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Production of high-value bioproducts enriched with γ-linolenic acid and β-carotene by filamentous fungi *Umbelopsis isabellina* using solid-state fermentations

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Abstract

Solid-state fermentation is a useful tool for utilizing different plant-based materials as cultivation substrates in order to produce potentially high-value fermented bioproducts. The aim of the present study was to successfully prepare various types of such bioproducts, using a zygomycetous strain *Umbelopsis isabellina* CCF2412. Various legume and cereal substrates were utilized effectively, while a few of them were obtained from agricultural waste, which is particularly advantageous from ecological and economic point of view. A common feature of the produced fermented materials was the increased content of different polyunsaturated fatty acids and carotenoid pigments in these bioproducts. Subsequent to the optimization of the solid-state fermentation process using cornmeal as the cultivation substrate, bioproducts enriched with γ-linolenic acid (11.45 mg γ-linolenic acid per gram of bioproduct), β-carotene (50.90 μg β-carotene per gram of bioproduct), and various microbial sterols were obtained. Appropriate n–6/n–3 acid ratio and enrichment of other microbial substances, such as the pigments and sterols mentioned above, in the fermented bioproducts widens the applicability of these bioproducts in different industries. The fermented cereal bioproducts produced in the present study from fermented wheat bran substrate were used for evaluating their application as feed for broiler chicken, and satisfactory results were obtained. Therefore, the present study creates novel opportunities for improving the quality of fermented bioproducts obtained during solid-state fermentation processes, especially for application in the feed industry.

Keywords: Solid-state fermentation, *Umbelopsis isabellina*, γ-Linolenic acid, β-Carotene

Introduction

Solid-state fermentation (SSF) has enormous potential for interesting applications in several industries. SSF plays an interesting role in economically efficient biofuel production, considering the fact that agro-energy expansion is occurring currently, as well as in food fermentation processes. SSF represents an alternative approach for the production of industrially important food because of the possibility of bioconversion of the agro-industrial residues into high value-added bioproducts (Farinas 2015). The importance of SSF for humankind dates back to thousands of years, especially in the field of food processing (bread and cheese in western cultures, Koji in eastern cultures, among others), while its importance in regard to population growth is increasing with each passing day.

SSF is defined as the process of fermentation using solid substrates in the absence or near-absence of free-flowing water. However, for this process to occur, the substrate must contain a minimal amount of moisture in order to support the growth and metabolism of the cultivated microorganisms which would cause the fermentation process. SSF offers several benefits, including low energy requirement while maintaining high product yields. In comparison to the traditional submerged fermentation, which involves microorganisms cultivated in a liquid medium, one of the main advantages of SSF is

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that the complete fermentation process is environment-friendly and the only a little wastewater is produced (Wang et al. 2014; Soccol et al. 2017). On the other hand, SSF also has certain limitations, such as the fact that only a small number of microorganisms are capable of growing in the conditions specific to this fermentation process, in addition to the requirements of high humidity, free oxygen transfer, airflow, and the necessity of removing the generated CO_2. In certain cases, pretreatment of solid substrates is necessary as well, which leads to increased financial and technological requirements (Pérez-Rodríguez et al. 2014).

Nowadays, SSF is being used for the production of several industrially relevant compounds, such as enzymes (xylanase, tannase, amylase, etc.), flavors and aromas, phenolic anti-oxidants, biofertilizers, biopesticides, and organic acids (Graminha et al. 2008; He and Chen 2013; Thomas et al. 2013).

As stated above, only a limited number of microorganisms could be involved in SSF. Zygomycetes fungi have been known to be highly successful in SSF (Stredanský et al. 2000; Čertík et al. 2013; Takó et al. 2015). Zygomycetes have been well described as the producers of wide spectra of polyunsaturated fatty acids (PUFAs), such as γ-linolenic acid (GLA), docosahexaenoic acid (DHA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) (Klempová et al. 2013; Vadivelan et al. 2017). Owing to their structure and other specific properties, PUFAs are responsible for a broad spectrum of cellular processes, such as maintaining cell membrane fluidity, decreasing the secretion of pro-inflammatory cytokines, inhibiting the inflammatory processes and blood platelet aggregation, and decreasing triglyceride synthesis in the liver. Long-chain PUFAs, such as ω–6 AA and ω–3 EPA, also have important functional roles as the precursors of eicosanoids (i.e., prostaglandins, thromboxanes, lipoxins, and leukotrienes) that are involved in the physiological functioning of several systems (Bellou et al. 2016). PUFAs are also able to induce platelet aggregation and vasoconstriction or exhibit anti-inflammatory, antiplatelet, and anti-arrhythmic properties (Wiktorowska-Owczarek et al. 2015). PUFAs serve as essential nutrients for neonatal babies and are involved in the development of retinal and neural functions (Uemura 2012).

β-Carotene is one of the most abundant carotenoids, which is responsible for the yellow-orange color of several fruits and vegetables, such as carrot, papaya, pumpkin, and tomato (Avalos and Limón 2015). β-Carotene is important mainly because of the myriad of health benefits associated with it. β-carotene is the most effective precursor of vitamin A. It has potent antioxidant capacity and also lowers the risk of heart diseases and certain types of cancers. It also enhances the immune system and provides protection from age-related macular degeneration and the loss of vision associated with it (Gul et al. 2015). β-Carotene production through extraction from plants or through chemical synthesis involves the limitation of inadequate yields, which is detrimental economically. According to the World Health Organization (WHO), synthetic β-carotene may be responsible for increasing the risk of cardiovascular diseases (WHO 2003). The latest research has been focused on the microbial production of β-carotene (Mata-Gómez et al. 2014). β-Carotene production is a typical trait in the order Mucorales, and its production in several species of Zygomycetes such as *Blakeslea trispora* (Avalos and Cerdá-Olmedo 2004) or *Mucor circinelloides* (Fraser et al. 1996), Basidiomycetes such as *Sporidiobolus pararoseus* (Han et al. 2012) or *Ustilago maydis* (Estrada et al. 2010), and Ascomycetes such as *Penicillium* sp. (Han et al. 2005) has been well described. However, it is noteworthy that only the fungi belonging to Zygomycetes are capable to form both GLA and carotenes simultaneously (Klempová et al. 2013).

