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Douglas Evangelista Braga

Micotoxinas e aminas bioativas em salames: métodos analíticos, ocorrência, alteração ao longo do armazenamento e bioacessibilidade

Belo Horizonte

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Douglas Evangelista Braga

MICOTOXINAS E AMINAS BIOATIVAS EM SALAMES: métodos analíticos, ocorrência, alteração ao longo do armazenamento e bioacessibilidade

Tese apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal de Minas Gerais como requisito para obtenção do título de Doutor em Ciência Animal.

Orientadora: Profa. Dra. Maria Beatriz de Abreu Glória
Coorientadores: Prof. Dr. José Eduardo Gonçalves e Prof. Dr. Bruno Martins Dala Paula

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Dr.(a). Maria Beatriz de Abreu Gloria - Orientador(a)

Dr.(a). Renan Campos Chisté

Dr.(a). Débora Cristina Sampaio de Assis

Dr.(a). Bruno Martins Dala Paula

Dr.(a). Flávia Beatriz Custódio

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RESUMO

O salame é um produto cárneo fermentado e desidratado nutritivo, com vida útil longa e valor agregado valorizado no Brasil e para exportação. Entretanto, este produto pode ter contaminantes, dentre eles, as aminas bioativas e micotoxinas. Neste contexto, este trabalho teve como objetivo, determinar a ocorrência destes contaminantes em diferentes tipos de salames e investigar fatores que afetam a sua formação e acúmulo, assim como a sua bioacessibilidade. Para tal, métodos analíticos foram otimizados e validados. Um método usando 'dilute and shot' e LC-MS/MS foi desenvolvido, demonstrando ser adequado para a análise simultânea de aflatoxinas (B1, B2, G1 e G2) e ocratoxina A em salames. Amostras de diferentes tipos de salame industrial (n=27) foram analisadas e nenhuma das micotoxinas foi detectada, demonstrando a inocuidade destes alimentos á saúde humana. Um método por HPLC-FL foi otimizado e validado para a análise de 10 aminas bioativas em salame. Amostras de diversos tipos de salame foram analisadas (n=148). Todos os tipos de salame apresentaram teores totais elevados de aminas, sendo que apenas o tipo Serrano apresentou menor teor ($p<0,05$). Todas as aminas foram detectadas nos salames tipo Hamburguês, Italiano e Salaminho; o tipo Mini-Hamburguês apresentou apenas quatro (feniletilamina, espermidina, espermina e tiramina), Italiano gourmet sete, Serrano oito e Milano nove. Espermidina, espermina e tiramina estavam presentes em todos os tipos de salame. Os teores médiosde tiramina e histamina foram de 1154,3 e 1301,0 mg/kg, respectivamente, e, portanto, seriam capazes de causar efeitos adversos à saúde humana. Quatro tipos de salames (Hamburguês, Mini-Hamburguês, Italiano gourmet e Salaminho) foram caracterizados físico-química e microbiologicamente. As dez aminas foram analisadas nestas amostras de 45 a 90 dias de armazenamento refrigerado (5,5 ± 1,5 °C). Foi observada uma alteração nos teores de aminas ao longo do armazenamento, inclusive das aminas que podem causar efeito adverso a saúde. Amostras de salame Milano, com diferente perfil e teores de aminas foram submetidos a estudo de bioacessibilidade pelo método de digestão gastrointestinal simulada *in vitro*, seguindo o protocolo INFOGEST. O índice de bioacessibilidade foi calculado pelo percentual de aminas livres no salame digerido, em relação ao não digerido. Uma das amostras apresentou índice de bioacessibilidade de 27% para histamina, 26% de tiramina e ~16% de aminas totais. A segunda apresentou índice de bioacessibilidade de 62% para cadaverina, 63% para histamina e ~39% de aminas totais. Estes resultados sugerem a diferente bioacessibilidade em função da composição do produto, o que merece mais estudos.

Palavras chave: aminas biogênicas, tiramina, histamina, cromatografia, salame, micotoxina, bioacessibilidade

ABSTRACT

Dry ferdmented sausage is a nutritious fermented and dehydrated meat product, with longer shelf life and added value appreciated in Brazil and for exportation. However, this product may contain contaminants, including bioactive amines and mycotoxins. In this context, this work aimed to determine the occurrence of these contaminants in different types of dry fermented sausages and to investigate factors that affect their formation and accumulation, as well as their bioaccessibility. For that, analytical methods were optimized and validated. A method using 'dilute and shot' and LC-MS/MS was developed, being suitable for the simultaneous analysis of aflatoxins (B1, B2, G1 and G2) and ochratoxin A in sausage. Different types of industrial sausages (n=27) were analyzed and none of the mycotoxins were detected, demonstrating the harmlessness of these foods to human health regarding these mycotoxins. An HPLC-FL method was optimized and validated for the analysis of 10 bioactive amines in sausage. Samples of different types were analyzed (n=148). All types showed high total levels of amines, with only Serrano showing lower levels ($p<0.05$). All amines were detected in Hamburgues, Italiano and Salaminho; the Mini-Hamburgues type had only four (phenylethylamine, spermidine, spermine and tyramine), Italian gourmet seven, Serrano eight and Milano nine. Spermidine, spermine and tyramine were present in all types of sausages. The mean levels of tyramine and histamine were 1154.3 and 1301.0 mg/kg, respectively, and therefore, they would be capable of causing adverse effects to human health. In four types of salami (Hamburgues, Mini-hamburgues, Italian gourmet and Salaminho), the samples were characterized physico-chemically and microbiologically. The ten amines were analyzed in these samples from 45 to 90 days of refrigerated storage at 5.5 ± 1.5 °C. A change in amine levels was observed during storage, including amines that can cause adverse health effects. Samples of Milano, with different profiles and levels of amines, were submitted to bioaccessibility study by the *in vitro* simulated gastrointestinal digestion, following the INFOGEST protocol. The bioaccessibility index was calculated by the percentage of free amines in digested salami in relation to undigested salami. One of the samples showed a bioaccessibility index of 27% for histamine, 26% for tyramine, and ~16% for total amines. The second had a bioaccessibility index of 62% for cadaverine, 63% for histamine and ~39% for total amines. These results suggest different bioaccessibility depending on the composition of the product, which deserves further studies.

Keywords: biogenic amines, tyramine, histamine, chromatography, salami, mycotoxin, bioaccessibility

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INTRODUÇÃO

Os produtos cárneos fermentados representam a forma mais antiga conhecida de conservar a carne para obter um produto microbiologicamente estável com características sensoriais particulares que podem ser mantidos por vários meses (García-Díez et al., 2021). Os salames fazem parte destes produtos cárneos fermentados com maior vida de prateleira e valor agregado valorizados no Brasil e para exportação. Entretanto, este produto pode ter contaminantes, dentre eles, as micotoxinas e as aminas bioativas.

As micotoxinas são uma preocupação global de saúde pública já que algumas são carcinogênicas como as Aflatoxinas e Ocratoxina A (Comi e Iacumin, 2013; Montanha et al., 2017). Especiarias, produtos agrícolas, carnes e laticínios são as principais fontes de micotoxinas (Darwish et al., 2014). Podem estar presentes em salames por estarem presentes em carnes, principalmente a suína (principal matéria prima dos salames) caso a ração utilizada esteja contaminada com as micotoxinas, as quais irão depositar na carne (tecido muscular), ou pela ação de fungos micotoxigênicos (Montanha et al., 2017). Os efeitos negativos da exposição a micotoxinas podem ser mitigados através do uso de conhecimento e práticas de saúde pública, como o processamento adequado do produto e armazenamento (Atherstone et al., 2014).

As aminas bioativas são compostos nitrogenados de baixo peso molecular que são formadas principalmente pela descarboxilação de aminoácidos (Ozogul et al., 2019). O perfil e os teores de aminas no produto final são relevantes, pois podem refletir condições higiênico-sanitárias inadequadas durante o processamento.

O acúmulo de certas aminas em alimentos e no salame pode causar efeitos adversos à saúde humana (EFSA, 2011). As aminas biogênicas podem causar efeitos adversos e intoxicações, dependendo da resposta individual de cada indivíduo e da presença simultânea de cofatores como outras aminas, consumo de álcool ou fármacos que podem atuar sinergicamente ou como antagonistas (Torre et al., 2020).

As aminas mais frequentemente encontradas na carne fresca são as poliaminas espermina e espermidina (Jairath et al., 2015; Custódio et al., 2018). Ao longo do processamento de salames, ocorre uma diminuição nos teores das poliaminas e a formação de aminas biogênicas (Ruiz-Capillas et al., 2019).

No entanto, os efeitos tóxicos e biológicos de uma amina podem estar relacionados também à sua bioacessibilidade, isto é, à quantidade que é liberada da matriz alimentar e é considerado disponível para absorção através da parede intestinal (Minekus et al., 2014; Barba et al., 2017; Castaldo et al., 2020). Poucos estudos sobre a bioacessibilidade de aminas bioativas estão

disponíveis, tendo sido investigado apenas nas matrizes cogumelo e chocolate (Reis et al., 2020; Dala-Paula et al., 2021a).

Pelo fato das micotoxinas e aminas bioativas poderem causar efeitos adversos à saúde além de prejuízos econômicos é importante a pesquisa destes contaminantes em alimentos cárneos fermentados como os salames.

REVISÃO DA LITERATURA

1. Salames

1.1. Histórico do salame

Os produtos cárneos fermentados representam a forma mais antiga conhecida de conservar a carne para obter um produto microbiologicamente estável com características sensoriais particulares que podem ser mantidos por vários meses (García-Díez et al., 2021). As primeiras noções sobre linguiças e salames fermentados são de 3000 a.C. na China e na área do Mediterrâneo de 2.000 anos atrás (Petäjä-Kanninen et al., 2007).

A história diz que os salames originaram na Itália e começaram a ser fabricados por camponeses. A origem da palavra salame vem do latin "salumen", que descreve uma mistura de carnes salgadas. O salame tem uma longa história, mesmo pré-datando a Roma antiga. Ao longo destes séculos variações regionais, bem como técnicas de preparo criaram vários tipos de embutidos (Sinha, 2007; Toldrá et al., 2014; Portal São Francisco, 2023)

A receita moderna de salame provavelmente originou na Itália no início do século XVIII. Foi adotado posteriormente em outros países, principalmente na Europa Central, com procedimentos de fabricação adaptados. Naquela época, era amplamente consumido pelos ricos, pois a carne, em geral, era muito cara. Em todo o mundo, há muitas versões diferente de salames que possuem seus próprios perfis culturais e de sabor. Além disso, cada salame tem seu próprio tipo de tempero e quantidade de sal, tornando cada sabor e textura únicos. Esta vasta gama de embutidos fermentados, sobretudo o salame, revela o seu carácter exclusivo. Por exemplo, devido à imigração para a América do Norte, os colonos europeus trouxeram muitas tradições, incluindo carnes fermentadas como o pepperoni, tipos semelhantes de embutidos encontrados no Oriente Médio, onde são usadas várias carnes, como a bovina, ovina e caprina (Sinha, 2007; Toldrá et al., 2014).

No Brasil, a produção de salames se concentra na região sul, representando cerca de 3% dos produtos cárneos industrializados no país. Os tipos mais conhecidos são italiano, milano, hamburguês, friolano, calabrês, alemão, salaminho e napolitano e se assemelham aos salames produzidos no sul da Europa (Terra, 2005).

Segundo o Observatório da Complexidade Econômica (OEC), o Brasil em 2020 exportou US$ 116 milhões em embutidos cárneos, tornando-se o 12º maior exportador de embutidos do mundo. No mesmo ano, o salame era o 152º produto mais exportado do Brasil. Os principais

destinos das exportações brasileiras são: Angola (US$ 21,5 milhões), Venezuela (US$ 17,3 milhões), Japão (US$ 10,7 milhões), Cuba (US$ 6,44 milhões) e Gana (US$ 6,14 milhões). Somente em 2019, a demanda por salames brasileiros aumentou, com variação de 8,49% em relação a 2018.

1.2. Definição

A Instrução Normativa nº 22 de 2000 do Ministério da Agricultura Pecuária e Abastecimento - MAPA estabelece o Regulamento Técnico de Identidade e Qualidade - RTIQ (BRASIL, 2000) para carnes e produtos cárneos. Neste RTIQ o salame é definido como produto cárneo industrializado obtido de carne suína ou carne suína e bovina, adicionado de ingredientes, embutido, curado, fermentado, maturado, defumado ou não, e dessecado. São considerados ingredientes obrigatórios a carne suína, o toucinho, o sal e o nitrito e/ou nitrato de potássio e/ou de sódio. Ainda, podem ser utilizados na elaboração destes produtos a carne bovina, leite em pó, açúcares, vinho, condimentos, aromas, especiarias e substâncias glaceantes, sendo estes considerados opcionais. Além destes ingredientes podem ser usados coadjuvantes de tecnologia como culturas iniciadoras (Brasil, 2000). Na figura 1 pode-se observar ao fluxograma da fabricação do salame.

Os ingredientes obrigatórios para a fabricação do salame incluem a carne suína numa quantidade não inferior a 60%, exceto para o salame tipo Hamburguês que o mínimo de carne suína é de 50%. A presença de "mofos" característicos, é consequência natural do seu processo tecnológico de fabricação. Entretanto, as culturas starters ou iniciadoras iniciam a fermentação do salame (BRASIL, 2000).

O nitrito e nitrato são amplamente usados na cura dos salames, devido à sua ação antimicrobiana, que previne o crescimento do *Clostridium botulinum* e a produção de esporos que podem, eventualmente, estar presente na carne. Além deste fato, o nitrito ainda possui outras funções como participar do processo de cor e flavor característicos e ação antioxidante (Di Nunzio et al., 2022). Como estes aditivos estão associados à formação de N-nitrosaminas, a adição de ácido ascórbico ou eritórbico é recomendada com o objetivo de prevenir a formação destas substâncias. O ácido ascórbico, por exemplo, é um agente inibidor de nitrosação, reagindo rapidamente com o radical nitroso formando ácido deidroascórbico e óxido nítrico, o qual não é um agente nitrosante (Dutra, 2007; Brasil 2023).

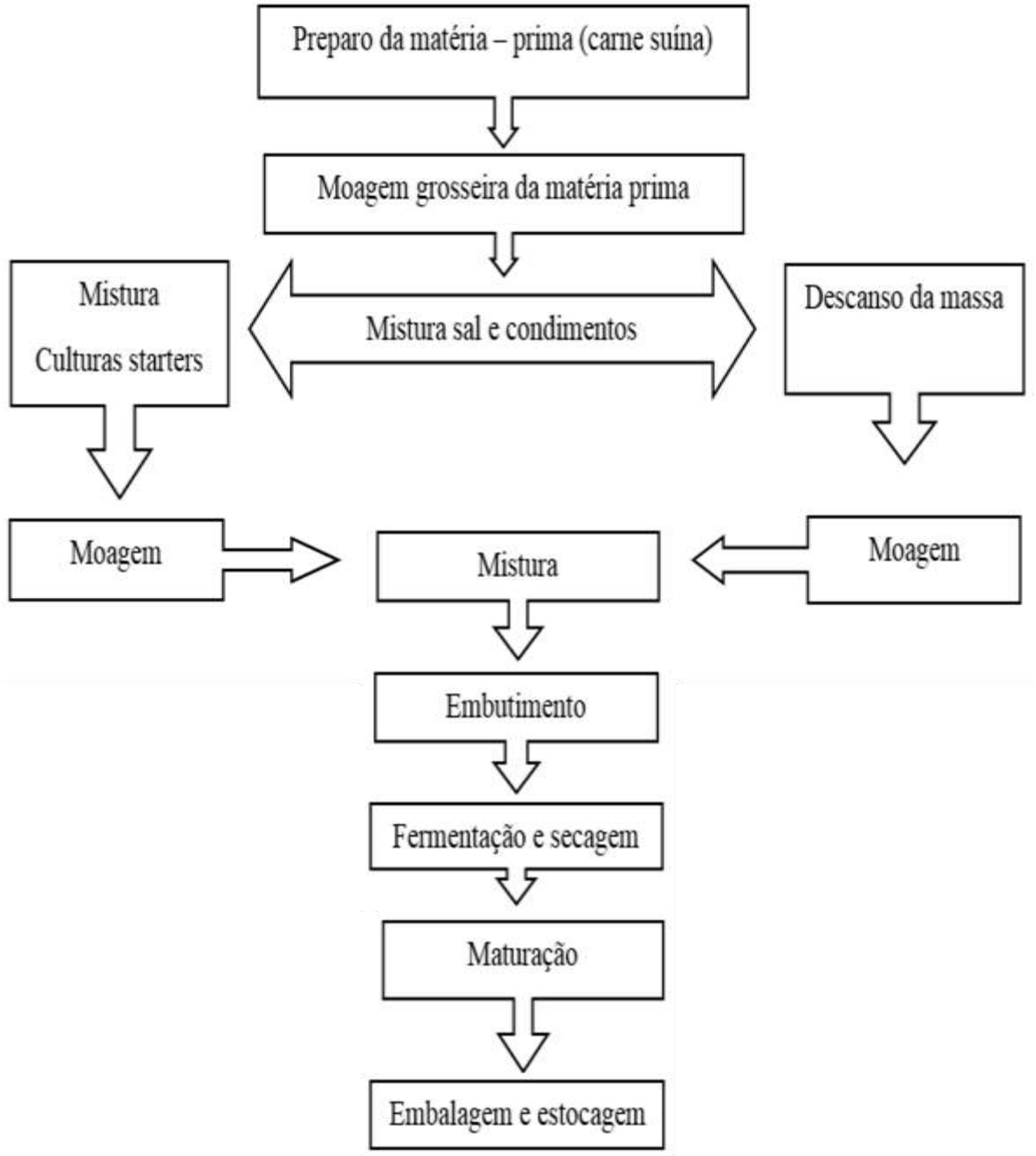

Figura 1. Fluxograma do processamento de salame. Fonte: Adaptado de Terra (2005).

1.3. Classificação

Segundo o Regulamento Técnico de Identidade e Qualidade de Salames (Brasil, 2000), o salame é designado ou denominado como Salame, seguido ou não das expressões que caracterizem sua origem ou processo de obtenção (Brasil, 2000). São exemplos os Salame Tipo Italiano, Salame Tipo Milano, Salame Tipo Hamburguês, Salame Tipo Friolano, Salame Tipo Calabrês, Salame Tipo Alemão, Salaminho.

As características físico-químicas dos diferentes tipos de salame estão de acordo com a designação do produto em seus respectivos regulamentos técnicos. De modo geral são parâmetros legislados com os respectivos máximos ou mínimos, conforme descrito na Tabela 1.

As características microbiológicas dos diferentes tipos de salames seguem os parâmetros contidos na legislação brasileira para produtos cárneos maturados, dessecados (presuntos crus, copas, salames, linguiças dessecadas, charque, "jerked beef") (Brasil 2022) conforme descrito na tabela 2.

Tabela 1. Características físico-químicas de salames segundo o Regulamento Técnico de Identidade e Qualidade de Salames

Características Físico-químicas (g/100g)	**Tipos de Salames**			
	Hamburguês	**Salaminho**	**Italiano**	**Milano**
Lipídeos Totais (máx.)	35,00	35,00	32,00	35,00
Proteínas (mín.)	23,00	20,00	24,00	23,00
Umidade (máx.)	40,00	40,00	35,00	35,00
Carboidratos	1,5	1,50	1,50	4,00
Atividade de Água (Aw) (máx.)	0,920	0,920	0,900	0,900

Fonte: Adaptado de (Brasil, 2000).

Tabela 2. Características microbiológicas para salames segundo a legislação brasileira.

Microrganismo	**n**	**C**	**M**	**M**
Salmonela / 25g	5	0	Ausente	-
Estafilococus coagulase positiva / 25g	5	1	10^2	10^3
Aeróbios mesófilos / g	5	3	10^5	10^6
Escherichia coli / g	5	2	Menor que 10	10^2

n: número de unidades a serem colhidas aleatoriamente. **m**: limite mínimo, em um plano de três classes, separa o lote aceitável do produto ou lote com qualidade intermediária aceitável; **M:** limite máximo, em um plano de duas classes, separa o produto aceitável do inaceitável. **c**: número máximo aceitável de unidades de amostras com contagens entre os limites de **m** e **M**.
Fonte: Adaptado de Brasil (2022).

1.4. Processamento

1.4.1. Fermentação em salame: objetivo, relevância, e fatores que afetam

A fermentação é considerada a etapa mais importante do processamento de salames, pois é durante esta etapa que ocorre a produção de ácido lático e, como consequência, a redução do pH

do produto (Petäjä-Kanninen et al., 2007; Piscane et al., 2015). Durante a fermentação ocorrem reações químicas e enzimáticas que degradam proteínas e lipídios (Leroy et al., 2004). O pH é reduzido de aproximadamente 5,7 para valores mais baixos, que podem variar de 4,6 a 5,5 em função da temperatura da fermentação. A fermentação dura de 12 horas a vários dias, dependendo do tipo de salame. Salames fermentados em temperaturas elevadas (≥37 °C) atingem rapidamente valores mais baixos de pH (Petäjä-Kanninen et al., 2007), por outro lado, em temperaturas mais baixas (próximas de 24 °C) geram um pH mais alto em uma velocidade mais lenta, resultando em um pH de 4,6 a 5,0 (Petäjä-Kanninen et al., 2007).

O objetivo da fermentação é causar uma redução do pH inicial da mistura cárnea, devido à multiplicação das bactérias ácido láticas presentes na carne ou pela adição de culturas iniciadoras. Estas bactérias têm a capacidade de produzir ácido lático pela fermentação dos carboidratos, acidificando o meio e promovendo reações que irão gerar o sabor e a textura característicos do produto cárneo fermentado, no caso os salames (Santa et al., 2014)

O uso de culturas iniciadoras permite a homogeneização da produção e evita possíveis defeitos. Além disso, elas melhoram a segurança de produtos cárneos fermentados pela produção de vários compostos, como ácido lático, ácido acético, ácido propiônico, ácido benzóico, peróxido de hidrogênio ou proteínas bactericidas (ou seja, bacteriocinas) (García-Díez et al., 2021). Assim, as culturas iniciadoras passam a ser a microbiota predominante, direcionando a fermentação e competindo com os microrganismos indesejáveis, diminuindo os riscos higiênicos e de fabricação por deficiências de origem microbiana. Existem muitos gêneros microbianos usados como culturas iniciadoras para produtos cárneos fermentados. Os microrganismos mais utilizados pertencem ao grupo das bactérias láticas e cocos Gram-positivos catalase-positivos, representadas principalmente por *Staphylococcus* spp. e *Kocuria* spp. (Laranjo et al., 2017; García-Díez et al., 2021). Entretanto, outras culturas iniciadoras pertencentes a *Lactococcus* spp., *Leuconostoc* spp., *Enterococcocus* spp. e *Pediococcus* spp. também são usadas (Franciosa et al., 2018; García-Díez et al., 2021). Além disso, leveduras e fungos filamentosos, que conferem características sensoriais específicas, também são adicionados como culturas iniciadoras. As leveduras e os fungos são representados principalmente por *Debaromyces* spp. e *Aspergillus* spp., respectivamente. Os fungos, por serem aeróbicos, são utilizados como microbiota de superfície com o objetivo de melhorar as características sensoriais e externas do salame (García-Díez et al., 2021).

Bioquimicamente, a fermentação é definida como uma reação em que adenosina trifosfato (ATP) é produzido em nível de substrato pela fosforilação, ou seja, o oxigênio não é necessário e, consequentemente nenhum carbono é perdido como CO_2. Os microrganismos utilizam ATP para manter suas funções celulares e para crescimento (Petäjä-Kanninen et al., 2007). A parte principal

do uso do ATP está relacionado ao gradiente de pH (força motriz de prótons) através da membrana celular, ou seja, o fluxo de prótons para a célula para a síntese de ATP (Petäjä-Kanninen et al., 2007).

Durante a produção de salame, há a adição de açúcares, cuja quantidade constitui um dos parâmetros de maior influência sobre o pH final, mas não na velocidade do abaixamento de pH. Geralmente o pH sofre redução em função do ácido produzido pelas bactérias láticas e durante a maturação sofre um aumento devido a produção de compostos amoníacos e aminas, período subsequente, que dura de algumas semanas a vários meses (Petäjä-Kanninen et al., 2007).

A fermentação pode ser realizada pela ação da microbiota nativa, fermentação natural, composta principalmente por bactérias lácticas e por espécies da família *Micrococcaceae* (Silva et al., 2017). Na fermentação natural, os microrganismos são provenientes da matéria-prima utilizada, dos ingredientes, bem como do ambiente de processamento (Lebert et al., 2007; Silva et al., 2017). Porém, a elaboração de salames por fermentação natural, muito comum em pequenas produções e denominados salames coloniais, pode causar grande variação na qualidade final dos produtos, em relação à suas características sensoriais, aspectos higiênicos e de segurança (Silva et al., 2017). Dessa forma, a utilização de culturas *starter* e de condições controladas na fabricação de salames, permite o controle do processo fermentativo resultando em um produto padronizado (Leroy et al., 2004; Silva et al., 2017).

1.4.1.1. Fermentação no processo de produção dos salames

O período de maturação do salame é de aproximadamente 30 dias, quando são gerados o aroma e o sabor característicos, com pH em torno de 5,3, e atividade de água (Aw) inferior a 0,920. A redução da Aw caracteriza o final do processo de maturação. Sua fabricação se dá em duas fases: na primeira, há a fermentação com a ocorrência simultânea de acidificação e formação da cor; a segunda fase é a maturação e consiste na desidratação como decorrência da fermentação (Terra et al., 2004; Vedovatto et al., 2019). A estabilidade microbiológica do salame se deve principalmente ao seu baixo pH (abaixo de 5,3) e atividade de água inferior a 0,920 (Terra et al., 2004; Piscane et al., 2015; Vedovatto et al., 2019).

A temperatura interna do salame na etapa de fermentação deve estar entre 20 e 30 °C devido à seleção que acontece de bactérias láticas passando a provocar a acidificação do produto. Essa temperatura também influencia no crescimento de *Micrococcus* (*Staphylococcus* coagulase positiva), que são responsáveis pela redução do nitrato a nitrito, contribuindo ainda para o desenvolvimento de sabor e aroma além de inibir o crescimento de microrganismos patogênicos

(Vedovatto et al., 2019). A redução do nitrato a nitrito acelera a formação e estabilização da cor de curado, além da formação de ânion que pode limitar a oxidação de lipídeos que podem resultar em sabores indesejáveis (Talon et al., 1999).

1.4.1.2. Microrganismos presentes em salame naturalmente fermentado

Durante a fabricação de salames, a fermentação pode ser natural, ou pode ser direcionada pela adição de culturas iniciadoras para a fermentação do produto. Durante a fermentação natural (espontânea), a contagem de bactérias ácido láticas normalmente aumenta nos primeiros dias de fermentação (Comi et al., 2005), e permanece constante durante a maturação em cerca de 7 a 9 log UFC/g (Cocolin et al., 2001a; Comi et al., 2005; Lebert et al., 2007). Rantsiou et al. (2005b) observaram que, em uma fermentação natural de salames italianos do nordeste da Itália, a população de bactérias ácido láticas aumentou mais devagar quando comparados com salames italianos fermentados com cultura iniciadora. Em salame Milano da Itália (Rebecchi et al., 1998) e em salames franceses (Chevallier et al., 2006) fabricados sob baixa temperatura, a população inicial de bactérias ácido láticas era geralmente baixa, compreendida entre 3,2 e 5,3 log UFC /g. Em salames tradicionais italianos, franceses e gregos, os cocos Gram positivos catalase positivos, constituíram a segunda maior população da microbiota no final da maturação (Lebert et al., 2007). Na maioria dos salames, estes cocos estavam em uma população de 6-8 log UFC /g, que geralmente era inferior a população de bactérias ácido láticas (Lebert et al., 2007). Os microrganismos encontrados em salames tradicionais, aqueles que são produzidos por fermentação natural, estão descritos na tabela 3.

O crescimento das bactérias ácido láticas está frequentemente correlacionado com a diminuição do pH na primeira fase de fermentação (Cocolin et al., 2001a; 2001b). Devido à boa adaptação das bactérias ácido láticas ao ambiente e sua taxa de crescimento mais rápida durante a fermentação e maturação de embutidos como os salames, elas tornam-se a microbiota dominante.

Entre as bactérias ácido láticas, *Lactobacillus sakei* é dominante no salame tradicional, seguido por *L. curvatus* e *L. plantarum* (Ritter et al., 2020). Reckem et al. (2019) e Cullere et al. (2020) relataram *Staphylococcus xylosus* como a espécie microbiana mais comum no salame tipo italiano.

1.4.1.3. Microrganismos presentes em salame produzido com culturas iniciadoras

As culturas iniciadoras estão sendo cada vez mais usadas na fabricação de salame, pois tem como vantagens a segurança, padronização da fermentação e do produto final, a inibição do crescimento e a atividade microrganismos indesejáveis (Rantsiou et al., 2005; Cullere et al., 2020).

Tabela 3. Microrganismos encontrados em salames tradicionais (fermentação natural)

Produto	Microrganismos	Referências
Bacterias ácido láticas		
Salame tradicional grego	*Lactobacillus plantarum; L. curvatus;* *L. pentosus; L.sakei;* *L. paracasei; L. buchneri;* *L. pentosus; L. rhamnosus*	Papamanoli et al., 2003; Drosinos et al., 2005; Greco et al., 2005; Aymerich et al., 2006
Salame tradicional italiano	*Lactobacillus. sakei; L. curvatus* *L. plantarum; L. paraplantarum;* *L. carnis*	Comi et al., 2005; Greco et al., 2005; Rantsiou et al., 2005a; Ursoet al., 2006
Cocos Gram positivos catalase positivos		
Salame tradicional grego	*Staphylococcus. saprophyticus; S. xylosus;* *S. simulans; S. capitis;* *S. haemolyticus; S. sciuri;* *S. caprae*	Papamanoli et al., 2003; Drosinos et al., 2005
Salame tradicional italiano	*Staphylococcus. xylosus; S. saprophyticus* *S. lentus; S. warneri* *S. succinus*	Rebecchiet al., 1998; Cocolin et al., 2001b; Iacumin et al., 2006
Salame tradicional francês	*Staphylococcus. equorum; S. succinus* *S. saprophyticus; S. warneri*	Morot-Bizot et al., 2006

1.4.1.3. Microrganismos presentes em salame produzido com culturas iniciadoras

As culturas iniciadoras estão sendo cada vez mais usadas na fabricação de salame, pois tem como vantagens a segurança, padronização da fermentação e do produto final, a inibição do crescimento e a atividade microrganismos indesejáveis (Rantsiou et al., 2005; Cullere et al., 2020).

As bactérias utilizadas como culturas iniciadoras na produção de salames são geralmente heterofermentativas facultativas, as quais produzem ácido lático a partir de hexoses e promovem a redução do pH a valores de aproximadamente 5,0 durante os primeiros dias da fermentação. Entre as bactérias ácido láticas frequentemente empregadas como culturas iniciadoras em produtos cárneos destacam-se *Lactobacillus spp. e Pediococcus spp* como pode-se observar na tabela 4.

Tabela 4. Bactérias ácido láticas utilizadas como culturas starters em produtos cárneos

Microrganismo	**Produto**	**Referência**
Lactobacillus casei	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Salame, linguiça fermentada	Rubio et al., 2014
Lactobacillus curvatus	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Linguiças fermentadas do sul da Europa	Reckem et al., 2019
	Salame de avestruz tipo italiano	Cullere et al., 2020
Lactobacillus pentosus	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
Lactobacillus plantarum	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Salame, linguiça fermentada	Rubio et al., 2014
	Linguiças fermentadas do sul da Europa	Reckem et al., 2019
	Salame de avestruz tipo italiano	Cullere et al., 2020
	Salame tipo italiano	Ritteret al., 2020
Lactobacillus sakei	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Salame italiano	Polka et al., 2015; Montanari et al., 2016
Pediococcus acidilactici	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Salame italiano	Polka et al., 2015
Pediococcus pentosaceus	Salame tradicional, linguiça seca fermentada	Työppönen et al., 2003
	Salame italiano	Polka et al., 2015
Staphylococcus xylosus	Salame tipo italiano	Reckem et al., 2019; Cullere et al., 2020

As bactérias ácido láticas degradam principalmente os carboidratos como a glicose a piruvato e, por sua vez, em lactato ou em ácido lático, sendo responsáveis pela acidificação do produto (Cullere et al., 2020).

Braga et al. (2013) observaram a influência da adição de cultura starter composta por *Lactobacillus plantarum* e *Staphylococcus xylosus* em salames tipo italiano inoculados com

Escherichia coli. Observaram que nos salames do grupo teste (com cultura starter) ocorreu decréscimo nas contagens de *E. coli* ao longo do processo de fermentação/maturação, do primeiro ao 19º dia após a fabricação, com concentração de *E. coli* menor que 1,0 x 10^2 UFC/g, que, segundo a legislação brasileira atual (Brasil, 2019), seria aceitável.

Os membros da família *Micrococcaceae*, como *Staphylococcus carnosus, Staphylococcus xylosus, Micrococcus varians, Micrococcus candidus e Micrococcus aquatilis* são também adicionados como culturas starter além dos *Lactobacillus spp*. Os *Staphylococcus spp*. são microrganismos aeróbicos facultativos, ou seja, em condições anaeróbicas produzem ácido lático a partir de glicose e, em condições aeróbicas, ácido acético e CO_2. A aplicação destes microrganismos visa a produção das enzimas nitrato-redutases e catalases, que estabilizam a cor e previnem a rancidez oxidativa, através do consumo de peróxido de hidrogênio produzido pelas culturas heterofermentadoras (Hammes et al., 2003; Terra, 2005). *Staphylococcus spp*. e *Micrococcus spp*. apresentam atividade lipolítica e proteolítica que contribuem significativamente para o desenvolvimento do aroma característico de salame (Demeyer et al., 2000; Demeyer et al., 2002). A maioria dos microrganismos utilizados na fabricação dos salames como culturas starters para a fermentação e suas características pode ser observado na tabela 5.

Além das bactérias ácido láticas também podem ocorrer associações entre estas e leveduras na fabricação dos salames para um melhor controle da fermentação (Flores et al., 2015; Wang et al., 2018). As leveduras são utilizadas pela ação proteolítica e lipolítica, sendo seus principais efeitos a promoção de aumento de pH, a utilização de lactato, e a formação de aromas desejáveis no produto (Flores et al., 2015). As principais leveduras utilizadas na produção dos salames e produtos cárneos fermentados podem ser observadas nas tabelas 5 e 6.

Os bolores e leveduras usados normalmente em salames são microrganismos dos gêneros *Penicillium e Debaromyces*, como o *Penicillium nalgiovense e Debaromyces hansenii* (Demeyer et al., 2002) além dos gêneros *Candida, Yarrowia, Rhodotorula* e *Saccharomyces*, como *Candida zeylanoides, Candida parapsilosis, Yarrowia lipolytica, Rhodotorula mucilaginosa* e *Saccharomyces cerevisiae* (Mi et al., 2021). Estes microrganismos são utilizados em salames para proteger contra a influência de O_2, estabilizando a cor, bem como pela ação das proteases e lipases de alguns destes microrganismos, que metabolizam o ácido lático em compostos relacionados positivamente com o aroma/sabor do produto (Corral et al., 2018). Os mofos, além da redução da tensão de O_2, também impedem a exposição da superfície do produto, à luz assim retardando o desenvolvimento da rancidez (Terra, 2005; Feiner, 2006). *Saccharomyces cerevisiae* isolada em salame exibiu atividade proteolítica sobre a proteína da carne e favoreceu a formação de aminoácidos livres que podem ser transformados em aldeídos, cetonas, aminas e ácidos (Mi et al.,

2021). As leveduras são capazes de inibir a oxidação lipídica, favorecendo a formação de ésteres (Mi et al., 2021). Além disso, as leveduras contribuem para a inibição do crescimento de microrganismos patogênicos, como o mofo ocratoxigênico e o aflatoxigênico *Aspergillus parasiticus* (Peromingo et al., 2019). Estas micotoxinas constituem uma preocupação, tendo em vista que são carcinogênicas causando danos à saúde humana, animal e produtos agrícolas além dos prejuízos econômicos (Abd-Elghany e Sallam, 2015).

Tabela 5. Microrganismo atividade metabólica e benefícios na fabricação de salames

Microrganismo	**Gênero e espécie**	**Atividade metabólica**	**Benefícios**
Bactérias ácido láticas	*Pediococcus acidilactici* *Pediococcus pentosaceus* *Pediococcus cerevisae* *Lactobacillus plantarum*	Formação de ácido lático	Inibição de bactérias patogênicas e deteriorantes
	Lactobacillus sakei *Lactobacillus curvatus* *Lactobacillus pentosus* *Lactobacillus acidophilus* *Lactobacillus casei*	Formação de ácido lático	Aceleração da formação da cor e tempo de secagem
	Micrococcus varians *Micrococcus lutens* *Micrococcus roseus*	Redução de nitrato em nitrito e consumo de O_2	Formação e estabilização da cor e retarda a oxidação
	Staphylococcus xylosus *Staphylococcus carnosus* *Streptomyces griséus*	Destruição de peróxidos	Formação de aroma, Remoção de nitrato em Excesso
Leveduras	*Debaryomyces hansenii* *Cândida famata*	Consumo de oxigênio; Lipólise	Retardamento da oxidação; Desenvolvimento de aroma.
Fungos	*Penicilium nalgiovense* *Penicillium crysogenum*	Consumo de O_2; Destruição de peróxidos; Oxidação de lactato; Proteólise, Lipólise	Estabilidade da cor; Retarda oxidação; Desenvolvimento de aroma

Fonte: Adaptado de Abunyewa et al., 2000; Demeyer et al., 2000; Demeyer et al., 2002; Flores et al., 2015; Mi et al 2021.

