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MISE AU POINT ET CARACTÉRISATION D'UNE BOISSON FERMENTÉE ENRICHIE EN BACTÉRIES PROBIOTIQUES ET EN PROTÉINES D'INSECTES

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Résumé

La préoccupation des consommateurs s'oriente de plus en plus vers les aliments fonctionnels, particulièrement les produits probiotiques qui connaissent un grand succès commercial et présentent aujourd'hui un marché en essor. Les probiotiques possèdent divers effets bénéfiques pour la santé. Ils peuvent, par exemple, prévenir les infections intestinales, la diarrhée et les infections urogénitales. Actuellement, les recherches se concentrent sur le développement des produits fermentés enrichis en protéines pour fournir aux consommateurs des aliments dotés des propriétés nutritionnelles et fonctionnelles élevées et pour lutter contre la carence en protéines et en acides aminés essentiels, en particulier pour les enfants, les sportifs et les personnes âgées. Les insectes apparaissent comme une source alternative durable appropriée pour l'alimentation humaine et la nutrition animale. Ils sont une source de protéines complètes et équilibrées en acides aminés. Cependant, les insectes ont une faible digestibilité, du fait de la présence de la chitine qui offre une certaine rigidité aux protéines et les rend plus récalcitrantes à l'hydrolyse enzymatique. De plus, la présence de forte teneur en acides aminés hydrophobes tels que la méthionine, la phénylalanine, le tryptophane et la valine, peut réduire certaines propriétés fonctionnelles comme la solubilité qui est directement lié à la digestibilité. Ces points négatifs freinent la valorisation des protéines d'insectes dans des formulations alimentaires. De ce fait, La dégradation de la chitine semble une alternative valable afin d'améliorer la disponibilité des protéines et leur propriétés fonctionnelles et nutritionnelles. Pour ce faire, plusieurs techniques peuvent être testées. Certains traitements peuvent favoriser une meilleure libération de peptides. Parmi ces traitements, on note la prédigestion enzymatique, la sonication et l'irradiation.

L'objectif de ce travail est de développer une boisson probiotiques enrichie en protéines dans le but d'offrir aux consommateurs des aliments dotés des propriétés nutritionnelles élevées et une bonne digestibilité.

Tout d'abord, le traitement de la poudre de criquet par irradiation gamma (IR), ultrason (US) et irradiation gamma assisté par ultrason (US-IR), hydrolyse enzymatique assisté par ultrason suivi ou non de centrifugation (US-E et US-EWC, respectivement) et hydrolyse enzymatique assistée par irradiation gamma et ultrason (US-IRE) et l'étude de leur impact sur les propriétés physicochimiques, structurales et la digestibilité *in vitro* a été effectuée.

Les résultats ont montré qu'un traitement par US, IR et US-IR favorisait une amélioration de la solubilité, une augmentation de la teneur en groupe sulfhydryle (SH) et des modifications de la

structure primaire et secondaire des protéines. Des breuvages fermentés ou non fermentés par la combinaison de probiotiques *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus* (*Lactobacillus*) *casei* LBC80R et *Lacticaseibacillus* (*Lactobacillus*) *rhamnosus* CLR2, enrichis ou non enrichis en protéines de criquet traitées par US-IR, US-E, US-EWC et US-IRE ont été élaborés. La digestibilité *in vitro* et la caractérisation des profils peptidiques au cours de la digestion ont été réalisés. Les résultats ont montré que le traitement combiné US-IRE et US-E ont eu un effet très positif en améliorant i) la digestibilité des boissons fermentées de 55% pour le contrôle à 94% pour US-IRE et US-E et ii) l'efficacité de l'hydrolyse des peptides de haut poids moléculaire en peptides de faibles poids moléculaire (80% à la fin de la digestion pour le traitement US-IRE), comparé aux boissons enrichies en protéines non traitées et aux boissons non-enrichies fermentées et non fermentées. La qualité protéique et la digestibilité *in vivo* des boissons probiotiques enrichies en protéines de criquet non traitées (CP) et enrichies en protéines de criquet traitées par US-E (CPH) ont été évaluées. Les résultats ont montré que l'incorporation de protéines de criquet sous forme d'hydrolysat (CPH) a augmenté significativement le Coefficient d'Efficacité Protéique (CEP) et le Coefficient d'Efficacité Protéique nette (CEPN) ($P \leq 0,05$) par rapport au CP, de 1,7 à 2,0 et de 0,4 à 1,0, respectivement. Également, une augmentation de la digestibilité *in vivo* a été observé pour la boisson CPH (96%) par rapport à la boisson CP (85%). De plus, l'effet d'un régime alimentaire à base de protéines de criquet sur la composition du microbiome intestinal a été étudié et les résultats ont montré que les protéines de criquet entiers ou sous forme d'hydrolysats peuvent maintenir une composition équilibrée et réduire certaines bactéries pathogènes dans l'intestin à savoir *Enterococcaceae* et *Erysipelatoclostridium.*

La qualité physico-chimique, nutritionnelle et microbiologique de la boisson fermentée enrichie (CPH) été évaluée pendant 147 jours de conservation réfrigérée à 4°C et comparée avec la boisson non-fermentée et fermentée non-enrichie (boisson Bio-K+^{MD}). Les résultats ont montré que l'enrichissement de boisson en CPH a assuré l'augmentation de la viscosité, la réduction du pH et l'augmentation de l'acidité titrable accompagnées du maintien d'une concentration élevée de cellules viables de probiotiques par rapport à la boisson non enrichie avec 8,45 log/mL après 147 jours. Ainsi, la fermentation a permis d'améliorer la viscosité qui a été augmentée significativement ($P \leq 0,05$) au cours du stockage. De plus, les probiotiques ont présenté une grande résistance aux conditions gastro-intestinales *in vitro* particulièrement pour la boisson enrichie en CPH et a montré un pourcentage de 83% de probiotiques viables après 147 jours.

Les résultats obtenus pendant ce projet ont mis en évidence la possibilité de développer une boisson probiotique enrichie en protéines non-conventionnelles de bonne qualité nutritive et

fonctionnelle. Les protéines de criquet prétraitées via l'hydrolyse enzymatique et ultrason peuvent être une nouvelle alternative prometteuse pour le développement des aliments fonctionnels.

Le produit obtenu pourrait répondre aux désirs de consommateurs en un produit de haute valeur nutritive, fonctionnelle et de bonne digestibilité.

Abstract

Consumer concerns are increasingly shifting towards functional foods, particularly probiotic products, which are successful and present today a continuously growing market.

Probiotics have various health benefits, such as prevention of intestinal infections, diarrhea and urogenital infections. Currently, research is focused on the development of fermented products enriched with protein to provide consumers with foods with high nutritional and functional properties and to combat protein and essential amino acid deficiency, especially for children, athletes and the elderly. Insects are emerging as a sustainable alternative source of protein suitable for human food and animal nutrition. They are a source of complete protein and balanced in amino acids. However, insects have a poor digestibility, due to the presence of chitin, which gives proteins a certain rigidity and makes them more recalcitrant to hydrolysis by digestive enzymes. In addition to the presence of high content of hydrophobic amino acids like methionine, phenylalanine**,** tryptophan and valine, that can reduce some functional properties of these proteins, namely solubility, which reduce their valuation in food formulations. Therefore, the degradation of chitin seems a valid alternative. To improve the nutritional properties and digestibility of proteins, several techniques have been tested. Some treatments can favor the release of higher quantities of peptides, such as enzymatic pre digestion, sonication and irradiation.

The objective of this work is to develop a protein-enriched probiotic beverage with the aim to provide consumers with foods with high nutritional properties and good digestibility. First, the upstream treatment of the cricket powder by gamma irradiation (IR), ultrasound (US) and ultrasound-assisted gamma irradiation (US-IR), enzymatic hydrolysis assisted by ultrasound followed or not by centrifugation (US-E and US-EWC, respectively) and enzymatic hydrolysis assisted by gamma irradiation and ultrasound (US-IRE), and the study of their impact on physicochemical, structural and *in vitro* digestibility properties were performed.

The results showed that the treatment with US, IR and US-IR promoted an improvement in the solubility, an increase in the content of sulfhydryl (SH) group and changes in the primary and secondary structure of proteins were observed of the treated samples. Non-fermented and fermented beverages containing the combination of probiotics *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus* (*Lactobacillus*) *casei* LBC80R et *Lacticaseibacillus* (*Lactobacillus*) *rhamnosus* CLR2, non-enriched and enriched with US-IR, US-E, US-EWC and US-IRE processed cricket

proteins have been developed. *In vitro* digestibility and the characterization of peptide profiles during digestion were performed. The results showed that the combined treatment US-IRE and US-E had a very positive effect by improving i) the digestibility of fermented beverage from 55% for the control to 94% for US-IRE and US-E and ii) the efficiency of peptide hydrolysis of high molecular weight in low molecular weight peptides (80% at the end of digestion for US-IRE treatment), compared to the non-fermented and fermented non-enriched beverage.

The protein quality and *in vivo* digestibility of probiotic beverage enriched with non-treated cricket protein (CP) and enriched with US-E processed cricket protein (CPH) were evaluated. The results showed that the incorporation of cricket protein in the form of hydrolysate (CPH) increased significantly the Protein Efficiency Ratio (PER) and the Net Protein Ratio (NPR) ($P \leq 0,05$) compared to CP, from 1.7 to 2.0 and 0.4 to 1.0, respectively. Also, an increase in the *in vivo* digestibility was observed for the CPH beverage (96%) compared to the CP beverage (85%). Additionally, the effect of a cricket protein diet on the composition of the gut microbiome was determined and the results showed that the whole cricket protein or its hydrolysates could maintain a balanced composition and reduce certain pathogenic bacteria in the intestine such as *Enterococcaceae* and *Erysipelatoclostridium.*

The physicochemical, nutritional and microbiological quality of the enriched fermented beverage (CPH) was evaluated during 147 days of refrigerated storage at 4◦C and compared with the non-fermented and non-enriched fermented beverage (Bio-K+^{MD} drinkable probiotic). The results showed that the fortification of the beverage with CPH increased the viscosity, reduced the pH and increased the titratable acidity along with a high concentration of viable cells of probiotics compared to the non-enriched beverage with 8.45 log / mL after 147 days. The fermentation made it possible to improve the viscosity which was significantly increased ($P \leq 0,05$) during storage. In addition, probiotics showed high resistance to the *in vitro* gastrointestinal conditions particularly for the beverage enriched with CPH, and showed a percentage of 83% of viable probiotics after 147 days.

The results obtained during this project highlighted the possibility of developing a probiotic beverage enriched with non-conventional proteins with a good nutritional and functional quality. Cricket protein pretreated via enzymatic hydrolysis and ultrasound may build a promising new alternative for the development of functional foods.

The product obtained could meet the wishes of consumers in a product with a high nutritional value, functional, good digestibility and accepted and preserved organoleptic quality over time.

