RESERCHES REGARDING STABILITY OF THE FUNCTIONAL PRODUCT *HONEYLACT* DURING STORAGE

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Abstract
Regarding functional foods, it has been observed that modern consumers are increasingly interested in their personal health, expecting the food they eat to be healthy or capable of preventing illnesses. Probiotic bacteria are the most important microorganism group which it comes to improve consumers health. The functional product Honeylact seeks to combine the therapeutic virtues of three types of honey (mint honey, lime honey, multi-flower honey) and commercial pollen with the beneficial effects of four probiotic lactic acid bacteria (Lactobacillus brevis 16GAL, Lactobacillus plantarum, Enterococcus faecium VL28 and Lactobacillus johnsonii La1). The aim of this study was to research in which way probiotic lactic acid bacteria evolved (concentration of viable cells) to storage during shelf-life. It has been discovered that all 24 Honeylact samples does not lose its functional properties during storage. Romanian honey used in this study was obtained from local beekeepers The fermentation was conducted at 37^0/48 h in sterile closed plastic recipients. After fermentation, all samples were stored at 4^0C for 7 days. The stability of the probiotic cells throughout time was assessed by monitoring the viability. The present study demonstrates that honey and pollen had a significant effect in protecting the viability of the probiotic strains during 7 days of storage at 4^0C (10^6 CFU/g).

Keywords: honeylact, honey, pollen, viability, probiotic lactic acid bacteria

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1.INTRODUCTION

The popularity of dose delivery systems for probiotic products has resulted in research efforts targeted to developing probiotic foods outside the dairy sector. New product categories, and thus novel and more complex raw materials with regard to probiotics technology, are certainly the key research and development area for functional foods market. Therefore, researches are important to develop new matrices for probiotic development, increasing the number of products with functionality in the marketplace, and offering new options for all types of consumer's demand and desire (Coman *et al*., 2012).
It is commonly accepted that the incorporation of probiotics into a food product increases its commercial value, as shown by recent market studies (Euromonitor, 2010) which describe an increasing awareness and preference of the general consumer for functional foods that incorporate probiotics. Functionality of probiotics has been associated with adhesion to enterocytes in order to block potential adhesion sites for pathogens, immunomodulatory potential and also to antagonistic effects against pathogens, namely by the production of antimicrobial substances (Liu *et al.*, 2011).
Functional foods are defined as foods that affect specific functions or systems in the human body, providing health benefits beyond energy and nutrients. The market of functional dairy products has developed markedly during the last decade (Granato *et al*., 2010).
New food products have been formulated with the addition of probiotic cultures. Different types of food matrices have been used such as various types of cheese, ice creams, milk-based desserts, powdered milk for newborn infants, butter, mayonnaise, powder products or capsules and fermented food of vegetable origin (Soccol *et al.*, 2010). Due to the large number of beneficial effects that honey, propolis and royal jelly could be considered as potential ingredients for different foods (Viuda-

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Martos *et al.*, 2008). The application of honey as a food additive is based on its manifold properties. Honey enhances the growth of dairy starter cultures in milk and milk products (Slacanac *et al.*, 2011).

Due to the variation of botanical origin honey differs in appearance, sensory perception and composition. An aromatic sweet food, honey possesses many valuable nutritional benefits to our body. There is evidence in the literature that honey has many beneficial health effects such as antibacterial, antioxidant, anti-tumour, anti-inflammatory and anti-viral activity (Bogdanov, 2014).

Pollen grains, a fine powder-like material, are the male seeds of a flower blossom which has been gathered by the bees and to which special elements from the bees has been added. Honeybee collects pollen and mixes it with its own digestive enzymes (Crane, 1990).

Bee pollen is a rich source of protein (25%); essential amino acids; oil (6%), containing more than 51% PUFA of which 39% linolenic, 20% palmitic and 13% linoleic acids; more than 12 vitamins; 28 minerals; 11 enzymes or co-enzymes; 11 carbohydrates (35–61%) which are mainly glucose, fructose and sucrose; flavonoids and carotenoids; phytosterols (Xu *et al*., 2009). Pollen bread was found to possess an antibacterial activity. In a recent study with 80 % ethanol extracts of Brazilian pollen antibacterial activity was exibited against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Klebsiella* sp. (Carpes, 2007). Pollen has also significant antifungal activity against different pathogens (Koc *et al*., 2011).