Samelis et al. (1994) observaram uma predominância de *Debaryomyces* spp. em salames tradicionais, e menores contagens de *Candida* spp. e *Pichia* spp., e menor ocorrência de

Cryptococcus spp., *Torulopsis* spp. e *Trichosporon* spp. Metaxopoulos et al. (1996) isolaram 100 cepas de leveduras durante a fermentação e estágios de maturação do salame grego seco e *Debaryomyces* spp. foi o mais abundante representando 66% do total. *Candida famata, C. zeylanoides, C. guilliermondii, C. parapsilosis* e *C. kruisii* foram outras espécies identificadas. Devido à prevalência de *D. hansenii*, esses autores sugeriram seu uso potencial como cultura starter (Selgas et al., 2007). Wolter et al. (2000) identificaram os mesmos gêneros em salames na África do Sul além de *Sporobolomyces* spp., *Torulaspora* spp. e *Saccharomyces spp* (Selgas et al., 2007).

Tabela 6. Principais leveduras encontradas em produtos cárneos e salame

Microrganismo	Produto	Referência
Candida haemulonii *Candida vinaria* *Candida zeylanoides*	Salame italiano tradicional, salame tipo italiano	Flores et al., 2015 Wang et al., 2018
Cryptococcus albidus *Cryptococcus hungericus* *Cryptococcus laurentii*	carne crua *in natura*, linguiça seca fermentada, salame italiano tradicional	Flores et al., 2015
Debaryomyces hansenii *Lipomyces tetrasporus* *Rhodotorula minuta* *Rhodotorula mucilaginosa* *Trichosporon beigelli* *Yarrowia lipolytica*	carne crua *in natura*, linguiça seca fermentada, linguiça seca fermentada espanhola, salame comercial, salame italiano tradicional	Abunyewa et al., 2000 Flores et al., 2015

2. Micotoxinas em produtos cárneos (salames)

2.1. Micotoxinas

As micotoxinas podem estar presentes em salames pela ação de fungos micotoxigênicos. Além disto, as micotoxinas podem estar presentes na carne, principalmente a suína (predominante em salame) caso a ração utilizada esteja contaminada com as micotoxinas, as quais irão depositar na carne (tecido muscular) (Montanha et al., 2017). As micotoxinas são uma preocupação global de saúde pública, sendo as especiarias, produtos agrícolas, carnes e laticínios as principais fontes de micotoxinas (Darwish et al., 2014). Entre as micotoxinas mais importantes estão as aflatoxinas e a ocratoxina, que são um problema mundial, causando milhões de dólares em perdas anualmente, além de estarem relacionadas a danos à saúde humana, saúde animal e produtos agrícolas (Abd-Elghany e Sallam, 2015). O impacto econômico e social dessas micotoxinas inclui perdas e mortes

além de doenças de animais, aumento com custos médicos e veterinários, redução na produtividade animal, perda de meios de subsistência, perdas econômicas para os agricultores através das perdas e desperdícios de alimentos e rações devido à contaminação (Atherstone et al., 2014; Montanha et al., 2017). Os efeitos negativos da exposição a micotoxinas pode ser mitigada através do uso de conhecimento e práticas de saúde pública, como o processamento adequado do produto e armazenamento (Atherstone et al., 2014). O problema das micotoxinas é de suma importância, pois algumas são carcinogênicas, imunológicas, alergênicas (Comi e Iacumin, 2013; Montanha et al., 2017), toxigênica, nefrotóxica, hepatotóxica, imunossupressora, mutagênica (Martín et al., 2006), estrogênica e teratogênicas, dependendo do nível de exposição (Iqbal et al., 2014; Montanha et al., 2017).

As micotoxinas contribuem significativamente para as perdas de alimentos em países em desenvolvimento (Udomkun et al., 2017b). De acordo com o Departamento de Alimentação e Agricultura Organização Mundial da Saúde (FAO), perde-se cerca de um terço de todos os alimentos produzidos, totalizando cerca de 1,3 bilhão de toneladas métricas por ano (FAO, 2011; Montanha et al., 2017). Estima-se também que aproximadamente cinco bilhões de pessoas em todo o mundo estão expostas a micotoxinas, como aflatoxinas (Atherstone et al., 2014). No entanto, formas para avaliar o impacto econômico global da presença de micotoxinas em alimentos têm sido extremamente difíceis de serem desenvolvidas (Montanha et al., 2017).

As micotoxinas são metabólitos secundários de fungos filamentosos tóxicos para humanos e animais (Eckhardt et al., 2014; Iqbal et al., 2014; Montanha et al., 2017; Vipotnik et al., 2017). A maioria dessas toxinas tem pesos moleculares relativamente baixos, entre 300-700 Da (Eckhardt et al., 2014; Iqbal et al., 2014; Singh & Metha, 2020) e geralmente são termicamente estáveis (Iqbal et al., 2014; Turner et al., 2015) demonstrando níveis elevados de bioacumulação (Turner et al., 2015; Montanha et al., 2017). Mais de 400 tipos de micotoxinas foram identificados, no entanto, apenas cerca de 10 a 15 são consideradas de interesse para a saúde pública (Turner et al., 2015), como aflatoxinas (AFs), ocratoxinas (OTA), desoxinivalenol, alcalóides do ergot, fumonisinas (FBs), patulina (PAT), zearalenona (ZEN) e tricotecenos (T-2 e HT-2). Estes são os mais proeminentes devido à sua alta incidência em alimentos (Iqbal et al., 2014; Udomkun et al., 2017b). Ocratoxina e aflatoxinas podem ser produzidas por fungos associados a produtos cárneos curados secos (Abd-Elghany e Sallam, 2015; Montanha et al., 2017). A estrutura química de algumas micotoxinas podem ser observadas na figura 2.

Devido à sua ocorrência comum em produtos cárneos fermentados desidratados, bem como sua toxicidade, as micotoxinas de maior interesse são a ocratoxina A e aflatoxinas. A ocratoxina A é classificado pela Agência Internacional para Pesquisa em Câncer (IARC) como pertencente

ao Grupo 2B possível carcinógeno humano e foi considerada o contaminante dominante em produtos cárneos (IARC, 1993; Roncada et al., 2020). A aflatoxina B1 pertence ao Grupo 1 da IARC de carcinógenos comprovados e ocorre produtos cárneos em concentrações mais baixas (IARC, 2002; Leši´c et al., 2021).

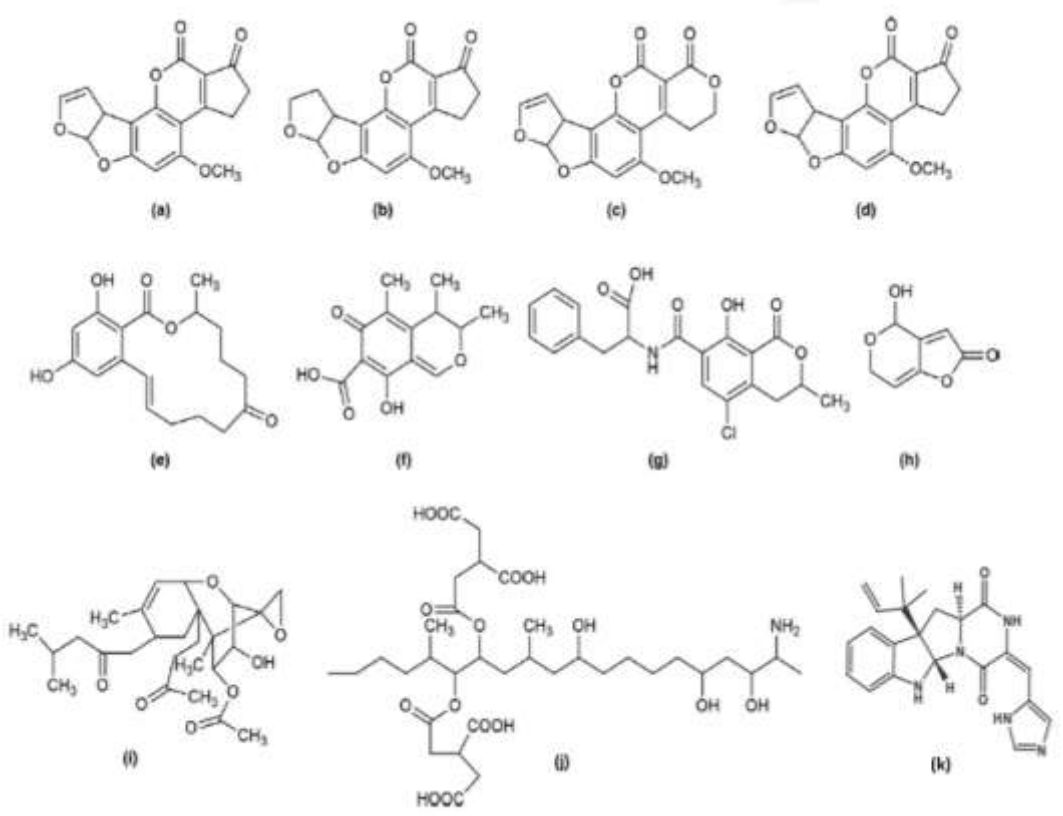

Figura 2. Estrutura química de algumas micotoxinas: (a) Aflatoxina B1, (b) Aflatoxina B2, (c) Aflatoxina G1, (d) Aflatoxina G2, (e) Zearalenona, (f) Citrinina, (g) Ocratoxina, (h) Patulina, (i) Tricotecenos, (j) Fumonisina B1, e (k) Roquefortina C.
Adaptado de Singh & Metha, 2020.

2.1.1. Aflatoxinas

As aflatoxinas são derivadas de difuranocumarinas produzidas por várias cepas de *Aspergillus parasiticus* e *Aspergillus flavus* (Atherstone et al., 2014; Bernáldez et al., 2014; Iqbal et al., 2014; Udomkun et al., 2017a; Montanha et al 2017). Podem também ser produzidas por *Aspergillus parvisclerotegenus* e *Aspergillus minisclerotigenes* (Udomkun et al., 2017a), e menos comumente *Aspergillus nomius* (Udomkun et al., 2017a). Normalmente, o *Aspergillus flavus*

produz apenas aflatoxina tipo B, enquanto outras espécies de Aspergillus produzem tipos B e G (Atherstone et al., 2014; Eckhardt et al., 2014; Udomkun et al., 2017a).

As quatro mais importantes aflatoxinas encontradas são Aflatoxina B1 (AFB1), Aflatoxina B2 (AFB2), Aflatoxina G1 (AFG1) e Aflatoxina G2 (AFG2) que podem ser diferenciadas de acordo com sua fluorescência sob luz UV (verde ou azul) e movimento cromatográfico comparativo durante cromatografia em camada fina (Singh & Metha, 2020).

A aflatoxina B1 (AFB1) é carcinogênica para humanos, pertence ao Grupo 1 segundo o IARC e pode exercer efeitos imunossupressores. Há também incertezas relacionadas aos efeitos causados por exposição a longo prazo a baixos níveis de micotoxinas únicas e/ou múltiplas e a relação com doenças crônicas (Singh & Metha, 2020). Na tabela 7 pode-se observar os efeitos tóxicos relacionados a aflatoxina B1.

Tabela 7. Efeitos tóxicos relacionados às micotoxinas

Micotoxina	Efeitos tóxicos	Mecanismo de ação
Aflatoxina B1	Hepatotoxico Genotóxico Carcinogênico Imunomodulador	Bioativação pelo citocromo P450 Peroxidação lipídica Mutagênico
Ocratoxina A	Nefrotóxico Genotóxico Imunomodulador	Efeitos na sítese protéica Inibição da produção de ATP

Adaptado de Bailly et al. (2009).

2.1.2. Ocratoxina

Além das aflatoxinas, a ocratoxina A (OTA) é uma importante micotoxina encontrada em carnes curadas secas (Volkel et al., 2011). É um policetídeo derivado acoplado a β-fenilalanina da família das dihidrocumarinas. A OTA é opticamente ativa e é caracterizada espectralmente por métodos de detecção UV-visível, fluorescência, IR e NMR e MS (Singh & Metha, 2020).

A OTA foi primeiramente isolada em 1965 de uma cultura de *Aspergillus ochraceus* (Vipotnik et al., 2017). A OTA é produzida por cepas do gênero *Aspergillus e Penicillium* (Volkel et al., 2011; Comi e Iacumin, 2013; Domijan et al., 2015; Vipotnik et al., 2017). Considerando o gênero Aspergillus, *A. carbonarius, A. westerdijkiae, A. steynii, A. niger* e *A. ochraceus* são os principais responsáveis pela ocorrência de ocratoxina A em produtos alimentícios juntamente com duas espécies de Penicillium, *P. verrucosum* e *P. nordicum* (Schmidt-Reydt et al., 2011). OTA pode ser transferida de alimentos contaminados com micotoxinas dados a suínos que são

posteriormente consumidos por humanos, ou por contaminação direta por mofo nas camadas externas da carcaça (Iacumin et al., 2011). Consequentemente, os fungos toxigênicos têm atraído atenção da indústria alimentícia (Comi e Iacumin, 2013; Montanha et al., 2017).

A OTA foi identificada em salames italianos (Iacumin et al., 2009) e AFB1 e AFB2 em salame do Egito (Aziz e Youssef, 1991). A OTA também foi encontrada em linguiças de sangue, e salsichas de fígado na Alemanha (Gareis e Scheuer, 2000), presunto de Parma em Dinamarca (Sorensen et al., 2010) e presunto ibérico curado a seco na Espanha (Rodríguez et al., 2015). Em um estudo no Cairo, hambúrgueres e salsichas tiveram as maiores contagens de fungos em comparação com os produtos frescos e carnes enlatadas. Esta contaminação pode estar relacionada com a adição de especiarias contaminadas com AFB1 (Darwish et al., 2014)

A ocratoxina A afeta enzimas envolvidas no metabolismo da fenilalanina, e enzimas que utilizam a fenilalanina como substrato, como por exemplo a fenilalanina hidroxilase que catalisa a hidroxilação de fenilalanina em tirosina. Sua ação tóxica acontece sobre o sistema renal. A nefrotoxicidade manifesta-se desde a alteração do volume dos rins, alterações na osmolaridade e volume da urina, alterações na função renal, até o desenvolvimento de adenomas e tumores renais (Pereira et al., 2011). Na tabela 7 pode-se observar os efeitos tóxicos relacionados a ocratoxina A.

3. Aminas bioativas em salames

3.1 Aminas bioativas

As aminas bioativas são compostos nitrogenados básicos nos quais um, dois ou três dos átomos de hidrogênio da amônia são substituídos por grupos alquil ou aril (Ozogul et al., 2019). A formação desses compostos ocorre por meio de quatro reações enzimáticas: descarboxilação, transaminação, aminação por redução e degradação de certos compostos amino precursores e por hidrólise em tratamentos térmicos drásticos (Ozogul et al., 2019).

A descarboxilação de aminoácidos é a forma mais comum de síntese de aminas biogênicas em alimentos, e ocorre pelo metabolismo dos micro-organismos via descarboxilação de aminoácidos livres ou pela descarboxilação térmica de aminoácidos livres. A descarboxilação de aminoácidos ocorre pela remoção do grupo α-carboxílico para gerar a amina correspondente. Os nomes das aminas se originam dos nomes de seus aminoácidos precursores; por exemplo histidina histamina, tirosina tiramina, arginina agmatina, fenilalanina feniletilamina e triptofano triptamina (Ozogul et al., 2019).

As aminas mais frequentemente encontradas na carne fresca são as poliaminas espermina e espermidina (Jairath et al., 2015; Custódio et al., 2018). Entretanto, durante armazenamento, processamento, contaminação microbiana (condições higiênico-sanitárias inadequadas), ou armazenamento em temperaturas elevadas, pode ocorrer diminuição das poliaminas e formação de aminas biogênicas (Custódio et al., 2018; Schirone et al., 2022).

O perfil e os teores de aminas no produto final são relevantes, pois podem refletir condições higiênico-sanitárias inadequadas durante o processamento. A cadaverina e a histamina estão entre as principais aminas bioativas utilizadas para prever as condições sanitárias do processamento de alimentos e o frescor das matérias-primas, pois estão relacionadas ao aumento de bactérias deteriorantes (Molognoni et al., 2018). Por exemplo, um acúmulo de cadaverina e putrescina pode indicar contaminação por enterobactérias. Altos níveis dessas aminas podem prejudicar as características sensoriais do produto, com sabor pútrido, proveniente do material em decomposição, prejudicando a aceitabilidade do produto (Molognoni et al., 2018).

3.2.1 Efeitos adversos das aminas bioativas

O acúmulo de certas aminas em alimentos e no salame, como tiramina e histamina, pode causar efeitos adversos à saúde humana (EFSA, 2011). Na verdade, essas são as aminas mais associadas a surtos de intoxicação por aminas. As aminas biogênicas podem causar efeitos adversos e intoxicações, dependendo da resposta individual de cada indivíduo e da presença simultânea de cofatores como outras aminas, consumo de álcool ou fármacos que podem atuar sinergicamente ou como antagonistas (Torre et al., 2020). Outros fatores que interferem na toxicidade das aminas estão relacionados à eficiência dos mecanismos de desintoxicação do organismo de cada indivíduo, à presença de substâncias potencializadoras nos alimentos (como a presença de outras aminas), ao uso de drogas inibidoras da amino-oxidase, ao álcool e à presença de ambos doenças gastrointestinais inflamatórias e neoplásicas (gastrite, síndrome do intestino irritável, doença de Crohn, neoplasias colorretais, úlceras de estômago e cólon) devido à menor atividade de oxidases no intestino em comparação com indivíduos saudáveis (EFSA, 2011).

A histamina pode causar alguns distúrbios de neurotransmissão devido a sua ação como um falso neurotransmissor liberando adrenalina e noradrenalina. A liberação destas estimula neurônios sensoriais e motores que podem atuar na excitação da musculatura lisa do útero, intestino, trato respiratório, controle da secreção gástrica, além de atuar como vasodilatador (Jairath et al., 2015; Eisenberg et al., 2016; Dala-Paula et al., 2021). De acordo com a EFSA (2011), um indivíduo sensível pode apresentar efeitos adversos ao consumir 50 mg de histamina

por refeição. No entanto, a ausência de histamina nos alimentos é necessária para indivíduos sensíveis (intolerância a histamina).

Tiramina, triptamina e feniletilamina têm ação vasoconstritora e têm sido descritas como iniciadoras de enxaqueca induzida por dieta. Além disto, a tiramina pode causar crises hipertensivas em alguns pacientes em uso de medicamentos inibidores da monoaminoxidase (IMAO). Os efeitos fisiológicos da tiramina incluem: vasoconstrição periférica, aumento do débito cardíaco, aumento da respiração, elevação da glicemia e liberação de norepinefrina; crise hipertensiva (Jairath et al., 2015). De acordo com a EFSA (2011), a dose tóxica da tiramina é de 600 mg por refeição; no entanto, para indivíduos em uso de inibidores da IMAO clássicos, essa dose agora é de 6 mg por refeição, e para indivíduos que tomam medicamentos IMAO de terceira geração, a dose agora é de 50 mg de tiramina por pessoa por refeição.

Além disso, aminas biogênicas e outros compostos nitrogenados podem favorecer a formação de N-nitrosaminas, descritas como potencialmente carcinogênicas (Bover-Cid et al., 2001; Komprda et al., 2004; De Mey et al., 2014). No entanto, para evitar a formação dessas substâncias, deve-se utilizar ácido ascórbico ou eritrórbico em produtos cárneos (De Mey et al., 2014).

3.2.2. Características das aminas bioativas

A histamina é uma monoamina heterocíclica, seu aminoácido precursor é a histidina (Wójcik et al., 2021). As legislações brasileira (Brasil, 1997) e europeia (European Comission, 2013) recomendam teores de histamina abaixo de 100 mg/kg para produtos à base de pescados. Já Food and Drug Administration (FDA) recomenda níveis de histamina em peixes e produtos de pescado, até 50 mg/kg (Ly et al., 2020). Não existem padrões específicos para o teor de histamina em outros produtos alimentícios (Wójcik et al., 2021). A histamina é uma amina biogênica associada à neurotransmissão e vasodilatação no sistema nervoso central e cardiovascular. A alta ingestão pode causar hipotensão, palpitações, náuseas, eritema, enxaqueca, dor abdominal, dificuldade respiratória, taquicardia e problemas cardíacos (Silva et al., 2020; Dala-Paula et al., 2021).

A reação à toxicidade da histamina é referida como “envenenamento escombroide” ou “envenenamento por histamina” (Ruiz-Capillas et al., 2019; Wójcik et al., 2021). Os sintomas mais comuns de alta ingestão de histamina são formigamento na língua, erupção cutânea, vômito, diarréia, sensação de queimação, dor de cabeça e tontura, náusea, diminuição da pressão arterial, vasodilatação, sangramento intracraniano, palpitações ou dificuldades respiratórias. O efeito de intoxicação do organismo aparece após algumas horas do consumo de histamina, embora possa se

manifestar até vários dias após o consumo (EFSA, 2011; Ruiz-Capillas et al., 2019, Ekici et al., 2020; Wójcik et al., 2021). Histamina, também é chamada de amina psicoativa, que pode ser percebida pelo sistema nervoso como falsos neurotransmissores, com efeito na pressão arterial (Wójcik et al., 2021). Os efeitos tóxicos da histamina são potencializados na presença das diaminas alifáticas putrescina e cadaverina e também por monoaminas (EFSA, 2011; Wójcik et al., 2021). O metabolismo da histamina compartilha metabólitos (piridoxal 5-fosfato e S-adenosina metionina), atividades enzimáticas (diamina oxidase, monoamina oxidase, aldeído desidrogenase e transglutaminase) e transportadores de membrana com as vias metabólicas de outras aminas biogênicas (a diamina putrescina, as poliaminas espermidina e espermina, dopamina e serotonina) (Moya-García et al., 2021).

A tiramina é uma monoamina aromática, tendo a tirosina como aminoácido precursor (Wójcik et al., 2021). Os queijos estão entre os produtos ricos em tiramina; motivo pelo qual a intoxicação por tiramina é referida como "efeito do queijo" ou "reação do queijo" (Wójcik et al., 2021). Entretanto, teores elevados de tiramina também têm sido relatados em salames e outros produtos fermentados. No entanto, se consumida em quantidades excessivas, esta amina pode causar efeitos adversos à saúde humana, como hipertensão, vasoconstrição, dilatação da pupila, hemorragias cerebrais e enxaquecas (Doeun et al., 2017; Silva et al., 2020; Dala-Paula et al., 2021).

Os primeiros sintomas de intoxicação ocorrem entre 1 e 2 horas após o consumo. Os sintomas de envenenamento incluem enxaqueca, queixas gastrointestinais, taquicardia, aumento de açúcar no sangue, ejeção de norepinefrina e hipertensão (EFSA, 2011; Ruiz-Capillas et al., 2019; Wójcik et al., 2021). Além disso, estudos em ratos mostraram que a tiramina na presença de nitrito de sódio se transforma na substância mutagênica 3-diazotiramina, que induz o câncer (EFSA, 2011; Wójcik et al., 2021). Os efeitos tóxicos da tiramina são potencializados na presença das monoaminas alifáticas (EFSA, 2011; Wójcik et al., 2021).

A tiramina mostrou propriedades anti-inflamatórias inibindo produção do fator de necrose tumoral alfa, interleucina 6, prostaglandina E2 e atividade da ciclooxigenase-2 (Dala-Paula et al., 2021). A tiramina foi identificada na urina de pacientes com síndrome metabólica e correlacionaram-se inversamente com múltiplos biomarcadores de inflamação e fatores de risco cardiometabólicos, contribuindo para novos insights sobre sua patogênese (Patel et al., 2019; Dala-Paula et al., 2021).

As mono aminoxidases catalisam a desaminação oxidativa da tiramina em 4-hidroxifenilacetaldeído, que é então convertido em ácido 4-hidroxifenilacético (Rafeh et al., 2019). A monoamina oxidase A (MAO-A) provavelmente é responsável pela baixa biodisponibilidade

intestinal da tiramina, e a tiramina sistêmica é eliminada via MAO hepática (Gillman, 2018; Rafeh et al., 2019).

A triptamina é uma monoamina heterocíclica com seu aminoácido precursor triptofano que pode causar aumento da pressão arterial. A triptamina e a feniletilamina causam sintomas de enxaqueca, resultante da constrição dos vasos sanguíneos (Ekici et al., 2020; Ruiz-Capillas et al., 2019; Wójcik et al., 2021).

A feniletilamina é uma monoamina aromática cujo aminoácido precursor é a fenilalanina (Wójcik et al., 2021). Essa amina atua como neuromodulador e neurotransmissor, participa da liberação de norepinefrina e aumenta a pressão arterial, além de estimular quase todos os processos em que o sistema límbico está envolvido, sendo, portanto, responsável pelas emoções. É um antidepressivo e facilitador das relações interpessoais (agindo de forma semelhante às anfetaminas (Luengo et al., 2020). Além disso, protege os neurônios do estresse oxidativo causado pelos radicais livres (Luengo et al., 2020). Baixas concentrações desta amina têm sido associadas a déficit de atenção, estados depressivos e doença de Parkinson (Luengo et al., 2020).

A putrescina é uma diamina alifática que possui a ornitina como aminoácido precursor (Wójcik et al., 2021). A relevância da putrescina reside no fato de esta diamina ser a precursora obrigatória das poliaminas. É uma amina vasoativa com efeitos adversos agudos como aumento do débito cardíaco, trismo e paresia de extremidades (Ladero et al., 2010; del Rio et al., 2019). A putrescina pode ser formada em altas concentrações em produtos cárneos, como os embutidos fermentados (até 1550 mg/kg) (EFSA, 2011; del Rio et al., 2019). A putrescina é metabolizada nas poliaminas, espermidina e espermina. Por outro lado, a diamina oxidase desamina diaminas para produzir aldeídos, amônia e peróxido de hidrogênio (Barua et al., 2019).

A cadaverina é uma diamina alifática cujo aminoácido precursor é a lisina (Wójcik et al., 2021). O odor fétido do tecido em decomposição do cadáver é devido à cadaverina (Rajpal et al., 2020). A cadaverina pode formar-se em altas concentrações em queijos (até 3170 mg/kg), peixes e derivados (até 1690 mg/kg), embutidos fermentados (até 1250 mg/kg) e molhos de peixe (até 1150 mg/kg) (EFSA, 2011; del Rio et al., 2019). Em alimentos em putrefação, condições higiênico sanitárias inadequadas contaminadas com enterobactérias. Assim como a putrescina e a cadaverina podem reagir com os nitritos e produzir nitrosaminas, a cadaverina em relação aos nitritos forma a nitrosopiperidina, um composto carcinogênico (Ladero et al., 2010; Ladero et al., 2017).

A serotonina é uma monoamina heterocíclica cujo aminoácido precursor é o hidroxitriptofano (Wójcik et al., 2021). A serotonina, também conhecida como 5-hidroxitriptamina (5-HT), é um importante neurotransmissor chave no sistema nervoso central, estando relacionada a funções cerebrais como regulação do humor, sono e apetite, além de fator

de crescimento. Em mamíferos, a serotonina é sintetizada a partir do aminoácido essencial triptofano (Jones et al., 2020).

A agmatina é uma poliamina alifática cujo aminoácido precursor é a arginina (Wójcik et al., 2021; Dala-Paula et al., 2021). Essa poliamina atua como um neuromodulador que imita as propriedades funcionais de outros neurotransmissores (Barua et al., 2019). A enzima arginina descarboxilase (ADC) sintetiza agmatina pela descarboxilação da L-arginina. Por outro lado, estudos sugeriram que no mamífero sem ADC, a descarboxilação da L-arginina é catalisada pela enzima ornitina descarboxilase (Barua et al., 2019). A sua atividade *in vivo* é mínima em mamíferos, indicando que deve ser fornecido através da dieta ou microbiota intestinal, que são fontes importantes deste poliamina (Akasaka & Fujiwara, 2020; Dala-Paula et al., 2021). A agmatina exógena também é relatada como um agente antiproliferativo que reduz a biossíntese e aumenta a degradação do crescimento celular e poliaminas como putrescina, espermidina e espermina em diferentes linhagens de células cancerígenas (Barua et al., 2019). A agmatina pode ser metabolizada em putrescina pela enzima agmatinase ou oxidada em γ-guanidinobutiraldeído pela diamina oxidase (Barua et al., 2019). A agmatina tem efeitos positivos nas complicações associadas ao sistema nervoso, como a depressão, por meio de seus efeitos do tipo antidepressivo em camundongos semelhantes à fluoxetina (Olescowicz et al., 2018); ansiedade, através da atividade ansiolítica (Gawali et al., 2017); e convulsão por exercer efeitos neuromoduladores e neuroprotetores (Bahremand et al., 2018). Agmatina tem mostrado resultados positivos no tratamento da obesidade, melhorando a oxidação ácida da gordura (Nissim et al., 2014).

A espermina é uma poliamina alifática cujos aminoácidos precursores são a arginina e a ornitina (Wójcik et al., 2021). A biossíntese da espermina é realizada através de duas vias principais (Hasan et al., 2019). A arginase hidrolisa o aminoácido arginina em ornitina como parte do ciclo da ureia. A descarboxilação da arginina dá origem à putrescina, que é então convertida em espermidina e espermina pelas inserções sequenciais de grupos aminopropil. A espermidina/espermina-N1-acetiltransferase (SSAT) catalisa a transferência de um grupo acetil para a espermina ou para a espermidina, que então se tornam substratos para a acetilpoliamina oxidase. A espermina pode ser oxidada pela espermina oxidase e, assim, reconvertida em espermidina (Proietti et al., 2020).

A espermina tem importantes propriedades antienvelhecimento, devido a muitos papéis relacionados à capacidade de induzir autofagia citoprotetora, importante para a manutenção da homeostase celular e vida útil (Madeo et al., 2018). Também são essenciais para a síntese e estabilização de DNA, RNA e proteína (Munoz-Esparzã et al., 2019; Dala-Paula et al., 2021)

A espermidina é uma poliamina alifática que possui arginina e ornitina como aminoácidos precursores e que na presença de enzimas consegue transformar putrescina em espermidina (Wójcik et al., 2021). É um composto catiônico trivalente encontrado em células eucarióticas, particularmente abundante em espermatozoides. Ao interagir com ácidos nucleicos, proteínas, ATP e outros poliânions por meio de ligação eletrostática, a espermidina é indispensável na divisão e proliferação celular, mantendo a homeostase do DNA genômico, regulando a transcrição e tradução gênica e modulando a autofagia, apoptose, estresse oxidativo, angiogênese e comunicação celular (Fan et al., 2020).

3.2.3. Aminas bioativas em salames

As aminas mais frequentemente encontradas na carne fresca são as poliaminas espermina e espermidina (Jairath et al., 2015; Custódio et al., 2018). Ao longo do processamento de salames, ocorre uma diminuição nos teores das poliaminas e a formação de aminas biogênicas (Ruiz-Capillas et al., 2019). As aminas mais estudadas nos salames fermentados são tiramina e histamina (Caccioppoli, 2002; Caccioppoli et al., 2006; Giroto et al., 2010; De Mey et al., 2014; Mologni et al., 2018). Na tabela 8 são apresentados alguns tipos de salames e as aminas bioativas mais frequentemente presentes nos mesmos.

Tabela 8. Aminas presentes em alguns tipos de salame comercializados no Brasil

Tipo de Salame	Aminas	Referência
Friolano	agmatina, cadaverina, histamina, tiramina, feniletilamina, serotonina	Caccioppoli, 2002; Mologni et al., 2018
Hamburguês	agmatina, cadaverina, tiramina, serotonina, espermidina	Caccioppoli, 2002
Italiano	agmatina, cadaverina, histamina, tiramina, feniletilamina, serotonina, espermina, espermidina, putrescina, triptamina	Caccioppoli et al., 2006; Giroto et al., 2010; De Mey et al., 2014; Roselino et al., 2020
Milano	agmatina, cadaverina, tiramina, serotonina espermidina	Caccioppoli, 2002; De Mey et al., 2014
Brianza	cadaverina, histamina, tiramina, serotonina	Caccioppoli, 2002

A seguir, na tabela 9, estão resumidas as informações sobre a concentração média de aminas bioativas em salames comerciais comercializados na Bélgica e Brasil (sem especificação de tipos de salames) e salames tipo italiano. Caccioppoli et al. (2006), em estudo com salames tipo italiano,

encontraram uma grande diversidade de aminas. Encontraram também teores elevados de tiramina – 19,02 mg/100 g, valores estes capazes de provocar intoxicação caso estes salames fossem consumidos por indivíduos em tratamento com IMAO clássicos, além de 0,55 mg/100 g de triptamina e 1,39 mg/100 g de feniletilamina. De Mey et al. (2014) encontraram aminas biogênicas em vários tipos de salames, sendo que no tipo italiano observaram um teor médio de 14,99 mg/100 g de tiramina chegando a um máximo de 20,10 mg/100 g desta amina, concentrações consideradas elevadas por EFSA (2011). Molongnoni et al. (2018) encontraram teores similares de cadaverina, histamina, putrescina, espermidina, espermina e tiramina em concentrações médias entre 2,64 e 26,12 mg/100 g em amostras de salame tipo italiano

O perfil e os teores de aminas no produto final são relevantes pois podem refletir as condições higiênico-sanitárias inadequadas durante o processamento. Cadaverina e histamina estão entre as principais aminas bioativas usadas para prever as condições sanitárias do processamento de alimentos e o frescor das matérias-primas por estar relacionadas com o aumento de bactérias deteriorantes, contribuindo para a manutenção da segurança dos alimentos (Molognoni et al., 2018). Por exemplo, um acúmulo de cadaverina e putrescina pode indicar contaminação por enterobactérias. Teores elevados destas aminas podem prejudicar as características sensoriais do produto, com odor pútrido, de material em decomposição (Molognoni et al., 2018).

Tabela 9. Teores médios de aminas bioativas em salame tipo italiano e salame comercial

Aminas (mg/kg) por tipo de salame										**Referências**
EPM	**EPD**	**AGM**	**PUT**	**CAD**	**TIM**	**HIM**	**TRM**	**FEM**	**SRT**	
Italiano										
33,5	40	-	98,8	60,8	17,8	21,09	-	3,4	-	Giroto et al., 2010
37,47	4,01	1,88	87,45	50,54	190,2	30,54	5,5	13,9	0,68	Caccioppoli et al., 2006
5,4	0,7	-	170,4	7,8	149,9	1,4	47	24	-	De Mey et al., 2014
124,53	54,72	-	161,05	79,91	129,14	87,31	45,06	116,74	-	Roselino et al., 2020
Comercial										
6,3	1,6	-	67	1,9	27,3	Nd	5	0,9	nd	De Mey et al., 2014
63,4	164,1	nd	124	151	135	Nd	-	-	-	Molognoni et al., 2018

- : não informado; nd - não detectado; EPM: espermina; EPD: espermidina; AGM: agmatina; PUT: putrescina; CAD: cadaverina; TIM: tiramina; HIM: histamina; TRM: triptamina; FEM: feniletilamina; SRT: serotonina.

Ainda, o acúmulo de certas aminas em salames, como a tiramina e a histamina, pode indicar risco potencial de causar efeitos adversos a saúde (EFSA, 2011). De fato, estas são as aminas mais associadas a surtos de intoxicação por aminas.

A tiramina é a amina mais comumente relacionada as bactérias ácido láticas em salames. Segundo Masson et al. (1996) os microrganismos produtores de tiramina, em salames pertenciam a *Carnobacterium, L. curvatus e L. plantarum*. Komprda et al. (2004) e Izquierdo et al. (2006) observaram que lactobacilos (*L. divergens, L. carnis*), carnobacteria (*Carnobacterium divergens*) e enterococos (*Enterococcus faecalis)* eram os microrganismos responsáveis pela presença de tiramina em salames. Kurt et al. (2010) observaram que o aumento da concentração de nitrito, em salames turcos, promoveu a redução dos teores de tiramina, sendo este o resultado do efeito antimicrobiano do nitrito sobre microrganismos proteolíticos capazes de levar a formação da tiramina.

Além da presença de tiramina, a cadaverina, putrescina, podem ocorrer devido às altas concentrações de aminoácidos livres expostos à degradação microbiana (Bover-Cid et al., 2001; Izquierdo et al., 2006). A putrescina e a cadaverina têm sido relacionadas à atividade da ornitina e lisina descarboxilases, respectivamente, de *Enterobacteriaceae*. A produção de putrescina requer a ação combinada de Bactérias Ácido Láticas e *Enterobacteriaceae*, pelo fato de seu precursor, a ornitina ser sintetizada a partir de uma bactéria BAL a partir de outros aminoácidos, como os *Lactobacillus* e *Pediococcus* (Bover-Cid et al., 2001; Izquierdo et al., 2006).

Os teores de feniletilamina encontrados em salames turcos foram menores quando foram adicionados sais de cura como o nitrito ao seu processamento. A feniletilamina geralmente ocorre quando um alto teor de tiramina está presente (Kurt et al., 2010).

3.2.3.1. Impacto da microbiota na formação de aminas bioativas em salames

As enzimas capazes de descarboxilar aminoácidos, oriundas de microrganismos presentes nos salames desempenham um papel fundamental na formação de aminas bioativas e, portanto, os microrganismos que as produzem são elemento de extrema importância (Figura 3). Têm-se procurado estabelecer uma relação entre a formação de aminas bioativas em carnes e produtos cárneos e a presença de vários tipos de microrganismos (Tabela 10) (Masson et al., 1996; Pisacane et al., 2015; Custódio et al., 2018).

Em geral, a atividade de descarboxilases em produtos cárneos é atribuída principalmente a microrganismos das famílias Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae e bactérias ácido lácticas. Na carne fresca, microrganismos da família Enterobacteriaceae foram identificados como os principais produtores de cadaverina (Guerrero-Lejarreta et al., 1991; Bover-Cid et al., 2001a). Por outro lado, a putrescina tem sido associada a altas contagens totais de aeróbios viáveis (Özogul et al., 2019; Ruiz-Capillas et al., 2019).

Diversos estudos sobre as bactérias ácido láticas e as aminas bioativas já foram realizados e mostram que as bactérias ácido láticas são as principais produtoras de aminas biogênicas em produtos fermentados como os salames (Özogul et al., 2019). Como exemplo, altas contagens de *Lactobacillus* sp. foram associadas à formação de altas concentrações de histamina. No entanto, a formação de aminas parece ser uma característica apenas de algumas espécies de *Lactobacillus sp.*, já que outras não as produzem (Maijala et al., 1993; Özogul et al., 2019).

