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CELLULAR AND MOLECULAR BIOLOGY

The Comparisons of Fatty Acid Composition in Some Anaerobic Gut Fungi *Neocallimastix, Orpinomyces, Piromyces,* and *Caecomyces*

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Abtract: The objective of this study were to identify the fatty acid composition for decanoic (C10:0), tridecanoic (C13:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), linoleic (C18:2n6c), arachidic (C20:0), arachidonic (C20:4n6), heneicosanoic (C21:0), erucic (C22:1n9) and Cis-4,7,10,13,16,19-docosahexaenoic (C22:6n3) acids by Neocallimastix, *Orpinomyces*, *Caecomyces* and *Piromyces* species of rumen fungus during in vitro culture. Fatty acid (FA) profile of anaerobic fungi comprises carbon chains of length ranging from 10 to 22 were analyzed as methyl esters. Analysis of fatty acids was performed using Gas Chromatography-Mass Spectrophotometer (GC-MS). FA measures are presented as proportions of relative amounts (% total fatty acid). The highest amounts of fatty acids for all samples were found as myristic (C14:0) acid. The tridecanoic (C13:0) acid represented the second abundant FA in the fungi in all experimental groups. Stearic acid (C18:0) was the third major fatty acid for isolates investigated in the current study. In addition, another fatty acid was palmitic (C16:0) acid with relative amount representing >20 % of total FA in all samples. Pentadecanoic (C15:0) acid could not be found in any other samples except *Orpinomyces* sp. (GMLF5). It is concluded that biohydrogenation of fatty acid composition by anaerobic gut fungi are very variable.

Key words: *Caecomyces*, Fatty acid, *Neocallimastix*, *Orpinomyces*, *Piromyces*, Rumen Gut Fungi.

INTRODUCTION

Anaerobic gut fungi (AGF) are robust degraders of plant biomass in the guts of ruminants and other large monogastric herbivorous mammals (Theodorou et al. 1996). They have also been identified using microscopy and molecular methodologies in the digestive tract of herbivorous reptiles (Liggenstoffer et al. 2010). AGF, which live in the digestive tract of many herbivore mammals and reptiles, participate in biodegradation of plant material ingested by host animals (Trinci et al. 1994, Giménez et al. 2017). AGF degrade the structural polysaccharides located in plant cell wall with the aid of their highly active polysaccharides. The participation of AGF to cellulose and hemicellulose digestion is seen as the most important role of these microorganisms for host animals. AGF is the microorganisms that provide efficient digestion of foodstuff taken by animals and play important roles in the food of animal origin and improvement of tissue (Ekinci et al. 2006).

Cell fatty acid composition is one of the methods used routinely for the identification of microorganisms and manifestation of their differences today (Tighe et al. 2000, Whittaker et al. 2005, 2007. The composition of fatty acids of rumen fungi is also an indicator reflects

their anaerobic developments (Nam et al. 2007, Koppova et al. 2008). AGF diverges from other fungi because they contain monoenoic fatty acids at a higher-level relatively. Moreover, oleic acid comprises 70% of fatty acids (Orpin 1988). Although AGF is distinguished from other more than 100 aerobic filamentous fungi investigated in terms of the presence of very-long-chain fatty acids (Stahl & Klug 1996), it has also been reported that differences in fatty acid compositions among AGF can be used as taxonomic criteria (Koppova et al. 2008). Among the biochemical data used for all taxonomies of fungi, fatty acid compositions have an important role (Stahl & Klug 1996, Bentivenga & Morton 1996), on the other hand, there is no taxonomy decided or evaluated for AGF in terms of fatty acid compositions yet (Koppova et al. 2008).