Vamanu *et al*., (2008) presents the multiplication of a *Bifidobacterium bifidum b1* strain on media containing pollen and honey.

The effect of prebiotics on the multiplication of some probiotic strains used in a pollen and honey medium was investigated in laboratory conditions, aiming to obtain a bee bread-like product (Vamanu *et al*., 2010).

Ionescu *et al*., (2013) presents the results obtained concerning a probiotic manufacture on media with pollen and honey, using some selected bacterial strains and several essential factors for direct and effective microbial biomass production.

2. MATERIAL AND METHODS

The stability of the juices depend on the chemical composition of the juice and the dose of lactic acid bacteria with which are inoculated these.

2.1. Samples

Honey origin

Romanian honey used in this study was obtained from local beekeepers (Luncaviţa - Tulcea country, Vizantea - Vrancea country and Cuca - Galaţi country) in 2012. The samples had different botanical origins: mint honey (M), linden honey (T) and multi-flower honey (P). Melissopalynological analysis was performed in order to confirm the botanical source. Honey physicochemical properties were determined according to the national standard SR 784-3:2009/C91:2012.

Pollen origin

Polifloral romanian bee pollen used in this study was obtained from SC. LASTING PREST.

Probiotic lactic acid bacteria

In the practical experiments we used the *Lactobacillus brevis* 16 GAL and *Lactobacillus plantarum* (probiotic strains from the MIUG collection of the Faculty of Food Science Galati), *Enterococcus faecium* VL 28 and *Lactobacillus johnsonii* La1 (probiotic strains from the MICROGEN collection of the Faculty of Biology Bucharest).

The API 50CHL test (BioMerieux, France) was applied for identification by fermentation patterns. *Lactobacillus plantarum* was isolated from the commercial pollen and conserved in the SANYO Freezer Biomedical, at -82^{0}C, on a protective medium, enriched with 20% glycerol, and then revitalized on a medium containing MRS. Surviving bacteria were counted by Breed method. Viable cell counts were determined by preparing serial decimal dilutions which were subsequently plated on MRS agar (Merk) on Petri dishes. The plates were incubated for 48 h, at 37°C. Plates containing 30–300 colonies were selected and CFU/mL was recorded. All plate counts were carried out in duplicates.

Experimental design

As can see in table 1 each sample has been obtained by mixing honey (50g) with pollen (5%, respectively 10%), just enough to break the pollen grains and obtain a relatively homogenous medium and adding a minimal quantity of inoculum. The levels were based on a preliminary study. Inoculum from each probiotic strain containing 6 - 6,5 x 10^6 CFU/mL. The fermentation was conducted at 37^0/48 h in sterile closed plastic recipients. After fermentation, all samples were stored at 4^0C for 7 days. The stability of the probiotic cells throughout time was assessed by monitoring the viability. It was decided to evaluate the cell viability for three time in this period.

3. RESULTS AND DISCUTION

3.1. Physicochemical properties of honey

Honey is considered a potential complete food, regarding nutritional standards, being a natural product, rich in simple, easy assimilable sugars (fructose, glucose), enzymes (invertase, glucose oxidase, catalase, phosphatase), amino and organic acids (proline, gluconic acid, acetic acid), vitamins (ascorbic acid niacin, riboflavin), volatile oils, phenolic acids and flavonoids, minerals and carotenoid like substances (Sudhanshu *et al*., 2010). In the past three decades, the large number of published studies concerning the physicochemical characteristics of honeys of different botanical and geographical origins illustrates the importance of determining honey's quality.

