydrate content of psyllium. In addition, the solution of standard sugars included mono. sugars (xylose, mannose, arabinose, fructose, glucose), oligosac-charide (sucrose and raffinose) and Polysacc- harides inulin were prepared, according to the method described by (Reiffova, and Nemcova 2006).

The R*f* values (retention factor) for the separated spots were calculated using the equation:

$$R_f \text{ value} = \frac{\text{Distance traveled by spot (cm)}}{\text{Distance traveled by solvent (cm)}}$$

FT-IR spectra of carbohydrate components from psyllium powder extracted

Carbohydrate mucilage of psyllium seed was extracted and purified as method described by (Shazia *et al*., 2006), in order to identify the chemical bonding and its active group by using FT-IR (BOMEM MB- Series) of the mucilage of psyllium were recorded in KBr pellets within spectral range of 500-4000 cm^{-1}, with a spectral resolution of 2 cm^{-1}, and a scan of 10 (Zhou *et al.,* 2012).

Study some properties of psyllium seed powder as prebiotic

This experiment was carried out to identify the psyllium seed powder as a prebiotic potential to enhance probiotics growth, by fortifying MRS broth by adding 0.5w/v of commercial psyllium seed (Thornthan *et al.,* 2018) and inoculated by 10% *L. rhamnosus* (GG) probiotic after autoclaved, and incubated for two periods (24 and 96 hours) and compared to control which was unfortified MRS broth.

Effect of psyllium seed powder in probiotics viable logarithmic number in pure and co-culture fermented milk

Pure and co-culturing of *S. boulardii* (CS) and *L. rhamnosus* (GG), was carried out in skimmed milk by the starters were added 5% and 1% of the probiotics respectively, after the addition 0.125% and 0.50% of both commercial and

local psyllium seed powder individually, and the inculcated milk were incubated at 37 until the completion milk coagulation and then kept in the refrigerator for counting viable logarithm numbers of probiotic.

Statistical Analysis: Statistical Analysis System (SAS) (2012) was used to study the effect of different coefficients on the studied traits, the mean differences between the averages was compared by a Least Significant Difference test (LSD), significant level at (P<0.05) (SAS, 2012).

Results and Discussion

Chemical composition of psyllium seed

The results in table (1) shown the chemical composition for commercial and local psyllium seed powder, which included reducing, non-reducing sugars, (% protein, % fat, % moisture and % ash) were respectively, (2.27, 53.94, 15.74, 16.22, 7.10 and 4.10) and (2.58, 51.43, 14.70, 15.68, 10.90 and 4.10), which showed no significant differences in the Chemical composition in spite there were variety of both as shown in the same table.

Table 1 : Chemical composition of psyllium seed powder

Variety	Local psyllium	Comer. psyllium	Chemical composition% / dry matter	
12.48 %	**2.58**^{A}	**2.27**^{A}	Reducing	Total sugars
4.65 %	**51.43**^{A}	**53.94**^{A}	Non-Reducing	
6.60 %	**14.70**^{A}	**15.74**^{A}	Protein	
3.32 %	**15.68**^{A}	**16.22**^{A}	Fat	
34.86 %	**10.90**^{B}	**7.10**^{A}	Moisture	
6.81 %	**4.40**	**4.10**^{A}	Ash	
–	**99.69**	**99.37**	**Total**	
* (P<0.05), NS: Non-Significant.				

*The averages with different letters within the same column significantly between them.

The % of total sugars of psyllium seed powder for both, were lower than mentioned by Alicja et al. (2018), who reported that The % total sugars was 79.27%, but the result of reducing sugars was agreed with and was 2.04%. It was found that the % protein for the local powder was in line with Alicja *et al*. (2018, which recorded the protein content was 15.45%. The % fat in the commercial and local powder are not consistent with of Alicja *et al*. (2018), which recorded % fat 3.55.

The variance in the results of the chemical composition for both commercial and local seed can be attributed to several factors, including (crop species, harvest time, genetic, agricultural techniques, pre-harvest and post-harvest climate conditions as well as its wild or cultivated crops) (Gao *et al*., 2011).

