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Utilization of Lignin Monomer Isolation from Black Liquor of Empty Palm Oil Bunches as a Prebiotic

Upotreba izoliranog monomera lignina iz crnog luga dobivenoga od praznih grozdova palmina ploda kao prebiotika

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ABSTRACT • *Waste is generated in the form of black liquor during pulp processing. Furthermore, lignin, the main ingredient of black liquor, contains a phenylpropanoid compound with prebiotic and antimicrobial activity. Also, it is a component of lignocellulose that has prebiotic and antimicrobial activity effects due to its indigestible nature and it comprises phenylpropanoid components. Therefore, this study examines and identifies the lignin purification and test results of its prebiotic activity. The technique used to identify lignin fraction is called Thin Layer Chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GC-MS) plates. Prebiotic activity tests were performed using the calculation of total bacteria on the growth of Lactobacillus casei. The results showed that the purification process using CHCl3, 3 % MeOH:CHCl3, 20 % MeOH:CHCl3, and MeOH yielded 10.68 %, 6.34 %, 11.38 % 44.85 %, respectively. The 3 % MeOH:CHCl3 fraction contained benzaldehyde, 4- hydroxy-3,5-dimethoxy, 1-methylbutyl hexadecanoate, oleic acid, and di-2-ethylhexyl phthalate. The 3 % MeOH:CHCl3 fractionate with a concentration of 15 % also showed a prebiotic activity for L. casei at 6.1 x 102 colonies/mL.*

KEYWORDS: *black liquor; lignin monomer; empty palm oil bunch; prebiotic*

SAŽETAK • *Tijekom prerade pulpe nastaje otpad u obliku crnog luga. Lignin kao glavni sastojak crnog luga sadržava spoj fenilpropanoid prebiotičkoga i antimikrobnog djelovanja. Također, on je i dio lignoceluloze koja zbog svoje neprobavljivosti ima prebiotičko i antimikrobno djelovanje, a sastoji se od fenilpropanoidnih komponenata. Stoga se u ovom istraživanju ispituju i razmatraju pročišćivanje lignina i rezultati prebiotičke aktivnosti. Za identifikaciju frakcija lignina primijenjene su tankoslojna kromatografija (TLC) i plinska kromatografija s masenom spektrometrijom (GC-MS). Ispitivanje prebiotičke aktivnosti provedeno je korištenjem izračuna ukupnog broja bakterija na rast Lactobacillus casei. Rezultati su pokazali da je proces pročišćivanja uz pomoć $CHCl_3$, 3 % $MeOH:CHCl_3$, 20 % $MeOH:CHCl_3$, i MeOH dao prinos od 10,68 %, 6,34 %, 11,38 % i 44,85 %. Frakcija 3 %*

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MeOH:$CHCl_3$ sadržavala je benzaldehid, 4- hidroksi-3,5-dimetoksi, 1-metilbutil heksadekanoat, oleinsku kiselinu i di-2-etilheksil ftalat. Frakcija 3 % MeOH:$CHCl_3$ koncentracije 15 % također je pokazala prebiotičku aktivnost za L. casei pri 6,1 × 10^2 kolonija/mL.

KLJUČNE RIJEČI: *crni lug; monomer lignina; prazni grozdovi palmina ploda; prebiotik*

1 INTRODUCTION
1. UVOD

During pulp processing, liquid waste is generated in black liquor, which has lignin content with a complex structure that is difficult to decompose; thus, it can pollute the environment (Lara *et al.*, 2003). However, the lignin content is approximately 25-35 % of the total black liquor (Lara *et al.*, 2003; Goujon *et al.*, 2003). This implies that it can be isolated for further use. Currently, people want to use black liquor as a source of raw material for lignin (Min *et al.*, 2013), which is further used as biomass (Schorr *et al.*, 2014), adhesives (Ramires *et al.*, 2010; Uraki and Koda, 2015), dispersants, surfactants, antioxidants in plastics and rubbers, dyes, synthetic floors, thermosets, paints, and fuel for road maintenance (Laurichesse and Averous, 2013; Mankar *et al.*, 2012; Magnus and Hakan, 2014; Padkościelna *et al.*, 2017; Baurhoo *et al.*, 2008).