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AAE: acide aminé essentiel

AAS: amino acid score

AD: apparent digestibility

AOAC: association of official analytical chemists

CEP: coefficient d'efficacité protéique

CEPN: coefficient d'efficacité protéique net

CFU: colony forming unit

^{60}CO: Cobalt 60

EAA: essential amino acid

FAO: Food and Agriculture Organization

LAB: Lactic acid bacteria

NPR: net protein efficiency ratio

OMS: Organisation Mondiale de la Santé

PDCAAS: protein digestibility corrected amino acid score

PER: protein efficiency ratio

SEC-HPLC: size exclusion-high performance liquid chromatography

TA: titratable acidity

TCA: trichloroacetic acid

TD: true digestibility

WHO: World Health Organization

kGy: kiloGray

kGy h-1: KiloGray par heure

CHAPITRE 1: REVUE DE LITTÉRATURE

1. Introduction

Le marché des produits alimentaires est très diversifié et en plein essor. En effet, les intervenants dans le domaine alimentaire (chercheurs, industriels) tentent inlassablement d'innover et de développer des nouveaux produits de haute qualité pour répondre aux besoins et aux désirs des consommateurs d'un produit sain, salubre et de bonne qualité fonctionnelle, nutritionnelle et organoleptique (Anderson et al., 2019). En effet, avec le changement du rythme de vie et l'émergence des maladies liées à l'alimentation à savoir le diabète, les maladies gastro-intestinales et les maladies cardiovasculaires, la préoccupation de consommateurs s'oriente de plus en plus vers les aliments fonctionnels et particulièrement les produits probiotiques (Goetzke et al., 2014; Szakály et al., 2012). Ces produits ont démontré une efficacité pour prévenir les infections intestinales, la diarrhée et les infections dû à *Clostridioides (Clostridium) difficile* et la modulation du système immunitaire (Plummer et al., 2004 ; Chabot et al., 2001 ; Michail and Kenche, 2011; Rafter et al., 2007).

Actuellement, les recherches se focalisent sur le développement des produits fermentés enrichis en protéines pour combiner les effets bénéfiques des probiotiques et des protéines. Les produits enrichis en protéines pourraient contribuer dans la lutte contre la carence en protéines et en acides aminés essentiels, en particulier pour les enfants, les sportifs et les personnes âgées. Ils pourraient aider les personnes âgées à prévenir les maladies chroniques et aider les plus vulnérables souffrant d'immunodéficience, de perte osseuse et musculaire (Douglas et al., 2013) tout en établissant un régime alimentaire équilibré. Cependant, face aux contraintes environnementales et aux difficultés associées à la production des protéines conventionnelles, la recherche d'une alternative durable est devenue une nécessité (Guéguen et al., 2016; Huis, 2013).

Les insectes sont considérés comme l'une des sources de protéines les plus intéressantes pour l'avenir. Des nombreux avantages de l'élevage et de consommation des insectes ont été démontré, tels que l'impact environnemental inférieur par rapport aux autres ressources protéiques existantes et la valeur nutritionnelle élevée (Madau et al., 2020). De plus, les insectes répondent aux exigences de la FAO (2007) en acides aminés essentiels (Rumpold and Schlüter, 2013) avec une teneur élevée en protéines. Cependant, les insectes ont une faible

digestibilité, du fait de la présence de la chitine qui rend les protéines plus récalcitrantes à l'hydrolyse par les enzymes digestives. Par conséquent, des précipités insolubles peuvent se former, ce qui réduit la biodisponibilité des oligo-éléments et diminue la digestibilité des protéines dans l'intestin (Marono et al., 2015). Elles sont souvent riches en acides aminés hydrophobes tels que la méthionine, la phénylalanine, le tryptophane et la valine qui affectent certaines propriétés fonctionnelles des protéines à savoir la solubilité qui est considérée comme la propriété la plus intéressante dans les industries des boissons (A., 2011; Kinsella and Melachouris, 1976). Néanmoins, il a été démontré que les procédés de transformations tels que la cuisson et le blanchiment (Melgar- Lalanne et al., 2019) permettent l'amélioration de la qualité sensorielle, la valeur nutritionnelle ainsi que la durée de conservation des protéines d'insectes mais ils peuvent réduire sa solubilité et sa digestibilité.

D'autres procédés émergents comme l'ultrasons et l'irradiation gamma qui peuvent être appliqués comme des pré-traitements pour les protéines, ont démontré une capacité à affecter positivement les propriétés fonctionnelles, structurelles et la digestibilité (Körzendörfer et al., 2019; Kuznetsova et al., 2014 ; Han et al., 2018 ; Qu et al., 2018). La fermentation et l'hydrolyse enzymatique *via* des protéases ont également été appliquées pour augmenter la disponibilité des protéines et faciliter la digestibilité des protéines tout en améliorant leur valeur nutritive et leur qualité organoleptique (Sinha et al., 2007 ; Calbet and Holst, 2004 ; Ali et al., 2003; Beausoleil et al., 2007; Raveschot et al., 2018; Afify et al., 2012).

2. Aliments fonctionnels

Les aliments fonctionnels ont pour rôle de bénéficier à la santé humaine dépassant les propriétés nutritives des aliments de base (van der Zanden et al., 2014 ; Menrad, 2003). Ils sont situés entre les aliments qui fournissent les fonctions physiologiques de base et les médicaments qui traitent les maladies. Les aliments fonctionnels sont utilisés pour maintenir une bonne santé et contrebalancer les petits troubles physiologiques que les hôtes sains peuvent éprouver. En plus des ingrédients fonctionnels bien établis tels que les vitamines, les minéraux, les polyphénols et les micronutriments (Crowe and Francis, 2013), les probiotiques font aussi partie de la génération émergente d'ingrédients actifs (Rodrigues et al., 2020).

Ainsi, avec l'augmentation de la sensibilisation de consommateurs à la nutrition et aux aliments naturels ayant des bienfaits pour la santé, synchronisée avec l'augmentation du coût des soins de santé et le désir des personnes âgées d'améliorer la qualité de leur vie, une hausse de production et de consommation des aliments fonctionnels a été notée (Kotilainen et al., 2006).

Parmi les aliments fonctionnels, on trouve les fruits riches en polyphénols et les légumes comme les crucifères, riches en glucosinolates et en isothyocyanates (Andrade et al., 2020). Les céréales comme le lin, riche en lignanes et en fibres insolubles à savoir les oligosaccharides tels que les galacto et les fructo-oligosaccharides et le bêta-glucane, ont été suggérés comme des prébiotiques, appliqués surtout dans les industries laitières (Brennan and Cleary, 2005; Charalampopoulos et al., 2002; Abdi and Joye, 2021) .

Les polyphénols, particulièrement les catéchines, les anthocyanes et les proanthocyanidines, peuvent augmenter l'abondance de *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Roseburia* et *Faecalibacterium spp*. De plus, l'enrichissement des produits alimentaires en polyphénols a été démontré pour leur effet positif sur la santé. Il permet l'augmentation de la production d'acides gras à chaînes courtes, impliqué dans la prévention des maladies comme le cancer (Alves-Santos et al., 2020).

Plusieurs bactéries lactiques tels que *Lactobacillus sakei*, *Lactobacillus rhamnosus*, *Lactobacillus lactis*, des levures comme *Saccharomyces boulardii* ainsi que leurs composantes membranaires insolubles ont montré plusieurs propriétés nutraceutiques comme des activités anticancer, antioxydantes, antimicrobiennes et la régulation immunitaire nécessaires pour maintenir la santé de l'hôte (Gunaratnam et al., 2021; Fortin et al., 2018; Desrouillères et al., 2016).

Les boissons fonctionnelles sont devenues de plus en plus populaires et ont connu une grande demande durant les dernières années dans le monde entier. En effet, selon Statista (Statista, 2018), les ventes d'aliments et des boissons fonctionnelles dans le marché nord-américain, représentent 45 milliards de dollars US en 2019 et une prévision et sont estimées à 68 milliards de dollars pour 2024.

Parmi les boissons fonctionnelles ayant le plus grand potentiel de croissance mondiale, on trouve les boissons hypocholestérolémiantes (avec combinaison d'oméga-3 et de soja), boissons santé (avec lutéine) ou les boissons « santé des os » (avec calcium et inuline) et les boissons riches en polyphénols. Les yaourts probiotiques sont considérés comme des produits populaires au Canada avec une forte consommation tels que Activia, DanActive de Danone et le lait fermenté de Bio-K+^{MD}.

2.1. Les probiotiques

Les probiotiques représentent un domaine à évolution rapide. Ils sont définis comme des micro-organismes vivants qui, lorsqu'ils sont consommés en quantité adéquate, confèrent un bénéfice pour la santé de l'hôte (Hill et al., 2014). Les bactéries probiotiques ont la capacité de produire une large variété des métabolites avec des avantages pour la santé humaine. Les composés

bioactifs produits par les bactéries probiotiques comprennent, par exemple, les bactériocines, les enzymes métaboliques, les acides aminés, les peptides, les acides gras à chaînes courtes, les vitamines, les antioxydants, les agents anti-inflammatoires et immunomodulateurs et les exo-polysaccharides. Certains produits probiotiques peuvent jouer un rôle dans l'amélioration du métabolisme, la diminution du taux de cholestérol dans le sang, la stimulation du système immunitaire, la désintoxication des cancérogènes potentiels, etc. (Didari, 2015; Michail and Kenche, 2011; Rafter et al., 2007). En plus des avantages pour la santé, il a été démontré que l'hydrolyse enzymatique bactérienne améliore la biodisponibilité des protéines en augmentant la production d'acides aminés libres, ce qui peut améliorer l'état nutritionnel de l'hôte, en particulier si l'hôte présente un déficit en production de protéases endogènes (Arora et al., 2010). La consommation de probiotiques est plus associée à la consommation de produits laitiers, comme les yaourts et les fromages. Habituellement, le yaourt est préparé en présence des cultures pures spécifiques de bactéries lactiques (*Streptococcus thermophilus* et *Lactobacillus bulgaricus*). De plus en plus, les yaourts ont été enrichis avec des probiotiques dont la viabilité varie grandement au cours du temps de conservation (McFarland et al., 2018). Ainsi, la viabilité et les propriétés fonctionnelles de ces bactéries sont liées aux caractéristiques du produit alimentaire y compris sa composition chimique (Ranadheera et al., 2010).

Bien qu'une grande variété de genres et d'espèces de microorganismes soient considérés comme des probiotiques potentiels, les genres utilisés commercialement dans les aliments probiotiques sont principalement des bactéries classées comme *Lactobacillus* et *Bifidobacterium,* qui sont des habitants naturels du microbiote intestinal. Cependant, les espèces appartenant aux genres *Lactococcus*, *Enterococcus*, *Propionibacterium et Saccharomyces* (*Saccharomyces cerevisiae* et *Saccharomyces boulardii*) et les champignons filamenteux comme *Aspergillus oryzae* sont également utilisés comme des probiotiques en raison de leurs effets bénéfiques pour la santé (Min et al., 2019). En revanche, le développement d’un produit alimentaire probiotique nécessite, dans un premier temps, la sélection des souches probiotiques appropriées et le choix de la dose adéquate. Ainsi, la viabilité pendant les opérations de transformation et le stockage, la survie pendant le transit intestinal, la résistance aux antibiotiques, l’exclusion compétitive des bactéries pathogènes, la capacité d’adhésion à la muqueuse intestinale et les bienfaits potentiels pour la santé des consommateurs sont les principaux critères de sélection des souches appropriées des bactéries probiotiques (Ventura and Perozzi, 2011).

Les souches probiotiques *Lactobacillus acidophilus* CL1285, *L.casei* LBC80R et *L. rhamnosus* CLR2 (Bio-K+) sont commercialisées dans des capsules et des boissons fermentées en Amérique du Nord depuis 1996. Ces bactéries ont démontrées une forte capacité à réduire l’incidence des

diarrhées associées aux antibiotiques et à *Clostridioides difficile* (ICD) (Auclair et al., 2015; McFarland et al., 2018). Également, l'évaluation du surnageant du lait fermenté Bio-K+ contenant les trois souches probiotiques en synergie, a montré une inhibition de la croissance des bactéries pathogènes telles que *Escherichia coli, Listeria monocytogenes* et *Enterococcus faecium* en produisant des acides organiques et des substances inhibiteurs de type bactériocine comme des agents antimicrobiens (Millette et al., 2007). De même, les probiotiques de Bio-K+ ont démontré leur efficacité à réduire des lésions précancéreuses dans un modèle animal (Desrouillères et al., 2016) et à l'amélioration de la qualité de la santé du microbiote intestinal (Preston et al., 2018).

