**JOICE RAÍSA BARBOSA CUNHA**

# FERMENTADOS DE MACROFUNGOS CULTIVADOS EM TORTAS DE SEMENTES DE ALGODÃO OU PINHÃO-MANSO COMO FONTES DE BIOATIVOS, ENZIMAS E NUTRIENTES PARA PRODUÇÃO DE BACTÉRIAS PROBIÓTICAS

**LAVRAS-MG**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de Doutor.

Prof. Dr. Eustáquio Souza Dias

Orientador

Dr. Félix Gonçalves de Siqueira

Coorientador

LAVRAS-MG

2021

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# MACROFUNGI FERMENTED CULTIVATED IN COTTON SEED PIE OR JAW PINION AS SOURCE OF BIOACTIVES, ENZYMES AND NUTRIENTS FOR THE PRODUCTION OF PROBIOTIC BACTERIA

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de Doutor.

APROVADA em 29 de setembro de 2021
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*Aos meus amados pais, Regina Barbosa e Joilson Cunha e à minha irmã, Jeysa Cunha pelo apoio e carinho.*

*Dedico*

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## RESUMO

A adoção de novas matérias-primas para produção de biodiesel depende, entre outras coisas, do valor comercial de seus coprodutos, já que o mercado da torta de soja é importante na indústria de ração animal. Os óleos de algodão e de pinhão-manso têm boas qualidades para o uso como biodiesel, mas suas tortas residuais são tóxicas e a destoxificação fúngica delas pode possibilitar o seu uso na nutrição animal, agregando valor a esses coprodutos. Este trabalho teve o objetivo de avaliar a eficiência dos macrofungos das coleções do Instituto Nacional de Pesquisas do Amazônas (INPA) e da Embrapa Agroenergia na destoxificação de torta de caroço de algodão (CSC) e torta de semente de pinhão-manso (JSC), por meio de fermentação em estado sólido (FES), a caracterização química dessas biomassas, e as bioatividades dos fermentados quanto a potencial aplicação como antimicrobiano e aditivo para nutrição animal. Dos 26 fungos viáveis, 16 foram capazes de crescer nas duas biomassas vegetais (CSC e JSC). Quatro desses fungos foram capazes de reduzir os níveis de ésteres de forbol na TSPM para níveis considerados atóxicos (<0,09 mg/g) e pelo menos um degradou eficientemente o gossipol livre na CSC. Os fermentados dos macrofungos *Coriolopsis sp.* INPA1646 e *Tyromyces sp.* INPA1696 foram selecionados para as demais caracterizações químicas quando crescidos em JSC e CSC, respectivamente. Os fermentados apresentaram aumento das concentrações de ergosterol, redução da atividade antioxidante destes coprodutos e produção eficiente de enzimas protease e lacase, principalmente pelo *Tyromyces sp.* na CSC. As análises químicas mostraram que não houve degradação de lignina pelos fungos e, por isso, não reduziu o teor de fibras das biomassas durante o tempo de fermentação (15 dias). No entanto, os fermentados apresentaram aumento nas concentrações de proteínas solúveis, pela ação proteolitíca das enzimas secretadas pelos macrofungos. As análises de atividade prebiótica dos fermentados fúngicos (sólidos e submersos) e biomassas vegetais mostraram que o sobrenadante seco de JSC não fermentada é mais eficiente do que glicose, FOS e inulina em estimular o crescimento de *L. acidophilus*, *B. lactis*, *L. plantarum* e *L. rhamnosus*. Além disso, o uso de CSC na fermentação submersa de *Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *Panus lecomtei* CC40 e *Pleurotus pulmonarius* EF88 apresentou sobrenadantes mais favoráveis ao crescimento de *L. acidophilus.* Estes mesmos macrofungos geraram sobrenadantes mais favoráveis ao crescimento de *B. lactis* na fermentação estado sólido utilizando JSC. Para atividade antimicrobiana, nenhum tratamento apresentou halo de inibição das bactérias enteropatogênicas igual ou maior ao antibiótico. No entanto, os extratos aquosos dos fermentados de *P. lecomtei* CC40 e de *Coriolopsis* sp. INPA1646, na condição de cultivo sólido em CSC, na concentração de 100 mg/mL, apresentaram halos de inibição com diâmetros de 1,2 e 0,73 cm, respectivamente, para a cepa de *Salmonella enterica*. Além disso, os sobrenadantes do crescimento de *L. acidophilus, B. lactis* e *L. rhamnosus* cultivados nos extratos aquosos e sobrenadantes de todos os macrofungos, nas diferentes condições, foram mais eficientes na inibição do crescimento de *S. aureus* que os controles, o que indica possível atividade antimicrobiana.



**Palavras-chaves:** Basidiomicetos. Bactérias probióticas. Atividade antimicrobiana.

## ABSTRACT

The adoption of new raw materials depends, among other things, on the commercial value of its co-products, since soybean cake market is important in the animal feed industry. Cotton and Jatropha oils have good qualities for use as biodiesel, but their residual cakes are toxic and fungal detoxification can allow its use in animal nutrition, adding value to these co-products. This study aimed to evaluate the efficiency of macrofungi (Basidiomycetes) from the collections of the Instituto Nacional de Pesquisas do Amazonas (INPA) and Embrapa Agroenergia in the detoxification of cotton seed cake (CSC) and jatropha seed cake (JSC) by solid state fermentation (SSF), as well as the bromatological characterization of these biomasses, the bioactivities of the fermented ones and the potential application as an antimicrobial and additive for animal nutrition. From 26 viable fungi, 16 of them were able to grow in the both biomasses (CSC and JSC). Four of these fungi were able to reduce the levels of phorbol esters in JSC to levels considered non-toxic (<0.09 mg/g) and at least one efficiently degraded free gossypol in CSC. The fermented macrofungi *Coriolopsis* sp. INPA1646 and *Tyromyces* sp. INPA1696 were selected for the other characterizations and their growth in CSC and JSC, respectively, resulted in increased concentrations of ergosterol, reduced antioxidant activity in these co-products and efficient production of cellulose enzymes, laccases and proteases, mainly by *Tyromyces* sp. in the CSC. The bromatological and structural characterization analysis showed that there was no degradation of lignin by the fungi and, therefore, did not reduce the fiber content of the biomass during the fermentation time (15 days), but confirmed its high protein concentrations and the growth of the fungi resulted in solubilization of most of these proteins. The prebiotic activity analysis of the solid and submerged fermented of these biomasses showed that the dry supernatant of unfermented JSC is more efficient than glucose, FOS and inulin in stimulating the growth of *L. acidophilus*, *B. lactis*, *L. plantarum* and *L. rhamnosus*. In addition, the submerged fermentation of CSC by *Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *Panus lecomtei* CC40 and *Pleurotus pulmonarius* EF88 made the supernatant of this biomass more efficient for the growth of *L. acidophilus* and the solid fermentation of JSC by these fungi produced a more efficient aqueous extract for the growth of *B. lactis*. About antimicrobial activity, no treatment presented an inhibition halo of enteropathogenic bacteria equal to or greater than that of Ampicillin. However, the aqueous extracts of CSC fermented by *P. lecomtei* CC40 and *Coriolopsis* sp. INPA1646, at 100 mg/mL concentration, showed inhibition halos with diameters of 1.2 and 0.73 cm, respectively, for the tested *S. enterica* strain. In addition, the growth supernatants of *L. acidophilus*, *B. lactis* and *L. rhamnosus* in the fermentation products of the biomasses were more efficient in inhibiting the growth of *S. aureus* than their supernatants in the control media, which indicates possible antimicrobial activity.



**Keywords:** Basidiomycetes. Probiotic bacteria. Antimicrobial activities.

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## LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

| | |
|---|---|
| A | Absorbância |
| ABTS+ | Ácido 2,2'-azinobis-(3-etilbenzotiazolino-6-sulfônico) |
| ADF | Acid detergent fiber |
| ASE | Extrator Acelerado por Solvente |
| ATP | Adenosina trifosfato |
| ATCC | American Type Culture Collection |
| BCA | Bicinchoninic acid |
| BG | Bound gossypol |
| BOD | Biochemical Oxygen Demand |
| CC40 | Código do fungo 40 da coleção CC |
| CF | Crude fiber |
| CFU | Unidades formadoras de colônia |
| CMC | Carboximentilcelulose |
| CMMABio | Microorganisms and Microalgae Collection applied to the biorefinery |
| $CO_2$ | Dióxido de carbono |
| CP | Crude protein |
| CSC | Cottonseed cake |
| DNS | Ácido 3,5-dinitrosalicílico |
| D.P. | Desvio padrão |
| DPPH | 2,2–Difenil–1–picril–hidrazila |
| EDTA | Ethylenediamine tetraacetic acid |
| EE | Ether extract |
| EF | Elongation Factor |
| EF88 | Código do fungo 88 da coleção EF |
| FG | Free gossypol |
| FDA | Food and Drug Administration |
| $FeCl3.6H_2O$ | Cloreto de ferro III |
| FOS | Fructooligosaccharides |
| GOD-POD | Glucose oxidase/peroxidase |
| HCl | Hydrochloric acid |
| $H_2O_2$ | Peróxido de hidrogênio |
| HPLC | Cromatografia Líquida de Alto Desempenho |
| IgA | Imunoglobulina A |
| Il-2 | Interleucina-2 |
| INF- γ | Interferon-γ |
| INPA | Instituto Nacional de Pesquisas do Amazonas |
| ITS | Internal Transcribed Spacer |
| IU | Unidade Internecional |
| JSC | Jatropha seed cake |
| kg/ha | Quilômetros por hectare |
| KOH | Hidróxido de potássio |
| LiP | Lignin peroxidase |
| M | Molar |
| mg/g | Miligramas por grama |
| µg/g | Microgramas por grama |
| mg/mL | Miligramas por mililitros |
| mL | Mililitros |

| | |
|---|---|
| µL | Microlitros |
| MM | Mineral matter |
| MnP | Manganese peroxidase |
| ng | Nanogramas |
| µmol | Micromolar |
| mbar | Milibar – Unidade de pressão |
| Mm | Milímetros |
| mM | Milimolar |
| MRS | Meio de Man Rogosa & Sharpe |
| MRS(-C) | Meio de Man Rogosa & Sharpe sem glicose |
| NaCl | Cloreto de sódio |
| NDF | Neutral detergent fiber |
| NREL | Laboratório Nacional de Energia Renovável |
| OD | Densidade ótica |
| OD 600 | Densidade ótica num comprimento de onda de 600nm |
| PCR | Reação em cadeia da polimerase |
| PDA | Potato Dextrose Agar |
| pH | Potencial hidrogeniônico |
| PKC | Proteína quinase C |
| PNPB | Programa Nacional de Produção e Uso de Biodiesel |
| ppm | Partes por milhão |
| rpm | Rotações por minuto |
| SCFA | Ácidos graxos de cadeia curta |
| SD | Desvio padrão |
| SLC | Lignin/Cellulose Selectivity |
| SLH | Lignin / Holocellulose Selectivity |
| SmF | Submerged fermentation |
| SMS | Spent mushroom substrate |
| SSF | Solid-state fermentation |
| TAA | Total antioxidant activity |
| TCA | Ácido tricloroacético |
| TDN | Total digestible nutrients |
| TNF-α | Fator de necrose tumoral-α |
| TPC | Total phenolic compounds |
| Tris-HCl | Tris(hydroxymethyl)aminomethane hydrochloride |
| Trolox | 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid |
| U | Unidade internacional – Unidade de atividade enzimática |
| UHPLC | Ultra High Performance Liquid Chromatography |
| UnB | Universidade de Brasília |
| W | Watts – Unidade de potência |
| x g | Gravidade - Unidade de força centrífuga |

**SUMÁRIO**

## INTRODUÇÃO GERAL

O Brasil é reconhecido pelo seu esforço na diversificação da matriz energética. O Programa Nacional de Produção e Uso de Biodiesel (PNPB), implantado em dezembro de 2004, evoluiu de forma bastante eficiente, superando as metas iniciais, que previa adição de 5% de biodiesel ao diesel de petróleo, e já atingiu 12% (ANP, 2017). Os incentivos à inclusão da agricultura familiar na cadeia produtiva do biodiesel (CONCEIÇÃO *et al*., 2015) podem ter corroborado para esse resultado.

Mas como qualquer agroindústria, esta também gera grandes volumes de coprodutos. Neste caso, os coprodutos são farelos e tortas, obtidos após a extração química ou mecânica, respectivamente, do óleo das sementes oleaginosas, que representam mais de 50% da massa inicial das sementes e a utilização desses resíduos é fundamental para agregar valor à cadeia produtiva do biodiesel e minimizar os impactos ambientais (RODRIGUES e RONDINA, 2013).

A principal matéria-prima para produção de biodiesel no Brasil é a soja, cujo subproduto, a torta ou farelo proteico, tem grande importância comercial para nutrição animal (SEDIYAMA *et al.*, 2013). A inserção de novas matérias-primas na produção de biodiesel depende não só do rendimento e das características favoráveis do óleo, mas também do valor agregado de seus coprodutos (PATIL e DENG, 2009).

Oleaginosas como algodão (*Gossypium hirsutum* L.) e pinhão-manso (*Jatropha curcas* L.) têm quantidade e qualidade de óleo satisfatórios para produção de biodiesel (LAVIOLA *et al*., 2012; GOMES, 2015; EDRISI *et al*., 2015). No entanto, os coprodutos da extração destes óleos (tortas ou farelos) tem menor valor agregado que os gerados pela soja. A torta de algodão (CSC) é usada, apenas em pequenas quantidades para a suplementação da alimentação de animais ruminantes não reprodutores (BELTRÃO *et al*., 2000; CARNEIRO *et al.*, 2007; KONG, DAUD, ZHU, 2010), e a de pinhão-manso (JSC) é usada apenas como adubo orgânico, na India, por exemplo (MAKKAR e BECKER, 2009).

A utilização destes coprodutos na alimentação animal não é viável devido à presença de compostos tóxicos e antinutricionais, que causam efeitos negativos no crescimento, reprodução e desempenho dos animais que a consome, como o gossipol livre na torta de caroço de algodão (CSC) (LIU *et al*., 2008; KONG, DAUD, ZHU, 2010) e os ésteres de forbol na torta de semente de pinhão-manso (JSC) (HE *et al*., 2011; INSANU *et al*., 2013).

Neste sentido, a degradação destes compostos e destoxificação destas tortas agregaria valor a esses coprodutos, aumentando e viabilizando a inserção destas oleaginosas ao programa de biodiesel do Brasil.

Tratamentos químicos e físicos foram, e ainda são estudados e utilizados para destoxificar essas tortas. Porém, no geral, não são totalmente eficazes, ou econômica ou ecologicamente viáveis. No entanto, processos fermentativos têm indicado que macrofungos da classe dos basidiomicetos são capazes de degradar os compostos tóxicos destes coprodutos (RAJARATHNAM, SHASHIREKHA, BANO, 2001; AREGHEORE *et al*., 2003; BELUWE e SAM, 2010; KASUYA *et al*., 2012; DA LUZ *et al*., 2013; GOMES, 2015; CUNHA, 2017; ARAÚJO, 2018). Aparentemente, por meio das enzimas extracelulares liberadas durante o seu crescimento (SOARES NETO, 2019; GOMES, 2019).

Além de destoxificarem eficientemente essas biomassas, o crescimento destes macrofungos pode aumentar a digestibilidade destas biomassas. Isso ocorreria por degradação da lignina, digerindo parcialmente as fibras, e enriquecendo nutricionalmente. O enriquecimento ocorre pelo aumento do teor proteico, solubilizando a proteína bruta e/ou adicionando as moléculas bioativas fúgnicas comumente encontradas nesses macrofungos (MENDONCA, 2010; GOMES, 2015).

Os compostos bioativos presentes em muitos macrofungos formadores de cogumelos comestíveis, como polissacarídeos (beta-glucanas), ergosteróis, proteínas, fibras, lectinas, fenóis, polifenóis, terpenóides e compostos orgânicos voláteis são os responsáveis pelas estudadas bioatividades, que são usados há anos na medicina tradicional chinesa e como alimentos nutracêuticos (EL ENSHASY e HATTIKAUL, 2013).

Estes compostos químicos com atividades prebiótica, antioxidante, antimicrobiana, anti-inflamatória, imunomoduladora, antitumoral, antiviral, hipocolesterolemiante e anti-hiperglicêmica estão presentes não só nos cogumelos, mas também no micélio vegetativo destes fungos (GIBSON *et al*., 2004; EL ENSHASY e HATTIKAUL, 2013; KALAČ, 2013; MATTILA, MARNILA e PIHLANTO, 2017).

Neste sentido, a utilização destes micélios na alimentação animal, por meio da fermentação estado sólido em biomassas vegetais ricas em proteínas, tais como as tortas de sementes oleaginosas, apresentam potencial auxilio na saúde, favorecendo significativamente a microbiota intestinal dos animais. Tais compostos podem estimular e aumentar as populações de *Lactobacillus* e Bifidobactérias. Estas por sua vez, podem ajudar na modulação do sistema imunológico com aumento da expressão e secreção de imunoglobulina A (IgA),

interleucina-2 (Il-2), interferon-γ (INF- γ) e fator de necrose tumoral-α (TNF-α) (SHANG *et al*., 2016; CHEN *et al*., 2017). O uso de produtos fermentados por basidiomicetos na alimentação animal já é uma realidade comercial, com apresentação de benefícios nutraceuticos (FYSAL SOLUTE, 2020).

## OBJETIVO GERAL

Avaliar fermentados de macrofungos cultivados em tortas de sementes olegionosas como pontenciais fontes para obtenção de compostos químicos bioativos em vista à aplicação na nutrição animal.

## OBJETIVOS ESPECÍFICOS

➔ OEI: Determinar macrofungos capazes de destoxificar as JSC e/ou CSC, degradando ésteres de forbol e gossipol;

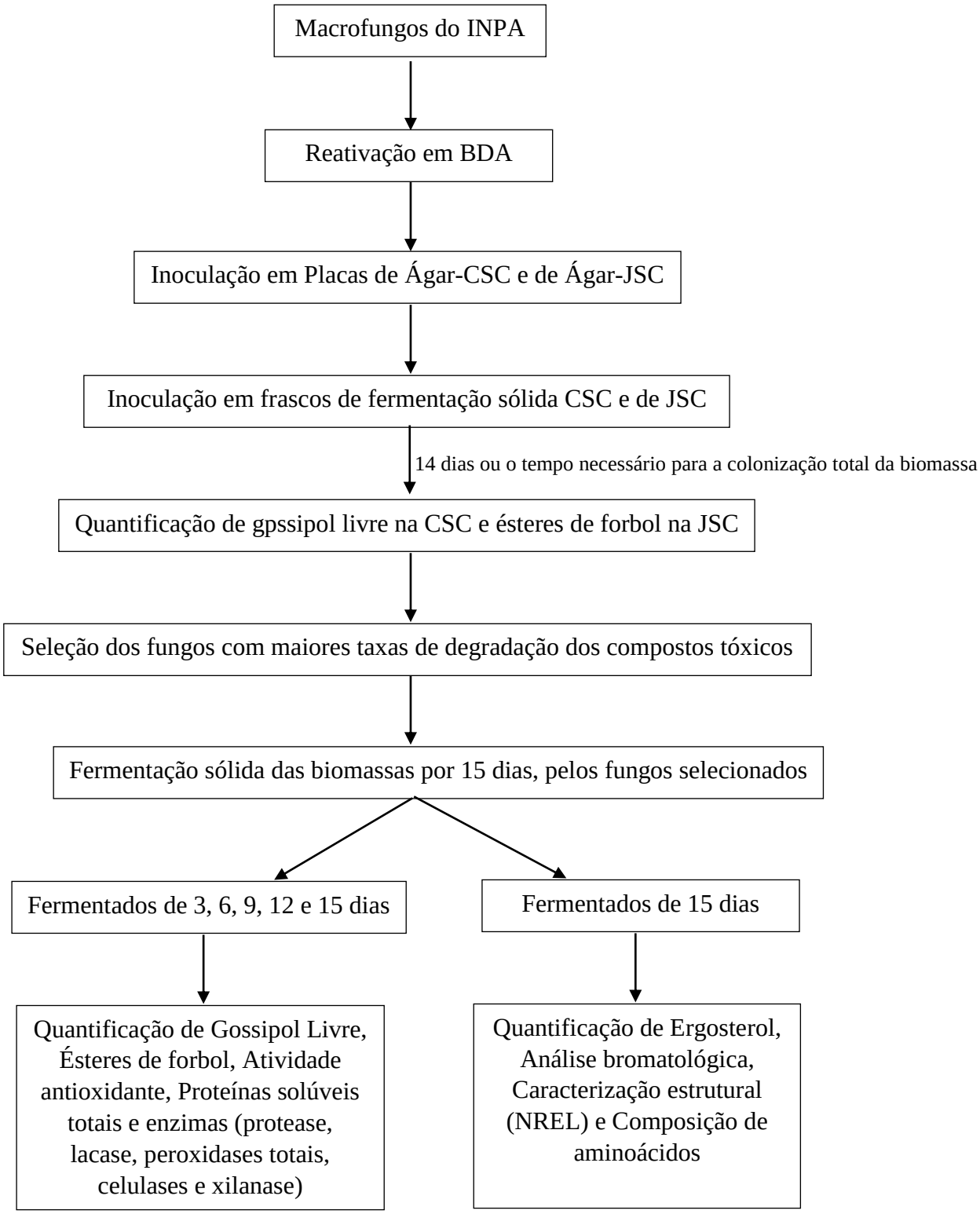
➔ OE2: Avaliar as atividades enzimáticas (ligninocelulolíticas e proteolíticas) dos fermentados fúngicos capazes de degradar compostos tóxicos destas tortas de sementes oleaginosas.

➔ OE3: Caracterizar quimicamente esses fermentados quanto a presença de compostos bioativos e nutricionais;

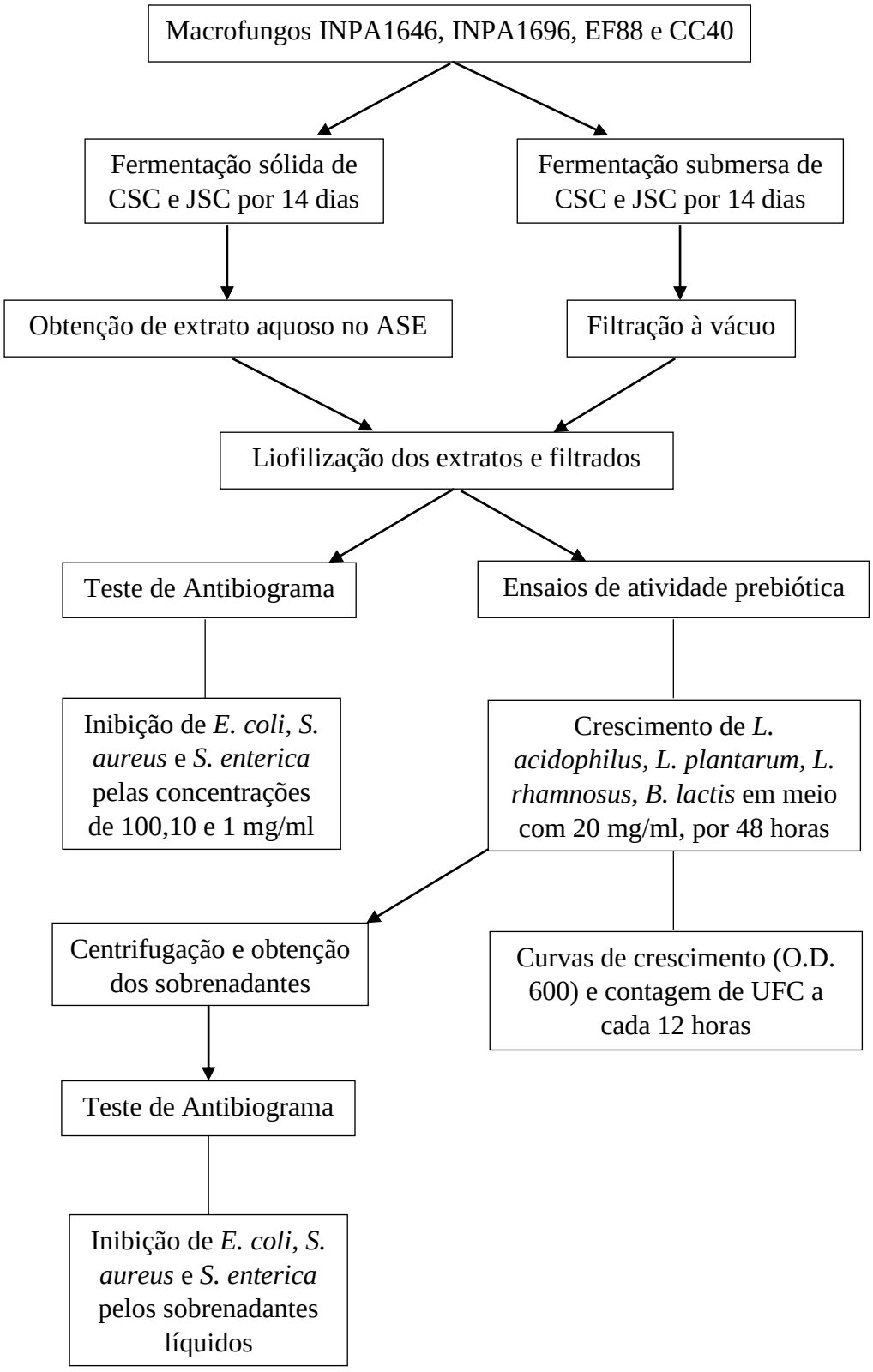
➔ OE4: Avaliar extratos aquosos desses fermentados quanto atividade antimicrobiana e como fonte de carbono para bactérias probióticas.

➔ OE5: Avaliar atividades antibacteriana dos sobrenadantes das bactérias probióticas cultivadas em meios com extratos brutos oriundos desses fermentados dos macrofungos.

FLUXOGRAMA do Capítulo II



FLUXOGRAMA do Capítulo III



**CAPÍTULO I**

# DESTOXIFICAÇÃO FÚNGICA DE TORTAS DE SEMENTES OLEAGINOSAS (BIODIESEL) E A INCLUSÃO DOS FERMENTADOS PARA ALIMENTAÇÃO ANIMAL: REVISÃO

## RESUMO

A busca por fontes alternativas de óleo para produção de biodiesel é um desafio no Brasil e em vários países. A inserção de novas culturas para este fim só será bem-sucedida se os seus coprodutos tiverem algum valor agregado. No entanto, oleaginosas como algodão e pinhão-manso têm características de óleo favoráveis para a produção de biodiesel e suas tortas residuais (coprodutos) são tóxicas para inserção na nutrição de monogástricos, por exemplo. A presença de gossipol livre na torta de caroço de algodão (CSC) limita o seu uso na alimentação à apenas animais ruminantes não reprodutores, e em pequenas quantidades. Enquanto que os ésteres de forbol na torta de semente de pinhão-manso (JSC) limitam o seu uso à apenas adubo orgânico, pela sua composição nutricional e mineral. Esses compostos tóxicos estão envolvidos no sistema de defesa das plantas, mas quando ingeridos podem causar injurias, intoxicações e até óbito dos animais. Essas tortas têm excelentes características proteicas e sua destoxificação para utilização na alimentação animal agrega valor a esses resíduos e, consequentemente, pode impactar na viabilidade do uso destas oleaginosas para a produção de biodiesel. Os métodos físicos e químicos de destoxificação destas biomassas, por vezes não são economicamente viáveis ou não eficientes. O pré-tratamento biológico, por macrofungos por exemplo, tem se mostrado eficiente para degradar gossipol e ésteres de forbol, além de agregar valor nutricional às tortas, ora fermentadas. Os micélios destes fungos, assim como os seus basidiomas (cogumelos) apresentam ainda moléculas bioativas, que podem conferir a essas biomassas fermentadas atividades antioxidante, antimicrobiana, prebiótica, entre outras. A suplementação de ração animal com micélios de basidiomicetos podem resultar em aumento no desempenho de animais, como suínos e aves, melhora na resposta imunológica e na microbiota intestinal. A oferta de biomassas vegetais com micélio fúngico (fermentados) contém altas concentrações de β-glucanas e outras biomoléculas (compostos químicos bioativos) que podem ser uma alternativa ao uso de antibióticos, como promotores de crescimento na alimentação animal, podendo substituir parcial ou totalmente as fontes proteicas tradicionais. Outra vantagem além da destoxificação e a presença de bioativos, é o aumento da digestibilidade destas biomassas, pela degradação da lignina realizada pelas enzimas lignocelulósicas liberadas pelos macrofungos durante a fermentação.

**Palavras-chaves:** Sementes oleaginosas, gossipol livre, ésteres de forbol, digestibilidade, fermentação fúngica.

# 1 INTRODUÇÃO

A produção de biodiesel foi iniciada no Brasil em 2004, a partir do Programa Nacional de Produção e Uso de Biodiesel (PNPB), como alternativa energética autossuficiente de menor impacto ambiental (DA LUZ *et al*., 2014), gerando resíduos que podem ser usados como combustíveis para caldeiras, polímeros, condicionadores de solo, forragem para animais, fertilizantes, adubo, substratos para indústria têxtil ou automobilística, e na alimentação humana e animal (COSTA *et al*., 2014).

A principal fonte de óleo para biodiesel no Brasil é a soja, cujo coproduto, a torta proteica, é usada na alimentação humana e animal como fonte de proteína vegetal. A utilização de outras oleaginosas poderá ser viável se seus coprodutos tiverem um mercado tão promissor quanto o das cadeias produtivas da soja, e das outras culturas utilizadas (RODRIGUES e RONDINA, 2013).

Os coprodutos são farelos e tortas, obtidos após a extração química ou mecânica, respectivamente, do óleo das sementes oleaginosas. Eles representam mais de 50% da massa inicial das sementes utilizadas na cadeia agroindustrial, por isso a utilização desses resíduos é fundamental para agregar renda à cadeia produtiva do biodiesel e minimizar os impactos ambientais (RODRIGUES e RONDINA, 2013). Infelizmente, culturas com características de óleo favoráveis têm resíduos tóxicos ou com fatores antinutricionais, como é o caso da mamona, do algodão e do pinhão-manso.

O caroço ou semente de algodão (*Gossypium hirsutum* L.) é a segunda oleaginosa mais usada para produção do biodiesel no Brasil. A torta resultante da prensagem deste caroço (CSC) é aproveitada principalmente para a alimentação de animais ruminantes não reprodutores, que são capazes de tolerar pequenas quantidades do seu composto tóxico, o gossipol, que pode causar infertilidade em machos (CARNEIRO *et al.*, 2007).

O pinhão-manso (*Jatropha curcas* L.) é considerado a oleaginosa com maior potencial para a produção de biodiesel pelo seu alto teor de óleo (GOMES, 2015; LAVIOLA *et al*., 2012), porém os coprodutos resultantes da extração do seu óleo possuem substâncias tóxicas/antinutricionais, como os ésteres de forbol, que causam inflamações agudas e a indução de tumores (MENDONÇA, 2010; BELEWU *et al*., 2010), limitando o seu uso a apenas adubo orgânico, por ser rica em minerais como nitrogênio, fósforo e potássio (GOMES, 2015).

Como esses coprodutos são ricos em proteínas de alta qualidade nutricional, diversas estratégias químicas e biológicas têm sido abordadas na tentativa de resolver o problema da toxicidade desses resíduos, para utilizá-los na alimentação animal, no entanto algumas delas não são economicamente viáveis, ou resolvem apenas parcialmente o problema da contaminação (GOMES, 2015; GOMES *et al*., 2018; MENDONÇA *et al*., 2019).

Diversos trabalhos comprovam que gossipol, ésteres de forbol, curcina, lectina, saponina, tripsina e inibidores de tripsina podem ser eficientemente degradados por agentes biológicos, principalmente fungos (macrofungos – Basidiomicetos) em fermentações em estado sólido (RAJARATHNAM, SHASHIREKHA, BANO, 2001; AREGHEORE *et al*., 2003; BELUWE e SAM, 2010; KASUYA *et al*., 2012; DA LUZ *et al*., 2013; GOMES, 2015; CUNHA, 2017; ARAÚJO, 2018; GOMES *et al*., 2018; MENDONÇA *et al*., 2019). Estes tratamentos podem representar uma alternativa à utilização dos coprodutos da extração de óleo das sementes de pinhão-manso e algodão.