A putrescina e a cadaverina têm sido relacionadas à atividade da ornitina e da lisina descarboxilase, respectivamente, de Enterobacteriaceae. A produção de putrescina requer a ação combinada de bactérias ácido láticas e Enterobacteriaceae, pois seu precursor, a ornitina, é sintetizado a partir de bactérias ácido láticas de outros aminoácidos, como *Lactobacillus* e *Pediococcus* (Bover-Cid et al., 2001; Izquierdo et al., 2006).

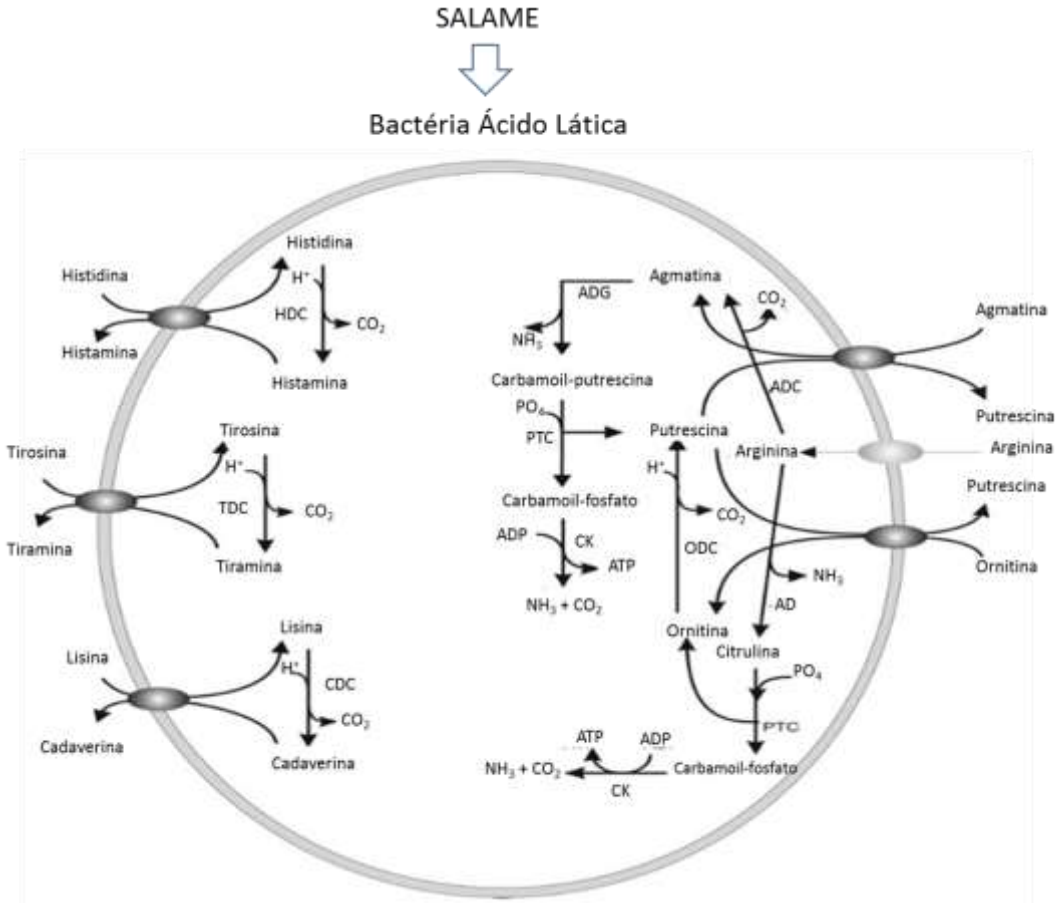

Figura 3. Vias de biossíntese de aminas bioativas.
Arginina decarboxilase (ADC), agmatina deiminase (AGD), arginina deiminase (AD), histidina decarboxilase (HDC), lisine decarboxilase (LDC), tirosine decarboxilase (TDC), ornitina decarboxilase (ODC), carbamato quinase (CK), putrescina carbamoil transferase (PTC)
Fonte: Adaptado de Linares et al., 2011.

Tabela 10. Microrganismos e aminas encontrados em carne de porco *in natura* e em salames

Produto / Microrganismo	**Amina**	**Referência**
Carne de porco *in natura*		
Carnobacterium sp. *Lactobacillus curvatus* *Lactobacilus plantarum*	Tiramina	Masson et al., 1996
Salame		
Lactobacillus sp.	Histamina	Maijala & Eerola, 1993
Carnobacterium *Lactobacillus curvatus*	Tiramina	Masson et al., 1996
Lactobacillus plantarum	Tiramina	Pisacane et al., 2015

A alta ingestão e acúmulo de algumas aminas bioativas no organismo podem gerar efeitos adversos à saúde. Os salames, por serem ricos em proteínas animais, podem ser fontes de aminas bioativas, são amplamente consumidos em todo o mundo, podem levar à intoxicação por aminas bioativas quando estas estão em grandes proporções e quando o indivíduo faz uso de inibidores da monoaminoxidase. Por esses motivos, as aminas bioativas são substâncias importantes a serem constantemente estudadas em diferentes alimentos, como os salames.

4. Bioacessibilidade

Os métodos *in vitro* que simulam processos de digestão são amplamente utilizados para estudar o comportamento gastrointestinal de alimentos ou produtos farmacêuticos. Estudos nutricionais humanos tem sido considerados o "padrão ouro" para abordar problemas relacionados à dieta mas os métodos *in vitro* têm a vantagem de serem mais rápidos, menos dispendiosos, menos trabalhosos e não possuem restrições éticas. Isto permite um número relativamente grande de amostras a serem medidas em paralelo para fins de triagem (Minekus et al., 2014).

Os métodos de digestão simulada normalmente incluem as vias oral, fases gástrica e do intestino delgado e, ocasionalmente, fermentação intestinal. Esses métodos tentam imitar a fisiologia e as condições *in vivo*, levando em conta a presença de enzimas digestivas e suas concentrações, pH, tempo de digestão, e concentrações de sal, entre outros fatores (Minekus et al., 2014).

São diversos os modelos usados para a simulação da digestão. Alguns modelos são informatizados e sofisticados como o modelo de trato gastrointestinal holandês TNO (Minekus, 1998), o modelo do Instituto Inglês Food Research (Wickham et al., 2009) ou pelo francês INRA (Méenard et al., 2014) e têm permitido a simulação de aspectos dinâmicos da digestão, como transporte de refeições digeridas, concentrações variáveis de enzimas e mudanças de pH ao longo tempo (Minekus et al., 2014).

Modelos estáticos de digestão humana têm sido usados para abordar questões científicas tão diversas como a digestibilidade e bioacessibilidade, ou seja, a quantidade de um composto que é liberado da matriz e é considerado disponível para absorção através da parede intestinal (Minekus et al., 2014; Barba et al., 2017; Castaldo et al., 2020). Diversos alimentos e mesmo produtos farmacêuticos (Kaukonen et al., 2004) têm sido avaliados com relação a algum componente natural ou contaminantes (Minekus et al., 2014). Os estudos sobre aminas bioativas são escassos, tendo sido investigado apenas nas matrizes cogumelo e chocolate (Reis et al., 2020; Dala-Paula et al., 2021a).

Já a biodisponibilidade é a porção dos nutrientes digeridos ou fitoquímicos que são absorvidos e metabolizados por vias normais e normalmente é medida por métodos *in vivo* (Barba et al., 2017). Consequentemente, informações sobre o conteúdo de nutrientes e compostos bioativos em produtos alimentícios disponíveis em bancos de dados, por exemplo do USDA, pode ser alto mesmo não sendo nocivo à saude, exigindo assim a determinação de sua biodisponibilidade ou pelo menos sua bioacessibilidade. Nesse sentido, é mais importante saber a quantidade de compostos bioativos biodisponíveis (Carbonell-Capella et al., 2014) do que apenas saber quanto está presente no produto alimentício (Barba et al., 2017). Além disso, para entender melhor as tecnologias de processamento, condições de armazenamento e/ou matriz alimentar, entre outros fatores, que podem influenciar e até melhorar a biodisponibilidade de compostos bioativos é de extrema importância (Barba et al., 2015b, Barba et al., 2015a; Barba et al., 2017).

Várias metodologias podem ser utilizadas para avaliar a bioacessibilidade e biodisponibilidade de compostos bioativos: modelos *in vitro* (digestão gastrointestinal simulada, membranas artificiais, células Caco-2, membranas celulares, etc.); modelos *ex vivo* (órgãos gastrointestinais em condições de laboratório); modelos *in situ* (perfusão intestinal em animais); e modelos *in vivo* (estudos em animais e humanos) (Carbonell-Capella et al., 2014; Minekus et al., 2014; Barba et al., 2017). Dentre essas metodologias, a digestão *in vitro*, por exemplo, acoplada a experimentos de cultura de células Caco-2, pode simular a captação de compostos bioativos no intestino delgado, sendo a mais comumente utilizada (Barba et al., 2017).

O protocolo INFOGEST (Dupont et al., 2011) para análises de bioacessibilidade *in vitro* é um método de digestão estática que avalia os desfechos resultantes da digestão de alimentos analisando os produtos da digestão e avaliando a liberação do analito (micronutriente, compostos não nutrientes, contaminantes etc.) da matriz alimentar. Usa proporções constantes de refeição para fluidos digestivos e um pH constante para cada etapa da digestão. Isso torna o método simples de execução, mas possui a desvantagem de não simular a cinética real da fisiologia digestiva. Usando este método, as amostras de alimentos são submetidas à digestão sequencial (oral, gástrica

e intestinal), enquanto parâmetros como eletrólitos, enzimas, bile, diluição, pH e tempo de digestão são baseados em dados fisiológicos disponíveis na literatura (Minekus et al., 2014; Brodkorb et al., 2019).

O protocolo INFOGESTE é composto por três fases, oral, gástrica e intestinal como é descrito na figura 4. Para isto são utilizados fluidos de digestão simulados. O Fluido Salivar Simulado (SSF), Fluido Gástrico Simulado (SGF) e Fluido Intestinal Simulado (SIF) são constituídos por soluções estoque de eletrólitos, enzimas, $CaCl_2$ e água (Minekus et al., 2014).

A mastigação de alimentos sólidos é simulada pela trituração de uma amostra. A solução estoque de eletrólito SSF é adicionada para criar uma consistência pastosa fina. Se necessário, a solução estoque de eletrólito pode também ser adicionado durante a moagem. Uma proporção final de alimentos para SSF de 50:50 (p/v) é o alvo. Para alimentos líquidos, uma fase oral pode ser incluída, especialmente se a amostra contiver amido. Neste caso a proporção final de 50:50 (v/v) é o alvo. A α-amilase salivar humana é adicionada, seguido de $CaCl_2$ e a quantidade necessária de água para diluir a solução estoque de SSF. O tempo recomendado de contato com a enzima é de 2 minutos a 37 °C, o que requer pré-aquecimento de todos os reagentes para 37 °C (Minekus et al., 2014).

Alimentos líquidos podem ser expostos à fase oral (opcional) ou diretamente para a fase gástrica. Cinco partes de alimento líquido, é misturado com 4 partes de solução de eletrólito estoque SGF para obter uma proporção final de alimento para SGF de 50:50 (v/v). A pepsina suína é adicionada para atingir 2000 U/mL na mistura, seguido de $CaCl_2$ para atingir 0,075 mM. na digestão final. HCl é adicionado para reduzir o pH para 3,0. Finalmente, a quantidade necessária de água é adicionada à mistura para diluir a solução estoque de SGF. O tempo recomendado de a digestão é de 2 horas a 37 °C (Minekus et al., 2014).

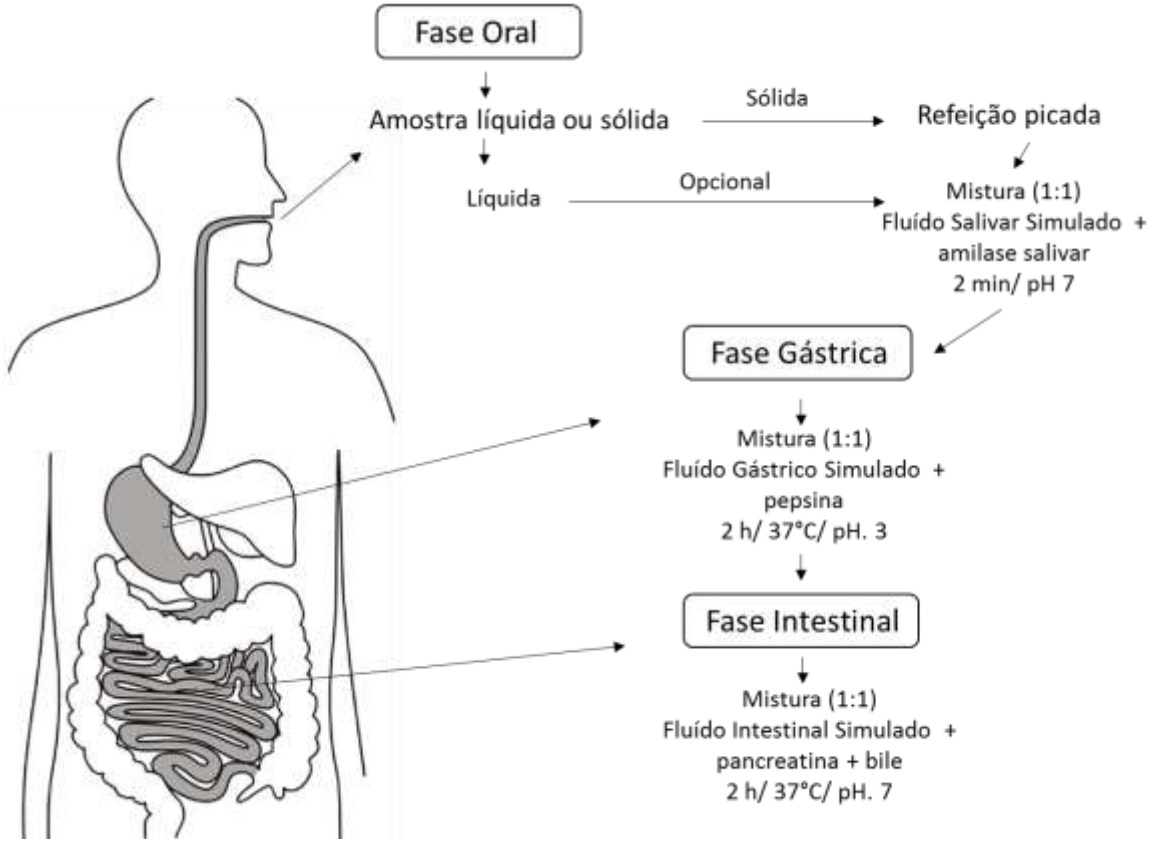

Figura 4. Fases do protocolo INFOGEST.
Adaptado de Minekus et al., 2014 e Dala-Paula et al., 2021a

Cinco partes de quimo gástrico são misturadas com 4 partes de eletrólito SIF solução estoque para obter uma proporção final de quimo gástrico para SIF de 50:50 (v/v). As amostras gástricas – quimo são misturadas com estoque de eletrólito SIF solução eletrolítica. A adição de base (NaOH) é necessária para neutralizar a mistura para pH 7,0. Enzimas digestivas podem ser adicionadas como pancreatina de pâncreas suíno ou enzimas individuais. Os sais biliares são adicionados na mistura final. O tempo recomendado de digestão intestinal é de 2 horas a 37 °C (Minekus et al., 2014)

Estudos sobre a bioacessibilidade de poliaminas nos alimentos são escassos. Entre os estudos publicados existe um sobre a bioacessibilidade *in vitro* de aminoácidos e aminas bioativas em chocolate amargo 70% (Dala-Paula et al., 2021a). Foram investigadas dez aminas bioativas no chocolate, porém estavam presentes apenas sete (cadaverina, 2-feniletilamina, putrescina, espermina, espermidina, triptamina e tiramina). As poliaminas foram predominantes antes da digestão *in vitro*, enquanto tiramina, cadaverina e espermidina após a digestão. Todas as aminas apresentaram alta bioacessibilidade com leve influência de enzimas digestivas. Os autores levantaram a hipótese de que o aumento do total de aminas na fase gástrica foi devido ao baixo valor do pH (2,0), sugerindo que essa condição pode ter sido responsável pela quebra das formas conjugadas de aminas com proteínas e compostos fenólicos. Foi mostrado que na fase gástrica

ocorreu a maioria das alterações nas aminas individuais: tiramina aumentou mais (8,7 vezes), seguido por espermidina (5,9 vezes), espermina (2,9 vezes) e feniletilamina (2,6 vezes). Além disso, os conteúdos de espermina e espermidina foram maiores em relação ao controle (processo realizado sem adição das enzimas digestivas), reforçando a atividade da pepsina na bioacessibilidade das aminas em chocolate (Dala-Paula et al., 2021a).

Reis et al. (2020) avaliaram a bioacessibilidade de poliaminas, em especial da espermidina, após simulação *in vitro* das etapas de digestão (gástrica e intestinal) *in vitro* de cogumelo (*Agaricus bisporus*) fresco e processado. Os autores observaram que os níveis de espermidina não foram afetados com o processamento (cozimento e enlatamento) quando comparados com o cogumelo fresco após a digestão gástrica e intestinal simulada *in vitro*, demostrando que esta poliamina possui alta bioacessibilidade.

Pelo fato de existirem poucos estudos de bioacessibilidade de aminas bioativas em matrizes alimentares e estas poderem causar efeitos adversos no organismo humano, é importante desenvolver novos estudos sobre a biodisponibilidade de aminas bioativas em outros produtos alimentares.

OBJETIVOS

Objetivo geral

O objetivo geral deste trabalho foi investigar o perfil e teores de micotoxinas e de aminas bioativas livres em salames brasileiros, investigar a ocorrência deste contaminantes durante armazenamento refrigerado e determinar a bioacessibilidade *in vitro*.

Objetivos específicos

Os objetivos específicos foram:

1) desenvolver métodos rápidos para análise de micotoxinas por LC-MS/MS em salames e analisar amostras de salame do mercado varejista de Belo Horizonte, MG;
2) desenvolver métodos rápidos para análise de aminas bioativas livres por HPLC-fluorescência em salames e analisar amostras de salame do mercado varejista de Belo Horizonte, MG;
3) selecionar os tipos de salame mais relevantes e investigar sua qualidade físico-química, microbiológica, e a presença de contaminantes durante armazenamento refrigerado dos salames
4) investigar a bioacessibilidade das aminas bioativas *in vitro*

MATERIAL, MÉTODOS, RESULTADOS E DISCUSSÃO

Cada objetivo específico foi elaborado na forma de capítulos (artigos submetidos ou em submissão a jornais de impacto da área). Cada capítulo (artigo) contém os respectivos Material e métodos e Resultados e discussão. Importante ressaltar que não foram encontradas micotoxinas nas amostras de salame, desta forma, os demais objetivos específicos associados a micotoxinas não puderam ser realizados.

CAPÍTULO I - Determination of aflatoxins B1, B2, G1, G2 and ochratoxin A in Dry fermented sausages Using a dilute and shoot Method and LC-MS/MS

Abstract

The presence of mycotoxins in meat and meat products can result from contaminated feed carry-over, from contamination with toxigenic moulds during processing and storage, and from contaminated spices and ingredients. Aflatoxins are carcinogenic to humans whereas ochratoxin A is a possible human carcinogen. The occurrence of mycotoxins in dry fermented sausage is described worldwide, however scarce information is available for Brazilian products. Therefore, the objective of this study was to investigate the occurrence of aflatoxins (B1, B2, G1 and G2) and ochratoxin A (OTA) in dry fermented sausage. For that, a simple and accurate 'dilute and shoot' method by liquid chromatography–tandem mass spectrometry was validated in accordance with the criteria set by European and Brazilian guidelines. Fat removal from the samples did not improve recoveries. The recoveries at three different concentration levels (4, 9, and 15 µg/kg) after outlier treatment ranged from 81% to 94%. The method showed good linearity in the matrix ($R^2 \geq 0.9974$), the limits of detection varied from 0.96 up to 0.97 µg/kg, and the limit of quantification ranged from 3.18 up to 3.22 µg/kg. Twenty-seven samples of industrial dry fermented sausage from the market were analyzed and none of the mycotoxins of interest were detected. The absence of mycotoxins can result from the use of good management practices at the farm, industry, and distribution of the products; monitoring and controlling the factors which can affect contamination with toxigenic moulds and mycotoxin production and accumulation.

Keywords: Mycotoxin, salami, figures of merit, mass spectrometry, validation, dilute and shoot

1. INTRODUCTION

Mycotoxins contaminate food commodities worldwide, posing a major challenge to public health and food trade (Sivamaruthi et al., 2018). Mycotoxins are secondary fungal metabolites with diverse structures and toxicological properties that induce a variety of toxic effects in humans and animals (Pleadin et al. 2021; Ulusoy et al., 2022). Several types of mycotoxins have been identified, however, aflatoxins and ochratoxin are the most widely distributed in nature. The problem of these mycotoxins is more serious in tropical and subtropical regions of the world, due to favorable climatic conditions (Pleadin et al., 2021; Lešić et al., 2022). Aflatoxins are by-products from some species of *Aspergillus*, especially *Aspergillus flavus* and *Aspergillus parasiticus.* There are four major groups of aflatoxins: B1, B2, G1 and G2 (AFB1, AFB2, AFG1, and AFG2, respectively) (Montanha et al., 2018; Lešić et al., 2022; Ulusoy et al., 2022; Sartori et al., 2023). Aflatoxins are genotoxic and are associated with liver cancer and cirrhosis; and AFB1 can cause hepatocellular carcinomas in humans (EU 2010b; EFSA 2020a). They are classified as Group 1, carcinogenic to humans, by the International Agency for Research on Cancer (IARC), in special aflatoxin B1 (EFSA 2020a). Ochratoxin A (OTA) is produced by several species of *Penicillium* and *Aspergillus* (Pleadin et al., 2021; Delfino et al., 2022). OTA is hepatotoxic, nephrotoxic, teratogenic neurotoxic, genotoxic, carcinogenic and immunotoxic for several animal species (EFSA 2020b; Lešić et al., 2022). The IARC has classified OTA as a possible human carcinogen - Group 2B (EFSA, 2020b).

Aflatoxins and ochratoxin are ubiquitous in nature, and they can readily contaminate several food commodities at any step along the chain, from farm to fork (EFSA 2020a,b; Pleadin et al., 2021; Lešić et al., 2022; Ulusoy et al., 2022). They can contaminate animals' feed resulting in undesirable mycotoxins in animal-derived food (meat and meat products) due to the carry-over effect (Pleadin et al., 2021; Vlachou et al., 2022). Mycotoxins have been reported in animal feed at high levels. OTA has been found up to 224 µg/kg in corn (Rosa et al., 2009) whereas aflatoxin was reported in maize (≤560 µg/kg), sorghum (≤33 µg/kg) and soybean (≤11 µg/kg) (EFSA 2020a). In pig feed, OTA was found up to 120 µg/kg (Rosa et al., 2009; Li et al., 2014; Leiva et al., 2019; EFSA 2020b; Vlachou et al., 2022), with several samples exceeding the OTA limit of 50 µg/kg established by the European Community (EU 2010a) and also the OTA Chinese limit of 100 µg/kg for pig feed (Li et al., 2014). Pigs are the most susceptible to OTA exposure, due to its high bioavailability and long half-life (Pleadin et al., 2021). The levels and the duration of feeding with OTA-contaminated feed affected OTA levels in the kidney, liver, muscles, and fat of pork (Perši et al., 2014; Altafani et al., 2017). Several cases of aflatoxin carry-over in pig have been reported for aflatoxin B1, and may occur in liver, muscles, and adipose tissue (Pleadin et al., 2021).

According to EFSA (2020b), the mean OTA level in pork edible tissues is 0.20 µg/kg. However, after OTA administration in the feed, ~2 µg/kg was found in the muscle tissue (Jørgensen 2005).

As mentioned previously, when present in animals' feed, aflatoxins and OTA can result in animal and animal-derived food products (carry-over effect) (Altafini et al., 2019; Delfino et al., 2022; Lešić et al., 2022; Vlachou et al., 2022). Mycotoxins are also present in spices and other food ingredients (Altafini et al., 2019; EFSA 2020a,b; Pleadin et al. 2021; Vlachou et al., 2022). In addition, toxigenic molds can contaminate food products during fermentation and ripening and can grow on the surface and diffuse into the product (Markov et al., 2013; Pleadin et al., 2021; Delfino et al., 2022; Vlachou et al., 2022). Furthermore, toxigenic fungi can contaminate food products during processing and storage when proper manufacturing practices are not observed (Markov et al., 2013; Rodríguez et al., 2015; Sivamaruthi et al., 2018; Perrone et al., 2019; Kudumija et al., 2020; Ricci et al., 2021; Lešić et al., 2022). This is worrisome since mycotoxins show high stability and are resistant to acidity and high temperatures, therefore, food manufacture, e.g., heating, ripening, drying, and storage have little or no effect on mycotoxins (EFSA 2020b; Delfino et al., 2022). Consequently, mycotoxins can be present in products of animal origin (Markov et al., 2013; Pleadin et al., 2021; Mitchell et al., 2017; Altafini et al., 2019).

OTA and aflatoxins have been found as a contaminant in dry fermented meat products (Merla et al., 2018; Vlachou et al., 2022). OTA was found at levels up to 621 µg/kg in Italian sausages (Merla et al., 2018; Altafini et al., 2019; EFSA 2020b; Roncada et al., 2020); Croatian salami (Kudujima et al. 2020; Lešić et al., 2022), and Egyptian salami (Shaltout et al., 2014). Aflatoxin B1 has been reported in dried cured meat products at levels up to 11.1 µg/kg (Markov et al., 2013; Pleadin et al., 2022; EFSA 2020a); whereas total aflatoxins have been found up to 12.5 µg/kg (EFSA 2020a; Ulusoy et al., 2022). Aflatoxins have been reported in different types of Croatian sausages at levels ≤ 3.0 µg/kg (Markov et al., 2013). Other mycotoxins have also been reported sporadically in sausages, including citrinin (Markov et al., 2013; Lešić et al., 2022), sterigmatocystin (Lešić et al., 2022), and cyclopiazonic acid (Lešić et al., 2022), but their impact on the safety of meat products and human health needs to be ascertained (Pleadin et al., 2021).

Dry fermented sausages are well appreciated worldwide. They are industrialized meat product obtained from meat (pork or pork and beef), with added pork fat, and other minor ingredients (e.g., salt, seasonings, spices, wine) embedded in natural and/or artificial casings, cured, fermented, matured, smoked or not and dried (Brasil 2000). There are several types of sausage worldwide, which can differ based on the origin of the meat, the methods of preparation of the lean and the fatty parts, the ratio between the different parts, the salting, the addition of different spices, the type of casing, the size of the final product, the colonization of fungi on the

casing, and the seasoning methods (Merla et al., 2018; Altafani et al., 2019; Pleadin et al., 2021). Even though there are reports on the occurrence of mycotoxins in dried fermented sausages, there is no tolerable limit established by European, North American, and Brazilian regulations. Nevertheless, according to the European Union (EU 2010b), the maximum levels of total aflatoxins and aflatoxin B1 should be 4 μg/kg and 2 μg/kg, respectively, for products for direct human consumption or use as an ingredient in foodstuffs. However, a few countries (FAO 2004) have established a limit of 5 μg/kg for AFB1 for meat, meat products and sausages e.g., Estonia, Serbia, Montenegro, Slovakia, and Ukraine. As for OTA, maximum limits of 3 μg/kg have been established for cereal products intended for direct human consumption (EU 2006). Limits for meat and meat products have been implemented in some countries, e.g., 10 μg/kg in Denmark and Estonia, and 5 μg/kg in Romania and Slovakia. In Italy, a guideline value of 1 μg/kg in pork meat and derived products for OTA since 1999 (Italian Ministry of Health, 1999). However, according to EU (2022), additional monitoring on the presence of ochratoxin A is appropriate before establishing maximum levels.

Several methods are available for the analysis of mycotoxins in sausages. HPLC, and among the immunochemical methods, ELISA have traditionally been applied. The most widely used are high performance liquid chromatography combined with fluorescence - HPLC and immunochemical methods, e.g., enzyme-linked immunosorbent assay – ELISA (Meulenbeg et al., 2012). ELISA kits are commercially available and are for specific each mycotoxin, however they are expensive. Meat and products are a complex matrix and pose difficulties during mycotoxin analysis due to the presence of fat and protein (Merla et al., 2018; EFSA 2020a,b; Delfino et al., 2022; Lešić et al., 2022; Vlachou et al., 2022). In general, the mycotoxins are extracted using methanol and/or acetonitrile with water acidified with acetic acid (Merla et al. 2018); and the fat is removed using hexane (Merla et al., 2018; EFSA 2020a,b). Samples are usually cleaned up and purified using immunoaffinity columns (Delfino et al., 2022) and they need to be derivatized for fluorescence detection (EFSA 2020a,b). These methods are laborious, require organic solvents and expensive reagents/immunoaffinity columns. Disadvantages of fluorescent assays are the background fluorescence (Meulenbeg et al., 2012). Among analytical innovations, there has been a shift toward generic sample clean-up techniques such as ‘dilute and shoot’ seeking expansion of analytical capabilities and create multi-class, multi-analyte methods (Greer et al., 2021). High-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) has been reported as a promising tool in the analysis of several mycotoxins simultaneously (Vlachou et al., 2022; Sartori et al., 2023). However, sample purification and clean-up have been used for sensitive analysis.

There is scarce information on the occurrence of mycotoxins in Brazilian dry fermented sausages. In this context, the objective of this study was to investigate the occurrence of some mycotoxins including aflatoxins B1, B2, G1 and G2 and ochratoxin A in fermented sausages. For this, a simple, rapid, and green dilute and shoot LC-MS/MS method-was optimized and validated.

2. MATERIALS AND METHODS

2.1. Materials

Samples

A total of twenty-seven samples of dry fermented sausage were obtained for this study, including Italian (n=3), Italian gourmet (n=6), Hamburgues (n=6), Hamburgues mini (n=6) and Salaminho (n=6). The samples were purchased from the local market (Belo Horizonte, MG, Brazil). Most of the sausages (n=24) were from industries under the Brazilian Federal Inspection System (SIF), whereas the other three (Italian) sausages were from industries under inspection by the State inspection system. Additional Italian sausage (SIF inspected) samples were obtained from the local market and used in the validation of the method.

Reagents

The reagents used were LC grade (Merck, Darmstadt, Germany), except hexane, which was analytical grade. Ultrapure water was obtained from Milli-Q Synthesis (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for LC analysis were filtered through 0.22-μm pore size membranes (Millipore Corp). The mycotoxins standards were purchased from Merck/Supelco (Darmstadt, Germany), and included: ochratoxin A - certified reference material (50 μg/mL) in benzene:acetic acid (99:1); Aflatoxin B1, B2, G1 and G2 - certified reference material (3 μg/mL) in benzene:acetonitrile (98:2).

2.2. Methods

Sample preparation

Dry fermented sausages were grinded in a blender and homogenized thoroughly and aliquots of 5 g each were used for the analysis of the mycotoxins. The extraction of the mycotoxins was performed in triplicate (Fig 1). Briefly, the 5 g samples were placed into a 50 mL Falcon tube along with 20 mL solution of methanol:acetonitrile:water (60:20:20, v/v/v). After 30 minutes in a

multi-wrist shaker (Lab-line™, Cairo, Egypt), the samples were centrifuged (Jouan BR4i, Saint-Herblain, France) at 1800 *g* for 10 min at room temperature (25 °C). An aliquot of 250 µL of the supernatant was transferred to an Eppendorf vial and 750 µL solution of methanol:water (1:1, v/v) was added. After brief agitation, the sample was centrifuged again at 15000 *g* for 10 min at 4 °C. The supernatants were filtered through a 13 mm diameter syringe filter with a PVDF 0.22 µm pore size membrane (GE Uniflo™ – Whatman, Amersham, UK) immediately prior to LC-MS/MS analysis.

Chromatographic separation of the mycotoxins

Chromatography was performed using an Acquity I-Class UPLC coupled with a Xevo-TQ-S tandem quadrupole mass spectrometry detector. The column used was a reverse phase (Acquity UPLC BEH, Waters, Massachusetts, USA), 130 Å, 1,7 µm, 2.1 x 100 mm (Paschoal et al. 2017). The separation of the five mycotoxins was possible using two mobile phases: (A) 0.2 % formic acid and (B) 0.2 % formic acid in acetonitrile. The gradient which allowed optimum resolution in a shorter run time (15 minutes) was: initial - 90% A: 10% B; 3.0 min. - 90% A: 10% B; 10.0 min. - 30% A: 70% B; 10.1 min. - 10% A: 90% B; 12.0 min. - 10% A: 90% B; 12.1 min. - 90% A: 10% B and 15.0 min. - 90% A: 10% B. The flow rate was set at 0.4 mL/min.

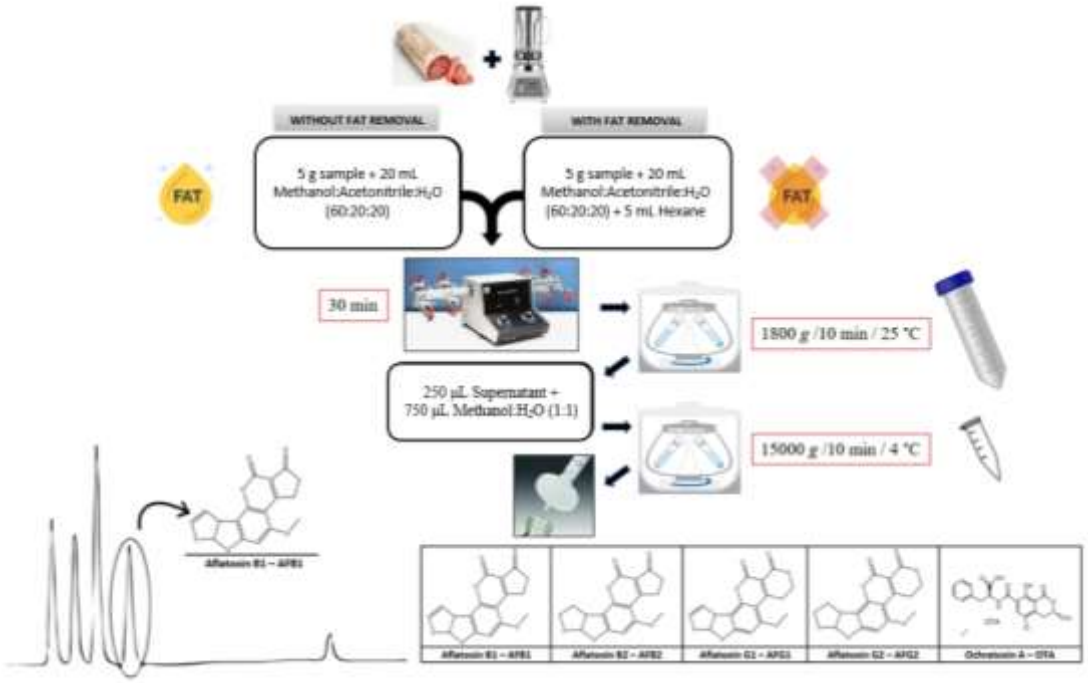

Fig. 1. Flow diagram of the extraction of mycotoxins from dry fermented sausage with and without fat removal using hexane.

Mass spectrometry conditions

Mass spectrometry analysis was performed using electrospray ionization in the positive mode (ESI^+) using Multiple Reaction Monitoring (MRM). The operation conditions were as follows: the capillary voltage applied to the electrospray was 3.5 kV; the source temperature was 150 ºC and the nebulizer gas (Nitrogen) pressure was 7.0 bar; the desolvation temperature was 600 ºC, the desolvation gas (Nitrogen) flow was 1200 L/h and the cone gas flow of 150 L/h. Argon was used as the collision gas.

The working parameters for each analyte of interest were determined through individual standard solution injection directly into the mass spectrometer. The two most abundant ions were selected for detection, one used for quantification and the other for confirmation. The selected transitions and their optimum conditions are described in table 1. The cone voltage was 50 V for AFB1, 40 V for AFB2, and 30 V for AFG1, AFG2 and OTA. The collision energies for the quantification and confirmation transitions were 22 V and 36 V for AFB1; 25 V and 30 V for AFB2; 27 V and 25 V for AFG1; 30 V for both AFG2 transitions; and 23 V and 35 V for OTA, respectively.

Influence of fat removal during extraction of the mycotoxins

For the investigation of the influence of fat removal on mycotoxins recovery, a pool of samples spiked with 8 µg/kg of each of the mycotoxins of interest were tested. Extraction was performed in triplicate, with and without fat extraction using hexane (Fig 1). For the removal of fat, 5 mL hexane was added to the Falcon tube containing 5 g sample along with 20 mL solution of methanol:acetonitrile:water (60:20:20, v/v/v). The procedure was followed as previously described starting from 30 min agitation in a multi-wrist shaker. The supernatants were filtered through a 13 mm diameter syringe filter with a PVDF 0.22 µm pore size membrane (GE Uniflo™ – Whatman, Amersham, UK) immediately prior to LC-MS/MS analysis. The influence of fat removal on the analysis of mycotoxins in dry fermented sausages was determined by the recovery of the mycotoxins and the correlation coefficients of the matrix-matched standard curve.

Validation of the method

The fitness of the optimized method for the analysis of five mycotoxins (AFB1, AFB2, AFG1, AFG2 and OTA) in sausage was investigated according to the Commission Decision 2002/657/EC (EC 2002) and the Analytical Quality Assurance Manual (Brasil 2015). The

following parameters were determined: selectivity, linearity of the calibration curves, accuracy, precision, and limits of detection and quantification.

Selectivity

The selectivity of the method was investigated by analyzing a blank sample of sausage. The existence of interfering peaks that could affect detection in the range of retention times of the mycotoxins was also investigated.

