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PREBIOTIC CONTENT AND PROBIOTIC EFFECT OF KOMBUCHA FERMENTED POLLEN

Elena UTOIU[1, 2], Anca OANCEA[2], Ana-Maria STANCIUC[2], Laura M. ŞTEFAN[2], Agnes TOMA[2], Angela MORARU[3], Camelia Filofteia DIGUȚA[1], Florentina Matei[1], Călina Petruța CORNEA[1], Florin OANCEA[1, 4]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2]National Institute of Research and Development for Biological Sciences (INCDSB), 296 Spl. Independenței, 060031, District 6, Bucharest, Romania
[3]S.C. LaboratoareleMedica SRL, 11 Frasinului Street, Otopeni, Ilfov County, Romania
[4]The National Institute for Research and Development in Chemistry and Petrochemistry (ICECHIM), 202 Spl. Independenței 060021, District 6 Bucharest, Romania

Corresponding author email: elena.utoiu@gmail.com

Abstract

Bee pollen contains high levels of bioactive compounds but their bioavailability is restricted to human's diet due to complex external structure of cellular wall. SCOBY fermented pollen contains compounds with prebiotic effects, such as carbohydrates, polyphenols, flavonoids and dietary fibres, but also lactic bacteria and yeasts with probiotic characteristics. In order to increase the bioavailability of pollen grains, we used an innovative pollen fermentation biotechnology using SCOBY consortium (Kombucha). The aim of this study was to assess the release of different prebiotic compounds using HPLC, spectrophotometric and enzymatic methods in both unfermented and Kombucha fermented pollen. Furthermore, the probiotic effect of fermented pollen was assessed through adesivity test of lactic acid bacteria to Caco-2 intestinal cells and observed by light microscopy. The highest content of prebiotics was obtained between 5-7 days of fermentation. Our results also showed that lactobacilli isolated from pollen fermented broth adhered to intestinal cells in a time depending manner, suggesting a probiotic effect. Overall, Kombucha fermentation increased the release of pollen prebiotics and exhibited in vitro probiotic potential.

Key words*: Kombucha fermented pollen, prebiotics, probiotics.*

INTRODUCTION

Pollen is recognized for its nutritional values and its high content in bioactive compounds, including proteins (ranging between 10% and 40%), essential amino acids (around 10.4%), digestible carbohydrates (approx. 30.8%), lipids and fatty acids (ranging between 1% and 10%), phenolic compounds (approx. 1.6%), enzymes, coenzymes, vitamins and bio elements (Vassev et al., 2015; Villanueva et al., 2002; Asafova et al., 2001; Campos et al., 2008; Campos et al., 2010). However, the bioavailability of pollen bioactive compounds is limited by the complex structure of the double-layered cell wall of the pollen grains. The external layer of the pollen grains, namely exine, exhibits a strong resistance to physic-chemical factors due to sporopolenin, a biopolymer with high stability and resistance to enzymatic biodegradation (Wiermann et al., 2005).

Several studies showed that, in animals, pollen nutritional content was released by digestive juices (Roulston and Cane, 2000), while in humans was just partly digested (approx. 15% for carbohydrates and 53% for proteins) (Franchi et al., 1997).

In order to increase the pollen digestibility of the current products available on the market, several approaches have been developed, such as grinding or soaking in warm water. For example, the pollen grains maintained in water for several hours swell, crack and release their content (Vassev et al., 2015).

While the uncrushed pollen is used by the organism in proportion of 10-15%, the accessibility of pollen content increases up

too60-80%, after mechanical crushing or natural release (Bogdanov, 2014; Rimpler, 2003).

Our approach was to increase the bioavailability of pollen bioactive ingredients, through a microbial fermentation of pollen using a SCOBY consortium, commonly known as Kombucha. Kombucha is a sugared black tea, fermented by a symbiotic culture of yeast and acetic acid bacteria. This beverage provides a source of both probiotic bacteria (such as lactic acid bacteria) and yeast, as well as prebiotics (micro cellulose) (Greenwalt et al., 2000; Malbasa et al., 2008; Kozyrovska and Foing, 2010). Generally, traditionally fermented foods are the best places to look for probiotic microorganisms with potential applications in food industry and health (Zamfir et al., 2014). Kombucha prebiotics support the growth of beneficial microorganisms in digestive track also by enhancing adherence to intestinal cell surfaces and, therefore, protecting the host organism from pathogen invasion (Salminen, 1996).