Hidayati *et al.* (2018) isolate lignin from black liquor of empty palm oil bunches by using NaOH at a concentration of 30 % yielded of 5.67 %, with a pH of 5.42, total solid content of black liquor 65.11 %, methyl lignin level of 14.61 %, and equivalent weight 1787.23. Prayuwidayati *et al.* (2016) showed that lignin is used as a prebiotic, which is considered to have an antimicrobial effect by reducing the growth rate of pathogenic bacteria in the cattle rumen. According to Baurhoo *et al.* (2008), the tested, purified lignin product has a prebiotic effect. Furthermore, prebiotic is a beneficial component of human food because it enhances the growth and activity of several bacteria in the colon, thus improving the health of the human digestive tract (Toma and Pokrotnieks, 2006). This study aims to identify the components contained in lignin purification products by TLC, Chromatography Column, and GC-MS as testing the prebiotic activity.

2 MATERIALS AND METHODS
2. MATERIJALI I METODE

2.1 Tools and materials
2.1. Oprema i materijali

The materials used were the Empty Palm Oil Bunches (EPOB) black liquor resulting from formacell pulping, acetic acid, formic acid, HCl, $CuSO_4$, H_2O_2, NaOH, pyridine, MeOH, $CHCl_3$, and equates, as well as NA (Nutrient Agar), NB (Nutrient Broth), media de Man, Rogosa and Sharpe Agar (MRSA), and media de Man, Rogosa and Sharpe Broth (MRSB). Black liquor was obtained from the formacell pulping process using the method used by Hidayati *et al.* (2017). The advantages of formacell pulping compared to other pulping methods are that it is more environmentally friendly, the pulp yield is high, the pulp produced is sulfur free, and it has high brightness and good strength. The pulping process was carried out in a 1.3 liter autoclave with 20 g of EPOB fiber and liquid for a sample ratio of 15:1 flat bottom, and a wide-mouth boiling flask equipped with a condenser. The solvent ratio of acetic acid: formic acid (85:15) and HCl catalyst was given 0.5 % for 1 hour of cooking time at 130 °C. After cooking, the remaining boiled liquid was separated and collected by filtering. The microbial cultures used were *L. casei*. Furthermore, the tools used were oven, Buchner funnel, silica gel 60 F_{254} TLC plate, column chromatography, chamber, autoclave, micropipette, incubator, calipers, capillary pipette, GCMS (Varian/CP- 3800 GC and Saturn 2200 MS), and supporting glasses.

2.2 Study method
2.2. Ispitne metode

The study was conducted with a Completely Randomized Design (CRD) in which black liquor was extracted until lignin was obtained twice. Subsequently, lignin was fractionated until a monomer fraction was obtained. TLC analysis identified each lignin monomer fraction, and the prebiotic activity was screened for *L. casei*. The fraction with the highest prebiotic activity was analyzed using GC-MS to determine the chemical composition. Furthermore, they were generated at several concentrations of 0 %, 2.5 %, 5 %, 7.5 %, 10 %, 12.5 %, and 15 %, and they were to be tested for antimicrobial and prebiotic activity for three replications. The data obtained were analyzed descriptively and presented in figures and tables.

2.3 Study implementation
2.3. Provedba ispitivanja
2.3.1 Lignin degradation
2.3.1. Degradacija lignina

The lignin obtained from black liquor due to the pulp cooking with the raw material of EPOB was precipitated and degraded using $CuSO_4$, pyridine, and H_2O_2. A total of 2 g of lignin precipitate was dissolved with 20 mL NaOH solution to a pH of 12. Afterward, a total of 25 mL of 10^{-2} M $CuSO_4$ and 5 mL pyridine were added to the lignin solution, and then it was stirred with a magnetic stirrer for 30 minutes. 10 ml of