Table 1. Mass spectrometry conditions for the analysis of aflatoxins B1, B2, G1 and G2 and ochratoxin A by LC-MSMS

MS parameters	**Mycotoxins**				
	AFB1	**AFB2**	**AFG1**	**AFG2**	**OTA**
Precursor ion (m/z)	313.3	315.3	329.1	331.1	404.1
Retention time (min)	6.81	6.48	6.47	6.11	8.74
Cone voltage (V)	50	40	30	30	30
Quantification					
Product ion (m/z)	285.1	287.1	243.1	245.1	329.0
Collision energy (eV)	22	25	27	30	23
Confirmation					
Product ion (m/z)	241.1	259.1	283.1	257.1	221.0
Collision energy (eV)	36	30	25	30	35

AFB1, AFB2, AFG1, AFG2 – Aflatoxins B1, B2, G1 and G2, respectively. OTA – ochratoxin A.

Linearity of the calibration curves

The linearity of the method was assessed according to the procedure described by Souza and Junqueira (2005). The existence of matrix effect was assumed. Therefore, the calibration curves were constructed in sausage extract from the blank sample. For preparation of the standard curve, two stock solutions were prepared, one pool of standard for AFB1, AFB2, AFG1 and AFG2 and a standard solution for OTA, both containing approximately 1000 ng/mL. An intermediate solution was prepared containing all five mycotoxins. Aliquots of 5 µL of the aflatoxins and 10 µL of the ochratoxin stock solutions were placed into a flask, protected from light, and the solvent was evaporated under a mild stream of nitrogen at room temperature until dryness. The solid residue was resuspended in 5 mL of the sausage extract. Working standard solutions for five different concentrations were prepared by adding different amounts of the intermediate solution to the appropriate amount of the sausage extract to obtain the concentration of all five mycotoxins,

evenly spaced, as described in Table 2. Three replicates of each matrix-matched standard were used. Outliers were investigated using the Jackknife test. For each mycotoxin, a plot of the analyte signal versus concentration was built, and the linear equations and the correlation coefficients were calculated by the ordinary least squares linear regression. Normality, homoscedasticity, independence of residuals, significance of regression, and fitness to the linear model were also evaluated.

Accuracy and precision

Known levels of the analytes were added to 5 g samples of sausage to determine accuracy and precision (repeatability and reproducibility). The samples were fortified at three different levels (spiked with 4, 9 and 15 µg/kg) the day before analysis. For each level of fortification six samples were prepared (six replicates per concentration level). The mean recoveries were estimated for each concentration level after outlier treatment. Accuracy was calculated as (100×mean concentration found)/fortification level (EC 2002). Repeatability was established through evaluation of the coefficient of variation for each replicate.

Table 2. Concentrations of aflatoxins B1, B2, G1 and G2, and ochratoxin A used in the construction of the standard curve in the matrix (dried fermented sausage extract)

Standard	**Mycotoxin concentration (ng/mL)**				
	Aflatoxin B1	**Aflatoxin B2**	**Aflatoxin G1**	**Aflatoxin G2**	**Ochratoxin A**
1	3.22	3.19	3.19	3.22	3.18
2	6.43	6.38	6.39	6.44	6.36
3	9.67	9.58	9.58	9.66	9.53
4	12.86	12.77	12.78	12.88	12.72
5	16.08	15.96	15.97	16.11	15.90

Limits of detection and quantification

The limit of detection (LOD) was determined as the smallest concentration of the compounds that the method was capable of distinguishing from zero. The limit of quantification (LOQ) was the smallest concentration that the method was capable of quantifying in an acceptable way (Brasil 2015). LOD and LOQ were calculated based on the analytical curves. LOD and LOQ were defined as three and ten times the standard deviation of the slope divided by the intercept of the analytical curve, respectively (Thompson et al. 2002; Souza et al. 2007).

2.3. Analysis of real samples

Samples (n=27) of different types of sausages (Italian, Italian gourmet, Hamburgues, Hamburgues mini, and Salaminho) were analyzed using the developed method for the detection of the five mycotoxins of interest and determination of their occurrence.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS characteristics

The working parameters for each analyte of interest were determined through individual standard solution injection directly into the mass spectrometer. The two most abundant ions were selected for detection, one used for quantification and the other for confirmation. The selected transitions and their optimum conditions are described in table 1.

3.1.2. Influence of fat removal on mycotoxins recovery

Two different parameters were used to compare the influence of fat removal from sausages on the extraction of mycotoxins: recovery and correlation coefficient of the matrix-matched standard curve (Tables 3 and 4, respectively). The recoveries obtained during the extraction of the mycotoxins (Table 3) varied from 85.3% for ochratoxin A up to 99.5% for aflatoxin G1 without fat extraction and it ranged from 81.9% for ochratoxin A up to 96.3% for aflatoxin G2 with fat extraction. The recoveries obtained for both extraction procedures are within the range of 70% to 110% established by validation/analytical quality guidelines (EC 2002; Brasil 2015). When comparing the recoveries from the extractions with and without fat removal, there was no significant difference between the two extraction procedures (Anova, $p>0.05$). When considering the relative standard deviations (RSD) calculated for each analyte (Table 3), the percentages varied from 4.15% for aflatoxin B2 up to 6.57% for aflatoxin B1 without fat extraction and from 1.21% for ochratoxin A up to 7.14% for aflatoxin B1 with fat extraction. The RSD complied with Brasil (2015) and InMetro (2020) for the mycotoxins, as for the spike levels of 8 µg/kg, the RSD values should be lower than 20% and 15%, respectively. Based on these results, when comparing the extractions of the mycotoxins with and without the aid of hexane for fat removal, there was no significant difference ($p>0.05$) for the recoveries between the extraction procedures.

Based on the recoveries and respective relative standard deviations, on the correlation coefficients of the matrix-matched standard curves results, as well as on the absence of interferents in the chromatograms, the extraction without fat removal using hexane was selected for the multiclass mycotoxin analysis performed on this study, since it was faster, simpler, and more environmentally friendly. This method constitutes an easy and simple dilute and shoot procedure, which is convenient for mycotoxins analysis as it has been reported and valued in the literature for several multi analytes methods (Greer et al., 2021).

Table 3. Influence of fat removal from dried fermented sausages using hexane on the recovery of aflatoxins B1, B2, G1 and G2 and ochratoxin A from samples spiked with ~8 ng/kg of each mycotoxin

Mycotoxin	**% Recovery during extraction (mean ± sd) [RSD]**	
	without fat removal	**With fat removal**
Aflatoxin B1	95.40 ± 9.65 [6.28] [a]	88.9 ± 6.35 [7.14] [a]
Aflatoxin B2	94.40 ± 2.82 [4.15] [a]	92.14 ± 1.95 [2.11] [a]
Aflatoxin G1	99.50 ± 4.60 [5.18] [a]	95.98 ± 4.49 [4.68] [a]
Aflatoxin G2	97.10 ± 2.97 [6.57] [a]	96.30 ± 2.91 [3.58] [a]
Ochratoxin A	85.27 ± 2.24 [4.33] [a]	81.90 ± 0.76 [1.21] [a]

Mean values with the same superscript in the same line are not significantly different (Anova, p>0.05).
sd – standard deviation; RSD – relative standard deviation.

Table 4. Influence of fat removal from dried fermented sausages with hexane on the linearity of the standard curves of the aflatoxins B1, B2, G1 and G2 and ochratoxin A

Mycotoxin	**Linear equation [determination coefficient – R^2]**	
	no fat removal	**fat removal**
Aflatoxin B1	y=106344y+4697.48 [0.9889] [a]	y=83694.5x+1916.61 [0.9895] [a]
Aflatoxin B2	y=53332.1-1130.34 [0.9976] [a]	y=39443.8x-1482.59 [0.9898] [a]
Aflatoxin G1	y=47252.8x+810.531 [0.9924] [a]	y=40752.5x-1951.84 [0.9926] [a]
Aflatoxin G2	y=9674.42x+691.729 [0.9964] [a]	y=9280.72x+6.4464 [0.9907] [a]
Ochratoxin A	y=112761y-2636.1 [0.9957] [a]	y=92179.4-798.337 [0.9988] [a]

Mean values with the same superscript in the same line are not significantly different (Anova, p>0.05).
y=ax+b – linear equation: a – inclination; b – intercept.

3.1.3. Validation of the method

Selectivity was achieved as no interfering peaks in the chromatogram of the blank sample were seen, as indicated in Fig 2. The retention times of AFB2 and AFG1 are very close (6.46 and

6.45 min, respectively), however, their precursor ion (m/z) are different (315.3 and 329.1, respectively), allowing adequate quantification of both.

The method demonstrated linearity for all the mycotoxins in the concentration ranges investigated with confirmation of all assumptions of the linear regression model. The outliers detected were lower than the recommended limit of 20% of the original data (Brasil 2015). The residues showed independency and followed a normal distribution for all the five analytes (p>0.10). The variability of the residues was homogeneous (p>0.05) for all analytes along the concentration ranges. The regression was significant and there was no deviation from linearity for all five mycotoxins. The linear equations parameters - intercept (b values) and the inclination (a values) of the curves are indicated in Table 5. The coefficients of determination were ≥0.9773.

The accuracy (repeatability) and the precision (reproducibility) of the method were investigated by analyzing six repetitions of the blank sample of sausage spiked with three different concentrations (approximately 4, 9 and 15 µg/kg) of a pool of all five mycotoxins. The analysis was performed by the same analyst in the same day. The recoveries varied from 81.1% for OTA at the lower spike level (4 µg/kg) up to 98.8% for aflatoxin G1 at the higher spike level – 15 µg/kg (Table 6), which is in accordance with the limits of 80 up to 110% recommended by EC (2002) and Brasil (2015) for analytes at concentrations above 0.1 µg/kg. The recoveries observed are similar to values reported for Aflatoxin B1 (Markov et al., 2013) and for OTA (Markov et al., 2013; Altafani et al., 2019) in fermented meat products.

The precision of the method is associated with the dispersion of the recovery results from independent assays of a sample under defined conditions (Brasil 2015). These values are expressed as the relative standard deviations – RSD of the recoveries of six repetitions of the blank sample of sausage spiked with three different concentrations of a pool of all five mycotoxins (approximately 4; 9 and 15 µg/kg), analyzed by the same analyst in the same day (Table 6). The RSD varied from 0.01% for OTA at 15 µg/kg up to 1.22% for aflatoxin B1 at 9 µg/kg. For the spike levels of 4 and 9 µg/kg, the RSD were ≤1.22%. In a similar way, when spiking samples with 15 µg/kg, the CV were ≤0.95%. These coefficients of variation agree with the InMetro guidelines (InMetro 2020), that determines a maximum of 21% precision for samples spiked with 4 to 9 µg/kg and a maximum of 15% for samples spiked with 15 µg/kg. The results also agree with the requirements established by Brasil (2015), that set a limit of 20% CV. In addition, the relative standard deviation obtained for ochratoxin (0.01% – 0.28%) were lower compared to the maximum RSD for ochratoxin A of 40% established by CE no. 401 (EC, 2006). Therefore, the method was precise, providing results that agreed with the Brazilian and European guidelines.

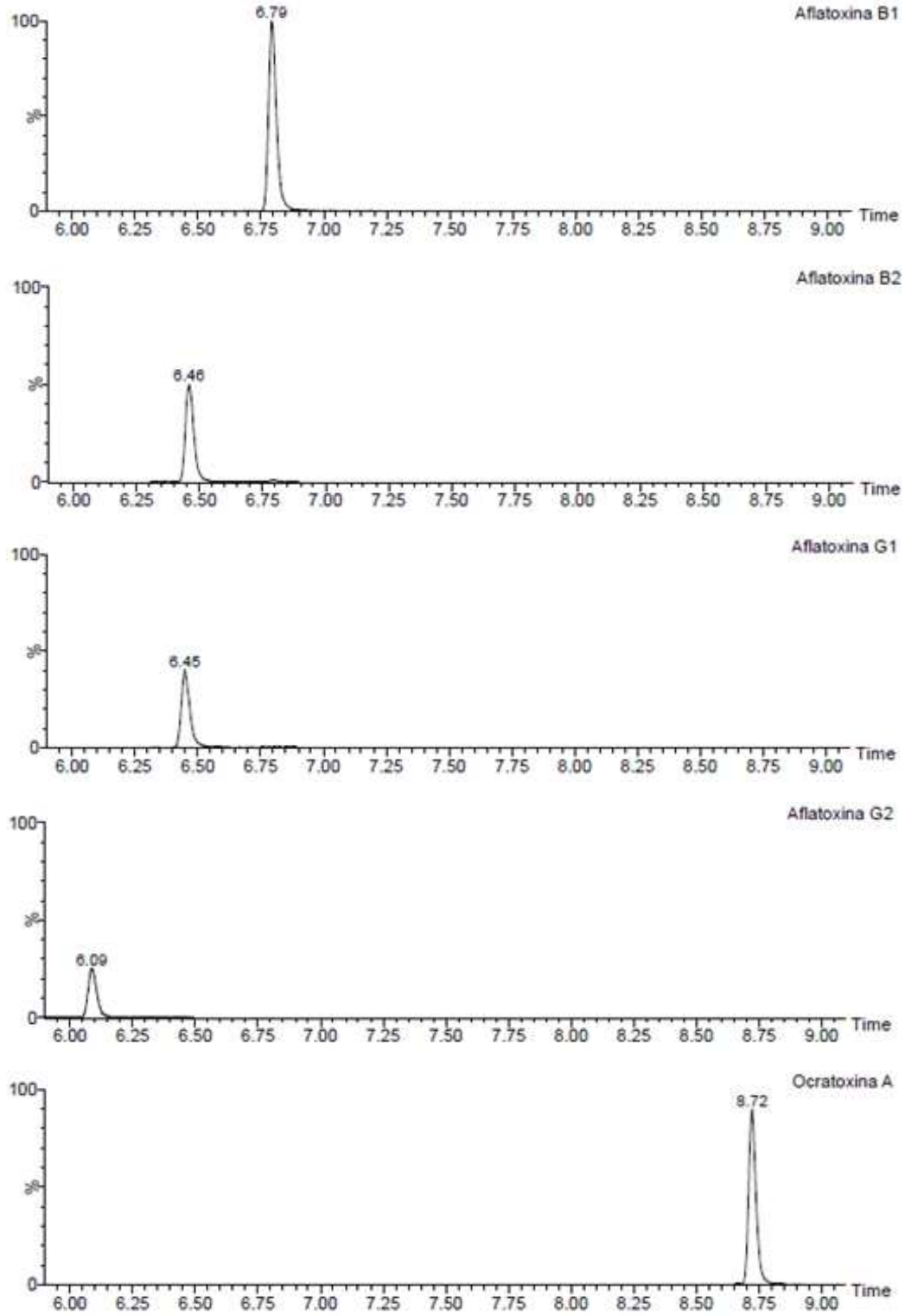

Fig. 2. LC-MS/MS chromatograms showing the elution of mycotoxins in dry fermented sausage (Italian type salami) spiked with aflatoxins (B1, B2, G1and G2) and ochratoxin A.
Sausage spiked with mycotoxin; Retention times (minutes): aflatoxin G2 – 6.09 min; aflatoxin G1 – 6.45 min; aflatoxin B2 – 6.46 min; Aflatoxin B1 – 6.79; min; and Ochratoxin A – 8.73 min. Relative intensity (%)

Table 5. Parameters describing the linearity of the analytical curves of aflatoxins B1, B2, G1 and G2 and ochratoxin A in the dried fermented sausage matrix

Mycotoxins	Parameters of the analytical curves		
	Inclination (a)	Intercept (b)	R^2
Aflatoxin B1	112934.026	-6480.039	0.9987
Aflatoxin B2	45987.011	-1676.978	0.9986
Aflatoxin G1	43566.615	-999.264	0.9974
Aflatoxin G2	9872.329	147.862	0.9988
Ochratoxin A	94269.329	-97.499	0.9989

y= ax + b.
R^2 - Determination coefficient.
n = 3, injected randomly in triplicate

Table 6. Outliers, mean recoveries, standard deviation and relative standard deviation of the concentration of the mycotoxins in dry fermented sausage sample spiked with 4, 9 and 15 µg/g

Mycotoxin	Spike level (µg/g)	N – outliers	Outlier (%)	Mean recovery (%)	sd	RSD
Aflatoxin B1	4	6	0	93.0	1.73	0.20
	9	6	0	87.1	9.27	1.22
	15	5	16.7	94.4	8.45	0.95
	Overall	17	5.5	91.5	7.18	0.86
Aflatoxin B2	4	6	0	92.9	1.50	0.17
	9	6	0	94.1	1.47	0.17
	15	5	16.7	95.4	0.50	0.06
	Overall	17	5.5	94.13	1.15	0.13
Aflatoxin G1	4	6	0	92.9	1.31	0.15
	9	6	0	98.0	1.00	0.10
	15	6	0	98.8	4.91	0.53
	Overall	18	0	96.6	3.47	0.40
Aflatoxin G2	4	6	0	95.7	3.50	0.38
	9	5	16.7	96.1	2.84	0.31
	15	6	0	91.3	0.85	0.10
	Overall	17	5.5	97.7	3.26	0.37
Ochratoxin A	4	6	0	81.1	1.48	0.23
	9	5	16.7	84.0	2.00	0.28
	15	5	16.7	83.8	0.10	0.01
	Overall	16	11.1	82.9	2.03	0.22

N – outliers - number of observations after outlier treatment; sd - standard deviation; .RSD - relative standard deviation.

The limits of quantification (LOQ) and detection (LOD) determined for the mycotoxins are indicated in Table 7. When considering the theoretical LOQ values calculated from the standard

deviation of the slope divided by the intercept, the obtained values were below the lowest concentration used for the analytical curve for all the aflatoxins (AFB1, AFB2, AFG1 and AFG2), In this way, the LOQ values the aflatoxins were assumed as the lower levels of the calibration curves (Paschoal et al., 2017). The calculated LOQ for OTA was at the lowest standard concentration of the calibration curve, and this value was assumed. Based on these results, the LOQ for the mycotoxins in sausages varied from 3.18 µg/kg for OTA up to 3.22 µg/kg for AFB1 and AFG2. The LOD values varied from 0.96 up to 0.97 µg/kg, for the same analytes.

Table 7. Limits of detection (LOD) and of quantification (LOQ) for the analysis of aflatoxins B1, B2, G1 and G2, and ochratoxin A in dried fermented sausages by LC-MS/MS

Mycotoxin	**Limits (µg/kg)**	
	LOD	**LOQ**
Aflatoxin B1	0.97	3.22
Aflatoxin B2	1.09	3.19
Aflatoxin G1	1.22	3.19
Aflatoxin G2	1.38	3.22
Ochratoxin A	1.23	3.18

The LOQ found for OTA are higher compared to the values reported for LC-MS/MS of dry fermented sausages by Zhao et al. (2015) and Kudumija et al. (2020), e.g., 0.1 and 1.44 µg/kg, respectively, in methods specific for OTA. Markov et al. (2013) found similar LOQ values (0.89 µg/kg) during HPLC-FL for OTA in fermented meat products. The method used by Markov et al. (2013) was simultaneous for AFB1 and the LOQ was 1.01 µg/kg, which is also lower compared to the method proposed in this study. In all these studies (Markov et al., 2013; Zhao et al., 2015; Kudumija et al., 2020), the extraction procedure was laborious and included a purification and/or concentration step. Therefore, when considering that the proposed method is multiclass mycotoxin (4 aflatoxins and OTA), using a simple dilute and shot method, the LOQ found are adequate. In addition, the LOQ found in this study are adequate for the analysis of dry fermented sausages compared to Brazilian legislation for these mycotoxins in foods for consumption – tolerable upper limits of 20 and 30 µg/kg for aflatoxins for ochratoxin A, respectively (Brasil, 2022). However, when considering the limits recommended in Italy for OTA (1.0 µg/kg), this method would be a reliable screening method, as the LOD is near 1.0 µg/kg. Therefore, samples with a LC-MS/MS signal, even though below the LOQ, should be submitted to a more sensitive method, e.g., ELISA or a more laborious sample preparation should be used.

3.1.4. Analysis of real samples

The validated method proved to be suitable for the routine analysis of aflatoxins (B1, B2, G1, G2) and OTA in dry fermented sausage and the removal of fat was not needed. The method was used in the analysis of 27 samples of industrial dry fermented sausages; however, the mycotoxins of interest were all below the LOD of the method (no signal was observed at all). Some additional sampling, considering only the outside layer of the sausage (0.5 cm thick), were investigated but the mycotoxins were still below the LOD. The hypothesis was that the outside layer was supposed to have a higher concentration of mycotoxins as the moulds grow on the surface of the sausages.

OTA at levels below the LOD are similar to those from Ulusoy et al. (2022), using ELISA, which did not find OTA in dry fermented sausages from Cyprus. However, according to the literature, OTA was detected in dry fermented sausage from Croatia (Pleadin et al., 2021; Zadravec 2020; Kudujima et al., 2020; Lešić et al., 2022); China (Zhao et al., 2015), Italy (Jorgensen 1998; Altafani et al., 2019; Roncada et al., 2020), and Denmark (Meulenberg et al., 2012).

Similar results for aflatoxins were reported by Lešic et al. (2022), which found no detected levels of AFB1 in dry fermented sausage from Croatia. However, they are different from Shaltout et al. (2014), which detected the presence of aflatoxins B1, B2, G1 and G2 at concentrations up to 26.1, 9.0, 5.6 and 3.5 µg/kg, respectively, in Kaliobia sausage from Egypt.

The presence of mycotoxins in sausage has been linked to several factors, including pork muscle and fat carry-over from contaminated feed Altafini et al., 2019; Delfino et al., 2022; Lešić et al., 2022; Vlachou et al. 2022; contaminated ingredients (mustard, garlic, Rosemay); contaminated spices, e.g., pepper, paprika, chilli (Altafini et al., 2019; EFSA 2020a,b; Pleadin et al., 2021; Lešić et al., 2022; Vlachou et al., 2022); from contamination during processing and storage with toxigenic moulds; growth of toxigenic moulds on the surface and diffusion into the product (Markov et al., 2013; Pleadin et al., 2021; Delfino et al., 2022; Vlachou et al., 2022); use of inadequate manufacturing practices (Markov et al., 2013; Rodríguez et al., 2015; Sivamaruthi et al., 2018; Perrone et al., 2019; Kudumija et al., 2020; Ricci et al., 2021; Lešić et al., 2022); lack of biocontrol of filamentous fungi which can inhabit sausage maturation chambers and lack of control of temperature and relative humidity (Franciosa et al., 2021; Stefanello et al., 2022). On the other side, the control of all these parameters can prevent occurrence of mycotoxins. In addition, the constant monitoring and control of mycotoxins in feed, ingredients, and spices; the constant biocontrol of the industry environment (Vlachou et al., 2022). The practice of some processing steps, including the washing and brushing of dry fermented sausages surfaces prior to

commercialization are useful in preventing mycotoxins contamination (Pleading et al., 2021). Moreover, the use of starter cultures during sausage processing can prevent the contamination and presence of other filamentous fungi capable of producing mycotoxins, among them *Penicillium nalgiovense* (Franciosa et al., 2021; Stefanello et al., 2022).

The absence of mycotoxins in Brazilian dry fermented sausages can result from good management practices at the farm, industry, and distribution of the products, monitoring and controlling all the factors which can affect contamination with toxigenic moulds and mycotoxin production and accumulation. This is possible because the dry fermented sausages included in this study are from large and well-established industries, and not from artisanal production, which is typical of most of the production in Brazil, in detriment to production in other countries.

4. CONCLUSION

A LC-MS/MS procedure for the separation and quantification of four aflatoxins (B1, B2, G1 G2) and ochratoxin A in dry fermented sausage was optimized and validated. Extraction was possible by a simple liquid-liquid extraction, using methanol, acetonitrile, and water. The LC-MS/MS run was short (15 min) and efficiently separated the mycotoxins. The removal of fat from the sausages using hexane did not improve mycotoxin recovery and therefore was not needed. The standard curve was built in the matrix. The figures of merit for the dilute and shot multiresidue method indicated that it was fit for the purpose. Twenty-seven samples of commercial sausages were analyzed and none of the mycotoxins investigated were detected (detection limit from ~1.0 µg/kg). These results suggest that industries in Brazil are monitoring and controlling mycotoxins occurrence from farm to fork. No evidence has been found to indicate that the consumption of these type of meat product can impose a risk regarding the exposure of aflatoxins B1, B2, G1 and G2 and ochratoxin A.

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CAPÍTULO II - Free bioactive amines in dry fermented sausages using a dilute and shoot method and HPLC-fluorescence

Abstract

Dry fermented sausages have been considered a significant source of amines. There are different types of dry fermented sausages in Brazil, but scarce information is available regarding their profile and contents of amines. In this context the objective of this study was to develop a dilute and shoot procedure for multi amines analysis in sausages by HPLC-fluorescence. The method was simple and fast. It did not require fat extraction, and did not show matrix effect. Mean recoveries of the amines varied from 86.1% up to 109.9% for phenylethylamine and histamine respectively. The analytical curves were linear ($R^2 \geq 0.9811$) in the range of 0.5 and 25.0 μg/g. The method was sensitive with LOQ from 1.56 to 4.08 mg/kg for phenylethylamine and histamine, respectively. A total of 148 dry fermented sausages were analyzed. All 10 amines were found in Hamburgues and Salaminho, whereas Mini Hamburgues had only four amines. The mean total levels of amines were high (107.8 – 385.5 mg/kg, $p>0.05$), except for Serrano (40.73 mg/kg). Tyramine contributed the most to total amines, representing 33.2% in Salaminho up to 81.4% in Mini-Hamburgues. It was followed by putrescine which varied from 25.8% up to 31.7% in Hamburgues, Salaminho, Italian, Gourmet Italian and Milano. However, in Mini-Hamburgues, tyramine was followed by spermine (17.4%); in Serrano, it was followed by serotonin (34.0%); and in Gourmet Italian it was followed by histamine (28.9%). Hamburgues had higher amines levels compared to Mini-Hamburgues, suggesting that a smaller sausage diameter can decrease amine formation. Gourmet Italian sausage had high histamine levels compared to Italian can result from the wine added to the Gourmet sausage. Principal components analysis and hierarquical cluster analysis were able to differentiate the seven types of sausage into four clusters, differentiating Gourmet Italian, Serrano and Hamburgues from the other sausages. These results indicate the need of sausage industries to mitigate amine formation in their products.

Keywords: Biogenic amines, HPLC, sausage, Italian, Hamburgues, Milano, Serrano

1. INTRODUCTION

Dry fermented sausage production is an economic and interesting way to preserve meat without requiring refrigeration for storage and adding desirable flavor characteristics to the product (Suliman et al., 2014; Toldrá et al., 2017). According to the Technical Regulation of Identity and Quality (Brasil, 2000), dry fermented sausage is the industrialized meat product obtained from pork or pork and beef, added ingredients, embedded in natural and/or artificial wraps, cured, fermented, matured, smoked or not, and dried. Mandatory ingredients are pork, bacon, salt, and potassium and/or sodium nitrite and/or nitrate. Pork meat is included in an amount of no less than 60%, except for Hamburgues type sausage with a minimum of 50% pork meat. Starter cultures can be used to initiate the fermentation of the sausage, and are auxiliary in the technology (Brasil, 2000). In addition, beef, powdered milk, sugar, wine, condiments, aromas, spices and glazing substances can be used in the preparation of these products, as optional ingredients (Brasil, 2000). In Brazil, sausage production is concentrated in the southern region, representing about 3% of the industrialized meat products in the country. Brazilian exports of sausages (made with pork meat) in 2021 were 15,428 tons, representing an increase of 34% when compared to the year 2020 (ABPA, 2022). There are several types of dry fermented sausages available, however, the most common types are Italian, Milano, Hamburgues, Friolano, Calabrian, German, Salaminho and Serrano, different raw meat types, formulations, additives, processes, casing, and drying periods are used (Terra, 2005; Toldrá et al., 2007; Degenhardt et al., 2021).

Sausages are known to be rich in free bioactive amines. Some of them are naturally present in meat and in some ingredients. Fresh meats are rich in spermine and spermidine (Ruiz-Capillas et al., 2004; Jairath et al., 2015; Custódio et al., 2018). These amines are relevant, as they participate in the synthesis of DNA and RNA, they have anti-inflammatory and antioxidant activities (Jeong et al., 2016; Soda et al., 2022); they protect cells and genes from harmful stimuli such as radiation (Douki et al., 2000; Soda et al., 2022), ultraviolet rays (Pothipongsa et al., 2012; Soda et al., 2022), toxic chemicals (Zhou et al., 2018; Soda et al., 2022) and other stresses (Soda et al., 2022). In addition, they promote autophagy, a natural regulatory mechanism within cells to remove degenerate or dysfunctional cell components (Chae et al., 2013; Zhou et al., 2018; Djajadikerta et al., 2020; Soda et al., 2022). However, during sausage processing, there can be a significant change on the profile and levels of amines. There can be loss of polyamines and formation and accumulation of biogenic amines (Roselino et al., 2020). Polyamines can be used as nitrogen sources by the microorganisms present (Krysenko et al., 2022); whereas the formation of biogenic amines can result from the activity of microbial enzymes (free amino acid

decarboxylases), from the added starter cultures or from contaminants during processing. Therefore, the presence and the concentration of some amines in sausage can be a useful index of quality and hygienic conditions prevalent during processing (Feddern et al., 2019). For example, high concentrations of putrescine and cadaverine can be used to predict the freshness of raw materials and the sanitary conditions during processing, as they are related to deteriorating bacteria (Mologноni et al., 2018). In addition, these amines can lead to a putrid flavor to the product (Mologноni et al., 2018), thereby affecting food acceptability. High levels of histamine and tyramine can be an index of safety, as these amines can cause adverse effects to human health (Triki et al., 2018; Ruiz-Capillas et al., 2019). In fact, these amines are associated with food outbreaks (EFSA, 2011). Histamine, at high levels, can cause the release of adrenaline and noradrenaline, stimulate sensory and motor neurons, it can act on the excitation of the smooth muscles, control of gastric secretion, and it also has vasoactive properties acting as a peripheral vasodilator (EFSA, 2011, Jairath et al., 2015; Eidenberg et al., 2016; Dala-Paula et al., 2021). Tyramine has vasoconstrictive activity, and it can trigger diet-induced migraine and hypertensive crises in individuals taking monoaminoxidase inhibitor (MAOI) drugs. The physiological effects of tyramine include vasoconstriction, increased cardiac output, increased respiration, elevation of blood glucose and release of norepinephrine (EFSA, 2011). According to EFSA (2011), no adverse health effects (NOAEL) were established as 50 mg histamine per meal for healthy individuals, but below detectable limits for those with histamine intolerance, whereas for tyramine, the NOAEL is 600 mg/meal for healthy individuals, but 50 mg/meal for those taking third generation MAOI or 6 mg/meal for those taking classical MAOI drugs. Furthermore, biogenic amines and other nitrogenous compounds may lead to N-nitrosamines formation, which are potential carcinogenic compounds (Bover-Cid et al., 2001; Komprda et al., 2004; De Mey et al., 2014). However, to avoid the formation of these substances, ascorbic or erythrorbic acids should be used (De Mey et al., 2014).

The occurrence of amines in sausages is widely investigated worldwide; however, there is scarce information on the levels of amine in Brazilian sausage. Cacciopoli et al. (2006) described the occurrence of ten bioactive amines in 42 samples of Italian-type sausage in the Brazilian market. They found high total levels of amines (≤532.7 mg/kg). Tyramine was present in every sample at high levels (≤229.9 mg/kg), followed by putrescine and cadaverine. Histamine was also present at high levels in some samples (≤120.7 mg/kg). In 2015, Santos et al. analyzed six amines in six Italian-type sausages, and they found even higher levels of total amines, tyramine, and histamine (≤997.6, 339.2 and 169.9 mg/kg, respectively). The increased levels of amines in the

sausage throughout the years are worrisome. In addition, there are several other types of sausage in the Brazilian market which have not been investigated.

Several methods are available for the determination of amines in dry fermented sausages and the most widely used are HPLC with UV or fluorescence detection after amines' derivatization with dansyl chloride, *o*-phthalaldehyde, or dabsyl chloride (Caccioppoli et al., 2006; Coloretti et al., 2008; Bomke et al., 2009; Mazzucco et al., 2010; De Mey et al., 2014; Santos et al., 2015). However, these methods are laborious, require organic solvents and expensive reagents and their fitness for the analysis of amines in sausage was seldom investigated. In addition, sausages are rich in fat (up to 32% fat) and its impact on the recovery of amines and on the HPLC base line was scarcely investigated. Nowadays, there has been a shift toward green and generic sample clean-up such as 'dilute and shoot' techniques, improving analytical capabilities and creating multi-analyte methods (Greer et al., 2021).

In this context, the objective of this study was to investigate the efficiency of a dilute and shoot HPLC/FL method in the determination of ten bioactive amines in sausage. The method was used in the quantification of different types of sausage available in the Brazilian market.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Samples and reagents

Sausage (dry fermented or salami) produced under the Brazilian Federal Inspection System (SIF) were purchased from supermarkets. The samples included nine Italian-, nine Gourmet Italian-, 39 Hamburgues-, nine Mini Hamburgues-, 41 Salaminho-, 37 Milano- and four Serrano-types of sausage. The samples were ground using a Black & Decker HC32-BR mini processor (Towson, MD, USA) prior to analysis. A pool of different types of sausages was prepared and used in the validation of the method.

The bioactive amines standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and included spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride. Analytical grade sodium acetate trihydrate, glacial acetic acid, octane sulfonic acid sodium salt, boric acid, Brij-35 (30% w/v), β-mercaptoethanol, potassium

hydroxide and *o*-phthalaldehyde (OPA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol and hexane were LC grade. Ultrapure water was obtained from Milli-QTM (Millipore Corp., Milford, MA, USA). Organic and aqueous solvents for HPLC analysis were filtered through 0.45 µm pore size HVPL Membranes (Millipore Corp., Milford, MA, USA).

2.2. Methods

2.2.1. Extraction of bioactive amines

The extraction of bioactive amines was based on the method described by Silva and Gloria (2002) and Custódio et al. (2016). The amines were extracted from 5 g samples with trichloroacetic acid (TCA, 5% w/v) in three 7-mL successive extractions. The mixture was shaken (Ovan, Barcelona, Spain) at 280 rpm for 10 min, followed by centrifugation at 10,000 *g* for 20 min at 4°C (MR23I Jouan, refrigerated centrifuge, Saint Herblain, France). The supernatants were filtered through qualitative filter paper and collected into a 25-mL volumetric flask which completed with 5% TCA. After homogenization, an aliquot of the extract was filtered (syringe + swinnex filter holder + cellulose ester membrane) immediately prior to HPLC analysis.

2.2.1.1. Influence of fat removal

The influence of fat removal using hexane on the recovery of amines was investigated before the extraction of amines with TCA. Aliquots (5 g each) from the pool of sausages were spiked with three different levels of amines (5, 15, and 30 µg/g). After homogenization, fat was extracted using 20 mL hexane. The mixture was shaken at 280 rpm for 10 minutes, followed by centrifugation at 10,000 *g* for 20 minutes at 4 °C (Jouan MR23I refrigerated centrifuge). The supernatant (hexane layer) was removed. Afterwards, the fat free sausage samples underwent extraction of amines with 5% TCA as previously described. The final extracted volume was homogenized, filtered (0.45 µm membrane) and used for HPLC injection.

2.2.2. Chromatographic conditions

The chromatographic apparatus consisted of a Shimadzu LC-20AD Prominence high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with three

pumps (Shimadzu LC-20AD, a post-column bypass system consisting of a low-pressure mixing chamber with zero dead volume, installed between the column outlet and the detector and a 2-m long x 0.25 mm diameter Teflon tubing protected from light, connected between the mixing chamber and the spectrofluorimetric detector (Shimadzu RF-10AXL). A Luna C18 Phenomenex column (4.6 x 250 mm, 5 μm) and a C18 (4x3 mm) pre-column were used in an oven (CTO-10 ASvp, Shimadzu, Kyoto, Japan) at 30 °C. An auto-injector (SIL-20AHT), with the capacity of 105 1.5-mL vials and a HPLC interface control unit (CBM-20A) were also used.

The mobile phases used were A solution of 0.2 M sodium acetate and 15 mM octane sulfonic acid sodium salt, pH adjusted to 4.9 with acetic acid; and B, acetonitrile. The gradient was: 0.01-17.99 min/2% B; 18.00-18.99 min/20% B; 19.00-39.99 min/5% B; 40.00-49.99 min/23% B; 50.00-50.49 min/35% B; 50.50-60.00 min./2% B. The post column derivation reagent, delivered at 0.3 mL/min., consisted of 1.5 mL Brij-35, 1.5 mL β-mercaptoethanol and 0.2 g OPA dissolved in 500 mL solution of 25 g boric acid and 22 g KOH (pH adjusted to 10.5 with 3% KOH). The post-column reaction took place at 30 °C. The fluorescence detector was set at 340 nm excitation and 450 nm emission. The identification of the amines was performed by comparison of the retention time of the analyte peaks in the sample with those of the standard solution and by addition of the suspected amine to the sample. Analyte concentrations were calculated by interpolation in the respective external analytical curves. The total running time was 60 minutes.

2.3. Fitness of the method for the analysis of amines

The suitability of the method for the analysis of ten bioactive amines in sausage was evaluated in accordance with the Commission Decision 2002/657/EC (EC 2002). The following figures of merit were determined: specificity, linearity of the calibration curves, accuracy, precision and limits of detection and quantification.

For determination of the specificity, a pool of the different types of sausage was used. The existence of any interference (possible peaks) that could affect the detection in the range of retention times of the ten amines was investigated. Samples spiked or not with the ten amines were analyzed to confirm peak identity; samples were enriched with each standard to certify identity by increasing the signal from the suspect peak to the respective added standard.

The existence of a matrix effect was expected since sausages are a natural source of free bioactive amines (Debadé et al., 2020; Roselino et al., 2020), therefore the matrix effect was assumed. Matrix calibration curves were constructed using eight different concentrations of all ten

amines (0.5; 2.5; 5.0; 10.0; 15.0; 20.0; 25.0 and 30.0 μg/ g) in a pool of fermented dry sausages. The samples were filtered and randomly injected.