Os resíduos ou coprodutos agroindustriais têm características úteis para aplicações nas formulações de rações para alimentação animal, e não apenas fertilizantes ou combustível de caldeiras. A indústria de ração animal tem buscado outras fontes de ingredientes além da soja e do milho. O uso de coprodutos agroindustriais, inclusive os da produção de biodiesel, como ração animal parece ser a melhor estratégia para agregar valor à cadeia de coprodutos agrícolas, diminuir a geração de resíduos sólidos, e substituir parcialmente ou completamente as proteínas vegetais de alto custo (GODOY *et al*., 2018).

Este capítulo apresenta uma revisão bibliográfica sobre a cadeia produtiva do biodiesel, as características energéticas dos óleos de algodão e de pinhão-manso, e as características agronômicas e nutricionais de seus coprodutos, além do potencial de destoxificação de fungos basidiomicetos, suas características bioativas e possível viabilização destas biomassas fermentadas na alimentação animal.

## 2 TORTAS OU FARELOS COPRODUTOS DA CADEIA PRODUTIVA DO BIODIESEL

As principais oleaginosas vegetais utilizadas no Brasil para a produção de biodiesel são: soja, algodão, girassol, palma-dendê, mamona e amendoim, e desde 2005 a produção e o uso de biodiesel só aumenta (ANP, 2017; ARAÚJO, 2018). Á medida que essa produção se

intensifica, aumenta a quantidade de coprodutos e resíduos gerados, fomentando assim a busca por aplicabilidades destas biomassas vegetais residuais (VIANNA *et al*., 2016).

Esses coprodutos/resíduos são fontes potenciais para geração de produtos biotecnológicos, agregando valores econômicos na cadeia produtiva. O beneficiamento da soja para biodiesel gera um farelo ou uma torta altamente proteica (aproximadamente 45% de proteína), com grande demanda na indústria de nutrição animal (TIBURCIO, 2011; RODRIGUES e RONDINA, 2013) e a ampliação da utilização de outras biomassas poderá ser viável quando os seus coprodutos tiverem um maior valor agregado, seja para a alimentação como a soja, seja para a obtenção de produtos biotecnológicos como polímeros, enzimas e moléculas bioativas.

## 2.1 Algodão (*Gossypium hirsutum* L.)

A cadeia produtiva do algodão permite integração de diferentes segmentos industriais como a indústria têxtil, a de nutrição animal com farelo/tortas, de cosméticos, indústria farmacêutica, de celulose e de biocombustíveis, com um variado número de produtos primários e secundários (BARBOSA, 2016).

O primeiro levantamento da safra de algodão 2021/2022 pela Conab apresenta uma expectativa inicial de crescimento de 10,2% na área plantada (1,51 milhão de ha) e 13,7% na produção de pluma (2,67 milhões de toneladas), enquanto que a estimativa divulgada pela ABRAPA em setembro de 2021 foi de um aumento de 12,6% na área plantada e de 20,3% na produção de pluma (ABRAPA, 2021).

A torta originada da extração física do óleo, por esmagamento mecânico, apresenta em média 5% de óleo residual, sendo mais energética e com menor teor de proteína quando comparada ao farelo, da extração química com solvente, que tem menos de 2% de óleo residual e, portanto, maior teor de proteína. Esses coprodutos têm como mercado principal o setor de nutrição animal, principalmente de animais ruminantes, que são capazes de tolerar determinados níveis de gossipol (BELTRÃO *et al*., 2000; CARNEIRO *et al.*, 2007).

A utilização destes coprodutos para alimentação animal fortalece a cadeia produtiva sustentável, no entanto, a presença de compostos tóxicos e antinutricionais, como o gossipol limitam o uso destes à nutrição de animais ruminantes não reprodutores, e em quantidade limitada (ARAÚJO, 2018; BARBOSA, 2016). Se forem estabelecidos processos de destoxificação e melhoria da digestibilidade para animais monogástricos como suínos, aves e

peixes, o mercado de torta de caroço de algodão (CSC) pode ser ampliado, agregando valor a esse coproduto.

Processos físicos, químicos e biológicos de destoxificação de CSC para alimentação de monogástricos têm sido relatados na literatura, mas não há um método eficaz em escala industrial. É necessário promover a redução do gossipol na CSC para níveis considerados seguros, comprovando sua destoxificação não apenas com análises químicas, mas com testes toxicológicos em modelos animais, como *Artemia salina* e roedores, culminando com ausência de alterações no organismo das cobaias, como comprometimento das funções hepáticas, a taxa de respiração e a capacidade de transporte de oxigênio pelos eritrócitos (ARAÚJO, 2018).

O crescimento de um microrganismo na CSC pode resultar em aumento da concentração de gossipol livre, por meio da liberação do gossipol ligado, mas alguns fungos são eficientes da eliminação deste composto. A eficiência da eliminação do gossipol livre depende da espécie fúngica, do tempo de fermentação/cultivo e da composição do substrato, podendo variar de apenas 50% do gossipol livre até a total eliminação da substância indesejável para geração de insumos para nutrição animal, como pode ser observado na Tabela 1.1 (CONCEIÇÃO, 2018).

**Tabela 1.1** Resultados descritos na literatura sobre a degradação de gossipol livre em TCA, por meio do cultivo de algumas espécies de fungos, utilizando fermentações em estado sólido (SSF).

| AUTORES | MICRORGANISMO | DEGRADAÇÃO | TEMPO |
|---|---|---|---|
| ZHANG *et al*., 2006a | *Candida capsuligena* | 73,50% | 2 dias (FES) |
| | *Candida tropicalis* | 94,57% | |
| | *Saccharomyces cerevisae* | 88,51% | |
| | *Aspergillus terricola* | 82,91% | |
| | *Aspergillus oryzae* | 67,51% | |
| | *Aspergillus niger* | 85,16% | |
| ZHANG *et al*., 2006b | *C. tropicalis x A. niger* | 91,72% | 2 dias (FES) |
| KHALAF; MELEIGY, 2008 | *C. tropicalis* | 86,18 % | 3 dias (FES) |
| | *S. cerevisae* | 81,04% | |
| | *A. oryzae* | 64,16% | |
| | *A. terreus* | 50,83% | |
| | *A. niger* | 73,95% | |
| SUN; LIU; DU, 2008 | *Geotrichum candidum* | 78,9% | 2 dias (FES) |
| ARAÚJO, 2018 | *Pleurotus ostreatus* | 99,1% | 60 dias (FES) |
| SOARES NETO, 2019 | *Panus lecomtei* | 99,47% | 15 dias (FES) |
| | *Pleurotus ostreatus* | 99,43% | |
| | *Schizophyllum commune* | 80,6% | |

| | | | |
|---|---|---|---|
| GUIMARÃES, 2020 | *Pleurotus pulmonarius* | 97,3% | 30 dias (FES) |
| | *Fistulina hepatica* | 94,9% | |
| | *Panus lecomtei* | 95,5% | |
| | *Ganoderma lucidum* | 90,5% | |

Fonte: adaptado de CONCEIÇÃO, 2018

O gossipol está presente em várias partes do algodoeiro, em diferentes concentrações (Tabela 1.2). As concentrações de gossipol nas tortas de caroço de algodão (CSC) apresentam variações de até 66% para amostras obtidas de diferentes locais do Brasil, em função da variedade, das condições ambientais de cultivo e armazenamento das sementes, variando de 0,04 a 1,77% (ROMERO, 2013).

**Tabela 1.2** Valores médios de gossipol em subprodutos de semente/caroço de algodão.

| **Substância (%)** | **Torta de algodão** | **Farelo de algodão** | **Caroço de algodão** | **Cascas de algodão** |
|---|---|---|---|---|
| Gossipol total | 1,09 | 1,16 | 0,66 | 0,107 |
| Gossipol livre | 0,06 | 0,14 | 0,68 | 0,049 |

Fonte: Araujo (2018)

O uso de CSC na ração de ruminantes é uma prática já estabelecida no mercado brasileiro, mas em quantidade moderada em função de efeitos antinutricionais, quando inseridos em maiores quantidades ou em qualquer quantidade para monogástricos, podendo afetar o crescimento, provocar anormalidades nos órgãos reprodutores, infertilidade e morte (KONG, DAUD, ZHU, 2010).

A concentração de gossipol livre em produtos alimentares não deve exceder 0,045% (450 ppm) pela *Food and Drug Administration* (FDA), 0,060% (600 ppm) pela *Protein Advisory Group of the United Nations Food and Agriculture*, e 1,2% (12.000 ppm) pela *Organization and World Health Organization* (LIADAKIS *et al*., 1993). No Brasil, a única referência sobre a regulamentação data de 1988, mas foi revogada, não existindo regulamentações vigentes no país até o momento para limite de concentração de gossipol em produtos alimentares (ARAÚJO, 2018).

Existem métodos físicos, químicos, biológicos e combinados para degradação de gossipol livre em CSC, como adição de sulfato de ferro, óxido ou hidróxido de cálcio, ou peletização, mas esses processos oneram o custo da produção ou alteram significativamente as

características proteicas do CSC, principalmente a disponibilidade da lisina, e não solucionam o problema por completo (BARBOSA, 2016; NETO *et al*., 2021).

Os tratamentos biológicos de CSC com microrganismos apresentaram resultados positivos na degradação de gossipol livre e são considerados eficientes e economicamente viáveis, e isso inclui os basidiomicetos, em meio líquido e sólido (RAJARATHNAM, SHASHIREKHA, BANO, 2001; ARAÚJO, 2018; NETO *et al*., 2021).

## 2.2 Pinhão-manso (*Jatropha curcas* L.)

*Jatropha curcas* L. não é usada para produção de biodiesel no Brasil, mas está entre as espécies mais estudadas para obtenção de óleo para esse fim, graças à sua resistência à seca e à estresse biótico e abiótico, seu crescimento rápido, fácil propagação, pequeno período de gestação, baixo custo de sementes, boa adaptação e alto teor de óleo (DA LUZ *et al*., 2014; EDRISI *et al*., 2015).

O gênero *Jatropha* tem cerca de 170 espécies de pequenas árvores, arbustos, subarbustos ou ervas amplamente distribuídas nos trópicos, e algumas destas espécies, como *J. platyphylla*, são comestíveis no México, por exemplo, com cerca de 60% de óleo livre de éster de forbol. *Jatropha curcas* L. é uma árvore perene que mede de 5 a 7m de altura, pertencente à família Euphorbiaceae, nativa do México e América Central, com distribuição pantropical, por toda a América Latina, África, Índia e Sudeste Asiático e é bem adaptada, crescendo em vários climas e solos, como áreas marginais, degradadas e contaminadas, com baixos requerimentos nutricionais (EDRISI *et al*., 2015).

Existem perspectivas promissoras nos programas de biodiesel com relação à *Jatropha* sp. em vários países. Países como a Indonésia, Malásia e Índia já adotaram o pinhão-manso, no programa de biocombustível, sendo a preferência secundária na Indonésia, país no qual o óleo de palma ainda é a principal fonte (EDRISI *et al*., 2015). No Brasil, o pinhão-manso foi uma promessa no lançamento do Plano Nacional de Biodiesel, que tinha a mamona e o girassol como destaque, porém apresentando espaço para outras oleaginosas.

As sementes de pinhão-manso representam entre 53 e 79 % do peso do fruto e apresentam teor de óleo entre 33 e 38 %, com rendimento de óleo/grão superior ao das oleaginosas tradicionais, como a soja, além de ter características físico-químicas de óleo favoráveis à produção de biodiesel, chegando a 1500 kg/ha de óleo, na idade adulta (LAVIOLA *et al*., 2012; GOMES, 2015; GOMES *et al*., 2018; MENDONÇA *et al*., 2019).

O óleo de semente de pinhão-manso tem sido tradicionalmente usado, para a produção de sabão ou vela, iluminação e lubrificante, mas a sua composição de ácidos graxos é adequada para nutrição humana e excelente para utilização como combustível (INSANU *et al*., 2013). Apesar da sua composição altamente nutritiva, o óleo de *J. curcas* não é comestível em razão de sua toxicidade, o que faz dele uma ótima opção para a produção de biodiesel, já que não compete com a indústria alimentícia (BOSE e KEHARIA, 2014).

Para cada tonelada de óleo de semente de pinhão-manso, são geradas no mínimo 2,76 toneladas de torta ou farelo, coprodutos da extração mecânica ou extração por solvente, respectivamente, ricos em proteína de boa qualidade, que são usados como adubo orgânico, por serem ricos em minerais e nutrientes, mas compostos tóxicos, como ésteres de forbol, impedem a sua utilização na alimentação animal (MAKKAR e BECKER, 2009).

Para melhorar a sustentabilidade econômica da cadeia de biodiesel de *J. curcas*, é importante investir na exploração adequada dos coprodutos, após a extração do óleo (torta e farelo de sementes), uma vez que esta representa cerca de 70% (p/p) das sementes processadas e tem um teor nutricional elevado, com altos níveis de aminoácidos essenciais e proteínas (MAKKAR e BECKER, 2009; HE *et al*., 2011).

A composição química da torta de semente de pinhão-manso (TSPM) desidratada é 60% de proteína, 0,6% de gordura, 9% de cinzas, 4% de fibra e 26% de carboidratos (RAKSHIT *et al*., 2008), ou 22,41% de proteína bruta, 19,14% de gordura, 5,8% de cinzas, 48,7% de fibras e 25,5% de carboidratos (GOMES, 2015), dependendo da origem das sementes e da eficiência da extração do óleo. Ela tem mais nutrientes orgânicos que cama de frango e estrume de gado, com altos teores de nitrogênio, fósforo e matéria orgânica, sendo altamente eficiente como fertilizante (FRANCIS, EDINGER, BECKER, 2005). A JSC também pode ser usada para a produção de biogás, por digestão anaeróbia, com rendimento de até 0,6 $m^3$/kg de JSC, utilizando consórcios microbianos como inóculo (RADHAKRISHNA, 2007).

Os sinais de intoxicação por ésteres de forbol em animais ou pessoas incluem diarreia, dispneia, desidratação e perda de peso, além de aumento de aspartato aminotransferase, amônia e potássio, diminuição da proteína total no soro sanguíneo, hemorragia nos rins, pulmões, retículo, baço e rúmen e necrose e degeneração do fígado (INSANU *et al*., 2013). Em crianças os principais sintomas são vômito (95% dos casos), diarreia (50%) e cefaleia (40%) (INSANU *et al*., 2013).

Os ésteres de forbol são substâncias tóxicas termoestáveis que se mantem presentes na torta, mesmo após a extração do óleo, tornando-a inviável para nutrição animal, sem destoxificação prévia. Elas têm afinidade pelos receptores da membrana de fosfolipídios e ativam a proteína quinase C (PKC), levando a uma resposta miogênica, ampliando a eficácia de agentes cancerígenos, agindo, assim, como cocarcinógenos (GOMES 2015). Eles são o principal impedimento comercial de torta e farinha de *J. curcas* como matéria-prima para nutrição animal.

Métodos químicos e físicos de destoxificação de JSC são considerados economicamente inviáveis, ou resolvem apenas parcialmente o problema, enquanto que processos biológicos, de fermentação microbiana, principalmente fermentação sólida utilizando os macrofungos (basidiomicetos), são considerados eficientes na conversão desse coproduto em torta proteica atóxica para alimentação animal (BELUWE e SAM, 2010; BARROS *et al*., 2011; KASUYA *et al*., 2012; DA LUZ *et al*., 2013 e 2014; BOSE e KEHARIA, 2014; GOMES, 2015; CUNHA, 2017; GOMES et al., 2018).

**Tabela 1.3** Resultados descritos na literatura sobre a degradação de ésteres de forbol em JSC, por meio do cultivo de algumas espécies de fungos, utilizando fermentação em estado sólido (SSF).

| AUTORES | MICRORGANISMO | DEGRADAÇÃO | TEMPO |
|---|---|---|---|
| Da LUZ *et al*., 2014 | *Pleurotus ostreatus* | 37% | 15 dias |
| | | 60% | 45 dias |
| | | 99% | 60 dias |
| Da LUZ *et al*., 2013 | *Pleurotus ostreatus* | 91% | 30 dias |
| | | 99% | 45 dias |
| | | 99% | 60 dias |
| KASUYA *et al*., 2012 | *Pleurotus ostreatus* | 58% | 15 dias |
| | | 85% | 30 dias |
| | | 99% | 45 dias |
| BOSE e KEHARIA, 2014 | *Ganoderma lucidum* | 100% | 20 dias |
| | *Trametes zonata* | 100% | |
| | *Trametes gibbosa* | 91,7% | |
| | *Trametes versicolor* | 89% | |
| | *Trametes hirsute* | 81,6% | |
| | *Pleurotus sapidus* | 76% | |
| | *Pleurotus ostreatus* | 72,5% | |
| | *Pleurotus florida* | 67,9% | |
| | *Pleurotus sajor-caju* | 67,9% | |
| | *Phanerochaete chrysosporium* | 44,9% | |
| BARROS *et al*., 2011 | *Phlebia rufa* | 97% | 30 dias |
| | *Bjerkandera adusta* | 91% | |
| | *Ganoderma resinaceum* | 20% | |
| BELUWE e | *Aspergillus niger* | 76,9% | 7 dias |

| | | | |
|---|---|---|---|
| SAM, 2010 | *Rhizopus nigricans* | 23,1% | |
| | *Penicillium chrysogenum* | 15,4% | |
| | *Trichoderma longibrachitum* | 15,4% | |
| | *Rhizopus oligosporus* | 7,7% | |
| GOMES, 2015 | *Aurantioporus pulcherrimus* | 41% | 30 dias |
| | *Ganoderma lucidum* | 42% | |
| | *Agaricus ssp.* | 54% | |
| | *Agaricus fuscofibrillosus* | 62% | |
| | *Agaricus mediofuscus* | 71% | |
| | *Ascopolyporus sp* | 76% | |
| | *Panaeolus antillarum* | 78% | |
| | *Lentinus strigellus* | 88% | |
| | *Amylosporus ssp.* | 95% | |
| | *Pleurotus pulmonarius* | 97% | |

Fonte: Cunha (2017)

## 3 DESTOXIFICAÇÃO FÚNGICA DE TORTAS OLEAGINOSAS E PROTEÍNAS MICROBIANAS PARA NUTRIÇÃO ANIMAL

Os macrofungos (ascomicetos e basidiomicetos), chamados de fungos de podridão branca (do inglês, White-rot fungi) ou podridão marrom (do inglês, brown-rot fungi) são exímios produtores de enzimas extracelulares capazes de degradar substâncias complexas e recalcitrantes, como lignina e outros compostos. Fungos de podridão branca como *Pleurotus ostreatus, Trametes versicolor, Phanerochaete chrysosporium* e *Pycnoporus sanguineus*, por exemplo, são capazes de degradar vários poluentes, como substâncias aromáticas, policíclicas, xenobióticos, polifenólicos, corantes sintéticos, hidrocarbonetos, tinitrotoluenos e fitatos (ALBERTS *et al*., 2009; KASUYA *et al*., 2012; DA LUZ *et al*., 2014).

Tratamentos biológicos utilizando microrganismos são eficientes para reduzir substâncias tóxicas em plantas, como gossipol em farelo e torta de sementes de algodão utilizando *Geotrichum candidum* e *Panus lecomtei* (SUN, LIU, DU, 2008; ARAÚJO, 2018; SOARES NETO, 2019; GUIMARÃES, 2020; NETO *et al*., 2021), ésteres de forbol de torta de semente de pinhão-manso por *Pleurotus pulmonarius* (GOMES, 2015; CUNHA, 2017; GOMES *et al*., 2018; MENDONÇA *et al*., 2019), e ricina em sementes de mamona por *Paecilomyces variotii* (MADEIRA, MACEDO, MACEDO, 2011).

Pesquisas recentes demonstraram que a fermentação sólida de tortas de semente de algodão ou pinhão-manso pelos macrofungos do gênero *Pleurotus* spp. e *Panus lecomtei* levaram a diminuição de quase 100% da toxicidade destas (GOMES, 2015; CUNHA, 2017; ARAÚJO, 2018, SOARES NETO, 2019; MENDONÇA *et al*., 2019; NETO *et al*., 2021).

Durante a degradação da lignocelulose, os basidiomicetos liberam enzimas hidrolíticas e oxidativas, como celulases, β-glicosidases, xilanases, lacases, manganês peroxidases e lignina peroxidases que podem estar envolvidas na destoxificação dessas biomassas (QINNGHE *et al.*, 2003; GOMES, 2015; CUNHA, 2017; ARAÚJO, 2018, SOARES NETO, 2019).

Muitos trabalhos na literatura correlacionam a degradação de compostos tóxicos com atividades enzimáticas (BOSE e KEHARIA, 2014; DA LUZ *et al*., 2014; GOMES, 2015), porém nenhum deles comprovou qual ou quais enzimas microbianas são responsáveis pela biotransformação dos ésteres de forbol ou gossipol, apesar de Soares Neto (2019) ter indicado uma lacase como a responsável pela degradação de gossipol por *Panus lecomtei* e Gomes (2019) ter concluído que esterases e metaloproteases estavam envolvidas na degradação de ésteres de forbol por *Pleurotus pulmonarius*, além de proteínas hit-shock.

O tratamento biológico de destoxificação de JSC por fungos pode ser mais eficiente e gerar outros bioprodutos de maior valor agregado durante o processo, como enzimas, cogumelos comestíveis e insumos para nutrição animal (DA LUZ *et al*., 2013). A Tabela 1.3 apresenta uma compilação de trabalhos que testaram fermentação sólida de fungos para destoxificação de JSC (CUNHA, 2017).

## 4 MACROFUNGOS: COGUMELOS COMESTÍVEIS E MEDICINAIS

### 4.1 Propriedades bioativas

Não é à toa que os cogumelos têm sido utilizados há centenas de anos, em diferentes países, como alimento nutracêuticos e aditivo medicinal. Como parte do processo de adaptação e do seu sistema de defesa, os cogumelos produzem metabólitos secundários com funções antimicrobianas, antioxidantes e anti-inflamatórias (ZJAWIONY, 2004; SHEN *et al.*, 2017). Eles possuem uma série de substâncias bioativas, como fibras, polissacarídeos (β-glucanas e quitina), compostos fenólicos e polifenólicos, lectinas, terpenóides, esteróis, vitaminas, aminoácidos, proteínas e compostos orgânicos voláteis. Estes compostos possuem atividades antioxidante, anti-inflamatória, antimicrobiana, antitumoral, antiviral, imunomoduladora, hipocolesterolemiante, reguladora de níveis glicêmicos, entre outras (EL ENSHASY e HATTIKAUL, 2013; KALAČ, 2013; MATTILA, MARNILA e PIHLANTO, 2017).

Cogumelos e micélios fúngicos são conhecidos pela sua alta atividade antioxidante, graças à presença de compostos fenólicos, tocoferóis, vitamina E e polissacarídeos como β-glucanas. Eles retardam as reações de degradação oxidativa, reduzindo a velocidade da oxidação por inibição de radicais livres e/ou complexação de metais. Doenças como aterosclerose, doenças cardiovasculares, câncer, cirrose, diabetes, doenças pulmonares, doenças neurológicas, doença de Parkinson, entre muitas outras, estão relacionadas à produção não controlada de radicais livres (HALLIWELL e GUTTERIDGE, 1999).

Os principais compostos fenólicos presentes nos macrofungos são ácido gálico, ácido cafeico, quercetina, rutina, vanilina, flavonoides e polifenóis. Os compostos fenólicos são compostos aromáticos hidroxilados com anéis aromáticos com um ou mais grupos hidroxila (APAK, 2007; BARROS, BAPTISTA e FERREIRA, 2007). Os tocoferóis e tocotrienóis são compostos biológicos essenciais presentes na membrana dos macrofungos, capazes de quelar fisicamente o singleto de oxigênio. Existem quatro isômeros dos tocoferóis e tocotrienois (α-, β-, γ-, δ-), sendo o α-tocoferol o que possui a maior atividade antioxidante (KAGAN, 1989).

### 4.1.1 Atividades antibacteriana e prebiótica

Das 2000 espécies de cogumelos conhecidas no mundo, 148 espécies de 88 gêneros diferentes são conhecidas por ter atividade antimicrobiana (BALAKUMAR *et al*., 2011), sendo que para algumas espécies, as propriedades antibacterianas podem variar conforme a maturação dos basidiomas (cogumelos). A maioria das espécies possui mais atividade contra bactérias gram-positivas, enquanto menos espécies agem contra gram-negativas. Os metabólitos presentes em cogumelos responsáveis por atividade antimicrobiana são os compostos fenólicos, as β-glucanas, esteróis, ácidos graxos, os terpenóides, sesquiterpenóides e triterpenóides, proteínas e enzimas (SHEN *et al*., 2017).

Estes compostos têm polaridades diferentes. A água é capaz de extrair os compostos antimicrobianos de maior polaridade, como compostos fenólicos de baixo peso molecular, sacarídeos e polissacarídeos. A temperatura da água é importante, pois aumenta a eficiência da extração, mas pode resultar na degradação de compostos termossensíveis. Por exemplo, extratos aquosos de *Russula vesca* e *Pleurotus squarrosulus* apresentaram maiores atividades antibacterianas quando a água é quente (4 horas de fervura) do que quando é morna (28ºC durante 26 horas) (NWACHUKWU e UZOETO, 2010).

Além destas, os basidiomicetos têm atividade prebiótica, principalmente graças aos seus oligossacarídeos e polissacarídeos complexos. Prebióticos são compostos não-digestíveis que estimulam seletivamente o crescimento e/ou atividade de uma ou mais espécies de bactérias probióticas, melhorando a saúde, podendo inclusive inibir o crescimento de microrganismos patogênicos endógenos no trato gastrointestinal e aumentar a capacidade do sistema imunológico de resistir a patógenos exógenos (DE SOUSA, DOS SANTOS e SGARBIERI, 2011).

Os principais polissacarídeos dos basidiomicetos são quitina, α-glucanas e β-glucanas, eles são resistentes à digestão gastrointestinal superior, chegando ao cólon intactos, onde são fermentados seletivamente por bactérias probióticas, como lactobacilos e bifidobactérias (GIBSON *et al*., 2004). Estas por sua vez aumentam a expressão de ácidos graxos de cadeia curta (SCFA, do inglês *short-chain fatty acids*), reduzindo um pouco o pH do lúmen do cólon, aumentando o bolo fecal, reduzindo produtos com nitrogênio terminal e enzimas redutivas, aumentando a expressão de proteínas de ligação ou carreadores associados com a absorção mineral e modulação do sistema imune, além da supressão de microrganismos patogênicos (DOUGLAS e SANDERS, 2008).

## 4.2 Fermentados de Macrofungos e outros microrganismos na alimentação e sanidade animal

Considerando que além da capacidade de destoxificar esses coprodutos (CSC e JSC) altamente proteicos, os basidiomicetos apresentam uma variedade de moléculas bioativas, com atividades antioxidante, antimicrobiana e prebiótica, estudos do cultivo em estado sólido de basidiomicetos em tortas oleaginosas tóxicas visando a formulação de aditivos para a alimentação animal pode ser uma alternativa para o uso indiscriminado de antibióticos (GUIMARÃES, 2020), substituindo total ou parcialmente os volumosos proteicos tradicionais e mais caros.

O uso de prebióticos como moduladores da microbiota intestinal é uma alternativa para o uso de antibióticos na alimentação animal (BIRMANI *et al*., 2019; KHALIQUE *et al*., 2020; HASSAN *et al*., 2020). Os antibióticos são amplamente utilizados na alimentação animal, mas seu uso a longo prazo induz a resistência microbiana, ameaçando a saúde dos consumidores e o meio ambiente. Por isso o uso deles como promotores de crescimento na alimentação animal foi banido pela União Europeia desde 2006, mas em vários países do

mundo, incluindo o Brasil, esse uso ainda é permitido, mesmo depois das recomendações da Organização Mundial da Saúde (OMS, 2017).

O pré-tratamento fúngico para aumento da digestibilidade é necessário em diversos resíduos agroindustriais, devido ao seu alto teor de fibras não digeríveis. O crescimento de *Coprinopsis cinerea*, por exemplo, é uma abordagem prática, econômica e ambientalmente correta para melhorar o valor nutritivo e a digestibilidade da palha de arroz para alimentação animal (ZHANG *et al*., 2017).

A suplementação da dieta com micélio de basidiomicetos é uma boa estratégia para avaliar a influência das biomoléculas fúngicas no desempenho e na sanidade animal. Chen *et al*. (2017) demonstraram melhor desempenho de crescimento de leitões desmamados precocemente com dietas contendo 0,1% e 0,3% de micélio de *Armillariella tabescens* seco em pó. Essa suplementação também aumentou a secreção de imunoglobulina A (IgA) na mucosa jejunal em 2,6 e três vezes, respectivamente.

Os leitões que receberam suplementação de micélio de *A. tabescens* na dieta também apresentaram elevação da expressão de interleucina-2 (Il-2), interferon-γ (INF- γ) e fator de necrose tumoral-α (TNF-α) na mucosa jejunal, indicando estimulação da resposta imunológica. Além de quantidades aumentadas de *Lactobacillus* spp. e *Bifidobacterium* spp. no jejuno, e quantidades diminuídas de *Escherichia coli* no jejuno e íleo, indicando atividade prebiótica deste micélio (CHEN *et al*., 2017).

A suplementação da ração de frangos com 6, 12 e 18 g/kg de micélio de *Hericium caput-medusae* também resultou em aumento no ganho médio diário de peso, além de aumento significativo na contagem de Lactobacilos e Bifidobactérias e redução significativa na contagem de *E. coli* na digesta do intestino delgado e do ceco. A digestibilidade da proteína bruta e a altura das vilosidades e a relação entre a altura das vilosidades e a profundidade da cripta no duodeno, jejuno e íleo dos frangos de corte também aumentaram, melhorando o crescimento dos frangos (SHANG *et al*., 2016).

A utilização de fermentados fúngicos, com micélios de macrobasidiomicetos já é uma realidade comercial. A Trouw Nutrition, por exemplo, desenvolve e comercializa o Fysal® Solute, que é um novo produto alimentar à base de centeio fermentado pelo macrofungo *Agaricus subrufescens*, comercialmente conhecido como “cogumelo do sol”. A Trouw Nutrition é conhecida por fornecer soluções nutricionais específicas para cada espécie animal, com especialidades inovadoras para rações, pré-misturas e serviços nutricionais para a indústria de nutrição animal. A empresa afirma que as mudanças na produção de gado e aves

nas últimas décadas exigem que os agricultores aumentem o foco na saúde animal, principalmente na saúde intestinal. Deste modo, haverá maiores garantias no desempenho animal. A presença de aditivos funcionais pode ajudar significativamente a manter a saúde e o desempenho do intestino dos animais, com foco no controle da microbiota, integridade intestinal e aprimoramento do sistema de defesa natural, visando a redução de antibióticos, segurança alimentar, incluindo controle de *Salmonella* e melhoria de desempenho (FYSAL SOLUTE, 2020; TROUW NUTRITION, 2020).

Outro exemplo é o ingrediente proteico ME-PRO®, principal produto da Prairie AquaTech, que fabrica ingredientes para rações formuladas com proteínas vegetais. ME-PRO é a abreviação de "proteína microbianamente aprimorada". É uma proteína para aquacultura, à base de plantas que tem dois objetivos principais: sustentabilidade e digestibilidade. Esta tecnologia da Prairie AquaTech pode lidar com muitos tipos de proteínas vegetais, como a farinha de soja, que tem uma série de fatores antinutricionais que precisam ser tratados, como proteínas alergênicas, oligossacarídeos e carboidratos complexos, e esse processo aumenta o conteúdo de proteína, tornando-a também mais digerível (WRIGHT, 2020).