Table 1. Experimental design of Honeylact product

Probiotic strain	Code sample	Honey types	Pollen (%)
Lactobacillus johnsonii	V1	M	5
	V2	M	10
	V3	T	5
	V4	T	10
	V5	P	5
	V6	P	10
Enterococcus Faecium	V7	M	5
	V8	M	10
	V9	T	5
	V10	T	10
	V11	P	5
	V12	P	10
Lactobacillus Brevis	V13	M	5
	V14	M	10
	V15	T	5
	V16	T	10
	V17	P	5
	V18	P	10
Lactobacillus plantarum	V19	M	5
	V20	M	10
	V21	T	5
	V22	T	10
	V23	P	5
	V24	P	10

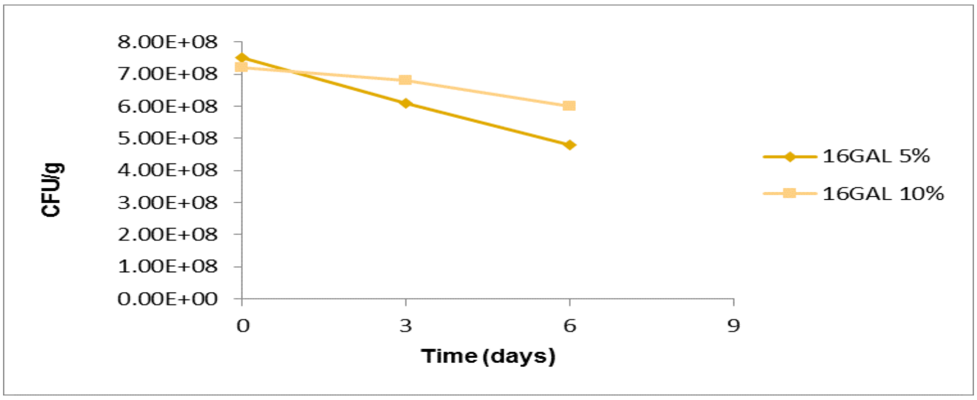
Table 2. Physicochemical properties of honey

Physicochemical properties	Honey types		
	T	P	M
Water, %	16,6	16,2	15,8
Ash, %	0,24	0,38	0,15
Acidity, ml NaOH 0,1N/100g honey	0,5	0,3	0,2
HMF, mg/100g	1,03	1,17	1,10
Invert sugar, %	86	83	82
Saccharose, %	4,6	4,4	4,3
Diastazic index	10,9	6,5	8,3
Pollen, %	33	-	55

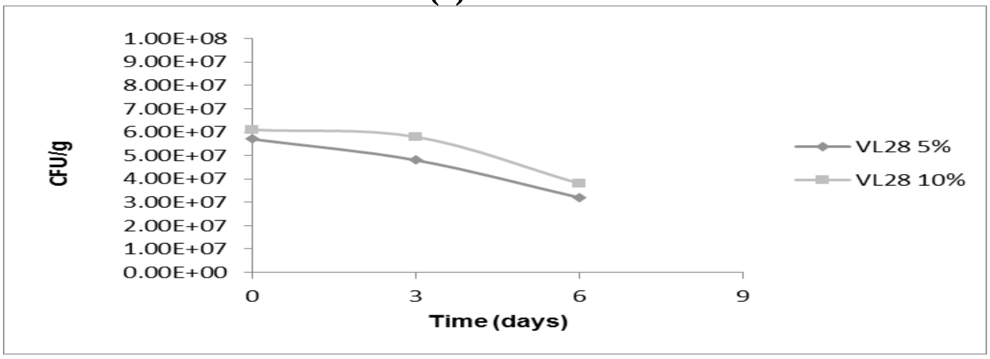
The obtained data are presented in Table 2 and show a good quality of studied honey samples.
Experimental results show that the honeys properties values were in the range of approved limits (according to SR 784-3:2009/C91:2012). Similar results were reported by Cimpoiu *et al.*, (2013) for 26 Romanian honey samples from different botanical source.
Romanian honeys contain important level of proteins, amino-acids and phenolics, showing a substantial antioxidant capacity which may be used as a natural source of compounds with these properties. The quality of Romanian honey differs on account of various factors like geographical, seasonal and processing conditions, floral source, packaging and storage conditions (Mărghitaș *et al.*, 2009).