The AGF are classified in the phylum Neocallimastigomycota, class Neocallimastigomycetes, order Neocallimastigales, family Neocallimasticaceae and 18 genera (Chang & Park 2020). This classification is supported by morphological analysis (Barr 1988, Li et al. 1993) as well as rDNA analysis (Dore & Stahl 1991, Bowman et al. 1992, Li & Heath 1992, Fliegerova et al. 2004). The genera in the family *Neocallimasticaceae* have been classified based on the morphological characteristics including zoospore flagellation (uniflagellate vs. polyflagellate), the sporangia development (monocentric vs. polycentric) and the thallus morphology (filamentous vs. bulbous) (Ho et al. 2000). Filamentous rhizoidal development is observed for all anaerobic fungal genera (n=18) (apart from *Cyllamyces spp* and *Caecomyces spp*) identified and reported so far, and they are divided into polycentric (*Orpinomyces*, *Anaeromyces, and Cyllamyces*) and monocentric (*Agriosomyces, Akiloshbomyces, Buwchfawromyces, Caecomyces, Capellomyces, Feramyces, Ghzallomyces, Joblinomyces, Khoyollomyces, Liebetanzomyces, Neocallimastix, Oontomyces, Pecoramyces, Piromyces and Tahromyces*). The two genera, *Caecomyces* and *Cyllamyces,* have spherical rhizoidal systems, designated as a bulbous body, instead of filamentous rhizoidal structure (Ozkose et al. 2001). While *Caecomyces* genus shows monocentric development, a polycentric reproduction manner is observed for the genus *Cyllamyces.* In this study, the molecular identification of AGF deposited in the culture collection of BİGEM (Biotechnology and Gene Engineering Laboratory) AGF Culture Collection was made and a phylogenetic tree was formed. The differences between determined phylogenetic structures of rumen fungi in terms of the composition of volatile fatty acids involved by them were revealed, and they were compared with their current generations. *Orpinomyces* sp., *Neocallimastix* sp. and *Caecomyces* sp. cultivated on microcrystalline cellulose (avicel) together with *Clostridium* sp. CHK5, which is a chitinolytic bacteria, and it was suppressed these fungi' digestion with avicel, short-chain fatty acid generation and endoglucanase release significantly (Kopečný et al. 1996).

The objective of this study were to identify the fatty acid composition for decanoic (C10:0), tridecanoic (C13:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), linoleic (C18:2n6c), arachidic (C20:0), arachidonic (C20:4n6), heneicosanoic (C21:0), erucic (C22:1n9) and Cis-4,7,10,13,16,19-docosahexaenoic (C22:6n3) acids by *Neocallimastix*, *Orpinomyces*, *Caecomyces* and *Piromyces* species of rumen fungus during in vitro culture.

MATERIALS AND METHODS

Microorganisms

The AGF used in this study were obtained from Anaerobic Gut Fungal Culture Collection established within the Biotechnology and Gene Engineering Laboratory (BIGEM) of Kahramanmaras Sutcu Imam University Faculty of Agriculture Department of Animal Science. Fungal strains, culture medium (Orpin 1977) and growth conditions (Griffith et al. 2009). Culture medium composition was: rumen fluid of cattle, 150 ml/l; $NaHCO_3$, 6 g/l; yeast extract, 2.5 g/l; peptone from pancreatic digest, 10 g/l; Lcystein hydrochloride, 1 g/l and resazurin 0.001 g/l. Mineral solutions used in anaerobic medium was prepared separately and added 150 ml/l. Mineral solution I contained 0.3% K_2HPO_4 (w/v). Mineral solution II contained (w/v): 0.3% KH_2PO_4, 0.6% NaCl, 0.6% $(NH_4)_2SO_4$, 0.06% $CaCl_2$, and 0.06% $MgSO_4$. Medium was completed to 1 l with distilled water after the addition of mineral solutions. Medium was boiled for 1 hour and dispensed into 10 ml Hungate tubes under the CO_2 (99% purity) stream. Hungate tubes containing medium was sterilised by autoclaving at 121 °C for 15 min. Six strains belonging to four genera (*Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Piromyces*) of rumen anaerobic fungi were examined. 1 ml of fungal culture was inoculated into Hungate tubes by injection method under anaerobic conditions. The fungus was incubated anaerobically at 39 °C with a substrate of chopped either glucose (5 mg ml^{-1}) or wheat straw (50 mg ml^{-1}) and was transferred every 3 days.

Fatty acid analysis

For lipid extraction AGF were grown on glucose (0.5% w/v) containing medium (Orpin 1977) for 3 days at 39 °C and the fungal biomass was harvested by centrifugation at 1250 g for 10 min. Then, the cells washed using deionized water (diH_2O) followed by precipitated utilizing centrifugation for 10 min at 1250 g. This extraction process was repeated thrice then, the cells were stored in Eppendorf tubes at -20 °C for fatty acid extraction.

