Biological Environment and Pollution
Vol. 1(2) 2021 | 67 - 75
ISSN 2798-2378
ISSN 2798-2777

Methane reduction from ruminant microbiota of sheep using polyphenols extract of different olive mill wastewater

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ARTICLE INFO

Article history
Submission
May 11, 2021
Revision
July 28, 2021
Accepted
August 20, 2021

Keyword
Methane
Olive mill wastewater
Polyphenols
Ammonia
Protozoa

ABSTRACT

Olive oil production generates considerable quantities of olive mill wastewater, a powerful pollutant dumped in nature without any prior treatment. Olive mill wastewater is considered as a potential source of natural products of high additive value, due to their content of phenolic compounds and other natural antioxidants. This study aims to investigate the impact of phenolic substances, extracted from different olive mill wastewater of different olive varieties, on the composition of the ruminal microbiota of sheep. The results of the quantification of phenolic compounds show the richness of the three varieties (Sigoise, Azzeradj and Chemlal) in polyphenols with respective values of 26.3, 23.97 and 20.09 g/L. Moreover, a stimulation in the fermentative activity was reported, which caused a significant reduction in methane production (24 hours of incubation) of 7.31, 39.36 and 30.06% for Sigoise, Azzeradj and Chemlal, respectively. In addition, this decrease creates a reduction in the production of ammonia and the number of protozoa.

Conflict of interest: The authors declare that they have no conflicts of interest.

Introduction

In recent decades, the world's olive oil crop has increased dramatically. Knowing that the edible olive was cultivated as early as 3500 B.C. Where it is mainly concentrated in the Mediterranean countries which hold about 95% of the world's production, including 11.62% for Maghreb countries in 2020[1]. Like all agro-industries, the extraction process requires large quantities of water, and therefore this industry generates large quantities of liquid effluents named olive mill wastewater (OMW). Only press or 3-phase centrifuge systems generate OMW. Although these two processes are less environmentally friendly, they are still widely used, especially around the Mediterranean area where they lead to the production of large volumes of this effluent, estimated at 3×10^7 m^3 per year[2].

OMW is considered to be one of the most harmful effluents produced by the food industry due to their pollutant load and their toxicity towards ecosystems (plants, microorganisms and aquatic and aerial organisms), also due to their acidic pH, and their richness in organic matter, especially polyphenols[3,4]. Studies have identified more than fifty phenolic in OMW such as cinnamic acid, p-coumaric acid, caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid, vanillic acid, gallic acid, syringic acid, sinapic acid, homovanillic acid, 4-hydroxyphenyl acetic acid, etc[5]. Generally, the OMW composition depend on several parameters like variety and maturity of olive fruits (Chemlal, Azzeradj, etc.), type of soil, method extraction (presse or 3-phase), and pesticides and fertilizers used[1]. Therefore, numerous studies have been conducted to detoxify or valorise this effluent by using different physical, chemical and biological techniques[6-9]. However, most of these methods are limited because they are either are either too costly to be widely applied or inefficient to meet stringent effluent standards and could generate huge amounts of sludge that are even more difficult to manage[10].

Hence, a particular attention was given to polyphenols of OMW as highly valorisable bioactive products, which can be used in the synthesis of nanoparticles[11]. Also as a potent antibacterial agents and as effective candidate to reduce the production of methane (CH_4) from ruminants, which is a major source of atmospheric methane with an estimated contribution of 16%. It should be noted that methane is a powerful greenhouse gas. It has 28 times the warming power of that CO_2[12].

In attempt to valorise the polyphenols of OMW, a possibility to add different phenolic compounds from different varieties such as Azzeradj, Chemlal and Sigoise to ruminal microbiota of sheep on the gas emission, methane production, and the composition of microbiota were studied.

Method

Samples

The olive mill wastewaters were collected from olive mill located in the East and North-East of Algeria (Batna, Guelma and Skikda). The effluents were derived from the extraction of oil from olives of different varieties (Azzeradj, Chemlal and Sigoise). Each of these samples is taken from two types of oil mills: traditional and industrial (three phases). The samples were stored at -20°C and defrosted before use.