The aim of this study was to increase the bioavailability of pollen grain bioactive ingredients by using Kombucha pollen fermentation and to demonstrate the probiotic effect of this fermented pollen through adhesion of lactic acid bacteria to Caco-2 intestinal cells.

MATERIALS AND METHODS

Sample preparation. An infusion of black tea (0.5%) was prepared in boiled water and filtered after 15 minutes of extraction. Then, 70 g of sugars and 50 g of fresh frozen bee collected pollen were added to the infusion. This mixture was inoculated with 100 mL/L of fermentation liquid containing the symbiotic culture of bacteria and yeast (SCOBY). As control, we used a 7% sweetened black tea infusion with bee collected pollen. The fermentation process was conducted at 28^{o}C, for a period of 18 days and samples were collected at different time intervals (0, 3, 4, 5, 6, 7, 10 and 12 days).

Quantitative carbohydrates determination. Hexoses, reducing sugars and pentoses were spectrophotometrically quantified (Iordachescu et al., 1988).

Chromatographic determination of monosaccharides by HPLC. For this determination we used an Agilent HPLC 1200 system (Agilent USA), comprising a refractive index detector, isocratic pump, and a thermostatic auto sampler. Samples were analysed on a chromatographic carbohydrate analysis column Agilent (column size: Φ 4.6 x 150 mm). 25 mL from each sample were brought to dryness at 40°C and 40 mg of dry sample was eluted in 1 mL of purified water and sonicated for 30 minutes. The monosaccharide compounds detection was made at 30^{o}C on RID detector and column, with a mobile phase of 75% acetonitrile and 25% purified water, a 0.5 mL/min. flow, and 5 µL injection volumes. Quantification of the compounds was carried out from the pick area, in comparison with monosaccharide analytical standards (Monosaccharide Kit from SUPELCO) Standard curves were used to quantify monosaccharides concentrations using a Chemstation software.

Determination of total phenolic and flavonoids compounds. The total phenolic compounds from pollen sample were measured by a slightly modified Folin-Ciocalteu method (Craciunescu et al., 2012). 150 µL of sample were mixed with 750 µL of Folin-Ciocalteu reagent, for 5 minutes, at room temperature, then added 4 ml of 15% Na_2CO_3 and distilled water until a final volume of 15 ml. Absorbance was measured at 765 nm after 2 h of incubation at room temperature with a UV/vis spectrophotometer (Jasco V530, Japan). The total phenolic compounds were expressed as caffeic acid equivalents. Total flavonoid content was measured through colorimetric method (Alexandru et al., 2007; Chang et al., 2002) mixing 0.5 mL of sample, 1.5 mL methanol, 0.1 mL 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water and then incubated 30 minutes at room temperature. Absorbance was measured at 415 nm and flavonoid content was expressed as quercetin equivalents.

Antioxidant activity. DPPH scavenging activity was measured as described by Huang (2005). 150 µL DPPH solution (0.25 mM) in methanol, 15 µL of samples and 90 µL 0.1 M Tris HCl were mixt, shaken and incubated at 37^{o}C for 30 minutes in the dark. BHT was used as positive

control. Sample absorbance (A_{sample}) at 520 nm was measured against methanol blank (A_{blank}) using a UV/visible using a microplate reader (Sunrise Tecan, Austria). Inhibition (%) was calculated using the following formula and the results were reported per gram dry weight: % Inhibition = $(1-A_{sample}/A_{blank})*100$.