In the present study, the potential of the Zygomycetes fungi named *Umbelopsis isabellina* as a producer of GLA and β-carotene during SSF using a wide variety of substrates has been described. The usage of *U. isabellina* in fermentations may represent a novel approach to extend the possibilities of preparing high-value bioproducts, which may be utilized for applications in various fields including the food industry as well as pharmaceuticals or the cosmetic segment. The present report describes the production of economically interesting bioproducts with relatively low setup costs.

Materials and methods

Organisms and growth conditions

Umbelopsis isabellina CCF2412 strain was obtained from the Culture Collection of Fungi (Charles University, Prague). The strain was maintained on potato dextrose agar (PDA) slants (Carl Roth, Germany) at 4 °C, and was periodically re-inoculated every 3 months. Static SSF cultivations were performed in high-density polyethylene bags (20 × 30 cm) containing 10 g of different dry cereal substrates. Distilled water (10 mL) was added to each bag, in which the substrates were soaked for 2 h at room temperature. The substrates were autoclaved at 105 °C for 30 min, followed by cooling to room temperature and subsequent inoculation through spore suspension using the final concentrations of 10^7 spores/mL obtained from a 7-day-old culture. Cultivation was performed at 28 °C for 5 or 7 days, and the obtained bioproducts were collected and dried at 65 °C until a constant weight was achieved.

The cereal substrates used were crushed corn, corn waste, and cornmeal (Amylum Slovakia, Boleráz, Slovak Republic), oat flakes, barley meal, barley flakes, wheat

bran, wheat germ, and rye bran (mill, Pohronský Ruskov, Slovak Republic), and amaranth (Plant Research Institute Piešťany, Slovak Republic).

The legume substrates used were bean (white, red, and color-spotted), soya, pea, lentil, and chickpea.

Lipid/pigment extraction and analysis

Lipid and the carotenoid pigment in the obtained bioproducts were extracted using a modified Folch method (Folch et al. 1957). One gram of dried bioproduct was homogenized, followed by two rounds of extraction using a mixture of chloroform and methanol in a ratio of 2:1 (v/v) for 1.5 h at room temperature with occasional stirring. Post extraction, the mixture was filtered to remove solid particles, and the extracts were mixed with distilled water (1.2-folds of the total extract volume). The mixture was stirred for 1 min and then centrifuged to achieve phase separation. The chloroform layer containing the lipids and pigments was dried using anhydrous Na_2SO_4 and then subjected to evaporation under vacuum. Lipid extract with carotenoid pigments was suspended in 1 mL of the mixture of hexane and chloroform in a ratio of 9:1 and used for analysis.

Carotenoid pigment analysis was performed using HPLC as described by Klempová et al. (2013). The carotenoid pigments were identified using authentic standards (Sigma, Germany) and quantified using ChemStation B 01 03 (Agilent Technologies). Quantification of β-carotene was performed by using known amounts of β-carotene standards (Sigma, Germany) measured under the conditions identical to those used for the samples.

Fatty acid methyl esters required for the analysis were prepared from a total lipid sample, with heptadecanoic acid as an internal standard, using the method reported by Christopherson and Glass (1969). Fatty acid methyl esters were analyzed through gas chromatography using the method reported by Gajdoš et al. (2015). Identification of the fatty acid methyl ester peaks was performed by comparison with the authentic standards of C4–C24 fatty acid methyl ester mixtures (Supelco, USA). Quantitative evaluation of the individual fatty acids was performed using ChemStation B 01 03 (Agilent Technologies).

Sterol analysis was performed using GC-MS. Lipid sample (15 mg) was saponified using 1 mL of 25% (w/v) KOH in ethanol at 90 °C for 60 min. The sample was subsequently diluted with distilled water (1 mL), followed by extraction with two portions of hexane (1 mL each). The mixture was then centrifuged (5000 rpm, 5 min), and the organic extract obtained was subjected to evaporation. The sterol extract was analyzed using a GC (Agilent Technologies 6890 N) coupled to an Agilent Mass-selective detector. The instrument was equipped with a 30 m HP–5 column (film thickness 0.25, i.d. 320 μm). Sample (1 μL) was injected into the injection port of the equipment at 300 °C. Hydrogen was used as the carrier gas at a velocity of 40 cm/s in constant flow mode. The temperature program initiated at 280 °C (1 min) was ramped to 290 °C at the rate of 5 °C/min and held for 22 min. Subsequently, the temperature was raised up to 310 °C at the rate of 20 °C/min) and finally held for 2 min. The data obtained were evaluated and quantified using the ChemStation software (Agilent Technologies).

Preparation of chicken feed

The substrate used was wheat bran (obtained from mill, Pohronský Ruskov, Slovak Republic).

Static SSF cultivations were performed in HDPE bags (30 × 40 cm) containing 100 g of dry wheat bran. Distilled water (100 mL) was added to each bag, and the substrates were soaked in the distilled water for 2 h at room temperature. Subsequently, the substrates were autoclaved at 105 °C for 45 min, cooled to room temperature, and inoculated through spore suspension with a final concentration of 10^7 spores/mL obtained from a 7-day-old culture. Cultivation was performed at 28 °C for 7 days. The obtained bioproducts were collected and dried at 65 °C until a constant weight was achieved. After 72 h of fermentation, all of the submerged culture of *U. isabellina* in the Erlenmeyer flasks was added into the solid-state system.

Submerged fermentation was performed in 250-mL Erlenmeyer flasks containing 50 mL media composed of glucose (15 g/L) and yeast extract (5 g/L); the media were inoculated through spore suspension with the final concentrations of 10^7 spores/mL. Cultures were grown at 28 °C for 72 h with constant shaking at 165 rpm.

The prepared feed was administered to broiler chicken according to the methods described by Marcinčák et al. (2018) and Mudroňová et al. (2018).

Data analysis

One-way analysis of variance (ANOVA) was performed on the obtained data using Microsoft Excel (Microsoft Office 365 software pack) equipped with a data analysis tool. Post hoc testing was performed for the ANOVA results using Tukey's HSD test in programming language R and in Python v. 3.7 using StatsModels libraries.