3.2. The effect of storage on the viability of probiotic bacteria

The effect of storage on the viability of probiotic bacteria during Honeylact shelf life is an important factor that must be studied to ensure viability of tested probiotic bacteria is mentained in excess of recommended level of 10^6 CFU/g in the time of consumption.
It was observed that medium with multi-flower honey (P) is optimal for the cultivation of probiotic strains. The results obtained are shown in Figure 1 (a-d).
The microorganisms viability on Honeylact samples V5, V6, V11, V12, V17, V18, V23, V24 is influenced obviously by the medium composition (honey type), the viability having values of 6 x 10^7 and 7,5 x 10^8 CFU/g.
It should be noticed the evolution of *Lb. brevis* 16GAL and *Lb. johnsonii* La1 strains which had a maximum evolution in the case of all probiotics. Samples with 10% pollen maintains relatively constant viability values. Viability loss of samples with 10% pollen did not exceed 20%. The results indicated that higher pollen content did provide better protection to the tested probiotic microorganisms. The weakest viability is observed for *E. faecium* VL28 (Figure 1 - b). Honeylact V5 sample made with 5% pollen (*Lb. johnsonii* La1) had significantly decreased viability after 6 days of refrigeration (50%).
Honeylact product made with mint honey (V1, V2, V7, V8, V13, V14, V18, V19), is medium for the cultivation of probiotic strains. The results obtained are shown in Figure 2 (a-d). After fermentation at 37^0/48 h the values of viable probiotic bacteria were between 4 x 10^7 CFU/g (*Enterococcus faecium* VL28) and 9 x 10^7 CFU/g (*Lb. johnsonii* La1). *Lb. johnsonii* La1 strain had a maximum multiplication and better stability (80% after 6 days of refrigeration) as can see in Figure 2 - c. The weakest viability is observed for *E. faecium* VL28, but stable in refrigeration period (Figure 2 - b). *Lb. plantarum* strain had a medium multiplication (6,5 x 10^7CFU/g), but after 6 days of refrigeration, 50% of viability decreased. Lime honey is not favourable for the cultivation of probiotic strains. The microorganisms viability on Honeylact product made with mint honey (V3, V4, V9, V10, V15, V16, V21, V22), is not favourable for the cultivation of probiotic strains. Only *Lb. plantarum*, strain isolated from pollen, had a maximum viability (1,2 x 10^8 CFU/g) and stability in medium made with 10 % pollen.



(a)



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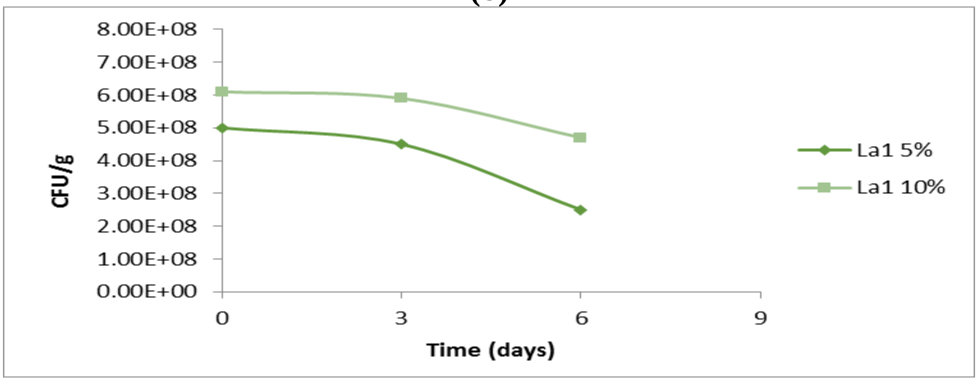