Quantification of phenolic compounds

After ethyl acetate extraction using Hamimed method[11]. The extracts were subjected to various methods of polyphenols determination. Makkar used to determine the total of polyphenols using Folin ciocalteu colorimetric technique and results were expressed as gallic acid equivalent[13]. The condensed tannins were measured by n-butanol-HCl method described by Porter et al., and results were expressed to quebracho as equivalent[14]. The total flavonoids were determined using Ayoola method and the concentrations were expressed to quercetin as equivalent[15].

Fermentation procedure

The *in vitro* gas production technique, developed by Menke, is a simulation of digestion in the rumen[16]. The fermentation was carried out in polypropylene syringes of 60 mL under anaerobic conditions. The fermentation medium was obtained by mixing one volume of filtered rumen juice, obtained from slaughterhouses, with two volumes of artificial saliva constituted of 0.12 mL of solution A ($CaCl_2.2H_2O$, 13.2 g; $MnCl_2.4H_2O$, 10g; $CoCl_2.6H_2O$, 1g; $FeCl_3.6H_2O$, 0.8 g), 240 mL of solution B ($NaHCO_2$, 39 g), 240 mL of solution C ($Na_2HPO_4.12H_2O$, 5.7 g; KH_2PO_4, 6.2g; $MgSO_4.7H_2O$, 0.6 g), 1.22 mL of solution D (Résazurine ($C_{12}H_6NO_4$), 100 mg), and 95 mL of solution E ($Na_2S.9H_2O$, 0.625 g; NaOH (1N), 4 mL).

200 mg of fed was introduced into each syringe, at the same time, 100µL of each phenolic extract was added to each syringe. All syringes were then inoculated with 30 mL of fermentation medium. In parallel, syringes containing 200 mg of fed (without extract) and syringes without fed (rumen juice + artificial saliva) were prepared for use as blank. The syringes were incubated horizontally in a water bath with rotary agitation (type GFL 1083) at 39°C for 48 hours. Fermentation kinetics were monitored by measuring the total volume of gas produced during fermentation. For each experiment, six replicates were performed.

Analytical methods

The gas production was measured at different times: 3, 6, 9, 24, and 48 hours according to the following equation:

$$GP = V_t - V_0 - V_b \quad (1)$$

GP (mL): Net gas production at each incubation time ;

Vt (mL): Volume of gas recorded at each incubation time;

V0 (mL): Volume of gas recorded at time t_0;

Vb (mL): Average volume of gas recorded in the control syringes.

Subsequently, the gas volumes at each incubation time were treated by the exponential model proposed by Orskov and Mc Donald[17]

$$Y = a + b\left(1 - e^{-ct}\right) \quad (2)$$

Y (mL): Gas production after each incubation time;

a (mL): Gas production from the soluble fraction;

b (mL): Gas production from the insoluble fraction;

a + b (mL): Potential gas production;

c (%/h): Rate of gas production.

The volumes of carbon dioxide and methane produced (CO_2, CH_4) were determined chemically using (NaOH, 4 mL, 10N) according to the procedure described by Jouany[18]. In addition, The pH of the medium was measured using the Nahita pH Meter Model 903.

The Ammonia ($N\text{-}NH_3$) was determined according to the method of Chaney and Marbach and concentrations were expressed to ammonium sulphate $(NH_4)2SO_4$ as equivalent[19].

After 24 hours of fermentation, a volume of 100 µL of the contents of each syringe was mixed with 100 µL of the MFS solution (methylgreen"0.6 g"-formalin"35%"-saline"8 g"). The treated samples were incubated for 30 minutes in the dark. Enumeration of protozoa was performed on a Malassez cell[20].

Statistical analysis

The experimental data are statistically processed by SPSS version 25 software and subjected to a one and two factor analysis of variance ANOVA (nature of treatment and OMW variety). The differences are considered significant at the 5% level. Means are classified according to Dunett's classification and Fisher's LSD at (α=5%). The calculation of the kinetic parameters is carried out by the DEG and Excel computer software.