Trolox equivalent antioxidant capacity (TEAC assay) was measured using Re et al (1999) method with some modifications. The ABTS radical cation was generating by reacting a 7 mM 2.2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution with 2.45 mM potassium persulfate solution (1: 1, v/v). The mixture was preserved in dark at room temperature for 16 h. The absorbance of ABTS radical solution was equilibrated to a value of 0.7 ± 0.02 at 734 nm. 1 mL ABTS radical solution was mixed with 0.1 mL test sample and after incubation at room temperature for 6 minutes, the absorbance was measured at 734 nm. TroloX (0-250 μM) was used to achieve a calibration curve. Absorbance was converted to equivalent activity of Trolox per g of dry weight (mg Trolox/gd.w.) based on a standard curve.

Fiber content determination was performed by gravimetric method (Standard AACC 32-07) using the Megazyme Total Dietary Fiber Kit and according to the manufacturer's instructions. This method determines soluble (SDF), insoluble (IDF) and total dietary fiber (TDF) content and the results were expressed as g/100 g of tested sample.

Adhesivity test to intestinal Caco-2 cells. The *in vitro* assays were performed on Caco-2 cell line, derived from human Caucasian colorectal adenocarcinoma and purchased from ECACC (Sigma). The bacterial population was isolated from Kombucha fermented tea (SCOBY consortium), from which a strain of lactic acid bacteria (L5) was further used for adhesivity test. Caco-2 cells were grown in MEM culture medium (Sigma), supplemented with 10% fetal bovine serum (Biochrom) and 1% antibiotics (penicillin, streptomycin and neomycin) at 37°C and in a humidified atmosphere (5% CO2). For the experiments, Caco-2 cells were seeded on circular glass lamellae (∅19 mm) placed in 12 multiwell plates at a density of 5 x 104 cells/mL and incubated in standard conditions for seven days. The culture medium was refreshed every second days. Before the addition of the bacterial suspension the cell culture medium was discarded and replaced with antibiotics free medium.

In the adhesion study has been used a lactic acid bacteria, named L5, isolated from Kombucha and identified by molecular tools as *Pediococcus pentosaceus* (Matei et al., 2018). The bacteria have been cultivated in MRS broth during 24 hours at 37°C. The bacterial cultures (1 x 10^8 CFU/mL) were washed twice with sterile PBS and equal volumes (1 mL) of the bacterial suspension were added to each well containing the cellular monolayer and incubated at 37°C. From the control well, the bacterial suspension was immediately removed and replaced with culture medium without antibiotics. After 1 h and 4 h of incubation, the unattached bacteria were removed by washing the cellular monolayer 5 times with sterile PBS, fixed in methanol for 5 minutes and Giemsa stained for 30 minutes. The lamellae with the Caco-2 monolayer and adhered bacteria were mounted in Canada balm and microscopically examined under immersion oil using Zeiss AxioSkop 40.

RESULTS AND DISCUSSIONS

Determination of carbohydrates. The release of carbohydrates from both unfermented and fermented pollen grains varied in a time dependent manner (Table 1).

Table 1. Carbohydrates content in unfermented and fermented pollen samples.

Sample	Time intervals (days)	Hexoses (μg/mg d.w.)	Reducing sugars (μg/mg d.w.)	Pentoses (μg/mg d.w.)
Unfermented pollen	0	260.503	531.335	93.01
	3	649.791	580.68	348.107
	4	762.158	560.241	396.234
	5	685.812	524.786	409.741
	6	762.365	580.386	498.959
	7	678.233	664.063	612.369
	10	441.798	558.983	602.045
	12	472.996	519.943	483.683
Kombucha fermented pollen	0	234.783	525.514	88.93
	3	249.177	203.327	71.385
	4	70.783	151.263	60.365
	5	81.697	188.187	89.771
	6	66.387	172.832	129.538
	7	71.331	145.341	77.341
	10	58.567	157.042	63.223
	12	45.333	251.621	67.997

The obtained results exhibited an increase of the hexoses, reducing sugars and pentoses