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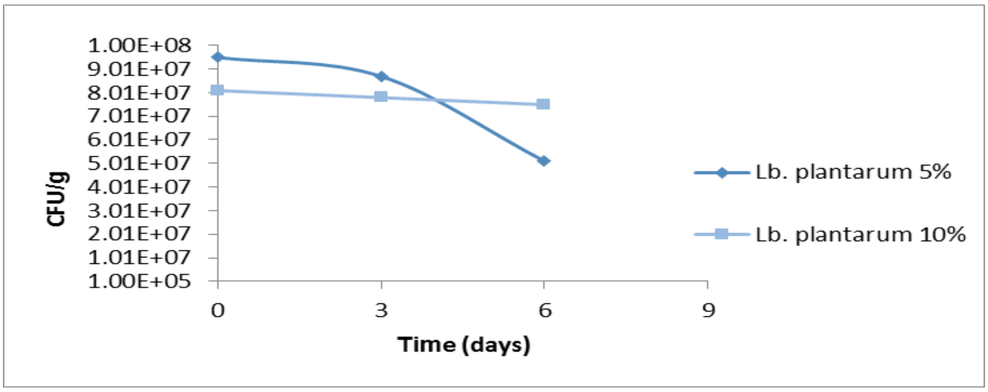
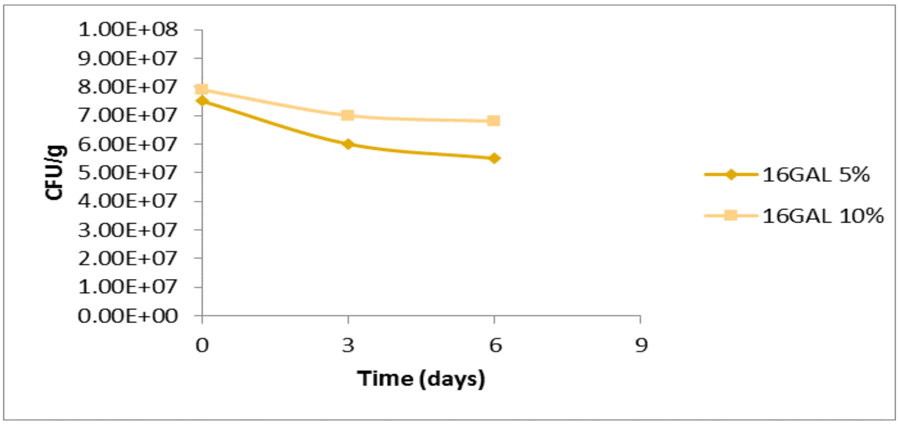


(d)

Fig. 1. Viabilitaty of probiotic strains in Honeylact product made with multi-flower honey during storage at 4^{0}C (a) *Lb. brevis* 16GAL; (b) *Enterococcus faecium* VL28; (c) *Lb. johnsonii* La1; (d) *Lb plantarum*



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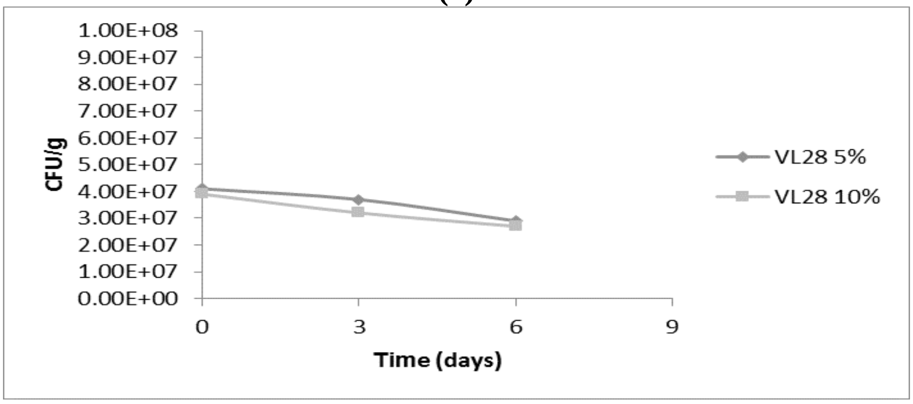

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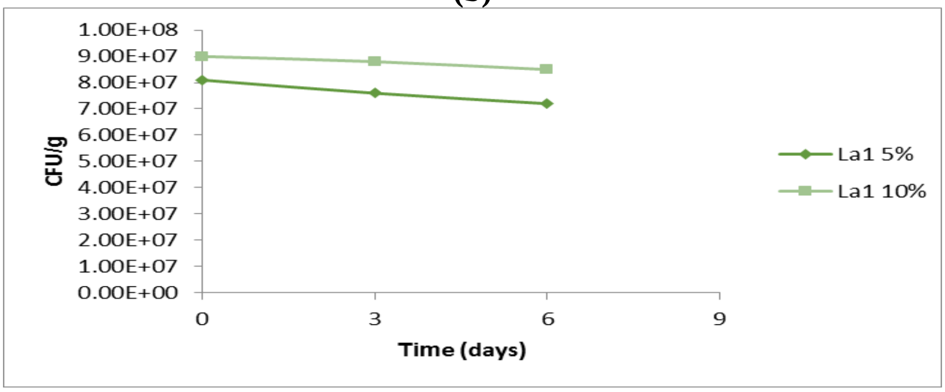