Results and discussion

Verification of co-production and substrate screening

The *Umbelopsis isabellina* CCF2412 strain has been described previously as an efficient producer of both GLA and β-carotene in submerged fermentation processes (Klempová et al. 2013). So far, no study has been conducted on the possibility of this co-production in a solid-state fermentation process. Therefore, the first experiment in the present study was aimed at the verification of the co-production of GLA and β-carotene during SSF. Several common cereal substrates, which are well

known as a good matrix for SSF and are easily accessible, were selected and screened for suitability of using them in SSF (Table 1) (Bogar et al. 2003; Čertík et al. 2013; de Cassia Pereira et al. 2015; El-Naggar et al. 2009). In general, owing to their composition, cereals offer all the components necessary for fungal proliferation. Cereals serve as sources of assimilable carbon which is involved in cell growth and lipid storage, organic nitrogen, and other important nutrients (Čertík et al. 2013; Jangbua et al. 2009). The strain used in the present study was observed to utilize all the substrates with varying degrees of effectiveness. As depicted in Fig. 1, GLA and β-carotene were produced in sufficient amounts. The only exception was the cultivation performed on corn waste, a substrate that was determined to be unsuitable for the cultivation of the strain used.

Increasing amounts of total lipid content were recorded, indicating the accumulation of a wide spectrum of different fatty acids in the obtained fermented bioproduct. The results are presented in Table 1.

Unexpected results were achieved in case of cultivation on spent malt grain. Spent malt grain has been reported to serve as an inert carrier for SSF cultivations and to provide internal support in order to increase the interparticle space (Čertík et al. 2006; Čertík et al. 2008). The assumption that this substrate would serve as an inert carrier was not confirmed, although sufficient growth and co-production of both metabolites were observed for this substrate.

As depicted in Fig. 1, several substrates were determined to be suitable for the production of both reference metabolites. Analysis of the obtained data confirmed that the mean values differed significantly ($P < 0.0001$) when different fermentation substrates were used. Statistically substantial changes were observed with the use of several cultivation substrates, i.e., cornmeal, amaranth, rye bran, and barley meal. Corn waste was determined to be the only inappropriate one, as only trace amounts of GLA and β-carotene were observed in the case of this substrate. Statistically significant increase in the GLA content was achieved with cultivation on barley flakes (2.87 mg/g BP) and amaranth (2.63 mg/g BP). These results were comparable to a previous study conducted by Čertík et al. (2013), in which the amount of GLA obtained after the cultivation of the Zygomycetes strain *Mucor circinelloides* CCF2617 reached 2.0 mg/g BP.

Table 1 Content of lipids and profile of fatty acids in lipids of cereal substrates and fermented bioproducts

Substrate		TL (%)	PA (%)	POA (%)	SA (%)	OA (%)	LA (%)	GLA (%)	ALA (%)
Crushed corn	C	2.56 ± 0.01	12.61 ± 0.63	0.11 ± 0.01	2.10 ± 0.11	29.36 ± 1.47	52.79 ± 2.64	nd	0.99 ± 0.05
	F	34.19 ± 0.62	12.25 ± 0.11	0.13 ± 0.00	1.96 ± 0.03	29.60 ± 0.02	52.64 ± 0.10	0.52 ± 0.02	0.91 ± 0.02
Corn waste	C	1.06 ± 0.05	17.37 ± 0.87	0.27 ± 0.02	7.25 ± 0.36	15.08 ± 0.75	49.30 ± 2.46	nd	4.36 ± 0.22
	F	0.66 ± 0.07	16.86 ± 0.19	0.19 ± 0.01	7.93 ± 0.55	15.98 ± 0.30	49.41 ± 0.55	0.23 ± 0.04	3.90 ± 0.04
Cornmeal	C	2.94 ± 0.10	12.22 ± 0.61	0.13 ± 0.01	2.58 ± 0.13	28.40 ± 1.42	52.80 ± 2.64	nd	1.20 ± 0.06
	F	5.60 ± 0.16	17.59 ± 0.53	1.05 ± 0.12	3.92 ± 0.05	41.52 ± 0.32	24.17 ± 0.55	7.58 ± 0.19	0.55 ± 0.08
Oat flakes	C	0.14 ± 0.14	19.02 ± 0.95	0.20 ± 0.01	4.44 ± 0.22	27.73 ± 1.39	42.61 ± 2.13	nd	1.39 ± 0.07
	F	6.24 ± 0.55	21.88 ± 0.14	0.77 ± 0.07	4.05 ± 0.09	34.68 ± 0.32	30.11 ± 0.99	2.62 ± 0.27	0.79 ± 0.01
Barley meal	C	0.57 ± 0.03	22.75 ± 1.14	0.19 ± 0.01	3.21 ± 0.16	16.94 ± 0.85	48.68 ± 2.43	nd	4.15 ± 0.21
	F	3.44 ± 0.20	23.00 ± 0.06	1.46 ± 0.02	5.49 ± 0.06	43.36 ± 0.29	15.08 ± 0.37	6.73 ± 0.05	0.69 ± 0.07
Barley flakes	C	0.35 ± 0.02	20.36 ± 1.02	0.14 ± 0.01	4.17 ± 0.21	22.56 ± 1.13	47.23 ± 2.36	nd	1.67 ± 0.08
	F	3.47 ± 0.22	22.38 ± 0.08	1.91 ± 0.04	5.00 ± 0.14	45.15 ± 0.38	12.32 ± 0.16	8.28 ± 0.04	0.25 ± 0.01
Wheat bran	C	1.67 ± 0.08	25.52 ± 1.28	0.23 ± 0.01	2.88 ± 0.14	23.87 ± 1.19	39.06 ± 1.95	nd	2.72 ± 0.14
	F	2.40 ± 0.02	18.17 ± 0.16	0.60 ± 0.02	5.53 ± 0.00	36.54 ± 0.46	26.60 ± 0.45	6.68 ± 0.07	1.05 ± 0.08
Wheat germ	C	14.54 ± 0.73	20.70 ± 1.03	0.16 ± 0.01	2.20 ± 0.11	9.24 ± 0.46	55.73 ± 2.79	nd	7.83 ± 0.39
	F	13.03 ± 0.42	19.14 ± 0.98	0.18 ± 0.01	1.09 ± 0.06	11.52 ± 0.12	56.11 ± 0.75	0.31 ± 0.02	7.70 ± 0.10
Amaranth	C	2.77 ± 0.14	23.40 ± 1.17	0.09 ± 0.01	4.70 ± 0.23	21.87 ± 1.09	36.88 ± 1.84	nd	0.61 ± 0.03
	F	8.00 ± 0.31	20.85 ± 0.05	0.48 ± 0.00	4.96 ± 0.05	32.63 ± 0.38	26.36 ± 0.09	3.28 ± 0.05	0.32 ± 0.01
Spent malt grain	C	7.24 ± 0.36	25.20 ± 0.02	0.17 ± 0.01	2.52 ± 0.13	11.91 ± 0.60	50.83 ± 2.54	nd	5.16 ± 0.26
	F	3.60 ± 0.10	22.37 ± 0.09	0.21 ± 0.00	2.96 ± 0.03	17.02 ± 0.15	47.05 ± 0.34	3.24 ± 0.07	2.94 ± 0.04
Rye bran	C	0.44 ± 0.02	22.48 ± 1,12	0.24 ± 0.01	5.53 ± 0.28	20.01 ± 1.00	39.57 ± 1.98	nd	2.93 ± 0.15
	F	2.58 ± 0.20	22.75 ± 0.03	1.02 ± 0.01	8.51 ± 0.62	28.98 ± 0.11	25.20 ± 0.07	5.19 ± 0.13	1.79 ± 0.05