To prepare the standard curve, two stock solutions were prepared (100 μg/mL), a standard pool for the ten amines (agmatine, cadaverine, histamine, phenylethylamine, putrescine, serotonin, spermidine, spermine, tryptamine and tyramine) in hydrochloric acid (0.1 mol/L) and another with the pool of 10 amines in dry fermented sausage extract (dry fermented sausage pool extracted with TCA 5%). Intermediate solutions were prepared from the solution of the 10 amines in HCl (0.1 mol/L). Working standard solutions for five different concentrations of amines were prepared by adding different amounts of the intermediate solution (0.5 a 30 μg/mL) to the appropriate amount of the sausage extract to obtain the concentrations of all ten amines. Three replicates of each standard matched matrix were used. Outliers were investigated using the Jackknife test. For each amine, a plot of analyte signal versus concentration was constructed, and linear equations and correlation coefficients were calculated by ordinary least squares linear regression. Normality, homoscedasticity, independence of residuals, regression significance and adequacy to the linear model were also evaluated (Souza and Junqueira, 2005).

Precision was determined by adding known amounts of amines to five aliquots of 5 g sausage. These aliquots were divided into three groups with six repetitions, each, and were fortified at the beginning of the extraction with 5, 15 and 25 μg/g of amines. The samples were analyzed, the concentrations were determined and the recoveries at each level were calculated. Repeatability was established by evaluating the coefficient of variation of the recoveries for each spike level. Different analysts and different days were not used to assess reproducibility. Reproducibility was calculated as 100× mean concentration/fortification level (EC, 2002).

The limit of detection (LOD) was determined as the lowest concentration of the analyte, corresponding to three times the signal-to-noise ratio. The limit of quantification (LOQ) was the lowest concentration of the analyte that could be precisely and accurately determined. It was assumed to be the first analyte concentration in the calibration curve (EC, 2002).

2.4. Analysis of real samples

Dry fermented sausage samples under the Federal Inspection System (SIF) were obtained from the consumer market. The samples included Italian-, Italian Gourmet-, Hamburgues, Mini Hamburgues, Salaminho, Milano and Serrano type fermented dry-sausages in a total of 148 samples.

2.5. Statistical analysis

The fitness of the matrix curves was evaluated by ordinal least squares regression, visual inspection of residuals, Dublin-Watson and Breusch-pagan tests at 5% probability (Minitab Inc., PA, USA). The standard curves were submitted to normality and significance tests (Shapiro-Wilk normality test, one-way ANOVA followed by Tukey's test at 5% probability). Minitab® 17.3.1 software (Minitab Inc., PA, USA) was used. Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were used for the characterization of the dry fermented sausage types. In PCA, all detectable amines were used as active variables in the derivation of the principal components. PCA was done using correlation as the type of matrix. The dendrogram for HCA analysis was obtained by clustering variables (the same used for PCA). McQuitty's linkage was used for the distance matrix and Euclidean's method to calculate the distance between observations.

3. RESULTS AND DISCUSSION

The chromatographic conditions of the method developed by Silva and Gloria (2002) and Custódio et al. (2018) for the separation and quantification of amines in poultry and pork, respectively, were adequate for the analysis of sausage. Therefore, they were used with a few modifications, e.g., the previously used columns (μBondapak and Novapak C18, respectively), were replaced by Luna C18 column (Phenomenex, Torrance, CA, USA) as it provided faster regeneration and stability of the ion-pair.

3.1. Influence of fat removal

Amine recovery was used to investigate the influence of fat removal using hexane on the extraction of bioactive amines in sausages. For that, the pool of different types of sausages was spiked with three different concentrations of the ten amines. The recoveries obtained during extraction of the amines (Table 1) ranged from 10.9% for phenylethylamine at the lowest fortification level (5 μg/g) up to 87.65% for serotonin at the intermediate fortification level (15 μg/g). Overall, mean recoveries were higher for serotonin (67.9%), followed by histamine (63.6%) and tryptamine (60.3%), whereas the lowest recoveries were observed for phenylethylamine (25.6%). In most of the samples, the recoveries were below the 70% limit established by the Analytical Quality Assurance Manual (Brasil, 2015) and EC (2002).

As for the extraction of amines without fat removal (Table 2), the recoveries were higher. They varied from 86.1% for phenylethylamine at the intermediate spike level (15 µg/g) up to 115.4% for putrescine at the lowest spike level. Acceptable recoveries should be within 70% and 110%, as established by the Analytical Quality Assurance Manual (Brasil, 2015) and EC (2002). Therefore, only in for conditions, the limit was extrapolated, e.g., putrescine at the lowest spike level (115.4%), tyramine (115.1%) and tryptamine (112.6%), both at the intermediate spike level (15 µg/g), and tyramine at the upper spike levels (110.6%). However, when considering the average recoveries of the three spike levels, the values were all within the established range, with higher recoveries for tryptamine (~107%), putrescine (106.3%), tyramine, agmatine (~105%) and lower recoveries observed for spermidine (94.0%), phenylethylamine (96%), and spermine (97%). Overall. The mean recoveries ranged from 94% for spermidine up to ~107% for tryptamine and the mean total recovery was approximately 102%.

Table 1. Recovery of ten bioactive amines in dry fermented sausage spiked at three different levels (5 to 30 µg/g) during 5% TCA extraction with fat removal using hexane

Amines	**Mean recovery ± standard deviation (%) at different spike levels**			
	5 µg/g	**15 µg/g**	**30 µg/g**	**Mean**
Agmatine	12.57 ± 0.40 ^{c}	71.36 ± 4.36 ^{a}	53.18 ± 0.14 ^{b}	45.70 ± 30.10^{d}
Cadaverine	11.97 ± 0.61 ^{c}	67.25 ± 4.75 ^{a}	52.17 ± 0.75^{b}	43.79 ± 28.58 ^{d}
Histamine	64.02 ± 74.30 ^{a}	72.39 ± 3.37 ^{a}	54.45 ± 0.37 ^{a}	63.62 ± 8.97 ^{d}
Phenylethylamine	10.94 ± 0.50 ^{b}	19.80 ± 2.21 ^{b}	46.16 ± 9.07 ^{a}	25.64 ± 18.32 ^{d}
Putrescine	11.98 ± 0.16 ^{c}	66.91 ± 1.29 ^{a}	52.74 ± 0.12 ^{b}	43.88 ± 28.52 ^{d}
Serotonin	54.98 ± 62.38 ^{a}	87.65 ± 1.35 ^{a}	61.07 ± 0.19 ^{a}	67.90 ± 17.37 ^{d}
Spermidine	12.70 ± 0.27 ^{c}	64.84 ± 2.49 ^{a}	50.22 ± 0.96 ^{b}	42.59 ± 26.90 ^{d}
Spermine	30.49 ± 36.06 ^{a}	49.76 ± 0.82 ^{a}	35.08 ± 0.06 ^{a}	38.44 ± 10.07 ^{d}
Tyramine	12.71 ± 0.22 ^{c}	71.18 ± 0.26 ^{a}	58.28 ± 0.19 ^{b}	47.39 ± 30.72 ^{d}
Tryptamine	64.07 ±10.85 ^{a}	63.00 ± 3.68 ^{a}	53.81 ± 7.14 ^{a}	60.29 ± 5.64 ^{d}
Mean	28.64 ± 32.85 ^{a}	63.41 ± 17.61 ^{a}	51.71±7.42 ^{a}	47.97 ±15.90$^{a\ d}$

n = 6

TCA – trichloroacetic acid.

Mean values with the same superscripts ^{abc} in a line and ^{d} in a column (mean recoveries) are not significantly different (Tukey test, $p>0.05$).

When comparing the mean recoveries obtained for all amines with and without fat removal, values of 48.0% and 101.9% were observed, respectively. Furthermore, when considering the chromatograms, the presence of fat in the extract did not affect the baseline or the separation of the peaks. Therefore, fat removal of the dry fermented sausages was not recommended as it negatively affected amines' recovery. In addition, without the fat removal step, the method is more environmentally friendly as fewer organic solvents are used. Furthermore, the method is faster and very simple, typical of a 'dilute and shoot' procedure.

Table 2. Recovery of ten bioactive amines in dry fermented sausage spiked at three different levels (5 to 30 µg/g) during 5% TCA extraction without fat removal

Amines	Mean recovery ± standard deviation (%) at different spike levels			
	5 µg/g	**15 µg/g**	**30 µg/g**	**Mean**
Agmatine	109.65 ± 0.77 [a]	109.51 ± 0.36 [a]	96.03 ± 4.42 [b]	105.06 ± 7.82 [d]
Cadaverine	101.01 ± 0.01 [a]	106.30 ± 1.83 [a]	103.18 ± 3.76 [a]	103.50 ± 2.65 [d]
Histamine	101.18 ± 1.75 [b]	109.94 ± 1.06 [a]	103.02 ± 5.12 [ab]	104.72 ± 4.61 [d]
Phenylethylamine	106.29 ± 4.67 [a]	86.09 ± 5.41 [b]	96.29 ± 9.03 [ab]	96.22 ± 10.10 [d]
Putrescine	115.37 ± 0.89 [a]	99.37 ± 1.04 [b]	104.09 ± 4.44 [b]	106.28 ± 8.22 [d]
Serotonin	104.60 ± 3.17 [a]	98.72 ± 7.18 [a]	97.88 ± 1.40 [a]	100.4 0± 3.66 [d]
Spermidine	86.71 ± 3.84 [b]	103.55 ± 0.58 [a]	91.74 ± 4.06 [b]	94.00 ± 8.64 [d]
Spermine	86.75 ± 3.28 [b]	96.67 ± 0.85 [ab]	107.43 ± 7.82 [a]	96.95 ± 10.34 [d]
Tryptamine	99.62 ± 9.75 [a]	112.59 ± 3.44 [a]	108.39 ± 6.03 [a]	106.87 ± 6.62 [d]
Tyramine	89.46 ± 1.29 [b]	115.11 ± 0.42 [a]	110.62 ± 4.44 [a]	105.06 ± 13.69 [d]
Mean	100.06 ± 2.27 [a]	103.79 ±0.79 [a]	101.87 ± 4.38 [a]	101.91±1.86 [a d]

n = 6

TCA – trichloroacetic acid.

Mean values with the same superscripts [abc] in a line and [d] in a column (mean recoveries) are not significantly different (Tukey test, $p>0.05$).

3.2. Adequacy of the method for the analysis of amines in dry fermented sausage

3.2.1. Selectivity

Chromatographic conditions were optimized to provide the shortest possible run time with adequate peak resolution for all the ten analytes of interest. The optimum chromatographic run was 60 minutes. The ten analytes eluted within 56 min, with additional 4 min for regeneration and stabilization of the ion-pair, which is needed prior to the following sample injection. The shortest retention time was observed for putrescine (36.2-37.2 min), which showed the highest affinity for the mobile phase - acetonitrile and the lowest interaction with the stationary phase. The longest retention time was observed for tryptamine (55.2-55.8 min), all eight other amines were in between. The method was selective for all analytes as indicated in the chromatogram (Fig. 1). The proposed sample preparation procedure and the HPLC conditions provided peaks with good resolution, attesting for the selectivity of the method. When comparing the chromatograms in the solvent and in the sausage extract, there was no significant change on selectivity.

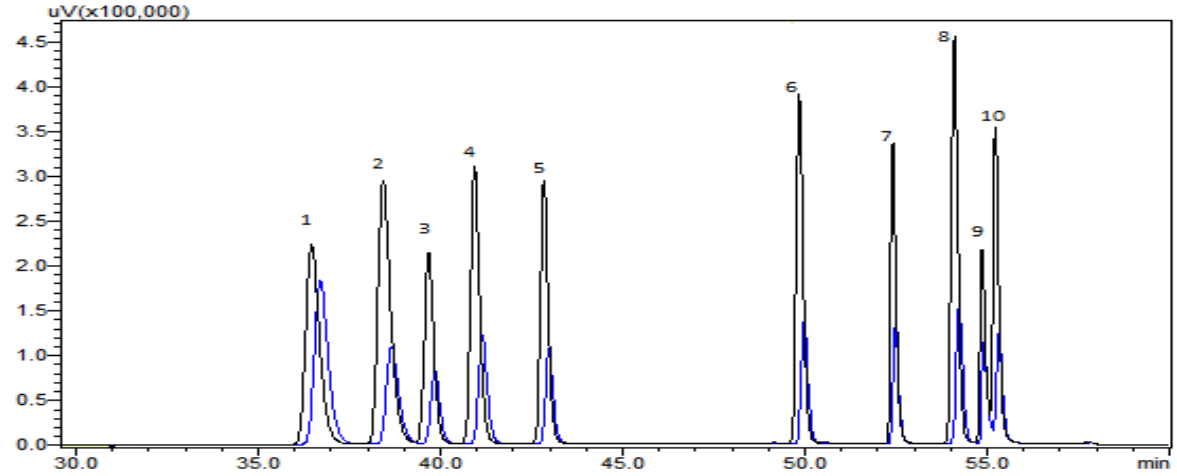


Figure 1. HPLC-fluorescence chromatograms of the calibration curves of ten bioactive amines in solvent - 0.1 M HCl (black line) and in the dry fermented sausage extract (blue line).
Peak numbers: 1- Putrescine, 2- Cadaverine, 3- Tyramine, 4- Histamine, 5 -Serotonin, 6- Agmatine, 7- Spermidine, 8- Phenylethylamine, 9- Spermine, and 10- Tryptamine.

3.2.2. Analytical curves / Linearity

The analytical curves using eight different concentrations of the ten amines (0.5; 2.5; 5.0; 10.0; 15.0; 20.0; 25.0 e 30.0 μg/g) were prepared in 0.1 M HCl and in the extract of a pool of dry fermented sausage. Five replicates of each concentration were used. As indicated in Table 3, the analytical curves, both in solvent and in the extract of the sausage were linear in the range investigated. In solvent (HCl), the correlation coefficients of the analytical curves varied from 0.9811 for agmatine up to 0.9977 for phenylethylamine, whereas in the sausage extract, the correlation coefficients ranged from 0.9810 for agmatine up to 0.9982 for phenylethylamine. Based on these results, both analytical curves (in solvent and in the matrix) were linear in the concentration range investigated, showing correlation coefficients ≥0.981. In addition, the chromatograms for the standard curves in solvent and in the sausage extract were not affected, considering the baseline and the separation among amines. Therefore, the analytical curve in solvent was used, as it is simpler, faster and more stable, to further investigate linearity.

All assumptions of the linear regression model were confirmed (Table 3). The outliers varied from 0 to 16.6%, and, therefore, are lower than the 20% recommended limit (Brasil, 2015). The residues were independent and followed a normal distribution for all ten amines (p>0.10). The variability of the residues was homogeneous (p>0.05) for all the amines along the concentration range (0.5 – 25.0 μg/g). There was no deviation from linearity for all ten bioactive amines. The linear equations parameters, e. g., the intercept (b values), the inclination (a values) and the coefficients of determination (R^2) of the analytical curves in solvent are indicated in Table 4. The correlation coefficients were ≥0.9811 and fitted a linear regression model.

Table 3. Outliers, mean recoveries, standard deviation and relative standard deviation (RSD) of the concentration of the amines in dry fermented sausage sample spiked with 5, 15 and 25 µg/g

Amine	Spike level (µg/g)	Number without outliers	Outlier (%)	Mean recovery (%)	Standard deviation	RSD
Agmatine	5	5	16.6	109.7	0.77	0.35
	15	6	0	109.5	0.36	0.28
	25	6	0	109.6	0.57	0.21
	Overall	17	5.6	109.6	0.57	0.28
Cadaverine	5	6	0	101.0	0.01	1.22
	15	5	16.6	106.3	1.83	0.09
	25	6	0	103.7	0.92	0.35
	Overall	17	5.6	103.7	0.92	0.55
Histamine	5	6	0	101.2	1.75	0.16
	15	6	0	109.9	1.06	0.23
	25	6	0	105.6	1.41	0.09
	Overall	18	0	105.6	1.41	0.16
Phenylethylamine	5	5	16.6	106.3	4.67	0.49
	15	6	0	86.1	5.41	1.15
	25	6	0	96.2	5.04	0.35
	Overall	17	5.6	96.2	5.04	0.66
Putrescine	5	6	0	115.4	0.89	0.94
	15	6	0	99.4	1.04	0.10
	25	6	0	107.4	0.97	0.73
	Overall	18	5.6	107.4	0.97	0.59
Serotonin	5	6	0	104.6	3.17	0.52
	15	6	0	98.7	7.18	2.77
	25	5	16.6	101.7	5.18	3.79
	Overall	17	5.6	101.7	5.18	2.36
Spermidine	5	6	0	86.7	3.84	0.48
	15	6	0	103.6	0.58	0.43
	25	6	0	95.1	2.21	0.24
	Overall	18	0	95.1	2.21	0.39
Spermine	5	6	0	86.8	3.28	0.63
	15	5	16.6	96.7	0.85	0.44
	25	6	0	91.7	2.07	0.43
	Overall	17	5.6	91.7	2.07	0.50
Tryptamine	5	6	0	99.6	9.75	0.81
	15	6	0	112.6	3.44	0.35
	25	6	0	106.1	6.60	0.07
	Overall	18	5.6	106.1	6.60	0.41
Tyramine	5	6	0	89.5	1.29	0.91
	15	6	0	115.1	0.42	0.34
	25	5	16.6	102.3	0.86	0.73
	Overall	17	5.6	102.3	0.86	0.66

According to Brasil (2015), the precision of the method is related to the dispersion of the recoveries from independent assays. It is described as the relative standard deviations – RSD of the recoveries of six repetitions of the blank sample of sausage spiked with the three different concentrations of a pool of all five amines. The RSD ranged from 0.07% for tryptamine at the higher spike levels (25 µg/g) to 3.79% for serotonin also at the higher spike level. These values are in accordance with Brazil (2015), with a maximum RSD of 20%, for samples enriched with 5 to 30 µg/g. Therefore, the method was precise, providing results that agreed with the Brazilian guidelines and AOAC (2016).

Table 4. Parameters describing the linearity of the analytical curves in solvent (HCl) and in the matrix during determination of ten bioactive amines in dry fermented sausage by ion-pair HPLC-Fluorescence

Amines	Linearity of the analytical curve					
	In solvent (HCl, 0.1M)			In the matrix (sausage extract)		
	Inclination (a)	Intercept (b)	R2	Inclination (a)	Intercept (b)	R2
Agmatine	246516.73	361202.67	0.9811	256878.81	444289.64	0.9810
Cadaverine	427604.24	275266.87	0.9942	419295.04	515503.85	0.9941
Histamine	367788.18	924144.21	0.9967	316943.90	1529455.01	0.9962
Phenylethylamine	283852.86	43346.11	0.9977	315331.47	19429.47	0.9982
Putrescine	745806.39	1103141.08	0.9968	921118.09	1096224.36	0.9965
Serotonin	242798.23	164968.16	0.9933	256131.96	232071.64	0.9935
Spermidine	225890.70	114999.15	0.9960	234669.54	114157.60	0.9965
Spermine	225482.35	402827.67	0.9974	226681.35	450005.45	0.9972
Tryptamine	201405.08	99734.66	0.9920	209285.26	85642.39	0.9916
Tyramine	429727.67	407839.21	0.9962	425605.11	524227.38	0.9960

n = 6
Linear equation: y=ax+b.
R^2: correlation coefficient.

3.2.4. Limits of detection and quantification

The limits of detection (LOD) and quantitation (LOQ) for each bioactive amine are shown in table 5. The LOD was established as the lowest concentrations of each analyte corresponding to three times the signal-to-noise ratio. They ranged from 0.47 to 1.22 mg/kg, with the lowest value for phenylethylamine and the highest for histamine. The LOQ, established as the first points of the calibration curves, ranged from 1.56 to 4.08 mg/kg, with the lowest for phenylethylamine and the highest for histamine. When comparing these quantification limits for the amines with those described in the literature for sausages (Caccioppoli et al., 2006; Giroto et al., 2010; De Mey et al., 2014; Mologni et al., 2018; Debadé et al., 2020; Roselino et al., 2020), similar results were found for most of them, however, lower LOQ were found in this study for phenylethylamine, tryptamine and spermidine.

There is no legislation for amines in dry fermented sausages; however, according to EFSA (2011), the NOAEL levels for histamine in a meal are 50 mg for normal individual, and no

detectable levels for histamine intolerant individuals. The NOAEL for tyramine in a meal is 600, 50 and 6 mg for normal, for individuals which are normal, taking third generation and classical MAOI. Therefore, the LOQ values achieved are adequate for the analysis of these compounds in dry fermented sausages and are sensitive enough to quantitate amines to warrant safety regarding histamine and tyramine. However, even though the limit of detection for histamine (1.22 mg/kg), which is compatible or even lower compared with literature values (Anderegg et al., 2020) cannot warrant that the product is histamine free as needed for histamine intolerant individuals.

Table 5. Limits of detection (LOD) and quantification (LOQ) during analysis of ten bioactive amines in dry fermented sausage by ion-pair HPLC and fluorescence detection

Amines	**Limits (mg/kg) of**	
	Detection	**Quantification**
Agmatine	0.85	2.84
Cadaverine	0.72	2.40
Histamine	1.22	4.08
Phenylethylamine	0.47	1.56
Putrescine	1.17	3.91
Serotonin	0.67	2.25
Spermidine	0.54	1.80
Spermine	0.62	2.07
Tryptamine	0.50	1.67
Tyramine	0.62	2.07

3.3. Analysis of real samples

Overall, 148 dry fermented sausages were analyzed, including Hamburgues, Mini Hamburgues, Salaminho, Italian, Gourmet Italian, Milano, and Serrano (Table 6). These types are the most widely available in the Brazilian market. The largest number of samples was of Salaminho (41; 27.7%), Hamburgues (39; 26.4%), Milano (37; 25.0%). A smaller number of Italian, gourmet Italian and Mini Hamburgues (9; 6.1% each) were found, and even smaller for serrano (4; 2.7%).

All ten amines were found only in the Hamburgues and Salaminho sausages (Table 6). Nine amines were detected in Milano, eight in Serrano, seven in gourmet Italian and four in mini Hamburgues. Spermidine and tyramine were present in all seven types of sausages; cadaverine, histamine, putrescine and spermine were detected in six of the sausage; phenylethylamine was

present in 5 types, tryptamine in five and agmatine and serotonin in three. Based on these results, agmatine and serotonin were the less frequent amines in the dry fermented sausages. Agmatine was only detected in Hamburgues, Salaminho, and Milano at low levels (≤16 mg/kg); whereas serotonin was present only in Hamburgues and Salaminho at low levels (≤5.0 mg/kg), and in Serrano at higher levels ≤42.12 mg/kg. Regarding the polyamines, spermine was also present in all (nd-409.00 mg/kg), except for Italian whereas spermidine was present in all types of sausage (nd-14.00 mg/kg). The presence of spermine and low levels of spermidine in sausages is expected, since these polyamines are present in meat (Silva & Gloria, 2002; Custódio et al., 2018, Muñoz-Esparza et al., 2021). Low levels of putrescine are also expected, as it is an obligate precursor of polyamines (Muñoz-Esparza et al., 2021). It was present in most sausages (nd-1116.4 mg/kg), except Mini-Hamburgues. However, high putrescine and cadaverine levels are suggestive of poor hygiene conditions during processing, or poor raw material quality and/or contamination during processing (Durak-Dados et al., 2020; Saewan et al., 2021; Wójcik et al., 2021). Cadaverine was present in most of the samples (nd-232.63 mg/kg) except for Mini-Hamburgues. Other amines can be formed during fermentation by the activity of microbial enzymes (amino acid decarboxylase) that can decarboxylate free amino acids forming amines (Durak-Dados et al., 2020; Muñoz-Esparza et al., 2021; Dasa et al. 2022).

The prevalence of amines in the sausages varied as indicated in Fig. 2. The prevalent amine was tyramine, for all the types of dry fermented sausage analyzed, at percentages that varied from 33.2% in Salaminho up to 81.4% in Mini-Hamburgues. The second prevalent amine was putrescine in Hamburgues, Salaminho, Italian and Milano, at values from 25.8% in Milano up to 31.7% in Hamburgues. Gourmet Italian, Serrano, and Mini Hamburgues had a different profile compared to the others. In the first, histamine was the second prevalent amine, at 28.9%, followed by putrescine with 22.0%. In Serrano, the second prevalent amine was serotonin (34.0%), followed by histamine (20.3%). Whereas in Mini Hamburgues, tyramine encompassed 81.4% of the total amine levels followed by spermine. Based on the prevalence of amines, for main patterns can be observed: Gourmet Italian, Serrano, Mini-Hamburgues and the other.

Interesting to observe that the Mini Hamburgues sausages contained only four amines (phenylethylamine, spermidine, spermine, and tyramine), whereas Hamburgues contained all ten. Both sausages are produced in a similar way, using the same ingredients. The only difference is the diameter of the sausage, which is smaller for the Mini Hamburgues (5 cm) compared to the typical Hamburgues (7 cm). It is possible that, due to the smaller diameter of the Mini Hamburgues

sausage, dehydration can occur faster, changing the conditions and decreasing the time available for the formation of amines. According to the literature (Bover-Cid et al., 1999; Komprda et al., 2004; Komprda et al., 2009), the diameter of the sausage can affect proteolysis and amino acid transformations during sausage preparation. Another interesting difference was observed for the Italian sausages. The gourmet Italian sausage contained more amines - seven (cadaverine, histamine, putrescine, spermidine, spermine, tryptamine and tyramine), compared to the Italian sausage (only five amines). The difference between these two products is that wine was added to the gourmet Italian formulation and may have contributed with amines typical of wine.

When comparing the average total amine contents by type of sausage (Table 6), there was no significant difference between the types of sausage (probably due to the varying levels of amines found), except for Serrano, which had significantly lower levels (40.73 mg/kg) compared to the others. Total levels ranged from 3.61 mg/kg (in hamburger type) to 1973 mg/kg (Italian gourmet). Compared to the literature, similar levels of amines have been reported for different dry fermented sausages (Caccioppoli et al., 2006; Giroto al., 2010; De Mey et al., 2014; Molognoni et al., 2018; Roselino et al., 2020). When comparing the individual amines for the different types of sausages, significant difference ($p \leq 0.05$) was observed in the means only for histamine, phenylethylamine, spermine and tyramine (Table 6). Gourmet Italian sausages had higher levels of histamine and tyramine ($p \leq 0.05$), compared to the others. This may be because the gourmet Italian sausage has wine in its composition, which is a product rich in histamine and tyramine (Pérez-Magariño et al.,2020; Shimoji et al., 2020). Lower levels of spermine were found in Serrano, compared to the other sausages analyzed. These lower values are similar to those found by Li et al. (2019), for fermented dry Chinese sausages.

To better understand the similarities and differences among the dry fermented sausages regarding amines, principal component analysis (PCA) and hierarchical cluster analysis (HCA) of the mean contents of free bioactive amines were used. In the PCA analysis (Fig. 3a), PC1 represented 55.1% of the variance, whereas PC2 represented 20.9% of the variance, providing a total of 76.0% of the variance. Fig. 3b shows the loading graph (factorial load) of the main components of the amines in the seven types of dry fermented sausages. Cadaverine, spermidine, putrescine, tyramine, spermine and histamine affected positively the first component, whereas phenylethylamine, serotonin, tyramine and agmatine contributed in a negative way. For the second principal component phenylethylamine, cadaverine and spermidine contributed in a positive way, and serotonin, tryptamine, agmatine, histamine, spermidine, tyramine, and putrescine contributed in a negative way to this component.

Table 6. Occurrence (%), (number of positive/analyzed samples) and mean levels ± standard deviation (range) of ten free bioactive amines in different types of dry fermented sausage from the Brazilian market

Amines	**% Occurrence (number positive/total) and Mean levels ± standard deviation (Minimum-maximum) in mg/kg**						
	Hamburgues	**Mini-hamburgues**	**Salaminho**	**Italian**	**Gourmet Italian**	**Milano**	**Serrano**
Agmatine	53.8% (21/39) 3.19 ± 6.20 [a] (nd-15.70)	(0/9) nd [a]	51.2% (21/41) 1.46 ± 0.37 [a] (1.20-1.90)	(0/9) nd [a]	(0/9) nd [a]	91.9% (34/37) 1.37 ± 0,79 [a] (nd-2.56)	(0/4) nd [a]
Cadaverine	76.9% (30/39) 22.92 ± 30.15 [a] (nd-82.62)	(0/9) nd [a]	58.5% (24/41) 21.61 ± 29.30 [a] (nd-77.36)	33.3% (3/9) 25.79 ± 50.48 [a] (nd-232.6)	66.7% (6/9) 34.72 ± 55.97 [a] (nd-99.30)	81.1% (30/37) 57.94 ± 76.63 [a] (nd-227.0)	75% (3/4) 0.52 ± 0.65 [a] (nd-1.47)
Histamine	38.5% (15/39) 39.78 ± 99.37 [b] (nd-285.26)	(0/9) nd [b]	73.2% (30/41) 15.37 ± 43.08 [b] (nd-130.2)	66.6% (6/9) 44.12 ± 79.97 [b] (nd-185.5)	66.7% (6/9) 514.6 ± 691.8 [a] (nd-1301)	43.2% (16/37) 36.08 ± 67.08 [b] (nd-194.6)	100% (4/4) 20.79 ± 22.58 [b] (1.16-41.38)
Phenyletylamine	74.4% (29/39) 1.74 ± 1.94 [a] (nd-4.30)	33.3% (3/9) 2.97 ± 5.15 [a] (nd-8.93)	43.9% (18/41) 2.9 ± 2.16 [a] (nd-4.20)	(0/9) nd [b]	(0/9) nd [b]	86.5% (32/37) 3.08 ± 3.25 [a] (nd-8.40)	50% (2/4) 2.31 ± 2.74 [a] (nd-5.40)
Putrescine	76.9% (30/39) 158.5 ± 387.9 [a] (nd-1116.4)	(0/9) nd [a]	90.2% (37/41) 93.76 ± 160.6 [a] (nd-501.66)	66.6% (6/9) 68.0 ± 94.51 [a] (nd-232.6)	66.7% (6/9) 391.4 ± 472.4 [a] (nd-916.1)	100% (37/37) 98.39 ± 116.3 [a] (3.91-366.6)	100% (4/4) 1.60 ± 1.54 [a] (0.26-2.98)
Serotonin	30.76% (12/39) 0.60 ± 1.42 [a] (nd-3.51)	(0/9) nd [a]	14.63% (6/41) 1.69 ± 2.91 [a] (nd-5.05)	(0/9) nd [a]	(0/9) nd [a]	(0/37) nd [a]	100% (4/4) 34.80 ± 6.31[a] (28.00-42.12)
Spermidine	100% (39/39) 3.73 ± 4.38 [a] (0.43-14.00)	66.7% (6/9) 1.08 ± 1.54 [a] (nd-2.90)	56.1% (23/41) 1.89 ± 1.95 [a] (nd-6.77)	55.5% (5/9) 1.24 ± 1.79 [a] (nd-5.62)	66.7% (6/9) 2.78 ± 4.32 [a] (nd-7.77)	81.1% (30/37) 0.80 ± 1.04 [a] (nd-2.90)	100% (4/4) 0.59 ± 0.68 [a] (nd-1.27)
Spermine	84.6% (33/39) 94.99 ± 83.59 [a] (25.60-211.6)	33.3% (3/9) 62.72 ± 108.6 [a] (nd-188.2)	78.0% (32/41) 80.92 ± 78.02 [a] (5.50-162.3)	(0/9) nd [a]	100% (9/9) 180.2 ± 198.7 [a] (49.45-409.0)	91.9% (34/37) 22.89 ± 11.37 [a] (nd-34.46)	100% (4/4) 2.18 ± 0.36 [b] (1.89-2.67)
Tryptamine	53.8% (21/39) 1.58 ± 3.03 [a] (nd-7.70)	(0/9) nd [a]	51.2% (21/41) 1.57 ± 2.31 [a] (nd-5.85)	(0/9) nd [a]	33.3% (3/9) 0.78 ± 1.35 [a] (nd-2.35)	91.9% (34/37) 15.55 ± 22.52 [a] (nd-69.64)	(0/4) nd [a]
Tyramine	100% (39/39) 174.2 ± 223.0 [b] (0.95-709.4)	100% (9/9) 292.6 ± 266.3 [ab] (11.83-541.49)	100% (41/41) 109.6 ± 89.42 [b] (24.02-325.8)	100% (9/9) 95.2 ± 74.85 [b] (29.38-224.9)	100% (9/9) 657.7 ± 453.3 [a] (266.20-1154)	10.8% (4/37) 146.4 ± 98.44 [b] (nd-268.1)	100% (4/4) 39.44 ±44.23 [b] (1.09-79.62)
Total	385.5 ± 538.9 [a] (36.15-1418)	107.8 ± 276.9 [a] (37.97-877.9)	272.7 ± 382.5 [a] (8.70-1002)	367.8 ± 507.2 [a] (10.8-1617)	352.3 ± 752.7 [a] (82.91-1973)	352.3 ± 422.4 [a] (81.28-1162)	40.73 ± 61.77 [b] (61.17-157.8)

nd - not detected (≤LOQ – limit of quantification.

Mean values with different superscripts in the same line are significantly different (Tukey test, $p \leq 0.05$).

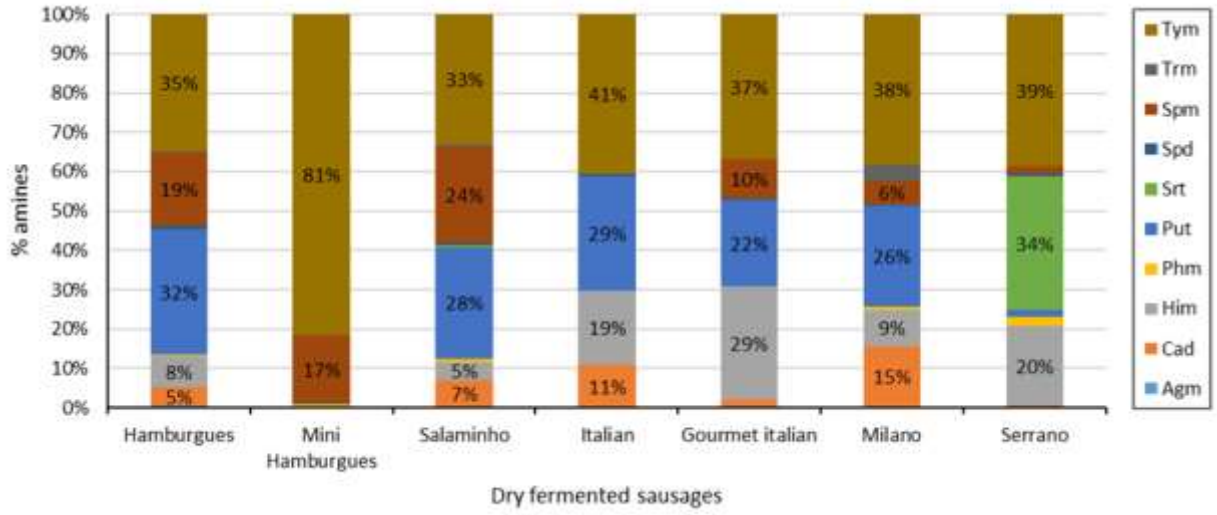
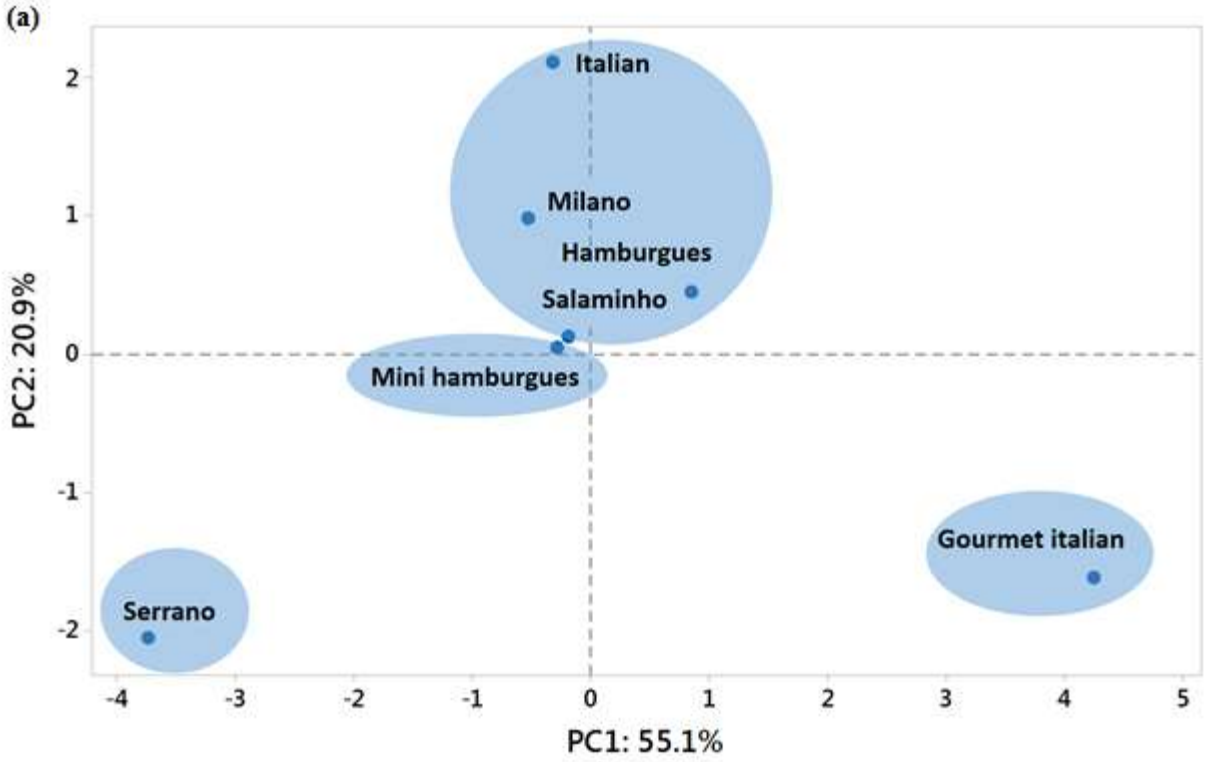
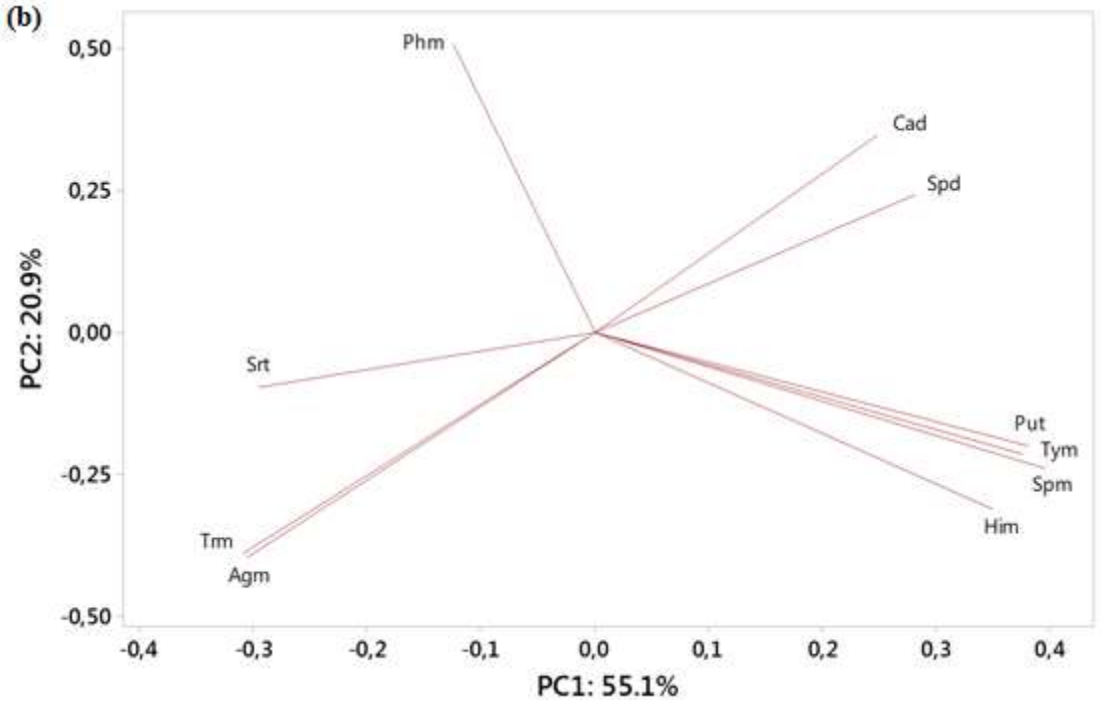


Figure 2. Occurrence of free bioactive amines in seven different types of Brazilian dry fermented sausage
Tym – Tyramine; Trm – Tryptamine; Spm – Spermine; Spd – Spermidine; Srt – Serotonin, Put – Putrescine; Phm – Phenylethylamine; Him – Histamine; Cad – Cadaverine; Agm – Agmatine.