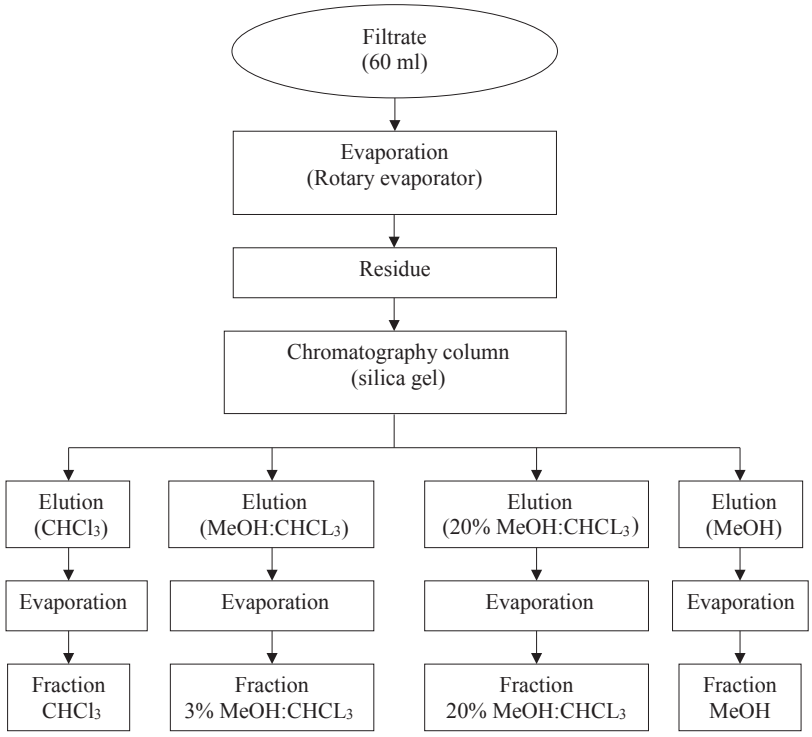


Figure 1 Lignin monomer fractionation flow diagram
Slika 1. Dijagram toka frakcioniranja monomera lignina

H_2O_2 1 M was also added five times for 30 minutes, stirred, and stored in an unlighted room for 72 hours.

2.3.2 Lignin purification faction
2.3.2. Pročišćivanje frakcija lignina

The fractionated filtrate was evaporated until a residue was obtained and dissolved in 500 mL H_2O. After that, extraction was performed with EtOAc of 500 mL for three times to obtain H_2O and EtOAc layers that were evaporated until a residue was obtained. The residue was then put into the chromatographic column silica gel and eluted with $CHCl_3$ (1L) solution. Furthermore, the $CHCl_3$ fraction was then evaporated, and the obtained residue was purified by preparative thin-layer chromatography (PTLC). The lignin monomer isolation process can be seen in Figure 1.

2.3.3 Lignin fraction identification
2.3.3. Identifikacija frakcija lignina

Identification of lignin monomers obtained was made using TLC and GC-MS plates. Subsequently, the isolates were dissolved in hexane and injected into GC-MS devices. The analysis was performed using GC-MS CP-3800 variant with MS Saturn 2200 detector, and VF-5 ms column 30 mm × 0.25 mm by manual injection method at 240 °C for 40 minutes (Suroso *et al*., 2018).

2.4 Prebiotic activity test for lignin fraction
2.4. Ispitivanje prebiotičke aktivnosti frakcija lignina

Prebiotic tests were performed microbiologically using *L. casei* grown on MRSA media, and the measured variable was the number of bacteria counted using the living bacterial colonies method. The lignin monomer fraction was composed of media such that each cup was 0 %, 2.5 %, 5 %, 7.5 %, 10 %, 12.5 %, and 15 %. *L. casei* that had been refreshed on MRSB media was diluted as much as 0.05 mL into 4.95 mL of physiological solution. Then, 1 mL of physiological solution was inoculated into MRSA media, and it was poured into 20 petri cups of 19.5, 19, 18.5, 18, 17.5, and 17 mL and 3 % MeOH:$CHCl_3$ fraction of 0, 0.5, 1, 1,5, 2, 2.5, and 3 mL were added. Furthermore, the bacteria were incubated for 24 hours in an incubator. The number of bacteria was calculated by counting the living bacterial colonies using the following method of Ogimoto and Imai (1981).

$$Microbe\ population = \frac{Colony\ account\ (kol)}{0.05\ x\ 10^{x}\ x\ 0.1\ (ml)} \quad (1)$$

Note: x: tube xth retail series

3 RESULTS AND DISCUSSION
3. REZULTATI I RASPRAVA

3.1 Lignin isolation
3.1. Izoliranje lignina

The black liquor produced by formacell pulping was then isolated to obtain lignin isolates. Furthermore, the extraction process of lignin from black liquor was performed by adding water to 500 mL of the sample in a 2 L glass beaker and allowed to form a precipitate which was then separated by filtering and dried for three days at room temperature; afterwards, it was observed for its characteristics (Table 1).