2.2. Les produits enrichis en protéines

Les aliments enrichis en protéines peuvent être considérés comme un sous-type parmi les aliments fonctionnels. Aujourd'hui, les produits alimentaires, particulièrement les produits laitiers, enrichis en protéines sont considérés comme une partie intégrante du développement alimentaire pour combler les carences en certains nutriments essentiels, surtout pour les enfants et les personnes âgées souffrant de malnutrition ou souffrant d'un manque d'énergie et de problèmes musculaires (Douglas et al., 2013). En effet, certaines études ont montré que les protéines alimentaires, en plus de l'activité physique, jouent un rôle dans la prévention et la gestion de la sarcopénie (Bauer et al., 2013; Nieuwenhuizen et al., 2010; Song et al., 2019). En outre, des produits enrichis en protéines sont utilisés pour contrôler l'appétit, la satiété et empêcher la reprise de poids après une perte de poids (Hursel et al., 2010; Leidy et al., 2007; Westerterp-Plantenga et al., 2009).

3. Les protéines, besoins et valeur nutritionnelle

Les protéines sont des composants importants de l'alimentation humaine. Elles jouent un rôle essentiel en tant que composants structurels et fonctionnels de systèmes vivants. Les protéines sont formées d'acides aminés liés par des liaisons peptidiques et qui constituent la principale source d'azote dans l'alimentation (Millward et al., 2008).

Le corps humain étant incapable de maintenir des réserves de protéines, un approvisionnement constant en protéines de bonne qualité s'avère nécessaire pour maintenir la croissance et d'autres fonctions physiologiques (Boye et al., 2012). En effet, un faible apport en protéines, par exemple pendant les périodes de croissance et de développement, peut affecter tous les organes du corps, y compris le cerveau, le cœur, le système immunitaire et d'autres organes vitaux. De ce fait, la

qualité des protéines alimentaires est donc un critère important d'une alimentation adéquate et le maintien d'une bonne santé (Ghaly, 2010).

La qualité des protéines peut être définie comme la capacité d'une protéine alimentaire à répondre à la demande métabolique du corps en acides aminés et en azote. Elle est définie par la composition en AA et la digestibilité de la protéine ainsi que la biodisponibilité des AA. La digestibilité est généralement définie en termes d'équilibre des AA dans l'intestin grêle ou à travers l'intestin entier. D'autre part, la digestibilité et la biodisponibilité des AA sont affectés par la matrice alimentaire comme, les concentrations et les types de graisses, glucides et composés antinutritionnels. D'autres facteurs pouvant influencer la qualité des protéines sont liés à l'individu qui consomme la nourriture, comme l'âge, la santé, l'état physiologique et le bilan énergétique (Boye et al., 2012) .

La détermination de la qualité nutritionnelle pourrait être effectuée par des études cliniques chez l'homme par l'évaluation de la croissance et le bilan azoté des individus. Cependant, pour des raisons de coûts et d'éthiques, cette méthode n'est pas adaptée pour la détermination régulière de la qualité d'une protéine. Ainsi, d'autres techniques ont été élaborées en utilisant un modèle animal, généralement le rat mâle en croissance, ou des techniques de détermination *in vitro* (WHO et FAO, 1991). Les techniques fréquemment utilisées comprennent le score d'acides aminés (AAS), digestibilité des protéines *in vivo* (digestibilité apparente, digestibilité réelle), Coefficient d'Efficacité Protéique (CEP), Coefficient d'Efficacité Protéique Net (CEPN) ou encore la PDCAAS (*Protein Digestibility Corrected Amino Acid Score*) (tableau 1).

La digestibilité des protéines *in vitro* est une méthode largement utilisée. Il existe deux types de digestion *in vitro*, la digestion statique et la digestion dynamique. Cette dernière consiste à créer un milieu similaire du système digestif réel. Elle se base sur deux étapes : une digestion dans un mélange pepsine-HCl suivie d'une neutralisation puis une digestion en présence de pancréatine, trypsine ou chymotrypsine-trypsine (Corgneau et al*.,* 2019 ; Lacroix et al*.,* 1983). La digestion doit être effectuée à 37 °C, les échantillons sont mis sous agitations à une durée varie grandement d'une étude à l'autre et en fonction de la nature de l'aliment étudié. Cette méthode a montré une bonne corrélation avec la méthode *in vivo* (Butts et al., 2012). Une méthode multienzymatique qui mesure la baisse du pH après 10 minutes de digestion, a été développée. Elle a montré une reproductibilité et une bonne corrélation avec la méthode *in vivo.*

Cette approche suppose que le taux de variation du pH est corrélé à la digestibilité des protéines reflétant une relation directe entre la baisse du pH et le degré d'hydrolyse des protéines (Butts et al., 2012). Cependant, une méthode séquentielle de la digestion en présence de la pepsine à un

pH proche de 2 pendant 1-3 h puis la trypsine pendant 2-4 h à un pH proche de 7-8, est considérée comme la procédure la plus appliquée (Wang et al., 2008; Li et al., 2013; Minekus et al., 2014; Wen et al., 2015).

Tableau 1. Méthodes utilisées pour l'évaluation de la qualité nutritionnelle d'une protéine

Méthode	Expression des résultats	Principe/Norme
AAS	Teneur en acides aminés essentiels/Acides aminés essentiels recommandés	Lorsque l'AAS est inférieur à 1, l'AAE correspondant est considéré comme limitant et donc la protéine ne fournit pas assez de cet acide aminé.
CEP	Gain de poids (g)/Protéines ingérées (g)	Les sources de protéines avec un CEP inférieur à 1,5 sont de mauvaise qualité
CEPN	Gain de poids (g) + perte de poids groupe sans protéines (g)/Protéines ingérées (g)	Une cote protéique entre 20 et 40 est considérée comme une « une bonne source de protéines » et une cote protéique > 40 comme une « excellente source de protéines »
Digestibilité apparente	AD=Azote consommé – azote excrété/Azote consommé	Correspond à la quantité d'azote excrété présente dans les fèces qui est soustraite à la quantité d'azote ingéré dans la nourriture
Digestibilité réelle	TD= Azote consommé – (azote excrété – azote excrété groupe sans protéines) /Azote consommé	Considère les pertes endogènes en prenant en compte un groupe consommant un régime dépourvu de protéines
PDCAAS	(Teneur en acides aminés essentiels/Acides aminés essentiels recommandés) × TD (%)× 100	Les protéines avec des scores supérieurs à 100 % sont arrondies à 100 %.

(WHO et FAO, 1991 ; Boye et al., 2012 ; Agence canadienne d'inspection des aliments, 2019).

3.1. Protéines conventionnelles

Les protéines utilisées pour l'enrichissement des produits laitiers sont diverses et différentes selon leurs origines et leurs états. En effet, les protéines d'origine animale à savoir le lait et les œufs ont

été largement étudiés (Damin et al., 2009; Garcés-Rimón et al., 2016; Karam et al., 2013). Par exemple, la lactoferrine a été ajoutée en tant que composant dans le yaourt, le lait et les compléments alimentaires et son effet sur la fabrication et les caractéristiques physicochimiques a été étudié (Franco et al., 2010). De plus, les isolats et les concentrés de protéines de lactosérum sont les protéines les plus utilisées pour l'enrichissement des produits laitiers; ils ont une influence significative sur la texture et le processus de fermentation du produit (Morell et al., 2017; Nastaj et al., 2019).

Dans le même contexte, l'ovalbumine a été ajoutée dans les produits laitiers pour sa fonctionnalité, sa valeur nutritionnelle et ses activités biologiques à savoir l'activité antioxydante et antimicrobienne (Nimalaratne et Wu, 2015). Son incorporation a amélioré les propriétés fonctionnelles tout en maintenant les caractéristiques rhéologiques et organoleptiques des produits formulés (Menéndez et al., 2006). Cependant, au cours des dernières années, des protéines végétales et d'autres protéines d'origine animale non conventionnelles, sont devenues plus populaires (Ainis et al., 2018; Alves and Tavares, 2019) en raison de l'état actuel de l'environnement et en réponse à l'intérêt croissant des consommateurs pour un système durable avec une alimentation plus économique et plus respectueuse de l'environnement.

3.2. Protéines alternatives

Les protéines alternatives ont des propriétés nutritionnelles comparables et parfois meilleures par rapport aux protéines animales conventionnelles et peuvent apporter des avantages pour la santé des consommateurs (Barac et al., 2012). De plus, ces protéines peuvent contribuer à l'amélioration des propriétés technologiques des produits alimentaires. L'exemple le plus courant est le soja utilisé comme matière première pour obtenir des boissons au soja. Il est considéré une alternative pour les personnes souffrant d'intolérance aux protéines laitières (Vij et al., 2011). En outre, les graines de lupin et les isolats de protéines de graines de lupin ont été utilisés dans la fabrication d'un breuvage fermenté (Kuznetsova et al., 2014). Cet intérêt est lié à sa haute teneur en protéines qui sont considérées comme une bonne source de lysine (El-Adawy et al., 2001; Pollard et al., 2002).

L'arachide a également été ajoutée dans le yaourt et évaluée pour son effet sur les paramètres physico-chimiques (Isanga and Zhang, 2009). Le pois et la fève représentent une alternative potentielle aux protéines d'origine animale (Ainis et al., 2018; Alves and Tavares, 2019). Le *Moringa oleifera* Lam. (Moringa) a fait récemment l'objet des recherches en raison de ses propriétés nutritionnelles et son efficacité comme agent de coagulation naturel dans les industries alimentaires. La capacité coagulante des graines de Moringa peut être liée à la présence de

protéines et de peptides qui représentent 45% de sa composition (Baptista et al., 2015; Cardines et al., 2018).

La digestibilité et la valeur biologique des protéines de riz ont été rapportées comme supérieures à celles des autres céréales à savoir le blé, le maïs et l'orge (Amagliani et al., 2017). Aussi, les protéines de riz sont généralement considérées comme hypoallergéniques, avec plusieurs études suggérant que les protéines de riz, particulièrement leurs hydrolysats ont des activités antioxydante (Burris et al., 2010), antihypertensive, anticancéreuse (Kannan et al., 2010) et anti-obésité (Yang et al., 2012). De ce fait, le riz représente une source intéressante de protéines pour le développement des produits enrichis en protéines et de bonne qualité nutritionnelle.

D'autres voies explorées sont les champignons, les algues, les microorganismes, les plantes aquatiques, les insectes et la viande synthétisée *in vitro,* à partir de cellules souches. Ces sources alternatives représentent un intérêt en raison de leur richesse en nutriments et particulièrement en protéines, de leur effet positif sur l'environnement, de leur production élevée et économique et de leur faible exploitation de surfaces comparativement aux sources traditionnelles (Tableau 2).

Tableau 2. Présentation des différentes sources protéiques

Source protéique	Valorisation en alimentation	Particularités	Références
Insectes	Animale/humaine	Avantage écologique, valorisation des sous-produits, richesse nutritionnelle	Koeleman, 2016; (Huis, 2013)
Végétales	Animale/humaine	Bénéfiques pour la santé humaine, faible teneur en cholestérol, faible coût	(Marti-Quijal et al., 2019 ; 2018)
Algues	Animale/humaine	Faible coût, durable, développement très rapide à partir de nutriments simples	(Jung et al., 2013); (Marti-Quijal et al., 2019)

3.3. Source animale non conventionnelle : Insectes

3.3.1. Impact et consommation

Les insectes sont considérés comme une nourriture traditionnelle dans des nombreux pays depuis des milliers d'années, notamment dans les zones tropicales qui trouvaient dans les insectes récoltés dans la nature une source de protéines abondante et très abordable (Barre et al., 2014).