Results and Discussion

1. Evaluation of polyphenols content in OMW

The results of the determination of phenolic compounds from three varieties of OMW are showed in Table 1. Results indicated that the extraction process (pressed and 3 phases) does not affect the concentrations of total phenols, total flavonoids, and condensed tannins of the the varieties Azzeradj, Chemlal and Sigoise (P > 5%). For both pressing processes, the Sigoise variety has the highest total phenol content (26.3 g/L), followed by the Azzeradj and Chemlal varieties (23.97 and 20.09 g/L, respectively). Regarding total flavonoids, the three varieties have statistically similar concentrations (P > 5%), with values of 0.019, 0.016 and 0.017 g/L for the three varieties of Sigoise, Chemlal and Azzeradj respectively. For condensed tannin content, a highly significant difference is noted between the three varieties (P < 0.0001). The highest concentration is observed for the Sigoise variety (5.29 g/L) and the lowest for the Chemlal variety (3.93 g/L). This variability in the concentrations of the different phenolic compounds of the three varieties depends on several factors represented essentially by the extraction process (technological processes)[21-23]. It may also depend on the difference in variety between samples, the degree of ripeness of the fruit, the harvesting period, and the storage time (duration of storage before extraction)[1]. A notable difference exists in the composition between the three varieties, which are attributed to a series of chemical and enzymatic changes of some phenols during oil extraction[24].

Table 1. Phenolic content (g/L) of olive mill wastewater from the Azzeradj, Chemlal and Sigoise varieties pressed by traditional method and three phases.

	Traditional mode			**3 phases mode**		
	Azzeradj	**Chemlal**	**Sigoise**	**Azzeradj**	**Chemlal**	**Sigoise**
PT	24.65^{a}	19.83^{a}	28.50^{a}	23.29^{a}	20.35^{a}	24.1^{a}
FT	0.016^{a}	0.017^{ab}	0.022^{b}	0.018^{ab}	0.015^{a}	0.016^{a}
CT	5.79^{a}	2.91^{c}	5.31^{ab}	4.67^{b}	4.95^{b}	5.28^{b}

PT: total phenols, FT: total flavonoids, CT: condensed tannins, a,b,c: means in the same column with different letters are significantly different ($P < 0.05$)

2. Influence of phenolic content on the fermentation process of the ruminal microbiota

2.1 Volume of gas produced

The impact of phenolic content on gas production during *in vitro* fermentation of fed is shown in Table 2. The addition of phenolic content extracted from the OMW of the three varieties to the fermentation medium stimulated gas production at different incubation times (3h to 24h) compared to the control. After 24 hours of incubation, this increase, although not significantly different from the control (without addition), is respectively 20.17, 17.42 and 6.41% for the varieties Chemlal, Sigoise and Azzeradj from the three-phase process. A different trend is observed for the traditional process where the increase in gas production is only noted for the Chemlal variety (33.03%) ($P < 0.05$). These results could be explained by the capacity of the ruminal microbiota in the degradation of phenolic compounds. According to McSweeney et al., certain rumen microorganisms, notably *Sterptococus*, possess tannases that are capable of degrading hydrolysable tannins[25]. These enzymes are involved in the hydrolysis of the ester bonds of hydrolysable tannins by producing gallic acid and glucose.

Table 2. Impact of phenolic content extracted from olive mill wastewater of Azzeradj, Chemlal and Sigoise varieties on *in vitro* cumulative ruminal gas production.

	Control	**Traditional mode**			**3 phases mode**		
		Azzeradj	**Chemlal**	**Sigoise**	**Azzeradj**	**Chemlal**	**Sigoise**
3h	$\mathbf{7.66^{a}}$	7.66^{a}	9^{a}	8.33^{a}	6.33^{a}	12^{a}	9^{a}
6h	$\mathbf{12.66^{a}}$	11.33^{a}	14.66^{a}	14.33^{a}	9.66^{a}	20^{a}	14^{a}
9h	$\mathbf{18.33^{a}}$	15.66^{a}	18.33^{a}	19.33^{a}	13.33^{a}	26.33^{a}	17.33^{a}
24h	$\mathbf{36.33^{a}}$	38.66^{a}	43.66^{a}	42.66^{a}	33.66^{a}	48.33^{a}	36.66^{a}
8h	$\mathbf{46.33^{c}}$	61^{ab}	69^{b}	69^{b}	54.33^{bc}	75^{a}	72.33^{b}

a,b,c: means in the same column with different letters are significantly different ($P < 0.05$).

At the end of fermentation (after 24 hours of incubation), the addition of phenolic extracts caused a slight and insignificant decrease for the Azzeradj and Sigoise varieties, which could be explained by a slowdown in the degradation of phenolic compounds. This could be attributed to the depletion of biodegradable phenolic compounds in the culture medium and/or their bioconversion to stable, non-biodegradable phenolic compounds[11]. In contrast, the increase in the Chemlal variety can be explained by the methanogenic effect produced by the condensed tannins (CT), which depends on the concentration and structure of the existing CT in the extract[26].