Table 1 Characteristics of isolated lignin
Tablica 1. Svojstva izoliranog lignina

Parameter / *Parametar*	**Characteristic** / *Svojstvo*
Color / *boja*	Black / *crna*
Form / *konzistencija*	Solid / *čvrsta*
pH (25 °C)	4.5
Yield / *prinos*	1.74%
Water content / *sadržaj vode*	0.24%

Lignin isolates obtained have a black appearance, and they are solids in the form of fiber crumbs. They have a moisture content of 0.24 %. The yield of lignin obtained is 1.74 %, and this is presumed because there was no addition of acid to the precipitation process.

3.2 Lignin monomer fraction screening for *L. casei*
3.2. Probir frakcije monomera lignina za *L. casei*

The process of lignin degradation was performed by dissolving it into a solution of $CuSO_4$, pyridine, and NaOH, after which it was stirred and H_2O_2 was added. The obtained fraction was then added to the chromatographic column silica and eluted with $CHCl_3$, 3 % MeOH: $CHCl_3$, 20 % MeOH: $CHCl_3$, and MeOH, respectively. Then, each fraction was dried. The fraction of $CHCl_3$, 3 % MeOH:$CHCl_3$, 20% MeOH:$CHCl_3$, and MeOH yielded 10.68 %, 6.34 %, 11.38 %, and 44.85 %, respectively. The results of each fraction were identified on a TLC plate using UV light to examine the content of chemical compounds qualitatively.

Black spots on the TLC showed the presence of chemical compounds. Furthermore, the spot on TLC of each fraction consists of different compounds based on their retention time. For example, the spots generated at fractions of $CHCl_3$, 3 % MeOH: $CHCl_3$, 20 % MeOH: $CHCl_3$, and MeOH sequentially produced 4, 4, 2, and 1 spot, which was considered to be the dominant compound in each lignin fraction. Figure 2 and Table 2 show the black spot on TLC and the screening results for *L. casei* growth, respectively (Table 2).

Table 2 Screening lignin fraction as a prebiotic for *L casei*
Tablica 2. Probir frakcije monomera lignina za *L. casei*

Fraction *Frakcija*	**Number of microbes, 10^2colony/mL** *Broj mikroba,* 10^2 *kolonija*/mL
$CHCl_3$	1.48 ± 0.29
3 % MeOH:$CHCl_3$	4.52 ± 0.10
20 % MeOH:$CHCl_3$	2.58 ± 0.23
MeOH	2.41 ± 0.34

The 3 % MeOH:$CHCl_3$ fraction showed higher prebiotic activity than other fractions. It showed prebiotic activity against *L casei* at 4.52 x 10^2 colonies/mL, while the $CHCl_3$ fraction, 20% MeOH:$CHCl_3$, and MeOH showed prebiotic activity against *L. casei* at 1.48 x 10^2 colonies/mL, 2.58 × 10^2 colonies/mL, and 2.41 × 10^2 colonies/mL, respectively.

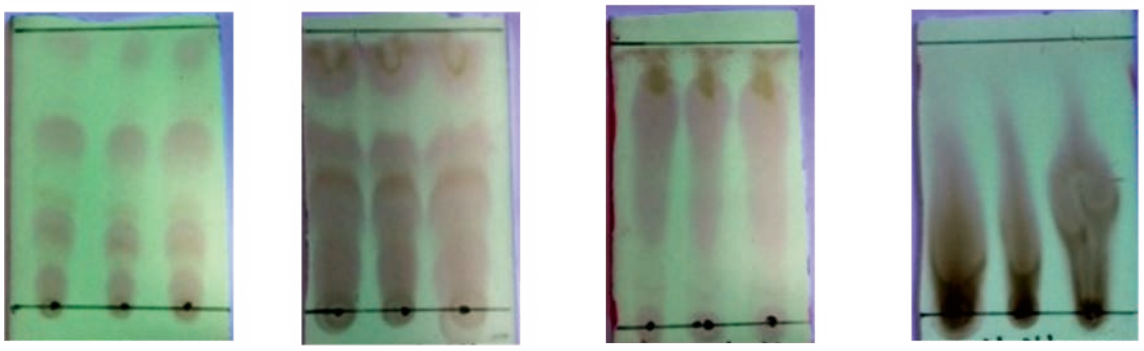
3.3 Identification of 3 % MeOH:$CHCl_3$ fraction compound content
3.3. Identifikacija spojeva u frakciji 3 % MeOH:$CHCl_3$

The identification process was performed by injecting a 3 % MeOH:$CHCl_3$ fraction into the GC-MS (Figure 3).