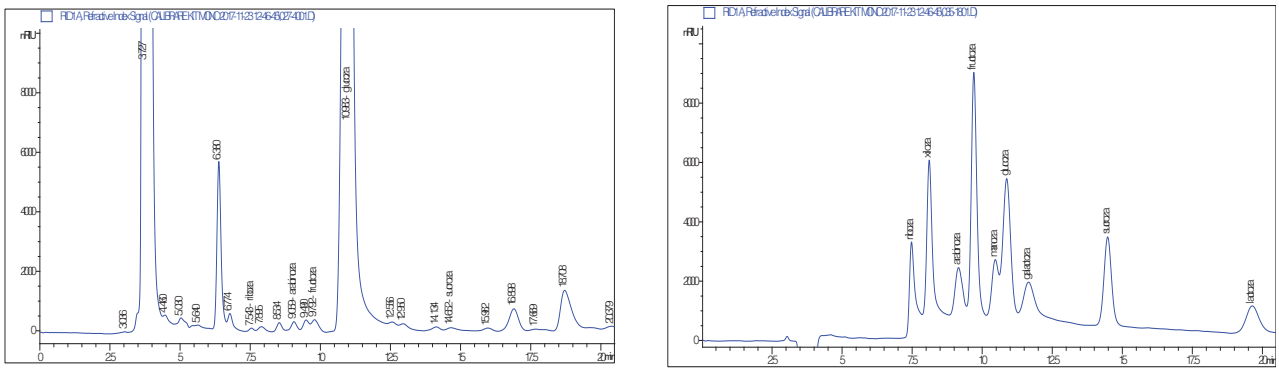
amounts in unfermented pollen samples. The highest quantities of hexoses were obtained between 4th and 7th days of maintaining pollen in sweetened black tea (678.233-762.365 µg/mg d.w.), while the maximum amount of reducing sugars and pentoses was reached on 7th day of the experiment (612.369 µg/mg d.w.). The increased quantities of carbohydrates suggest that pollen grains were broken, releasing their content into the media. For the Kombucha fermented pollen, the hexose quantities decreased significantly, with the lowest value registered on 12th day (45.33 µg/mg d.w.). Also, the quantities of reducing sugars and pentoses decreased through the fermentation process compared to the control samples, due to yeast and bacteria metabolism from the fermented broth (Table 1).

Furthermore, major monosaccharides, such as ribose, xylose, arabinose, fructose and glucose, present in investigated samples were identified and quantified by HPLC (Figure 1).

The content of all tested monosaccharides increased in a time dependent manner in the unfermented pollen samples, except fructose, whose content decreased from 729.234 µg/mg d.w. to 175.423 µg/mg d.w. (see Table 2). In the Kombucha fermented pollen samples, lower quantities of ribose, fructose and glucose were found compared to control samples, while the amount of xylose and arabinose slightly increased (Table 2).

Studies showed that 34.06% of sucrose from Kombucha fermentation broth remained unfermented after 7 days, while after 21 days a reduced value (19.28%) was observed (Chen and Liu, 2000). Yeasts and bacteria in Kombucha are involved in certain metabolic activities that utilize this substrate. Via glycolysis, yeasts hydrolyse sucrose into glucose and fructose and produce carbon dioxide and ethanol, with a preference for fructose as a substrate (Carpes et al., 2009).

In fermented pollen samples, the fructose content decreased during fermentation process probably due to its oxidation to acetaldehyde by the bacteria found in the broth. The same fructose profile exhibited by the unfermented pollen samples, but with significantly lower values, could indicate the damaging of double-layered cell wall of the pollen grains and the releasing of its content.



A) B)

Figure 1. Chromatographic sample profile: a) Kombucha fermented pollen at 10 days; b) monosaccharide analytical standards (4 mg/mL)

Furthermore, the decreasing glucose content observed during Kombucha pollen fermentation could be explained by the fact that acetic acid bacteria metabolize glucose and ethanol to gluconic acid and acetic acid (Jayabalan et al., 2014), whereas in unfermented pollen samples, the glucose content accumulates due to the absence of the bacteria.

Time-dependent composition of the total phenolic compounds and flavonoids in unfermented and fermented pollen. Plant polyphenols constitute a class of compounds that can also meet the criteria of prebiotics (Gibson et al., 2017). The contents of phenolic compounds and flavonoids in both fermented and unfermented pollen samples varied depending on the experiment time intervals, as shown in Figure 2.