Fatty acid extraction was made by the usage of Zivak brand (in blood/serum for 500 samples) fatty acid analysis kit. Lipid extraction was performed according to the protocol of the manufacturer based on the methodology reported by Folch (Folch et al. 1957) and incubation of AGF with linoleic acid was prepared according to Kim et al. (2000). The obtained supernatant parts of the samples were transferred/loaded (2 µl) to the Gas Chromatography-Mass spectrophotometer (GC-MS) via automatic sampler equipped with fatty acids column. Helium was used as the carrier gas with a flow rate of 1 ml/min. The airflow is 350 ml/min. The flow rate was determined as 30 ml/min helium. Injection split is 1:10, 260 °C and 2 µl. The program was applied as 1 minute at 100 °C, temperature increase was 10 °C/min, and 10 min at 250 °C. Detector starting temperature was adjusted as 290 °C. Teknoroma brand (60m x 0.25 mm x 0.20 µm) column was used (Column TR-CN100). All analyses were conducted by Shimadzu GC-MS QP 2010 model device.

A reference standard of decanoic (C10:0), tridecanoic (C13:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), linoleic (C18:2n6c), arachidic (C20:0), arachidonic (C20:4n6), heneicosanoic (C21:0), erucic (C22:1n9) and Cis-4,7,10,13,16,19-docosahexaenoic (C22:6n3) acids (Sigma, St. Louis, MO, USA) were used to compare and identify the peaks.

Statistical analysis

Fatty acids of fungal isolates were compared by analysis of variance using the SPSS v17.0 statistical package program and statistical significance was declared at $P < 0.05$ and Duncan

multiple range test was used to further compare means at $P < 0.05$ and trends were declared at $0.05 \leq P \leq 0.10$. Mean values and standard deviation of the mean are shown (mean ±SEM). All incubations were performed in triplicate.

RESULTS AND DISCUSSION

The screening of AGF for fatty acids carried out using GC-MS (standards as shown in Figure 1) revealed that AGF is a rich source of fatty acids. To the determination of fatty acids concentrations, *Caecomyces* sp. (GMLF12), *Neocallimastix* sp. (GMLF1, GMLF23, GMLF25), *Piromyces* sp. (GMLF17) and *Orpinomyces* sp. (GMLF5) samples were examined in Gas Chromatography-Mass spectrophotometer (GC-MS). The comparison of the results of the obtained fatty acids concerning their significance levels was presented in Table I. The highest amounts of fatty acids for all samples were found as Myristic Acid ($C_{14:0}$). In contrast to our study, Kemp & Lander (1984) reported that the highest fatty acid concentration in their study conducted for *Caecomyces, Neocallimastix,* and *Orpinomyces*, were Oleic Acid ($C_{18:1n9c}$) and Palmitic Acid ($C_{16:0}$). Body & Bauchop (1985) also reported similar results in the study they carried out on *Piromyces communis* and *Neocallimastix frontalis*.

It was found that the highest fatty acid amount in the *Caecomyces sp.* (GMLF12) sample is Myristic Acid with a ratio of 27,43%, while the lowest fatty acid amount is Decanoic Acid with the ratio of 1%. While the highest amount of Decanoic Acid was found in *Caecomyces* sp. (GMLF12), the lowest amounts were determined in *Neocallimastix* sp. Samples for this fatty acid concentration. Decanoic Acid ($C_{10:0}$) averages