C control, *F* fermented bioproduct, *TL* total lipids, *PA* palmitic acid, *POA* palmitoleic acid, *SA* stearic acid, *OA* oleic acid, *LA* linoleic acid, *GLA* γ-linolenic acid, *ALA* α-linolenic acid, *nd* not detected

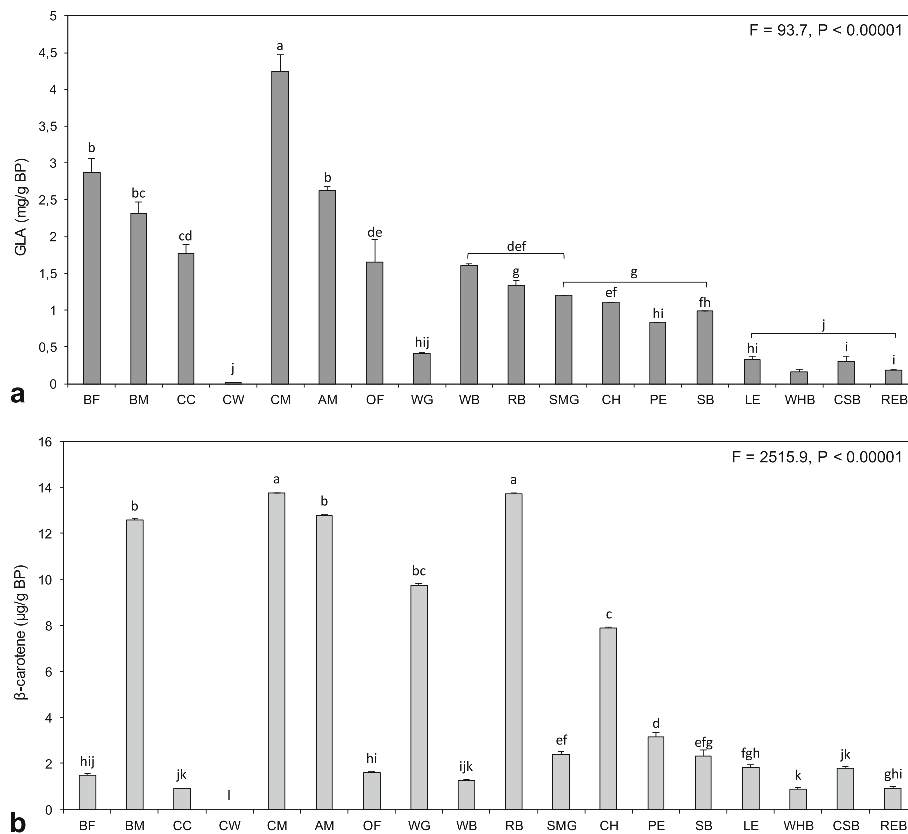

Fig. 1 Yield of GLA (mg/g BP) (**a**) and β-carotene (μg/g BP) (**b**) after solid-state fermentation using strain *Umbelopsis isabellina* CCF2412 on various types of cereal and legume substrates: BF, barley flakes; BM, barley meal; CC, crushed corn; CW, corn waste; CM, cornmeal; AM, amaranth; OF, oat flakes; WG, wheat germ; WB, wheat bran; RB, rye bran; SMG, spent malt grain; CH, chickpea; PE, pea; SB, soybeans; LE, lentil; WHB, white bean; CSB, color-spotted bean; REB, red bean. Columns bearing different letters are significantly different

Specific to β-carotene production, rye bran (13.73 μg/g BP) and amaranth (12.78 μg/g BP) were determined to be suitable. However, the highest amounts of GLA and β-carotene were reached with cultivation on cornmeal (GLA 4.23 mg/g BP; β-carotene 13.75 μg/g BP).

The use of different substrates may itself serve as an alternative for increasing the yields of the required metabolites. The aim of this experiment was, therefore, to confirm the ability of *U. isabellina* to utilize different legume substrates (Table 2). Legumes have been used traditionally as substrates in fermentation for the production of interesting bioproducts (such as Indian Kinema from spontaneously fermented soybeans or African Soumbala from locust beans), using both submerged fermentation and SSF processes (Sarkar et al. 2002).

Utilization of the legume substrates evaluated in the present study was, in general, several times lower than the cultivation using cereal substrates, which also produced low yields of GLA and β-carotene after the fermentation process (Fig. 1). Seven different legume substrates were evaluated, which revealed chickpea as the most suitable one (GLA 1.11 mg/g BP; β-carotene 7.88 μg/g BP). Nevertheless, the amounts of both the metabolites were significantly lower than those obtained after cultivation with cereal substrates. Among the evaluated substrates, all three types of beans were determined to be unsuitable. The lowest yields of GLA and β-carotene (GLA 0.16 mg/g BP; β-carotene 1.78 μg/g BP) were obtained with cultivation on white beans.

On the basis of these findings, legume substrates were determined to be unsuitable for SSF cultivation with the tested strain of *U. isabellina* (statistically important changes in the monitored values were observed only after cultivation on chickpea). In the present study, the next step of optimization of the cultivation conditions was not performed, and the study was focused on the optimization of fermentation conditions using different cereal substrates.