En plus de leur rôle dans l'alimentation animale et humaine, les insectes fournissent des nombreux services écologiques fondamentaux pour l'humanité. En effet, ils jouent un rôle important dans la reproduction végétale par la pollinisation, améliorent la fertilité des sols par bioconversion des déchets, contrôlent les nuisibles et fournissent divers produits de valeur à savoir le miel, la soie, ou médicinaux comme l'asticothérapie. En plus, FAO et l'UE, indiquent que les insectes comestibles pourraient devenir l'une des solutions aux problèmes de l'approvisionnement alimentaire mondial. Toutes ces raisons poussent la FAO à recommander dans son rapport « *Edible Insects* » d'envisager l'élevage d'insectes à grande échelle (**Avis de l'Anses Saisine n° 2014-SA-0153).** Toutefois, le concept d'élevage d'insectes pour l'alimentation est relativement nouveau. En Amérique du nord, la production et la consommation d'insectes comestibles sont en plein essor. Ces insectes représentent une source alternative et innovante de protéines. Actuellement, ils se présentent dans les épiceries sous plusieurs formes (farine, des barres énergétiques ou protéinée). Généralement, les insectes les plus consommés sont les coléoptères (*Coleoptera*; 31%), les chenilles (*Lepidoptera*;18%), les abeilles, les guêpes et les fourmis (*Hymenoptera*; 14%), viennent ensuite les sauterelles et criquets (*Orthoptera*;13%), les cochenilles (*Hemiptera*; 10%), les termites (*Isoptera;* 3%), les libellules (*Odonata; 2%*) et les mouches (*Diptera*; 2%) (Huis, 2013). Ces insectes sont consommés dans certains pays pour leurs caractéristiques organoleptiques et leurs propriétés nutritionnelles. Les criquets ont attiré une attention particulière. Ils se sont avérés être une bonne source de protéines, de matières grasses, de minéraux et de fibres (Montowska et al., 2019).

3.3.2. Enjeux législatifs

Les insectes comestibles ne sont toujours pas largement utilisés pour l'alimentation animale et la nutrition humaine dans plusieurs pays, principalement en raison de difficultés législatives.

Les limitations règlementaires de la consommation d'insectes sont liées à des dangers physiques, chimiques, microbiologiques et danger d'allergène (Huis, 2013). De ce fait, les législations qui concernent l'utilisation d'insectes sont bien strictes et divergent d'un pays à un autre. Au Canada,

aucune réglementation pour l'élevage, la production ou la transformation d'insectes comestibles n'existe encore. Également, l'exercice de cette activité ne demande aucune permission. Cependant, des règles de salubrité et d'hygiène s'appliquent pour la transformation ou la préparation d'insectes destinée à la consommation humaine afin de garantir l'innocuité de produit et d'assurer qu'ils sont appropriés pour la consommation humaine et répondent aux normes d'évaluation de Santé Canada (Santé Canada, 2007).

3.3.3. Composition biochimique

Les insectes constituent une source alimentaire très importante pour l'alimentation animale ainsi qu'humaine. Cependant, les valeurs nutritionnelles des insectes comestibles sont très variables, notamment en raison de la grande variété d'espèces. Même au sein du même groupe d'espèces, les valeurs peuvent différer selon le stade métamorphique, ainsi que leur habitat et leur régime alimentaire (Piccolo et al., 2017). En effet, les insectes possèdent une teneur élevée en protéines, en matière grasse et en acides gras essentiels insaturés notamment C18, y compris les acides linoléique et α-linolénique (Kouřimská and Adámková, 2016), pouvant avoir des effets bénéfiques en transportant le cholestérol (Jandacek, 2017) et en réduisant la pression sanguine (Sales-Campos et al., 2013). Également, ils constituent une source riche en minéraux tels que le fer, le zinc, le potassium, le sodium, le calcium, le phosphore, le magnésium, le manganèse et le cuivre, et en vitamines tels que B2 (riboflavine), B5 (acide pantothénique), B7 (biotine) et B9 (acide folique) (Nowak et al., 2016), mais aussi en fibres insolubles notamment la chitine (Finke, 2013). Ainsi, des travaux de recherches ont suggérés que la consommation d'insectes pourrait avoir un impact positif sur la santé humaine. Wang et al. (2016) and Stull et al. (2018) indiquent que les insectes peuvent stimuler la croissance du microbiote intestinal, améliorer les fonctions immunitaires et diminuer le taux sanguin du facteur de nécrose tumorale (TNF-α), une cytokine qui contribue à la création d'un environnement inflammatoire. D'autre part, les insectes ont été démontrés pour leur richesse en peptides antimicrobiens qui ont un effet sur différentes bactéries et pour lesquels le risque de résistance bactérienne est très faible (Chernysh et al., 2015). En effet, Ji et al. (2016), ont signalé une baisse marquée de diarrhée chez les porcs sevrés en raison de la supplémentation alimentaire en protéines de ténébrion jaune, en ver de farine géant et en mouche domestique, et ont attribué ce résultat à l'effet des peptides antimicrobiens contenus dans les farines d'insectes.

3.3.4. Protéines et acides aminés

Les insectes comestibles représentent une source protéique intéressante. En effet, des travaux de recherches ont démontré que les insectes présentent des teneurs élevées en protéines. Par exemple, les criquets ont une teneur en protéines supérieure à celle du poulet et du soja et comparable à celle du bœuf et de poisson (tableau 3), avec un apport important en acides aminés essentiels, et plus digestibles que les protéines végétales (Makkar et al., 2014).

Tableau 3. Teneur en protéines (g/100 g de matière sèche) d'insectes et produits utilisés comme source de protéines dans l'alimentation

Organismes	Protéines (g/100g)
Farine de criquet	62,5
Larves de ver de farine	68,1
Farine de poisson	70,6
Farine de soja	51,8
Bœuf	62,3
Poulet	39,0

(Makkar et al., 2014; Rumpold and Schlüter, 2013)

Comme le montre le Tableau 4, les protéines d'insectes ont une composition équilibrée et complète en AAE et qui répondent bien aux besoins nutritionnels d'un adulte en protéines indiqués par la FAO et l'OMS. De plus, ils sont comparables à celle des farines de poisson et de soja (Makkar et al., 2014; Rumpold and Schlüter, 2013). D'autre part, les céréales sont pour la plupart déficients en lysine et riche en méthionine, alors que les légumineuses sont souvent pauvres en méthionine, mais riches en lysine (Kannan et al*.,* 2001; Mensa-Wilmot et al*.,* 2001). Ceci affecte la qualité nutritive notamment le PDCAAS de ces protéines qui est par exemple proche de 50% pour le riz et varie entre 30 et 70% pour le pois (Boye et al*.,* 2012) tandis que les protéines d'insectes ont des valeurs proches de 100%.

Également, Ramos-Elorduy et al. (2009) ont trouvé qu'un insecte peut avoir une composition en AAE variant de 46 à 96 % avec une digestibilité élevée entre 77 et 98 %. Ces valeurs pour les insectes avec un exosquelette sont de niveau inférieur en raison de leur teneur en chitine. Si le

squelette externe est enlevé, alors leur digestibilité s'améliore. En effet, les protéines d'insectes sont plus digestibles que certaines protéines végétales à savoirs les cacahuètes et les lentilles mais moins digestibles que les protéines animales telles que le bœuf et le blanc d'œuf (Rumpold and Schlüter, 2013).

Dans une autre étude, Hwangbo et al. (2009) ont suggéré que la substitution de 25% de farine de poisson par la farine d'insectes a permis un gain de poids rapide des poissons. De plus, les protéines d'insectes se sont révélées efficaces pour assurer une bonne croissance de poulet (Huis, 2013), du poisson (St-Hilaire et al., 2007 ; Ng et al., 2001) et du porc (Newton et al., 1977). Alors que, l'étude de Hardouin et Mahoux (2003) a montré qu'aucune différence significative n'était notée entre le gain de poids des poulets nourris avec des farines de maïs /grillon et celui de poulets nourris avec des farines maïs/soja.

Ces études révèlent l'intérêt d'insectes en tant qu'ingrédient pour l'alimentation animale. Cependant, leur utilisation pour l'alimentation humaine reste encore marginale et à petite échelle.

Tableau 4 : Composition en acides aminés (g/g de protéines) d'insectes et de farines de poisson et de soja (Rumpold et Schlüter, 2013a ; Makkar et al., 2014b ; WHO, 2007c)

Acides aminés (g/100 g de protéines)	Ver de farine (adulte)[a]	Criquet (adulte)[a]	Farine de poisson [b]	Farine de soja [b]	Besoins de l'homme [c]
Acides aminés essentiels					
His	2,87	2,27	2,40	3,06	1,50
Ile	4,35	3,64	4,20	4,16	3,00
Leu	8,27	6,67	7,20	7,58	5,90
Lys	4,43	5,11	7,50	6,18	4,50
Cystine	-	-	0,80	1,38	0,60
Met	1,27	1,96	2,70	1,32	1,60
Met+Cys	1,94	2,93	-	-	2,20
Phe+ Tyr	5,95	7,42	7,00	8,51	3,00
Thr	3,42	3,11	4,10	3,78	2,30
Trp	1,10	0,76	1,00	1,36	0,60
Val	6,33	4,84	4,90	4,50	3,90
Acides aminés non essentiels					
Arg	4,30	5,73	6,20	7,64	-
Gly	8,44	4,53	6,40	4,52	-
Glu	-	-	12,60	19,92	-
Asp	-	-	9,10	14,14	-
Pro	6,33	5,42	4,20	5,99	-
Ser	4,14	5,20	3,90	5,18	-
Ala	7,64	7,69	6,30	4,54	-

His : histidine, Ile : Isoleucine, Leu : leucine, Lys : lysine, Met : méthionine, Cys : cystéine, Phe : phénylalanine, Tyr : tyrosine, Thr : thréonine, Trp : tryptophane, Val : valine, Arg : Arginine, Gly : Glycine, Glu : Acide Glutamique, Asp : Acide aspartique, Pro : Proline, Ser : Serine, Ala : Alanine.

3.3.5. Applications alimentaires et limites

Actuellement, les insectes émergent comme une alternative pour l'alimentation humaine et animale. De façon générale, les insectes sont souvent consommés en entier, mais ils peuvent également être transformés en granules ou en pâtes et utilisés comme ingrédients. Grâce à leur teneur élevée en protéines, les insectes sont utilisés en alimentation animale (poissons, poulets et porcs) et ils commencent à s'intégrer de plus en plus dans les formulations alimentaires destinées pour l'alimentation humaine.

Les protéines d'insectes ont été incorporées dans des matrices alimentaires, à savoir les viandes, les céréales, le pain et les pâtes analogues (Azzollini et al., 2018; Duda et al., 2019; Kim et al., 2016; Osimani et al., 2018). Toutefois, la valorisation des protéines d'insectes reste toujours limitée dans les industries alimentaires. Ceci pourrait être dû à l'insuffisance des données liés à leur propriétés fonctionnelles par rapport aux protéines largement utilisées et à la faible digestibilité due à la présence de la chitine qui enveloppe les cuticules externes d'insectes, forme des liaisons avec les protéines et limite grandement la dégradation des protéines par les enzymes digestives (Marono et al., 2015).