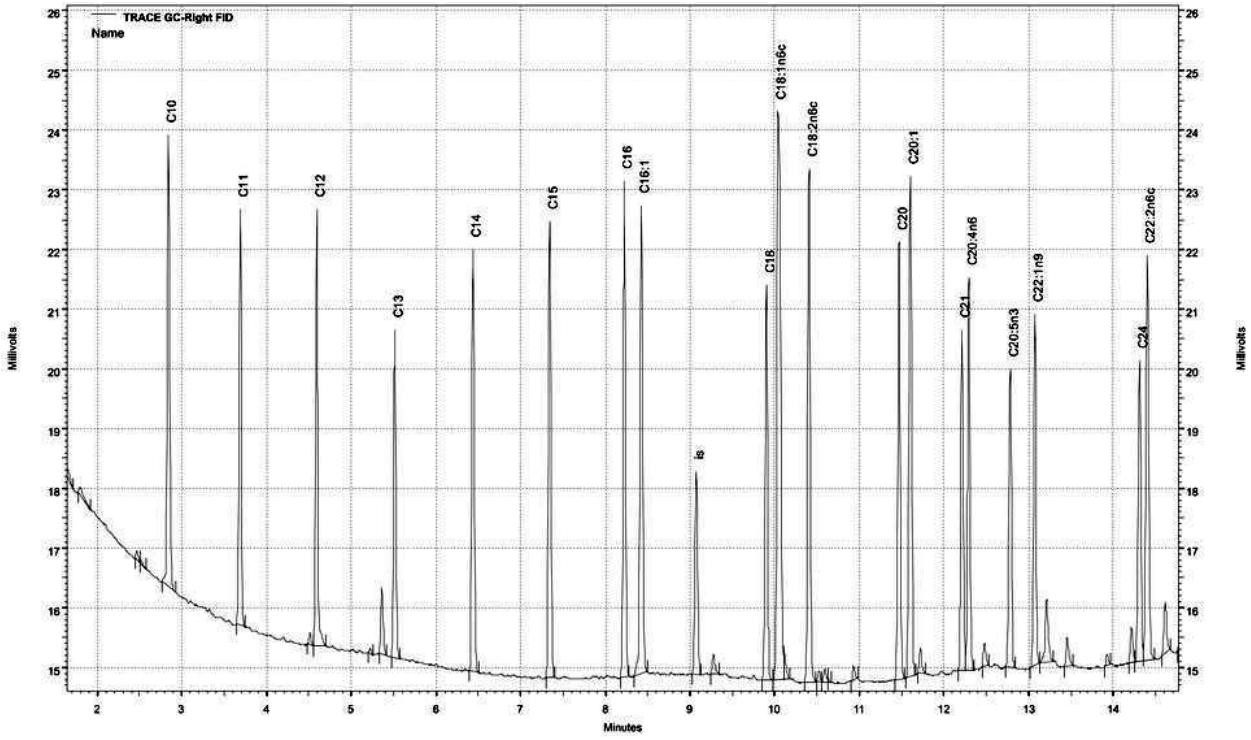

Figure 1. The internal standard fatty acids were processed to Methyl esters. GC-MS analysis was performed using Shimadzu GC-MS-QP-2010. Separation was performed on a teknoroma brand column (TR-CN100). Helium was used as the carrier gas with a flow rate of 1 ml/min. Free fatty acid methyl esters were separated at constant flow with the following temperature program: 100 °C (10 min) to 250 °C at 4 °C/min.

were considered as insignificant statistically for all fungal isolates tested in this study. While the highest amount of Tridecanoic Acid was found in *Piromyces* sp. (GMLF17), whilst the lowest amount was found in *Neocallimastix* sp. (GMLF25) (P<0.001). Tridecanoic Acid amounts belong to the *Neocallimastix* sp. samples were quite different from each other as stated in Table I.

Pentadecanoic Acid ($C_{15:0}$) could not be found in any other samples except *Orpinomyces* sp. (GMLF5). In the study conducted by Comlekcioglu et al. (2010), Pentadecanoic Acid was found approximately at the same proportions in *Neocallimastix* and *Caecomyces*, while *Orpinomyces* was quite different according to them. The highest amount of Palmitic Acid ($C_{16:0}$) was found in *Caecomyces* sp. (GMLF12), while the lowest amount was found for the isolates putatively identified as *Neocallimastix* sp. Palmitic Acid ($C_{16:0}$) and Oleic Acid ($C_{18:1n9c}$) proportions were quite different from each other in *Orpinomyces* sp. (GMLF5). In contrast to our study, Comlekcioglu et al. (2010) were found that the Palmitic Acid ($C_{16:0}$) and Oleic Acid ($C_{18:1n9c}$) proportions were very close to each other.

Stearic Acid ($C_{18:0}$) was the third major fatty acid for isolates investigated in the current study and these results were in parallel to the earlier report for *N. frontalis* (Body & Bauchop 1985), however, $C_{12:0}$ was more abundant than $C_{18:0}$ in *P. communis* (Kemp et al. 1984). The highest amount of Stearic Acid ($C_{18:0}$) was found in *Caecomyces* sp. (GMLF12), while the lowest amount was found in the *Neocallimastix* sp. (GMLF23) sample. The amounts of Stearic Acid were very close in all samples. The highest amount of Oleic Acid ($C_{18:1n9c}$), was found in *Piromyces* sp. (GMLF17), while the lowest amount was found in the *Neocallimastix* sp. (GMLF1) sample. The highest amount of Linoleic Acid ($C_{18:2n6c}$), was found in *Neocallimastix* sp. (GMLF23), while the lowest amount was found in the *Neocallimastix* sp. (GMLF25) sample. Linoleic Acid could not be found in GMLF12.