Optimization of GLA and β-carotene production

On the basis of the results of substrate screening, the next step of optimization of the fermentation conditions, which aimed at increasing the yield of both GLA and β-

Table 2 Content of lipids and profile of fatty acids in lipids of legumes substrates and fermented bioproducts

Substrate		TL (%)	PA (%)	POA (%)	SA (%)	OA (%)	LA (%)	GLA (%)	ALA (%)
Chickpea	C	5.70 ± 0.28	18.31 ± 0.92	nd	4.58 ± 0.23	23.97 ± 1.20	49.53 ± 2.48	nd	1.95 ± 0.10
	F	4.45 ± 0.22	10.81 ± 0.38	0.28 ± 0.05	1.88 ± 0.18	24.85 ± 0.49	54.44 ± 1.27	2.48 ± 0.14	2.69 ± 0.11
Lentil	C	1.09 ± 0.05	14.07 ± 0.70	nd	2.92 ± 0.15	31.70 ± 1.58	43.44 ± 2.17	nd	6.74 ± 0.34
	F	1.24 ± 0.26	12.76 ± 0.10	0.26 ± 0.04	2.56 ± 0.19	32.46 ± 0.38	39.72 ± 0.69	2.67 ± 0.18	6.51 ± 0.20
Soya	C	10.46 ± 0.52	17.11 ± 0.86	nd	8.64 ± 0.43	9.69 ± 0.48	58.48 ± 2.92	nd	6.08 ± 0.30
	F	18.41 ± 0.40	10.03 ± 0.08	nd	3.87 ± 0.10	19.35 ± 0.10	55.89 ± 0.16	0.54 ± 0.02	9.23 ± 0.01
Pea	C	1.00 ± 0.05	15.77 ± 0.79	nd	6.59 ± 0.33	27.57 ± 1.38	46.87 ± 2.34	nd	3.19 ± 0.16
	F	1.03 ± 0.03	14.42 ± 1.20	nd	5.00 ± 0.41	29.09 ± 0.08	38.33 ± 0.48	8.14 ± 0.15	4.12 ± 0.03
White bean	C	1.10 ± 0.05	27.44 ± 1.37	nd	6.81 ± 0.34	11.36 ± 0.57	25.23 ± 1.26	nd	27.30 ± 1.36
	F	1.48 ± 0.15	24.99 ± 0.36	nd	5.72 ± 0.43	13.63 ± 0.11	24.94 ± 0.15	1.26 ± 0.00	26.79 ± 0.01
Red bean	C	1.35 ± 0.07	28.64 ± 1.43	nd	4.47 ± 0.22	10.40 ± 0.52	28.80 ± 1.44	nd	25.78 ± 1.29
	F	0.87 ± 0.01	20.27 ± 0.36	0.32 ± 0.02	3.55 ± 0.01	12.70 ± 0.45	26.66 ± 0.91	2.14 ± 0.05	30.52 ± 0.17
Color-spotted bean	C	1.32 ± 0.07	30.17 ± 1.51	nd	4.44 ± 0.22	8.79 ± 0.44	33.51 ± 1.68	nd	19.76 ± 0.99
	F	0.98 ± 0.07	22.65 ± 0.28	0.49 ± 0.08	3.30 ± 0.24	14.84 ± 1.38	31.11 ± 0.74	3.06 ± 0.47	20.72 ± 1.42

C control, *F* fermented bioproduct, *TL* total lipids, *PA* palmitic acid, *POA* palmitoleic acid, *SA* stearic acid, *OA* oleic acid, *LA* linoleic acid, *GLA* γ-linolenic acid, *ALA* α-linolenic acid, *nd* not detected

carotene in the final fermented bioproduct, was performed. All the subsequent fermentations in this regard were performed on the cornmeal substrate.

It is well known that the membrane lipids in microorganisms are influenced by the cultivation temperature used. Microorganisms, including the Zygomycetes fungi, are able to adapt to environmental stress by regulating the fluidity of their membranes through lipid desaturation (Conti et al. 2001). In addition, cultivation temperature has been reported to affect the composition of cellular fatty acids in Mucorales (Michinaka et al. 2003; Dyal et al. 2005). In present study, fermentations were performed at two different temperatures (20 °C and 28 °C). In consideration of the energy requirements of the process operation, solid-state fermentation performed at 28 °C, which is close to the ambient temperature of the environment in which the evaluated strain dwells naturally (in balneological peloid) (Jangbua et al. 2009), resulted in the adequate values of biomass production and GLA content. After fermentation at 20 °C, GLA amount obtained was only 1.57 mg/g BP. On the contrary, the yield of GLA after cultivation at 28 °C was 5.48 mg/g BP, which was several times higher comparatively. Differences in the β-carotene content were of a similar character. Data analysis confirmed that only trace amounts of β-carotene (0.37 μg/g BP) were obtained after cultivation at 20 °C. However, in the case of fermentation at 28 °C, statistically significant increase in the β-carotene content was observed, as the obtained fermented bioproduct contained 27.43 μg/g BP of β-carotene. Therefore, the hypothesis that higher temperature is more suitable for the growth and metabolism of *U. isabellina* was confirmed. The results are presented in Fig. 2.

An additional possibility was to influence the production of the studied metabolites through the use of different amounts of water added to the substrate for swelling prior to the fermentation process. It is well known that the amount of water is one of the key features in the SSF process and depends on the material used (Oriol et al. 1988). In this study, six different amounts of water (50%, 75%, 100%, 125%, 150%, and 200% w/w distilled water per gram of substrate) were tested. Yields of GLA and β-carotene in the fermented bioproduct are shown in Fig. 3. As evident from the figure, the least suitable

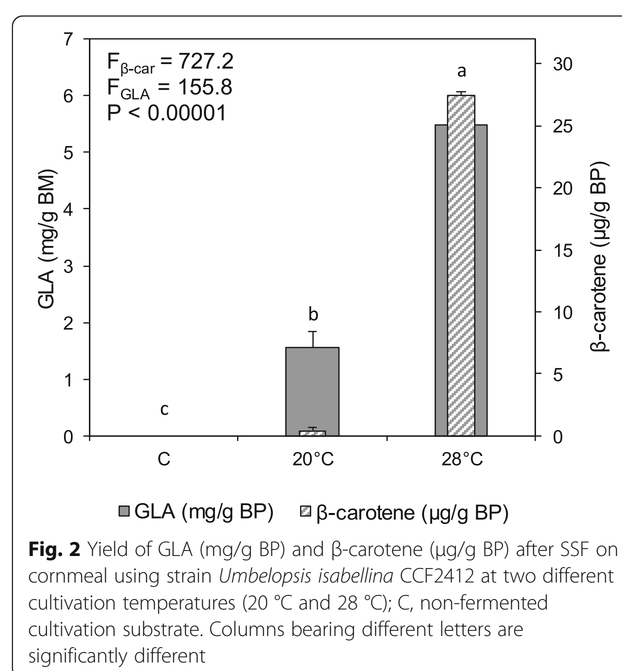

Fig. 2 Yield of GLA (mg/g BP) and β-carotene (μg/g BP) after SSF on cornmeal using strain *Umbelopsis isabellina* CCF2412 at two different cultivation temperatures (20 °C and 28 °C); C, non-fermented cultivation substrate. Columns bearing different letters are significantly different