Les insectes comestibles contiennent une quantité importante de fibres. La chitine insoluble est la forme de fibres la plus courante dans le corps des insectes, présente principalement dans leur exosquelette (Kouřimská and Adámková, 2016). La chitine est considérée comme une fibre indigeste, même si l'enzyme chitinase se trouve dans le suc gastrique humain (Schlüter et al., 2017). Cependant, il a été constaté que cette enzyme peut être inactive. D'autre part, l'interaction chitine-protéine pourrait affecter les propriétés techno-fonctionnelles (par exemple émulsification, moussage, solubilité, etc.), limitant ainsi son application dans des formulations alimentaires (Liceaga, 2019). De ce fait, l'élimination de la chitine peut améliorer la digestibilité des protéines d'insectes et donc leur qualité nutritionnelle (Finke, 2013).

4. Procédés du traitement de farines d'insectes

4.1. Procédés physiques

Plusieurs méthodes physiques ont été utilisées pour la modification de la structure des protéines dans le but d'élargir ses domaines d'application dans l'industrie alimentaire. Parmi ces méthodes, on trouve l'irradiation gamma et la sonication qui ont attiré une grande attention pour leurs capacités à modifier la structure et la fonctionnalité des protéines alimentaires et à dégrader la chitine (Abidin et al., 2017) tout en assurant l'efficacité énergétique, la sécurité alimentaire, la

désamination des protéines et des pertes minimales en nutriments (Ashraf et al., 2019 ; Kadam et al., 2015 ; Dogbevi et al., 2000).

4.1.1. Irradiation gamma

L'irradiation gamma est une technique non thermique utilisée pour la conservation des aliments et pour garder leur fraicheur et leur qualité tout en préservant les propriétés biologiques et sensorielles du produit traité (Farkas et Mohácsi-Farkas, 2011). L'irradiation des aliments consiste à traiter les aliments avec des rayonnements ionisants (rayons-γ, rayons-X et accélérateur d'électron) (Petersen, 2003). Lorsque les rayonnements ionisants pénètrent dans les aliments, les atomes et les molécules de l'aliment absorbent l'énergie. Selon la quantité d'énergie absorbée, les atomes et les molécules peuvent devenir excités (I), perdre un électron (II) ou se dissocier (III) pour former des ions ou libérer des radicaux (Fombang, 2005).

Les sources d'irradiation ionisantes autorisées pour le traitement des aliments sont les rayons gamma produits à partir de radioisotopes cobalt-60 (1,17 et 1,33 MeV), césium-137 (0,662 MeV), des faisceaux d'électrons (énergie maximale 4e10 MeV), Rayons X (énergie maximale 5 MeV) (Codex Alimentarius Commission, 2003). Récemment, le traitement par irradiation a suscité un intérêt croissant comme un procédé peu couteux et respectueux de l'environnement, pour des applications non conservatrices. En effet, l'irradiation a été utilisée pour modifier les caractéristiques physicochimiques, fonctionnelles, structurales mais aussi pour l'amélioration de la digestibilité des protéines (Afify et al., 2011; Baccaro et al., 2018). Généralement, les protéines peuvent être irradiées en solution ou bien à l'état solide. À l'état de poudre, les molécules de protéines peuvent absorber l'énergie de rayonnement directement, provoquant certaines altérations (de la Hoz and Netto, 2008), alors que dans le système humide, les molécules d'eau sont ionisées pour produire des espèces réactives qui peuvent se déplacer et réagir avec d'autres ions et radicaux libres provoquant des modifications des protéines. De ce fait, l'effet de l'irradiation devrait être plus prononcé dans les systèmes humides que dans les systèmes secs étant donné l'effet supplémentaire des produits de radiolyse de l'eau.

Des travaux de recherche ont rapporté des changements de structure primaire, secondaire et tertiaire sous l'effet de l'irradiation gamma (Moon and Song, 2001; Wolff et al., 1986). L'irradiation des protéines peut provoquer également certaines modifications permanentes telles que la désamination, la décarboxylation, la réduction des liaisons disulfures, l'oxydation des groupes sulfhydriles, la modification des groupements aminoacides, le clivage de la chaîne peptidique et l'agrégation (Dogbevi et al., 2000). De plus, le traitement par irradiation peut avoir un effet sur

propriétés fonctionnelles des protéines. En effet, Le Maire et al. (1990) ont montré que la fragmentation des protéines suite à un traitement par irradiation, diminue le poids moléculaire, conduisant à une amélioration de la solubilité, tandis que l'agrégation des protéines entraîne une diminution de la solubilité. De même, Liu et al. (2009) ont démontré que l'irradiation gamma pouvait réduire la viscosité et améliorer la capacité moussante du blanc d'œuf ainsi que leur capacité émulsifiante. Les propriétés nutritionnelles et digestives des protéines traitées par irradiation gamma ont également été étudiées (Hassan et al., 2018 ; Fombang, 2005). En outre, il a été démontré que le traitement par irradiation réduit les composants antinutritionnels comme les tannins, les phytates et les inhibiteurs de protéases tout en améliorant les propriétés nutritionnelles et fonctionnelles et la qualité globale des végétaux (Bhat et al., 2011). De plus, Osman et al. (2014) ont montré qu'à des faibles doses d'irradiation (0.5 et 1.0 kGy), la teneur en facteurs antinutritionnels a diminué significativement, ce qui a entrainé une augmentation de la digestibilité des protéines de graines de féverole. D'autre part, Fombang (2005) a montré que l'augmentation de la digestibilité pourrait être due à la modification de la structure de la protéine qui la rend plus accessible aux enzymes protéolytiques.

4.1.2. Ultrasons

Actuellement, la technologie des ultrasons attire une attention considérable notamment dans le domaine des industries alimentaires pour le traitement des produits liquides et même solides. Les ultrasons sont définis comme des ondes sonores dont la fréquence dépasse la limite d'audition de l'oreille humaine (~20 kHz), et qui ont des nombreux avantages comme la simplicité d'utilisation, faisabilité économique (économes en énergie) et respect de l'environnement (Ashokkumar, 2015).

Des systèmes de bain à ultrasons et de sonde sont utilisés pour le traitement des protéines. Les bains à ultrasons nécessitent plus du temps par rapport au système de sonde en raison de la faible énergie générée (Kadam et al., 2015). Les changements physiques et biologiques des matrices traitées par ultrasons sont dus aux effets de l'énergie mécanique, cavitationnels et thermiques générées par le procédé. Les ultrasons jouent un rôle clé dans la technologie alimentaire, comme dans le traitement, la conservation et l'extraction des composés d'intérêt à partir d'un produit naturel. Également, le traitement des protéines par ultrasons peut provoquer des changements physicochimique et structurel, se manifestant par des modifications des attributs fonctionnels des protéines, une réduction de la viscosité apparente, une augmentation de l'hydrophobicité et des améliorations dans la formation et la stabilité de l'émulsion, la solubilité des protéines et l'activité enzymatique (Awad et al., 2012). Vanga et al. (2020) ont révélé des

perturbations des liaisons non covalentes comme les liaisons covalentes disulfures et les liaisons hydrogène qui jouent un rôle principal dans la conformation structurale secondaire et tertiaire des protéines dues à l'énergie acoustique générée par les ultrasons. Cependant, l'effet des ultrasons sur la digestibilité et la structure secondaire des protéines d'insectes n'a pas encore été étudié profondément.

4.2. Traitements biologiques

4.2.1. Hydrolyse enzymatique

La biocatalyse est devenue un outil nécessaire dans la production industrielle de produits pharmaceutiques actifs, d'intermédiaires agrochimiques et pharmaceutiques, de produits chimiques en vrac et d'ingrédients alimentaires ainsi que pour la clarification des jus (Bilal and Iqbal, 2020; Thomas et al., 2002; Truppo, 2017).

Les enzymes protéolytiques sont utilisées dans la modification et le traitement des protéines. Il en résulte une solubilité, une stabilité à la chaleur et une résistance améliorée aux précipitations en milieu acide (García Arteaga et al., 2020). Les hydrolysats de protéines sont également utilisés comme agent émulsifiant dans diverses applications comme les vinaigrettes, les pâtes à tartiner, la crème glacée, le blanchisseur de café et les saucisses (Sharma et al., 2019). L'utilisation des hydrolysats de protéines laitières a été étudiée. En effet, les hydrolysats de protéines du lait préparés par hydrolyse enzymatique ont trouvé leur application dans les aliments nutritionnels (Mahmoud et al., 1992) et sont également utilisés pour remplacer les protéines natives destinées aux aliments et aux boissons pour nourrissons et adultes (Sinha et al., 2007). De même, les hydrolysats de protéines du lait peuvent diminuer l'allergénicité, augmenter l'adsorption (en raison de leur taille peptidique), améliorer les activités fonctionnelles et biologiques des protéines par la libération des peptides bioactifs (Li-Chan, 2015).

Des protéases de différentes sources peuvent être utilisées pour la préparation des hydrolysats de protéines. Les enzymes digestives (pepsine, trypsine et chymotrypsine), les enzymes d'origine végétale (comme la papaïne et la bromélaïne) et d'origine microbienne (alcalase, subtilisine) ont été utilisées isolément, en combinaison ou séquentiellement. L'alcalase a été largement exploitée. Il s'agit d'une protéase peu coûteuse et non spécifique utilisée pour la production d'hydrolysats de protéines de différentes origines comme les protéines de tournesol, de colza ou de pois chiche hautement solubles (Clemente et al., 1999; Villanueva et al., 1999). En outre, les hydrolysats de protéines obtenus par hydrolyse avec une alcalase sont caractérisés par des propriétés fonctionnelles élevées et une faible antigénicité (Meinlschmidt et al., 2016). Ainsi, l'alcalase s'est

avérée également utile pour la génération de peptides bioactifs (Byun and Kim, 2001; Park et al., 2001).

L'hydrolyse enzymatique de protéines d'insectes a été réalisée pour générer des peptides bioactifs et pour améliorer leurs propriétés techniques et fonctionnelles comme la lubrification, l'émulsification, le moussage et la stabilité thermique. (Nongonierma et FitzGerald, 2017).

4.2.2. Fermentation

La fermentation est l'un des plus anciens procédés de transformation et de conservation des aliments (Ross et al., 2002). De nombreux changements biochimiques se produisent pendant la fermentation, entraînant une modification du rapport des composants nutritifs et anti-nutritifs dans le produit, qui affectent les propriétés du produit telles que la bioactivité et la digestibilité. En effet, une modification de la structure des protéines plutôt que la décomposition des protéines en composants plus petits a été proposée comme mécanisme par lequel la fermentation améliorait la digestibilité des protéines (Taylor et Taylor, 2002). Outre la digestibilité, la fermentation pourrait également améliorer la solubilité des protéines, l'accessibilité aux glucides et aux acides aminés (Singh et al., 2012).

La fermentation lactique *via* des bactéries lactiques est l'un des processus de préparation des aliments et aurait de nombreux avantages et effets sur les propriétés technologiques, organoleptiques et nutritionnelles (Magala et al., 2013; Owusu-Kwarteng et al., 2015). Dans ce contexte, Hong et al. (2004) ont démontré que le processus de fermentation a amélioré la qualité nutritionnelle des graines et des tourteaux de soja tout en produisant un ingrédient alimentaire fonctionnel avec une digestibilité plus élevée. D'autre part, Roger et al. (2015) ont trouvé que la fermentation de pâte de maïs *via* des bactéries lactiques a pu réduire le taux de tannins et de phytates en plus d'améliorer la qualité nutritionnelle par l'augmentation de la biodisponibilité des minéraux et la teneur en protéine.