2.2 Kinetic parameters modelled at *in vitro* gas production

The kinetic parameters of the *in vitro* fermentation of fed supplemented with different phenolic extracts are given in Table 3. Generally, it was found that the addition of phenolic extracts of OMW of the varieties Azzeradj, Chemlal and Sigoise, pressed by traditional or industrial process, significantly improves the degradation of the soluble fraction (a) of fed. The improvement in gas production from the soluble fraction exceeds 100%. The highest values are recorded for the Chemlal and Sigoise varieties respectively in the case of the traditional method. The increase in gas production from fraction (a) probably results from ruminal metabolisation of phenolic compounds and glucose from the degradation of hydrolysable tannins[27]. The negative value of (a) for the control would be the consequence of a latency phase during which the micro-organisms attach and colonise the food particles before their possible degradation[28].

Table 3. Impact of phenolic content extracted from olive mill wastewater of Azzeradj, Chemlal and Sigoise varieties on *in vitro* modelled kinetic parameters of ruminal gas production.

	Traditional mode				3 phases mode		
	Control	Azzeradj	Chemlal	Sigoise	Azzeradj	Chemlal	Sigoise
a (mL)	-0.09^{c}	0.91^{b}	1.17^{b}	1.04^{b}	0.0049^{bc}	5.28^{a}	3.84^{a}
b (mL)	51.13^{b}	99.09^{a}	98.83^{a}	98.96^{a}	96.53^{a}	94.72^{a}	80.34^{ab}
c(%/h)	5.05^{a}	1.95^{a}	2.36^{a}	2.35^{a}	0.503^{a}	2.68^{a}	2.16^{a}

a : Gas production from the soluble fraction, b : Gas production from the insoluble fraction, c : gas production rate from b, a,b,c: means in the same column with different letters are significantly different ($P < 0.05$).

2.3. Methane production

The influence of phenolic extracts on the fermentative profile of fed, assessed by monitoring the kinetics of methane (CH_4) production during fermentation, is illustrated in Table 4. The addition of phenolic content obtained, independently of the crushing mode, from the OMW of the three varieties induced a significant decrease in methane production ($P < 0.05$). This decrease is significant at different incubation times, except for the Sigoise variety for the first 6 hours and after 24 hours of incubation ($P > 0.05$). The reduction rates in methane production are respectively 12.32, 45.64 and 48.45% for the varieties Sigoise, Azzeradj and Chemlal pressed by sedimentation mode. The same trend is observed for the three phases method where the reduction rates are respectively 33.08, 11.67 and 2.30% for the varieties Azzeradj, Chemlal and Sigoise.

The increase in CH_4 production, observed at the beginning of fermentation for some extracts, is probably due to the degradation of simple phenolic compounds. Indeed, some studies have been showed that the complete mineralisation of benzoate under rumen conditions results in the formation of 50% of the total gas as methane[29]. Furthermore, this result could be explained by the degradation of hydrolysable tannins and particularly gallic acid. Ruminal fermentation of this compound leads to the production of butyrate and acetate[25]. This fermentative profile is associated with a significant production of hydrogen, the elimination of which leads to an increased production of gas in the form of methane[30]. On the other hand, the decrease in CH_4 production noted in the three varieties obtained from the two modes (traditional and three-phase), could be due either to the direct activity of the phenolic content and/or to the accumulation of degradation products of certain phenolic compounds that may be more toxic than the initial compounds.

Table 4. Impact of phenolic content extracted from olive mill wastewater of Azzeradj, Chemlal and Sigoise varieties on ruminal methane production.

	Control	Traditional mode			3 phases mode		
		Azzeradj	Chemlal	Sigoise	Azzeradj	Chemlal	Sigoise
3h	**9.33^{a}**	4.33^{a}	5.67^{a}	11.33^{a}	5.67^{a}	6.33^{a}	5.00^{a}
6h	**17.00^{a}**	10.56^{b}	10.67^{ab}	17.33^{a}	10.33^{b}	11.67^{ab}	15.89^{a}
9h	**26.67^{a}**	14.89^{ab}	13.00^{b}	20.33^{a}	11.33^{b}	14.00^{ab}	22.78^{a}
24h	**43.34^{a}**	23.56^{b}	22.34^{b}	38.00^{ab}	29.00^{b}	31.67^{b}	42.34^{a}

a,b,c: means in the same column with different letters are significantly different ($P < 0.05$).