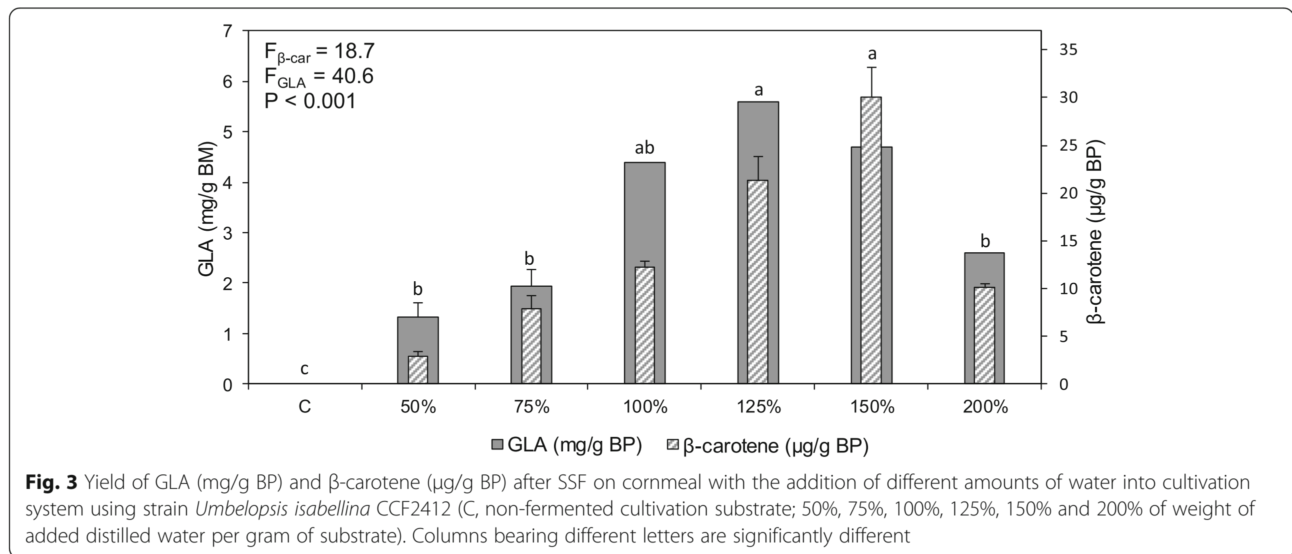

Fig. 3 Yield of GLA (mg/g BP) and β-carotene (μg/g BP) after SSF on cornmeal with the addition of different amounts of water into cultivation system using strain *Umbelopsis isabellina* CCF2412 (C, non-fermented cultivation substrate; 50%, 75%, 100%, 125%, 150% and 200% of weight of added distilled water per gram of substrate). Columns bearing different letters are significantly different

amounts observed were very low (50% and 75%) and very high (200%). This might have occurred because of a decrease in the metabolism of *U. isabellina* at these amounts of water, which resulted in the low production of the studied metabolites. The absolute minimum value of GLA, as well as β-carotene content, was reached after cultivation using 50% of distilled water (GLA 1.33 mg/g BP; β-carotene 2.89 μg/g BP). Statistically important changes in the GLA content were observed after fermentation on the substrate with 125% and 150% addition of distilled water. The highest amount of β-carotene content (30.13 μg/g BP) in the final fermented product was reached using 150% addition of distilled water. However, the highest GLA accumulation (5.59 mg/g BP) was obtained after fermentation using 125% addition of distilled water. Since the yields of β-carotene after fermentation with 125% water addition also reached 21.3 μg/g BP, 125% addition of distilled water was selected as an optimal condition for the co-production for both the major metabolites studied.

Kinetics of GLA and β-carotene formation

The aim of this experiment was to determine the rate of substrate utilization, fungal biomass (FBM) formation, and the biosynthesis rate of fatty acids. Furthermore, the experiment was focused on the yields of GLA and β-carotene in the obtained fermented bioproducts after fermentation. The fermentation lasted for 7 days, and sampling was performed every 24 h beginning from the second day.

Substrate utilization was observed to increase gradually, together with an increase in fungal biomass (Fig. 4a). The highest values were achieved on the seventh day when substrate utilization reached 33.2% and the fungal biomass levels reached 256.11 mg/g BP. Therefore, it could be concluded that the fungal strain of *U. isabellina* was able to efficiently utilize cornmeal as a substrate for its own metabolism. Furthermore, the strain was able to synthesize fatty acids, as it has been described as an oleaginous strain previously as well. The content of total fatty acids was raised from 3.26% to 19.17% after fermentation (Fig. 4b).

The yield of GLA increased every day during cultivation and reached 11.45 mg/g BP (44.71 mg/g of FBM) after 7 days of fermentation (Fig. 4c). As revealed by the data obtained, most of the GLA content in the fungal biomass was formed during the early stage of fermentation in the exponential growth phase of the strain. In comparison to the study conducted by Čertík et al. (2013), growth kinetics of *U. isabellina* were observed to be slightly different from those of the other Zygomycetes strain *Mucor circinelloides*. The amount of GLA obtained during cultivation using this strain reached its maximum level (3.4 mg/g BP) in just 5 days, following which a progressive step-down was observed in the GLA content. A similar profile of GLA content in the fermented products has been reported for *Mucor rouxii* as well, when cultivated on soybean meal (Jangbua et al. 2009). Culture, as well as GLA content, grew only for 1–2 days (maximum amount reached was just 1.8 mg/g BP), followed by a gradual decrease in both with prolonged cultivation. On the contrary, GLA content obtained with cultivation using the same strain and a different substrate (polished rice) in the same study was described to increase gradually during the cultivation period. Therefore, it may be stated that the GLA content profile during the cultivation period varies according to the selected fungal strain and is also dependent on the substrate used.