TLC technique for sugars diagnosing of psyllium seed powder

The results shown in figure (1) for separated sugars, commercial and local psyllium extract spots were appeared in blue, black and violet color on thin layer chromatography, were moved to different distance values of R*f*, depending on the molecular weight of each.

The R*f* values of sugars include (raffinose, sucrose, glucose, fructose, arabinose, mannose, xylose, inulin, commercial and local psyllium extract) were respectively, (0.08, 0.21, 0.24, 0.25, 0.25, 0.26, 0.29, 0.0, 0.0 and 0.0) as they shown in the table.(2), this was in agreement with Robyt (2000), who found that a higher DP leads to a decrease in the retention factor (R*f*), so sugars spots with the lowest molecular weight, moved with high R*f* values, then decreases with increasing of molecular weight.

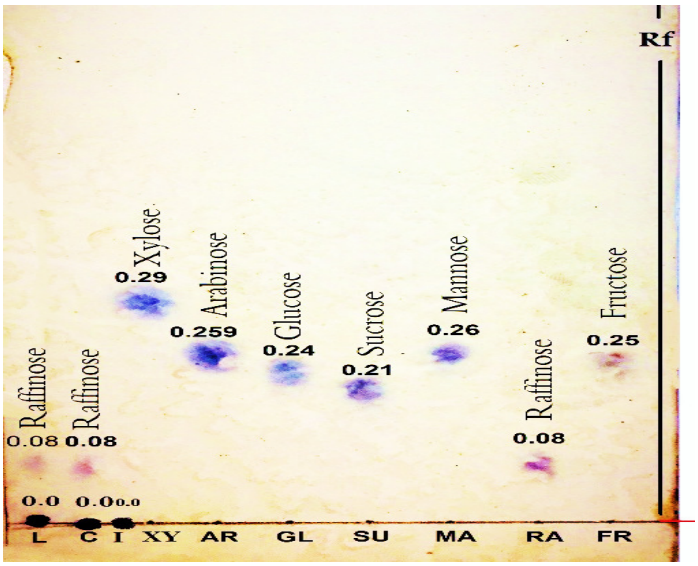

Fig. 1 : Separation and diagnosis of sugars by TLC (L: local psyllium extract. C: commercial psyllium extract. I: inulin standard)

The TLC figure shown, that aqueous extract for the commercial and local psyllium seed, was not contained monosaccharide and disaccharides compared with the standard solution of the same sugars, except of appearance of trisaccharides sugar spots that separated from the solutions in the same R*f* value to raffinose standard sugar. It should be noted that the polysaccharides of the both psyllium powder, stayed on the starting line without any displacement and their R*f* values were 0.0 compared to the R*f* of standard chicory inulin which was 0.0, this is due to their high molecular weight which prevented them from elution, and that matched with Reiffova and Nemcova (2006) results, which suggested that the sugar spots less 10 units, possible to move but more than 10 unites could not move.

Therefore psyllium seed powder could be prebiotic as it's a good source of branching polysaccharides as another prebiotic main structure, that indicated by Guo *et al*. (2008), and their low content of simple sugars (Saghir *et al*. (2008), and that is so necessary for probiotic enhancement, as well as promoting intestinal flora balance, and helped to limit the *firmicutes* phylum (Mendis *et al*., 2016).

Table 2 : The R*f* values of sugar spots on Thin layer chromatography

R*f* Value	**Signs**	**Compound**
0.00	L	Commercial psy. water extract
0.00	C	Local psyllium water extract
0.00	I	Standard chicory inulin
0.08	RA	Raffinose
0.21	SU	Sucrose
0.24	GL	Glucose
0.25	FR	Fructose
0.25	AR	Arabinose
0.26	MA	Mannose
0.29	XY	Xylose

FTIR spectroscopy of psyllium seed mucilage

The IR spectral in the figure (2) showed a significant similarity in the IR spectrum of (A) local and (B) commercial mucilage extract, within the spectral range (500-4000) cm^{-1}.