A Prairie AquaTech fermenta farelo de soja em biorreatores com um microrganismo de ocorrência natural, e afirma que o produto final é "garantido com 70% de proteína, altamente digerível" que pode ser incluído em dietas de salmonídeos, camarões e espécies marinhas com altas taxas de inclusão, como os 35% de inclusão na ração das trutas arco-íris vendidas atualmente. ME-PRO demonstrou reduzir drasticamente as descargas de fósforo nos resíduos dos peixes e é livre de contaminantes. O CEO da Prairie AquaTech, Mark Luecke, afirma que este produto pode substituir 100% da farinha de peixe utilizada, mas no momento ele é apenas uma extensão dela. Ainda segundo ele, "Precisamos de todas as fontes de proteína para vencer" e o foco está na fermentação (WRIGHT, 2020).

Ainda no campo das fermentações, a Fermentation Experts produziu proteínas complementares fermentadas com ácido lático, o produto EP100i, para substituir o óxido de zinco na produção de suínos sem aumentar a mortalidade, o custo de produção ou a quantidade de antibióticos usados. Segundo Jens Legarth, CEO da Fermentation Experts, o zinco prescrito parece prejudicar o sistema imunológico, dificultando a resposta à inflamação no intestino, mas a proteína vegetal fermentada faz exatamente o oposto, reforçando o sistema imunológico e reduzindo o risco de inflamação intestinal. A função do óxido de zinco na ração de suínos é aumentar a diversidade das bactérias e a concentração de bactérias láticas no intestino, reprimindo as bactérias que causavam doenças e criando um intestino equilibrado

que apoia a saúde dos suínos, controlando eficazmente a diarreia, e a proteína fermentada com ácido láctico alterou as bactérias intestinais de forma muito semelhante à do óxido de zinco (ALMIND, 2019).

Jens Legarth afirma que o uso de zinco só empurra o problema para um estágio posterior na produção de suínos. Pois, segundo ele, ainda haverá a necessidade contínua de antibióticos, que prejudicará o sistema imunológico. Os antibióticos contribuem para uma barreira intestinal mais fraca, aumentando as chances de as bactérias chegarem a corrente sanguínea, enquanto que (segundo o anúncio da empresa) a proteína vegetal fermentada EP100i pode substituir o óxido de zinco sem comprometer os resultados da produção, e ainda substitui as proteínas mais caras como farinha de peixe e plasma sanguíneo (ALMIND, 2019).

### 4.3 Bactérias probióticas e macrofungos

Os polissacarídeos de cogumelos comestíveis são capazes de passar pelo estômago inalterados e atingem o cólon, onde estimulam o crescimento de bactérias benéficas como *Lactobacillus acidophilus* e *Lactobacillus rhamnosus*. O crescimento de *L. acidophilus* nos polissacarídeos das 36 espécies de cogumelos analisadas aumentou de 3,87% a 37,96%, para *C. cinerea* e *P. involutus*, respectivamente. Quanto ao crescimento das duas cepas de *L. rhamnosus* isoladas de tratos gastrointestinais de pessoas saudáveis, o maior desempenho de estímulo de crescimento foi observado para os polissacarídeos de *P. capnoides* com 56,63%, e o menor para os de *P. betulinus* com 10,98% de aumento no crescimento de *L. rhamnosus* (NOWAK *et al*., 2017).

Os efeitos imunológicos e de crescimento da administração oral de substrato exaurido (SMS) de *Cordyceps militaris* com bactérias probióticas vêm sendo estudados em tilápia do Nilo. Os peixes alimentados com o fermentado fúngico com *L. plantarum* apresentaram um aumento significativo nas atividades de lisozima sérica, complemento alternativo, fagocitose, peroxidase sérica e atividades de explosão respiratória e peroxidase do muco da pele em comparação com o grupo de controle, após 8 semanas de alimentação, além de aumento significativo na taxa de crescimento específico, ganho de peso e peso final (DOAN *et al*., 2017).

Assim como os de outros macrofungos, os polissacarídeos do cogumelo medicinal *Cordyceps militaris* podem ser usados como antioxidantes e imunomoduladores seguros, sendo observado o aumento de até 80% da atividade antioxidante (DPPH) com administração

de 0,5 mg/mL desses polissacarídeos, bem como da produção de óxido nírico, forte indicador de aumento da atividade de macrófagos (ZENG *et al*., 2015). Outros autores também reconheceram a atividade antioxidante de *Cordyceps militaris* (JING *et al*., 2014) e de *Cordyceps sinensis* (DONG e YAO, 2008; YAN *et al*., 2009; HUANG *et al*., 2013).

## 5. CONSIDERAÇÕES FINAIS

A adoção de fontes alternativas para produção de biodiesel, principalmente oleaginosas que não competem com a indústria alimentícia, pode ser viável, desde que os seus coprodutos tenham valor econômico. Os óleos de algodão e de pinhão-manso são promissores para a produção de biodiesel, mas os resíduos/coprodutos (tortas/farelos) de sua extração são tóxicos ou limitados para nutrição animal e por isso são usados apenas em pequenas quantidades na alimentação de animais ruminantes (CSC) ou como adubo orgânico (JSC).

A biodestoxificação destas tortas, por meio de fermentação por fungos basidiomicetos podem transforma-las em biomassas proteicas altamente nutritivas e ricas em compostos fúngicos bioativos, com atividade antioxidante, antimicrobiana e prebiótica, que podem ser usadas como aditivos alimentares para animais ou como volumoso proteico na formulação de rações, pois apresenta alta concentração de proteínas de boa qualidade e pode ser menos onerosa do que as fontes tradicionais de proteínas para alimentação animal.

Os bioativos fúngicos presentes nas biomassas fermentadas podem atuar eficientemente no organismo animal, à ponto de substituir ou diminuir o uso de antibióticos, por exemplo. Isto tem sido apontado por alguns estudos, que podem ser ainda mais específicos e aprofundados sobre o comportamento dessas moléculas e dessas biomassas no trato digestivo de animais mono e poligástricos. Há trabalhos feitos com análises de desempenho e de sanidade dos animais com dietas suplementadas por micélios fúngicos, que comprovam melhorias na resposta imunológica e aumento das populações de bactérias probióticas intestinais. Deste modo, as fermentações em estado sólido de tortas oleaginosas com macrofungos podem ser insumos importantes na busca de novos bioprodutos para nutrição e sanidade animal, além de promover a economia circular junto as cadeias produtivas, transformando passivos ambientais das agroindústrias em ativos financeiros, com impactos ambientais e sociais.

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**CAPÍTULO II**

# BIOATIVOS E ENZIMAS EXTRACELULARES OBTIDOS DE FERMENTADOS DE MACROFUNGOS CULTIVADOS EM TORTAS DE SEMENTES DE ALGODÃO OU PINHÃO-MANSO

## RESUMO

Os fermentados fúngicos têm sido estudados e aplicados em formulações de alimentação animal, tendo como objetivos o aumento da digestibilidade das biomassas por meio das enzimas extracelulares e o enriquecimento com compostos químicos bioativos, tais como antioxidantes, beta-glucanas, entre outras. A toxicidade das tortas de semente de pinhão-manso (JSC) e de caroço de algodão (CSC) são um empecilho para a utilização destes coprodutos para a alimentação animal, apesar de sua alta concentração proteica. Neste sentido, processos eficientes de destoxificação podem contribuir para a valorização dessas oleaginosas usadas na produção de biodiesel. Os principais compostos tóxicos destes coprodutos, ésteres de forbol (em JSC) e gossipol livre (em CSC), foram mensurados antes e depois da fermentação em estado sólido (SSF) com os macrofungos selecionados, que foram cultivados por 14 dias. Os fermentados fúngicos com melhores resultados de degradação destes compostos foram selecionados, para avaliação da cinética de degradação. Também foram avaliadas as atividades antioxidante e enzimáticas ao longo da fermentação e, ao fim desta, foi realizada a quantificação de celulose, hemicelulose, lignina, proteína, lipídeos, cinzas, beta-glucanas e ergosterol, como também as análises de digestibilidade *in vitro* e composição de aminoácidos. A maioria dos macrofungos avaliados foram capazes de crescer nas duas tortas, sendo que seis deles foram capazes de reduzir os níveis de ésteres de forbol na JSC para níveis considerados atóxicos (<0,9mg/g) e pelo menos dois degradaram eficientemente o gossipol livre na CSC. Os tratamentos fermentados de *Coriolopsis sp.* INPA1646 e *Tyromyces sp.* INPA1696, aumentaram das concentrações de β-glucanas e ergosterol, respectivamente. Porém, apresentaram redução da atividade antioxidante em relação aos coprodutos (*in natura*). Mas, por outro lado, apresentaram alta atividade de celulases totais, lacases e proteases, principalmente por *Tyromyces sp.* na CSC. A análise bromatológica apresentou resultados significativos nas concentrações de proteína bruta nos tratamentos fermentados, como também a solubilização de parte destas (aminoácidos). No entanto, a degradação da celulose pelos fungos resultou em aumento da concentração da lignina e, consequentemente, redução da digestibilidade. Os fermentados fúngicos de *Coriolopsis* sp. INPA1646 e *Tyromyces* sp. INPA1696 em JSC ou CSC podem ser considerados insumos biológicos importantes para adição na alimentação animal de monogástricos e poligástricos. Ensaios toxicológicos e otimização do tempo de cultivo são necessários para obter melhores resultados na concentração proteica e de compostos bioativos, tais como beta-glucanas, como também para o aumento da digestibilidade, que não fora observado nos ensaios provavelmente em função do curto tempo de cultivo (14 dias) para este modelo de fermentação com macrofungos.

**Palavras-chaves:** Pinhão-manso. Semente de algodão. Fermentação em estado sólido.

Basidiomicetos. Nutrição animal.

# BIOACTIVES AND EXTRACELLULAR ENZYMES OBTAINED FROM FERMENTED MACROFUNGI CULTIVATED IN COTTON OR JATROPHA SEED CAKES

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**ABSTRACT**

Fungal fermented products have been studied and applied in animal feed formulations, aiming to increase biomass digestibility through extracellular enzymes and enrichment with bioactive chemical compounds, such as antioxidants, beta-glucans, among others. The toxicity of *Jatropha* seed cake (JSC) and cottonseed cake (CSC) is an obstacle to the use of these co-products for animal feed, despite their high protein concentration. In this sense, efficient detoxification processes can contribute to the valorization of these oilseeds used in the production of biodiesel. The main toxic compounds of these co-products, phorbol esters (in JSC) and free gossypol (in CSC), were measured before and after solid-state fermentation (SSF) with selected macrofungi, which were cultivated for 14 days. The fungal fermentations with the best degradation results of these compounds were selected for evaluation of the degradation kinetics. Antioxidant and enzymatic activities throughout the fermentation were also evaluated and, at the end of the fermentation, the quantification of cellulose, hemicellulose, lignin, protein, lipids, ashes, beta-glucans, and ergosterol was performed, as well as the *in vitro* digestibility and amino acid composition. Most of the evaluated macrofungi were able to grow in the two cakes, six of them were able to reduce the levels of phorbol esters in the JSC to levels considered non-toxic (<0.9mg/g) and, at least two efficiently degraded the free gossypol in the CSC. The fermented treatment of *Coriolopsis* sp. INPA1646 and *Tyromyces* sp. INPA1696, increased concentrations of β-glucans and ergosterol, respectively. However, antioxidant activity was reduced, compared to co-products (in *natura*). But, on the other hand, they showed high activity of total cellulases, laccases, and proteases, mainly *Tyromyces* sp. in CSC. The chemical analysis showed significant results in the concentrations of crude protein in fermented treatments, as well as the solubilization of part of these (amino acids). However, cellulose degradation by fungi resulted in increased lignin concentration and, consequently, reduced digestibility. *Coriolopsis* sp. INPA1646 and *Tyromyces* sp. INPA1696 in JSC or CSC can be considered important biological inputs for addition to animal feed of monogastrics and polygastrics. Toxicological tests and optimization of the cultivation time are necessary to obtain better results in the concentration of protein and bioactive compounds, such as beta-glucans, as well as to increase digestibility, which was not observed in the tests, probably due to the short cultivation time (14 days) for this model of fermentation with macrofungi.

**Keywords:** Jatropha, cottonseed. Solid state fermentation. Basidiomycetes. Animal nutrition.

# 1 INTRODUCTION

Sustainability of animal production can be developed by applying alternative lignocellulosic biomass for animal feed. Several physicochemical methods are used to improve the digestibility of these biomasses, such as hydrothermal treatment, ammonia fiber explosion, and acids/alkaline treatments. These methods increase accessibility of structural carbohydrates through breakdown of lignin. However, the environmental impact of these methods is still a major concern (ISROI *et al*., 2011; NAYAN *et al*., 2019). Biological pretreatments, mainly fungal, have gained popularity. Pretreatments that make use of white rot fungi have achieved high levels of efficiency, since these fungi selectively degrade lignin, therefore increasing the availability of carbohydrates (VAN KUIJK et al., 2015; NAYAN *et al*., 2019).

White rot fungi are a large group of basidiomycetes whose extracellular enzymes degrade lignin, selectively or not, leaving a white cellulosic and hemicellulosic wood (DA LUZ *et al*., 2013). As far as it is known, these fungi produce secondary compounds such as phenolic compounds, polysaccharides and enzymes and rarely produce toxic compounds (ZHAO et al., 2020). Fungal delignification occurs by oxidation of lignin, through action of enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Non-selective white rot fungi tend to degrade cellulose and hemicellulose, while selective fungi mainly secrete hemicellulolytic enzymes, resulting in higher concentrations of cellulose (DA LUZ et al., 2013). Therefore, white rot fungi with higher selectivity for lignin and lower selectivity for cellulose and hemicelluloses are more efficient in improving the digestibility of biomasses (ZHAO et al., 2020).

Cakes or bran that result from the oil extraction of cotton and Jatropha seeds have toxic/antinutritional compounds that limit their use. Among these toxic compounds, gossypol causes negative effects on the growth and reproduction of animals (HAVARTINE; FIRKINS; EASTYRIDGE, 2002); and phorbol esters can cause acute inflammation and tumors (MENDONÇA, 2010; BELEWU; BELEWU; OGUNSOLA, 2010; GOMES et al., 2018; MENDONÇA et al., 2019). Despite their toxicity, these co-products are of great interest to the feed industry, since they present a high protein content: from 25 to 63% in cotton cake (HAVARTINE; FIRKINS; EASTYRIDGE, 2002) and from 46 to 63% in Jatropha (MENDONÇA, 2010; LAVIOLA et al., 2012), or at least 16%, depending on the oil extraction method (MAKKAR and BECKER, 2009).

Several chemical or biological strategies have been tested in order to detoxify these residues. However, some of these are not economically viable, or only partially solve the problem of toxicity; this allows the addition of only exceptionally low percentages of the "detoxified" cake in the animal feed formulations. Cotton cake can be used in small amounts in ruminants nutrition (up to 30%), but is highly toxic for monogastrics (HAVARTINE; FIRKINS; EASTYRIDGE, 2002; XIAO et al., 2011; NETO et al., 2021). Jatropha cake is commonly used as an organic fertilizer, due to its high content in minerals such as nitrogen, phosphorus and potassium (GOMES, 2015).

This work was focused on cultivate white rot fungi in solid state fermentation in media containing JSC or CSC, to obtain fermented products with potential characteristics for the use in animal feed formulations, such as the presence of extracellular enzymes, bioactive chemical compounds (ergosterol and antioxidants) and potential of detoxification.

## 2 MATERIAL AND METHODS

### 2.1 Macrofungi

Basidiomycetes were provided by the Instituto Nacional de Pesquisas do Amazonas (INPA). After cryopreservation at -80°C in glycerol 30% (v/v), fungi were activated in petri dishes containing commercial Potato Dextrose Agar (PDA). Fungi that grew in this medium were considered viable. Agar-CSC and/or Agar-JSC media were prepared with 10% of the crushed dry biomass (following quality tests of biomass-agar medium in plates in Gomes, 2015) and 1.5% (w/v) of commercial agar, then sterilized for 30 minutes at 121°C. The viable fungi in PDA were inoculated in plates containing Agar-CSC, Agar-JSC or PDA (control), and incubated at 28ºC in triplicates. Radial growth of the fungi was measured with a caliper, every two days, and those that grew in medium containing CSC and/or JSC were selected for solid-state fermentation.

### 2.2 Solid-state fermentation in JSC and CSC

Solid-state fermentation (SSF) of macrofungi was carried out in 800 mL glass flasks covered with micropore tape. Culture media were prepared in triplicate with 40 g of JSC or 20 g of CSC (same volume). Moisture level of the biomasses was adjusted to approximatelly

60% (w/v) with distilled water. Flasks were sterilized at 121°C: JSC for 1 hour, for the elimination of endophytes (phorbol ester is thermostable), and CSC for 20 minutes (gossypol is thermolabile). After cooling, flasks were inoculated with 8 mm mycelial discs: the JSC flasks with 10 discs and the CSC flasks with 5 discs. Flasks were then kept at 28ºC for 15 days, or until complete colonization of the biomasses. Negative controls were performed in sterilized biomass flasks and incubated without any fungi.

**2.3 Quantification of toxic compounds**

For each of the biomasses (JSC and CSC), macrofungi with higher degradation of toxic compounds was selected. Fermentations of these biomasses were repeated and concentrations of the toxic compounds (phorbol ester in JSC and gossypol in CSC) were measured every 3 days of cultivation in solid-state, in order to establish a degradation curve from the beginning until 15 days of fermentation. Biomasses (*in natura*, sterilized and fermented) were dried at an oven at 60°C for 48 hours.

For determination of phorbol esters, 3 g of the dried samples were macerated with a mortar and pistil, according to Ribeiro et al. (2014). For determination of free gossypol, the dried samples were crushed and homogenized using a bench mill. Extraction and quantification of free gossypol (FG) was based on the methods described in Conceição et al. (2018), with acetone extraction and detection in an ultra-performance liquid chromatography (Acquity UPLC H-Class System, Waters, Massachusetts, USA) with PDA detector on Kinetex reverse phase column (100 x 2.1 mm x 2.6 μm), with pre-column kept at 35ºC. The PDA detector for acquisition 2D was set at 254nm, and 3D at the range of 210 to 400 nm. An elution gradient was employed and the total run time was 14 minutes. Phorbol esters and gossypol were detected as μg/g.

**2.4 Determination of the antioxidant activity**

From the solid-state fermentations, the best detoxification treatments for oilseed cakes (CSC and JSC) were determined. In order to obtain the antioxidant activity and phenolic compounds profiles, fermentations were repeated for the selected macrofungi. Samples analysis were performed every three days, by removing the flasks for quantification (in triplicate).

Extraction of total phenolic compounds (TPC) was performed according to Asolini, Tedesco and Carpes (2006), with modifications. 4 g of each dry sample were weighed in Falcon tubes, to which were added 35 ml of 80% (v/v) ethanol acidified with 0.5% (v/v) hydrochloric acid (HCl) P.A. in each extract. Tubes were bathed in boiling water for 30 minutes. Supernatant was removed and stored in another tube, while the precipitate was used for another extraction. The new supernatant was removed and kept together with the extract from the first extraction. The extract was centrifuged for 30 minutes at 6000 rpm and stored at 2ºC in the absence of light.

Total antioxidant activity (TAA) was performed using ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH (2,2–Difenil–1–picril–hidrazila) methods. ABTS method was carried out as according to Rufino et al. (2007), the $ABTS^{+}$ radical, generated during the oxidation of ABTS with potassium persulfate, captures hydrogen (electron donor of the antioxidant substance) by the radical, promoting discoloration of the solution. Discoloration degree is used to evaluate the antioxidant activity. The color of the extracts does not interfere in the analysis and the reagent is soluble in both polar and nonpolar solvents (ALAM et al., 2013).

DPPH method uses a purple and stable free radical, which after receiving the hydrogen atom of the oxidizing compounds is reduced and acquires a yellow color, measured by spectrometry (BRAND-WILLIAMS; CUVELIER; and BERSET, 1995). First, 150 µM DPPH solution, 2000 µM trolox (a synthetic antioxidant analogue to vitamin E) solution and extract dilutions at 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL were prepared. A standard trolox curve (40-360µM) was also prepared. TAA analyses were performed into wells of microplate, with 22 µL of each diluted extract and each point of the curve, together with 200 µL of the DPPH solution. As blank, 22µL of methanol was used in place of the sample and absorbance was taken at 520 nm in kinetics, every 15 minutes, for 1 hour, and every 1 hour, for 5 hours. DPPH oxidation inhibition data by time were plotted on a graph to determine the ideal time of antioxidant activity for each sample. The activity in trolox equivalent used at the ideal time determined in the previous step.

**2.5 Determination of total soluble proteins in the extracts**

The content of total soluble proteins in the crude extracts was determined through the bicinchoninic acid method (BCA) (SMITH et al., 1985), using ELISA plates according to the

kit manufacturer's protocol (Sigma-Aldrich®). A lineal regression curve was constructed using eight dilutions of known concentrations of bovine sereum albumin. From the linear regression line equation it was possible to calculate the concentration of total soluble proteins, in mg of proteins per ml of extract (mg/ml).

**2.6 Determination of enzymatic activities**

The best detoxification treatment for each of the oilseed cakes (JSC and CSC) were used for the determination of enzymatic activities throughout fermentation. The fermentation process was repeated for the selected fungi and three glass flasks containing the fermented media were removed for analysis, every three days.

Biomass of the biological triplicates for the different treatments (*in natura* cakes, sterilized cakes and cakes fermented every three days) were homogenized and 10 g (wet mass) of each sample were added to 50 mL of cold distilled water (1:5 m/v). Then, the mixture was homogenized in a shaker for 1 hour at 200 rpm and 5ºC, then centrifuged for 10 minutes at 8000 rpm and 4°C. After centrifugation, the supernatant was vacuum filtered using a 0.2 mm filter. Crude extracts were stored at 4ºC to determine the enzymatic activities. These enzymatic activities were expressed in IU/g of dry lignocellulosic substrate, IU is defined as the amount of reducing sugar (μmol) released per minute. All tests were carried out in triplicate.

Determination of proteolytic activities was done according to the protocol of Charney and Tomarelli (1947), with adaptations. Samples were centrifuged at 6000 rpm for 10 minutes at 4 ° C. Tests for proteases present in the extracts were carried out in ELISA micro plates, and sodium acetate buffer pH 5 (100 μL) was used as control.

Laccase activity was determined through oxidation of ABTS (Sigma-Aldrich) (WOLFENDEN; WILLSON, 1982) and readings were taken every 5 seconds at 420 nm (ε ABTS = 36000). Determination of total peroxidase activity was carried out in 96 well ELISA plates (HEINZKILL et al., 1998).

Determination of FPase activity followed the colorimetric method with DNS according to Xiao et al. (2011). Microassays were carried out to determine endoglucanase (CMCase) and xylanase activities using Carboxi-Methyl-Cellulose (CMC) 2% and xylan beechwood 2%, respectively (Sigma-Aldrich) (FILHO; PULS; COUGHLAN, 1993 modified; MILLER, 1959). The B-glucosidase activity was determined in a 96-well PCR plate, using

Cellobiose 15 mM as substrate (Sigma-Aldrich) (ADNEY; BAKER, 1996; GHOSE, 1987 modified). The released glucose was quantified on an ELISA plate using a commercial GOD-POD kit (Bioclin®), and absorbance was measured at 505 nm.

## 2.7 Biomass characterization

The oilseed cakes biomasses from the best detoxification treatments were chemically characterized and compared to the controls: cakes (substrates) without physical (sterilization using autoclave) and/or biological (macrofungus) treatments.

### 2.7.1 Bromatological characterization

CSC and JSC with and without physical and biological treatments were analyzed with respect to their dry matter content; mineral matter; neutral detergent fiber (NDF); acid detergent fiber (ADF); ether extract; and crude protein. Samples were dried for 48 hours with forced ventilation at 25°C and crushed in a Willey mill with a 60-mesh screen.

Dry matter content was determined using an oven at 105ºC for 12 hours and the mineral matter (ashes) were placed in muffle at 600ºC for 4 hours. Analyzes followed the methodologies of Van Soest (1994) for determination of the cell-wall components and quantification of NDF and ADF. Determination of the ether extract content was done according to the Am 5-04 method of (AOCS 2005) using an Ankon type equipment.

For determination of the crude protein (CP) content, the Kjeldahl method (AOCS, 2005) was used. This same method was used to determine the nitrogen concentration in the samples after aqueous extraction, using a Solvent Accelerated Extractor (Dionex ASE 350), in order to determine the content of structural proteins and measure the solubilization of proteins by the fungus growth. Concentration of soluble proteins was determined by the difference between crude protein (crude samples) and structural proteins (samples after the extraction process).

### 2.7.2 Determination of the strutural carbohydrate profile (cellulose, hemicellulose and lignin)

Samples were characterized in terms of cellulose, hemicellulose, lignin and extractives contents according to procedures recommended by the National Renewable Energy Laboratory (NREL, Golden, CO, USA) (SLUITER et al., 2008a; SLUITER et al., 2008b; SLUITE et al., 2008c). Molar absorption coefficient of the lignin from the JSC and CSC lignocellulosic substrates were determined in the Biomass and Biofuels Chemistry Laboratory at Embrapa Agroenergia, as well as other variables related to the biomasses.

**2.8 Determination of ergosterol**

The best detoxification treatments for JSC and CSC oil cakes were used to determine ergosterol levels at the end of fermentation. Ergosterol from biomasses without fungus was also quantified (control). The dried samples were macerated in a mortar with liquid nitrogen, and approximately 0.5 g of the obtained powder was weighed. To each sample tube, 2 g of KOH, 10 ml of methanol and 5 ml of ethanol were added and mixture by sonicator in a water bath for 30 minutes at 70ºC. Flasks were kept in the dark and, once they reached room temperature, 5 ml of distilled water and 10 ml of hexane were added. Mixture was then sonicated for 1 minute and the hexane phase (upper) was transferred to rotary evaporator flasks. The hexane extraction process was repeated three times, and the extracts were combined in one flask. The extracts were evaporated at 40ºC and 300 mbar and resuspended with 2 mL of methanol in sonicator for 1 minute. 1 mL of each sample was transferred to Eppendorf microtubes, centrifuged and finally transferred to vials for injection into ultra-efficient liquid chromatograph (Acquity UPLC HClass System, Waters, Massachusetts, USA) with Kinetex column PDA detector 2.6 µm C18 (STEUDLER and BLEY 2015).

**2.9 Determination of the aminoacids profile**

The best detoxification treatments for JSC and CSC oil cakes were selected for determination of the aminoacids profile. The same analyzes were carried out for unfermented biomasses (control). Determination of the tryptophan content was carried out according to the methodology of Hagen, Frost, Augustin (1989), and those of the other amino acids and total aminoacids were done through HPLC, according to White, Hart, Fry (1986), by the CBO group (https://www.grupocbo.com.br).

## 2.10 Statistical Analysis

The results of detoxification, enzymatic activities and characterization of the treated biomasses were subjected to analysis of variance, regression and correlation usingg SISVAR (FERREIRA, 2000). For the other tests, the means of the repetitions were compared using the Scott-Knott test, at 5% significance level.

# 3 RESULTS AND DISCUSSION

## 3.1 Selection of macrofungi

Preliminary identification of fungal species is shown in Table 2.1S of supplementary materials. Initially, macrofungi were analyzed with respect to their growth on JSC and CSC biomasses. The growth rate of macrofungi on JSC and CSC-based substrates is shown in Table 2.2S (supplementary material). From the 29 viable macrofungi on PDA, 19 were able to grow in petri dishes containg only Agar-JSC as carbon source. The mycelial growth rate varied from 1.92 to 7.37 mm/day, for macrofungi *Trametes elegans* INPA1698 and *Coriolopsis sp.* INPA1646, respectively. However, some of the fungi that grew on the plates with Agar-JSC or Agar-CSC did not grow on solid-state fermentation with JSC or CSC.

*F. flavus* INPA1739 and *Coriolopsis sp*. INPA1646 showed the highest growth-rate on the CSC and JSC-Agar plates, respectively. *Coriolopsis sp*. INPA1646 also presented the highest growth-rate in glass flasks containg SSF-CSC and SSF-JSC. The lowest growth-rate on Agar-CSC and Agar-JSC was observed for *Trametes elegans* INPA1698, while, on glass flasks, the lowest growth-rate was observed for *Trametes sp*. INPA 1719 on SSF-JSC and for *Trametes elegans* INPA 1698 on SSF-CSC.

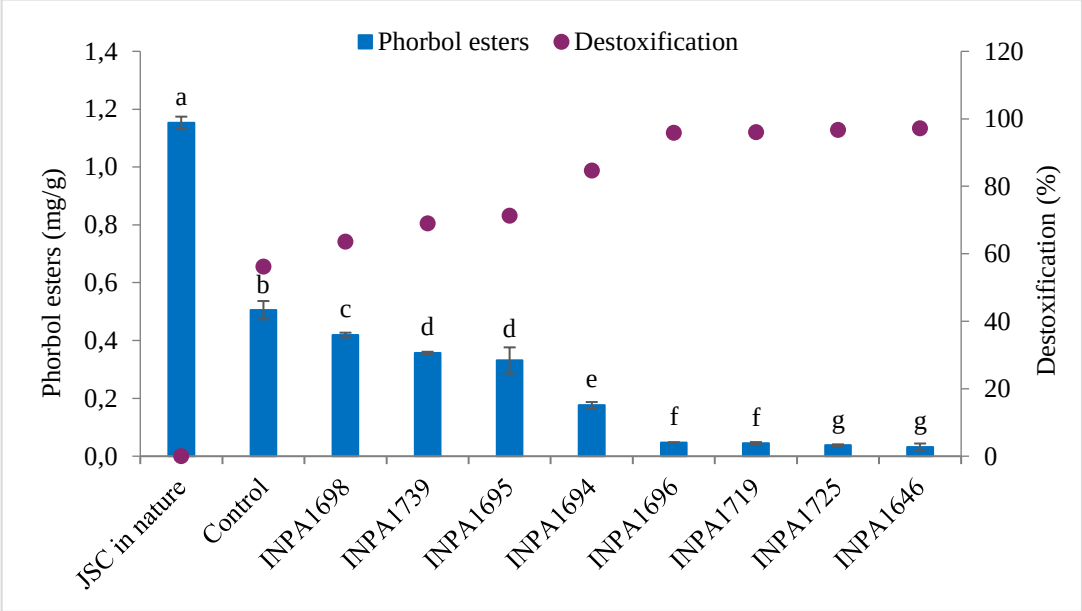
## 3.2 Cake biodetoxification through macrofungi solid-state fermentation

### 3.2.1 Jatropha Seed Cake – JSC

Ten macrofungi were able to grow on SSF-JSC and completely colonized the substrate after 14 days. Figure 1 shows the residual phorbol ester rate on the fermented cakes and shows the degradation rate of phorbol esters by macrofungi, compared to the *in natura* cake.

*In natura* JSC presented a concentration of 1.153 mg of phorbol ester per gram of cake, similar to the results of BOSE and KEHARIA (2014) of 1.072 mg/g.

**Figure 2.1** Concentration and degradation percentage of phorbol esters in jatropha seed cake after 14 days of fermentation by macrofungi. Data are presented in decreasing order of concentration. Control = unfermented autoclaved JSC. Tukey test ($p \leq 0,05$).




Fonte: da autora (2021)

For animal nutrition, 3 ppm of forbol esters are considered safe, according to the European Directorate-General for Health and Food Safety. For humans, however, there is not enough data yet to confirm safe levels of these substances (DG SANTE, 2016). Neverthelesse, a variety of non-toxic *J. curcas* was found in Mexico (KING et al., 2009; MAKKAR; BECKER, 1997). These seeds, once they are roasted, are consumed by the local population (KING et al., 2009), the only difference between toxic and non-toxic seeds is the concentration of phorbol esters between 0 and 0.09mg/g in non-toxic seeds. No differences were observed for the levels of amino acids, trypsin inhibitor, lectin, phytate, curcine and saponin when comparing toxic and non-toxic *J. curcas* varieties (HE et al., 2011; INSANU et al., 2013; MAKKAR; ADERIBIGBE; BECKER, 1998; MAKKAR; BECKER, 1997).