The results of Arachidic Acid show similarity to Linoleic Acid results. The highest amount of Arachidic Acid ($C_{20:0}$), was found in *Neocallimastix* sp. (GMLF23), while the *Neocallimastix* sp. (GMLF25) formed a remarkably lower amount of Arachidic Acid ($C_{20:0}$). In contrast, Comlekcioglu et al. (2010) did not report the presence of Arachidic Acid ($C_{20:0}$) in any samples in the study they made. The highest amount of Arachidonic Acid ($C_{20:4n6}$), was found in *Caecomyces* sp. (GMLF12), while the lowest amount was found in the *Neocallimastix* sp. (GMLF23) sample (P<0.01). Arachidonic Acid had not been observed in any tubes of the isolate GMLF1. The highest amount of Heneicosanoic Acid ($C_{21:0}$), was found in *Orpinomyces* sp. (GMLF5), while the lowest amount was found in the *Caecomyces* sp. (GMLF12) sample. Heneicosanoic Acid had not been determined in the samples belong to the isolates GMLF17, GMLF23 and GMLF25. The highest amount of Erucic Acid ($C_{22:1n9}$), was found in *Caecomyces* sp. (GMLF12), while the lowest amount was found in the *Neocallimastix* sp. (GMLF25) sample. The results of this study showed that the fungal isolates GMLF12, GMLF5 and GMLF1 cannot form the Cis-4, 7, 10, 13, 16, 19 Docosahexaenoic Acid ($C_{22:6n3}$). AGF, which constitutes one of the most important links of rumen microbiology, is a group of microorganisms in which the intense studies have been carried out in recent years due to both their functions in the gastrointestinal tracts of the herbivores and their potential usage, particularly in enzyme biotechnology.

Having metabolized (hydrolysis and biohydrogenation) of fats taken with ration in the digestive system by microorganisms is important for the absorption of unsaturated and essential fatty acids (linoleic and linolenic

Table I. Comparisons of fatty acid composition±SEM (n=3) expressed as total fatty acid methyl esters (% of total fatty acid) of anaerobic gut fungi.