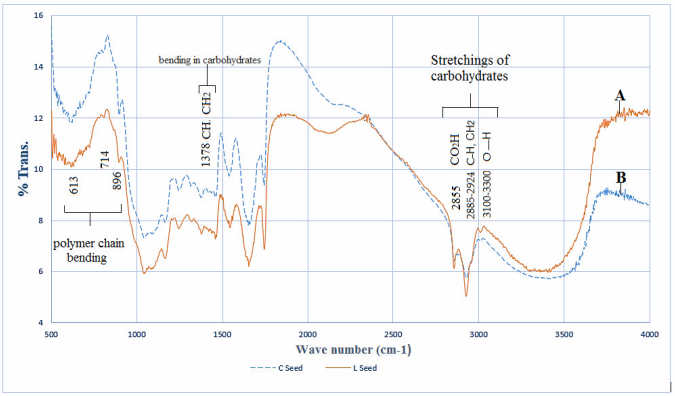

Fig. 2 : FT-IR spectroscopy of mucilage psyllium seed carbohydrate, (A): local seed, (B): commercial seed

Clear peaks were registered in the IR spectrum of psyllium mucilage at 3100 cm^{-1} and 3300 cm^{-1} (O–H stretchings of carbohydrates), IR spectrum of psyllium obtained peak at 2855.9 cm^{-1} (OH stretching of carboxylic acids) which refer to presence of galacturonic acid (Van-Creayveld *et al*., 2009). Peaks at 800 cm^{-1} and 900 cm^{-1} of (pyranose rings), that may be due to presence of pyranose monosaccharide (Srinameb *et al.*, 2015). The peaks were obtained at 896, 714 and 613 cm-1 may be due to (polymer chain bending).

The FT-IR spectral diagnosis of mucilage extracted from (A) and (B) psyllium seeds, were matched with the IR spectrum of Deepak *et al*. (2017), and Balbir and Kiran (2008), when they studying the FT-IR spectroscopy of the psyllium mucilage (arabinoxylan) extracted from the seed husk, and therefore, it is possible to prove that the mucilage extracted from the psyllium seeds is also arabinoxylan.

Properties of psyllium seed powder as prebiotic boost prebiotics growth

The results shown in table (3) of psyllium fortifying effect on the *L. rhamnosus* (GG) viable logarithmic number, for the two incubation periods (24 and 96) hour, compared to control treatment (MRS broth - non-fortified), were respectively, (14.39 and 15.93) and (14.45 and 14.10). As psyllium fortified broth was promoted to increase the number of probiotic viable count with significant increase at (P· 0.05), and with an increase reaches 11.48%, compared to the same unfortified MRS broth,

Table 3 : Effect of psyllium seed powder on *L. rhamnosus* (GG) logarithmic count

***L. rhamnosus* (GG) logarithmic count/ml**			**Carbon source**	Method of adding
Incubation time				
LSD	**96 hr.**	**24 hr.**		
1.305 *	$^{a\,B}$15.93	$^{a\,A}$14.39	psyllium seed	Fortifying
1.084 NS	$^{b\,A}$14.10	$^{a\,A}$14.45	Non fortified	
--	**11.48%**	**0.0%**	**Percent increase**	
	1.283 *	1.77 NS	**LSD**	
* (P<0.05), NS: Non-Significant.				

*The averages with different letters significantly between them. (**A**: for rows, **a**: for columns)

It was clear from the results, that the psyllium seed powder can be used as prebiotics source for promoting probiotic growth, and these results are not agree with Elli *et al*. (2008), which found that the psyllium powder, must partially digested to increase the number of *Bifidobacteria*, while recent studies have shown that the arabinoxylan-oligosaccharides (AXOS) are polysaccharides present in psyllium plant, it can be considered as potential new prebiotic as inulin and fructo-oligosaccharides (Deepika *et al.,* 2017) these results are also agree with Cremon *et al.* (2018), which suggested that psyllium husk can be considered as prebiotics which supporting growth of probiotic bacteria in the host and increase the production of fatty acids short chain (SCFA).