The amount of 11.45 mg/g BP of GLA obtained in the fermented bioproduct of the present study may be useful

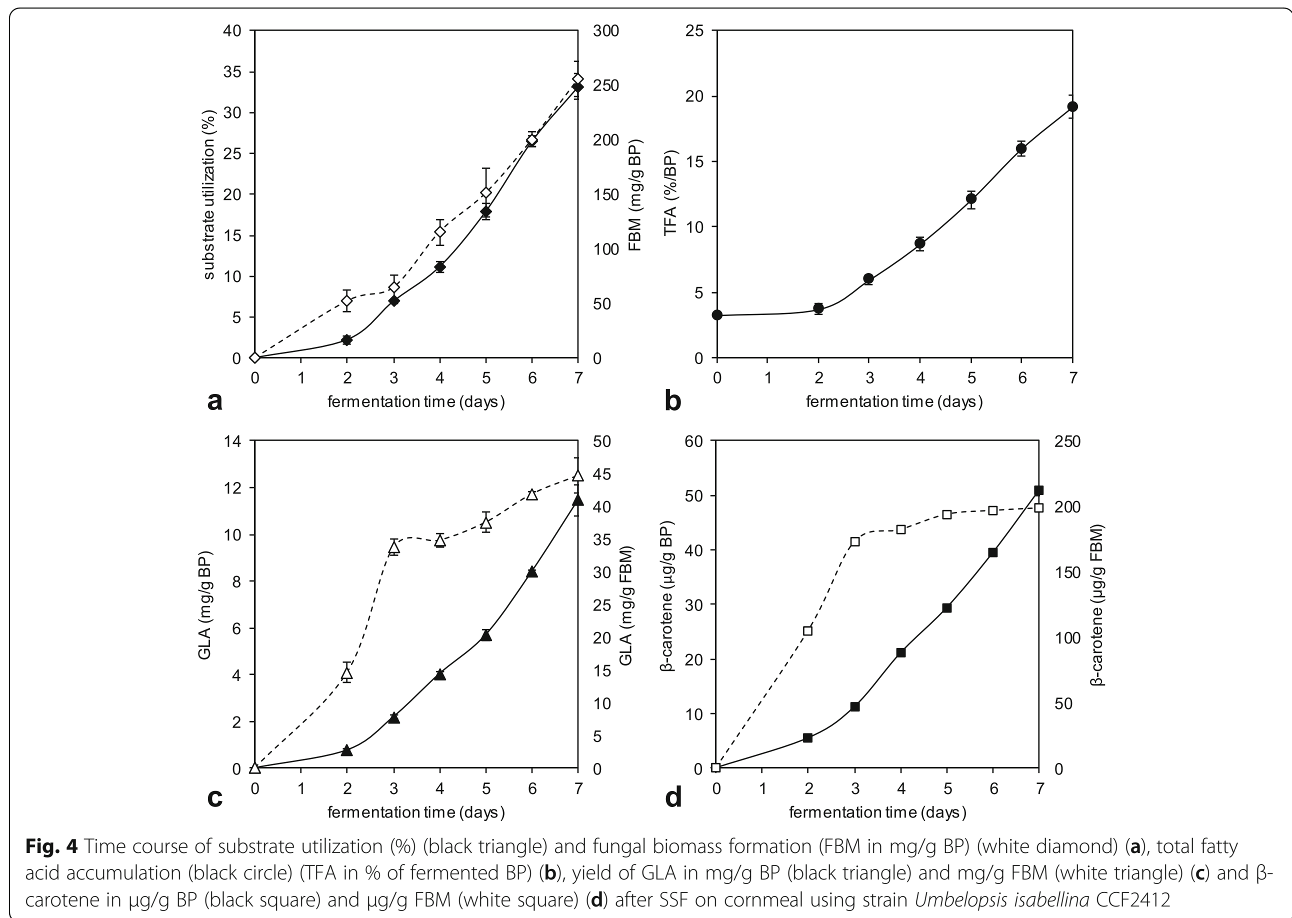

Fig. 4 Time course of substrate utilization (%) (black triangle) and fungal biomass formation (FBM in mg/g BP) (white diamond) (**a**), total fatty acid accumulation (black circle) (TFA in % of fermented BP) (**b**), yield of GLA in mg/g BP (black triangle) and mg/g FBM (white triangle) (**c**) and β-carotene in μg/g BP (black square) and μg/g FBM (white square) (**d**) after SSF on cornmeal using strain *Umbelopsis isabellina* CCF2412

for a possible industrial application. In a research conducted by Stredanský et al. (2000), in which the usage of *Thamnidium elegans* CCF 1456 under optimized conditions was described, GLA yields as high as 3.50 mg/g substrate were obtained, which is almost 3.5 times lower than the yields obtained in the present study.

When the results of GLA accumulation in the fungal biomass of the very same strain obtained in case of submerged fermentation (17.5 mg of GLA/g DCW fungal biomass) were compared to the results of the present study, it was apparent that solid-state fermentation process is far more suitable for fungal strain metabolism (Klempová et al. 2013).

The yield of β-carotene was also observed to increase during the fermentation. Similar to GLA formation, β-carotene biosynthesis also occurred mostly during the exponential growth phase. The final value reached after 7 days of cultivation was 50.90 μg/g BP (198.73 μg/g FBM) (Fig. 4d), which was almost six times higher than the value of β-carotene production by *M. circinelloides* in a study conducted by Čertík et al. (2013).

Since it is possible that sterols play an essential role in the physiological processes of almost all living organisms including Zygomycetes, the next parameter which was assessed was the amount of microbial sterols produced (McDonald et al. 2012). As depicted in Fig. 5, the fermented bioproduct obtained through cultivation using *U. isabellina* contained several microbial sterols. The level of individual sterols changed at different time points of the cultivation process. A gradual loss of a plant sterol, namely, β-sitosterol, which occurs naturally in cornmeal, was observed (Wrigley et al. 2015). At the same time, the amounts of several microbial sterols increased progressively, especially the amount of an industrially significant sterol, ergosterol. It was observed that the fermented bioproduct contained another microbial sterol, ergosta-5,8-dien-3-ol, which was present in a relatively high proportion, i.e., up to 45% of all the sterols present. Since limited information regarding this sterol is available in the literature, the biological functions performed by this sterol in the organisms remain unknown (Giera and Bracher 2008). It was assumed that this sterol, which contained a complex system of conjugated double bonds, was a product of ergosterol degradation.