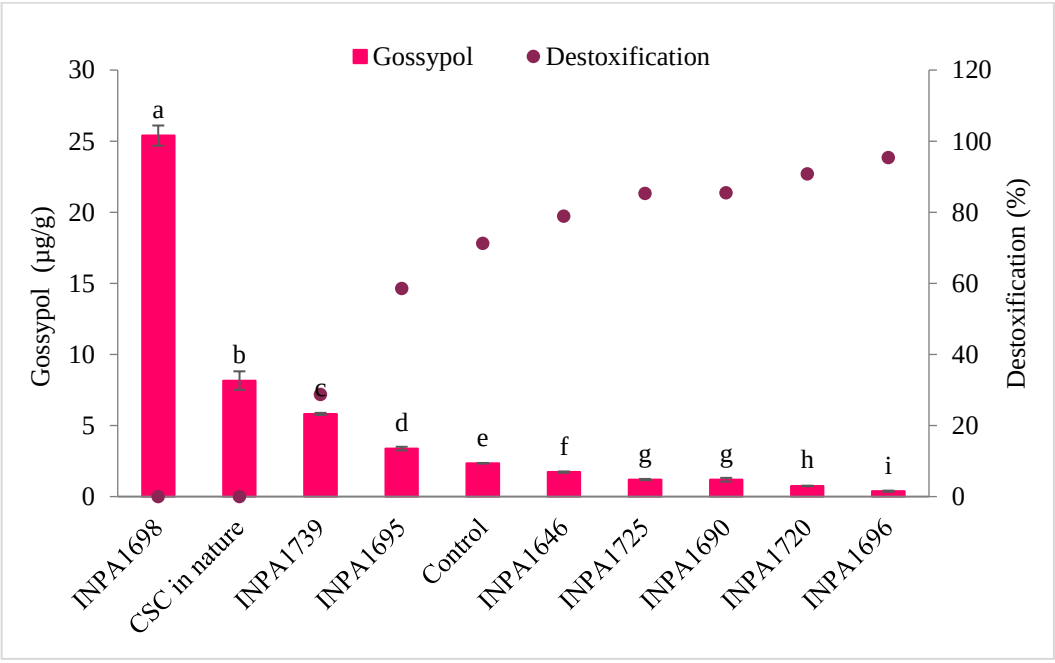
Degradation of phorbol esters through SSF-JSC by *Pleurotus* species is widely discussed in literature. A patent has even been filed, and it claims the biological detoxification of phorbol esters through fermentation using *P. ostreatus*, for 30 days (MANTOVANI et al.,

1989). Such potential detoxification is explained by the *P. ostreatus* ability to degrade lignin and substances such as chlorophenols and aromatic hydrocarbons, due to its laccase and manganese peroxidases activities (KASUYA et al., 2012; LUZ et al., 2013). However, in general, an efficient degradation process, using *Pleurotus* species, takes at least 30 days, while fermentation lasts for 15 days. And as a result, a maximum degradation of 58% of phorbol esters is achieved, much less than the 99.22% found in this work (Table 2.2). Other macrofungi have also been reported in the literature with the ability to degrade phorbol esters thorough SSF-JSC, as seen in Table 2.3S (suplementary material).

### 3.2.2 Cotton seed cake – CSC

Five macrofungi (INPA1646, INPA1695, INPA1696, INPA1720 and INPA1739) completely colonized CSC in glass flasks after 14 days. INPA1690 took 20 days for complete colonization, while INPA1698 and INPA1725 took 22 days. Figure 2.2 shows the rate of residual gossypol on the SSF-CSC and shows the degradation rate of gossypol by macrofungi and by sterilization using autoclave (physical treatment), compared to the *in natura* cake.

**Figure 2.2** Concentration and degradation percentage of free gossypol on sterilized cotton seed cake (control) and on SSF-CSC (fermented) between 14 and 22 days by INPA macrofungi (fungal names are showed in Table 2.1S). Control = unfermented autoclaved CSC. Tukey test ($p \leq 0,05$).




Fonte: da autora (2021)

Action of some macrofungi (INPA1698, INPA1739 and INPA1695), instead of degrading free gossypol, causes an increase in its concentration in the cotton seed cake (Figure 2.2). This is probably due to the release of bound gossypol by these fungi. Levels of gossypol from 200 and 300 ppm are toxic for non-ruminant animals. For adult pigs, for example, these levels are even lower: they do not tolerate more than 60 ppm of free gossypol, while ruminants tolerate higher levels of gossypol, from 350 to 500 mg/g for goats, which are the most sensitive among the ruminants (SAUNDERS, 2017).

Degradation of free gossypol in CSC is not exceedingly difficult to occur, since it is an unstable substance. Furthermore, degradation of this substance by fungal fermentation has been reported in literature, with reductions of gossypol up to 65.2%, by Ascomycetes such as *Fusarium thapsinum* (MAGESHWARAN and MAJEE, 2017), and of 94 and 93% by *P. ostreatus* and *F. hepatica*, respectively (ARAUJO, 2018). Araujo (2018) also carried out solid-state fermentation of CSC using *P. lecomtei* and achieved a reduction of 75% on the levels of free gossypol, after 20 days of cultivation, which corresponds to a longer fermentation time and a lower degradation rate than that achieved in the current work. No reports were found in literature regarding degradation of free gossypol by macrofungi of the genus *Tyromyces*.

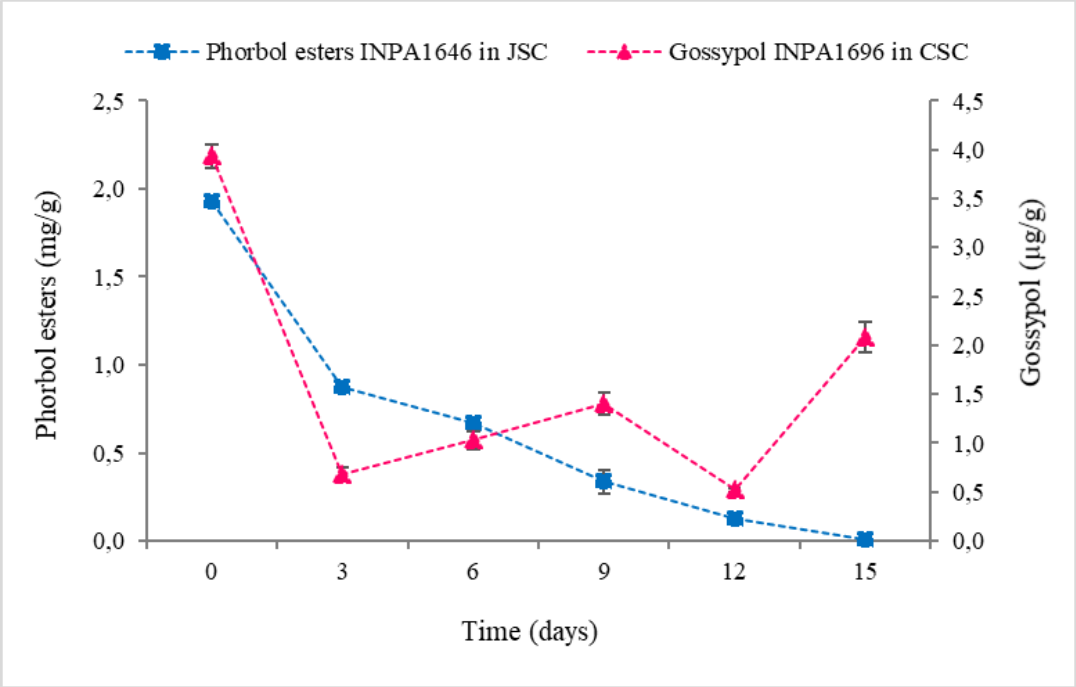
According to the work of Rajarathnam, Shashirekha and Bano (2001) *P. florida* could detoxify, after five days, up to 100% of gossypol, in medium containing 100 µg/g of gossypol. Treatment of CSC using other microorganisms, such as *C. tropicalis*, *S. cerevisae*, *A. niger* and *A. terricola* could also significantly degrade the free gossypol in the cake (up to 88.51%) after 15 days of incubation (ZHANG et al., 2006).

### 3.2.3 Degradation kinetics of toxic compounds

Fermentation of the cakes were repeated with the selected macrofungi, and biological triplicates were taken every three days of culture, to determine the levels of phorbol and gossypol ester on SSF-JSC and SSF-CSC (Figure 2.1S - Supplementary material).

Figure 2.3 illustrate the progressive degradation of phorbol and gossypol on SSF-JSC and SSF-CSC, respectively, for 15 days of fermentation, with the selected macrofungi. It is noteworthy that solid-state fermentation processes of agro-industrial residues by macrofungi generally occur for longer periods, as previously reported in literature.

**Figure 2.3** Concentration of phorbol esters in jatropha cake fermented by *Coriolopsis sp*. INPA1646 and of gossypol in cotton seed cake fermented by *Tyromyces sp.* INPA1696, every three days of cultivation.




Fonte: da autora (2021)

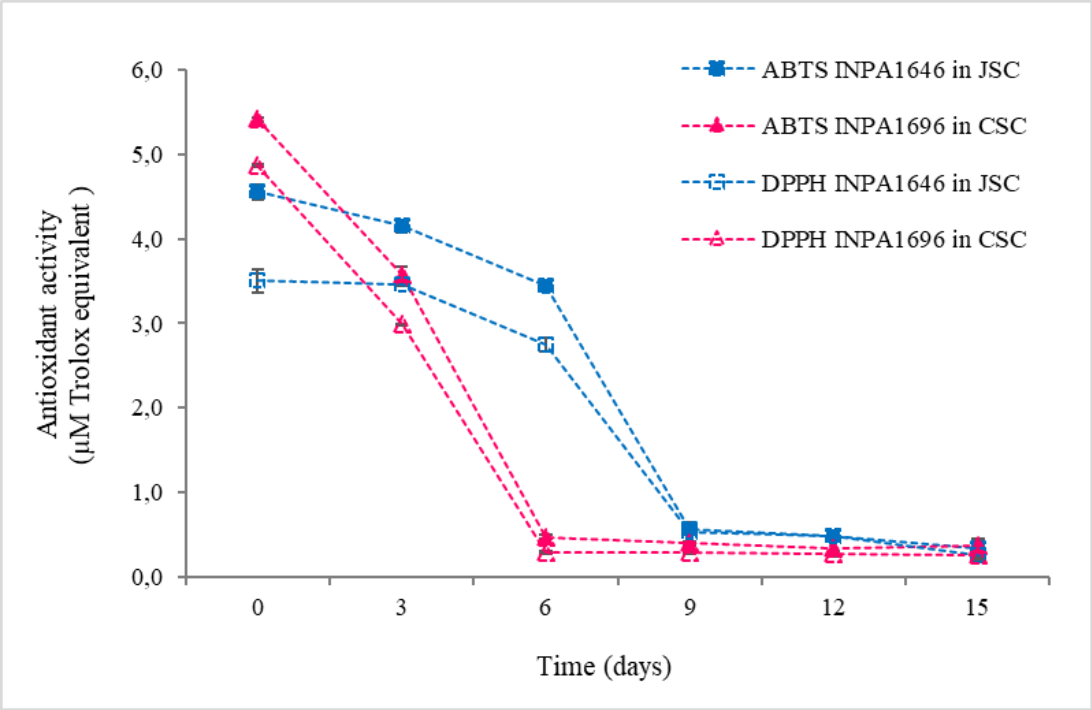
CSC autoclaved for 20 minutes degrades up to 89.4% of the free gossypol in the cake. However, during fermentation by *Tyromyces sp*. INPA1696, levels of free gossypol (FG) vary, increasing and decreasing its concentration, indicating that the fungus may degrade, but also release bound gossypol (BG) during fermentation (Figure 2.3). Despite that, previous work demonstrates that longer fermentation processes result in complete degradation of gossypol in CSC. However, despite the release of BG as FG, the degradation process continues to occur due to the enzymatic activities involved in the process. This corroborates the previous work of Soares Neto (2019), which indicates a series of genes and proteins from *P. lecomtei* CC40 that could be associated to the CSC detoxification process.

### 3.3. Determination of antioxidant activity

SSF-JSC and SSF-CSC, using *Coriolopsis sp*. INPA1646 and *Tyromyces* sp. INPA1696, respectively, for 15 days of cultivation, were also evaluated in terms of antioxidant activity. Every three days, biological triplicates were taken to determine

antioxidant activity by ABTS and by DPPH (Figure 2.4). Both methods of determination of antioxidant activity resulted in identical activity profiles, with slightly higher activities by the ATBS method.

**Figure 2.4** Determination of antioxidant activity, through ABTS and DPPH methods, for SSF-JSC and SSF-CSC using macrofungi *Coriolopsis sp*. INPA1646 and *Tyromyces sp*. INPA1696, respectively.




Fonte: da autora (2021)

In general, antioxidant activity on *in natura* cakes declines over the course of fermentation, probably due to the action of oxidative enzymes, such as laccases and peroxidases (KERSTEN and CULLEN, 2007), the action of other fungal bioactives secreted during fermentation and products formed by enzymatic reactions. Extracellular oxidative enzymes are involved in the process of depolymerization of lignin, caused mainly by the generation of free radicals by oxidases and peroxidases (KERSTEN and CULLEN, 2007).

Cakes, for themselves, present high antioxidant activity, as Guimarães (2020) and Nithiyanantham, Siddhuraju and Francis (2012) also observed for Jatropha seed cakes. Besides, peptides extracted from CSC also present antioxidant activity, which reduces catechin oxidation (KHASANOV, DAVRANOV and HASANOV, 2017). However, macrofungi are known to produce substances with antioxidant activity, such as phenolic

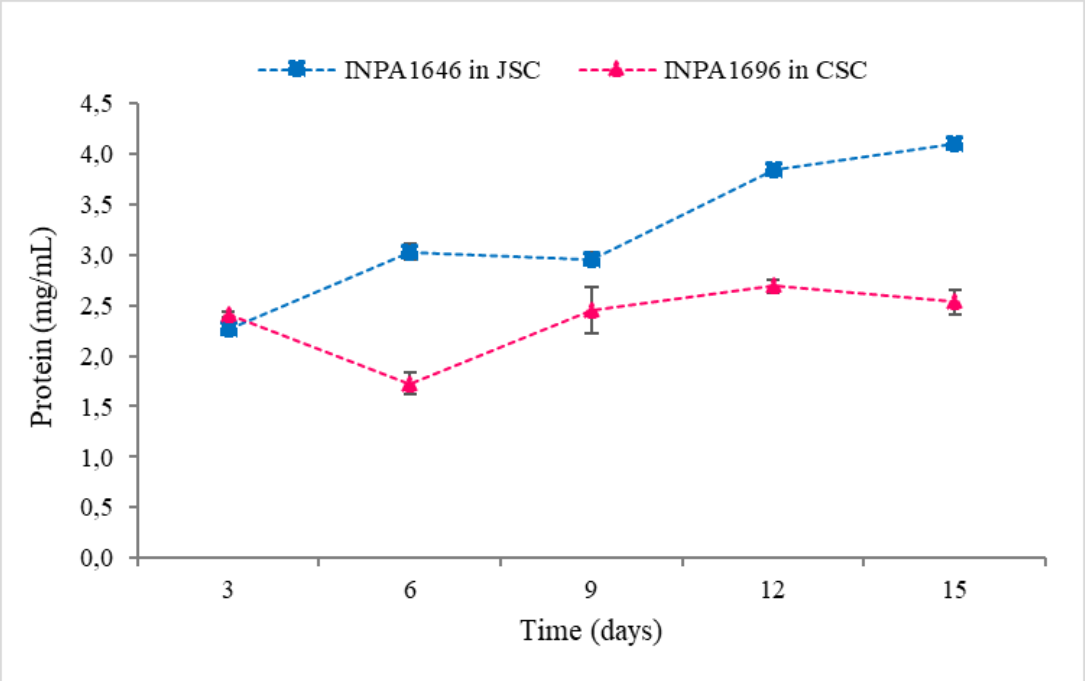
compounds, vitamins, flavonoids and tocopherols (MATTILA, MARNILA and PIHLANTO, 2018). Hence, under the studied contidions, reduction of the natural antioxidant activity of JSC and CSC cakes by fungal enzymes is clearly more significant than the production of any fungal compounds with antioxidant activity.

## 3.4. Determination of total soluble proteins and enzymatic activity

### 3.4.1 Kinetics of total soluble proteins

The extract that provided the highest concentration of total soluble proteins was the JSC extract fermented for 15 days by macrofungus INPA1646 (Figure 2.5). BCA method is used to quantify the fungal proteins released in the medium, including the enzymes. However, the aqueous extraction also removes the soluble vegetable proteins. Hence, a high content of total soluble proteins does not necessarily indicate a high concentration of secreted fungal enzymes.

**Figure 2.5** Total soluble protein kinetics in the crude extracts of SSF-JSC and SSF-CSC using *Coriolopsis sp*. INPA1646 and *Tyromices sp*. INPA1696, respectively.




Fonte: da autora (2021)

Throughout fermentation, levels of total soluble proteins on JSC were higher than in CSC. The highest concentration (4 mg/mL) was reached on the 15th day of fermentation. A fraction of fungal proteins produced during substrate colonization (proteolytic enzymes) is also responsible for the solubilization of vegetable proteins. Solubilization of vegetable proteins could be observed on the bromatological characterization of the cakes. Cunha (2017) observed that the submerged fermentation of JSC by *P. pulmonarius* EF88 and *P. lecomtei* CC40, for seven days, resulted in supernatants with 1.91 and 2.55 mg/mL of total soluble proteins.

### 3.4.2. Kinetics of extracellular enzymes

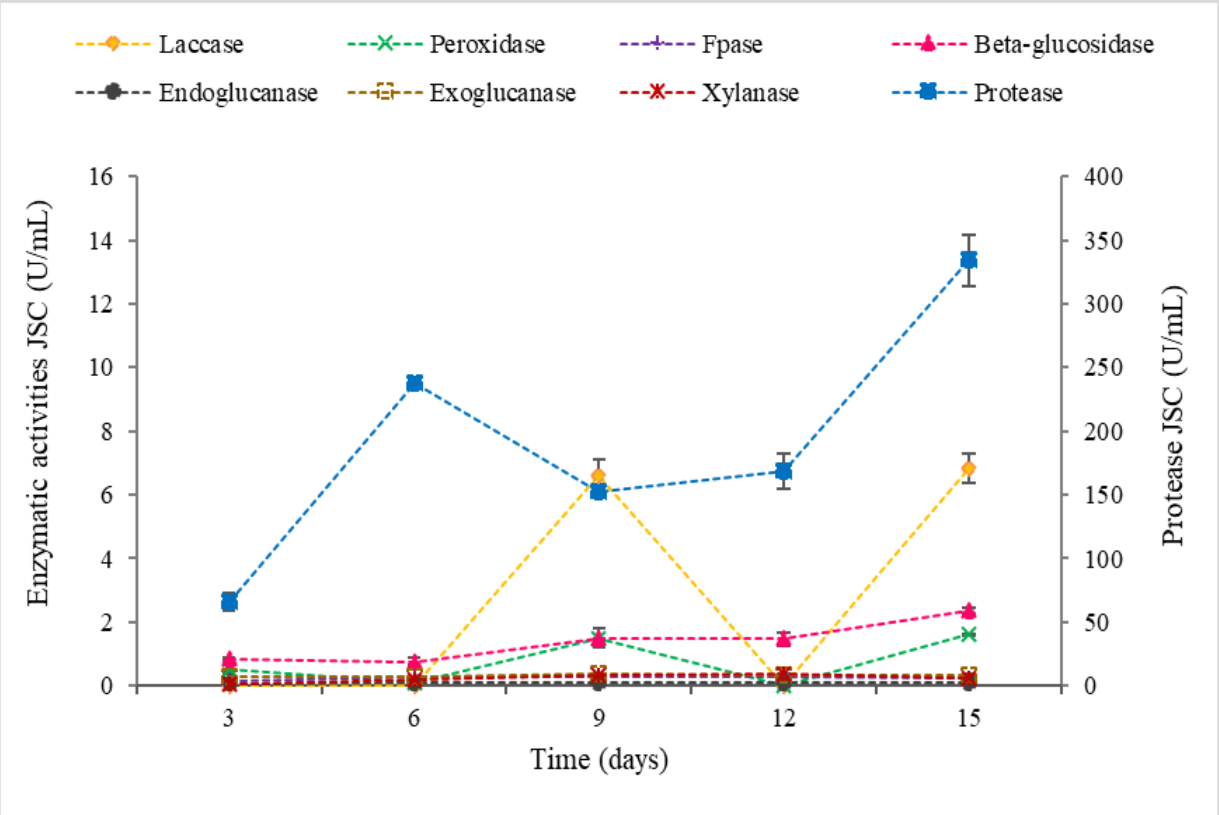
Proteases are enzymes that cleave the peptide bonds of proteins, releasing peptides or amino acids (LOPEZ-OTIN and OVERALL, 2002). They can be produced and released by a wide variety of microorganisms, such as bacteria, protozoa, yeasts and fungi. These enzymes can also be intra or extracellular, being linked or not to the membrane. Approximately 40% of the commercial proteases are of microbial origin, which are preferred to those of animal and plant origins, due to their lower production cost and more advantageous characteristics for biotechnological applications (FEDATTO, 2004).

Difference on the protease's activities for the two crude extracts from SSF-CSC and SSF-JSC was clear. *Tyromices sp*. INPA1696 (CSC) provided better results than *Coriolopsis sp.* INPA1646 (JSC) (Figure 2.6). The highest production of protease occurred between the 12th and 15th day of fermentation of SSF-CSC by *Tyromices sp*. INPA1696 (Figure 2.7), with an activity of 600 U/mL. For *Coriolopsis sp*. INPA1646 on SSF-JSC the maximum protease production was 350 U/mL, reached on the last day of fermentation (Figure 2.6). Yang et al. (2012) suggested that some proteases are closely linked to the degradation of free gossypol, then, probably because of this, the peak of protease activity for CSC was observed on the 12th day, the same day in which a significant reduction on the levels of free gossypol was observed, as well as a lower concentration of soluble proteins during cultivation (Figures 2.3 and 2.7). These might have been metabolized by macrofungus in the form of aminoacids or peptides.

Proteases were the enzymes with the highest activities observed, on the scale of hundreds of U/mL. The highest production of protease occurred on the 12th and 15th days of SSF-CSC by *Tyromices sp*. INPA1696, with an activity of 600 U/mL, indicating a high

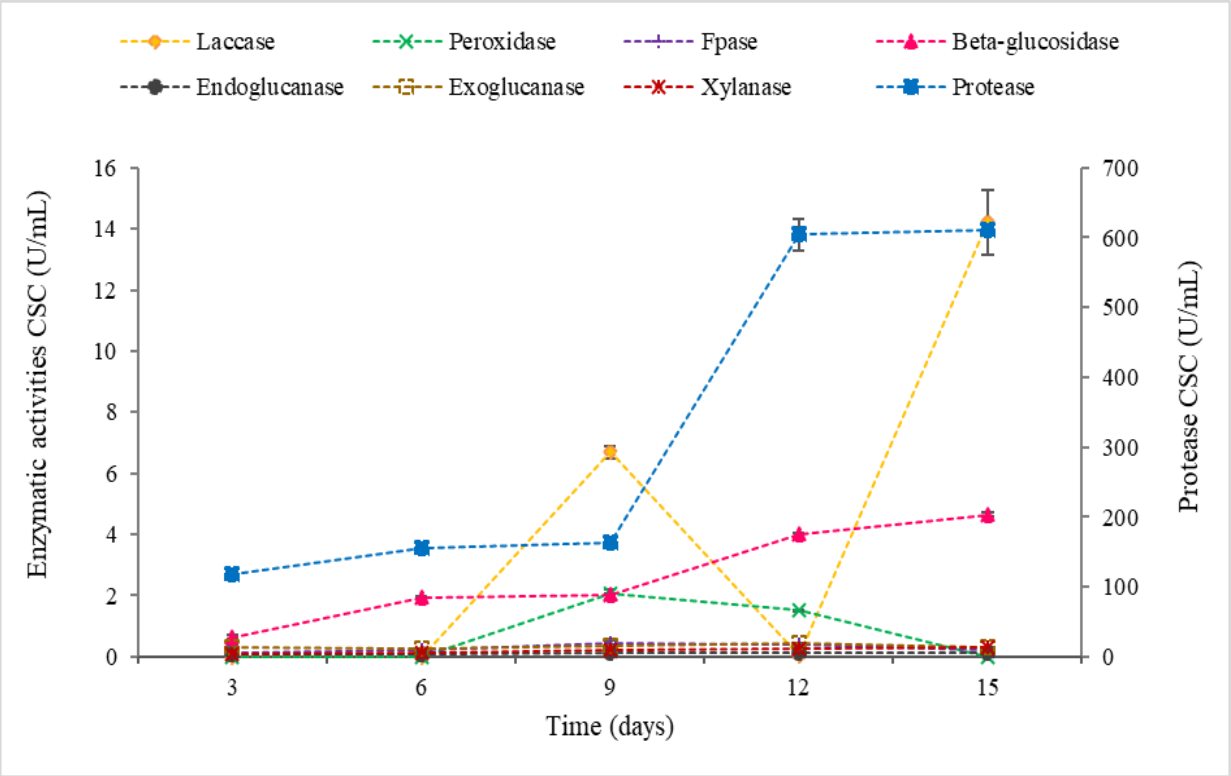
potential for biotechnological application. For *Coriolopsis sp*. INPA1646 on SSF-JSC, the highest activity was 350 U/mL, observed on the last day of fermentation (Figure 2.10). Perhaps, better results could have been achieved for cultivations longer than 15 days.

**Figure 2.6** Activity kinetics of soluble enzyme activities (laccase, peroxidase, Fpase, beta glucosidase, endoglucanase, exoglucanase, xylanase and protease) in the crude extracts of SSF-JSC using *Coriolopsis sp*. INPA1646.




Fonte: da autora (2021)

**Figure 2.7** Activity kinetics of soluble enzyme activities (laccase, peroxidase, Fpase, beta glucosidase, endoglucanase, exoglucanase, xylanase and protease) in the crude extracts of SSF-CSC using macrofungi *Tyromices sp*. INPA1696.




Fonte: da autora (2021)

The highest laccase activity was observed for SSF-CSC using *Tyromices sp.* INPA1696, for 15 days, with an activity of 15 U/mL (Figure 2.6). This is more than twice the highest activity observed using *Coriolopsis sp.* INPA1646 on SSF-JSC, which was of only 7 U/mL (Figure 2.7). In general, laccase activities were low for both fungi, during the whole fermentation, when compared with other macrofungi.

Cultivation of *P. ostreatus* CC389 on CSC, carried out by Araujo (2018), reported higher laccase activity on the crude extract, for the first five days of fermentation (166 U/mL) than those observed in the current work, using *Tyromyces sp*. Araujo (2018) observed a decrease of laccase activity between the 15th and the 20th day, with 20 U/mL. However, this activity is still higher than the maximum observed in the current work. Laccase activity observed by Gomes (2015) in the aqueous extract of SMS from JSC, during 60 days of cultivation, using *P. pulmonarius* EF88, was 9.01 U/mL. This result is similar to that observed in the present study, at the end of 15 days of cultivation. However, Cunha (2017) observed much higher laccase activities for the submerged fermentation (SSF-JSC) using *P. pulmonarius* EF88 and *P. lecomtei* CC40, for seven days, with activities of 583.99 and 101.89 U/mL, respectively.

The highest activity of total peroxidases was observed at the 9th day of SSF-CSC using *Tyromices sp*. INPA 1696. Until the sixth day no activity was observed. On the 6th and 12th days of SSF-JSC using *Coriolopsis sp*. INPA1646, neither. Only on the 9th and 15th day of fermentation it was observed some peroxidase activity (Figure 2.6). For both fungi, peroxidase activity was practically basal, throughout the whole fermentation, with a peak of 2 U/mL.

Cunha (2017), supernatants from the seven-day submerged fermentation of JSC by *P. pulmonarius* EF88 and *P. lecomtei* CC40 presented total peroxidase activities of 64.09 and 25.56 U/mL, respectively. And, in the work of Gomes (2015), the aqueous extract of SMS of *P. pulmonarius* EF88 on JSC, after 60 days, presented peroxidase activity of 22.2 U/mL, a result much higher than the activities observed in the current reserach, under the studied conditions for cultivation and production of the crude extracts from *Coriolopsis sp*. INPA1646.

*Tyromices sp*. INPA1696 presented high activity of total cellulases on SSF-CSC. The FPase assay determines the activity of total cellulases and, as can be seen in Figure 2.7, on the 12th day of CSC fermentation a peak on the production of cellulases by *Tyromices sp*. INPA1696 was observed. Araujo (2018) achieved even higher levels of FPase activity using *P. ostreatus* CC389 in CSC: it reached 0.90 U/mL on the 15th day of SSF. FPase activity for *Coriolopsis sp*. INPA1646 on SSF-JSC (Figure 2.6) was low and constant throughout fermentation, presenting activities similar to those observed in Gomes (2015), on the 60-day aqueous extract of SMS using *P. pulmonarius* EF88 on JSC (0.11 U/mL) and by Cunha (2017), on the supernatants of the submerged culture of JSC using *P. pulmonarius* EF88 (0.10 U/mL).

Beta-glucosidases are enzymes responsible for the cleavage of cellobiose beta-type bonds and are an important class of cellulases. *Tyromices sp*. INPA1696 in CSC presented high activity of this enzyme on the 12th and 15th days of fermentation (Figure 2.7). However, cellulase activity by *Coriolopsis sp*. INPA1646 in JSC remained at basal levels, as was previously observed in Gomes (2015), on day 60th aqueous extract of SMS using *P. pulmonarius* EF88 in JSC (0.08 U/mL). Behaviour of beta-glucosidase activity by *P. ostreatus* CC389 in CSC, observed by Araujo (2018), was the opposite of the behavior observed on the current work, using *Tyromyces sp*. INPA1696 (Figure 2.6). Araujo registered a gradual decrease on the activity levels from the tenth day of fermentation, achieving a peak of only 0.22 U/mL, on the $10^{th}$ day.

On the 12th day of CSC fermentation by *Tyromices sp*. INPA1696 it was observed the peak of endoglucanase production (Figure 2.7). This enzyme cleaves the internal bonds of cellulose into smaller molecules and, for this reason, CMC (carboxymethylcellulose) is used as a substrate to determine the activity of this enzyme. The high activity of this enzyme among the cellulolytic activities obtained on the extracts from this isolate indicated that this fungus has an excellent cellulolytic potential. CMCase (endoglucanase) activity by *Tyromyces sp*. INPA1696 in CSC was the opposite of those for *P. ostreatus* CC389 observed in the research of Araújo (2018), in which the peak of activity was observed on the first 5 and 10 days, reaching 0.98 U/mL on the tenth day, with further decrease to 0.55 U/mL on the 15th day. With respect to the activities of this enzyme in the extracts from *Coriolopsis sp*. INPA1646 on SSF-JSC (Figure 2.6), despite its low levels, even lower values were observed in Gomes (2015) on the SMS of *P. pulmonarius* EF88.

Exoglucanases, unlike endoglucanases, cleave bonds at the ends of cellulose and are also an important class of cellulases. Among the analyzed cellulases, these were the ones that presented the lowest activity during CSC fermentation. For both fungi and cake, activity drecreased throughout fermentation, reaching a maximum of only 0.17 U/mL, on the 9th day of the fermentation process, by INPA1696 (Figure 2.7). The low solubility of pectin, used as a substrate on this test, resulted in high standard deviations. Hence, it is not possible to affirm that there is a statistically significant difference on the activity levels observed.

Xylanase was the only among the holocellulases (cellulases and hemicellulases) that presented higher activity on SSF-JSC compared to SSF-CSC. *Coriolopsis sp*. INPA1646 showed increasing activities of xylanase until the 12th day of JSC fermentation, with a decrease on the 15th day (Figure 2.6). For *Tyromices sp*. INPA1696, activities decreased from the 3rd to the 6th day, after which an increase was observed, lasting until the end of the fermentation (Figure 2.7). In the work of Araújo (2018) the highest xylanase activity was 0.24 U/mL, on the tenth day of cultivation of *P. ostreatus* CC389 in CSC. On the 15$^{th}$ day, 0.19 U/mL were observed, less than the 0.35 U/mL achieved in the current work, using *Tyromyces sp*. INPA1696. Gomes (2015) with its 60-day SMS of *P. pulmonarius* EF88 in JSC observed an activity of 0.21 U/mL of xylanase.