En outre, la fermentation peut modifier certains paramètres physicochimiques à savoir le pH et l'acidité titrable (Chu et al., 2019), améliorer la qualité rhéologique (Hayta et al., 2001) et sensorielle du produit fermenté (Soukoulis et al., 2007). En effet, Salmerón et al. (2015) ont prouvé que la fermentation d'un breuvage à base de céréales avec des probiotiques notamment des lactobacilles, a amélioré l'acceptabilité du produit par le consommateur tout en modifiant les paramètres physicochimiques et nutritionnels de breuvages. La fermentation pourrait être également effectuée avec des souches fongiques (Bender and Doell, 1957; J. Yang et al., 2020) ou bien avec les *Bacillus* spp. (Zhu et al., 2008). Ainsi, il est important de mentionner que très peu

d'études sur l'effet de la fermentation sur les protéines de criquets ont été réalisées à l'heure actuelle. Par exemple, Klunder et al. (2012) ont montré que l'enrichissement des produits fermentés en protéines de criquets et d'autres insectes pourrait avoir des avantages sur les propriétés physicochimiques et l'amélioration de la stabilité du produit tout en empêchant la croissance de potentiel micro-organismes nuisibles. Dans une autre étude (Osimani et al., 2018), la farine de criquets a été utilisée pour l'enrichissement du pain et attribuée en un produit de haute qualité nutritionnelle.

4.3. Traitements combinés

La combinaison des traitements a pour but de réduire la consommation d'énergie, améliorer les performances de procédés et éviter des restrictions plus prononcées sur la matière traitée liées à l'élévation d'énergie et de la température. Récemment, la méthode enzymatique assistée par ultrasons a attiré une attention croissante pour son application dans la modification des structures et des propriétés des protéines alimentaires (Ozuna et al., 2015 ; Wu et al., 2018). En effet, Dong et al. (2019) ont montré que l'utilisation de traitement combiné d'hydrolyse enzymatique assisté par ultrasons a amélioré les propriétés fonctionnelles des protéines de poulet dont la solubilité et les propriétés émulsifiantes. De même, Yang et al. (2020) ont montré que le traitement par irradiation gamma précédé par l'ultrason a permis de réduire l'allergénicité de β-lactoglobuline tout en entrainant des modifications au niveau de la structure secondaire.

L'application d'ultrasons lors de la fermentation a permis de faciliter les traitements ultérieurs tels que le brassage et le cisaillement pour obtenir une texture onctueuse, augmenter la croissance bactérienne et réduire le temps de la fermentation (Ojha et al., 2017). Les ultrasons peuvent modifier positivement les propriétés du yaourt brassé, telles que la diminution de la viscosité apparente et la grossièreté perçue visuellement. Ce dernier effet est prometteur en termes de conception de produits riches en protéines et faciles à consommer (Körzendörfer et al., 2019; Körzendörfer and Hinrichs, 2019). La combinaison de la fermentation et de l'hydrolyse enzymatique *via* des protéases a été également étudiée. Une amélioration de la qualité des protéines de soja par l'augmentation de la teneur en acides aminés et en peptides de faibles poids moléculaire, a été constatée dans l'étude de Yang et al. (2020) tout en diminuant le temps de la fermentation. L'évaluation de l'effet de ces traitements combinés sur la digestibilité des protéines en général et sur les protéines d'insectes en particulier n'est pas encore investiguée.

5. Hypothèses, objectifs et moyens

5.1. Problématique

L'enrichissement des boissons en protéines pourrait être une solution efficace pour combler les carences en certains AAE et répondre aux besoins en un aliment protéiné afin d'améliorer certaines fonctions physiologiques et/ou lutter contre des maladies. Ceci est surtout pour les enfants, les sportifs et les personnes âgées (Keršienė et al., 2020). Les insectes constituent l'une des voies alternatives durables qui peuvent remplir ces besoins. Cependant, l'acceptabilité des produits enrichis en protéines peut être entravée par le problème de la digestibilité ce qui pourrait affecter la biodisponibilité des AA. Ainsi, les protéines d'insectes sont considérées comme des protéines à faible digestibilité dû à la présence des fibres insolubles notamment la chitine (Belforti et al., 2015; Fontes et al., 2019). De plus, les protéines d'insectes ont une solubilité faible en raison de leur teneur élevée en AA hydrophobes tels que la méthionine, la phénylalanine, le tryptophane et la valine, ce qui constitue un facteur limitant de leur valorisation dans les industries des boissons. En effet, l'ajout des protéines d'insectes dans une boisson peut donner un produit de texture très visqueuse voir pâteuse qui peut influencer l'acceptabilité du produit par le consommateur notamment le goût d'une part et affecter la faisabilité industrielle d'autre part par l'entrainement des endommagements des installations industrielles.

L'objectif général de ce projet est d'élaborer une boisson probiotique enrichie en protéines d'insectes (criquet) ayant une bonne qualité nutritionnelle, notamment la digestibilité avec des propriétés fonctionnelles améliorées et organoleptiques acceptées par le consommateur. Dans ce cadre, il est proposé de traiter les protéines de criquet en amont avec différents traitements physiques et biologiques et d'enrichir des boissons probiotiques avec les protéines prétraitées pour avoir un produit de haute fonctionnalité.

5.2. Hypothèses

1) Le traitement des protéines d'insectes en amont *via* des procédés physiques (ultrasons, irradiation gamma) et/ou des procédés biologiques (hydrolyse enzymatique) et/ou des traitements combinés améliore leurs propriétés fonctionnelles, structurelles et la digestibilité.
2) La fermentation *via* des bactéries probiotiques permet d'assurer une prédigestion et améliore la qualité protéique et nutritionnelle.

3) La fermentation d'une boisson enrichie en protéines d'insectes prétraitées a un effet bénéfique sur la qualité et l'efficacité protéique la rendant proche de celle de la caséine, la protéine animale de référence, et pourrait contribuer à un microbiote intestinal sain et équilibré.

4) L'enrichissement des boissons en protéines d'insectes permet d'améliorer les propriétés nutritionnelles, physicochimiques et organoleptiques, de protéger les probiotiques et d'améliorer leur résistance aux conditions gastrointestinales.

5.3. Objectifs

Le but du projet était la mise au point des technologies permettant d'améliorer les propriétés fonctionnelles, nutritionnelles et la digestibilité des protéines d'insectes, en combinaison avec la fermentation pour l'élaboration d'un breuvage enrichi en protéines de bonnes valeurs nutritives.

Les objectifs sont :

1) Mettre au point des procédés de pré-traitements des protéines tels que les ultrasons, l'irradiation gamma et la protéolyse seuls et en combinaison pour augmenter la valeur nutritive.
2) Évaluer les propriétés fonctionnelles et structurelles des protéines traitées suite aux pré-traitements mis au point dans objectif 1.
3) Mettre au point des boissons probiotiques (contiennent initialement 3% des protéines de riz) enrichies en protéines de criquet traitées suite aux pré-traitements mis au point dans l'objectif 1.
4) Évaluer la digestibilité *in vitro* et les profils peptidiques au cours de la digestion sur les boissons enrichies en protéines prétraitées selon les méthodes optimales mises au point dans 1.
5) Évaluer la digestibilité *in vivo* et les paramètres de la qualité protéique (CEP, CEPN) en fonction des résultats obtenus dans l'objectif 4.
6) Évaluer l'effet de l'enrichissement des boissons en protéines de criquet traitées sur le microbiote intestinal.
7) Analyser l'effet de l'entreposage à 4 °C sur la qualité physicochimique, microbiologique, nutritionnelle et sensorielle du breuvage mis au point.

5.4. Moyens pour atteindre les objectifs

Pour atteindre ces objectifs :

1) Des traitements tels que les ultrasons, l'irradiation gamma, et la protéolyse seuls et en combinaison ont été mis au point pour en vérifier leurs effets sur les propriétés fonctionnelles telle que la solubilité (Adebiyi et Aluko, 2011). Les propriétés structurelles ont également été analysées telles que la teneur en liaison et en surface sulfhydryle par spectrométrie UV-Vis à 412 mm, l'hydrophobicité par spectrophotométrie à fluorescence à une longueur d'onde d'excitation de 390 nm et une longueur d'onde d'émission de 468 nm, les structures primaires et secondaires par ATR-FTIR (Byler et Susi, 1988; Shimada et Cheftel, 1988; Kato et Nakai 1980).

Des boissons non fermentées et fermentées par des probiotiques ont été mises au point en utilisant la poudre de criquets traitée par les différents procédés cités ci-dessus, ajoutée aux boissons.

2) L'évaluation de la digestibilité *in vitro* a été effectuée selon une méthode de digestion enzymatique séquentielle (Wang et al., 2008) et a été calculée comme suit :

$$\%\ \text{Digestibilié} = \frac{\text{Azote dans le surnageant}}{\text{Azote dans l'échantillon}} \times 100$$

Les profils peptidiques des différentes boissons ont été caractérisés selon la distribution des poids moléculaires par HPLC *size exclusion* (SEC) en utilisant une colonne Biosep-SEC 2000 (Phenomenex, Torrance, Canada) selon la méthode de González-Olivares et al. (2014).

La boisson ayant les meilleures propriétés nutritionnelles (digestibilité et profils peptidiques) a été sélectionnée pour l'évaluation des propriétés nutritionnelles *in vivo*.

3) La digestibilité *in vivo* a été évaluée. Des jeunes rats Wistar de 20-23 jours ont été nourris pendant 14 jours avec une diète selon la méthode de AOAC 960.48, contenant 10 % des protéines de boisson sélectionnée, huile végétale, des vitamines, des minéraux, du saccharose, du cellulose et d'amidon pour ajuster les calories à 4.1 Kcal/g. La digestibilité *in vivo* a été déterminée par le calcul de la quantité d'azote ingérée par rapport à la quantité d'azote excrétée. Le CEP et le CEPN ont été évalués en rapportant la prise de poids de l'animal sur la quantité de nourriture ingérée selon la méthode de AOAC 960.48.
4) L'effet des boissons probiotiques enrichies en protéines de criquet sur le microbiote intestinal des groupes des rats testés a été évalué. L'extraction d'ADN microbien a été effectuée dans les fèces des rats récoltées au jour 14 et l'analyse de l'abondance microbienne et le profilage du microbiote intestinal ont été réalisés avec des méthodes bio-informatiques selon la méthode de Weiss et al. (2017).

5) Les propriétés physico-chimiques, nutritionnelles et microbiologiques de boissons nutraceutiques ont été évaluées pendant 147 jours de conservation à 4°C. La viscosité a été analysée à l'aide d'un viscosimètre Brookfield DVII+ (Ametek Brookfield, MA, USA), la couleur, à l'aide d'un colorimètre (Konica Minolta Sensing, Inc, Mahwah, NJ, USA) et le pH, à l'aide d'un pH-mètre (Accumet Basic AB15. Fisher Scientific. Ottawa. Canada). L'acidité titrable a été effectuée par titrage direct avec NaOH 0,1 M en utilisant un indicateur de phénolphtaléine selon la méthode officielle AOAC 942.15, et exprimée en pourcentage d'acide lactique. Les analyses microbiologiques ont été effectuées sur milieu MRS, dans le but d'évaluer le compte viable des probiotiques dans la boisson au cours de l'entreposage. L'évaluation de la résistance des probiotiques aux conditions gastro-intestinales a été effectuée dans des solutions gastriques et intestinales simulées *in vitro* et la viabilité a été exprimée comme suit (Guo et al., 2009) :

$$SR\ \% = \frac{\text{Log CFU N}}{\text{Log CFU N0}} * 100$$

6) Les profils peptidiques dans la boisson ont été évalués au cours du temps selon la distribution des poids moléculaires par HPLC *size exclusion* (SEC) (Manus et al., 2021). Les analyses sensorielles ont été effectuées. La boisson a été évaluée pour sa texture, son odeur, son goût et son apparence générale en utilisant une échelle hédonique à 9 points (Ben Fadhel et al., 2016).