The 3 % MeOH:$CHCl_3$ fraction contains (a) benzaldehyde, 4-hydroxy-3,5-dimethoxy- 2.76 %, (b) 1-methylbutyl hexadecanoate as much as 41.03 %, (c) oleic acid as much as 3.61 %, and (d) di-2-ethylhexyl phthalate as much as 31.25 %. Furthermore, the main product of the hydrolysis of lignin using NaOH catalysts is monomeric phenols and oligomers (Roberts *et al*., 2011). Figure 4 and Table 3 show the compound structure of the 3 % MeOH:$CHCl_3$ fraction and the identification results, respectively.

3.4 Prebiotic activity of 3 % MeOH:$CHCl_3$ fraction against *L. casei*
3.4. Prebiotičko djelovanje frakcije 3 % MeOH:$CHCl_3$ na *L. casei*

Prebiotic activity test uses a concentration between growth media with a fraction of 3% methanol-chloroform of 0, 2.5, 5, 7.5, 10, 12.5, and 15 (%). The



a b c d

Figure 2 Chromatographic profile of thin layers of each fraction: a) $CHCl_3$; b) 3 % MeOH:$CHCl_3$; c) 20 % MeOH:$CHCl_3$; d) MeOH
Slika 2. Kromatografski profil tankih slojeva pojedine frakcije: a) $CHCl_3$; b) 3 % MeOH:$CHCl_3$; c) 20 % MeOH:$CHCl_3$; d) MeOH

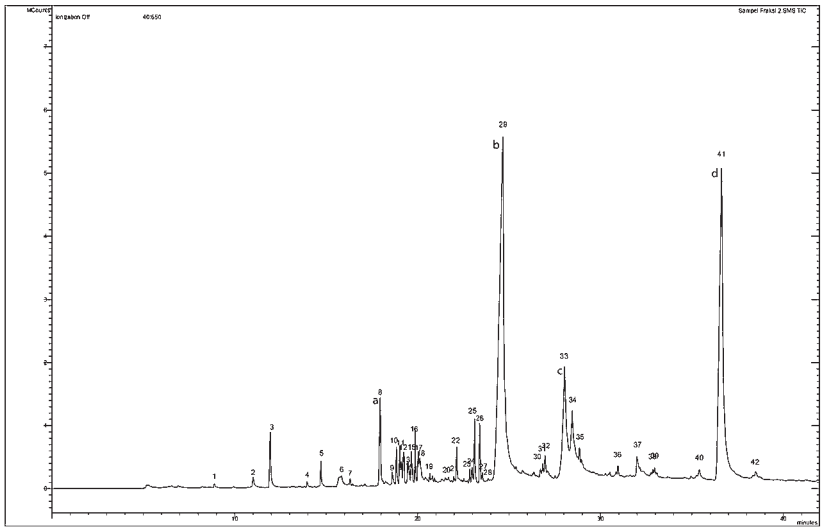


Figure 3 Fraction chromatogram of 3 % MeOH:$CHCl_3$: a) benzaldehyde, 4-hydroxy-3,5-dimethoxy; b) 1-methylbutyl hexadecanoate; c) oleic acid; d) di-2-ethylhexyl phthalate
Slika 3. Kromatogram frakcije 3 % MeOH:$CHCl_3$: a) benzaldehid, 4-hidroksi-3,5-dimetoksi; b) 1-metilbutil heksadekanoat; c) oleinska kiselina; d) di-2-etilheksil ftalat