Extracellular microbial enzymes FPase, CMCase, β-glucosidase and xylanase are of great biotechnological and industrial interest, due to their ability to degrade the plant cell wall. Fungi growth on agro-industrial residues is associated to their ability to produce holocellulases enzymes (cellulases and hemicellulases). Among fungi with such ability,

basidiomycetes stand out: they complement their oxidative activities and manage to completely deconstruct the plant cell wall (ARAUJO, 2018). It is common to quantify activities of these cellulolytic enzymes while carrying out fermentations of these biomasses by macrofungi. Araujo (2018) observed that the activities of xylanase (hemicellulase), CMCase (endoglucanase) and β-glucosidase of *P. ostreatus* on CSC had its peak on the first 10 days of fermentation, with gradual decrease over time. Total FPase activity (exoglucosidase, endoglucosidase and beta-glucosidase), however, had a different behavior, increasing during cultivation.

### 3.5 Characterization of the fermented SSF-JSC and SSF-CSC

The 14-day fermented SSF-JSC and SSF-CSC by macrofungi *Coriolopsis sp*. INPA1646 and *Tyromyces sp*. INPA1696, respectively, were characterized in terms of bromatological analysis and concentration levels of cellulose, hemicellulose and lignin (NREL).

#### 3.5.1 Bromatological characterization

After 14 days of fermentation, SSF-JSC and SSF-CSC presented a reduction of 10% reduction on their mass content (Table 2.1). This could be explained by the loss of carbon in the form of $CO_2$ during fungal respiration. It could also explain the increase on the levels of other components, such as ashes, which increased at least 0.43% on SSF-JSC. It might have occurred a proportional increase on the content of inorganic compounds to the detriment of organic matter, lost during fermentation. On SSF-CSC, all the other levels, except for ether extract (EE), increased with the growth of fungus *Tyromices sp*. INPA1696.

**Table 2.1** Bromatological analysis of JSC and CSC before and after fermentation by *Coriolopsis* sp. INPA1646 and *Tyromices* sp. INPA1696, respectively (%±SD).

| Samples | JSC *in natura* | JSC (autoclaved) | SSF-JSC | CSC *in natura* | CSC (autoclaved) | SSF-CSC |
|---|---|---|---|---|---|---|
| **DM** | 97.12±0.16a | 97.20±0.05a | 87.18±0.12d | 93.73±0.47c | 95.41±0.47b | 82.99±0.09e |
| **Ashes** | 4.66±0.05d | 4.52±0.10d | 5.33±0.19b | 5.47±0.05b | 5.00±0.08c | 6.80±0.15a |
| **CP** | 27.35±1.12a | 28.59±1.20a | 28.27±1.04a | 21.19±1.58b | 20.73±0.8b | 23.58±2.24b |

| | | | | | | |
|---|---|---|---|---|---|---|
| **EP** | 19.17±0.69b | 18.82±0.11b | 16.42±0.66d | 20.96+2.36a | 20.94+1.06a | 18.03+0.87c |
| **SP** | 8.18±0.48c | 9.77±0.85b | 11.85±0.97a | 0.23±0.05d | 0.21±0.06d | 11.50±1.14a |
| **NDF** | 52.74±1.24d | 53.64±0.55c | 56.89±0.83a | 51.10±1.99d | 54.55±1.65b | 54.03±1.96b |
| **ADF** | 40.54±0.76b | 35.25±0.44d | 45.22±0.62a | 34.89+0.56d | 36.96+0.32c | 40.29+1.47b |
| **CF** | 37.54±1.34a | 34.00±0.32b | 38.50±0.78a | 28.80±2.19cd | 28.82±0.47d | 30.90±0.66c |
| **Lig** | 23.36±0.41b | 19.21±0.45c | 28.72±0.55a | 9.19±0.51b | 10.09±0.35b | 14.27±0.35a |
| **EE** | 12.62±0.07a | 13.16±0.46a | 5.07±0.42b | 3.53±0.19c | 0.48±0.12e | 0.84±0.02d |

DM= dry mass; CP= crude protein; EP= Estructural protein; SP= Soluble protein; NDF= neutral detergent fiber; ADF= acid detergent fiber; CF= crude fiber; Lig= lignin; EE= ether extract.
Letters next to the values correspond to the comparisons of the averages on the same line and not between one line and another (Tukey test $p \leq 0.05$).

Fonte: da autora (2021)

NDF content on SSF-JSC and on SSF-CSC presented a slight increase, probably because of lignin concentration due to degradation of cellulose and other less complex carbohydrates. Structural carbohydrates with low digestibility, which could interfere on the animal diet, were found. Crude fiber content (CF) corresponds to cellulose, hemicellulose and lignin, as well as NDF, but their values are generally underestimated, while ADF represents only the cellulose and lignin fractions (NEUMANN, 2002). Gomes (2015), on his bromatological analysis of JSC and SMS fermented by *P. pulmonarius* EF88 on 100% of JSC, observed an increase on the levels of crude protein (from 22.41 to 26.11%), of NDF (from 48.7 to 57.19%) and of AFD (from 43.17 to 48.15%).

Analyzes of NDF and ADF calculate the percentage of soluble and insoluble fibers in the samples. The main insoluble fibers in plant samples are cellulose and lignin, and the the main soluble fiber is hemicellulose. NDF is the nutritional fraction corresponding to the sum of hemicellulose, cellulose and lignin. Hemicellulose is considered highly digestible, cellulose presents variable digestibility and lignin is practically indigestible. However, although there is no reduction in the concentration of lignin in the fermented biomass, the growth of the fungus may have modified the structure of this polymer, resulting in a less recalcitrant lignin. ADF is the sum of cellulose and lignin and the higher its content, higher is the chance of presenting high levels of lignin, which imply less digestibility. In conventional bromatological analyzes, hemicellulose contents are calculated by the difference between ADF and NDF, and cellulose by the difference between lignin and ADF (NOGUEIRA, 2005).

In terms of nutrition, ADF content allows to calculate total digestible nutrients (TDN), as it is directly related to digestibility and these digestible nutrients correspond to the energy

from the food. For such, the following formula should be applied: % TDN = 87.84 - (0.70 x% FDA). Hence, we have the following values for TDN: for *in natura* JSC, approximately 59.46%; for autoclaved JSC, approximately 63.16%; and, after fungus growth, approximately 56.19%.

The increase on lignin levels, observed after 14 days of fungus growth, can be explained by the following: degradation of hemicellulose during fermentation, release of cellulolytic enzymes released by the fungus (quantified on the previous topic) and concentration of the biomass, due to loss via fungal cell respiration.

Ether extract (EE) refers to the contents of oils and fats in the analyzed samples. Some microorganisms, including some known basidiomycetes, use lipids as an energy source during growth, through the release of lipases on the substrate (COLEN, 2006), resulting in a reduction on the levels of ether extract by the end of cultivation, as observed for both SSF-JSC and SSF-CSC samples (Table 2.3). Gomes (2015) demonstrated that the concentration of ether extract in the SMS of *P. pulmonarius* EF88 in 100% JSC was 6.73%, while in the unfermented substrate it was 19.14%, slightly higher than those observed in the current work. This is because the fat content of jatropha cakes vary according to the origin of the seeds and the efficiency of the pressing process for oil extraction. Thus, the evaluated macrofungi *Coriolopsis sp*. INPA1646 and *Tyromices sp*. INPA1696 did make use of the ether extracts present in JSC and CSC, respectively.

Unlike some bacteria, basidiomycetes do not have the ability to fix nitrogen. However, fermentation of plant substrates by these fungi has resulted in increase on the levels of proteins and amino acids (ZHANG et al., 2006), probably due to the consumption of other nutrients in the substrate, such as carbohydrates and lipids, during fungal metabolism. The loss of carbon, in the form of $CO_2$, via fungal respiration, increases the nitrogen concentration in the substrate, which increases CP because of concentration caused by that carbon loss (VAN KUIJK et al., 2015).

Despite the inevitable and known loss of carbon via fungal respiration, during biomass colonization, it seemed that there was no significant nitrogen concentration on the SSF-JSC, and, therefore, levels of CP did not show significant changes. On the SSF-CSC, however, crude protein was more concentrated, probably due to the loss of carbon through respiration. In the work of Araujo (2017), concentrations of crude protein on the SMS of *P. ostreatus* CC389 in CSC increased, when compared to unfermented substrates. Zhang et al. (2006) also demonstrated an apparent increase on the levels of crude protein in CSC colonized by the

fungi *Candida capsuligena*, *Candida tropicalis*, *Saccharomyces cerevisae*, *Aspergillus oryzae* and *Aspergillus niger*.

Although it does not cause a significant increase on the levels of crude protein, the fermentation process seems to solubilize part of this protein, and its nitrogen becomes more available. This can be noted by the reduction on concentrations of structural protein and the increase on soluble proteins (Table 2.3). Such solubilization of 3.6% of the crude protein on SSF-JSC and of 11.5% on SSF-CSC represents an advantage of the fermentation process. Almost none of the protein present in CSC was soluble before fermentation, hence it is possible to affirm that during growth of *Tyromices sp*. INPA1696 in the cake occurred solubilization of part of the structural protein present in the biomass. This was probably due to the production and release of proteolytic enzymes, which presented significant activity on SSF-CSC, when compared to the fermentation of JSC (Figure 2.7).

Protein levels in *in natura* JSC were of 27.35 + 1.12 g/100g, similar to the levels reported in literature, which vary from 28.87 to 37.82% (OMOJASOLA and BENU, 2016). Some studies indicate an increase on concentrations of crude protein on JSC, around 8%, when treated with *Absidia spinosa* and *Mucor rouxii* (SANUSI et al., 2013), or a reduction on these levels when treated with *Trichoderma longibrachiatum*, *Trichoderma harzanium* and *Aspergillus niger* (BELEWU, AHMED and IBRAHIM, 2011; OJEDIRAN et al., 2016).

### 3.5.2 Strutural composition (celulose, hemicellulose and lignin) by NREL analysys methods

Among the sugars investigated, only arabinan and galactan had their concentration reduced on SSF-JSC. For CSC, however, results were different: CSC does not have mannan and ramnan and, among the investigated sugars, only glucans (cellulose) did not have their concentrations reduced, as shown in Table 2.2, which presents the sugar profile quantified by the NREL method. According to scientific literature, reports of carbohydrates in mushrooms include β-glucans, D-galactose, D-mannose, D-xylose, L-fructose, L- (or D) -arabinosis, xylose, fructose, mannose, glucose, sucrose and mannose in mycelium of *P. ostreatus, P. eryngii, P. tuberregium, G. lucidum, P. baumii, A. bisporus, F. velutipes, L. edodes, A. blazei, S. crispa* and *I. obliquus*, among others (AIDA *et al*., 2009).

**Table 2.2** Concentrations of sugars, cellulose, hemicellulose and lignin in JSC and CSC before and after fermentation using macrofungi *Coriolopsis sp*. INPA1646 and *Tyromices sp*. INPA1696, respectively (%±SD).

| Component | JSC *in natura* | JSC autoclaved | SSF-JSC | CSC *in natura* | CSC autoclaved | SSF-CSC |
|---|---|---|---|---|---|---|
| **Glucan** | 17.67±0.12e | 19.99±0.54d | 19.87±0.48d | 24.65±0.61c | 26.37±1.18b | 38.31±1.67a |
| **Xylan** | 11.62±0.37[a] | 11.84±0.35a | 11.64±0.27a | 10.32±0.79b | 10.18±3.54b | 9.95±0.94b |
| **Mannan** | 1.61±0.09[a] | 1.53±0.01b | 1.52±0.05b | 0.0 | 0.0 | 0.0 |
| **Arabinan** | 1.64±0.16b | 1.24±0.04c | 0.31±0.01e | 3.11±0.05a | 1.09±0.32c | 0.44±0.06d |
| **Galactan** | 1.00+0.06[a] | 0.86±0.04b | 0.36±0.02e | 1.01±0.03a | 0.77±0.03c | 0.40±0.02d |
| **Ramnan** | 0.83±0.08b | 0.58±0.07c | 1.38±0.11a | 0.0 | 0.0 | 0.0 |
| **Cellulose** | 17.67±0.12d | 19.99±0.54c | 19.87±0.47c | 24.65±0.61b | 26.37±1.18b | 38.31±1.67a |
| **Hemicellulose** | 16.70±0.75[a] | 16.06±0.52a | 15.19±0.46b | 14.96±0.87b | 14.59±3.89bc | 11.83±1.02c |
| **Lignin** | 22.02±0.60d | 21.61±0.32e | 25.68±0.50c | 34.93±1.03a | 43.93±3.58a | 39.12±1.96b |

Letters next to the values correspond to the comparisons of the averages on the same line and not between one line and another (Tukey test $p \leq 0.05$).

Fonte: da autora (2021)

Based on the concentrations of sugars, it is possible to determine the contents of cellulose (glucan) and hemicellulose (other sugars). Besides, via NREL method it is possible to determine the concentration of lignin in the biomass (Table 2.4). Degradation of hemicellulose and cellulose produces free fermentable sugars, which stand out as the focus of researches concerning alternative fuel sources (second generation ethanol), indicating other potential applications of solid-state cultivation of basidiomycetes in lignocellulosic biomasses (HOUFANI et al., 2020).

Gomes (2015) observed that the levels of cellulose and hemicellulose in JSC were lower (19.97 and 5.52% respectively) than on SMS (more than 60 days of mushroom cultivation and fruiting) using *P. pulmonarius* EF88 in 100% of JSC (25.49 and 9.03%, respectively).

From the percentual loss (%) of these structural polysaccharides via degradation by the fungus during fermentation for 21 days, it is possible to calculate the selectivity of the basidiomycete for degradation of lignin rather than of cellulose ("Lignin/Cellulose Selectivity - SLC") and of holocellulose (sum of cellulose and hemicellulose) which is called "Lignin / Holocellulose Selectivity - SLH" (DA SILVA, 2019). However, since in the current work, for

any biomass, it was not observed any reduction on the levels of lignin, it is not possible to calculate these selectivities, but it is possible to infer that the isolates INPA1646 and INPA1696, under the established culture conditions, did not degrade lignin throughout JSC and CSC fermentations.

In addition to these results, NREL test makes it possible to determine structural inorganic compounds. For both biomasses, contents of structural inorganic substances decrease significantly at the end of fermentation, which indicates the solubilization of these by fungi during their growth and increases availability of these compounds in the fermented biomasses. For JSC, levels of structural inorganics compounds decreased from 3.57 ± 0.08% in *in natura* cake, to 3.23 ± 0.07% after autoclaving and to only 1.91 ± 0.06% at the end of fermentation by *Coriolopsis sp*. INPA1646. In CSC, levels were reduced from 2.06 ± 0.10% in *in natura* cake and 2.18 ± 0.04% in autoclaved (without statistical difference) to 1.34 ± 0.02% after the growth of *Tyromices sp*. INPA1696.

## 3.6 Determination of ergosterol

Ergosterol is a sterol that constitutes the cell membrane of fungi and is equivalent to the cholesterol of mammals. Ergosterol is also used as a marker and indicative of fungal growth and can be used as a method for mycelium quantification, as long as the specific concentration of ergosterol is determined per gram of mycelium grown in a given medium (KLAMER and BAATH, 2004).

This biomolecule is a precursor of vitamin D2, as it is transformed into viosterol by ultraviolet light and then into ergocalciferol, which is a form of vitamin D. Unfermented JSC presented ergosterol in the concentration of 22.78±3.93 µg/g of cake, probably due to the presence of some endophytic fungus considered a natural contaminant. At the end of the 14 days of cultivation, concentration of this sterol increased 10 times, reaching 201.88 ± 8.8 µg/g of fermented cake.

CSC without fungus did not show detectable ergosterol according to the method used in the current experiment. However, the cake fermented for 14 days by macrofungus INPA 1696 presented an ergosterol concentration of 147.12±20.37 µg/g.

## 3.7 Composition of aminoacids

Throughout fermentation, vegetable proteins are basically converted into fungal proteins. The total amount of protein, in general, does not change, since nitrogen in the substrate is not lost during fermentation neither is nitrogen in the environment fixed to the substrate by the fungus. However, what is observed is a decrease of nitrogen content content due to loss of carbon by respiration of the fungus; therefore, concentration of crude proteins might increase, even though the amino acid concentration has decreased, as can be seen in Table 2.3, below.

**Table 2.3** Amino acid composition in jatropha seed (JSC) and cotton seed (CSC) cakes before and after fermentation using *Coriolopsis sp*. INPA1646 and *Tyromyces sp*. INPA1696, respectively, for 14 days.

| **Aminoacids** | **JSC** | | | **CSC** | | |
|---|---|---|---|---|---|---|
| | **JSC *in natura*** | **JSC Ac** | **SSF-JSC** | **CSC *in natura*** | **CSC Ac** | **SSF-CSC** |
| Aspartic acid | 2.06% | 1.80% | 1.82% | 2.26% | 2.19% | 2.14% |
| Glutamic acid | 3.40% | 3.08% | 2.29% | 5.02% | 4.89% | 3.73% |
| Serine | 1.12% | 1.01% | 0.94% | 1.17% | 1.13% | 1.03% |
| Glycine | 1.01% | 0.90% | 0.89% | 1.08% | 1.09% | 0.98% |
| Histidine | 0.58% | 0.48% | 0.46% | 0.70% | 0.67% | 0.55% |
| Taurine | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Arginine | 2.51% | 2.09% | 1.38% | 2.78% | 2.70% | 2.01% |
| Threonin | 0.83% | 0.75% | 0.69% | 0.82% | 0.83% | 0.85% |
| Alanine | 1.05% | 0.95% | 0.86% | 0.97% | 0.98% | 0.96% |
| Proline | 0.94% | 0.84% | 0.73% | 0.94% | 0.93% | 0.88% |
| Tyrosine | 0.65% | 0.58% | 0.44% | 0.76% | 0.74% | 0.62% |
| Valine | 1.14% | 1.03% | 0.88% | 1.07% | 1.11% | 1.03% |
| Methionine | 0.28% | 0.27% | 0.22% | 0.36% | 0.36% | 0.31% |
| Cystine | 0.37% | 0.34% | 0.32% | 0.40% | 0.40% | 0.33% |
| Isoleucine | 0.97% | 0.86% | 0.73% | 0.84% | 0.83% | 0.79% |
| Leucine | 1.63% | 1.47% | 1.23% | 1.52% | 1.52% | 1.25% |
| Phenylalanine | 1.01% | 0.90% | 0.76% | 1.35% | 1.35% | 0.98% |
| Lysine | 0.77% | 0.55% | 0.63% | 0.99% | 0.96% | 0.91% |
| Tryptophan | 0.11% | 0.12% | 0.11% | 0.13% | 0.11% | 0.11% |

| | | | | | | |
|---|---|---|---|---|---|---|
| Total Aminoacids | 20.44% | 18.02% | 15.37% | 23.16% | 22.78% | 19.45% |
| Crude protein | 22.98% | 22.56% | 26.63% | 28.43% | 29.13% | 30.89% |

Ac= autoclaved

Fonte: da autora (2021)

Nayan et al. (2018) reported an increase in arginine content by approximately 56% and lysine by approximately 15%, after fungal colonization, while here there was a small reduction in arginine and a small increase in lysine. Overall, the amino acid content tended to decrease after fungal colonization.

The aminoacid profile is altered because plant and fungal proteins can have different amino acid profiles. As seen in Table 2.3, the most common aminoacid in the evaluated biomasses is glutamic acid, followed by arginine. Non-essential aminoacids are those synthesized by the body itself, and are involved in many metabolic functions, being glutamate a precursor to glutamine, which is required by several cells (NEWSHOLME et al., 2003).

## 4 CONCLUSION

The results reported in the current work show that 64% of viable INPA fungi (16/25) can grow in Petri dishes containing only JSC or CSC as a carbon source. Among these fungi, 62.5% (10/16) grew in glass flasks containing solid-state fermentation of biomasses. Four of the eight fungi that grew in JSC managed to detoxify this substrate (<0.09 mg/g) after 14 days of fermentation, with a degradation rate of up to 97.22% of phorbol esters. In CSC, the degradation rate of free gossypol reached 95.34%; however, due to the release of bound gossypol by some fungi, levels of free gossypol increased. When JSC and CSC were fermented by *Coriolopsis sp*. INPA1646 and *Tyromices sp*. INPA1696, respectively, antioxidant activity of the cakes decreased considerably on the 6th and 9th days of fermentation. *Tyromices sp*. INPA 1696 is an excellent producer of proteolytic and cellulolytic enzymes in CSC, with potential application on larger scales. Both fermented biomasses presented potential reduction of the quantified toxic/antinutritional factors and are both rich in proteins, aminoacids, enzymes, ergosterol and others bioactives chemical compounds, like carbohydrates (beta-glucan) promptly available for animal feed.

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## SUPPLEMENTARY MATERIAL FROM CHAPTER II

**Table 2.1S.** Taxonomic estimate of fungi, via Blast at NCBI, based on amplified ITS and EF1 gene sequences.

| Name in Collection | Code | ITS | | | | EF1 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Species** | **Accession** | **Query coverage** | **Percent identity** | **Species** | **Accession** | **Query coverage** | **Percent identity** |
| *Stereum sp.* | INPA1643 | *Stereum* sp. 4 RG-2014 | KJ832043.1 | 81% | 97,99% | | | | |
| | | *Stereum* sp. 4 RG-2014 | KJ832044.1 | 84% | 96,93% | | | | |
| *Schizophyllum commune* | INPA1644 | fungo não cultivado | GU053894.1 | 99% | 99,37% | | | | |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | KF573032.1 | 97% | 99,52% | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164886.1 | 96% | 97,33% |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164959.1 | 96% | 99,36% | *Trametes* aff. maxima FPRI101 | JN164884.1 | 98% | 87,79% |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164958.1 | 96% | 99,20% | *Trametes hirsuta* (Wulfen) Pilat | CP019371.1 | 98% | 87,42% |
| *Coriolopsis sp.* | INPA1646 | *Trametes polyzona* (Pers.) Justo 2011 | KC589124.1 | 98% | 98,80% | | | | |
| | | *Trametes polyzona* (Pers.) Justo 2011 | JN164979.1 | 95% | 99,96% | | | | |
| *Trametes sp.* | INPA1668 | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164946.1 | 96% | 99,69% | | | | |
| | | *Trametes membranacea* (Sw.) | JN164956.1 | 96% | 99,69% | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Kreisel 1971 | | | | | | | |
| | | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164927.1 | 95% | 99,69% | | | | |
| | | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164956.1 | 92% | 93,03% | | | | |
| *Trametes sp.* | INPA1681 | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164946.1 | 92% | 93,03% | | | | |
| | | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164956.1 | 98% | 99,20% | | | | |
| | | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164946.1 | 98% | 99,20% | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164886.1 | 96% | 97,33% |
| *Agaricus sp.* | INPA1687 | *Trametes membranacea* (Sw.) Kreisel 1971 | JN164927.1 | 98% | 99,20% | *Trametes* aff. maxima FPRI101 | JN164884.1 | 98% | 87,79% |
| | | | | | | *Penicillium solitum* Westling, 1911 | XM_04095 5243.1 | 83% | 91,54% |
| *Trametes* sp. | INPA1689 | | | | | *Penicillium expansum* (ATCC 24692/ NRRL 62431/ T01) | XM_01673 9759.1 | 83% | 91,12% |
| | | *Basidiomycota* sp. FPF38a | JX416577.1 | 99% | 100% | *Trametes hirsuta* (Wulfen) Pilat | XM_04079 1920.1 | 83% | 91,12% |
| NI | INPA1694 | *Leiotrametes lactinea* | MH910526.1 | 100% | 98,82% | *Trametes hirsuta* (Wulfen) Pilat | CP019371.1 | 100% | 90,60% |
| *Trametes sp.* | INPA1695 | *Daedalea microsticta* Cooke | FJ403209.1 | 98% | 97,77% | Trametes maxima (Mont.) A. | JN164885.1 | 100% | 90,38% |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1882 Cooke, 1882 | | | | David & Rajchenb. 1985 | | | |
| | | *Daedalea* sp. | MH267897.1 | 95% | 98,84% | | | | |
| | | *Trametes hirsuta* (Wulfen) Pilat | CP019375.1 | 98% | 96,01% | *Trametes* aff. maxima FPRI101 | JN164884.1 | 100% | 90,04% |
| | | *Trametes maxima* (Mont.) A. David & Rajchenb. 1985 | JN164932.1 | 98% | 96,01% | *Trametes cubensis* (Mont.) Sacc. 1891 | JN164883.1 | 98% | 91,30% |
| *Tyromyces sp.* | INPA1696 | Basidiomycota sp. FPF38a | JX416577.1 | 100% | 99,84% | *Trametes cubensis* (Mont.) Sacc. 1891 | JN164883.1 | 95% | 90,43% |
| *Trametes elegans* | INPA1698 | *Trametes elegans* | MW157265.1 | 99% | 99,84% | *Trametes polyzona* (Pers.) Justo 2011 | JN164881.1 | 95% | 86,98% |
| | | *Trametes elegans* | HQ248217.1 | 98% | 100% | | | | |
| | | fungo não cultivado | GQ999293.1 | 100% | 99,38% | | | | |
| | | *Fomitopsis* sp. | MH990633.1 | 99% | 95,89% | | | | |
| | | *Fomitopsis* sp. X1419 | KC595911.1 | 99% | 95,89% | | | | |
| | | *Fomitopsis* sp. CLF-T | AB505425.1 | 99% | 95,89% | | | | |
| | | *Fomitopsis* sp. 9V_3_1 | FJ372677.1 | 96% | 96,52% | | | | |
| NI | INPA1714 | fungo não cultivado | GU053894.1 | 98% | 99,37% | | | | |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | KF573032.1 | 97% | 99,36% | | | | |
| | | *Trametes* sp. DSM 9795 | AY840570.1 | 96% | 99,68% | | | | |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | MZ735400.1 | 96% | 99,36% | | | | |
| *Schizophyllum commune* | INPA1720 | *Schizophyllum commune* (4.8 streak/H4-8/Loenen | AF280759.1 | 96% | 99,68% | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | D/Tattone D/Tattone S) | | | | | | | |
| | | *Schizophyllum* sp. B179 | KR812266.1 | 96% | 99,52% | | | | |
| | | *Schizophyllum commune* (4.8 streak/H4-8/Loenen D/Tattone D/Tattone S) | MT466518.1 | 97% | 98,90% | | | | |
| | | *Fomes* sp. | MT611020.1 | 98% | 99,46% | | | | |
| | | *Hexagonia* sp. (in: Fungi) | MT939265.1 | 98% | 98,91% | | | | |
| | | *Fomes* sp. EUM1 | HM136871.1 | 96% | 99,44% | | | | |
| | | *Fomes* sp. | MT611014.1 | 97% | 98,36% | | | | |
| | | *Hexagonia hydnoides* | MH016937.1 | 92% | 99,71% | | | | |
| | | *Hexagonia apiaria* | KR049230.1 | 100% | 96,28% | | | | |
| | | *Hexagonia apiaria* | KX900635.1 | 100% | 95,74% | | | | |
| | | *Fomes* sp. EUM1 | HM136871.1 | 94% | 99,04% | | | | |
| *Hexagonia hydnoides* | INPA1725 | *Hexagonia* sp. (in: Fungi) | MT939265.1 | 91% | 99,01% | | | | |
| | | *Fomes* sp. | MT611020.1 | 89% | 99,49% | | | | |
| | | *Lentinus crinitus* | MH915574.1 | 98% | 97,68% | | | | |
| | | *Lentinus berteroi* (Fr.) Fr., 1825 | MK036389.1 | 91% | 99,38% | | | | |
| *Hexagonia hydnoides* | INPA1728 | *Lentinus berteroi* (Fr.) Fr., 1825 | MK890046.1 | 90% | 99,68% | | | | |
| | | fungo não cultivado | GU053894.1 | 98% | 99,85% | | | | |
| NI | INPA1734 | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | KF573032.1 | 97% | 99,85% | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164886.1 | 100% | 98,44% |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164959.1 | 96% | 99,53% | | | | |
| | | *Trametes pavonia* (Berk.) Fr., 1851 (Berk.) Fr. 1851 | JN164958.1 | 96% | 99,53% | *Phanerochaete sordida* | LC270920.1 | 98% | 89,74% |
| *Flavodon flavus* | INPA1739 | *Flavodon flavus* | KJ654386.1 | 99% | 99,85% | *Cutaneotrichosporon moniliiforme* | MK300109.1 | 87% | 90,77% |
| | | *Flavodon flavus* | JN710543.1 | 99% | 99,70% | *Trametes pavonia* (Berk.) | JN164886.1 | 96% | 86,81% |

Fonte: da autora (2021)

**Table 2.2S** Growth rate (mm/day) of macrofungi on Agar-JSC plates, on SSF in flasks containing JSC with 60% of moisture (SSF-JSC), on CSC-Agar plates and on SSF in flasks containing CSC with 60% of moisture.

| **Fungi** | | **Growth-rate (mm/day) ± SD** | | | |
|---|---|---|---|---|---|
| **Code** | **Name** | **Agar-JSC (Petri dish)** | **SSF-JSC (flask)** | **Agar-CSC (Petri dish)** | **SSF-CSC (flask)** |
| INPA 1643 | *Stereum sp.* | 3.62 ± 0.49c | NG | 3.53 ± 0.57b | NG |
| INPA 1644 | *Schizophyllum commune* | 3.70 ± 0.12c | NG | 3.78 ± 0.68b | NG |
| **INPA 1646** | ***Coriolopsis sp.*** | **7.37 ± 0.13a** | **2.97 ± 0.34a** | **6.96 ± 0.36[a]** | **2.42 ± 0.28a** |
| INPA 1668 | *Trametes sp.* | 3.66 ± 0.86c | NG | 3.47 ± 0.24b | NG |
| INPA 1681 | *Trametes sp.* | 3.63 ± 0.24c | NG | 3.70 ± 0.41b | NG |
| INPA 1689 | *Trametes sp.* | 3.66 ± 0.49 c | NG | 3.91 ± 0.67b | NG |
| INPA 1690 | *Trametes sp.* | 3.67 ± 0.93c | NG | 3.60 ± 0.11b | 1.98 ± 0.47a |
| INPA 1694 | NI | 3.61 ± 0.42c | 2.41 ± 0.65a | 3.71 ± 0.17b | NG |
| INPA 1695 | *Trametes sp.* | 3.55 ± 0.60c | 2.21 ± 0.71a | 3.91 ± 0.39b | 2.21 ± 0.39a |
| **INPA 1696** | ***Tyromyces sp.*** | **3.83 ± 0.48c** | **2.04 ± 0.43a** | **3.85 ± 0.48b** | **2.14 ± 0.34a** |
| INPA 1698 | *Trametes elegans* | 1.92 ± 0.31e | 1.65 ± 0.74b | 2.38 ± 0.78c | 1.37 ± 0.17b |
| INPA 1719 | *Trametes sp.* | 3.36 ± 0.22c | 1.43 ± 0.32b | 3.70 ± 0.42b | NG |
| INPA 1720 | *Schizophyllum commune* | 3.68 ± 0.13c | NG | 3.65 ± 0.00b | 2.16 ± 0.41a |

| | | | | | |
|---|---|---|---|---|---|
| INPA 1725 | *Hexagonia hydnoides* | 3.73 ± 0.52c | 2.40 ± 0.48a | 3.72 ± 0.72b | 1.46 ± 0.23b |
| INPA 1734 | NI | 2.79 ± 0.26d | NG | 3.28 ± 0.50b | NG |
| INPA 1739 | *Flavodon flavus* | 7.04 ± 0.02b | 2.56 ± 0.48a | 7.31 ± 0.20a | 2.34 ± 0.49a |

NI: no-identified, NG: no-growth. Letters next to each metric value correspond to comparisons of the averages in the same column and not between one column and another (Tukey test $p \leq 0.05$).