Table 2. Monosaccharide content (HPLC quantification)

Sample	Time intervals (days)	Monosaccharide content (μg/ mg d.w.)						
		D-(-) Ribose	D-(+) Xylose	D-(-) Arabinose	Fructose	D-(+) Manose	Glucose	D-(+) Galactose
Unfermented pollen (in sweet black tea)	0	6.533	2.505	ND	729.243	ND	654.135	ND
	3	10.991	3.812	5.333	420.150	ND	817.890	ND
	6	15.485	3.954	4.004	353.190	ND	896.112	ND
	10	19.831	4.031	9.366	223.3	ND	880.093	ND
	12	16.443	ND	6.442	175.423	ND	954.305	ND
Kombucha fermented pollen	0	10.741	ND	6.934	713.995	ND	561.095	ND
	3	9.032	3.898	11.115	2.662	ND	423.850	ND
	6	8.922	12.218	15.555	4.204	ND	588.808	ND
	10	8.267	5.795	15.259	6.714	ND	516.748	ND
	12	8.555	ND	18.559	5.043	ND	512.415	ND

In Kombucha fermented pollen samples, the amount of total phenolic compounds and flavonoids increased progressively up to 5-7 fermentation days, existing two possible explanations: 1) during fermentation process, a pollen grains breakage occurs, releasing the content and 2) degradation of complex polyphenols to small molecules due to secreted enzymes during fermentation, which is reflected in the increase of total phenolic compounds and flavonoids (Bhattacharya et al., 2011). A small increase in total phenolic compounds could also be observed in unfermented pollen, while the flavonoid content remained constant.

Antioxidant activity was determined by two complementary assays: DPPH and TEAC. The scavenger activity of the free radical DPPH was expressed as degree of inhibition (%) reported to 1 g of sample dry weight, higher values of this activity indicating better antioxidant capacity of the tested sample.

Overall, a better antioxidant capacity was found for Kombucha fermented pollen samples compared to control samples regardless the fermentation time. The highest values were observed between the 5th and the 7th day of fermentation (Table 3).

The Kombucha fermented pollen samples presented higher values in TEAC assay than unfermented pollen, with a maximum reached in the 5th fermentation day (8.16 mg Trolox/g dw) (Table 3). According to Campos et al. (2003), the antioxidant activity of pollen is largely assigned to phenolic compounds and flavonoids, but also, some proteins and vitamins may contribute to this biological activity. The antioxidant capacity results are correlated with the composition of the total phenolic compounds and flavonoids.

Dietary fiber content. Food fibers are a mixture of complex organic substances, including hydrophilic compounds (soluble and insoluble polysaccharides, non-edible oligosaccharides) and more or less hydrophobic compounds, such as cutin, suberin and lignin (Prosky et al., 1992).

The average TDF values for the unfermented pollen samples are around 14.8 g/100 g, in agreement with the findings of Fuenmayor et al. (2014). In Kombucha fermented pollen samples, it was observed that TDF content increased, reaching a maximum value on the 6th day of fermentation (19.75 g/100 g). Also the results showed that polysaccharides (cellulose and callose) constitute the most important fraction of the dietary fiber with IDS ranging between 13 g/100 g for control samples and 17.2 g/100 g for fermented samples (Table 4).