3. Impact of phenolic content on fermentation parameters

3.1 pH

The pH measurements taken after 24 hours are presented in Table 5 where it can be seen that the influence of phenolic content is statistically insignificant on the pH values ($P > 0.05$). A slight and non-significant increase is observed for the varieties Azzeradj and Chemlal pressed by the centrifugation process (7.01 and 6.96, respectively). These values remain within the range tolerated for effective microbial activity.

3.2 Ammonia production

The production of ammonia nitrogen ($N\text{-}NH_3$) from fed with added phenolic extracts is also reported in Table 5, which shows that the addition of phenolic content to the fermentation medium results in a non-significant decrease in ammonia nitrogen concentration after 24 h of incubation ($P > 0.05$). For the three-phase press mode, the highest reduction is recorded for the Azzeradj variety (27.8%) and the lowest for the Sigoise variety (7.08%). Concerning the traditional mode, a significant reduction was noted for the Chemlal variety (57.4%) and the lowest for Azzeradj (16.6%). This decrease in ammonia production may be the consequence of an effect of phenolic compounds on protozoa that play an important role in proteolysis where their specific activity of deamination of amino acids is three times higher than that of bacteria[31]. As it may be the consequence of the specific inhibition of deamination mainly by bacteria, and more specifically by ammonia hyper-producing bacteria and an intense assimilation of this macronutrient by the bacteria[32].

Table 5. Reduction rate (%), compared to the control, of the three varieties for pH, protozoa and ammonia.

	Traditional mode			3 phases mode		
	Azzeradj	Chemlal	Sigoise	Azzeradj	Chemlal	Sigoise
pH	0,15	0.06	0,01	0,017	0,135	0,020
Protozoa	5.55	24.3	2.56	9.61	2.08	2.56
Ammonia	16.6	57.4	16.9	27.8	18.4	7.08

3.3 Enumeration of protozoa

The influence of phenolic extracts with added fed on the quantitative composition of the ruminal fauna is illustrated in table 5. The addition of phenolic extracts from the OMW of the Azzeradj, Chemlal and Sigoise varieties induced a reduction in the protozoan population compared to the control. This reduction is more pronounced for extracts obtained from OMW from the traditional press than those obtained by three phases. For the first process, the greatest reduction is observed for the Chemlal variety (24.3%) and the lowest for the Sigoise variety with 2.56%. For the second pressing method, the highest reduction was noted for the Azzeradj variety (9.61%) and the lowest also for the Sigoise variety (2.56%). This decrease could be explained by the effects of phenolic compounds on protozoa. According to the literature, the decrease in the number of protozoa is mainly related to the effect of tannins and especially condensed tannins[33,34]. These molecules can act either directly on ruminal fauna (protozoa) by altering their metabolism or indirectly through their anitimethanogenic properties (reduction of methanogenesis)[35]. Indeed, between 9 and 25% of methanogenic archaea are associated with ciliated protozoa that provide them with molecular hydrogen (H_2) as an energy substrate.

Conclusion

This research revealed the richness of the three varieties in total phenols. Nevertheless, they contain a low content of condensed tannins and flavonoids. Furthermore, it seems clear that the difference between the three varieties (Azzeradj, Chemlal and Sigoise) influences the quantitative composition of phenolic compounds in the olive mill wastewater. In attempt to valorise these wasted compounds, they used as supplement in rumen diet and results showed an important impact on *in vitro* gas production especially in methane production after 24 hours of incubation and it was 12.32, 45.64 and 48.45% for Sigoise, Azzeradj and Chemlal, respectively pressed by traditional mode. As well, the same decrease is observed for the 3 phases where the reduction rates are of the order of 33.08, 11.67 and 2.30% for the respective varieties of Azzeradj, Chemlal and Sigoise. In the light of all the results obtained, it seems that the phenolic compounds are potent candidate in the reduction of methane emission by ruminants.

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Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by [**Selma Hamimed**] and [**Amani Kthiri**]. The first draft of the manuscript was written by [**Selma Hamimed**] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.