Fonte: da autora (2021)

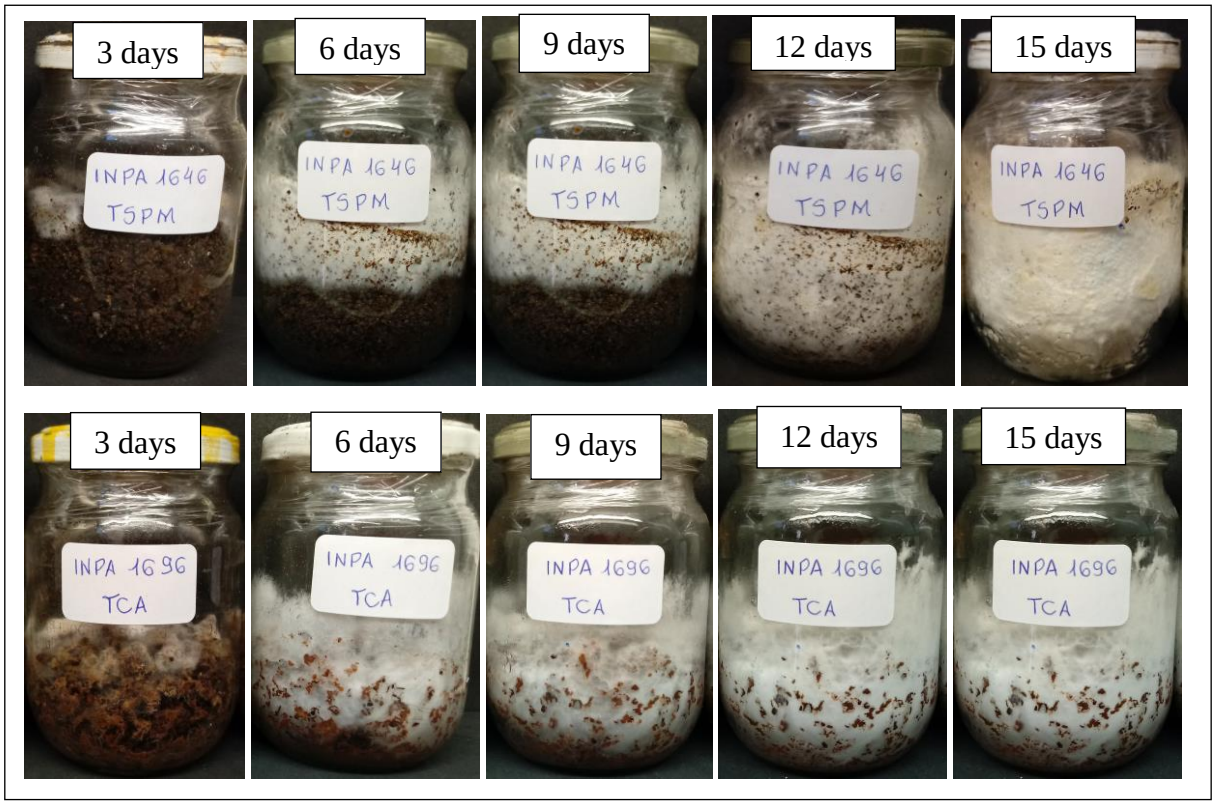
**Tabela 2.3S** Literature results for degradation of phorbol esters in JSC, via solid-state fermentation by macrofungi.

| Fungus | Degradation rate | Fermentation time | Reference |
|---|---|---|---|
| *Pleurotus ostreatus* | 37% | 15 days | LUZ et al., 2013 |
| | 60% | 45 days | |
| | 99% | 60 days | |
| *Pleurotus ostreatus* | 58% | 15 days | KASUYA et al., 2012 |
| | 85% | 30 days | |
| | 99% | 45 days | |
| *Pleurotus sapidus* | 76% | 20 days | BOSE; KEHARIA, 2014 |
| *Pleurotus ostreatus* | 72,5% | | |
| *Pleurotus florida* | 67,9% | | |
| *Pleurotus sajor-caju* | 67,9% | | |
| *Pleurotus pulmonarius* | 97% | 30 days | GOMES, 2015 |
| *Ganoderma lucidum* | 100% | 20 days | BOSE; KEHARIA, 2014 |
| *Trametes zonata* | 100% | | |
| *Trametes gibbosa* | 91,7% | | |
| *Trametes versicolor* | 89% | | |
| *Trametes hirsuta* | 81,6% | | |
| *Phanerochaete chrysosporium* | 44,9% | | |

| | | | |
|---|---|---|---|
| *Phlebia rufa* | 97% | 30 days | BARROS *et al*., 2011 |
| *Bjerkandera adusta* | 91% | | |
| *Ganoderma resinaceum* | 20% | | |
| *Aurantioporus pulcherrimus* | 41% | 30 days | GOMES, 2015 |
| *Ganoderma lucidum* | 42% | | |
| *Agaricus spp.* | 54% | | |
| *Agaricus fuscofibrillosus* | 62% | | |
| *Agaricus mediofuscus* | 71% | | |
| *Ascopolyporus ssp.* | 76% | | |
| *Panaeolus antillarum* | 78% | | |
| *Lentinus strigellus* | 88% | | |
| *Amylosporus spp.* | 95% | | |

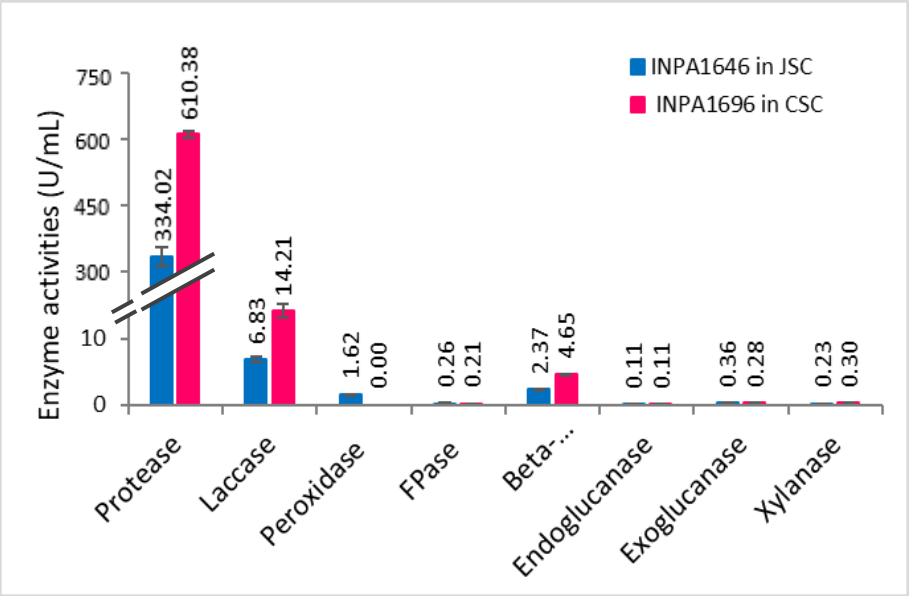
Fonte: da autora (2021)

**Figure 2.1S** Pictures of the glass flasks containing the fermented cakes after 3, 6, 9, 12 and 15 days of incubation at 28 ° C, to illustrate the level of colonization of JSC and CSC by macrofungi *Coriolopsis sp.* INPA1646 and *Tyromyces sp*. INPA1696, respectively.



Fonte: da autora (2021)

**Figure 2.2S.** Total enzyme activities in the crude extracts of SSF-CSC and SSF-JSC using macrofungi *Tyromices sp*. INPA1696 and *Coriolopsis sp*. INPA1646, respectively, after 15 days of fermentation.




Fonte: da autora (2021)

**CAPÍTULO III**

# EXTRATOS AQUOSOS DE FERMENTADOS DE MACROFUNGOS CULTIVADOS EM TORTAS DE SEMENTES OLEAGINOSAS COMO FONTE DE CARBONO PARA BACTÉRIAS PROBIÓTICAS E PONTENCIAL ATIVIDADE ANTIBACTERIANA

## RESUMO

Alguns fungos basidiomicetos e ascomicetos geram cogumelos, que podem ser comestíveis e/ou medicinais, alguns são utilizados há anos na medicina tradicional chinesa e também como alimentos e fontes de produtos nutracêuticos em todo o mundo, por conterem compostos químicos bioativos. Dados da literatura apontam que as substâncias presentes nos cogumelos também podem ser encontradas no micélio vegetativo destes fungos, neste sentido, biomassas vegetais colonizadas por estes macrofungos podem conter moléculas com propriedades bioativas, com benefício direto a saúde humana/animal ou indireta por meio da nutrição de bactérias probióticas presentes no trato digestivo ou ingeridas. O objetivo deste trabalho foi avaliar o potencial dos extratos aquosos dos fermentados fúngicos em tortas de sementes oleagionosas (tortas de semente de pinhão-manso (JSC) e de caroço de algodão (CSC) como fonte de carbono para cultivo de bactérias probióticas e atividades antibacteriana dos extratos fúngicos e sobrenadantes bacteriano. Os macrofungos utilizados foram *Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *Panus lecomtei* BRM 044603 e *Pleurotus pulmonarius* BRM 055674. A partir de fermentações sólidas e submersas dos macrofungos, foram obtidos extratos aquosos (fermentação fúngica) e sobrenadantes (fermentação bacteriana) para análise de atividade antibacteriana. O extrato aquoso da JSC não-fermentada (controle) foi mais eficiente do que a glicose em estimular o crescimento de todas as bactérias probióticas testadas. Os extratos aquosos dos fermentados dos quatro macrofungos avaliados em CSC (fermentação submersa) favoreceu o crescimento de *L. acidophilus.* Enquanto que na fermentação sólida os extratos aquosos dos macrofungos cultivados em JSC favoreceram mais o crescimento de *B. lactis*. Todos os extratos aquosos apresentaram maior estímulo de crescimento para quatro bactérias probioticas avaliadas, em comparação as fontes de carboidratos. Quanto às atividades antimicrobanas, nenhum extrato aquoso fúngico ou sobrenadantes bacterianos (probióticas) apresentaram halo de inibição de bactérias enteropatogênicas igual ou maior que o antibiótico controle (ampicilina). No entanto, os extratos aquosos dos fermentados de *P. lecomtei* BRM 044603 e *Coriolopsis* sp. INPA1646 no cultivo em CSC, na concentração de 100 mg/mL, apresentaram halos de inibição com diâmetros de 1,2 e 0,73 cm, respectivamente, para a cepa de *S. enterica* testada. Além disso, os sobrenadantes do crescimento de *L. acidophilus, B. lactis* e *L. rhamnosus* nos produtos de fermentação das biomassas resultaram em maior inibição do crescimento de *S. aureus* que os sobrenadantes desses nos meios controles, o que indica possível atividade antimicrobiana.

**Palavras-chaves:** Compostos bioativos de macrofungos, fermentados, bactérias probióticas, bactérias enteropatogênicas.

# AQUEOUS EXTRACTS OF FERMENTED MACROFUNGI CULTIVATED IN OILSEED CAKES AS A CARBON SOURCE FOR PROBIOTIC BACTERIA AND POTENTIAL ANTIBACTERIAL ACTIVITY

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**ABSTRACT**

Some basidiomycetes contain bioactive chemical compounds and literature data indicate that the substances present in mushrooms can also be found in the vegetative mycelium of these fungi, in this sense, plant biomass colonized by these macrofungi may contain molecules with bioactive properties with direct application to human/animal health or indirectly through nutrition of probiotic bacteria present in the digestive tract or ingested. The objective of this work was to evaluate the potential of aqueous extracts of fungal fermented in oil seed cakes (*Jatropha curcas* seed (JSC) and cottonseed (CSC) as a carbon source for the cultivation of probiotic bacteria and activities antibacterial from fungal extracts and bacterial supernatants. The macrofungi used were *Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *Panus lecomtei* BRM 044603 and *Pleurotus pulmonarius* BRM 055674. Solid and submerged fermentations of the macrofungi were carried out. Aqueous extracts (fungal fermentation) and supernatants (bacterial fermentation) were obtained for antibacterial activity. The aqueous extract of unfermented JSC (control) was more efficient than glucose in stimulating the growth of all probiotic bacteria tested. The aqueous extracts of the four macrofungi fermented evaluated in CSC (submerged fermentation) favored the growth of *L. acidophilus*. While in solid fermentation the aqueous extracts of macrofungi cultivated in JSC favored the growth of *B. lactis*. All aqueous extracts showed greater growth stimulus for the four probiotic bacteria evaluated, compared to carbohydrate sources. As for the antimicrobial activities, no aqueous fungal extract or bacterial supernatants (probiotics) showed a halo of inhibition of enteropathogenic bacteria equal to or greater than the control antibiotic (ampicillin). However, the aqueous extracts of *P. lecomtei* BRM 044603 and *Coriolopsis* sp. INPA1646 in CSC culture, at a concentration of 100 mg/mL, showed inhibition halos with diameters of 1.2 and 0.73 cm, respectively, for the strain of *S. enterica* tested. Furthermore, the growth supernatants of *L. acidophilus*, *B. lactis* and *L. rhamnosus* in the fermentation products of the biomass resulted in greater inhibition of the growth of *S. aureus* than the supernatants of these in the control media, which indicates possible antimicrobial activity.

**Keywords:** Bioactive compounds from macrofungi, fermented biomass, probiotic bacteria, enteropathogenic bacteria.

## 1 INTRODUCTION

Mushrooms are known to be rich in antioxidant compounds with anti-inflammatory and anticancer activity (ADEBAYO et al., 2012) and complex carbohydrates, which behave like intestinal fibers and which can help in the growth of probiotic microorganisms and in the production of short-chain fatty acids that favor these microorganisms over enteropathogens (AIDA, *et al.*, 2009).

The most commonly bioactive compounds found in mushrooms include polysaccharides (RUTHES; SMIDERLE; IACOMINI, 2016; CHEUNG, 2013), proteins (EL ENSHASY and HATTI-KAUL, 2013), terpenes (WANG *et al.*, 2012; LEE *et al*., 2011), unsaturated fatty acids (TEL-CAYAN *et al*., 2017) and phenolic compounds (HELENO *et al*., 2015) (MA *et al*., 2018). In mushrooms, these compounds provide activities as antioxidant, antimicrobial, antitumor, anti-inflammatory, antidiabetic, and hypocholesterolemia, among other important biological activities to mushrooms. (MA *et al.*, 2018; RATHORE *et. al.*, 2017).

There are more than 400 species of bacteria in the intestine, forming a complex and important microbiota, mainly composed of the genera *Bifidobacteria* and *Lactobacillus* (AZMI *et al.*, 2012), which are Gram-positive bacteria that ferment carbohydrates into acetic and lactic acids, respectively (KLEEREBEZEM and VAUGHAN, 2009). Some intestinal bacteria are homofermentative because they ferment glucose only into lactic acid, and others are heterofermentative which ferment hexoses and pentoses via phosphoketolase, also obtaining acetic acid, $CO_2$, and ethanol (ANJUM *et al.*, 2014).

These *Bifidobacteria* and *Lactobacillus* are known as probiotics, as they suppress the growth of enteropathogenic bacteria and improve food absorption and gastrointestinal tract function, and are also capable of modulating the immune system (MITSUOKA, 1996). Prebiotics are non-digestible food components that selectively stimulate the growth of these probiotic bacteria that, in addition to modulating the intestinal microbiota, attenuate health conditions such as diabetes, obesity, and cancer (JAYACHANDRAN, XIAO, and XU, 2017).

In addition to probiotic bacteria, other bacteria may be present in the gastrointestinal tract and some of them may be pathogenic, causing harm to hosts. Bacteria such as *Salmonella* sp., *Campylobacter*, *Escherichia coli*, *Listeria monocytogenes*, *Clostridium botulinum*, *Microsporidia*, and *Campylobacter jejuni* are frequently associated with intestinal

infections (ALVES, 2012a; ESCOBAR *et. al*., 2013). However, the inappropriate use of antibiotics over the years has selected resistant bacteria and this has been an increasing threat to health, so the search for alternative antimicrobials has gained the attention of researchers worldwide, and the chemical composition of some mushrooms suggests a potential antiviral and antibacterial activity (ANGELINI, 2019; WHO, 2021).

The present work aimed to evaluate the prebiotic (carbon source probiotics bacterias) and antibacterial potential of supernatants from submerged fermentation (SmF) and aqueous extracts from solid-state fermentation (SSF) of residues from cakes of seed oil cotton and *Jatropha* by *Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *P. lecomtei* BRM 044603, and *P. pulmonarius* BRM 055674.

## 2 MATERIAL AND METHODS

### 2.1 Obtaining supernatants and extracts

*Coriolopsis* sp. INPA1646, *Tyromyces* sp. INPA1696, *P. lecomtei* BRM 044603, and *P. pulmonarius* BRM 055674 belong to the INPA Macrofungi Collection (Manaus/AM) and the Microorganisms and Microalgae Collection applied to the biorefinery of Embrapa Agroenergia – CMMABio (Brasília/DF). These macrofungi were selected for this work because they showed the degradation activity of toxic compounds of plants such as gossypol and/or phorbol esters when grown by SSF in JSC (Jatropha seed cake) and CSC (cottonseed cake). The strains were reactivated on plates with PDA (Potato Dextrose Agar), incubated at 28°C for 15 days.

Submerged fermentation (SmF) was performed in flasks with 5.0 g of each of the dry biomasses and 100 mL of distilled water, and solid-state fermentation in flasks with 40 g of JSC or 20g of dry CSC, with moisture adjusted to approximately 70%. After sterilized, the liquid medium flasks were inoculated with 10 mycelial *plugs* (8 mm diameter) of each fungus and incubated at 28°C and 180 rpm for 14 days, and the solid culture flasks with 10 *plugs* in the JSC flasks and 5 in CSC flasks, and were incubated at 28 ºC for 14 days. Controls consisted of sterilized biomasses under the same conditions without fungal inoculation.

After incubation, submerged cultures were vacuum filtered to obtain supernatants and the solid-state fermented cultures were dried at 60ºC, crushed, and subjected to aqueous

extraction under pressure and temperature in an ASE - Accelerated Solvent Extractor (Dionex ASE 350). Supernatants and aqueous extracts were frozen and lyophilized.

**2.2 Determination of prebiotic activity**

The potential prebiotic activity (carbon sources probiotics bacteira) was evaluated by the ability of supernatants and aqueous extracts to stimulate the growth of *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus rhamnosus* and *Bifidobacterium lactis* strains in MRS (-C) (10.0 g/L of peptone + 10.0 g/L of meat extract + 5.0 g/L of yeast extract + 1.0 g/L of polysorbate 80 + 2.0 g/L of ammonium citrate + 5.0 g/L of sodium acetate + 0.1 g/L of magnesium sulfate + 0.05 g/L de manganese sulfate + 2.0 g/L of monopotassium phosphate) medium for 48 hours at 37°C. The strains, provided by the Central Public Health Laboratory of the Federal District (Lacen-UnB), were reactivated in MRS Broth and diluted in 0.9% saline solution, to obtain a concentration of $10^6$ CFU/mL according to McFarland standard.

The supernatants and aqueous extracts were diluted in sterile distilled water at a concentration of 40 mg/mL, filtered through a 0.22µm membrane, and added to MRS broth without glucose and twice the concentration of the other components (2xMRS-C) and 0.4% L-cysteine, in a 1:1 (v:v) ratio. Three positive controls were performed: glucose (Sigma-Aldrich), fructooligosaccharides (FOS) 90% purity (Newnutrition), and inulin from chicory (FiberBem), at 20 mg/mL. The negative control consisted of sterile distilled water.

To monitor bacterial growth, 2 mL of the medium was inoculated with 5 µL of inoculum and then transferred to two 96-well microplates (Elisa plates), 200 µL per well, in triplicate, and sterility controls consisted of wells with MRS medium without inoculum. The plates were sealed with highly translucent 2.0 polyethylene Platemax sealing membranes for PCR transport and storage (Axygen®), and incubated at 37ºC and 180 rpm, for 48h, with absorbance reading at 600 nm (OD 600), every hour for the first 12 hours and then every 2 hours, 48 hours in total.

To quantify viable cells, 100 µL of each treatment were collected at 12, 24, and 48 hours of incubation at 37ºC, for serial dilution and plating by spreading on plates of complete MRS medium with 0.2% L-cysteine, and colonies were counted after 48 hours of incubation. The growth of total cells was represented by the change in OD and the growth of viable cells in Petri dishes.

The Elisa plates, containing the remaining 800 µL of medium, were also incubated in the shaker at 180 rpm at 37ºC for 48 hours, to obtain the supernatants by centrifugation at 10000 rpm, for 15 minutes, which were also tested in the antimicrobial activity assays.

### 2.3 Determination of antimicrobial activity

*Escherichia coli* (ATCC 25922), *Salmonella enterica* subsp. *Enterica* serovar *Typhimurium* (ATCC 14028) and *Staphylococcus aureus* (ATCC 27154, enterotoxin-producing strain), from the Natural Products Laboratory of the University of Brasília (UnB), were reactivated in Agar Mueller Hinton, at 37°C for 48 hours. Bacteria were grown for 24 hours at 37°C and 180 rpm in Mueller Hinton broth and inoculated by spreading on Mueller Hinton Agar plates for an antibiogram. Supernatants and dry aqueous extracts were diluted in sterile distilled water at a concentration of 100 mg/mL and filtered through a 0.22 µm membrane. Autoclaved paper discs were prepared with 20 µL of the samples and their dilutions (1:10, 1:100, and 1:1000), as well as the supernatants from the above-mentioned prebiotic bacteria growth. After drying at room temperature, the discs were placed on the plates with the microorganisms, which were incubated at 35ºC for 24 hours, to assess the formation of inhibition halos. Ampicillin discs were used as a positive control.

## 3 RESULTS AND DISCUSSION

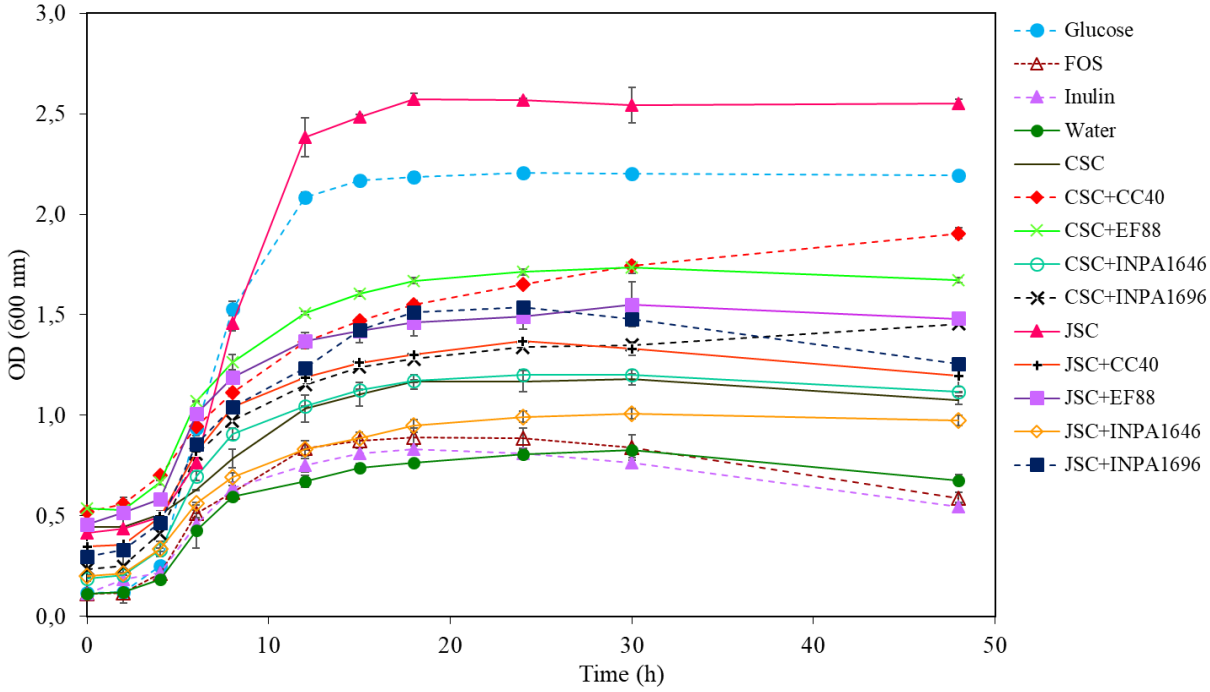
### 3.1 Prebiotic activity (carbon sources)

#### 3.1.1 *Lactobacillus acidophilus*

The highest increase of total cells of *L. acidophilus* was observed in MRS medium (-C) with 20 mg/mL of supernatant SmF-JSC incubated for 14 days at 28°C without fungus (figure 3.1), a variation of 2.139 ± 0.022A (Table 3.1), followed by medium containing glucose (2,081 ± 0,017A). The positive controls FOS and inulin provided similar results to the negative control (water), just as Rodrigues *et al*. (2016) observed to *Lactobacillus* sp. In the treatments of JSC fermented by fungi, the highest growth of *L. acidophilus* was obtained in the médium with the *P. pulmonarius* supernatant with 1.022 ± 0.015A after 48 hours of

incubation, followed by the supernatants of *Tyromyces* sp. INPA1696 (0.960 ± 0.058A), *P. lecomtei* CC40 (0.849 ± 0.053A) and *Coriolopsis* sp. INPA1646 (0.774 ± 0.028A).

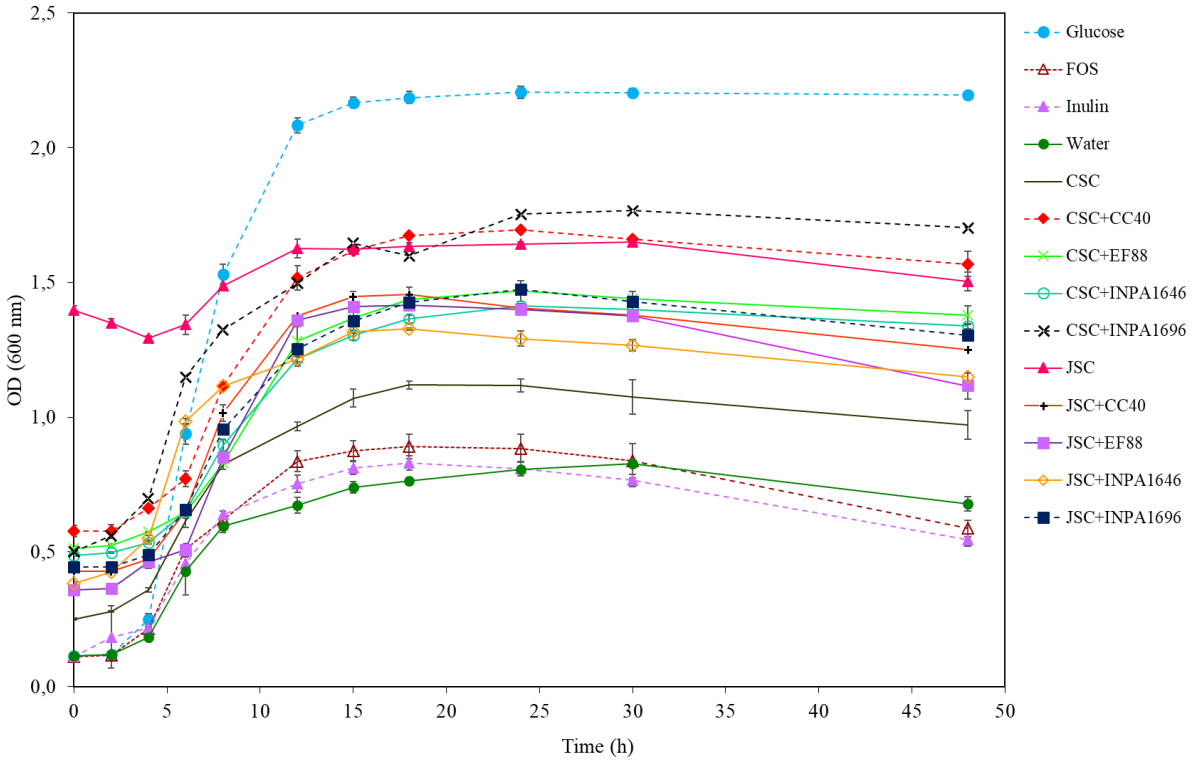
**Figure 3.1** Growth curves of *L. acidophilus* at 20 mg/mL of supernatants (carbon source – prebiotics) from submerged fermentations by macrofungi in cottonseed cake (SmF-CSC) and jatropha seed cake (SmF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

After 14 days of cultivation, the supernatants of SSF fermentations by *P. lecomtei* CC40, *Tyromyces* sp. INPA1696 and *P. pulmonarius* EF88 resulted in greater growth of *L. acidophilus*, when compared to unfermented SmF supernatant, probably due to the release of fermentable sugars (Figure 3.2). Likewise, the SSF supernatant fermented by *Coriolopsis* sp. INPA1646 showed greater total cell growth (0.922 ± 0.009A) than the unfermented CSC supernatant (0.630 ± 0.026A) and controls, however, its growth was lower when compared to the glucose treatment.

**Figure 3.2** Growth curves of *L. acidophilus* at 20 mg/mL of supernatants from solid-state fermentation by macrofungi in cottonseed cake (SSF-CSC) and jatropha seed cake (SSF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

The growth results of *L. acidophilus* in the aqueous extracts from solid-state fermentation of CSC were overall lower than those observed using the supernatants from the submerged fermentation (Table 3.1S). In this group, the highest growth of *L. acidophilus* was observed in the aqueous extract of SSF fermented by *Tyromyces* sp. INPA1696, followed by the CSC fermented by *P. lecomtei* CC40. The growth of *L. acidophilus* in the aqueous extract of JSC without fungus was the lowest observed among all samples tested. The opposite of that was observed with the JSC supernatant; this suggests that the treatment and extraction with agitation and 28°C after 14 days is more efficient than the aqueous extraction under pressure and temperature in ASE to obtain a *L. acidophilus* culture medium, probably due to the release of fermentable sugars.