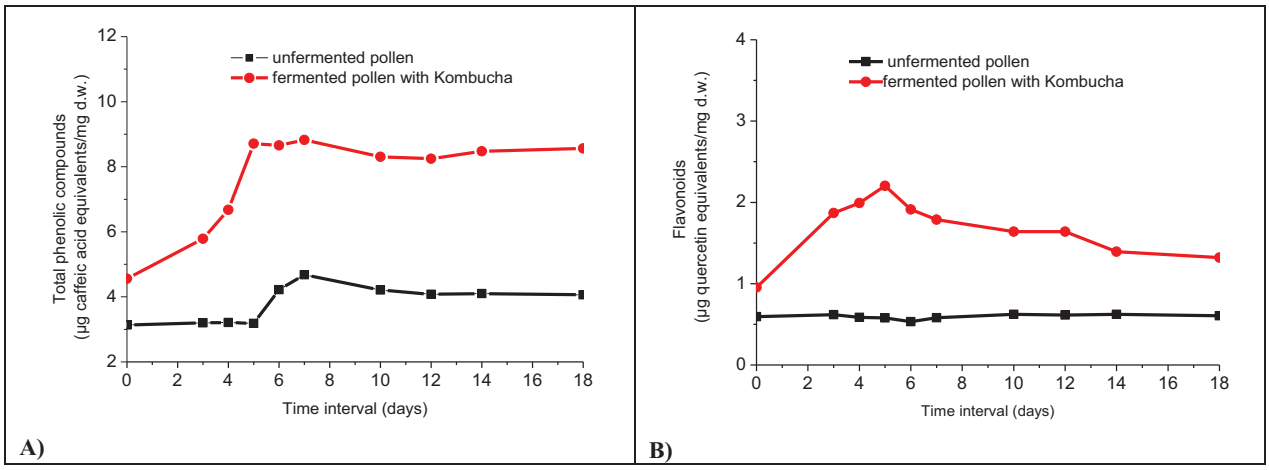


Figure 2. Quantitative determination of (A) total phenolic content expressed as mg caffeic acid equivalent/g dry weight (d.w.) and (B) total flavonoid content expressed as mg quercetin equivalent/g dry weight (d.w.)

Table 3. Antioxidant capacity of samples collected at different fermentation time points

Time intervals (days)	**TEAC** (mg Trolox /g d.w.)		**%DPPH** scavenging activity	
	unfermented pollen (control)	Kombucha pollen fermented	unfermented pollen (control)	Kombucha pollen fermented
0	4.21	4.95	0.91	1.23
3	3.96	6.13	0.92	1.87
4	4.21	6.79	0.92	1.88
5	4.58	**8.16**	0.93	**2.24**
6	4.32	**7.43**	0.96	**2.03**
7	4.14	**7.37**	0.96	**1.99**
10	4.03	7.36	0.89	1.97
12	3.89	7.23	0.87	1.76
14	4.02	7.06	0.83	1.73
18	4.12	6.98	0.81	1.72

Table 4. Dietary fiber content of unfermented and Kombucha fermented pollen

Dietary fibers **g/100 g**	**Unfermented pollen**			**Kombucha fermented pollen**		
	Time intervals **(days)**			Time intervals **(days)**		
	3	**6**	**12**	**3**	**6**	**12**
IDF (insoluble dietary fibers)	12.86	13.02	13.24	13.74	17.19	15.8
SDF (soluble dietary fibers)	2.07	2.14	1.98	2.7	2.67	2.84
TDF (total dietary fibers)	14.67	14.89	15.03	16.31	19.75	18.62

The fermentation process increased the bioavailability of dietary fibers found in pollen grains, suggesting a prebiotic potential.

Adhesion capacity to intestinal cells. Results obtained in the study showed that lactic acid bacteria L5 strain isolated from Kombucha has a good capacity to adhere *in vitro* to the surface of the Caco-2 cellular monolayer. The intestinal absorption by epithelial cells was already seen after 1 h of incubation. The light microscope images revealed a diffuse adhesion pattern (Figure 3) of the bacterial cells.

After 4 h of incubation a considerable increase in the percentage of adhered lactobacilli on the Caco-2 monolayer was observed (Figure 3). Light microscope images showed an enhanced adherence of L5 stain lactobacilli with a diffuse pattern. It is interesting to note that after 4 h the bacterial cells start to form aggregates (Figure 3), suggesting a diffuse-aggregative adherence pattern.

Caco-2 cell line has been used as a model of the absorptive and defensive properties of the intestinal mucosa due to the spontaneous differentiation process that leads to the formation of a monolayer expressing the morphological and functional characteristics of a mature enterocyte (Sambuy et al., 2005; Chauvière et al., 1992). Our results showed that lactobacilli isolated from pollen fermented broth, adhered to intestinal cells in a time depending manner, suggesting a probiotic effect.