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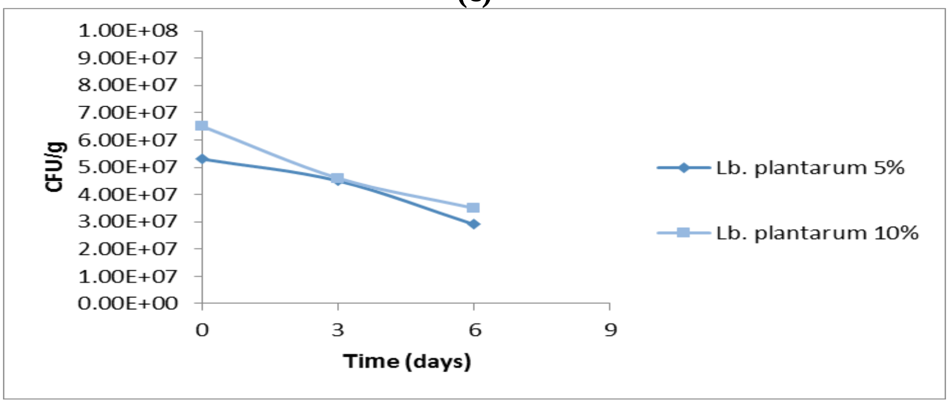
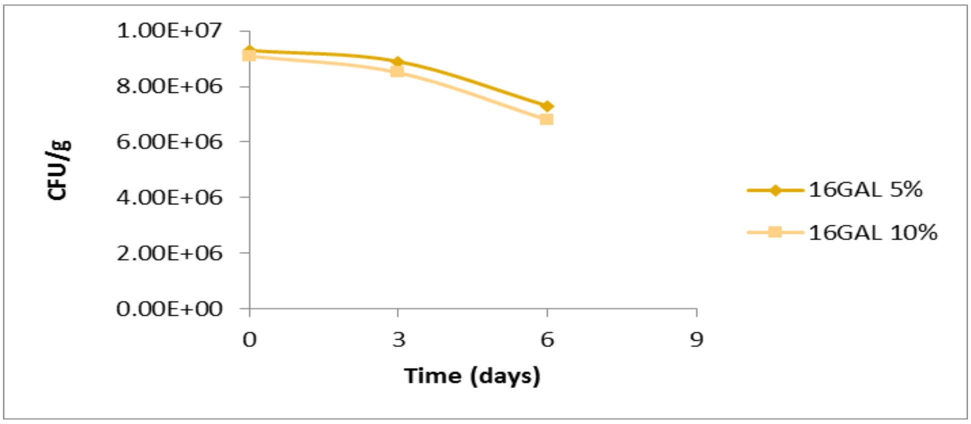
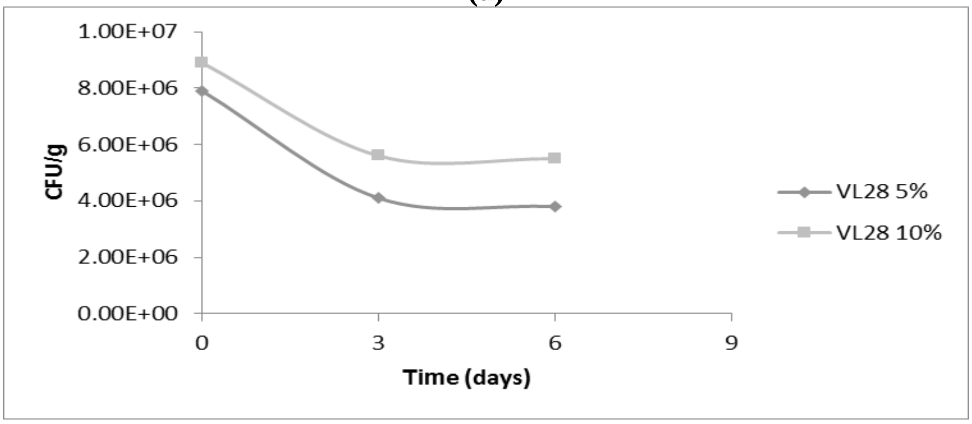