**Table 3.1** Total cell growth of *L. acidophilus* (OD 600) in 48 hours of incubation (mean±SD) in MRS medium with 20 mg/mL of the different supernatants from submerged cultures and aqueous extracts from solid-state fermentation using macrofungi grown in JSC and CSC. Tukey test ($p \leq 0,05$).

| Type of Fermentation | Fermented biomass | Variation of OD (Tf-Ti) |
|---|---|---|

| | | |
|---|---|---|
| Control (C-) | Water | $0.564 \pm 0.028^{j}$ |
| Controls (C+) | Glucose | $2.081 \pm 0.017^{b}$ |
| | FOS | $0.476 \pm 0.031^{k}$ |
| | Inulin | $0.432 \pm 0.026^{k}$ |
| Supernatants of submergded fermentations | CSC | $0.630 \pm 0.026^{i}$ |
| | CSC+CC40 | $1.383 \pm 0.028^{c}$ |
| | CSC+EF88 | $1.139 \pm 0.018^{d}$ |
| | CSC+INPA1646 | $0.922 \pm 0.009^{e}$ |
| | CSC+INPA1696 | $1.217 \pm 0.005^{d}$ |
| Aqueous extract of solid-state fermentations | CSC | $0.720 \pm 0.056^{h}$ |
| | CSC+CC40 | $0.991 \pm 0.026^{e}$ |
| | CSC+EF88 | $0.865 \pm 0.026^{f}$ |
| | CSC+INPA1646 | $0.851 \pm 0.007^{f}$ |
| | CSC+INPA1696 | $1.203 \pm 0.058^{d}$ |
| Supernatants of submergded fermentations | JSC | $2.139 \pm 0.022^{a}$ |
| | JSC+CC40 | $0.849 \pm 0.053^{f}$ |
| | JSC+EF88 | $1.022 \pm 0.015^{e}$ |
| | JSC+INPA1646 | $0.774 \pm 0.028^{h}$ |
| | JSC+INPA1696 | $0.960 \pm 0.058^{e}$ |
| Aqueous extract of solid-state fermentations | JSC | $0.107 \pm 0.046^{l}$ |
| | JSC+CC40 | $0.816 \pm 0.001^{g}$ |
| | JSC+EF88 | $0.748 \pm 0.062^{g}$ |
| | JSC+INPA1646 | $0.764 \pm 0.008^{g}$ |
| | JSC+INPA1696 | $0.861 \pm 0.019^{f}$ |

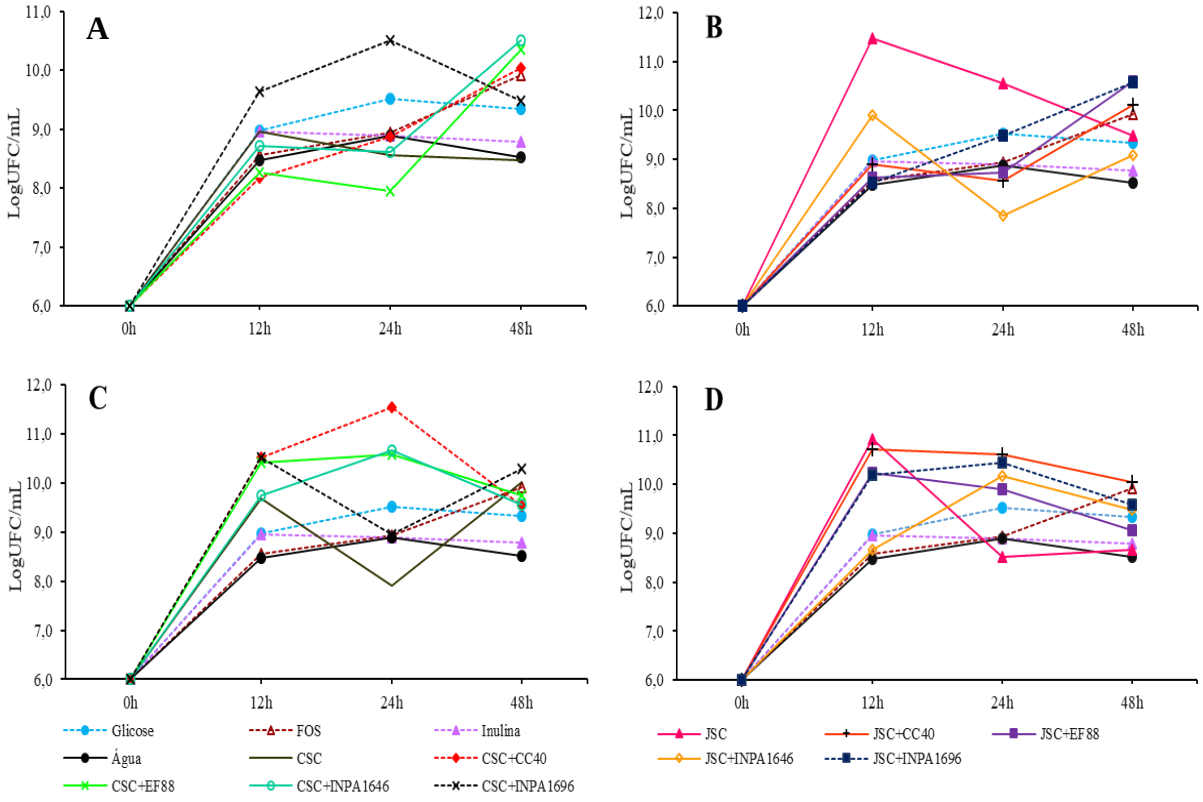
Fonte: da autora (2021)

Guimarães (2020) tested the growth of *L. acidophilus* in a medium with aqueous extracts of CSC fermented by *P. lecomtei* CC40 and observed that using extracts from this fermentation at 20 and 50%, the growth reached 1.5 OD in the first 12 hours, similar to the extract of the supernatant of *P. lecomtei* CC40 in CSC and the media with aqueous extracts of the fermented *P. pulmonarius* EF88 with JCS. The variation in OD of total *L. acidophilus* cell growth at the end of 48 hours of incubation allows us to conclude that the best culture

medium was MRS(-C) with 20mg/mL of JSC supernatant incubated for 14 days at 28ºC without fungal fermentation, having better results than the conventional MRS medium (with 20mg/mL of glucose). In general, supernatants from submerged fermentations are better for the growth of *L. acidophilus* than the aqueous extracts tested, and the effect of fermentation with macrofungi *P. lecomtei* CC40 and *Tyromyces* sp. INPA1696 indicates better growth of *L. acidophilus*.

The peak of total cells in the medium does not always occur in the 48th hour of incubation, but the maintenance of cell viability for long periods is an important characteristic to consider a prebiotic activity. The highest concentration of viable *L. acidophilus* cells is observed at the 12th hour of incubation in the medium containing the unfermented JSC supernatant (Figure 3.3B). Among the submerged CSC fermentation supernatants (Figure 3.3A), the highest concentration of viable cells was observed in the fermented medium with *Tyromyces* spp INPA1696 with 24 hours incubation, and *P. pulmonarius* EF88 and *Coriolopsis* sp. INPA1646 with 48 hours of incubation. For the solid CSC fermentation aqueous extracts (Figure 3.3B), the highest concentration occurs with the extract of *P. lecomtei* CC40 at 24 hours of incubation. In JSC treatments (Figures 3.3C and 3.3D), none surpasses the concentration in the medium with glucose, with 12 hours of incubation, but the extract of solid fermentation by *P. lecomtei* CC40 equals this concentration in 12 hours of cultivation.

**Figure 3.3** Growth of *L. acidophilus* in LogUFC/mL, with 0, 12, 24 and 48 hours of incubation in MRS-C medium with 20 mg/mL of CSC submerged fermentation supernatants (A), submerged fermentation supernatants of JSC (B), solid-state fermentation aqueous extracts of CSC (C), and solid-state fermentation extracts of JSC (D).

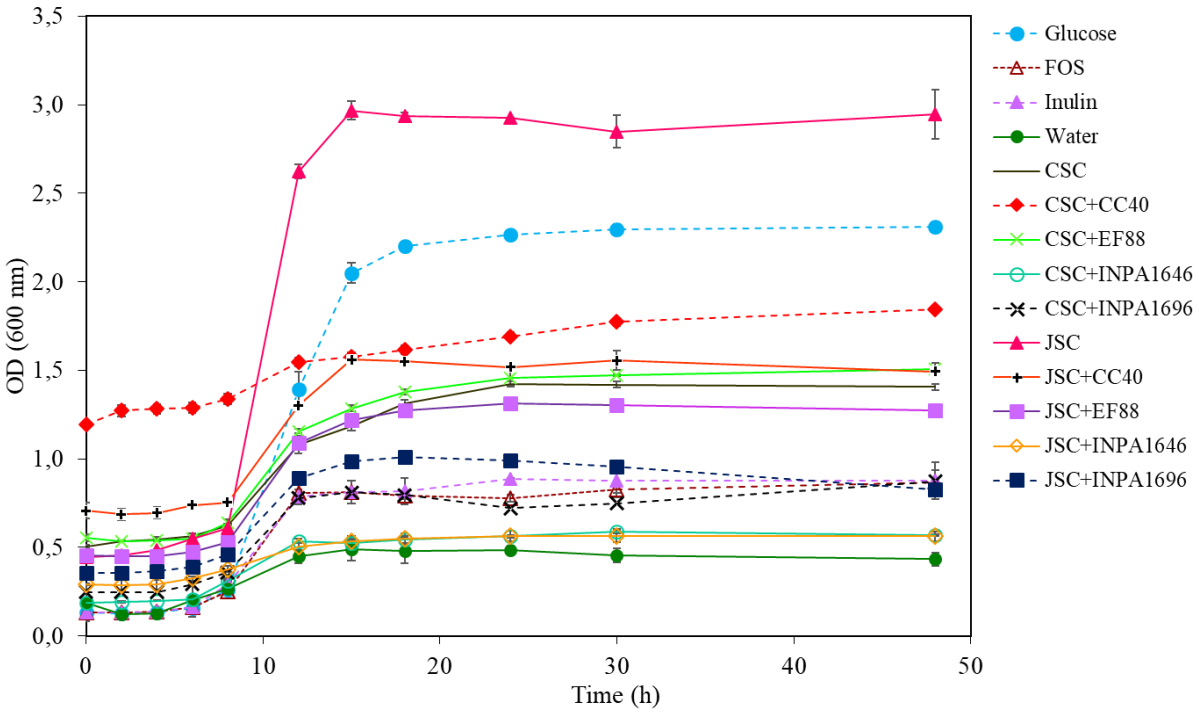



Fonte: da autora (2021)

### 3.1.2 *Bifidobacterium lactis*

In *B. lactis* cultures, the commercial prebiotics FOS and inulin did not show satisfactory growth, and the peak of growth in the medium with glucose was observed in the 15th hour of incubation (Figure 3.4), as in Candela *et al*. (2010) and Guimarães (2020) studies. However, Rodrigues *et al.* (2016) reported the growth of *Bifidobacterium* sp. higher in FOS than the negative control, with greater growth after 24h of incubation. The greatest growth of whole-cell *B. lactis* was also observed in the supernatant medium with JSC without fungal inoculation, with an increase of 2.501 ± 0.140A after 48 hours incubation, followed by the glucose treatment with 2.122 ± 0.086A.

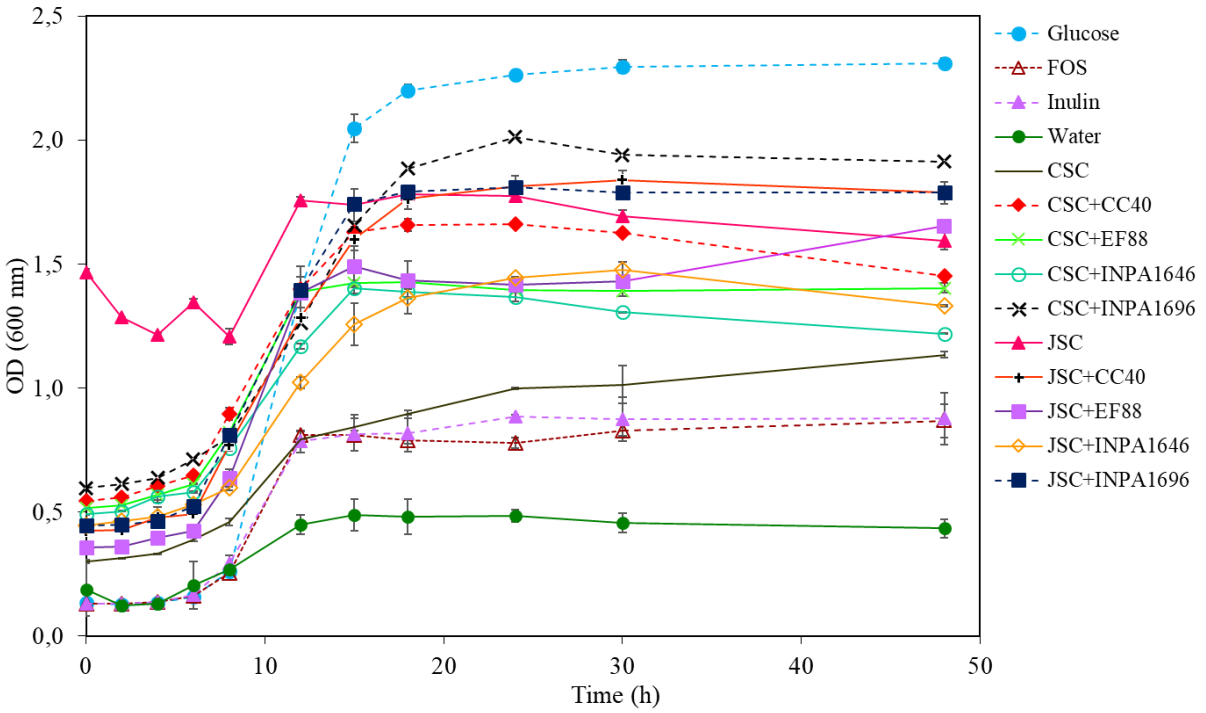
**Figure 3.4** Growth curves of *B. lactis* at 20 mg/mL of supernatants from submerged fermentations by macrofungi in cottonseed cake (SmF-CSC) and jatropha seed cake (SmF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

In cultures with supernatants obtained from CSC, the greatest cell growth after 48 h of incubation was observed in the treatment fermented by *P. pulmonarius* EF88, with an increase in OD of 0.949 ± 0.059, followed by CSC supernatant without fungus, with OD of 0.907 ± 0.012 (Table 3.2). The supernatant from CSC fermentation by *P. lecomtei* CC40 showed OD variation of 0.654 ± 0.003A, however, the dark color of the assay may interfere with absorbance reading and make it difficult to quantify the increase in total cells. The supernatant from the fermentation by *Tyromyces* sp. INPA1696 behaved similarly to the positive controls FOS and inulin in the growth of *B. lactis*, while the treatment with *Coriolopsis* sp. INPA1646 was similar to the negative control (water), with a OD of 0.379 ± 0.007A (Figure 3.5).

**Figure 3.5** Growth curves of *B. lactis* at 20 mg/mL of supernatants from solid-state fermentation by macrofungi in cottonseed cake (SSF-CSC) and jatropha seed cake (SSF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

On the other hand, in cultures with aqueous extracts obtained from CSC, the treatment with *Tyromyces* sp. INPA1696 showed OD variation of 1.318 ± 0.087 after 48 hours of incubation (Table 3.2). The treatment with CSC extract without fungus was similar to that observed in the media with CSC extracts fermented by *P. pulmonarius* EF88 and *P. lecomtei* CC40, while the absorbance in the medium with CSC extract fermented by *Coriolopsis* sp. INPA1646 was only 0.728 ± 0.005A. Guimarães (2020), reported the best growth rate of *B. lactis* in a medium with glucose, followed by the medium with the aqueous extract of the fermented *P. lecomtei* CC40 with 50% of CSC, with almost 1.7 of absorbance after the first 18 hours, similar to the profile observed in CSC fermented with *P. lecomtei* CC40 here.

In cultures with supernatants obtained with JSC, treatments fermented by *P. lecomtei* CC40 and *P. pulmonarius* EF88 showed similar results stimulating the growth of *B. lactis*, and higher when compared to the positive controls FOS and inulin. On the other hand, in cultivations with aqueous extracts, the treatments of JSC fermented by *P. lecomtei* CC40, *P. pulmonarius* EF88, and *Tyromyces* sp. INPA1696 showed greater growth stimulation of *B. lactis* (Table 3.2). Guimarães (2020) reported the growth of *B. lactis* in a medium with an aqueous extract obtained with 80% of JCS and fermented by *P. pulmonarius* EF88, with a OD profile similar to that observed in this work with the aqueous extract of JCS fermented with *P. pulmonarius* EF88, reaching OD ~ 1.5 in the first 18 h, higher than those fermented with 20

or 50%, indicating that higher concentrations of fermented JCS can favor the growth of this bacteria.

**Table 3.2** Growth of total *B. lactis* cells (OD 600) in 48 hours of incubation (mean ± SD) in MRS medium with 20mg/mL of different supernatants from submerged cultures and aqueous extracts from solid-state fermentation using macrofungi grown in JSC and CSC. Tukey test ($p \leq 0,05$).

| Type of Fermentation | Fermented biomass | Variation of OD (Tf-Ti) |
|---|---|---|
| Control (C-) | Water | $0.303 \pm 0.041$ |
| Controls (C+) | Glucose | $2.122 \pm 0.086^{b}$ |
| | FOS | $0.735 \pm 0.077^{e}$ |
| | Inulin | $0.745 \pm 0.120^{e}$ |
| Supernatants of submerged fermentation | CSC | $0.907 \pm 0.012^{d}$ |
| | CSC+CC40 | $0.654 \pm 0.003^{e}$ |
| | CSC+EF88 | $0.949 \pm 0.059^{d}$ |
| | CSC+INPA1646 | $0.379 \pm 0.007^{h}$ |
| | CSC+INPA1696 | $0.627 \pm 0.021^{f}$ |
| Aqueous extract of solid-state fermentation | CSC | $0.833 \pm 0.020^{e}$ |
| | CSC+CC40 | $0.906 \pm 0.001^{d}$ |
| | CSC+EF88 | $0.888 \pm 0.015^{d}$ |
| | CSC+INPA1646 | $0.728 \pm 0.005^{e}$ |
| | CSC+INPA1696 | $1.318 \pm 0.087^{c}$ |
| Supernatants of submerged fermentation | JSC | $2.501 \pm 0.140^{a}$ |
| | JSC+CC40 | $0.845 \pm 0.052^{d}$ |
| | JSC+EF88 | $0.817 \pm 0.020^{d}$ |
| | JSC+INPA1646 | $0.272 \pm 0.027^{i}$ |
| | JSC+INPA1696 | $0.478 \pm 0.067^{g}$ |
| Aqueous extract of solid-state fermentation | JSC | $0.126 \pm 0.020^{j}$ |
| | JSC+CC40 | $1.366 \pm 0.034^{c}$ |
| | JSC+EF88 | $1.295 \pm 0.017^{c}$ |
| | JSC+INPA1646 | $0.906 \pm 0.140^{d}$ |
| | JSC+INPA1696 | $1.342 \pm 0.022^{c}$ |

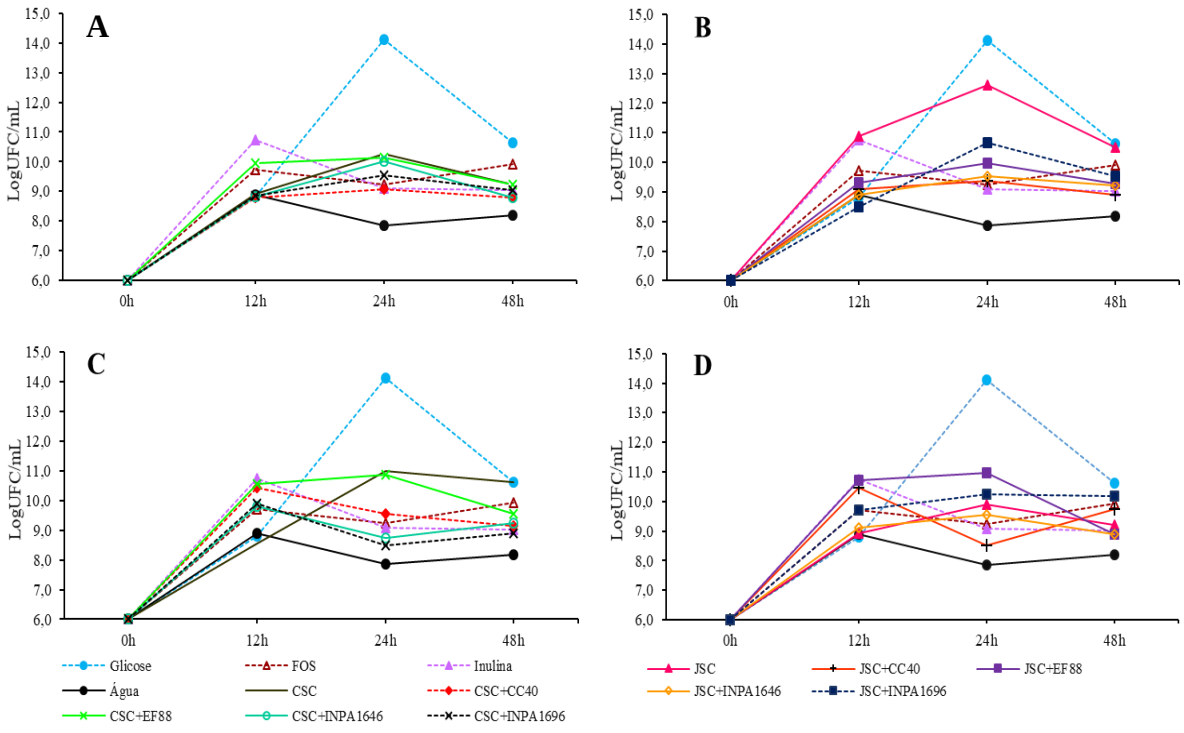
Fonte: da autora (2021)

The highest concentrations of viable cells occurred after 24 hours of incubation in the media with glucose (conventional MRS), followed by the medium containing the unfermented JSC supernatant. However, if shorter incubation times are considered (e.g. 12 h), the media with the supernatants and biomass extracts have higher concentrations of viable cells (figure 3.6).

### 3.1.3 *Lactobacillus rhamnosus*

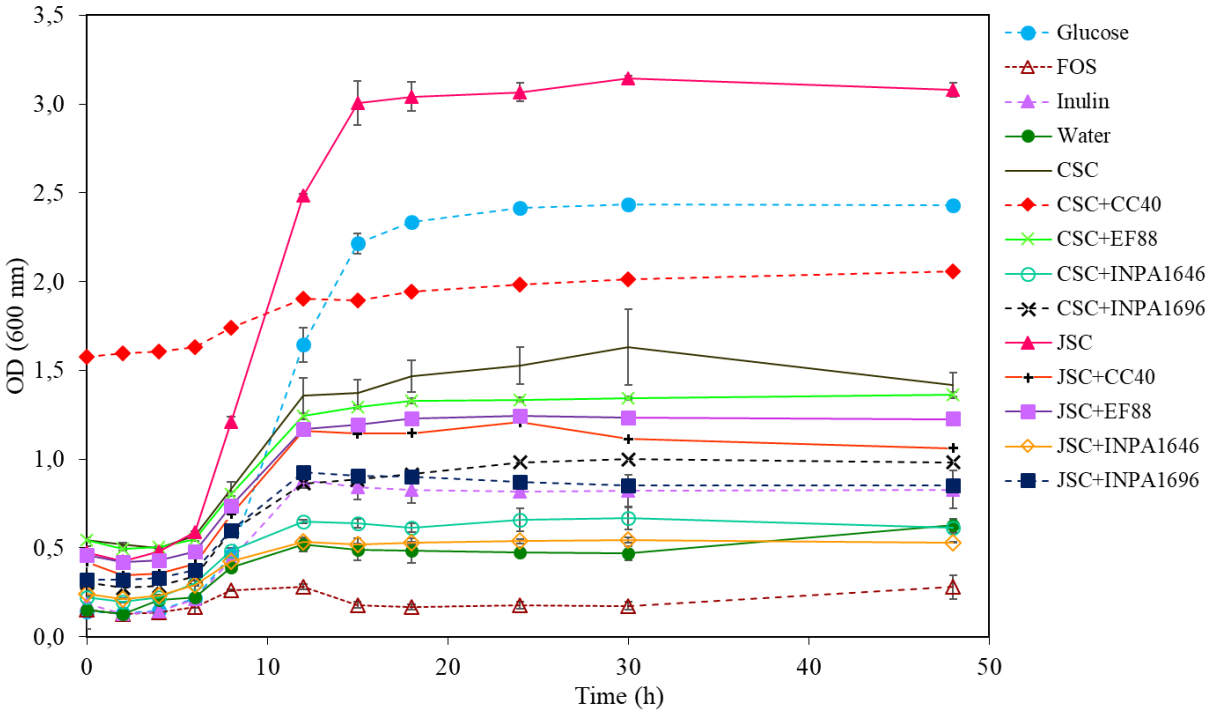
The growth of *L. rhamnosus* was strongly stimulated using JSC supernatant without fungus, reaching the exponential phase in a shorter time (6 – 12 h) (Figure 3.7), with an increase in OD of 2.570 ±0.057 at the end of 48 h of incubation, higher than the control with glucose (OD 2.279 ± 0.025A). Among the JSC supernatants tested, treatment with *Coriolopsis* sp. INPA1646 showed lower growth, with an increase in OD of 0.302 ± 0.032 at the end of 48 h of incubation.

**Figure 3.6** Growth of *B. lactis* in LogUFC/mL, with 0, 12, 24 and 48 hours of incubation in MRS-C medium with 20 mg/mL of CSC submerged fermentation supernatants (A), submerged fermentation supernatants of JSC (B), solid-state fermentation aqueous extracts of CSC (C), and solid-state fermentation extracts of JSC (D).



Fonte: da autora (2021)

**Figure 3.7** Growth curves of *L. rhamnosus* at 20 mg/mL of supernatants from submerged fermentations by macrofungi in cottonseed cake (SmF-CSC) and jatropha seed cake (SmF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

In the cultures with CSC supernatants, growth of *L. rhamnosus* showed greater variation in the treatment without fungus with OD 0.881 ± 0.059A. Among the fermented treatments, the highest growth value was observed in the treatment with *P. pulmonarius* EF88 (0.825 ± 0.002A), followed by *P. lecomtei* CC40 (0.481 ± 0.003A) and *Coriolopsis* sp. INPA1646 (0.404 ± 0.081A) (Table 3.3).

**Table 3.3** Total cell growth of *L. rhamnosus* (OD 600) in 48 hours of incubation (mean±SD) in MRS medium with 20 mg/mL of the different supernatants from submerged cultures and aqueous extracts from solid state fermentations using macrofungi grown in JSC and CSC. Tukey test ($p \leq 0,05$).

| Type of Fermentation | Fermented biomass | Variation of OD (Tf-Ti) |
|---|---|---|
| Control (C-) | Water | $0.438 \pm 0.113^{h}$ |
| | Glucose | $2.279 \pm 0.025^{b}$ |

| | | |
|---|---|---|
| Controls (C+) | FOS | $0.137 \pm 0.126^{i}$ |
| | Inulin | $0.677 \pm 0.046^{f}$ |
| Supernatants of submerged fermentation | CSC | $0.881 \pm 0.059^{ef}$ |
| | CSC+CC40 | $0.481 \pm 0.003^{fg}$ |
| | CSC+EF88 | $0.825 \pm 0.002^{e}$ |
| | CSC+INPA1646 | $0.404 \pm 0.081^{fg}$ |
| | CSC+INPA1696 | $0.636 \pm 0.038^{f}$ |
| Aqueous extract of solid-state fermentation | CSC | $0.913 \pm 0.089^{cd}$ |
| | CSC+CC40 | $0.802 \pm 0.003^{f}$ |
| | CSC+EF88 | $0.965 \pm 0.104^{de}$ |
| | CSC+INPA1646 | $0.825 \pm 0.137^{ef}$ |
| | CSC+INPA1696 | $1.199 \pm 0.091^{c}$ |
| Supernatants of submerged fermentation | JSC | $2.570 \pm 0.057^{a}$ |
| | JSC+CC40 | $0.641 \pm 0.025^{f}$ |
| | JSC+EF88 | $0.766 \pm 0.005^{e}$ |
| | JSC+INPA1646 | $0.302 \pm 0.032^{h}$ |
| | JSC+INPA1696 | $0.512 \pm 0.042^{g}$ |
| Aqueous extract of solid-state fermentation | JSC | $0.397 \pm 0.091^{h}$ |
| | JSC+CC40 | $0.725 \pm 0.029^{f}$ |
| | JSC+EF88 | $1.042 \pm 0.049^{d}$ |
| | JSC+INPA1646 | $0.922 \pm 0.051^{e}$ |
| | JSC+INPA1696 | $0.920 \pm 0.026^{e}$ |

Fonte: da autora (2021)

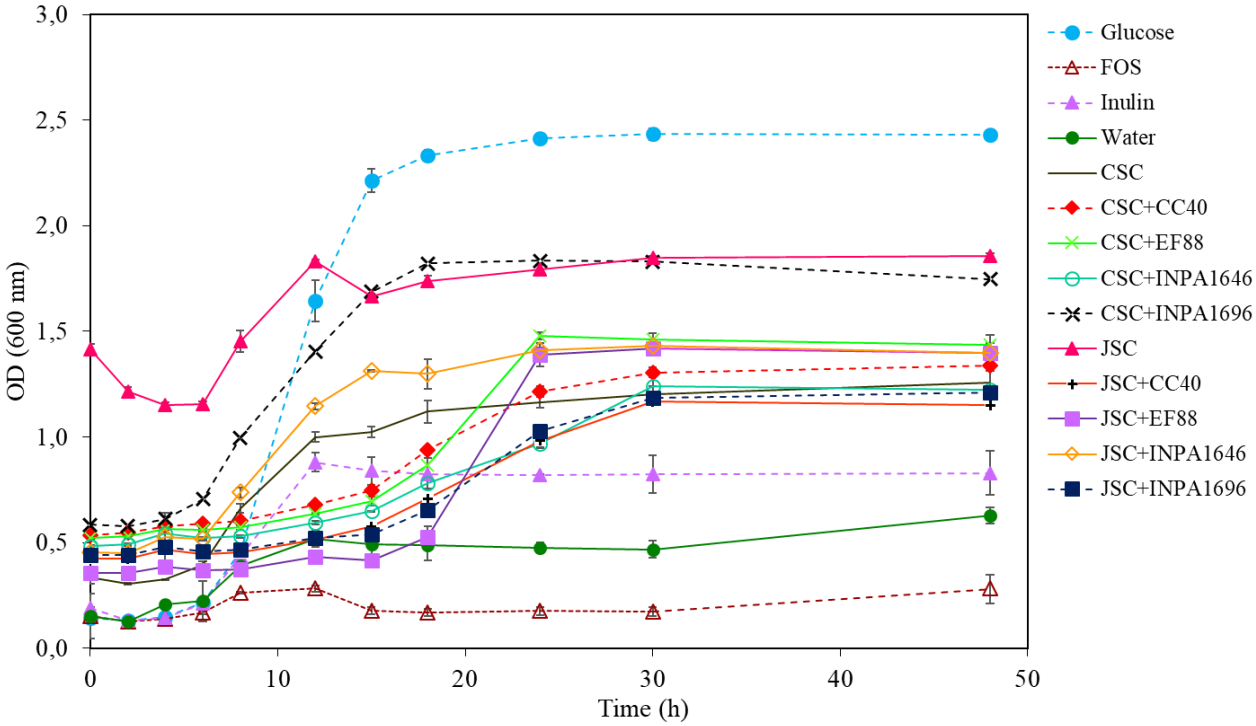
In aqueous extracts of CSC, the treatments fermented by *Tyromyces* sp. INPA1696 and *P. pulmonarius* EF88, showed greater growth of *L. rhamnosus* after 48 hours when compared to the aqueous extract of CSC without fungus. In Guimarães (2020) study, the media with aqueous extracts obtained from CSC fermented by *P. lecomtei* CC40 with any concentrations of CSC showed less variation in OD than the medium with CSC fermented by *P. lecomtei* CC40 in this work, indicating that this aqueous extract may have better stimulation of the growth of *L. rhamnosus*.

The results of this bacterium growth in the aqueous extracts obtained from solid-state fermentation with macrofungi did not have the same pattern as those observed in the

supernatants (figure 3.8), with the lowest growth in the JSC extract without fungus (0.397 ± 0.091A), and greater growth in the extract of CSC fermented by *Tyromices* sp. INPA1696 (1.199 ± 0.091), followed by JSC extract fermented by *P. pulmonarius* EF88 (1.042 ± 0.049A).