The dendrogram of the degree of similarity of the sausages is shown in Fig. 3c. It can be seen that with about 90% similarity, four groups are formed. Gourmet Italian, Mini Hamburgues and Serrano types of dry fermented sausages formed individual groups. The Gourmet Italian stands out for having very high levels of histamine, tyramine and spermine compared to the others. Putrescine, tyramine, spermine and histamine contributed positively to PC1 and negatively to PC2 of this type. The Mini Hamburgues stood out for having only phenylethylamine, spermidine, spermine and tyramine, with tyramine at the highest concentration. Tyramine and spermine contribute positively to PC1 and negatively to PC2. Spermidine contribute positively to PC1 and PC2. Phenylethylamine contributed negatively to PC1 and positively to PC2. Serotonin, tryptamine and agmatine contributed negatively to PC2. The Serrano type had the lowest concentration of tyramine among the other types. Tyramine contributed positively to PC1 and negatively to PC2. Phenylethylamine contributed negatively to PC1 and positively to PC2. Histamine, putrescine and spermine contributes positively to PC1 and negatively to PC2. Tryptamine, agmatine and serotonin contributed negatively to PC1 and PC2 on this type of sausage. Cadaverine and spermidine contributed positively to PC1 and PC2. Serotonin The Italian, Milano, Salaminho and Hamburgues sausages formed another cluster, in which cadaverine and spermidine contributed positively to PC1 and phenylethylamine to PC2.

Based on the results, dry fermented sausages are rich sources of free bioactive amines. Some of them are typical of fresh pork meat, including spermine, spermidine and agmatine (Custódio et al., 2018). And these amines can exert relevant roles in health (Dalla-Paula et al., 2021) and meat products stability due to their antioxidant potential (Xiong 2017). However, the formation and accumulation of tyramine and histamine is worrisome, as these amines are incriminated in food intolerance, food poisoning and hypertensive crisis due to MAOI drugs interaction (EFSA, 2011; Durak-Dados et al., 2020). High levels of putrescine and cadaverine can potentiate the toxicity of histamine and tyramine (EFSA, 2011; Durak-Dados et al., 2020). They can also impart a putrid flavor to the product, thereby affecting its acceptability (Westling et al., 2016; Lang et al., 2023). High levels of tryptamine and phenylethylamine can lead to vasoconstriction (Del Rio et al., 2020; Sanlier et al., 2021). Therefore, actions to mitigation the formation and accumulation of some amines in dry fermented sausages is a urgent need. Numerous factors can influence the formation of bioactive amines in dry fermented sausages. Among them, the use of good manufacturing practices; the use of good quality meat and its maintenance at low temperatures during storage and processing; the use of ingredients free of microbial contaminants and free bioactive amines; the assurance of hygienic conditions preventing the contamination of the raw material and throughout processing; the use of starter culture which does not have amino acid decarboxylating activity, especially for histidine and tyrosine (Gardini et al., 2016; Ruiz-Capillas et al., 2019). The manufacturing temperature of fermented dry sausages varies between 4 and 7 °C when preparing the mixture, rises between 18 and 24 °C during the fermentation period and is reduced from 12 to 15 °C during the drying and harvesting period. Therefore, it is necessary to select starter cultures that are able to grow in a wide range of temperatures, in addition to having tolerance to adverse conditions including the presence of sodium chloride, sodium nitrite and acidic pH, being essential that they adapt properly to the conditions of the food matrix (Agüero et al., 2020).


(a)
Italian
Milano
Hamburgues
Salaminho
Mini hamburgues
Gourmet italian
Serrano
PC2: 20.9%
PC1: 55.1%
2
1
0
-1
-2
-4
-3
-2
-1
0
1
2
3
4
5


(b)
Phm
Cad
Spd
Srt
Put
Tym
Spm
Him
Trm
Agm
PC2: 20.9%
PC1: 55.1%
0,50
0,25
0,00
-0,25
-0,50
-0,4
-0,3
-0,2
-0,1
0,0
0,1
0,2
0,3
0,4

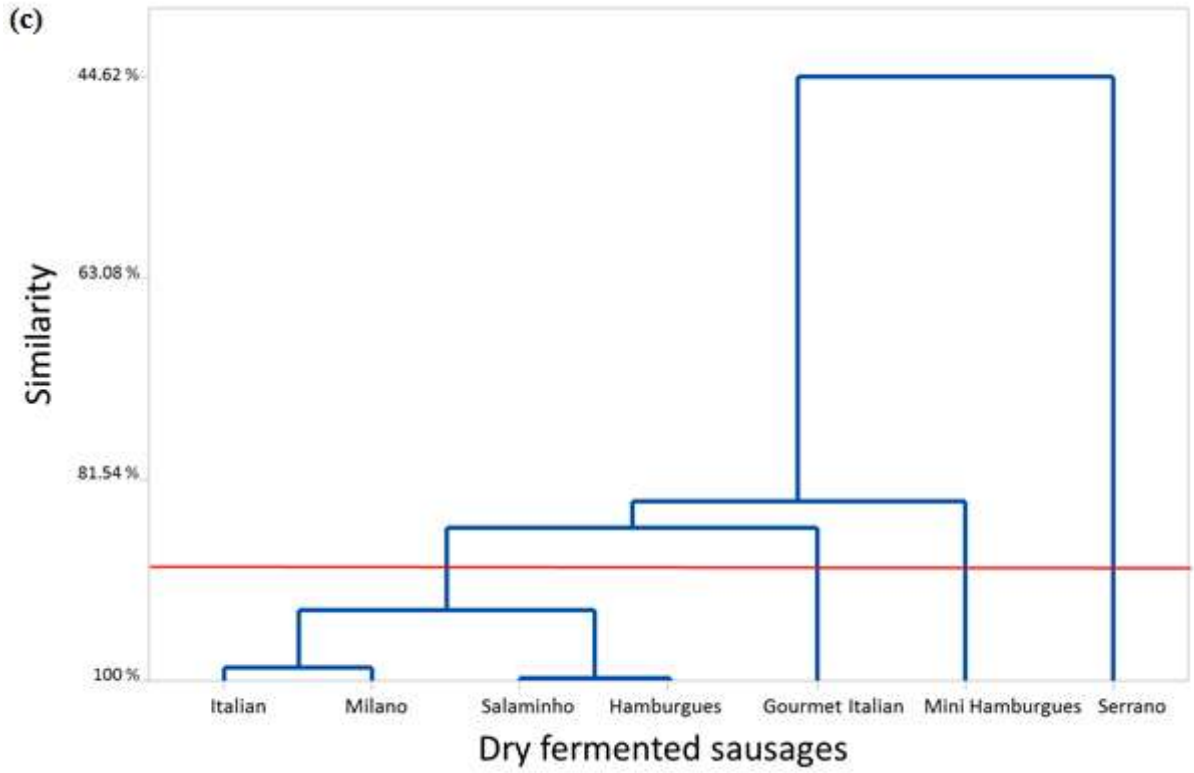


Figure 3. Principal component analysis of ten free bioactive amines in seven different types of dry fermented sausage: (a) Graph of scores of the main components; (b) loading graph (factorial load) of the main components; and (c) Similarity dendrogram of free bioactive amines of free bioactive amines in the seven types of sausages analyzed. Tym - Tyramine; Trm - Tryptamine; Spm - Spermine; Spd - Spermidine; Srt - Serotonin, Put - Putrescine; Phm – Phenylethylamine; Him – Histamine; Cad- Cadaverine; Agm- Agmatine.

4. CONCLUSIONS

A dilute and shoot HPLC-fluorescence method was proposed for the analysis of ten free bioactive amines in dry fermented sausages. No fat removal step was required. There was no matrix effect, therefore calibration curve in solvents could be used. The method was simple, fast, and fit for the purpose with adequate limits of quantification (1.56 – 4.08 mg/kg). The method was used to quantify ten amines in dry fermented sausages from the Brazilian market. The sausages had different amines profile: Hamburgues and Salaminho sausages had all 10 amines whereas Mini Hamburgues had the lower number of amines (4). Common to all sausages were tyramine and spermidine. The mean total levels of amines varied from 40.73 mg/kg in Serrano up to 385.5 mg/kg for Hamburgues, which did not differ from the others. Tyramine was the amine which contributed the most to total levels (33.2 up to 81.5%). It was followed by putrescine for most sausages, but by histamine in Gourmet Italian and by serotonin followed by histamine in Serrano. Gourmet Italian had significantly higher levels of tyramine and histamine. Serrano had lower levels of spermine ($p \leq 0.05$). PCA and HCA allowed distinction of sausages regarding

bioactive amines, confirming previous findings. Gourmet Italian, Serrano, and Mini Hamburgues stood alone, whereas the other types of sausage were clustered together due to their similarities. The high levels of undesirable amines found indicate an urgent need to ascertain factors affecting amines formation in dry fermented sausage to mitigate their formation.

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CAPÍTULO III - Influence of storage time on bioactive amines in different types of dried fermented sausage

Abstract

Sausages are described as being rich sources of biogenic amines. However scarce information is available about the formation and levels of amines in Brazilian sausages. The objective of this study was to investigate the changes on bioactive amines during 90 days of refrigerated storage of four types of dry fermented sausages. Four types of dry fermented sausages were obtained from an industry. The samples complied with the standards of identity and quality (physico-chemical and microbiological). The sausages, 45 days after processing, were rich in tyramine and spermine but Gourmet Italian was also rich in histamine. Agmatine, phenylethylamine and serotonin were not detected in any sample. Gourmet Italian had the largest diversity of amines (7), followed by Hamburgues (4), Mini Hamburgues and Salaminho (3). The mean total levels of amines varied from 95.72 mg/kg (Salaminho) up to 1358 mg/kg (Gourmet Italian). Tyramine (32.3-70.4%) contributed the most to total levels in most sausages except that putrescine was prevalent in Salaminho (38.6%). Histamine was only found in Gourmet Italian sausage suggesting that the wine added in the formulation contributed with this amine and putrescine. The levels of histamine and tyramine in the samples could cause adverse effects to human health. There was strong correlation between amines suggesting that their formation are affected by the same mechanism, for example tyramine and tryptamine, spermine and spermidine and histamine and putrescine. During refrigerated storage, there were significant changes on the amines, indicating that the decarboxylating enzymes are still active. Tyramine and tryptamine accumulate at the early days of storage or during processing; whereas histamine, phenylethylamine and putrescine are formed at later storage days. The change on amines followed polynomial regression. These results indicate that the formation of amines continues during refrigerated storage.

Key words: Biogenic amines, histamine, tyramine, HPLC-FL

1. INTRODUCTION

Meat is a component of high nutritional value for a balanced diet. However, it is perishable and conservation techniques have been widely used to offer stable meat products to the consumers with longer shelf life (Stefanello et al 2022). Dry fermented sausages, for example, are traditional products with different production processes determined by the origin and region with the use of different types of meat, ingredients, seasonings, and manufacturing process (EFSA, 2020; Stefanello et al., 2022).

According to the Observatory of Economic Complexity (OEC), Brazil in 2020 exported U$116 million in dry fermented sausages, making it the 12th largest exporter of sausages in the world. In the same year, sausage was the 152nd most exported product in Brazil. The main destinations for sausage exports from Brazil are Angola ($21.5 million), Venezuela ($17.3 million), Japan ($10.7 million), Cuba ($6.44 million) and Ghana ($ 6.14 million). In 2019 alone, the demand for Brazilian sausages increased, with a variation of 8.49% compared to 2018. Between 2017 and 2019, exports of sausages grew 10.48%, transporting US$ 112 to the exporter 0.57 million for the year 2019.

However, several studies have reported the occurrence of bioactive amines in dry fermented sausages (Durak-Dadoset al., 2020; Wójcik et al., 2022), in special tyramine and histamine, which, at high levels, are associated with adverse effect to human health (Latorre-Moratalla et al., 2017; Tabanelli, 2020). Histamine can cause histamine poisoning with symptoms like flushing, rash, nausea, vomiting, palpitation and sweating (Gonzalez et al., 2021; Tomaru et al., 2022). Histamine in foods is also associated with histamine sensitivity or intolerance, which is becoming more evident nowadays (Schnedl et al., 2021). In this case, any histamine present in food can cause histamine intolerance, with symptoms of flushing, rash and nausea (Gonzalez et al., 2021; Tomaru et al., 2022). According to EFSA (2011), the no adverse effect levels (NOAEL) for histamine is 50 mg/meal for normal individuals, however, for histamine intolerant individuals, no detected levels are required in the foods. With respected to tyramine, the symptoms of tyramine intoxication, also called cheese reaction, due to its prevalence in cheese, are migraines, visual changes, nausea and hypertensive crisis in individuals under monoaminoxidase inhibitor (MAOI) drugs (EFSA, 2011; Darnay et al., 2022). The NOAEL level for tyramine is described in three different situations. For normal individuals, the NOAEL is 600 mg/meal, for those taking third generation MAOI drugs, the NOAEL is 50 mg/meal, whereas for individuals taking classical MAOI drugs the NOAEL is 6 mg/meal (EFSA, 2011).

The occurrence of histamine and tyramine in dried fermented sausage has been reported in the literature. In Brazil, in a recent study, our research group found high levels of amines in the different types of sausage available in the market. Levels of histamine and tyramine up to 1301.0 and 1154.3 mg/kg, respectively, were found, which is worrisome. By consuming 50 g of sausages containing high levels of these amines, the intake of histamine would be 65 mg, which would be detrimental to human health. In the case of tyramine, the intake would be of 58 mg of tyramine, which would be enough to cause hypertensive crisis in individuals under classical and third generation MAOI drugs.

The formation and accumulation of bioactive amines in dried fermented sausage can result from several factors, including the type and quality of the raw materials (Ruiz-Capillas et al., 2019), added ingredients, starter cultures and microbial contamination during processing (Kononiuk et al., 2020). The intensity of bioactive formation depends on several factors, including proteolysis which liberates free amino acids, microbial growth with acidification of the media and activation of microbial amino acid decarboxylase activity (Kononiuk et al., 2020). The formation of amines is a consequence of the decarboxylation of free amino acids, which mainly depends on the activity of microbial decarboxylating enzymes (Kononiuk et al., 2020). The conditions during processing such as pH value, water activity or temperature as well as the speed of their change, may favor bacterial growth and microbial decarboxylase synthesis and activity (Suzzi et al., 2003, Kononiuk et al., 2020). Lactic acid bacteria (LAB) are associated with the formation of tyramine and, to a lesser extent, may also contribute to the production of histamine, phenylethylamine, tryptamine, putrescine and cadaverine (Bover-Cid et al., 2001; Kononiuk et al., 2020).

There is no legislation or recommendation for biogenic amines in dry fermented sausages, as established for histamine content in scombroid fish (FDA, 2011; Brasil 2011; FAO, 2012; Özogul et al., 2019), however, due to the adverse effects to human health, efforts should be taken by the industries to minimize the formation and accumulation of biogenic amines in their products. There is scarce information regarding the profile and levels of amines in the different types of sausage in Brazil as well as the factors affecting amine formation. In this context, it is important to determine amines levels and understand the changes on the profile and levels of amines during sausage processing and storage in order to mitigate amines formation and accumulation. Therefore, the objective of this study was to determine the profile and levels of amines in the most popular dry fermented sausages in Brazil and to investigate the changes during refrigerates storage. This information would be important to ascertain the factors affecting amine formation and propose mitigation steps to minimize amine formation in dried fermented sausages.

2. MATERIAL AND METHODS

2.1. Material

Samples of four different types of dried fermented sausages, including, Mini Hamburgues Hamburgues, Gourmet Italian, and Salaminho were provided by an industry under the Sistema de Inspeção Federal (SIF). The samples were obtained at 45 days after processing and fermentation. Immediately after processing and fermentation, the samples were analyzed for physico-chemical characteristics, including lipids, protein, carbohydrate, moisture content, and water activity. The samples were also analyzed for microbiological characteristics (aerobic mesophiles, coagulase positive staphylococci, *Escherichia coli*, *Salmonella* ssp and *Listeria monocytogenes*). Levels of free bioactive amines were also investigated. In addition, the samples were screened for the predominant microorganism in the final product. that can be used as starter cultures or contaminants in sausages.

The reagents were analytical grade (p.a.). The solvents were p.a, except the HPLC solvent which was LC grade (acetonitrile). Ultrapure water was obtained from Milli-QTM (Millipore Corp., Milford, MA, USA). Organic and aqueous solvents for HPLC analysis were filtered through 0.45 μm pore size HVPL Membranes (Millipore Corp., Milford, MA, USA). The standards for bioactive amines (spermine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium octane sulfonate, Brij-35, β-mercaptoethanol, and *o*-phthalaldehyde (OPA), all analytical grade, were also from Sigma-Aldrich Chemical Co.

The materials used for the microbiological analyzes were of analytical grade and the culture medium consisted of acriflavine, nalidixic acid, Listeria agar, modified Baird-Parker, 3M Petrifilm Rapid Aerobic Count Plate, 3M Petrifilm (modified red-violet bile medium) contains 2,3,5-triphenyltetrazolium chloride and glucuronidase indicator, phosphate buffered solution, 0.1% peptone saline solution.

2.2. Methods of analysis

2.2.1. Determination of the physico-chemical characteristics

The determination of total lipids was performed through organic solvent extraction. The method consists of mixing the sample with chloroform, methanol, and water, in order to form two distinct phases, one of chloroform containing lipids, and another of methanol plus water, containing non-lipid substances. The chloroform phase with the fat is submitted to chloroform evaporation and the amount of fat obtained by weighing (ME_LAB_9625 – Internal procedure, extraction with organic solvent).

The protein content was determined following the ISO 1871:2009 standard procedure. The method consists in digesting an aliquot of the sample with concentrated sulfuric acid in the presence of catalysts to convert the organic into ammonium sulfate. Excess sodium hydroxide is added to the cooled digest to release the ammonia. The released ammonia is distilled from excess boric acid solution and then titrated with a standard solution of sulfuric or hydrochloric acid. The protein content is calculated from the amount of ammonia produced.

The method used for carbohydrate determination is spectrophotometric, following the manual of official methods for the Analysis of Food of Animal Origin of the Ministry of Agriculture, Livestock and Supply (BRASIL 2022). The method is based on the spectrophotometric quantification at 620 nm of the colored compound formed by the reaction between anthrone and glucose, resulting from the hydrolysis of carbohydrates present in the sample.

The moisture content was determined according to ISO 1447:1997, which consists of mixing an aliquot of the sample with sand and then drying at 103 ± 2 °C until constant mass.

Water activity wass determined using the method described in ISO 18787:2019 and in accordance with the manufacturer's instructions for the equipment used. A portion of the sample prepared immediately before analysis was used. The equipment provides the direct measurement of water activity.

2.2.2. Determination of the microbiological characteristics

The presence of *Salmonella ssp* was determined following the 3M AFNOR methodology 01/16-11/16, which consists of a Salmonella pathogen test kit that is part of the 3M Molecular Detection System platform, which combines isothermal DNA amplification and bioluminescence detection.

The quantification of *Escherichia coli* was performed as described in the AOAC Microbiological Method 998.08. 21st ed. 2019. The 3M Petrifilm (modified red-violet bile

medium) contained 2,3,5-triphenyltetrazolium chloride and glucuronidase indicator that forms a blue precipitate around any *E. coli* colonies present. Gas is formed from lactose fermentation by coliform bacteria (including *E. coli*). Glucuronidase negative bacteria form red colonies from the reduction of 2,3,5-triphenyltetrazolium chloride.

Coagulase positive staphylococci counting was performed as described in ISO 6888-1/A1, 2015. The Petrifilm Staph Express Count Plate (PSE) is a sample-ready culture medium system which contains a cold-water-soluble gelling agent. The culture medium is modified Baird-Parker, selective and differential for *S. aureus*, inhibiting the proliferation of other bacteria. Red-violet colonies growing on the plate are characterized as *S. aureus*. The use of PSE eliminates the need to carry out additional biochemical tests, such as the coagulase test, simplifying the analysis with reduced laboratory work and allowing the processing of a greater number of samples in a shorter time.

Aerobic mesophiles counts were performed using a Petrifilm Rapid Aerobic Count Plate, following AOAC 2015.13. 21st ed. 2019. The 3M Petrifilm Rapid Aerobic Count (RAC) Plate is a sample-ready culture medium system that contains nutrients, a cold-water-soluble gelling agent, and an indicator system that facilitates aerobic bacterial enumeration. 3M RAC Petrifilm Plates are used for the enumeration of aerobic bacteria in less than 24 h for most food matrices. 3M™ Food Safety is ISO (International Organization for Standardization) 9001 certified for design and manufacturing.

The presence of *Listeria monocytogenes* was determined following the 3M AFNOR methodology 01/16-11/16, 3M™ Molecular Detection Assay 2 - Listeria monocytogenes Test Kit uses isothermal amplification of unique DNA target sequences; the amplified sequences are detected by bioluminescence.

2.2.3. Determination of free bioactive amines

The free bioactive amines were extracted according to Braga et al. (2023). Briefly, the amines were extracted from 5 g sausage samples by three successive extractions with 7-mL of trichloroacetic acid (TCA, 5% w/v). The mixture was shaken (Ovan shaker, Barcelona, Spain) for 10 min, followed by centrifugation at 10,000 *g* at 4 °C for 20 min (MR23I refrigerated centrifuge, Jouan, Saint Herblain, France). The supernatants were combined and filtered through qualitative filter paper into a 25-mL volumetric flask, and the volume was completed with 5% TCA. An aliquot of the extract was filtered (0.45 µm membrane) immediately prior to HPLC analysis.

HPLC was performed using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a post-column derivatization apparatus between the column outlet and the spectrofluorimetric detector (Shimadzu RF-10AXL). A C18 column (Luna, Phenomenex, 4.6 x 250 mm, 5 μm) and a C18 pre-column (4x3 mm) were used in an oven (CTO-10 ASvp, Shimadzu, Kyoto, Japan) at 30 °C. An auto-injector and a HPLC interface control unit (CBM-20A) were also used. The mobile phases used were a solution of 0.2 M sodium acetate and 15 mM octanesulfonic acid sodium salt, pH adjusted to 4.9 with acetic acid (mobile phase A), and acetonitrile (mobile phase B). The gradient was: 0.01-17.99 min/2% B; 18.00-18.99 min/20% B; 19.00-39.99 min/5% B; 40.00-49.99 min/23% B; 50.00-50.49 min/35% B; 50.50-60.00 min./2% B. The post column derivation reagent (1.5 mL Brij-35, 1.5 mL β-mercaptoethanol, and 0.2 g OPA dissolved in 500 mL solution of 25 g boric acid and 22 g KOH, adjusted to pH 10.5 with 3% KOH) was delivered at 0.3 mL/min. The post-column reaction took place in the oven at 30 °C. The fluorescence detector was set at 340 nm excitation and 450 nm emission. The amines were identified by comparison of retention times of the analyte peaks in the sample with those of the standard solution and it was confirmed by addition of the suspected amine to the sample. Analyte concentrations were calculated by interpolation in the respective external analytical curves ($R^2 \geq 0.9811$).

2.2.4. Isolation and identification of prevalent micoorganisms by Maldi-Tof

The identification of bacteria present in dry fermented sausages was carried out in the manufacturing industry itself. Dough samples from each batch of dry fermented sausage were collected. After growth of the colonies in MRS medium (36 ºC/48 h), they were selected up to three colonies with different morphological characteristics in terms of size, color and appearance. Then the material was analyzed by mass spectrometry technique by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-ToF) using the VITEK® MS system (VITEK® MS - BioMériux –SML). A single fresh bacterial colony was removed at a time from the Petri dishes and transferred to a stainless steel target plate with subsequent addition of 1 μL of formic acid (70%) and 1 μL of α-cyano-4-hydroxycinnamic acid, which, then, was attached to the equipment. The generated mass spectrum, according to the protein profile of the bacteria, was compared with information from the database. To interpret the scores, the criteria recommended by the manufacturer were used, which defines scores ≥ 2,000 as identification at the species level, from 1,700 to 2,000 indicating identification at the genus level, and scores below 1,700 were not associated with any microorganism (Assis et al., 2017).

2.3. Influence of the storage time on the levels of free bioactive amines in four different types of dry fermented sausages

Samples of four types of dry fermented sausage (Hamburgues, Mini hamburgues, Salaminho and Gourmet Italian) were stored under refrigeration at 5 ± 1.5 ºC. At intervals of 15 days (45, 60, 75 and 90 days of salami processing), aliquots of salamis (approximately 100g) were collected and frozen for analysis of free bioactive amines. The rest of the salami was again packaged and stored under refrigeration at 4 ± 2 ºC The changes on amines during storage time were submitted to regression analysis using Excel.

3. STATISTICAL ANALYSIS

The results were submitted to the Kolmogorov-Smirnov (K-S) normality test using Minitab® (v. 16.2.3). The results followed normal distribution (physico-chemical and microbiological characteristics and bioactive amines) and were submitted to analysis of variance and the means were compared by the Tukey test at $p = 0,05$.

4. RESULTS AND DISCUSSION

4.1. Physico-chemical characteristics of the four different types of dry fermented sausages

The physico-chemical characteristics of the four different types of dried fermented sausages are indicated in Table 1. The major components of the sausages were lipids and proteins and, then carbohydrates, at much lower levels. The mean contents of lipids, for the four types of dry fermented sausage varied from 26.6 g/100 g in Gourmet Italian and Mini Hamburgues up to 31.70 g/100 g in Hamburgues. These results (and also individual results) are in compliance with the Brazilian legislation (Brasil, 2000), that establishes maximum lipids contents of 35 g/100 g for Hamburgues, Mini Hamburgues and Salaminho and 32 g/100 g for Italian. The mean contents of protein varied from 24.13 g/100 g in Hamburgues up to 30.65 g/100 g in Mini Hamburgues. In the case of protein, there are minimum levels, which are 23 g/100 g for Hamburgues and Mini hamburgues, 20 g/100 g for Salaminho and 24 g/100 g for Italian. Every sample met the requirements. The mean contents of carbohydrates ranged from nd up to 2.50 g/100 g for Mini Hamburgues and Salaminho, respectively. All of them were below 4 g/100 g (Brasil, 2000).

The maximum limits for moisture content are 40 g/100 g for Hamburgues, Mini Hamburgues and Salaminho and 35 g/100 g for Italian (Brasil, 2000). The mean levels of moisture

contents (30.70 – 34,31 g/100 g) agreed with the established limits. However, Gourmet Italian had a sample (16%) with moisture content higher than 35 g/100 g, which was above required levels. Regarding water activity, all analyzed samples met the requirement (Brasil, 2000) which establishes water activity < 0.920 for the Hamburgues, Mini Hamburgues, and Salaminho but < 0.900 for the Gourmet Italian.

There was no statistical difference between the different types of sausage regarding water activity and moisture content. However, there were significant difference ($p<0.05$) for lipids, protein and carbohydrate contents. Lipids contents were higher in Salaminho and Hamburgues compared to the others, whereas the contents of proteins were higher Mini Hamburgues and Gourmet Italian compared to Hamburgues. Lower levels of carbohydrate were found in Mini Hamburgues compared to the others. Even though there are differences in the formulation of some sausages, these differences were not enough to significantly affect the physicochemical characteristics. For example, Hamburgues and Mini Hamburgues are prepared using a minimum of 50% pork meat, whereas the others must use a minimum of 60%; but this difference did not affect the macro composition of the final products.

Table 1. Physical-chemical characteristics of the different types of dried fermented sausage

Parameter (unity)	**Mean ± sd (min-max) per type**			
	Hamburgues	**Mini Hamburgues**	**Salaminho**	**Gourmet Italian**
Lipids (g/100 g)	31.70 ± 2.64 [a] (28.70–33.70)	26.63 ± 0.76 [b] (25.80–27.30)	30.06 ± 1.25 [ab] (29.20–31.50)	26.61 ± 1.58 [b] (24.79–27.63)
Protein (g/100 g)	24.13 ± 1.20 [c] (23.00–25.40)	30.65 ± 1.19 [a] (29.28–31.48)	26.69 ± 0.79 [bc] (25.80–27.31)	28.41 ± 0.64 [ab] (27.94–29.14)
Moisture (g/100 g)	34.00 ± 2.66 [a] (31.09–37.00)	34.16 ± 2.18 [a] (31.80–36.10)	30.70 ± 1.83 [a] (28.70–32.30)	34.31 ± 2.35 [a] (32.60–37.00)
Carbohydrate (g/100 g)	2.10 ± 0.17 [a] (1.90–2.20)	nd [b]	2.50 ± 0.26 [a] (2.30–2.80)	2.13 ± 0.11 [a] (2.00–2.20)
Water activity (aw)	0.885 ± 0.007 [a] (0.881–0.894)	0.870 ± 0.016 [a] (0.852–0.882)	0.871 ± 0.008 [a] (0.862–0.878)	0.873 ± 0.008 [a] (0.868–0.884)

n – 6 with analysis in triplicate.
sd – standard deviation.
nd – < LQ (1.0 g/100 g).
Mean levels with the same letters in the same line are not statistically different (Tukey test, $p>0.05$).

4.2. Microbiological characteristics of the four different types of dry fermented sausages

The microbiological characteristics of the four different types of dried fermented sausages are indicated in Table 2. All sausage samples were in accordance with the microbiological parameters required by Resolution of the Collegiate Board n° 331 (Brasil, 2019), current legislation in Brazil. Salmonella ssp and *Listeria monocytogenes* were not present in all samples.

The counts of *E. coli* and coagulase positive staphylococci were $<1.0x10^1$ cfu/g for all types of sausages. The only microbiological parameter that showed statistical difference between the types of sausage was the aerobic mesophilic counts, which were higher in Salaminho compared to the others.

Table 2. Microbiological characteristics of different types of dried fermented sausage

Parameter (unity)	Mean ± sd (min-max) per type			
	Hamburgues	**Mini Hamburgues**	**Salaminho**	**Gourmet Italian**
Aerobic mesophiles (cfu/g)	$1.7x10^6 \pm 1.8x10^{6\,b}$ ($9.8x10^3$–$3.7x10^6$)	$1.7x10^6 \pm 2.9x10^{6\,b}$ ($4x10^5$–$2x10^6$)	$7.2x10^6 \pm 1.58x10^{6\,a}$ ($6x10^6$–$9x10^6$)	$2.23x10^6 \pm 1.07x10^{6\,b}$ ($1x10^6$–$2.9\ x10^6$)
Coagulase positive staphylococci (ufc/g)	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
Escherichia coli (ufc/g)	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
Salmonela ssp.	Absent	Absent	Absent	Absent
Listeria monocytogenes	Absent	Absent	Absent	Absent

n – 6 with analysis in duplicate.
sd – standard deviation.
Mean levels with different letters in the same line are not statistically different (Tukey test, $p>0.05$).

4.3. Free bioactive amines in the four different types of dry fermented sausages

As indicated in Table 3, the profile of amines in the different types of sausage at 45 days after processing varied widely. Gourmet Italian was the sausage with the most diversity of amines (7), followed by Hamburgues (4) and by Mini Hamburgues and Salaminho (both with 3 amines). Agmatine, phenylethylamine and serotonin were not detected in any sample. Spermine and tyramine were the only amines present in every type of sausage analyzed. Gourmet Italian sausage had two amines which were not present in any of the sausages: histamine and tryptamine. Cadaverine was only detected in Hamburgues and Gourmet Italian. Putrescine was detected in Salaminho and Gourmet Italian types. Spermidine was not detected in Salaminho type.

Mean total amines levels varied from 95.72 mg/kg (Salaminho) up to 1358 mg/kg in Gourmet Italian. The amine which contributed the most to total levels was tyramine, with percentages that varied from 32.3 up to 70.4 for Salaminho and Mini Hamburgues, respectively (Fig. 1); except for Salaminho, in which the prevalent amine was putrescine. (38.6%). The second amine in contribution to total levels was spermine representing 46.2% and 29.2%, for Hamburgues and Mini Hamburgues, respectively. In Salaminho, the second prevalent amine was tyramine (32.3%), followed by spermine (29.1%). In Gourmet Italian, the second prevalent amine was histamine (20.3%) followed by putrescine and putrescine (~15%).

Table 3. Levels of free bioactive amines in the different types of dried fermented sausage 45 days after processing

Amines	Mean levels ± sd (min-max) in mg/kg per type			
	Hamburgues	**Mini Hamburgues**	**Salaminho**	**Gourmet Italian**
Agmatine	nd [a]	nd [a]	nd [a]	nd [a]
Cadaverine	16.67 ± 2.71 [a] (nd-46.57)	nd [a]	nd [a]	37.45 ± 58.01 [a] (nd-112.34)
Histamine	nd [b]	nd [b]	nd [b]	276.3 ± 240.9 [a] (nd-564.60)
Phenylethylamine	nd [a]	nd [a]	nd [a]	nd [a]
Putrescine	nd [b]	nd [b]	37.08 ± 57.57[a] (nd-117.10)	206.5 ± 160.4 [a] (nd-331.21)
Serotonin	nd [a]	nd [a]	nd [a]	nd [a]
Spermidine	5.20 ± 4.45 [a] (nd-9.91)	1.36 ± 0.50[a] (0.84-2.06)	nd [a]	3.92 ± 6.08 [a] (nd-11.76)
Spermine	207.0 ± 170.7 [a] (nd-375.29)	92.17 ± 142.8 [a] (nd-276.51)	28.04 ± 22.41 [b] (nd-50.30)	196.5 ± 238.0 [a] (nd-499.22)
Tryptamine	nd [a]	nd [a]	nd [a]	0.57 ± 0.88 [a] (nd-1.71)
Tyramine	219.0 ± 171.4 [b] (59.06-450.92)	221.9 ± 149.5 [b] (30.90-351.07)	30.60 ± 47.50 [b] (nd-96.63)	636.5 ± 573.4 [a] (226.37-1375.89)
Total	447.9 ± 30.31 [b] (402.20-497.50)	315.5 ± 22.28 [b] (284.82-353.13)	95.72 ± 83.58 [b] (36.11-213.73)	1358 ± 1012 [a] (366.62-2598.33)

n – 6.
nd – not detected, LOQ – Agmatine 2.84 mg/kg; Cadaverine 2.40 mg/kg; Histamine 4.08 mg/kg; Phenylethylamine 1.56 mg/kg; Putrescine 3.91 mg/kg; Serotonin 2.25 mg/kg; Spermidine 1.80 mg/kg; Spermine 2.07 mg/kg; Tryptamine 1.67 mg/kg; Tyramine 2.07 mg/kg.
Different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).

When comparing the levels of individual amines among different sausage types, no significant difference was observed among levels of spermidine and tryptamine, which were present at low levels (nd-5.20 and nd-0.57 mg/kg, respectively. Cadaverine was higher in Hamburgues (16.67 mg/kg), compared to Mini Hamburgues and Salaminho (nd, each). Histamine was detected only in Gourmet Italian at very high levels (276 mg/kg). Putrescine was only detected in Salaminho, and Gourmet Italian, and no significant difference was observed between them. Lower spermine levels were found in Salaminho, compared to the others. Tyramine levels were higher in Gourmet Italian, compared to the others. When comparing total levels, Mini Hamburgues and Gourmet Italian had higher levels ($p \leq 0.05$) compared to the others.