(d)

Fig. 2. Viabilitaty of probiotic strains in Honeylact product made with mint honey during storage at 4^{0}C
(a) *Lb. brevis* 16GAL; (b) *Enterococcus faecium* VL28; (c) *Lb. johnsonii* La1; (d) *Lb plantarum*



(a)



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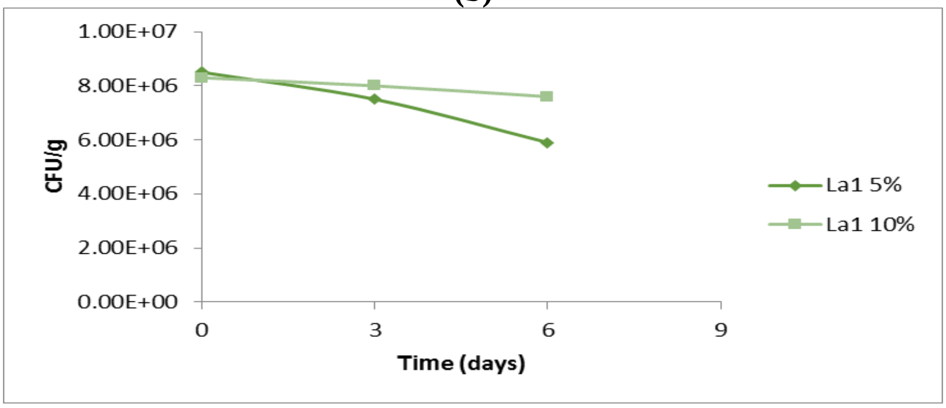

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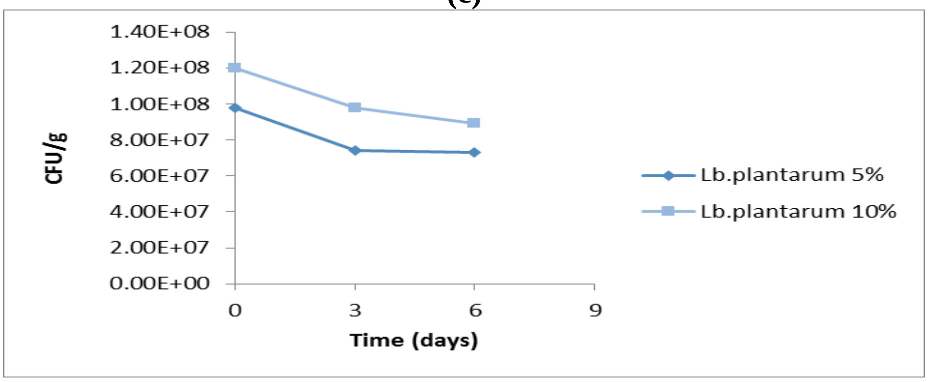

(d)

Fig. 3. Viabilitaty of probiotic strains in Honeylact product made with lime honey during storage at 4^0C (a) *Lb. brevis* 16GAL; (b) *Enterococcus faecium* VL28; (c) *Lb. johnsonii* La1; (d) *Lb plantarum*

The viability of *Lb. brevis* 16GAL and *Lb. johnsonii* La1 is 10^7 CFU/g. Honeylact sample V3 made with *Lb. johnsonii* La1 and 5 % pollen is stable. The weakest viability is observed for *E. faecium* VL28. After 3 days of refrigeration it was observed that 50% of viability decreased (Figure 3 - b).

4. CONCLUSIONS

We can conclude that the media with multi-flower honey and 10% pollen using an inoculum that contains *Lb. brevis* 16GAL or *Lb. johnsonii* is the most propitious alternative for the Honeylact product, with the biggest viability values (80%).

Honeylact product can be considered as a functional food. The synergic effect of the components (honey and pollen) and the viability having values of 10^6 CFU/g after 6 days at 4^0C in all samples ensures the functionality of the Honeylact product.

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