Effect of psyllium seed powder on the logarithmic count of pure and co-culturing probiotics

Results in table (4) shown that co-culturing fermented milk with psyllium seeds addition (commercial and local, 0.125% and 0.50%) individually, were led to the maximum increase in the cfu/ml logarithmic count of *S. boulardii* (CS) and *L. rhamnosus* (GG), which attained (9.24, 9.34, 9.10, 7.19) and (14.45, 14.65, 13.51, 10.01) respectively, compared to the both probiotics pure culturing, were (8.17, 8.30, 7.18, 7.79) and (14.01, 14.69, 13.01, 10.10) respectively.

The results in table. (4) shown also, that psyllium seed powders performed an important role in increasing the logarithmic count of *S. boulardii* (CS) and *L. rhamnosus* **(**GG) in the pure and co-cultured fermented milk, especially in the addition of 0.5% of the commercial powder, with logarithmic % increasing , 23.1%, 8.6%, 20.8% and 9.1%, respectively, compared to pure and co-cultured fermented without psyllium powder addition.

Deepika *et al.* (2017), demonstrated the fact that the carbohydrate polymer found in psyllium husk powder can be considered as a possible new version of prebiotic, and may be a good source of energy for probiotics (Mendis *et al*., 2016). The results shows that the effect of both psyllium seed (commercial and local) were various, because of the difference in their chemical composition as listed in table (1). In addition, the variance in local and commercial seed powder, can be attributed to the impact them by several factors, including (Species, harvest time, genetic, agricultural techniques, pre-harvest and post-harvest climate conditions as well as wild or cultivated crops) (Gao *et al*., 2011).

Table 4 : Effect of psyllium powder on the logarithmic cfu/ml viable count of pure and co-culturing probiotics

Co-culture		**Pure-culture***		**%**	**Psyllium Source**
CS	**GG**	**CS**	**GG**		
9.24	14.45	8.17	14.01	0.125	Comme. psyll. seed
22.2%	7.4%	19.5%	4.7%	Increase.	
9.34	14.65	8.30	14.69	0.500	
23.1%	8.6%	20.8%	9.1%	Increase	
9.10	13.51	7.18	13.01	0.125	Local psyll. seed
21.0%	0.9%	8.4%	0.0%	Increase	
7.19	10.01	7.79	10.10	0.500	
0.0%	0.0%	15.6%	0.0%	Increase	
7.18	13.38	6.57	13.35	0.00	control

* (GG): *L. rhamnosus* and (CS): *S. boulardii*

L. rhamnosus (GG) was found to be stimulated by *S. boulardii* (CS) growth through it could release (leucine, valine, vitamins and other nutrients, which they are necessary

to boost bacterial growth (Sauer *et al*., 2004). This interaction is also agreed with Sander *et al*., (2018), who mentioned that *S. cerevisiae* and *L. plantarum* stimulated each other in the presence of fructose, lactose and glucose as carbon source. Cheersilp *et al*., (2003), mention that the stimulation of *L. sanfranci- scensis* growth by *S. cerevisiae* was due to de-acidification caused by metabolized the lactic acid by yeast. In addition, it is possible that the increase in the yeast logarithmic viable count is due to the activity of *L. rhamnosus* to provision galactose to *S. boulardii*, as a carbon source derived from lactose metabolism (VIRGINIA and JOHN 1976).

According to the recommendations of (FAO and WHO 2001), that the total count to be considered as probiotics, should not be less than 10^7 cfu /ml or gm to achieve the specific therapeutic roles when consumed (Shah, 2000), and co-culturing of probiotics has performed an important role in the logarithmic count increase

The interaction between *S. boulardii* (CS) and *L. rhamnosus* **(**GG) during co-cultured fermented milk, is described as a co-operative interaction that called mutualism, which involves a relationship between two microorganisms that they stimulate each other, mutually (Bronstein, 2015). Moreover, this type of interaction is specifically termed as (non-symbiotic mutualism), which the microorganisms can growth individually, but without stimulation (Douglas *et al.,* 2014). And that to led to use this co-culture fermented milk as synbiotic products and that maybe s good solution for antibiotics and antifungal treatment during consumption probiotics products which its led to keep the healthy benefits for one of these probiotics kinds during treatment period.

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