The presence of spermine and spermidine in fresh pork meat has been described in the literature (Custódio et al., 2018), therefore they are typical of good quality meat. Custódio et al. (2018) also found that during storage, there were changes on spermidine and spermine levels, agmatine decreased and there was formation of putrescine, cadaverine and histamine. Therefore,

formation and accumulation of putrescine, cadaverine and histamine, can indicate raw material which has been stored, or the use of inadequate hygienic and sanitary conditions, or the contamination of the product with Enterobacteria (Durak-Dados et al., 2020). In fact, Enterobacteria are known to be prolific putrescine and cadaverine formation (Durak-Dados et al., 2020). The presence of tryptamine, tyramine and histamine can result from free amino acids decarboxylation activity from the added starter cultures, or contaminants during processing (Durak-Dados et al., 2020). Interesting to observe that, even though Hamburgues and Mini Hamburgues are made with the same percentage of pork meat (50%) ground to 3-6 mm, compared to the others (6-9 mm).

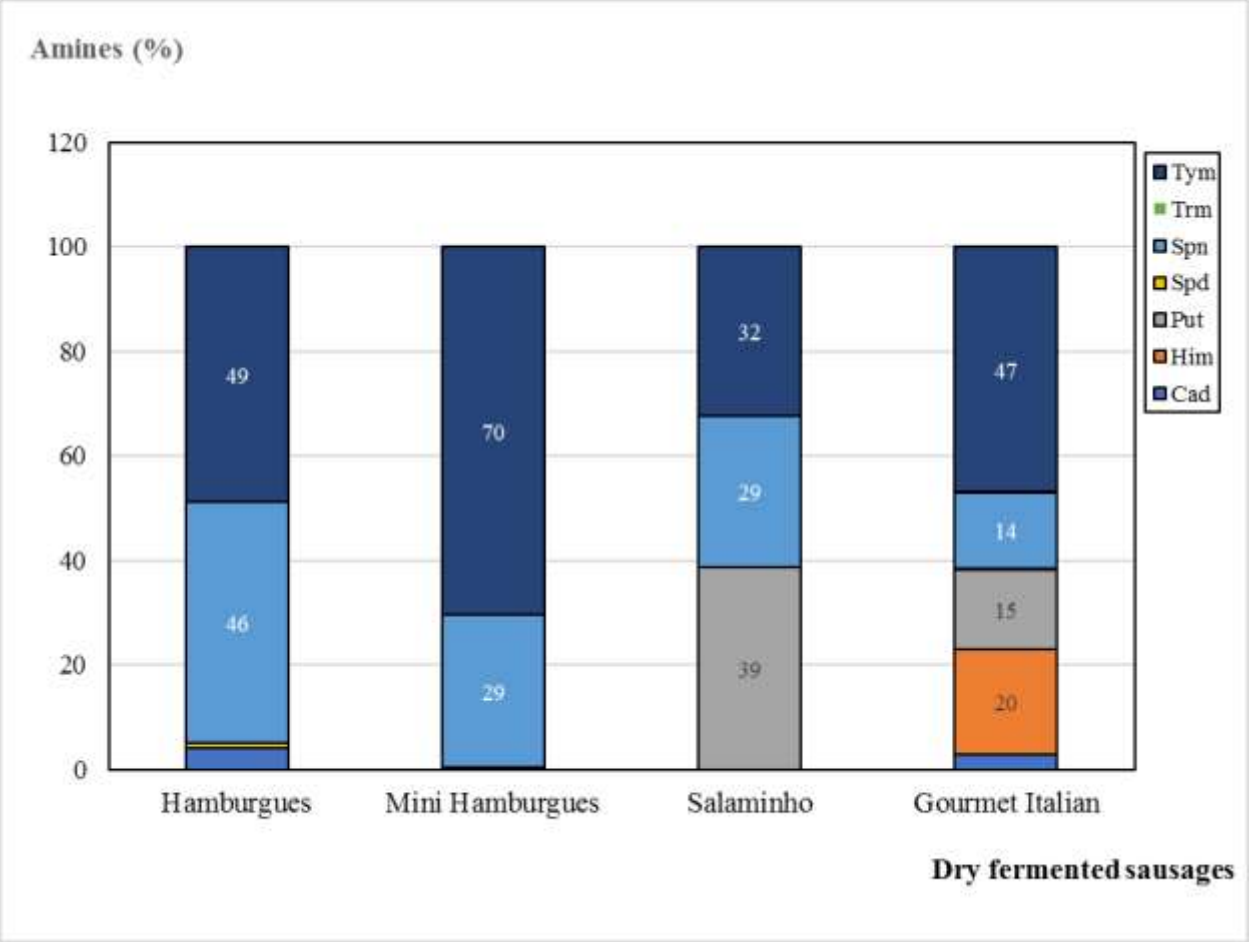


Figure 1. Contribution of free bioactive amines to total amines levels of different types of dry fermented sausage at 45 days of refrigerated storage (4 – 7 °C).
Cad – cadaverine, Him – histamine, Put – putrescine, Spd – Spermidine; Spn – Spermine, Trm – tryptamine, and tym – tyramine.

4.4. Correlation studies

Pearson's correlation was performed between the results of free bioactive amines 45 days after processing and physicochemical and microbiological characteristics. Pearson's correlation results are described in table 4. There was a very strong correlation ($\rho>0.90$) between histamine and putrescine, tyramine and cadaverine, tryptamine and cadaverine, tyramine and tryptamine, spermine and spermidine, tryptamine and total amines, and tyramine and total amines. These results suggest that some amines are affected by the same factors including, histamine and putrescine; tyramine, tryptamine and cadaverine; spermine and spermidine, tyramine and tryptamine. In addition, it suggests that tyramine and tryptamine are the amines which affect the most, total levels. Anderegg et al., 2020, evaluated sixty-two samples of Swiss dry fermented sausages with regard to the content of bioactive amines and found a significant correlation between the content of tyramine and putrescine. There was also correlation between total levels with putrescine and cadaverine (R^2 = 0.814 and 0.874, respectively). There was no significant correlation between the physico-chemical characteristics and the amines.

4.5. Identification of microorganisms

The predominant microorganisms identified in the dry fermented sausages using the Maldi-Tof technique are shown in table 5. Most of them are typical of meat and meat products. For example, *Staphylococcus saprophyticus*, *S xylosus* are commonly found in artisanal sausages (Talon and Leroy, 2014; Degenhardt, et al., 2021). Some have been used as a starter culture in the production of fermented meat, e.g., *Staphylococcus saprophyticus* (Laranjo et al., 2017; Organji et al. al., 2018).

Table 5. Microorganisms isolated from the four different types of dried fermented sausage and identified by Maldi-Tof

Sausage type	**Identified microorganisms by Maldi-Tof**
Hamburgues	*Staphylococcus saprophyticus,*
	Streptococcus mutans
Mini Hamburgues	*Staphylococcus xylosus,*
	Sphingomonas paucimobilis,
	Enterococcus faecalis
Salaminho	*Pediococcus pentosaceus*
Gourmet Italian	*Staphylococcus xylosus*

Table 4. Pearson's correlation between free bioactive amines and the physico-chemical and microbiological characteristics of dry fermented sausages at 45 days

	Fat	Protein	Moisture	Carbohyd.	Aw	Aerobic mesophile	Put	Cad	Tym	Him	Spd	Spm	Trm	Total amine
Put	-0.4260	0.1826	0.2548	0.2381	0.0491	0.0287	1.00							
Cad	0.0762	0.0081	-0.1022	0.1252	-0.1232	-0.0639	0.5465	1,00						
Tym	-0.1321	0.1971	0.0128	-0.0333	-0.2329	-0.2125	0.6087	**0.9357**	1.00					
Him	-0.4829	0.1505	0.3831	0.1910	0.1168	-0.1744	**0.9232**	0.3454	0.4309	1.00				
Spd	0.0492	-0.1034	0.1894	0.1025	0.1781	-0.1352	0.3011	0.5994	0.5783	0.1626	1.00			
Spm	-0.0275	0.0050	0.1602	0.0679	0.1925	-0.1447	0.2450	0.5625	0.4882	0.1157	**0.9004**	1.00		
Trm	-0.1298	0.1995	-0.0875	0.0961	-0.2089	-0.0013	0.6374	**0.9256**	**0.9235**	0.4152	0.6879	0.6666	1.00	
Total amine	-0.2650	0.1752	0.1802	0.0934	-0.0453	-0.1916	0.8141	0.8741	**0.9184**	0.6810	0.6623	0.6153	**0.9231**	1.00

n = 24.
Bold - Very Strong correlation; | | - Strong correlation; | | - Moderate correlation.
CAD – Cadaverine; HIM – Histamine; PHM – Phenylethylamine; PUT – Putrescine; SPD – Spermidine; SPM – Spermine; TRM – Tryptamine; TYM – Tyramine.

Some of the bacteria identified are lactic acid, e.g., *Pediococcus pentosaceus* and *Streptococcus mutans. Pediococcus pentosaceus* is Gram-positive, immobile, non-spore-forming cocci. It is commonly found in fermented products, such as pickled vegetables and silages. It is considered non-pathogenic and has been used in biotechnology and in the food industry (Chen et al., 2018). *Streptococcus mutans* is a Gran-positive cocci, it is usually present in milk serum, and can form biofilm (Jitpakdee et al., 2022). Since all the sausages included in this study use powdered milk as an optional ingredient, this microorganism can be identified in sausage. Furthermore, it *Enterococcus faecalis* is also a lactic acid bacterium, Gram-positive, facultative anaerobic, resistant to extreme environmental challenges and is usually found in the human oral cavity, and gastrointestinal tract (Almeida et al., 2018). It can produce bioactive amines through decarboxylation with tyrosine and phenylalanine as substrate (Perin et al., 2017). Burdychova et al. (2007) detected tyramine produced by Enterococcus faecalis in cheese samples (Barbieri et al., 2019).

Both *Pediococcus pentosaceus* and *Streptococcus mutans* contain the enzyme agmatine deiminase (AgDI) which breaks down agmatine as an initial substrate to produce putrescine via agmatine deiminase. Agmatine undergoes the sequential action of 3 enzymes: AgDI, putrescine carbamoyltransferase (PCT) and carbamate kinase (CK). AgDI acts on agmatine to produce an ammonium ion and N-carbamoyl putrescine which, in turn, is phosphorylated by (PCT) putrescine carbamoyl transferase to produce putrescine and carbamoyl phosphate (Barbieri et al., 2019).

In addition to lactic acid bacteria (LAB), species of the Micrococcaceae family, mainly belonging to the genus *Staphylococcus*, are commonly found in artisanal sausages, *S. saprophyticus*, *Staphylococcus xylosus*, are often predominant (Degenhardt, et al., 2021). *Staphylococcus saprophyticus* is a Gram-positive, coagulase-negative, non-hemolytic coccus that frequently colonizes humans and can be found in the gastrointestinal tract, vagina and perineum, it is also part of the intestinal and rectal microbiota of cattle, including pigs and cattle, it is a contaminant in meat and fermented meat products, therefore, it can be transferred to humans through the ingestion of these respective foods (Lawal et al., 2021). *Staphylococcus xylosus* belongs to the group of coagulase-negative staphylococci. It is a ubiquitous bacterium isolated throughout the food chain. It was isolated from animal skin (Nagase et al., 2002) and was one of the staphylococcal species found on cow teat skin (Leroy et al., 2010; Verdier-Metz et al., 2012). Consequently, *S. xylosus* has been frequently found in food products of animal origin such as raw milk and cheese, meat, and dry fermented sausages (Coton et al., 2010; Leroy et al., 2010). It has also been isolated in food manufacturing environments (Leroy et al., 2010), likely in relation to its potential to form biofilms (Planchon et al., 2009; Leroy et al., 2010). When this species is used as

a starter culture in the manufacture of cheeses and sausages, it can contribute to their flavor (Leroy et al., 2010; Talon and Leroy, 2014). Lu et al. (2015) demonstrated a synergistic action between *Latilactobacillus sakei* and *S. xylosus* and plant extracts to suppress the formation of tryptamine, putrescine, cadaverine, histamine and tyramine in traditional Chinese smoked horse meat and sausage during ripening and storage.*Sphingomonas paucimobilis* is a strictly aerobic and non-fermentative Gram-negative bacillus present in the environment – soil and water (Ionescu et al., 2022). It may have a flavin adenine dinucleotide (FAD)-dependent monoamine oxidase enzyme that promotes the conversion of tryptamine to indole-3-acetaldehyde (Lombardino et al., 2022).

4.6. Influence of storage time

The four types of sausages were analyzed at four storage times 45, 60, 75 and 90 days after manufacture (Table 6). During refrigerated storage of Hamburgues sausage, the total levels of amines remained the same for Mini Hamburgues and Gourmet Italian. However, it increased in Salaminho and Hamburgues reaching higher levels ($p<0.05$) at 60-90 days and at 75 days, respectively. When considering the amines which are inherent to the fresh raw pork meat, spermidine remained the same ($p>0.05$) throughout storage, for all the different types of sausage. Spermine also remained the same for Mini Hamburgues and Gourmet Italian, but increased in Salaminho and decreased and them increased in Hamburgues. During storage of fresh pork meat, Custódio et al. (2018) found changes in both spermine and spermidine. Spermine increased and them decreased whereas spermidine changed in opposite ways. The changes here were different probably affected by the fermenting microorganisms. According to the literature, microorganism require spermine and spermidine for development and growth, being able to produce them as needed (Tang et al., 2021). The differences observed may be associated with the prevalent microflora and their ability to synthesize the polyamines.

Even though tyramine is not typical of good quality pork meat, at the beginning of the study (45 days storage), high amounts of tyramine were already present in most of the sausages (219.0 – 636.5 mg/kg, respectively), except for Salaminho, which had lower levels (30.60 mg/kg). In a similar way, tryptamine was also present in Salaminho and Gourmet Italian (it was not present in the other sausages) at the beginning and remained constant throughout storage. In a similar way, cadaverine was present in Hamburgues at the beginning of the study, but it increased afterwards.

Other amine, e.g., phenylethylamine, was detected at 60 storage days in Mini Hamburgues, and at 75 days of storage in Hamburgues, both at low levels (≤ 1.52 mg/kg).

Table 6. Changes on the levels of free bioactive during refrigerated storage (4 – 7 °C) of different types of dry fermented sausage.

Sausage / Amines	Concentration in mg/kg (mean ± standard deviation)/days after processing			
	45	**60**	**75**	**90**
Hamburgues				
Cadaverine	16.67 ± 2.71 [a]	nd [b]	82.22 ± 63.69 [a]	nd [b]
Histamine	nd [b]	nd [b]	1343 ± 266.0 [a]	nd [b]
Phenylethylamine	nd [b]	nd [b]	0.62 ± 0.48 [a]	nd [b]
Putrescine	nd [b]	nd [b]	1073 ± 831.2 [a]	nd [b]
Spermidine	5.20 ± 4.45 [a]	nd [a]	1.52 ± 0.94 [a]	7.10 ± 1.19 [a]
Spermine	207.0 ± 170.7 [a]	23.33 ± 22.78 [b]	172.6 ± 6.50 [a]	191.6 ± 163.22 [a]
Tyramine	219.0 ± 171.4 [b]	277.5 ± 229.0 [b]	717.8 ± 483.6 [a]	192.36 ± 28.14 [b]
Total	447.9 ± 30.31 [b]	300.9 ± 238.8 [b]	2391 ± 1640 [a]	391.0 ± 186.3 [b]
Mini Hamburgues				
Phenylethylamine	nd [a]	0.70 ± 1.09 [a]	nd [a]	1.52 ± 2.35 [a]
Spermidine	1.36 ± 0.50 [a]	0.90 ± 1.39 [a]	1.16 ± 1.50 [a]	0.95 ± 1.47 [a]
Spermine	92.17 ± 142.8 [a]	209.6 ± 175.6 [a]	63.04 ± 97.7 [a]	110.4 ± 133.9 [a]
Tyramine	221.9 ± 149.5 [a]	521.7 ± 529.16 [a]	303.2 ± 243.2 [a]	662.8 ± 247.5 [a]
Total	315.5 ± 22.28 [a]	732.8 ± 648.4 [a]	367.4 ± 155.6 [a]	775.6 ± 371.8 [a]
Salaminho				
Putrescine	37.08 ± 57.57[a]	98.03 ± 135.61 [a]	176.88 ± 247.49 [a]	nd [b]
Spermidine	nd [a]	nd [a]	1.64 ± 2.55 [a]	2.50 ± 1.26 [a]
Spermine	28.04 ± 22.41 [b]	106.37 ± 43.39 [a]	121.48 ± 76.80 [a]	202.79 ± 54.45 [a]
Tryptamine	nd [a]	1.12 ± 1.73 [a]	1.25 ± 1.94 [a]	nd [a]
Tyramine	30.60 ± 47.50 [b]	122.9 ± 91.68 [a]	109.4 ± 136.2[a]	182.5 ± 206.07 [a]
Total	95.72 ± 83.58 [b]	328.37 ± 193.87 [a]	410.64 ± 482.06[a]	387.7 ± 261.3 [a]
Gourmet Italian				
Cadaverine	37.45 ± 58.01 [a]	18.90 ± 21.13 [a]	32.59 ± 42.75 [a]	39.64 ± 34.77 [a]
Histamine	276.3 ± 240.9 [b]	596.1 ± 895.4[a]	799.1 ± 1087.2 [a]	270.0 ± 271.2 [b]
Putrescine	206.5 ± 160.4 [a]	453.7 ± 691.0 [a]	591.4 ± 762.4 [a]	544.4 ± 675.0 [a]
Spermidine	3.92 ± 6.08 [a]	nd[a]	2.04 ± 2.23 [a]	5.47 ± 7.25 [a]
Spermine	196.5 ± 238.0 [a]	104.7 ± 77.05 [a]	171.5 ± 139.4 [a]	261.4 ± 309.0 [a]
Tryptamine	0.57 ± 0.88 [a]	nd [a]	1.02 ± 1.59 [a]	nd [a]
Tyramine	636.5 ± 573.4 [a]	246.7 ± 261.8 [b]	718.7 ± 356.3 [a]	264.9 ± 238.4 [b]
Total	1358 ± 1012 [a]	1420 ± 1343[a]	2316 ± 2097 [a]	1386 ± 716.7 [a]

n – 6.
nd – not detected, LOQ – Agmatine 2.84 mg/kg; Cadaverine 2.40 mg/kg; Histamine 4.08 mg/kg; Phenylethylamine 1.56 mg/kg; Putrescine 3.91 mg/kg; Serotonin 2.25 mg/kg; Spermidine 1.80 mg/kg; Spermine 2.07 mg/kg; Tryptamine 1.67 mg/kg; Tyramine 2.07 mg/kg.
Different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).

Histamine was only found in Gourmet Italian and Hamburgues types of sausage. In Hamburgues, it showed up late during storage - at 75 days however at very high mean levels (1343 mg/kg), decreasing afterwards. In Gourmet Italian, histamine was already present at high levels at the beginning of the storage period (45 days). In these two cases, the reasons for the formation of

histamine are probably different. In the first case, it seems that histamine was formed by a contaminant, whereas in the second, histamine can result from the addition of wine as an optional ingredient in the formulation. Him in wine?

The behavior of putrescine changes was different in the sausages. In Hamburgues, it showed up late (75 storage days) but at high mean levels (≤1073 mg/kg) probably due to contamination as it happened for histamine in Hamburgues type sausage. In Salaminho and Gourmet Italian, putrescine was already present at 45 days of storage (37.08 and 206.5 mg/kg, respectively). In both, there was a tendence of increase throughout storage (up to 75 days) followed by a decrease. The higher levels of putrescine at 45 days storage of Gourmet Italian sausage, can result from the addition of wine as optional ingredient as mentioned for histamine, suggesting that the addition of wine in sausage making, can bring amines and amino acid microorganisms or their enzymes typical of wine. This behavior was observed by Karwowska et al. (2022) that added liquid acid whey during manufacture of dry fermented sausage. The found that the addition of acid whey resulted in sausage with higher levels of biogenic amines. Another factor that can affect amine formation is the pH of the wine, which can lower the pH of the media, favoring amines formation to buffer the pH of media, as a mechanism of bacterial survival at low pH (Barbieri et al., 2019).

From the storage study of the four different types of sausage, some conclusions can be withdrawn. First, even though the water activity of the sausage is preventive of bacteria growth (Aw≤0.894), the microbial amino acid decarboxylating enzymes (and possibly other enzymes) are still active, allowing amines formation up to 90 days of storage. During sausage manufacture, some amines are formed during fermentation and at the beginning of the storage of sausages, including tyramine and tryptamine. However, other amines are formed at later storage times (≥75 days), for example, histamine, phenylethylamine and putrescine. Another factor which can affect amine changes during processing and storage, is the addition of optional ingredients which can incorporate additional biogenic amines and amino acid decarboxylating bacteria or their enzymes to the sausage. In the Gourmet Italian sausage that had wine as an optional ingredient the levels of biogenic amines were significantly higher compared to the others. Based on these results, to control the formation of tyramine and tryptamine in sausage, efforts are needed at the fermentation step, such as addition of desirable starter culture and control of hygienic conditions during manufacture, avoiding microbial contamination. The use of optional ingredients during sausage manufacture must be investigated prior to commercial use. This is so, because the optional

ingredient can bring to the sausage amines and microorganisms and possibly enzymes which can increase amine formation.

4.7. Changes on bioactive amines during dry fermented sausage storage

Kinetic modeling is a tool used in the study of changes in quality indices during the storage period (Ghosh et al., 2019; Klungboonkrong et al., 2019; Wang et al., 2020). It is commonly used to obtain information about reaction rate constants and to build mathematical models. Kinetic equations are developed and fitted to experimental data (Wang et al., 2020), and it has been used to predict the quality of beef (Dimakopoulou-Papazoglou et al., 2017), pork (Tango et al., 2016) and chicken meat (Schmidt et al., 2018). To study the kinetics of amines changes over storage time in the four types of fermented dry sausage, several regression models were investigated, and the one with the higher correlation coefficient was selected as the optimum regression. In Table 7 and figure 2, one can see the regression equation and coefficient of correlation and the regression lines, respectively.

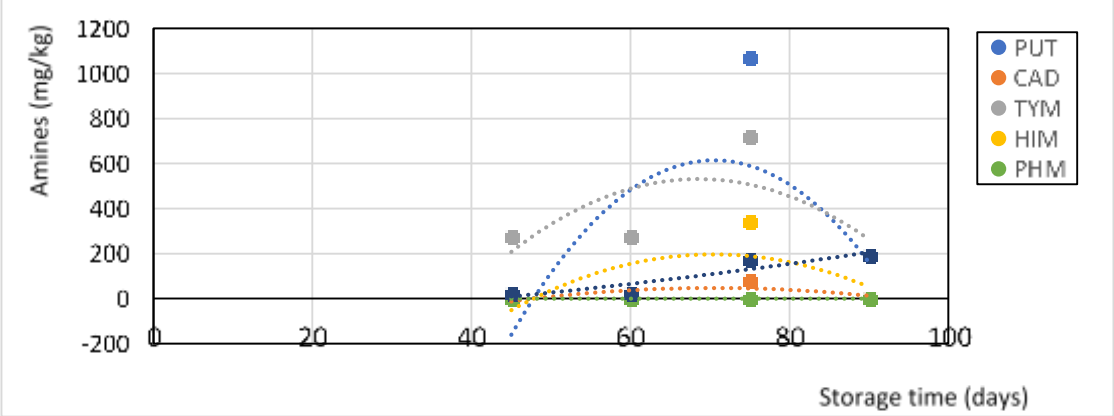
According to Table 7, the changes of amines in Salaminho followed polynomial regression, with $R^2 \geq 0.7913$. The changes in tyramine could also fit a logarithmic regression, suggesting the the changes could be associated with microbial changes that follow logarithmic regression (Li et al., 2018). The changes in Gourmet Italian followed polynomial regression ($R^2 \geq 0.7094$) except for tyramine ($R^2 \geq 0.1196$) which did not follow any of the models investigated. This result suggests that the formation and build up to tyramine in this type of sausage are affected by several factor. In Hamburgues sausage, the only significant regression was for spermine with $R^2 = 0.6461$. In Mini Hamburgues sausage, the fits showed poor regressions, with higher R^2 (≥ 0.5809) for polynomial regression. Therefore, most of the fits were polynomial, which means that the relationship between the independent variable (storage time) and the dependent variable (levels of amines) is modelled as the *2*th degree polynomial. It can also mean that the changes can be expressed by several linear regressions, e.g., there are different factors affecting amine formation throughout storage.

Studies on the changes of bioactive amines during processing and storage of dry fermented sausage are scarce in the scientific literature. It would be important to follow the whole process and determine amines to better understand their origin and, therefore, implement action to mitigate amines formation and accumulation in sausages. This study is of utmost importance as today, sausages are rich is amines which can cause adverse effects to human health.

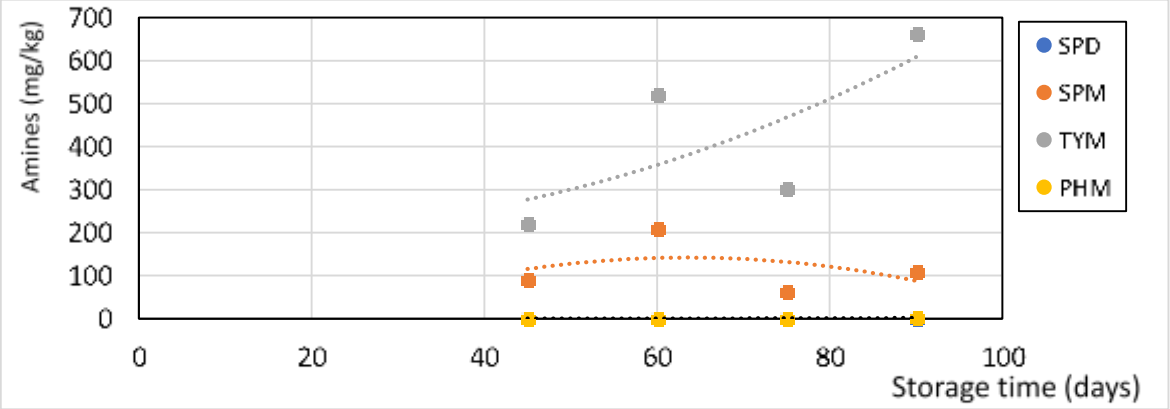
Table 7. Regression model for the changes on free bioactive amines in different types of dry fermented sausage during refrigerated storage (5.5 ± 1.5 °C)

Sausage / Amine	Regression		
	Equation	**R^2**	**Model**
Hamburgues			
Putrescine	$y = -1,1922x^2 + 168,11x - 5311,4$	0,4	Polynomial
Cadaverine	$y = -0,0914x^2 + 12,88x - 406,97$	0,4	Polynomial
Histamine	$y = -0,3815x^2 + 53,794x - 1699,6$	0,4	Polynomial
Tyramine	$y = -0,5839x^2 + 80,054x - 2212,9$	0,4171	Polynomial
Spermine	$y = 0,0211x^2 + 1,5083x - 101,28$	0,8461	Polynomial
Phenylethylamine	$y = -0,0007x^2 + 0,0969x - 3,061$	0,4	Polynomial
Mini Hamburgues			
Phenylethylamine	$y = 0,0009x^2 - 0,0968x + 2,6991$	0,5809	Polynomial
Spermine	$y = -0,0778x^2 + 9,8933x - 172,55$	0,1359	Polynomial
Tyramine	$0,0665x^2 - 1,6184x + 214,89$	0,5072	Polynomial
Salaminho			
Putrescine	$y = -0,2643x^2 + 35,459x - 1037,1$	0,7913	Polynomial
Spermine	$y = 0,0033x^2 + 3,1493x - 113,9$	0,9456	Polynomial
Spermidine	$y = 0,001x^2 - 0,0685x + 1,0195$	0,9374	Polynomial
Tryptamine	$y = -0,0026x^2 + 0,3558x - 10,709$	0,9943	Polynomial
Tyramine	$y = 192,73\ln(x) - 694,2$	0,8496	Logarithmic
	$y = -0,0213x^2 + 5,8248x - 178,74$	0,8422	Polynomial
Gourmet Italian			
Cadaverine	$y = 0,0284x^2 - 3,7041x + 144,6$	0,7094	Polynomial
Histamine	$y = -0,9432x^2 + 128,55x - 3629,4$	0,9057	Polynomial
Putrescine	$y = -0,3268x^2 + 51,788x - 1466,1$	0,9968	Polynomial
Spermidine	$y = 0,0082x^2 - 1,0572x + 34,74$	0,9373	Polynomial
Spermine	$y = 0,2019x^2 - 25,509x + 928,85$	0,9273	Polynomial
Tyramine	$y = -0,0712x^2 + 5,3216x + 451,78$	0,1196	Polynomial

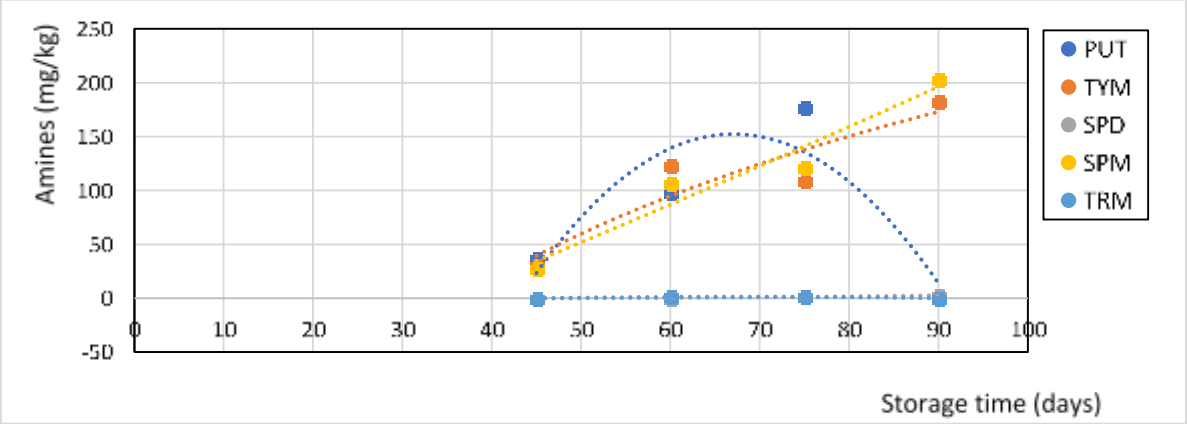
Hamburgues



Mini Hamburgues



Salaminho



Gourmet Italian

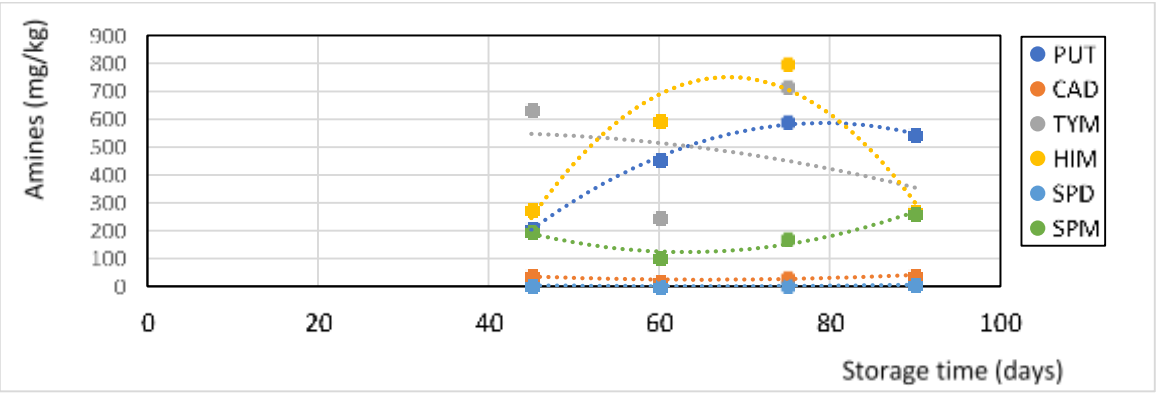


Figure 2. Changes on free bioactive amines different types of dry fermented sausage from 45 to 90 days of refrigerated storage (4 – 7 °C) of and the respective regression

5. CONCLUSIONS

The four types of dry fermented sausage included in this study complied with the microbiological parameters and most of them complied with physico-chemical characteristics, except one sample of Gourmet Italian that had higher moisture content compared to the Brazilian legislation. There was a different profile of amines in the different types of sausages at 45 days after proccessing. Italian gourmet had the largest diversity (7 amines) and Hamburgues and Salaminho had only three amines. Agmatine, serotonin and phenylethylamine were not detected. Total amines levels were higher in Gourmet Italian (1358 mg/kg) whereas Salaminho had the lower mean levels (95.72 mg/kg). The amine which contributed the most to total levels in most sausages was tyramine (32.3-70.4%), except for Salaminho, in which putrescine was the prevalent amine (38.6%) and tyramine the second one (32.3%). The second prevalent amine in Hamburgues and Mini Hamburgues was spermine and histamine in Gourmet Italian. Histamine was only found in Gourmet Italian sausage suggesting that the wine added in the formulation could contribute with amines. In a similar way, this type of sausage had also high levels of putrescine. The levels of histamine and tyramine in the samples are worrisome as they could cause adverse effects to human health. There was strong correlation between amines suggesting that the formation of some of them occur simultaneously or could be formed by the same mechanism, for example tyramine and tryptamine, spermine and spermidine and histamine and putrescine. The smaller diameter in Mini Hamburgues compared to Hamburgues sausages seems to diminish amine formation. The addition of wine as ingredient in Gourmet Italian affected the profile and levels of amines, introducing amines or free amino acids decarboxylating enzyme. During refrigerated storage of the sausages, there were significant changes on the amines, indicating that the decarboxylating enzymes are still active. Spermidine and to a certain extent spermine levels remained the same during storage. Tyramine and tryptamine seem to be primary metabolites formed, since its formation takes place at the fermentation/maturation step or at the first storage days. However, histamine, phenylethylamine and putrescine are secondary metabolites, being formed at later storage days (60-75 days after processing). The change on amines followed polynomial regression. Higher amines levels were found at 75 storage days, decreasing afterwards. These results indicate that the formation of amines continues during refrigerated storage. Therefore, even though there is low water activity and possibly no active microorganisms their decarboxylating enzyme are still active, affecting the levels of amines.

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CAPÍTULO IV - Levels and bioaccessibility of biogenic amines in Brazilian fermented sausages

Abstract

Dry fermented sausages are described as having considerable amounts of biogenic amines, however, for a better understanding of the impact of their ingestion on health, it is necessary to investigate their bioaccessibility in the sausage matrix. The aim of this study was to determine the bioaccessibility of biogenic amines and polyamines in two brands of Milano type sausage from Belo Horizonte, MG, Brazil. Samples were analyzed for bioactive amines by ion pair HPLC and fluorescence detection after extraction with trichloroacetic acid before and after in vitro digestion using the Infogest protocol. In brand 1, seven amines were detected, while in brand 2, eight amines were detected. The total amine contents in brands 1 and 2 of Mlano type sausage were 1140.70 and 356.73 mg/kg respectively. Putrescine was the prevalent amine, followed by tyramine, cadaverine and histamine. The proportion of amines in the sausages was similar, although the levels were different. Levels of tyramine and histamine were elevated and may cause adverse effects to human health. The bioaccessibility index (BI) of total amines ranged from 16.4 to 39.1%, histamine had the highest BI value, while agmatine and spermine had the lowest. The list of ingredients and additives and the processing conditions may have affected the bioaccessibility of the amines. This research also showed that digestive enzymes can significantly affect the release of amines.

Keywords: biogenic amines, putrescine, tyramine, Milano-type dry fermented sausage, INFOGEST, HPLC.

1. INTRODUCTION

According to the Technical Regulation of Identity and Quality (Brasil, 2000) dry fermented sausage is an industrialized meat product, made from pork, bacon, added ingredients, with an average particle size between 3 and 6 mm, embedded in natural or artificial casings, cured, smoked or not, fermented, matured, and dried for the time indicated by the manufacturing process. This type of sausage has pork meat as a mandatory ingredient at a minimum of 60%. In addition, it has, as ingredients, bacon, salt, nitrite and/or sodium and/or potassium nitrate. The manufacture is summarized in fig. 1. Even though several steps are needed, fermentation and ripening are of utmost relevance as they will impact on the sausage quality and safety. During fermentation and ripening, several biochemical transformations take place in parallel with complex microbiological activity. In the first days consist in lactic fermentation and with the ripening begins, the changes are due to activity of several microorganisms, including Micrococci, Staphylococci and lactic acid bacteria (LAB) (Tabanelli et al., 2012). Among the bacteria, coagulase-negative staphylococci (CNS), *Lactobacillus sakei*, *Lactobacillus curvatus*, and some yeasts such as *Saccharomyces cerevisiae* have been reported to be directly involved in meat proteolysis in dry fermented sausages (Serio et al., 2020). Proteolysis is one of the main phenomena that occur during ripening, in which myofibrillar proteins of the meat are converted into polypeptides by proteases, such as calpains and cathepsins and, and consequently, into oligopeptides and free amino acids (Pasini et al., 2018). The free amino acids are then catabolized, giving rise to different compounds such as ammonia, α-methylketones, and in low molecular weight organic compounds with biological activity and nitrogen content, called biogenic amines (Wang et al., 2022).

Biogenic amines can be formed due to the decarboxylation of free amino acids, and the amination or transamination of ketones and aldehydes with the coaction of bacterial enzymes (Durak-Dados, Michalski, & Osek, 2020). Dry fermented sausages are described as having considerable amounts of biogenic amines. Among amines present in sausages, the polyamines agmatine, spermine and spermidine are natural pork meat ingredients. Among biogenic amines, putrescine, cadaverine, histamine, tyramine, tryptamine, and phenylethylamine have been detected in sausages. Putrescine and cadaverine are formed from ornithine and lysine, respectively. They are associated with Enterobacteria, and therefore they can be an index of the hygienic sanitary conditions of the product. Histamine and tyramine result from the decarboxylation of histidine and tyrosine, respectively. Several microorganisms, including lactic acid bacteria can be associated with these amines. High levels of these amines are associated with food intoxications leading to adverse effects to human health.