Broiler chicken feed production

One of the most interesting applications in which fermented bioproduct may be effectively used is animal

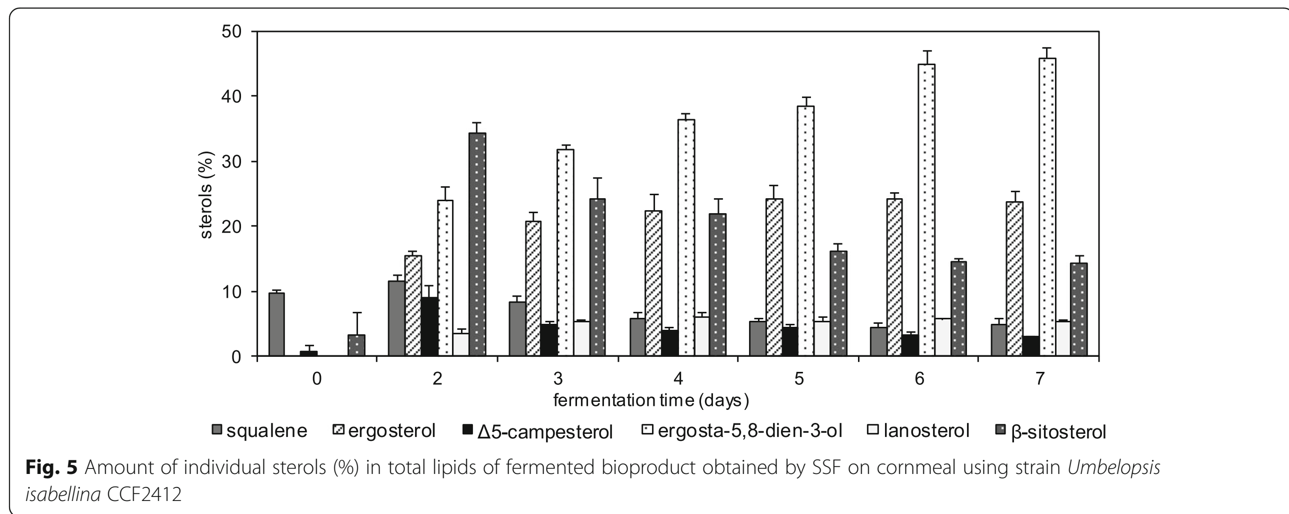

Fig. 5 Amount of individual sterols (%) in total lipids of fermented bioproduct obtained by SSF on cornmeal using strain *Umbelopsis isabellina* CCF2412

feed. Broiler chicken is a well-known species, which is used widely for breeding, mainly because of its ability to grow rapidly. This reflects in high production of chicken meat, which greatly favors the economics of the whole breeding process. Selecting a suitable feed may further enhance the growth of broilers, along with improving the quality of the meat itself.

Since cornmeal is a complex feed, with a wide range of applications, its usage for fattening chickens is quite expensive. Production of fermented bioproduct using cornmeal as a substrate, which could have been used for chicken feeding, is not suitable, mainly due to the high amounts of cornmeal spent for feeding. On the other hand, substrates that are essentially wastes of agricultural and agro-industrial productions are conveniently available, inexpensive, and represent a group of products that may be converted into high-value feed products through SSF. The main objective of using these bioproducts for broiler feeding is the production of meat enriched with ω−6 fatty acids and carotenoid pigments. Such meat is healthier for human consumption and adequately supplements the nutritional profile of the human diet.

Results from the screening of different cereal substrates (Fig. 1) indicated that adequate levels of GLA accumulation (1.60 mg/g BP) were reached using wheat bran as a substrate. The problem encountered while using this substrate was the low accumulation of β-carotene.

Since the strain *U. isabellina* CCF2412 has been previously described as an efficient producer of β-carotene in the submerged fermentation process (Klempová et al. 2013), different strategies in SSF process using fed-batch cultivation were applied in the present study. Given the necessity of high feed volumes required in the whole chicken feeding process, scaling-up of the fermented bioproduct production was performed. Wheat bran substrate was fermented for 7 days, and on the third day, mycelia obtained in submerged fermentation were added. The obtained final bioproduct was enriched in both GLA (1.75 mg/g BP) and β-carotene (1.39 μg/g BP) and was applied as a supplement for broiler chicken feed. The basic commercial feed available in the market lacks both of these metabolites. Improved quality of chicken meat was observed after feeding the chicken with a mixture of feed and the fermented bioproduct. In addition, the presence of these metabolites has been reported to improve the immune status of the produced chicken meat (Mudroňová et al. 2018).

The fact that the contents of GLA and β-carotene in the produced chicken feed were several times lower than those obtained after fermentations performed on laboratory scales warrants further research in this regard. The main objective of the present study was to reach comparable results in higher volumes of fermented feed production, which would lead to higher accumulation of both the studied metabolites and further improved quality of the chicken meat produced.

Concluding remarks

The present study is the pioneer in describing the co-production of GLA and β-carotene using the *Umbelopsis isabellina* CCF2412 strain in SSF. Legume substrates appeared to be unsuitable for the co-production of the studied metabolites in the SSF process using the tested fungal strain. On the contrary, after optimization of the fermentation conditions, the tested strain was determined to be an efficient producer of both GLA (11.45 mg/g BP) and β-carotene (50.90 μg/g BP) using cornmeal as a cereal substrate. It was possible to produce a potentially high-value bioproduct with a high content of the studied metabolites using inexpensive inputs. Adequate accumulation of both the studied metabolites, using cornmeal as an inexpensive substrate, encourages further experiments focusing on the possible

overproduction of biologically active compounds. The tested strain was subsequently applied for the production of fermented feed for broiler chicken, which resulted in the production of improved quality of meat, although further research is nonetheless required in this regard.

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Authors' contributions
OS carried out all cultivation experiments, run the sample analysis and data evaluation,preformed statistical analysis and draft the manuscript. TK conceived of the study and participated in its design and coordination, run data evaluation, reviewed and edited the manuscript, participated in funding acquisition. SM supervised the study and participated in funding acquisition. CM reviewed the manuscript, supervised the study and participated in funding acquisition. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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