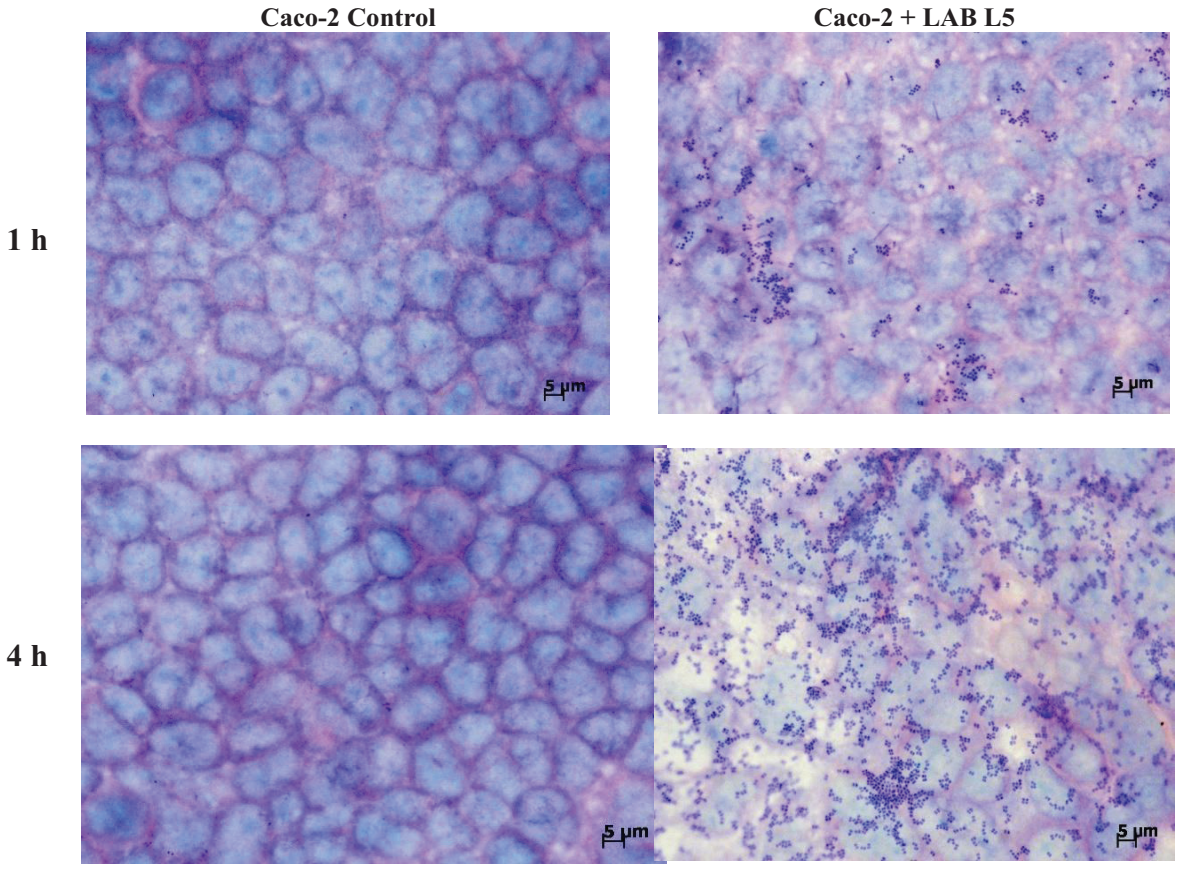


Figure 3. Light microscope images showing the adhesion of L5 lactobacilli strain to Caco-2 cellular monolayer compared with the control (Caco-2 cell culture) using Giemsa staining after 1 h and 4 h of incubation

CONCLUSIONS

Our results confirmed the cleavage of double-layered cell wall of the unfermented and fermented pollen grains, with an increased degree of prebiotic content release for the latter.

Overall, Kombucha fermentation increased the release of pollen prebiotics, such as polyphenols and dietary fibers, suggesting also a probiotic potential of the lactobacilli strain isolated from the SCOBY consortium.

The bioavailability of pollen grains for the human diet is supported by the Kombucha fermentation process.

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