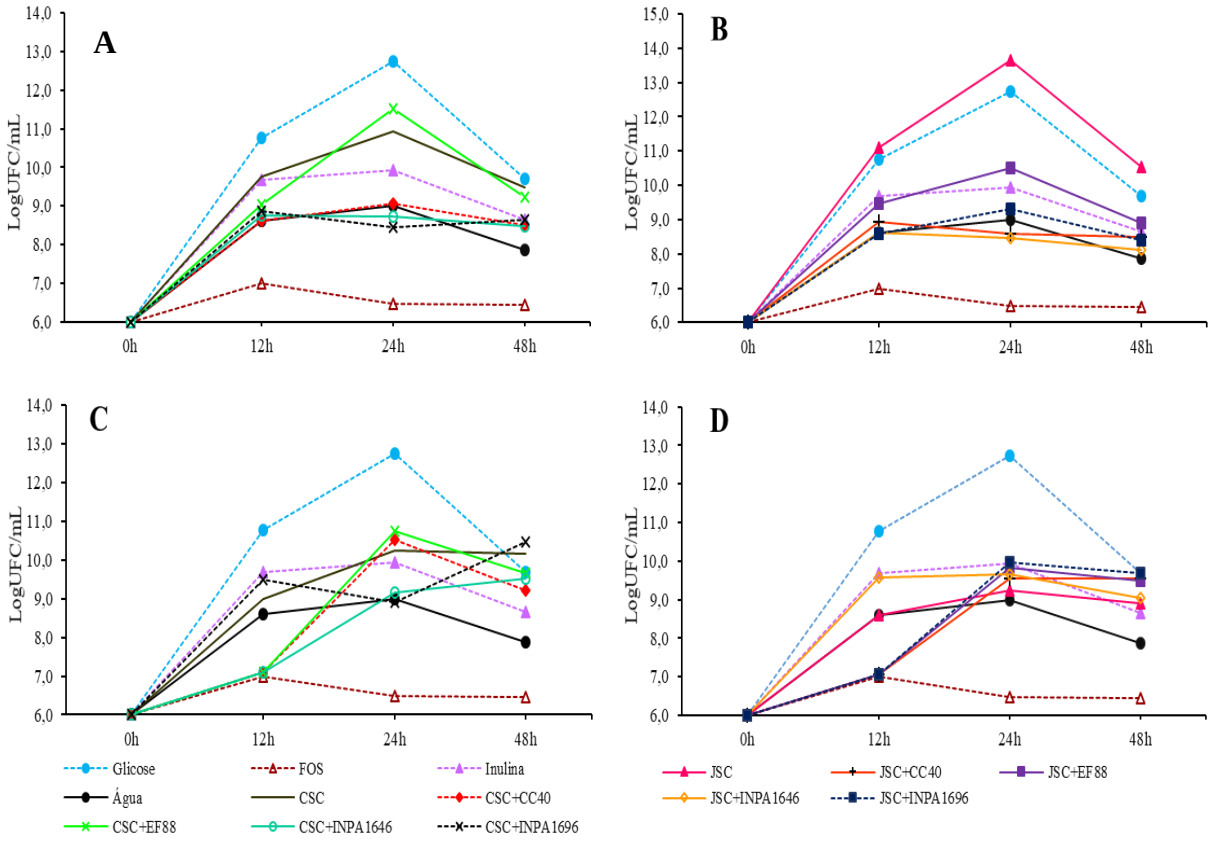
The highest concentration and viable cells were found after 24 hours of incubation in the medium with 20 mg/mL of unfermented JSC supernatant, and in the medium with glucose (conventional MRS), with 24 hours of incubation as well (figure 3.9).

**Figure 3.8** Growth curves of *L. rhamnosus* at 20 mg/mL of supernatants from solid-state fermentation by macrofungi in cottonseed cake (SSF-CSC) and jatropha seed cake (SSF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

**Figure 3.9** Growth of *L. rhamnosus* in LogUFC /mL, with 0, 12, 24 and 48 hours of incubation in MRS-C medium with 20 mg/mL of CSC submerged fermentation supernatants (A), submerged fermentation supernatants of JSC (B), solid-state fermentation aqueous extracts of CSC (C), and solid-state fermentation extracts of JSC (D).

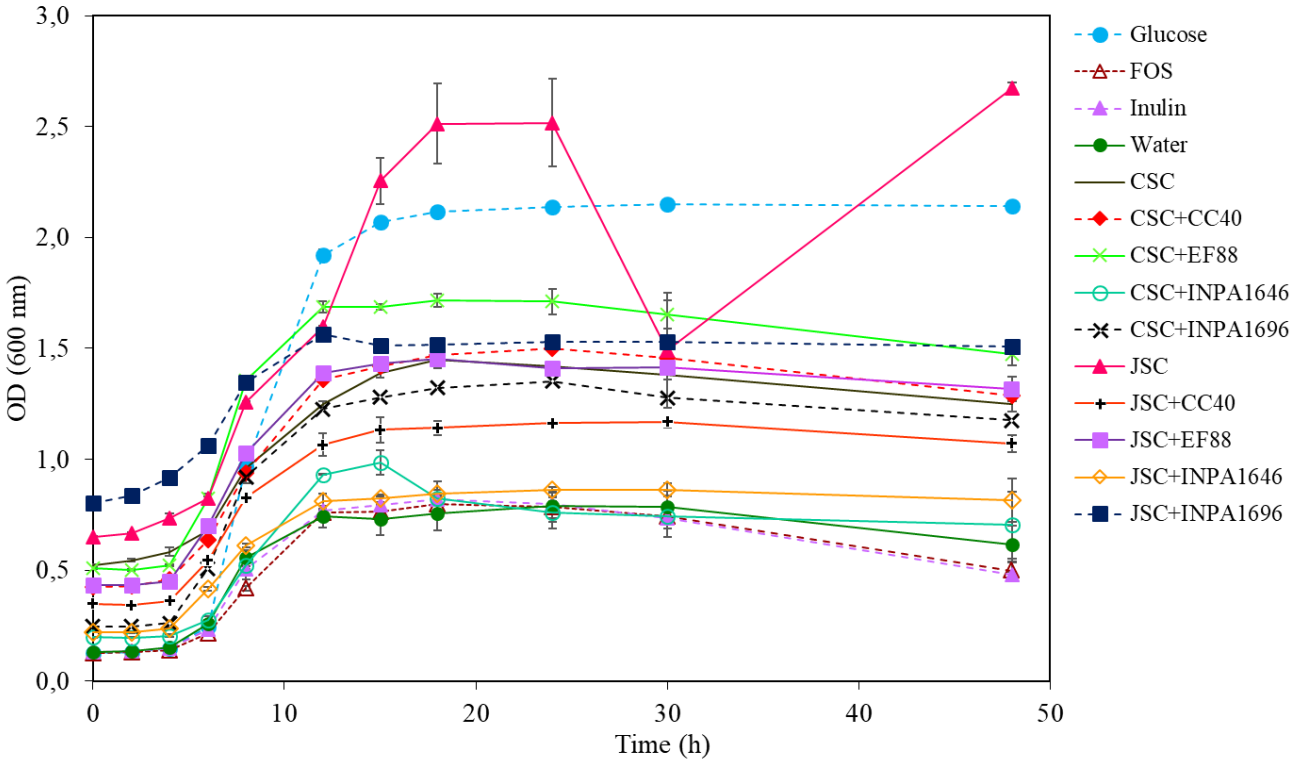



Fonte: da autora (2021)

### 3.1.4 *Lactobacillus plantarum*

The growth of the bacteria *L. plantarum* had a major stimulus, indicated with the variation of OD, using MRS media with glucose (2.014 ± 0.018A) and the supernatant JSC without fungus (2.026 ± 0.025A), indicating that this medium has potential in prebiotic activity (figure 3.10). Among the treatments with fermented cakes, the highest growth was observed in supernatants obtained in submerged fermentation of JSC by *P. pulmonarius* EF88, while the lower results of total cell growth were observed in the supernatants obtained from *Coriolopsis* sp. INPA1646 fermentations (Table 3.4).

**Figure 3.10** Growth curves of *L. plantarum* at 20 mg/mL of supernatants from submerged fermentations by macrofungi in cottonseed cake (SmF-CSC) and jatropha seed cake (SmF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

**Table 3.4** Total cell growth of *L. plantarum* (OD 600) in 48 hours of incubation (mean±SD) in MRS medium with 20mg/mL of different submerged culture supernatants and aqueous extracts of macrofungal solid-state fermentations of JSC and CSC. Tukey test ($p \leq 0,05$).

| Type of Fermentation | Fermented biomass | Variation of OD (Tf-Ti) |
|---|---|---|
| Control (C-) | Water | $0.462 \pm 0.105^{f}$ |
| Controls (C+) | Glucose | $2.014 \pm 0.018^{a}$ |
| | FOS | $0.370 \pm 0.041^{f}$ |
| | Inulin | $0.351 \pm 0.017^{f}$ |
| Supernatants of submerged fermentation | CSC | $0.728+0.023^{d}$ |
| | CSC+CC40 | $0.862+0.018^{c}$ |
| | CSC+EF88 | $0.965+0.048^{b}$ |
| | CSC+INPA1646 | $0.578+0.107^{e}$ |
| | CSC+INPA1696 | $0.931+0.011^{b}$ |
| Aqueous extract | CSC | $0.601+0.033^{e}$ |
| | CSC+CC40 | $0.922+0.026^{b}$ |

| | | |
|---|---|---|
| of solid-state fermentation | CSC+EF88 | 0.805+0.016[d] |
| | CSC+INPA1646 | 0.779+0.022[d] |
| | CSC+INPA1696 | 0.776+0.058[d] |
| Supernatants of submerged fermentation | JSC | 2.026+0.025[a] |
| | JSC+CC40 | 0.723+0.039[d] |
| | JSC+EF88 | 0.881+0.054[bc] |
| | JSC+INPA1646 | 0.593+0.095[e] |
| | JSC+INPA1696 | 0.706+0.033[d] |
| Aqueous extract of solid-state fermentation | JSC | 0.329+0.030[f] |
| | JSC+CC40 | 0.724+0.017[d] |
| | JSC+EF88 | 0.727+0.019[d] |
| | JSC+INPA1646 | 0.729+0.044[d] |
| | JSC+INPA1696 | 0.954+0.001[b] |

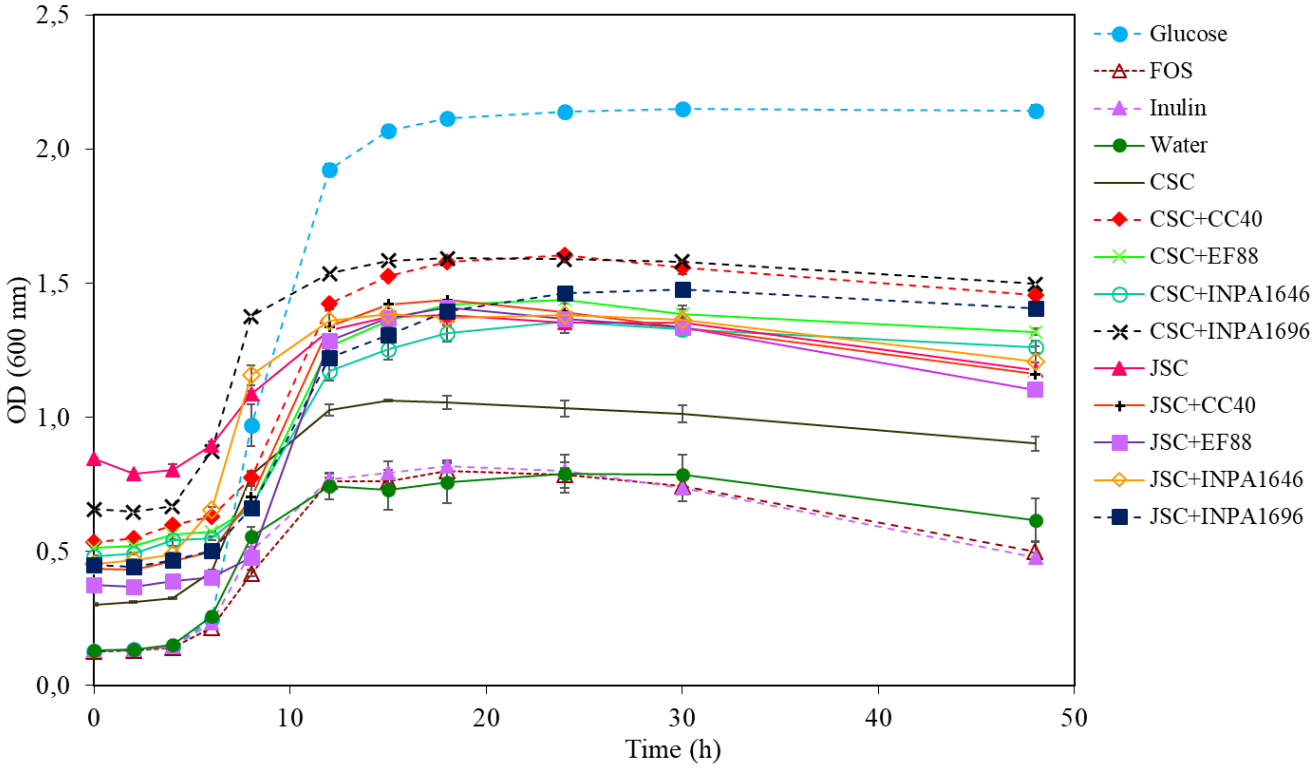
Fonte: da autora (2021)

In fermented JSC, the growth of macrofungi in this cake seems to reduce the efficiency of its supernatants for the growth of this bacteria by up to 70%, under the evaluated cultivation conditions. Longer cultivation times can generate more fungal mass structures, such as chitin, beta-glucans, mycosterols, among other chemical compounds, which can positively influence probiotic growth, such as *Ganoderma lucidum* polysaccharides, which increase the abundance of *Bifidobacteria*, *Lactobacillus*, *Roseburia, Lachnospiraceae,* and *Pleurotus eryngii*, which increase the proliferation of bacteria from the families Porphyromonadaceae, Rikenellaceae, Bacteroidaceae and Lactobacillaceae (MENG; LIANG; LUO, 2016). Nonetheless, the 14 - day fermentation supernatants showed less stimulus to the growth of *L. plantarum*, probably because fungi used available carbohydrates in the biomass for their growth.

When the growth rates are compared in extracts from solid-state fermentation, the medium with glucose also stands out, with the greatest increase in OD, followed by JSC fermented by *Tyromices* sp. INPA1696 and CSC fermented by *P. lecomtei* CC40, with OD variation of 0.954 ± 0.001A and 0.922 ± 0.026A, respectively (Figure 3.11). All other fermented solids in both biomasses resulted in similar total cell growth of *L. plantarum* after 48 hours of incubation. Guimarães (2020) reported a greater growth of *L. plantarum* in the media with all aqueous extracts obtained from the fermentation of *P. pulmonarius* EF88 and

*P. lecomtei* CC40 containing JSC and CSC, respectively, compared to the controls, but but a growth smaller thanglucose treatment.

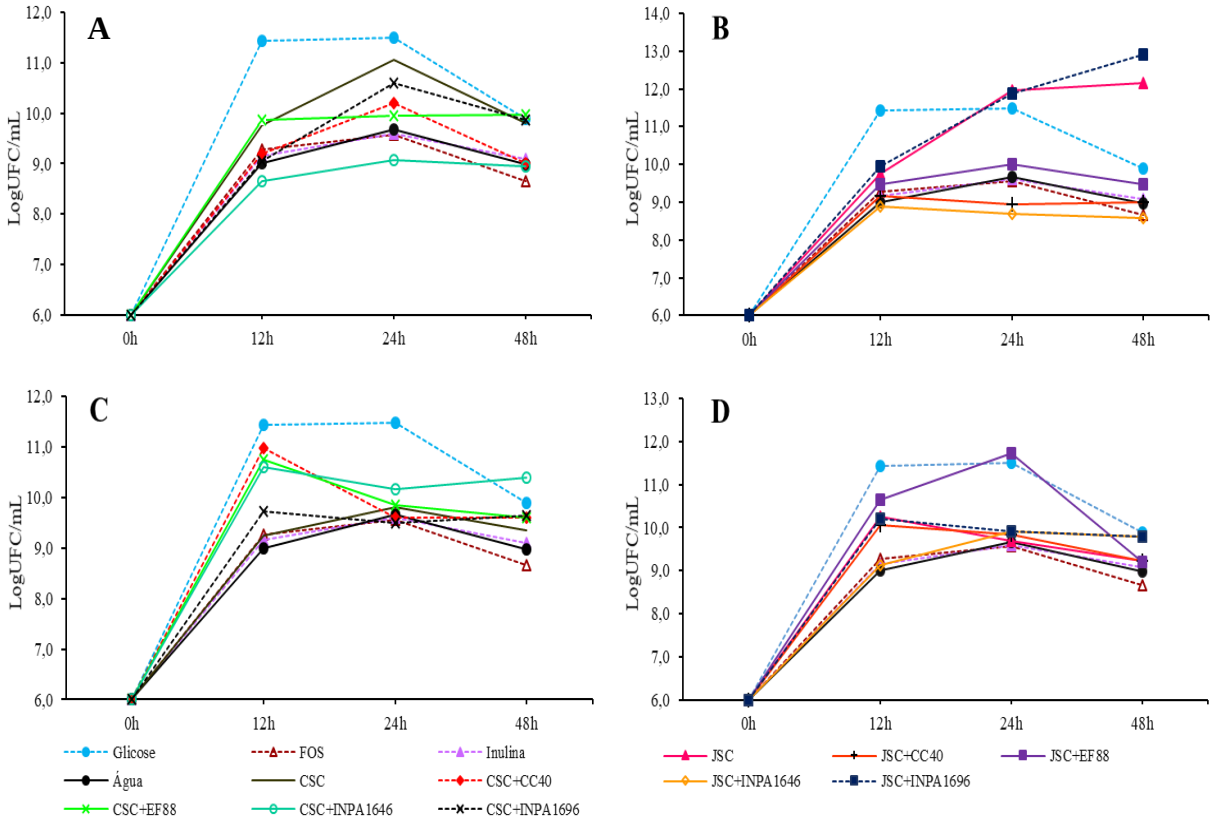
**Figure 3.11** Growth curves of *L. plantarum* at 20 mg/mL of supernatants from solid-state fermentation by macrofungi in cottonseed cake (SSF-CSC) and jatropha seed cake (SSF-JSC) for 48 hours at 37°C.




Fonte: da autora (2021)

The highest concentrations of viable cells were observed after 48 hours of incubation in MRS media with 20 mg/mL of supernatants from submerged fermentation of JSC by *Tyromices* sp. INPA1696 and *P. pulmonarius* EF88. It is important to note that at 48 hours of incubation in these media, the concentration of viable cells seems to continue to rise, maintaining cell viability longer (Figure 3.12).

**Figure 3.12** Growth of *L. plantarum* in LogUFC /mL, with 0, 12, 24 and 48 hours of incubation in MRS-C medium with 20 mg/mL of CSC submerged fermentation supernatants (A), submerged fermentation supernatants of JSC (B), solid-state fermentation aqueous extracts of CSC (C), and solid-state fermentation extracts of JSC (D).




Fonte: da autora (2021)

### 3.2 Antimicrobial activity

Table 3.5 shows all the results of the antibiogram performed with supernatants (SmF) and aqueous extracts (SSF) lyophilized and diluted to concentrations of 100, 10, and 1 mg/m. Strains were considered sensitive to aqueous extracts or supernatants when they presented inhibition halos greater than 10 mm, and resistant when there was no halo formation, or when it was smaller than 10 mm, as suggested by some authors (SOUZA *et al*., 2007; OLIVEIRA *et al*., 2008; AGUIAR *et al*., 2008). Only the aqueous extracts from solid-state fermentation of CSC by *P. lecomtei* CC40 and by *Coriolopsis* sp. INPA1646 showed a halo of inhibition against *S. enterica*, at a concentration of 100 mg/mL. The optimization of the cultivation time to evaluate the kinetics of supernatants on *Salmonella* should be done in a further study.

In Guimarães (2020), only methanol extracts obtained from CSC 80% fermented by *P. lecomtei* CC40 and JSC with 20 and 80% fermented by *P. pulmonarius* EF88 showed a halo of inhibition against *S. aureus*. This indicates that nonpolar molecules with antimicrobial activity may be present in fermented biomass in this work that were not present in the extracts and supernatants because they are not water-soluble. Some studies indicate that the

antimicrobial activity of mushrooms occurs mainly against gram-positive bacteria (VENTURINI *et al*., 2008; ALVES *et al*., 2012b; SHEN *et al*., 2017), which do not present the external lipid bilayer as in the gram-negative ones, which may indicate that compounds with antimicrobial activity are water-soluble ions and they are unable to cross a lipid bilayer.

In addition to the aqueous extracts and supernatants from the CSC and JSC fermentations, the probiotic bacteria growth supernatants were also tested in an antibiogram assay for inhibition of enteropathogenic strains of *E. coli* ATCC25922, *S. aureus* ATCC27154 and *S. enterica* ATCC14028. During their growth, in addition to lactic acid and acetic acid, probiotic bacteria produce short-chain fatty acids and other extracellular metabolites that can inhibit the growth of some enterobacteria (DOUGLAS and SANDERS, 2008).

**Table 3.5** Results of antibiogram tests to evaluate inhibition halos of *E. coli*, *S. aureus* and *S. enterica* strains when treated with submerged fermentation supernatants (SmF) and aqueous extracts from solid state fermentations (SSF) of macrofungi in CSC and JSC.

| Treatment | Inhibition halo diameter | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* | | | *S. aureus* | | | *S. enterica* | | |
| | 100mg/mL | 10mg/mL | 1mg/mL | 100mg/mL | 10mg/ml | 1mg/mL | 100mg/mL | 10mg/mL | 1mg/mL |
| Water | - | - | - | - | - | - | - | - | - |
| CSC (SmF) | - | - | - | - | - | - | - | - | - |
| CSC+CC40 (SmF) | - | - | - | - | - | - | - | - | - |
| CSC+EF88 (SmF) | - | - | - | - | - | - | - | - | - |
| CSC+INPA1646 (SmF) | - | - | - | - | - | - | - | - | - |
| CSC+INPA1696 (SmF) | - | - | - | - | - | - | - | - | - |
| JSC (SmF) | - | - | - | - | - | - | - | - | - |
| JSC+CC40 (SmF) | - | - | - | - | - | - | - | - | - |
| JSC+EF88 (SmF) | - | - | - | - | - | - | - | - | - |
| JSC+INPA1646 (SmF) | - | - | - | - | - | - | - | - | - |
| JSC+INPA1696 (SmF) | - | - | - | - | - | - | - | - | - |
| CSC (SSF) | - | - | - | - | - | - | - | - | - |
| CSC+CC40 (SSF) | - | - | - | - | - | - | 1.2 cm | - | - |
| CSC+EF88 (SSF) | - | - | - | - | - | - | - | - | - |
| CSC+INPA1646 (SSF) | - | - | - | - | - | - | 0.73 cm | - | - |
| CSC+INPA1696 (SSF) | - | - | - | - | - | - | | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| JSC (SSF) | - | - | - | - | - | - | - | - | - |
| JSC+CC40 (SSF) | - | - | - | - | - | - | - | - | - |
| JSC+EF88 (SSF) | - | - | - | - | - | - | - | - | - |
| JSC+INPA1646 (SSF) | - | - | - | - | - | - | - | - | - |
| JSC+INPA1696 (SSF) | - | - | - | - | - | - | - | - | - |
| **Antibiótic (C+)** | 2.1cm | | | 3.75cm | | | 3.6cm | | |

Fonte: da autora (2021)

The *S. aureus* strain is more sensitive than the other two bacteria tested, as it showed inhibition halos for several *L. acidophilus* supernatants (Table 3.6). The *S. enterica* strain showed a halo of inhibition only for *L. acidophilus* growth supernatants in the media with aqueous extracts of CSC fermented by *Tyromices* sp. INPA1696 and non-fermented JSC. These results suggest that during its growth, *L. acidophilus* releases some metabolic with antimicrobial activity to these strains of enteropathogens.

**Table 3.6** Results of antibiogram assays to assess halos of inhibition of *E. coli, S. aureus, and S. enterica* strains by probiotic bacteria growth supernatants in the samples tested in the prebiotic activity assays.

| | Sobrenadantes de *L. acidophilus* | | | Sobrenadantes de *B. lactis* | | | Sobrenadantes de *L. rhamnosus* | | | Sobrenadantes de *L. plantarum* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* | *S. aureus* | *S. enterica* | *E. coli* | *S. aureus* | *S. enterica* | *E. coli* | *S. aureus* | *S. enterica* | *E. coli* | *S. aureus* | *S. enterica* |
| Glucose | - | 0.95cm | - | - | - | - | - | - | - | - | - | - |
| FOS | - | 0.9cm | - | - | - | - | - | - | - | - | - | - |
| Inulin | - | 0.7cm | - | - | - | - | - | - | - | - | - | - |
| Water | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC (SmF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC+CC40 (SmF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC+EF88 (SmF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC+INPA1646 (SmF) | - | 0.8cm | - | - | - | - | - | 1.3cm | - | - | - | - |
| CSC+INPA1696 (SmF) | - | 0.7cm | - | - | - | - | - | - | - | - | - | - |
| JSC (SmF) | - | 1.0cm | - | - | - | - | - | 1.0cm | - | - | - | - |
| JSC+CC40 (SmF) | - | 0.8cm | - | - | 0.7cm | - | - | - | - | - | - | - |
| JSC+EF88 (SmF) | - | 1.0cm | - | - | - | - | - | - | - | - | - | - |
| JSC+INPA1646 (SmF) | - | 1.0cm | - | - | 1.05cm | - | - | - | - | - | - | - |
| JSC+INPA1696 (SmF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC (SSF) | - | 1.1cm | - | - | 0.95cm | - | - | - | - | - | - | - |
| CSC+CC40 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC+EF88 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |
| CSC+INPA1646 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CSC+INPA1696 (SSF) | - | - | 1.4cm | - | 1.15cm | 1.4cm | - | - | - | - | - | - |
| JSC (SSF) | - | 0.9cm | 1.3cm | - | 0.8cm | 1.3cm | - | - | - | - | - | - |
| JSC+CC40 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |
| JSC+EF88 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |
| JSC+INPA1646 (SSF) | - | 1.0cm | - | - | 1.2cm | - | - | - | - | - | - | - |
| JSS+INPA1696 (SSF) | - | - | - | - | - | - | - | - | - | - | - | - |
| **Antibiotic (C+)** | 2.1cm | 3.75cm | 3.6cm | 2.1cm | 3.75cm | 3.6cm | 2.1cm | 3.75cm | 3.6cm | 2.1cm | 3.75cm | 3.6cm |

Fonte: da autora (2021)

The growth supernatants of *B. lactis* showed less inhibition of the enteropathogens than those of *L. acidophilus*. Table 3.6 shows the inhibition of supernatants of *B. lactis*, where it can be seen that only the supernatant of the growth of the probiotic with the supernatants (SmF) of JSC fermented by *P. lecomtei* CC40 and *Coriolopsis* sp. INPA1646, CSC extracts without fungus, CSC fermented by *Tyromices* sp. INPA1696, JSC fermented by *Coriolopsis* sp. INPA1646 and JSC without fungus can inhibit *S. aureus*, and only CSC extract fermented by *Tyromices* sp. INPA1696 was able to inhibit *S. enterica*.

The growth supernatants of *L. rhamnosus* have even less antibacterial activity on the strains tested, inhibiting only *S. aureus*, and only when grown in the MRS with the CSC submerged fermentation supernatant (SmF) by *Coriolopsis* sp. INPA1646, and in the JSC supernatant without fungus. In *L. plantarum* supernatants, inhibition halos were not observed in any of the tested bacteria.

Although some supernatants have promoted significant inhibition halos in the antibiogram assays, mainly against *S. aureus*, it was not possible to perform MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) assays, as the exact concentration of these lactic fermentation supernatants is unknown, as they are the medium MRS, supplemented or not, with the extracellular metabolites of the tested probiotic bacteria. The optimization of macrofungal cultivation to obtain supernatants and crude extracts as a carbon source for probiotic bacteria is a step to be studied further to seek better results against enteropathogenic bacteria.

## 4 CONCLUSION

Variables such as fermentation methods, submerged or solid-state, and the consequent method of obtaining liquid extracts can alter the ability to stimulate (carbon source media) the growth of probiotic bacteria and inhibit the growth of enteropathogenic bacteria. In this work, the unfermented JSC supernatant showed better results in the growth of the four bacterial strains tested compared to the other treatments, indicating that the *Jatropha* cake *in natura* contains potential metabolites or carbohydrates benefits for probiotic bacteria on culture media development. The supernatants and aqueous extracts obtained from CSC fermentation can be used as culture media ingredients for the growth of probiotic bacteria, mainly *L. acidophilus*, and as possible antimicrobials in the case of extracts obtained from solid-state fermentation with *P. lecomtei* CC40 and *Coriolopsis* sp. INPA1646. It is important to know

the chemical characteristics of these extracts to answer why the probiotic and antimicrobial biological activity occurs, so it is necessary to explore secretory analysis in future studies, as well as to evaluate the best conditions and combinations of these extracts. It will also be important to optimize the macrofungal culture parameters to evaluate extracts or supernatants as a carbon source for probiotic bacteria with their respective activities against enteropathogenic bacteria.

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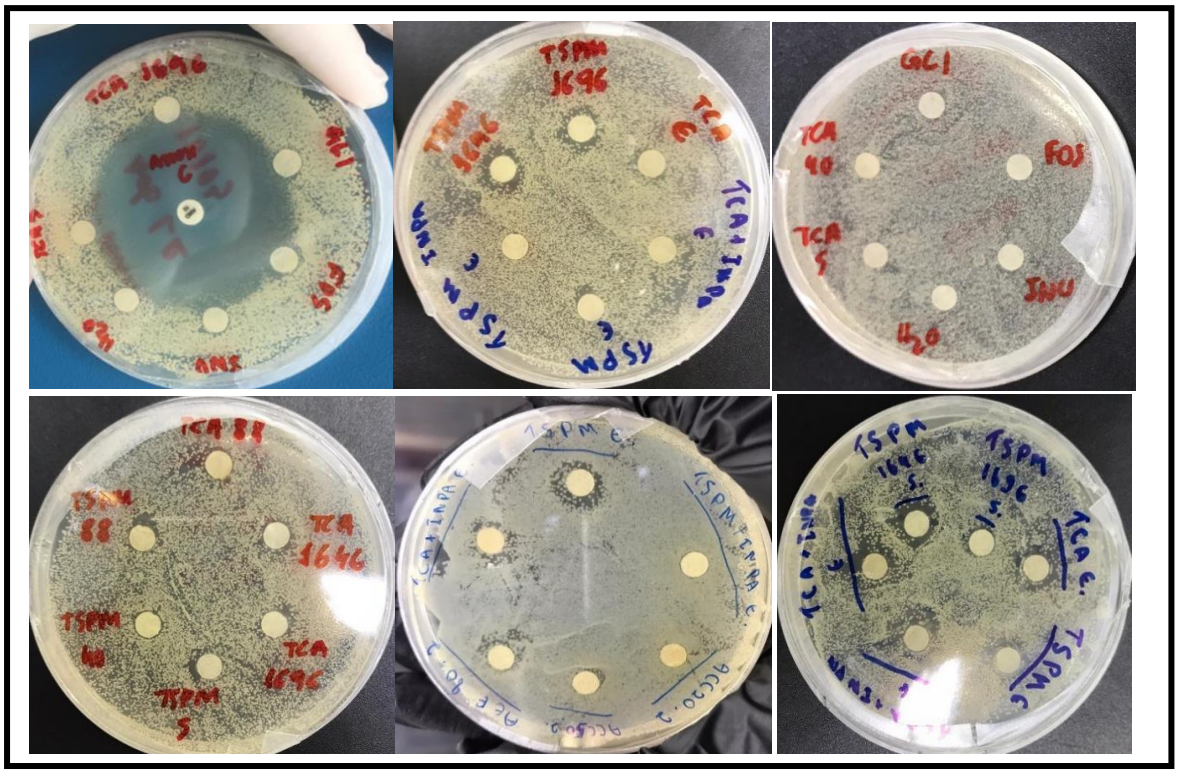
## SUPPLEMENTARY MATERIAL FROM CHAPTER III

**Figure 3.1S** Photos of antibiogram plates to illustrate the halo of inhibition of the positive control (amoxicillin), in the first photo, the absence of a halo in some samples, in the second photo, and the presence of some halos, in other samples, in the other photos.




Fonte: da autora (2021)

# CONSIDERAÇÕES FINAIS

É importante conhecer as características químicas dos extratos para responder por que ocorre a atividade biológica probiótica e antimicrobiana. Por isso, será necessário explorar atividades secretoras em estudos futuros, bem como avaliar as melhores condições e combinações desses extratos.

Uma otimização/concentração dos metabólitos presentes nos extratos aquosos de SSF-CSC por *P. lecomtei* CC40 e *Coriolopsis* sp. INPA1646 e/ou dos sobrenadantes do crescimento de *L. acidophilus* e *B. lactis* em meio com extrato de SSF-CSC fermentado por Tyromyces INPA1696 pode resultar em um produto com ação contra *S. entérica* Typhi (ATCC 14028).

A composição química dos coprodutos analisados é favorável para a alimentação animal, mas é necessário assegurar a eficiência da destoxificação, realizando testes toxicológicos *in vivo* em modelos animais. Além disso, as identidades dos fungos utilizados devem ser confirmadas molecularmente.