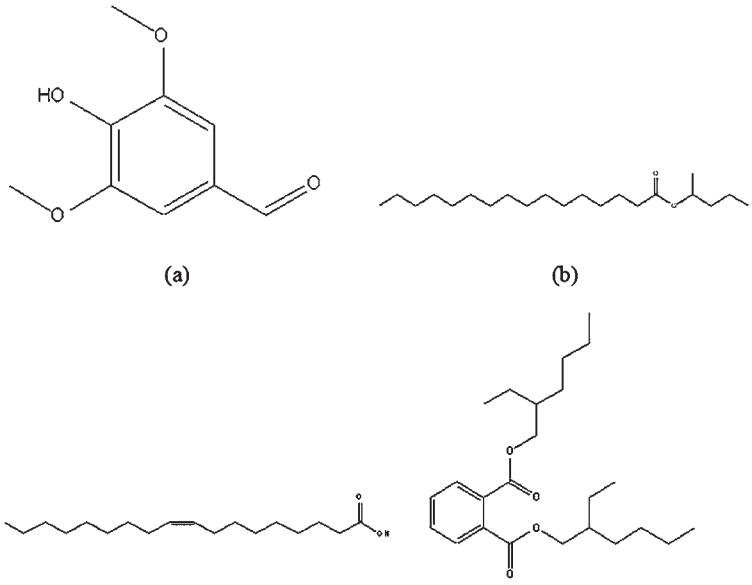


Figure 4 Fraction compound of 3% MeOH:$CHCl_3$ includes: a) benzaldehyde, 4-hydroxy-3,5-dimethoxy; b) 1-methylbutyl hexadecanoate; c) oleic acid and d) di-2-ethylhexyl phthalate
Slika 4. Spojevi u frakciji 3 % MeOH:$CHCl_3$ uključuju: a) benzaldehid, 4- hidroksi-3,5-dimetoksi; b) 1-metilbutil heksadekanoat; c) oleinsku kiselinu i d) di-2-etilheksil ftalat

number of colonies from the growth of *L. casei* for each concentration of 3 % MeOH:$CHCl_3$ fraction is shown in Figure 5.

The 3 % MeOH:$CHCl_3$ fraction at a concentration of 15 % showed the highest prebiotic activity of 6.10 x 102 colonies/mL against *L. casei*. In comparison, the 3 % MeOH:$CHCl_3$ fraction at a concentration of 2.5 % showed the lowest prebiotic activity of 2, 69 × 10^2 colonies/mL against *L. casei*. The prebiotic activity of the 3 % MeOH:$CHCl_3$ fraction on the growth of *L. casei* is shown in Figure 6.

The results of the analysis of the prebiotic activity of the 3 % MeOH:$CHCl_3$ fraction showed that the higher the concentration of the growth medium, the higher the growth of *L. casei*. Baurhoo *et al.* (2008) stated that the lignin monomer in the alcell process acts as a prebiotic. Prayuwidayati *et al*. (2016) reported that formacell purification products are generated from EPOB and oligomers, which are phenolic compounds that stimulate the growth of lactic acid bacteria. This shows that the isolated fraction of lignin and its derivatives can replace glucose in MRSA medium, and it is used as an energy source for the growth of *L. casei*. These also showed that the isolated lignin fraction could be used as a feed supplement and support the growth of *L. casei*. Lignin in the purified alcell

Table 3 Identification of lignin fraction monomer compounds of 3 % MeOH:$CHCl_3$
Tablica 3. Identifikacija spojeva u frakciji 3 % MeOH:$CHCl_3$ monomera lignina