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CHAPITRE 2: EFFECT OF PHYSICAL AND ENZYMATIC PRE-TREATMENT ON THE NUTRITIONAL AND FUNCTIONAL PROPERTIES OF FERMENTED BEVERAGES ENRICHED WITH CRICKET PROTEINS

Effet de prétraitement physique et enzymatique sur les propriétés nutritionnelles et fonctionnelles des boissons fermentées enrichies en protéines de criquet

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Chaima Dridi, Mathieu Millette, Blanca Aguilar, Johanne Manus, Stephane Salmieri, Monique Lacroix.

Le nombre de figures et de tableaux et le style des références ont été présentés selon le guide des auteurs du journal

Contributions des auteurs

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Chaima Dridi avec l'aide de Johanne Manus pour la mise en place des protocoles. Les discussions sur les résultats et tests statistiques ont été assistés par Stéphane Salmieri. L'article a été écrit par Chaima Dridi, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri, Dr. Mathieu Millette, Pr. Blanca Aguilar et Pr. Monique Lacroix.

Résumé

Le but de cette étude était d'évaluer les effets de l'irradiation (IR), des ultrasons (US) et des traitements combinés d'ultrasons suivis d'une irradiation (US-IR), d'ultrasons suivis d'une hydrolyse enzymatique avec et sans centrifugation (US-E et US-EWC respectivement) et d'ultrasons suivis d'une irradiation et d'une hydrolyse enzymatique (US-IRE), sur la digestibilité et la valeur nutritionnelle des boissons fermentées contenant des probiotiques. Les résultats ont montré que les traitements US (20 min), IR (3 kGy) et US-IR (t_{US} = 20 min, dose = 3 kGy) augmentaient la solubilité des protéines de 11,5 à 21,5, 24,3 et 29,9 % respectivement.

Selon nos résultats, ces traitements s'accompagnaient d'une augmentation de la quantité de groupes sulfhydryle totaux, d'une hydrophobie de surface et de modifications de la structure secondaire des protéines mesurées par FTIR. Des boissons probiotiques fermentées, non enrichies (C) et enrichies en protéine de criquet non traitée (Cr) ou traitée avec des traitements combinés ont également été évaluées pour leur digestibilité protéique *in vitro*. Les résultats ont montré que la fraction soluble de la boisson fermentée US-IRE avait la digestibilité la plus élevée (94 %) par rapport à l'ensemble des boissons fermentées testées. Le profil des peptides a démontré que l'US-IRE avait une faible proportion de peptides de poids moléculaire élevé (0,7 %) et la proportion la plus élevée de peptides de bas poids moléculaire de plus de 80 % par rapport aux autres traitements.

Mots clés : Protéines de criquet ; ultrasons ; irradiation ; hydrolyse enzymatique ; fermentation ; digestibilité.

Effect of physical and enzymatic pre-treatment on the nutritional and functional properties of fermented beverages enriched with cricket proteins

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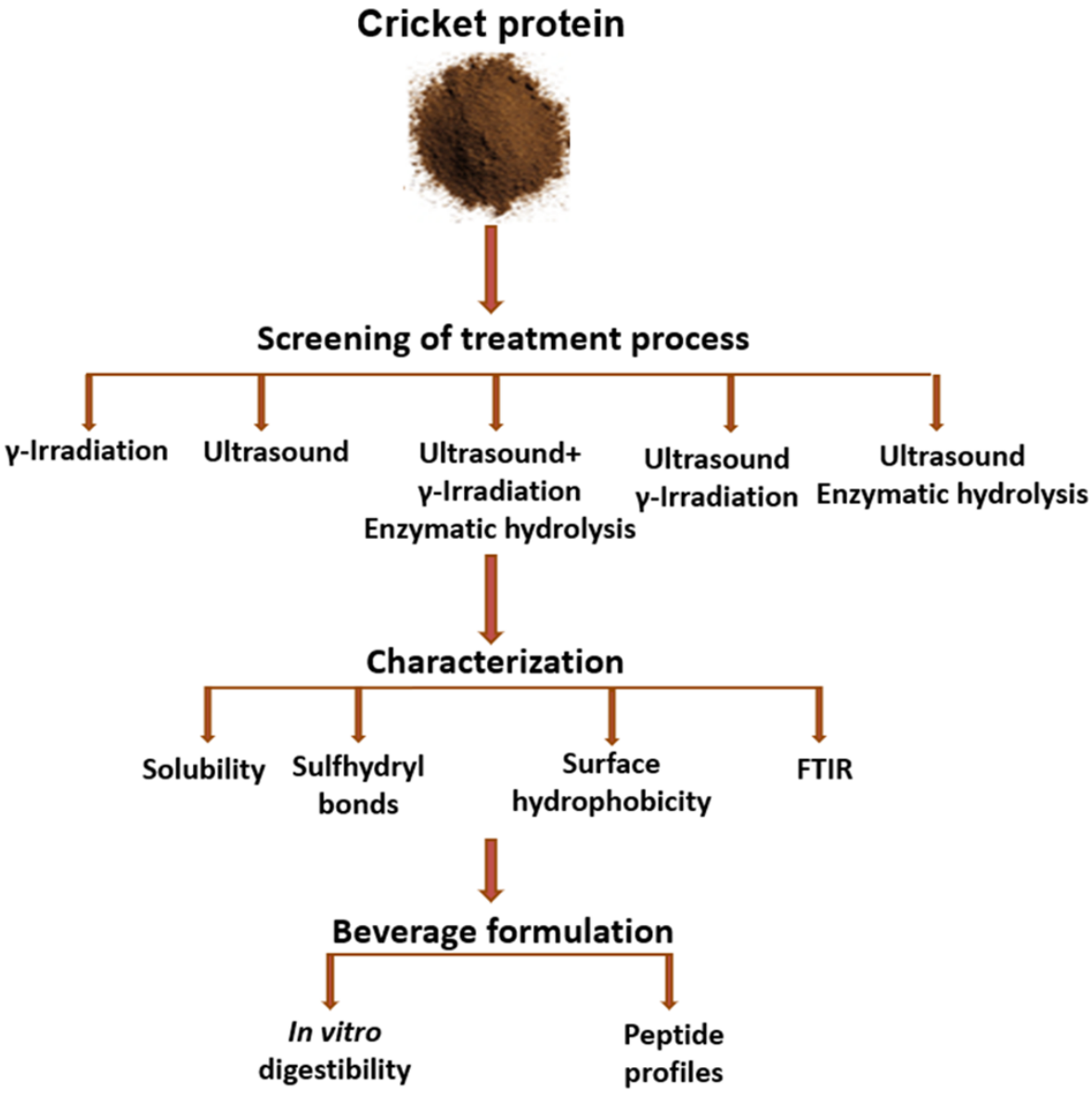
Abstract

The aim of this study was to evaluate the effects of γ-irradiation (IR), ultrasound (US), and combined treatments of ultrasound followed by γ-irradiation (US-IR), ultrasound followed by enzymatic hydrolysis with and without centrifugation (US-E and US-EWC, respectively), and ultrasound followed by γ-irradiation and enzymatic hydrolysis (US-IRE), on the digestibility and the nutritional value of fermented beverages containing probiotics. Results showed that US (20 min), IR (3 kGy) and US-IR (t_{US} = 20 min, dose = 3 kGy) treatments raised protein solubility from 11.5 to 21.5, 24.3 and 29.9%, respectively. According to our results, these treatments were accompanied by the increased amount of total sulfhydryl groups, surface hydrophobicity and changes to the secondary structure of the proteins measured by Fourier-transform infrared spectroscopy (FTIR). Fermented probiotic beverages, non-enriched (C) and enriched with untreated (Cr) or treated cricket protein with combined treatments were also evaluated for their in vitro protein digestibility. Results showed that the soluble fraction of US-IRE fermented beverage had the highest digestibility (94%) as compared to the whole fermented tested beverages. The peptides profile demonstrated that US-IRE had a low proportion of high molecular weight (M_W) peptides (0.7%) and the highest proportion of low M_W peptides by over 80% as compared to the other treatments.

Keywords: cricket proteins; ultrasound; irradiation; enzymatic hydrolysis; fermentation; digestibility.



Graphical abstract

1. Introduction

The concern of consumers is turning towards functional foods, particularly probiotic-based products, food enriched with proteins, and polyphenols, which are becoming more and more successful and present today within a market in continuous progress. Among these functional foods are fermented dairy products with probiotic bacteria. Probiotic bacteria may prevent gastrointestinal or urogenital infections, modulate the immune system, lower cholesterol to reduce the risk of cardiovascular diseases, control blood pressure and possess anti-carcinogenic ability (Desrouillères et al., 2020, 2015; Didari, 2015; Michail and Kenche, 2011; Rafter et al., 2007). The stakeholders of these products always seek to improve the quality and make them more functional in order to meet the requirements of consumers and to provide healthy, balanced, natural products. Currently, much research focuses on the development of enriched products with different protein sources to provide foods with high nutritional and functional properties and to counter protein deficiency, especially for kids and elderly people. Indeed, protein-enriched products could help the elderly to prevent chronic diseases and support the most vulnerable ones suffering from immunodeficiency, bone and muscle loss (Douglas et al., 2013; Song et al., 2019). Protein-enriched products could thus improve quality of life and contribute to the establishment of a well-balanced diet especially considering the uptake of the essential amino acids (Douglas et al., 2013). Furthermore, a particular interest in enriching dairy products with proteins has been proven due to the relationship between proteins and ferments (Felix da Silva et al., 2017). Indeed, the incorporation of proteins can decrease the fermentation time of the dairy products, inhibit post-fermentation acidification, increase the probiotic counts during the initial stage of fermentation and delay the decline of probiotic counts during storage (Zhao et al., 2006). However, with an increasing population and the high demand for nutrients, especially proteins, finding a sustainable alternative has become a great challenge and an urgent need. Indeed, Food and Agriculture Organization (FAO) experts (Durst et FAO, 2010) estimated that annual food production should rise from 8.4 billion tons to nearly 13.5 billion tons to be able to feed 9 billion people in 2050. In this perspective of using alternative protein resources, algae, legumes (soybeans, peas, etc.), and insects are proposed (Huis, 2013a). Insects represent a valuable protein resource compared to other conventional food resources such as beef or pork (Durst and FAO, 2010; Finke, 2013; Piccolo et al., 2017b; Rumpold and Schlüter, 2013b), with a content of protein ranging from 35% to 63% and a high content of essential amino acids (Makkar et al., 2014). However, insect proteins have a low digestibility, due to the presence of chitin which offers some rigidity to proteins and make them resistant to the hydrolysis by digestive enzymes. Hence, insoluble precipitates can be

formed, which reduces the bioavailability of trace minerals and decreases the digestibility of proteins in the small intestine (Marono et al., 2015). In addition, the presence of high levels of hydrophobic amino acids offers a low solubility and limits the use of insect proteins in food applications. To enhance the nutritional properties and the digestibility of the proteins, several techniques could be tested. Among them, gamma irradiation (γ-irradiation) is proven to improve the nutritive value and the functional properties of proteins (Ahmed et al., 2018; Ebrahimi et al., 2009; Liu et al., 2018). In addition, γ-irradiation was recognized as a safe method with a low energy cost (Gölge and Ova, 2008; Rooney et al., 2008). Ultrasound treatment (sonication) has also been used successfully to improve the efficiency of the enzymatic hydrolysis treatment and the functional properties of proteins (Chemat et al., 2011). These techniques have the ability to alter the protein structure, resulting in the improvement of the functionality properties and the nutritional value of the proteins, adding to the benefits of a better energy efficiency by the production of peptides with high nutritional value, resulting in a good protein digestibility (Kadam et al., 2015b; López-Ferrer et al., 2008), food safety and nutrient preservation (Han et al., 2018; Qu et al., 2018). Biological processes have also demonstrated their ability to facilitate protein digestibility and it is highly suitable for human consumption. Furthermore, protein hydrolysates obtained from enzymatic hydrolysis have better functional properties such as higher solubility, absorption, good gelling and foaming properties, and consequently, higher health effects (Tang et al., 2009). In addition, fermentation is considered as a process that decreases the levels of anti-nutrients in food grains, increasing the digestibility and their nutritive value (Ali et al., 2003; Beausoleil et al., 2007; Nkhata et al., 2018; Raveschot et al., 2018). Recently, combined treatments such as ultrasound pre-treatment assisted by enzymatic hydrolysis or γ-irradiation, has attracted increasing attention (Prajapat et al., 2016). These combined treatments can be applied to modify the protein structure and properties through hydrolysis of covalent bonds and an increase in free sulfhydryl groups. This could contribute to an increased solubility and hydrolytic sites of the substrate, making them more accessible to the enzyme (Ozuna et al., 2015; Zou et al., 2017) while avoiding the restrictions linked to the rise in energy and temperature and the need for a long treatment duration. It should also be noted that the use of combined treatment of ultrasound-assisted γ-irradiation and ultrasound-assisted enzymatic hydrolysis for the treatment of proteins from insects has never been studied. Furthermore, few studies were evaluated for their effect on the nutritional value and on the digestibility of protein in general.