Fatty acid composition																
	Kemp et al. [30]	Body and Bauchop [29]	Koppova et al. [10]				Comlekcioglu et al. [31]			In this study						Imp.
										Neocallimastix			*Orpinomyces*	*Caecomyces*	*Piromyces*	
Fatty Acids	Pir.[1]	Neo.[2]	Neo.[2]	Orp.[3]	Cae.[4]	Ana.[5]	Neo.[2]	Orp.[3]	Cae.[4]	GMLF1	GMLF23	GMLF25	GMLF5	GMLF12	GMLF17	
C10:0, Decanoic	-	-	-	-	-	-	-	-	-	0.103±0.011	0.080±0.010	0.073±0.003	0.074±0.022	0.053±0.003	0.072±0.003	NS
C12:0, Lauric	14.2	2.7	6.45	6.04	1.90	9.44	8.0	12.4	4.15	ND	ND	ND	ND	ND	ND	
C13:0, Tridecanoic	-	0.1	-	-	-	-	-	-	-	$36.844±1.447^{b}$	$69.501±0.314^{c}$	$102.707±0.291^{e}$	$38.657±1.959^{b}$	$91.086±0.181^{d}$	$7.377±0.027^{a}$	***
C14:0, Myristic	10.5	7.5	2.86	0.52	1.24	3.94	6.27	6.03	6.74	$47.439±1.986^{b}$	$77.426±0.414^{c}$	$108.672±0.177^{e}$	$49.350±2.095^{b}$	$93.587±0.684^{d}$	$39.326±0.229^{a}$	***
C15:0, Pentadecanoic	-	0.7	-	-	-	-	1	0.68	1.18	ND	14.157±0.108	ND	ND	ND	ND	ND
C16:0, Palmitic	20.3	20.7	3.07	2.85	2.23	1.72	21.54	27.97	27.04	$35.898±1.857^{e}$	$29.663±0.280^{d}$	$28.608±0.084^{cd}$	$24.427±1.214^{ab}$	$22.089±0.122^{a}$	$25.922±0.063^{bc}$	***
C16:1, Palmitoleic	1.5	2.8	-	-	-	-	1.13	1.75	0.56	ND	ND	ND	ND	ND	ND	ND
C18:0, Stearic	11	12.6	6.02	6.06	8.32	6.11	17.61	17.73	21.59	$30.795±1.484^{d}$	$28.210±0.143^{c}$	$24.646±0.115^{ab}$	$26.733±0.954^{bc}$	$23.478±0.056^{a}$	$26.186±0.095^{bc}$	***
C18:1n9c, Oleic	35.5	34.3	3.62	4.12	2.26	2.33	32.56	27.48	33.25	$7.998±0.980^{b}$	$10.367±0.049^{c}$	$11.304±0.155^{c}$	$6.228±0.180^{a}$	10.224±0.132c	$11.210±0.106^{c}$	***
C18:2n6c, Linoleic	-	0.3	0.44	0.35	0.32	0.37	1.26	1.38	3.18	ND	$5.241±0.108^{b}$	$5.254±0.124^{b}$	$5.543±0.325^{b}$	$15.564±0.131^{c}$	$3.654±0.051^{a}$	***
C20:0, Arachidic	0.3	1.2	6.50	7.07	8.58	7.24	-	-	-	ND	$6.666±0.064^{c}$	$6.365±0.036^{b}$	$4.718±0.097^{a}$	ND	$4.705±0.011^{a}$	***
C20:1, Eicosenoic	2	4.4	0.51	0.36	0.40	0.34	1.22	0.59	0.18	ND	ND	ND	ND	ND	ND	ND
C20:4n6, Arachidonic	-	-	-	-	-	-	-	-	-	$3.755±0.289^{b}$	$3.154±0.092^{a}$	$3.605±0.030^{b}$	-	$2.756±0.068^{a}$	$2.862±0.067^{a}$	**
C21:0, Heneicosanoic	-	-	6.41	7.56	8.55	7.70	-	-	-	$5.366±0.607^{a}$	$24.680±0.104^{c}$	ND	$13.126±0.170^{b}$	ND	ND	***
C22:1n9,Erucic	0.6	1.2	0.40	0.42	0.31	0.37	-	-	-	$4.746±0.470^{d}$	$3.409±0.026^{c}$	$3.125±0.053^{bc}$	$2.907±0.081^{abc}$	$2.465±0.061^{a}$	$2.654±0.050^{ab}$	***
C22:6n3, Cis-4,7,10,13,16,19-Docosahexaenoic	-	-	-	-	-	-	-	-	-	ND	ND	$2.945±0.035^{b}$	ND	$2.567±0.099^{a}$	$3.854±0.104^{c}$	*
C24:1, Nervonic	3.3	8.3	0.47	0.29	0.27	0.51	4.90	3.15	-	ND	ND	ND	ND	ND	ND	ND

Values represent the means of two samples produced from independent cultures, Fatty acid determination was carried out in triplicates (n = 3), a, b, c, d, e superscripts in the same row means that different superscripts are statistically significant (* P<0.05, ** P<0.01, P<0.001), NS: Insignificant; ND: Not detected, 1: *Piromyces* sp., 2: *Neocallimastix* sp. 3: *Orpinomyces* sp., 4: *Caecomyces* sp. and 5: *Anaeromyces* sp., Imp.: Importance.**

acid) for mammalian herbivores. Moreover, unsaturated fatty acids that ruminants store in their tissues (especially in conjugated linoleic acid –CLA- form) have a positive effect on human health through consumption. Today, it is well documented that heart diseases have been increasing in people with the consumption of fats containing an excessive amount of saturated fat. Metabolic activities of microorganisms, inhabiting the gastrointestinal tract of farm herbivores, therefore, play an important role in balanced/healthy food for humans as the main producer of health improvers such as CLA as side or end products of their metabolic pathways (Malmuthuge & Guan 2017).

CONCLUSION

The fatty acid composition is one of the methods used in identifying microorganisms and revealing their differences. Among the biochemical data used in my taxonomies of other fungi, it is possible to find available classifications according to fatty acid compositions. However, for anaerobic rumen fungi, it has not yet been determined or evaluated in terms of cell fatty acid compositions. The differences observed in the morphological data of the Romanian fungi occur exactly in the fatty acid compositions. The discovery of biochemical properties such as fatty acid properties will be important for anaerobic fungi that are difficult to classify and identify.

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Bulent KAR, Emin Özköse and M. Sait Ekinci contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.