Retention time *Vrijeme retencije*	**Molecule weight** *Molekulska masa*	**Compound** / Spoj	**%**
8.886	207	Phenol.2-(1-methylpropyl)-.methylcarbamate	0.12
11.008	212	Propanoic acid.3-chloro-.4-formylphenyl ester	0.38
11.942	152	Vanillin	1.96
13.958	166	Ethanone.1-(4-hydroxy-3-methoxyphenyl)-	0.15
14.709	166	Undecanoic acid.10-methyl-.methyl ester	0.47
15.839	214	Benzoic acid.4-hydroxy-3-methoxy-	1.04
16.313	168	Diethyl phthalate	0.13
17.952	222	Benzaldehyde.4-hydroxy-3.5-dimethoxy-	2.76
18.621	182	p-Anisic acid.4-nitrophenyl ester	0.32
18.838	273	p-Anisic acid.3.4-dichlorophenyl ester	0.90
19.022	413	Carbamic acid.N-[1.1- bis(trifluoromethyl)ethyl]-4.(1.1.3.3-tetra-methylbutyl)phenyl ester	0.95
19.114	220	4-Methyl-2-tert-octylphenol	0.54
19.235	296	m-Anisic acid.3.4-dichlorophenyl ester	1.10
19.447	270	Hexestrol	0.58
19.643	220	Phenol.2-methyl-4-(1.1.3.3- tetramethylbutyl)-	0.33
19.708	192	1.3-Dimethyl-5-ethyladamantane	0.74
19.870	413	Carbamic acid.N-[1.1- Bis (trifluoromethyl)ethyl]-4.(1.1.3.3- tetra-methylbutyl)phenyl ester	1.06
20.034	220	Phenol.2-methyl-4-(1.1.3.3- tetramethylbutyl)-	0.71
20.126	228	Tetradecanoic acid	1.17
21.690	268	2-Pentadecanone.6.10.14-trimethyl-	0.07
21.980	194	Caffeine	0.12
22.135	278	1.2-Benzenedicarboxylic acid.bis(2- methylpropyl) ester	0.70
22.520	338	Erucic acid	0.09
22.848	604	Tritetracontane	0.28
22.968	268	9-Hexadecenoic acid. methyl ester.(Z)-	0.41
23.116	276	7.9 Di-tert-butyl-1-oxaspiro(4.5)deca-6.9-diene-2.8 dione	1.60
23.395	270	Pentadecanoic acid.14-methyl-.methyl ester	1.37
23.358	292	Benzenepropanoic acid.3.5-bis(1.1-dimethylethyl)-4 hydoxy . methyl ester	0.20
24.659	326	1-Methylbutyl hexadecanoate	41.03
26.713	298	1-Eicosanol	0.23
26.837	294	9.12 Octadecadienoic acid (Z.Z)-.methyl ester	0.32
26.971	352	9.12.15 Octadecatrienoic acid.2.3 dihydroxypropylester.(Z.Z.Z)-	0.58
28.036	282	Oleic acid	0.89
28.453	282	Oleic acid	3.61
28.853	282	Oleic acid	0.33
30.953	604	Tritetracontane	0.21
31.997	324	4.8.12.16-tetramethylheptadecan-4-olide	0.57
32.860	298	1-Eicosanol	0.11
32.963	604	Tritetracontane	0.16
35.399	242	1-decanol.2-hexyl-	0.25
36.624	390	Di-2-ethylhexyl phthalate	31.25
38.491	592	1-Hentetracontanol	0.21

process showed a prebiotic effect on chickens, promoting the growth of beneficial bacteria and improving intestinal morphological structure, as measured by an increase in the number of villi and goblet cells (Baurhoo *et al.*, 2008). Cotta and Whitefield (1998) stated that all hemicellulolytic rumen bacteria could utilize xylooligosaccharides as growth substrates. Therefore, increased digestibility of crude protein and fiber supports ruminant protein and energy metabolism. The non-digestible carbohydrate prebiotics includes lactulose, inulin, resistant starch, and a number of oligosaccharides, a source of carbohydrates for beneficial bacteria in the digestive tract (Crittenden, 1999). Substrates such as inulin, fructooligosaccharides (FOS), and mannan-oligosaccharides (MOS), derived from yeast cells, absorbed the host and were hydrolyzed by endogenous digestive enzymes. The possible mechanism is a decrease in pH due to the

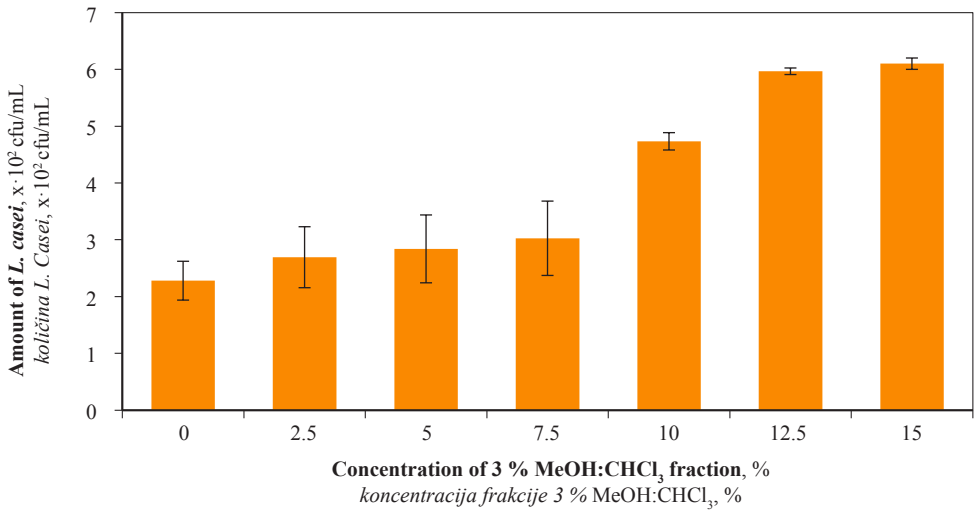


Figure 5 Effect of several concentrations of 3 % MeOH:$CHCl_3$ fraction as a prebiotic on the number of *L. casei* microbes
Slika 5. Utjecaj nekoliko koncentracija frakcije 3 % MeOH:$CHCl_3$ na broj mikroba *L. casei*