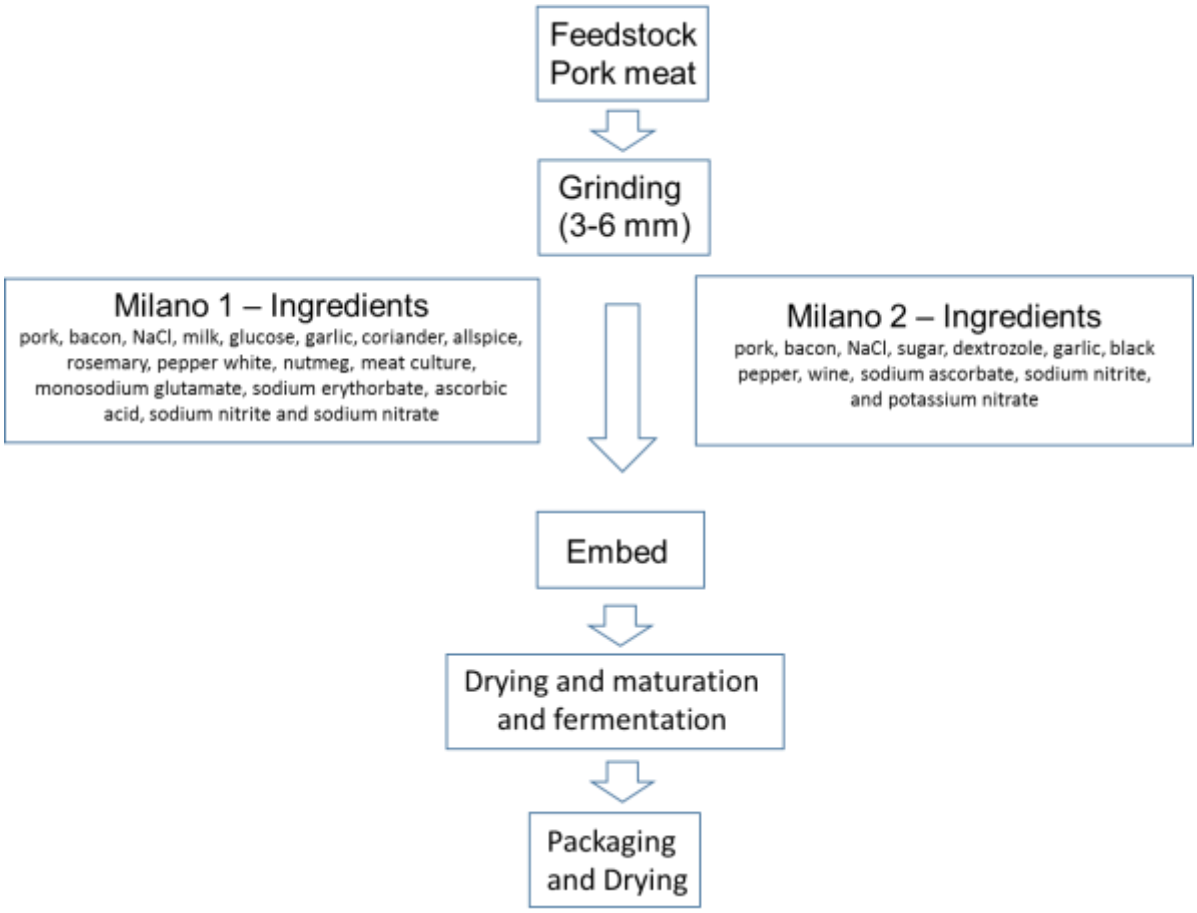

Fig 1. Production flowchart of Milano type dry fermented sausages and the ingredients used in the processing of brands 1 and 2.

The most significant biogenic amines occurring in dry fermented sausage are tyramine, cadaverine, putrescine (Pasini et al., 2018), and in minor occurrence and levels, histamine, tryptamine and 2-phenyletylamine (Latorre-Moratalla et al., 2012, Tabanelli et al., 2012). Biogenic amines have different physiological and biochemical effects, 2-phenylethylamine is related to cognitive functions, memory, and pleasurable sensations; tryptamine has antioxidant properties; histamine is associated with neurotransmission and vasodilatation in the central and cardiovascular nervous system, while tyramine is an anti-inflammatory and vasoconstrictor amine (Dala-Paula et al., 2021a, Dala-Paula et al., 2021b). However, the presence of biogenic amines in food is associated with toxicological reason and quality indicators. Histamine at high levels can cause headaches, low blood pressure, nausea, and heart palpitations (Silva et al., 2023); and high levels of tyramine and tryptamine are similar vascular causing hypertension, being the tyramine also related to headache, pupil and palpebral tissue dilatation, fever, and sometimes vomiting and sweating (EFSA, 2011, Dala-Paula et al., 2021b). The polyamines spermidine and spermine can

also be found in fermented sausages (Hu et al., 2023) because they are naturally present in meat and in some ingredients. Fresh pork meats are rich in spermine and spermidine (Custódio et al., 2018). Polyamines play an important role in DNA replication, in cell membrane permeability, providing cardiovascular protection, antioxidant and anti-aging activities (Eisenberg et al., 2016, Silva et al., 2023). However, during sausage processing, polyamines can be lost and there is formation and an accumulation of biogenic amines (Roselino et al., 2020).

However, for a better understanding of the impact of bioactive amines intake on health, it is necessary, in addition to characterizing its profiles and levels in foods, to study its bioaccessibility in different food matrices. The bioaccessibility of biogenic amines is the fraction present in a food that becomes accessible for absorption through the epithelial layer after gastrointestinal digestion of that food (Dima et al., 2020). There is scarce in the literature regarding the bioaccessibility of biogenic amines and polyamines in foods. Studies have been undertaken only with mushroom (Reis et al., 2019) and dark chocolates (Dala-Paula et al., 2021b; Silva et al., 2023). In these studies, the food matrix affected significant the bioaccessibility of the amines. In mushroom, there was high release of spermidine (Reis et al., 2020), whereas in chocolate, the higher bioaccessibility was found for tyramine, phenylethylamine, spermidine and putrescine (Silva et al., 2023). Thus, the aim of this study was to determine the profile, contents and bioaccessibility of biogenic amines and polyamines in two brands of Milano-type dry fermented sausages purchased in the market of Belo Horizonte, MG, Brazil.

2. MATERIAL AND METHODS

2.1. Sample and reagents

Analytical grade reagents were used, except HPLC solvents which were LC grade. Ultra-pure water was from Milli-Q Plus (Millipore Corp., Milford, MA, USA). Organic and aqueous solvents for HPLC analysis were filtered using HAWP and HVWP membranes, respectively (0.45 μm, Millipore Corp., Milford, MA, USA). Alpha-amylase (Sigma A-3176); bile salts (Sigma B-8756); pancreatin and pepsin from porcine gastric mucosa (Sigma P-3292 and P-7012, respectively); and bioactive amine standards (spermidine trihydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, serotonin hydrochloride, tryptamine hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. *In vitro* simulation of gastrointestinal digestion

Gastrointestinal digestion was performed as described by Minekus et al. (2014) and Brodkorb et al. (2019) with a few modifications. The protocol simulated the whole gastrointestinal digestion process: oral + gastric + intestinal, as described in Fig. 2. Briefly, the fermented sausage was chopped into small pieces, approximately 0.5 cm^2, with the aid of a stainless-steel knife, simulating the chewing process in the oral phase. An aliquot of about 5.0 g of sausage was mixed in a 50 mL Falcon tube with 4 mL of simulated salivary fluid [15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, and 1.1 mM HCl], 25 µL of 0.3 M $CaCl_2(H_2O)_2$, and 975 µL salivary amylase solution at a final enzyme activity of 75 U/mL, considering the final volume of sample and simulated fluids as 10 mL. The ratio of fermented sausage to simulated salivary fluid was 1:1 (w/w) ratio of fermented sausage to simulated oral fluid. The mixture was shaken (45 rpm) in an incubator at 37 °C for 2 min.

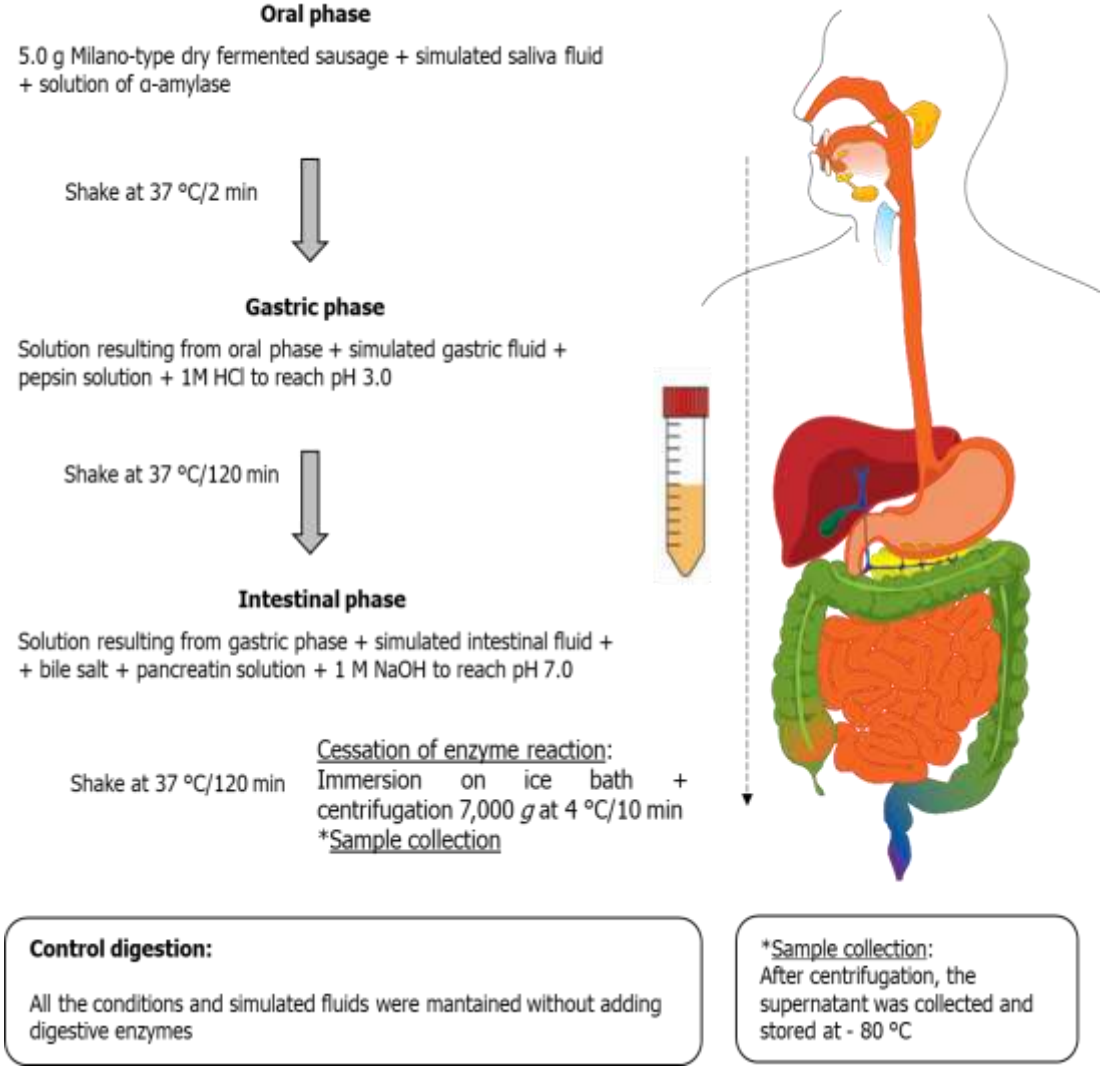

Fig 2. Sketch map of simulated in vitro gastrointestinal digestion of fermented sausage.

For the gastric phase, all the solution from the oral phase was adjusted to pH 3.0 (300 µL 1 M HCl) and mixed with 8.5 mL simulated gastric fluid [6.9 mM KCl, 0.9 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$ and 15.6 mM HCL], 5 µL of 0.3 M $CaCl_2(H_2O_2)_2$, and 1.195 µL pepsin to a final enzyme activity in a total volume of 20 mL equal to 2,000 U/mL. The mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h.

For the intestinal phase, all the solution from the gastric digestion was adjusted to pH 7.0 with 450 µL of 1 M NaOH and mixed with 11.51 mL of simulated intestinal fluid [6.8 mM KCl, 0.8 mM KH_2PO_4, 85.0 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, and 8.4 mM HCL], 40 µL of 0.3 M $CaCl_2(H_2O_2)_2$, 3 mL of bile salts and 5 mL of pancreatin solution necessary to reach the final bile concentration and enzymatic activity of trypsin in pancreatin of 10 mM and 100 U/mL, respectively, considering the final volume as 40 mL. This mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h. The interruption of the enzymatic reaction at the end of the *in vitro* digestion was performed by immersion of the test tube in an ice bath. The final mixture was centrifuged at 7,000 × *g* at 4 °C (MOD 280R, FANEN Excelsa 4, São Paulo, SP, Brazil) for 10 min. Then the supernatant was collected, filtered, and stored in Eppendorf type tubes at -80 °C until the analyses for bioactive amines. The digestion protocol was performed in triplicate and the samples were filtered through 0.45 µm membrane, prior to HPLC analysis. Control treatments were undertaken for the digestion, without addition of the respective enzymes, but with the addition of the bile salts, of all the simulated fluids and following all pH, temperature and shaken conditions.

2.3. Determination of bioactive amines by HPLC

For the analysis of amines in the sausage, around 5 g sausage was weighed, and the amines were extracted with 7 mL of trichloroacetic acid (TCA, 5% w/v). The mixture was shaken in a shaker (Ovan, Barcelona, Spain) at 280 rpm for 10 min, followed by centrifugation at 10,000 *g* for 20 min at 4 °C in a MR23I refrigerated centrifuge (Jouan, Saint Herblain, France). The process was repeated twice more and the supernatants were combined, filtered through qualitative filter paper and collected into a 25-mL volumetric flask which was completed with 5% TCA. After homogenization, an aliquot of the extract was filtered (syringe + swinnex + cellulose ester membrane) immediately prior to HPLC analysis (Custódio et al., 2016). The extracts from the bioaccessibility study and from the controls were filtered and submitted to HPLC analysis (Dala-Paula et al., 2021b; Silva et al., 2023).

HPLC-fluorescence analysis was performed using a Shimadzu LC-20AD Prominence high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a post-column derivatization system and a spectrofluorimetric detector (Shimadzu RF-10AXL), auto-injector (SIL-20AHT), and a HPLC interface control unit (CBM-20A). The column used was Luna C18 Phenomenex (4.6 x 250 mm, 5 μm) with a pre-column (C18, 4 x 3 mm) were placed in an oven (CTO-10 ASvp, Shimadzu, Kyoto, Japan) at 30 °C. A gradient of A (0.2 M sodium acetate and 15 mM octanesulfonic acid sodium salt, pH adjusted to 4.9 with acetic acid) and B (acetonitrile) was used at a flow of 0.8 mL/min: 0.01-17.99 min/2% B; 18.00-18.99 min/20% B; 19.00-39.99 min/5% B; 40.00-49.99 min/23% B; 50.00-50.49 min/35% B; 50.50-60.00 min./2% B. The post column derivation reagent (0.2 g *o*-phthalaldehyde, 1.5 mL Brij-35, 1.5 mL β-mercaptoethanol dissolved in 500 mL solution of 25 g boric acid and 22 g KOH, pH adjusted to 10.5 with 3% KOH) was delivered at 0.3 mL/min, and the reaction took place in the oven at 30°C. The fluorescence detector operated at 340 nm excitation and 450 nm emission. The identification of the amines was possible by comparing the retention time of the peaks from the samples with standards and by spiking the sample extract with the suspected amine. The concentrations of the amines were obtained by interpolation in the respective external standard curves ($R^2 \geq 0.992$).

2.4. Statistical analysis

Minitab® 17.3.1 software (Minitab Inc., PA, USA) was used. The levels of bioactive amines were submitted to normality and significance tests (Shapiro-Wilk normality test, Box-Cox data transformation, one-way ANOVA followed by Tukey's test at 5% probability).

Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were used for the characterization of the dry fermented sausage samples before and after the simulated digestion. In PCA, all detectable amines were used as active variables in the derivation of the principal components. PCA was done using correlation as the type of matrix. The dendrogram for HCA analysis was obtained by clustering variables (the same used for PCA). McQuitty's linkage was used for the distance matrix and Euclidean's method to calculate the distance between observations.

3. RESULTS AND DISCUSSION

3.1. Profile of bioactive amines

Among the ten amines investigated, seven were present in one brand of Milano-type dry fermented sausage (Milano 1) – agmatine, cadaverine, histamine, putrescine, spermine, tryptamine, and tyramine. In the other brand (Milano 2), eight amines were found, the same seven found in Milano 1 plus phenylethylamine. Spermidine and serotonin were not found in any sample. Dry fermented sausages are rich sources of bioactive amines. The most often investigated amines in sausage are histamine, tyramine, putrescine and cadaverine (Tabanelli et al., 2012; Pasini et al., 2018; Rocchetti et al., 2023). In some studies, spermine and spermidine were also included (dos Santos et al., 2015; Rocchetti et al., 2023). Agmatine, serotonin, tryptamine and phenylethylamine are seldom investigated in sausages (Caccioppoli et al., 2006). In Milano sausage, dos Santos et al. (2015) also detected spermidine at low levels, which was not detected in this study.

The predominant amine was putrescine (~31 and ~34%), followed by tyramine (24 and ~25%), cadaverine (~21% and ~15%), and histamine (~16 and ~10%) in Milano 1 and 2, respectively (Figure 3). Based on this result, even though there were differences on the levels of amines, they occurred at similar proportions. This same pattern of the amines was observed by dos Santos et al. (2015) for Milano sausage from Rio de Janeiro, and by Tabanelli et al. (2012); however, in their study, the predominant amine was tyramine, instead of putrescine.

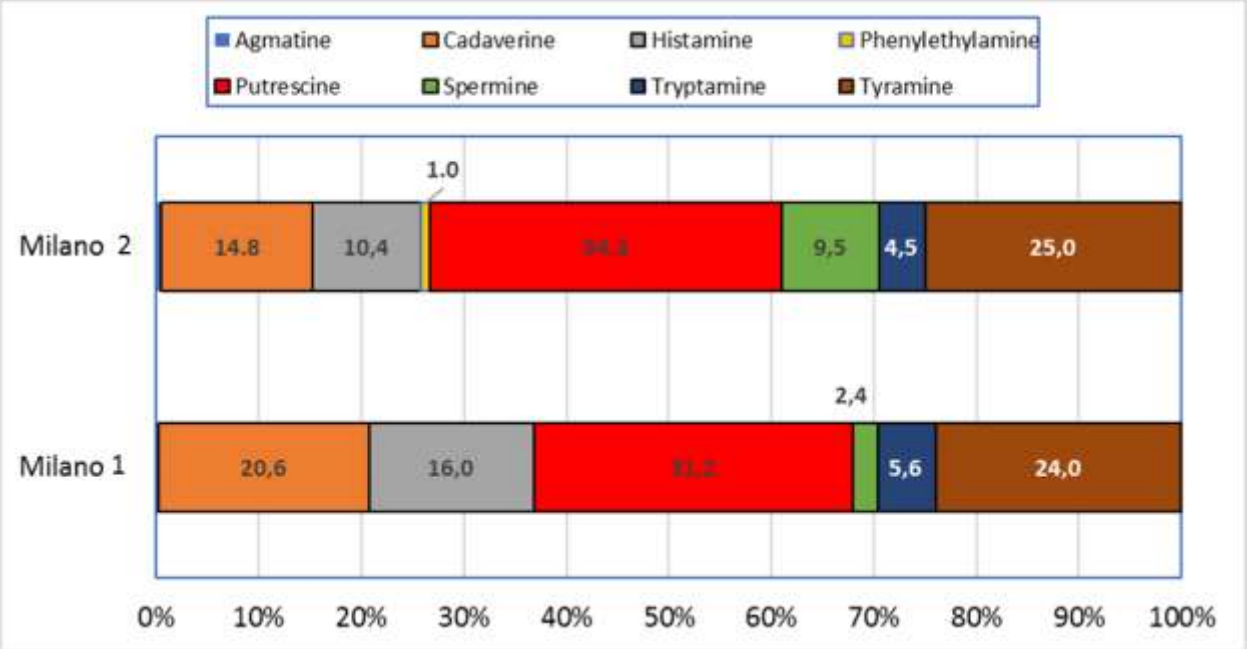

Fig 3. Contribution of bioative amines to the total content in two different brands of Milano-type dry fermented sausages.

The total levels of amines were significantly different, with higher levels in brand 1 (1140.70 mg/kg) compared to brand 2 (356.73 mg/kg). These levels are like those found in Brazilian (dos Santos et al., 2015) but higher than Italian Milano sausages (Tabanelli et al., 2012). When comparing individual amines, Milano 1 had significantly higher levels of all the amines, except for phenylethylamine which was only detected in Milano 2. Higher differences were observed for histamine (4.9-fold), followed by cadaverine (4.4-fold), tryptamine (4.0-fold), tyramine (3.1-fold), and putrescine (2.9-fold).

The high levels of amines in Milano sausage can result from several factors, including the quality of the raw meat, differences on the ingredients added, processing conditions and environment, fermentation conditions, use and type of starter culture, maturation conditions (temperature, relative humidity) and time. These factors can influence the biochemical changes (amino genesis), including microbial growth, acidification, proteolysis, pH drop, and activity of decarboxylases (Fernández et al., 2003; Latorre-Moratalla et al., 2012). In addition, the accumulation of bioactive amines is related to the presence of specific microorganisms and edaphoclimatic differences among countries and regions (González-Fernández et al., 2003; Latorre-Moratallaetal., 2012; Wang et al., 2022). In fact, there might be different raw materials, e.g., pork meat and bacon from different species, animals' age, cuts used, microbial quality, storage of the meat prior to processing (González-Fernández et al., 2003; Latorre-Moratalla et al., 2012). Differences in ingredients and additives may also affect amine formation (Wang et al., 2023). When looking at both labels, Milano 2 when compared to 1 used many more ingredients (10 x 5) and more additives (5 x 3). In Milano 2, milk was added as well as a 'meat culture' whereas in Milano 1, wine was used. It is well known that powdered milk (Moniente et al., 2022) and wine (Costantini et al., 2019) have their own profile and levels of amines, therefore leading to different profiles in the sausage. Wine for example can contain high histamine (23.1 ± 2.2 mg/L) levels (Papageorgiou et al., 2018). The use of starter culture (Brand 2) can lead the fermentation process providing specific situations, affecting the profile and levels of amines (Rocchetti et al., 2023). The amount of salt used can also affect amines levels (Liu et al., 2020). Some spices (e.g., cinnamon, cloves and anise) and the amount added can inhibit biogenic amine accumulation (Sun et al., 2018). In Milano Brand 1 the spices and additives included garlic, coriander, allspice, rosemary, pepper white, nutmeg, meat culture, monosodium glutamate, sodium erythorbate, ascorbic acid, sodium nitrite and sodium nitrate; whereas in Milano Brand 2, garlic, black pepper, wine, sodium ascorbate, sodium nitrite, and potassium nitrate were used. Differences were also observed in the type of sugar used; glucose in brand 1, and sugar and dextrozole in brand 2.

Spermine and agmatine, which are inherent to pork meat (Custódio et al., 2018) were present at low levels, spermine levels were 27.23 to 33.75 mg/kg, whereas agmatine were 2.3 and 1.84 mg/kg in Milano 1 and 2, respectively. Samples from Milano brand 1 showed higher levels of histamine (183.07 mg/kg) and tyramine (273.77 mg/kg), when compared to brand 2 (37.24 and 89.09 mg/kg, respectively). These amines are not inherent to good quality raw milk, and probably result from ingredients (e.g., wine), and the fermentation and maturation process. High levels of histamine and tyramine in foods can represent a serious public health issue, due to their adverse effects to health. The most common symptoms of histamine intoxication are rash, vomiting, diarrhea, headache and dizziness, nausea, palpitations or breathing difficulties (Ekici et al., 2020; Wójcik et al., 2021). Tyramine intoxication has symptoms such as migraine, gastrointestinal complaints, tachycardia, increased blood sugar, norepinephrine ejection, and hypertension (Ekici et al., 2020; Wójcik et al., 2021). According to EFSA (2011), the safe threshold for histamine, for healthy individuals is 25 mg/meal/person, as the most conservative level. However, for individuals with histamine intolerance, even small amounts of this amine in food may cause adverse effects. When considering tyramine, the safe threshold will also depend on the individual: 600 mg/meal/person for healthy individuals, but for patients under treatment with classical MAOI drugs, the safe threshold is 6 mg/meal/person for those taking traditional IMAO, but 50 mg/meal/person for patients under third generation MAOI drugs.

3.2. Bioaccessibility of biogenic amines and polyamine in Milano-type dry fermented sausages

To the best of our knowledge, this is the first insight on bioactive amines bioaccessibility from dry fermented sausage. In Table 1 one can see the results from the amines bioaccessibility in the two brands of Milano-type dry fermented sausage. Although Milano 1 presented total amines content about 3.2 times higher than Milano 2, at the end of the in vitro digestion the total amines content was similar (186.80 and 139.47 mg/kg, respectively) and the bioaccessibility index of Milano 1 (16.4%) was lower compared to Milano 2 (39.1%).

In Milano 1, the bioaccessibility indexes for all BA detected were lower than 30%, while in Milano 2 four amines had greater values than 30%, with two higher than 65%. The amine with greater bioaccessibility index in Milano 1 were histamine (29%), followed by tyramine (~26%), tryptamine (~16%), putrescine (~12%), and cadaverine (~5%); whereas in Milano 2 they were histamine (~72%), cadaverine (~65%), putrescine (~38%), tyramine (~34%), phenylethylamine (~23%), and tryptamine (3.3%). The polyamines agmatine and spermine were not detected in any

of the samples even after the simulated digestion, suggesting that these amines were not present in the sausage bound to other sausage components, like proteins. Therefore, the bioaccessibility of these compounds was equal to 0. Considering that polyamines have positive charges at physiological pH, electrostatic interactions with components of the food matrix may have occurred, such as waste from negatively charged protein digestion (Dala-Paula et al., 2021b, Silva et al., 2023).

The control assays for *in vitro* digestion, without the use of the digestive enzymes, but with the maintenance of other conditions, in addition to the presence of bile, were carried out to evaluate the impact of digestive enzymes on the bioaccessibility of the amines. The results indicated the contribution of digestive enzymes to the bioaccessibility of cadaverine, histamine, tryptamine in Milano 1, and of putrescine and tryptamine in Milano 2.

Multivariate analyses of the auto scaled data indicated that a two-principal components (PC) model explained 98.5% (Fig. 4A and B) of the correlation. According to PC1 loadings (Fig 4A), all BA except for phenylethylamine, were the components with similar positive impacts. PC2 explained 21.2% of the correlation and had high values of phenylethylamine and spermine, and negative low values of histamine, tyramine, cadaverine, tryptamine and putrescine with main loadings. This multivariate analysis separated Milano 1 and Milano 2 before digestion in the second and the first quadrants, respectively (Fig. 4D), while all digestion samples, including the control digestions (without digestive enzymes) were allocated in third quadrant.

Milano 2 after the *in vitro* digestion and its digestion control were clustered together with up to 95% similarity (Fig 4C), reinforcing the low impact of the digestive enzymes on the bioaccessibility of amines in this brand of Milano-type dry fermented sausage. However, Milano 1 after *in vitro* digestion and its digestion control were not clustered together with up to 90% of similarity (Fig 4C), in agreement with the significant differences in the levels of BA verified between the *in vitro* digestion and its control. The dendrogram (Fig. 4C) clustered together with up to 95% of similarity.

Table 1. Levels of free bioactive amines in two brands of Milano dry fermented sausage from the market of Belo Horizonte, MG, Brazil and in solutions resulting from in vitro digestion and the respective controls.

Milano	**Median levels ± standard deviation (mg/kg)**								
	Agmatine	**Cadaverine**	**Histamine**	**Phenylethylamine**	**Putrescine**	**Spermine**	**Tryptamine**	**Tyramine**	**Total**
Brand 1									
Sausage 1	2.30±0.27^{a}	234.45±7.47^{a}	183.07±11.56^{a}	nd^{c}	355.49±11.10^{a}	27.23±1.37^{b}	64.38±5.26^{a}	273.77±5.70^{a}	1140.70±13.65^{a}
Digested 1	nd^{c}	10.70±0.25^{d}	53.08±10.39^{b}	nd^{c}	42.04±3.11^{c}	nd^{c}	10.54±1.17^{c}	70.44±14.42^{bc}	186.80±25.09^{c}
C-D-Control Dig 1	nd^{c}	1.08±0.07^{e}	4.34±0.92^{e}	nd^{c}	44.19±2.31^{c}	nd^{c}	6.73±0.86^{d}	63.23±5.19^{c}	119.57±2.41^{d}
BI 1 (%)	0	4.6	29.0	0	11.8	0	16.4	25.7	16.4
Brand 2									
Sausage 2	1.84±0.01^{b}	52.73±2.63^{b}	37.24±5.35^{bc}	3.56±0.56^{a}	122.38±8.19^{b}	33.75±0.71^{a}	16.14±0.34^{b}	89.09±4.73^{b}	356.73±4.71^{b}
Digested 2	nd^{c}	34.41±1.35^{c}	26.95±0.70^{cd}	0.80±0.01^{b}	46.25±1.23^{c}	nd^{c}	0.54±0.08^{e}	30.52±1.00^{d}	139.47±3.64^{d}
C-D-Control Dig 2	nd^{c}	29.95±2.81^{c}	22.97±1.20^{d}	0.43±0.02^{bc}	36.15±1.77^{d}	nd^{c}	0.30±0.01^{f}	27.19±1.21^{d}	117.01±5.56^{d}
BI 2 (%)	0	65.3	72.4	22.5	37.8	0	3.3	34.3	39.1

Leg.: nd - not detected (≤LOQ – limit of quantification); D-: digestion *in vitro* (Minekus et al., 2014); C-D: control digestion (maintenance of all conditions of in vitro digestion but without the addition of digestive enzymes); BI: Bioaccessibility Index. Mean values with different superscripts in the same column are significantly different (Tukey test, $p \leq 0.05$).

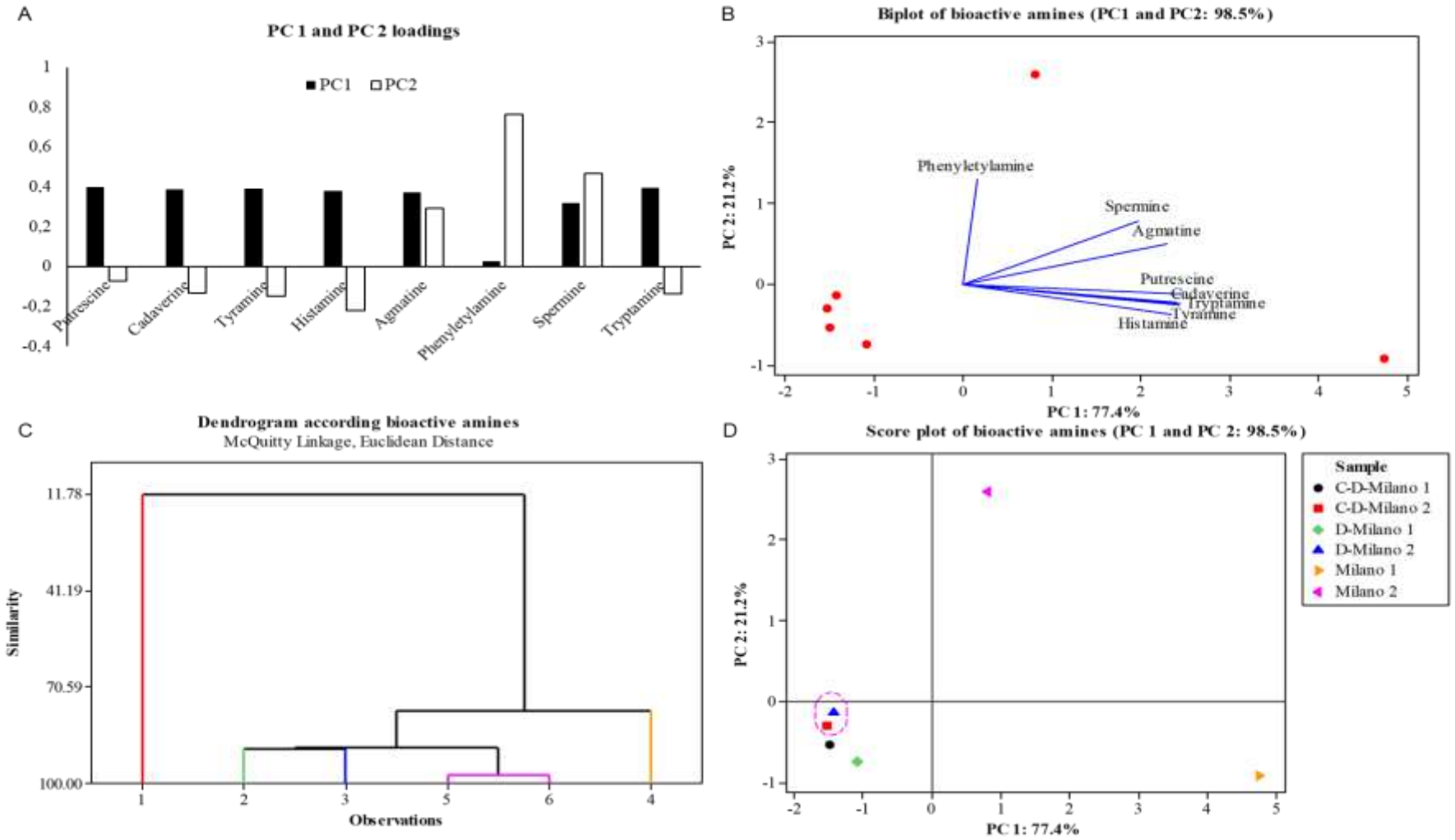

Fig. 4. Principal Component Analyses (PCA) and Hierarchical Cluster Analyses (HCA) of bioactive amines in two brands of Milano fermented sausage from the market of Belo Horizonte, MG, Brazil, 2022

4. CONCLUSION

Two different brands of Milano-type dry fermented sausages had similar profile of bioactive amines, however there are a significant difference in the levels between them. The bioaccessibility index of total amines ranged from 16.4 to 39.1%, showing a moderate release after *in vitro* digestion. Histamine had the highest bioaccessibility index in both sausages. Agmatine and spermine were not detected in sausages after the *in vitro* digestion. There was no relation between the bioaccessibility index of the other amines between the two investigated brands, which can indicate that ingredients, additives and the processing conditions may affect their bioaccessibility. Digestive enzymes can significantly affect the release of amines.

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CONCLUSÕES INTEGRADAS

O método validado para a detecção e quantificação das micotoxinas, Aflatoxinas (B1, B2, G1 e G2) e Ocratoxina A, atendeu as diretrizes contidas nas legislações brasileiras e internacionais. A extração das micotoxinas foi possível por uma extração simples usando metanol acidificado, acetonitrila e água. A corrida LC-MS/MS foi curta (15 min) e separou eficientemente as micotoxinas. A remoção de gordura dos salames usando hexano não melhorou a recuperação das micotoxinas e, portanto, não foi necessária. A curva padrão foi construída na matriz. As figuras de mérito para o método multirresíduo dilute & shoot indicaram que ele era adequado para o propósito. Vinte e sete amostras de salames comerciais foram analisadas e em nenhuma delas micotoxinas foram detectadas (limite de detecção de ~1,0 µg/kg). Esses resultados sugerem que as indústrias no Brasil estão monitorando e controlando a ocorrência de micotoxinas da fazenda à mesa.

O método dilute & shoot para análise de dez aminas em salame por fluorescência-CLAE foi satisfatório. Uma etapa de remoção de gordura não foi necessária. Não houve efeito de matriz, portanto a curva de calibração em solventes foi utilizada. O método foi simples, rápido e adequado ao objetivo com limites de quantificação adequados (1,56 – 4,08 mg/kg). O método foi utilizado para quantificar dez aminas em 146 amostras de salames brasileiros. Os salames apresentaram diferentes perfis de aminas. A tiramina e espermidina foram comuns a todos os tipos de salames. Os teores médios totais de aminas variaram de 40,73 mg/kg para o tipo Serrano até 385,5 mg/kg para o tipo Hamburguês, não diferindo dos demais. A tiramina foi a amina que mais contribuiu para o teor totais (33,2 até 81,5%). O salame tipo italiano gourmet tinha níveis significativamente mais elevados de tiramina e histamina. PCA e HCA permitiram a distinção dos salames quanto às aminas bioativas.

Quatro tipos de salames foram analisados quanto a aminas ao longo do armazenamento por até 90 dias. As amostras atenderam aos parâmetros microbiológicos e as características físico-químicas. Os quatro tipos de salame apresentaram diferente perfil e teores de aminas. O menor diâmetro do salame Hamburguês mini em relação ao tipo Hamburguês parece minimizar a formação de aminas. A adição de vinho como ingrediente no salame Italiano gourmet introduziu aminas ou aminoácidos livres e enzimas descarboxilantes. Durante o armazenamento refrigerado, houve alterações significativas nas aminas, indicando que as enzimas descarboxilantes ainda estão ativas. A tiramina e a triptamina parecem ser metabólitos primários formados, na fermentação/maturação ou nos primeiros dias de estocagem. No entanto, histamina, feniletilamina

e putrescina são metabólitos secundários, sendo formados em dias posteriores ao armazenamento. A alteração nos teores de aminas seguiu regressão polinomial. Maiores teores de aminas foram encontrados aos 75 dias de armazenamento. Esses resultados indicam que a formação de aminas continua durante o armazenamento refrigerado.

Duas marcas distintas de salame Milano com diferentes teores de aminas foram submetidas a estudos de bioacessibilidade *in vitro*. O índice de bioacessibilidade das aminas variou de 16,4 a 39,1%, apresentando liberação moderada após digestão *in vitro*. A histamina apresentou o maior índice de bioacessibilidade em ambos os salames. Não houve relação entre o índice de bioacessibilidade das demais aminas bioativas detectadas entre as duas marcas, sugerindo que os ingredientes, aditivos e as condições do processo podem afetar a bioacessibilidade.

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