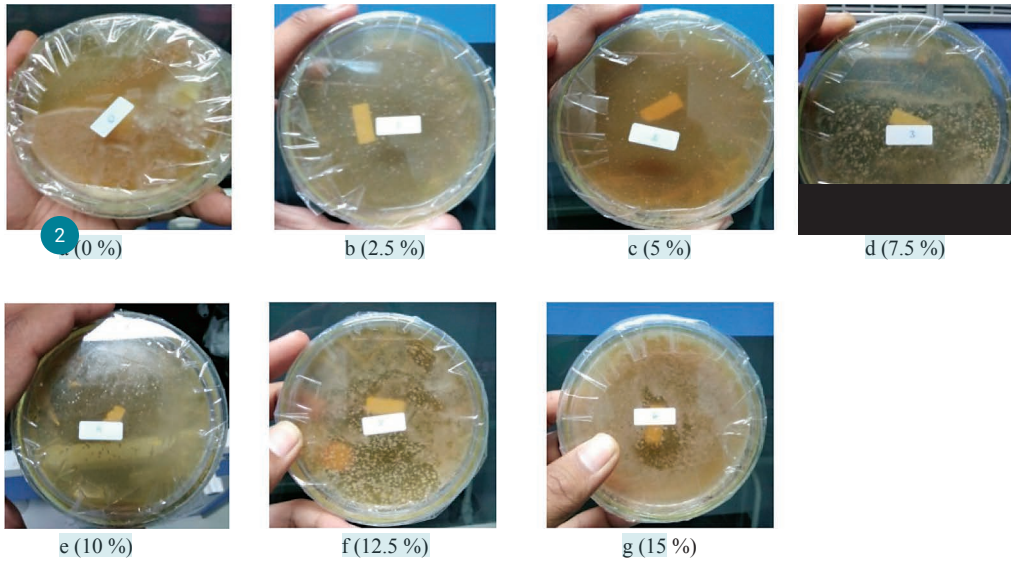


Figure 6 Prebiotic activity of 3 % MeOH:$CHCl_3$ fraction on the growth of *L. casei*
Slika 6. Utjecaj prebiotičke aktivnosti frakcije 3 % MeOH:$CHCl_3$ na rast *L. casei*

production of short-chain fatty acids, bacteriocin secretion, and immune stimulation.

Meanwhile, MOS as a prebiotic has a different mechanism that does not cause an increase in the population of beneficial bacteria, due to its ability to attach to mannose-specific lectins of G-negative type 1 fimbriae pathogens such as Salmonella and *E. coli,* which is excreted from the digestive tract (Toma and Prokotnieks, 2006; Crittenden, 1999). Similar to the effect of MOS and antibiotic-free diet supplemented with 1.25 % alcell lignin (LL) on Lactobacilli and Bifidobacteria, lignin at low levels is potentially classified as a prebiotic (Baurhoo *et al*., 2008). The results showed that using lignin in the alcell process (12.5 g) increases the concentration of intestinal Lactobacilli and Bifidobacteria in broilers (Baurhoo *et al*., 2008). In addition, the proliferation of gastrointestinal microbiota populations such as Lactobacilli and Bifidobacteria was increased through the consumption of prebiotics. The increase in the number of microbiotic in the feces ranges from 10-100 times (Thomas *et al*., 2004).

4 CONCLUSIONS
4. ZAKLJUČAK

Lignin, the main ingredient of black liquor generated from bunches of empty oil palm fruit, contains benzaldehyde, 4-hydroxy-3,5-dimethoxy, 1-methtylbutyl hexadecanoate, oleic acid, and di-2-ethylhexyl phthalate. Furthermore, its fraction of 3 % MeOH:$CHCl_3$ with a concentration of 15 % showed prebiotic activity against *L. casei* of 6.1 × 10^2 colonies/m. These results indicated that the lignin monomer is potentially prebiotic.

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