This study aimed to evaluate the ability of combination treatments involving sonication, γ-irradiation and pre-digestion of the protein sources with enzymes to improve the nutritional value and the functional properties of a fermented beverage with probiotics enriched with cricket

proteins. Three treatments on the cricket proteins were evaluated as follows: (i) γ-irradiation assisted by sonication, (ii) enzymatic hydrolysis with alcalase assisted by sonication, and (iii) enzymatic hydrolysis assisted by sonication and γ-irradiation as a pre-treatment on the digestibility and on the nutritive value of proteins. In this context, the optimal parameters of sonication, irradiation, enzymatic hydrolysis and their combined treatments were first studied by testing their effects on the physicochemical properties of proteins (solubility, surface hydrophobicity), their structure (molecular interactions, secondary structure, content of sulfhydryl groups, peptide profiles) and in vitro digestibility via the analyses of fermented beverages vs. non-fermented counterparts.

2. Materials and Methods

2.1. Materials

The fermented and the non-fermented beverages used in this study were produced by Bio-K Plus International Inc., a Kerry company (Laval, QC, Canada). Organic cricket flour (60% protein content) was produced by Nexxus Foods (Montreal, QC, Canada). Pepsin (lyophilized powder from porcine gastric mucosa ≥ 3200 units/mg protein), trypsin (from bovine pancreas 10,000 BAEE units/mg protein) and alcalase enzyme (from Bacillus licheniformis ≥ 2.4 Units/g of protein) and Ellman's reagent 5.50-dithiobis (2-nitrobenzoic acid (DTNB)) were supplied by Sigma-Aldrich (Oakville, ON, Canada). All other reagents were of analytical grade. 1-anilino-8-naphthalene sulfonate (ANS), Sodium Hydroxide (NaOH), Sulfuric Acid (H_2SO_4), Hydrochloric acid (HCl) and Kjeltabs Cu-1.5 were provided by Thermo Fisher Scientific (Saint-Laurent, QC, Canada).

2.2. Ultrasound Pre-Treatment

Ultrasound pre-treatment (US) was done using a sonicator, QSonica Q500 (model FB-505; Thermo Fisher Scientific, Ottawa, ON, Canada). US probe (model CL-334) was plunged into a flask, containing cricket powder diluted in distilled water to 40% (w/v) and the operation was conducted in a batch mode. The sonicator operated at a maximal power of 500 W and frequency of 20 kHz. Samples were treated for 10, 20, 30 and 40 min (pulsed mode: on-time 5 s and off-time 2 s), the amplitude was fixed at 70%. The flask containing the sample was immersed into a cooling bath to avoid heating induced by the US treatment. Ice-water bath was stirred every 10 min during treatment, then the suspension was freeze-dried (Labconco Freezone® 2.5 L, model 7670521, Thermo Fisher Scientific) and stored in polyethylene bags prior to analyses.

2.3. γ-Irradiation Treatment

Irradiation treatment (IR) was applied on an aqueous suspension of cricket powder (50%) using a Cobalt 60 Underwater Calibrator UC-15A (Nordion Inc., Kanata, ON, Canada), under a dose rate of 7.7 kGy h^{-1} and at room temperature and then kept at 4 °C. The samples were treated at doses of 3, 5 and 7 kGy. Untreated samples (0 kGy) served as a control and were prepared in the same way as described previously but without irradiation. After irradiation, the solutions were freeze-dried and then stored in polyethylene bags at room temperature until used for analysis.

The combination of the treatments was applied in this study. It included ultrasound (US) procedure for 20 min as a first step for cricket protein treatment and gamma irradiation (IR) at a dose of 3 kGy as the second step. These parameters have been selected based on the results of protein solubility and structure modifications. The whole suspension of US-IR treatment was then freeze-dried and stored prior to characterization and beverage enrichment.

2.4. Enzymatic Hydrolysis and Combined Treatments

Enzymatic hydrolysis was realized using alcalase enzyme according to Sousa et al. [35] with modifications. The enzyme/substrate (E:S) ratio was fixed at 1:10 (w/w) and the reaction was done under agitation 100 rpm at 55 °C, pH 8.0, for 180 min. At the end of the reaction, the mixture was heated at 95 °C for 10 min to inactivate the enzyme then cooled down quickly to room temperature in an ice bath, followed by centrifugation at 13,000× g for 20 min (Dong et al., 2019). The supernatant was collected and freeze-dried (Lacroix et al., 1983). The whole hydrolysate (without centrifugation) was used.

The combination of ultrasound (US), γ-irradiation (IR) and enzymatic hydrolysis (E) was also performed. Three (3) combinations were carried out: ultrasound followed by enzymatic hydrolysis with and without recovery of the whole hydrolysis product (US-E and US-EWC, respectively) and ultrasound followed by γ-irradiation and enzymatic hydrolysis (US-IRE). Conditions were optimized as follows: US pre-treatment, 15 min, power of 500 W, frequency of 20 kHz and amplitude of 60% followed by γ-irradiation at 3 kGy and hydrolysis treatment as described above (alcalase enzyme for 180 min at 55 °C, pH 8.0). The products resulting from these combinations were used for the enrichment of the probiotic-based beverage.

2.5. Beverage Preparation

A commercial Bio-K+ Blueberry fermented beverage containing L. acidophilus CL1285, L. casei LBC80R and L. rhamnosus CLR2 as probiotics was used for enrichment. The beverage has been enriched with cricket powder having a total protein content of 13% and the proteins were pre-treated with selected processes as described above. Other beverages non-enriched (Bio-K+ Blueberry, with 3% of protein) and non-fermented were produced and were used as controls for comparison.

2.6. Protein Solubility

Protein solubility was determined according to a method of Adebiyi and Aluko (Adebiyi and Aluko, 2011). A quantity of 100 mg of total proteins was dissolved in distilled water and then vortexed for 60 min at 25 °C. Samples were centrifuged for 15 min at 15,000× g. The protein content of the supernatant was determined by the Kjeldahl method using a Foss Kjeldahl system (Foss, Eden Prairie, MN, USA) with an automation consisting of 6 steps: (i) test portion and reagent addition, (ii) initial and final digestion, (iii) cooling and dilution, (iv) NaOH addition, (v) steam distillation and titration, (vi) automatic pumping of flask contents to waste. A volume of 4–6 mL of test portion was poured into a 250 mL Foss digestion tube. A number of 2 catalyst copper Foss Kjeltabs Cu-1.5 were added and 15 mL of concentrated H_2SO_4 was carefully poured into the tube. Digestion was performed at 420 °C for 2.5 h by using a Digestor Labtec™ Line DT208 (Foss). After cooling down, samples were diluted with 80 mL distilled water and distillation was performed using a Distillation Unit Kjeltec™ 8200.

Then, distillates were titrated with 0.1 M HCl and results were expressed in percent proteins (g/100 g), following calculations using AOAC 991.20 method, analysis and quality evaluation of milk and milk products. The protein content was obtained by using the following Equations (1) and (2):

$$\% \text{ Nitrogen} = [(V_{sample} \text{ (mL)} - V_{blank} \text{ (mL)}) \times N \times 14.007) / \text{wt test portion (mg)}] \times 100 \quad (1)$$

where V_{sample} is the volume of titrant used for titrating the sample (mL), V_{blank} is the volume of titrant used for titrating the blank (mL), N is the normality of titrant (standard HCl), 14.007 is the atomic weight of nitrogen element, and wt test portion is the weight (mg) of test portion.

$$\% \text{ Protein} = \% \text{ Nitrogen} \times 6.25 \quad (2)$$

where 6.25 is the protein factor.

Subsequently, the percentage of soluble proteins was calculated according to Equation (3):

% solubility = Quantity of total proteins in the supernatant/Quantity of total proteins in the sample * 100 (3)

2.7. Measurement of Sulfhydryl (SH) Bonds

The total sulfhydryl bonds (SH) content of cricket proteins was determined using Ellman's reagent 5.50-dithiobis (2-nitrobenzoic acid/DTNB) and according to Ellman's procedure 1959 with some modification (Shimada and Cheftel, 1988). Samples were prepared into 1% suspensions of phosphate buffer (86 mM, pH 7.0). A volume of 0.5 mL of sample suspension was then mixed with 5 mL of urea buffer (pH 8, dissolved 10.4 g of Tris, 1.2 g of EDTA, 6.9 g of glycine, and 480 g of urea in deionized Milli-Q water to 1 L) and 20 µL of 4 mg/mL DTNB then incubated for 30 min at room temperature. Absorbance was read at 412 nm using a UV-Vis spectrophotometer Cary 8454 (Agilent technologies, Mississauga, ON, Canada). The surface free SH groups were determined using the same method but without urea. The total SH group content was calculated as follows:

$$C_{SH} = 73.53\ A_{SH}/C_S \quad (4)$$

where C_{SH} is the content of sulfhydryl groups (µmol/g), A_{SH} is the absorbance at 412 nm, C_S is the sample concentration (mg/mL).

2.8. Surface Hydrophobicity

The surface hydrophobicity of cricket proteins was determined according to a method described by Kato and Nakai, (1980), using an ANS probe. A series of sample dilutions were prepared at 0.00125, 0.0025, 0.005, 0.01, 0.02% (w/w) with 0.01 M Na_2HPO_4 buffer (0.1 M, pH 7). A quantity of 20 µL of ANS (1-anilino-8-naphthalene sulfonate) solution (8 mM dissolved in 0.01 M phosphate buffer pH 7) was added into 4 mL diluted protein solution, shaken immediately and then the fluorescence intensity was measured at 25 °C with a Tecan Infinite M1000 Pro fluorescence microplate reader (Tecan Austria GmbH, Austria) set up to an excitation wavelength of 390 nm and an emission wavelength of 468 nm. Surface hydrophobicity (Ho) was expressed as the initial slope of the plot of fluorescence intensity versus protein concentration (mg/mL) plot.

2.9. Molecular Characterization by Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR analyses of treated cricket proteins were carried out by using an ATR-FTIR Spectrum One spectrometer (PerkinElmer, Woodbridge, ON, Canada), equipped with an attenuated total reflectance device for solids analysis and a high linearity lithium tantalate detector. Lyophilized samples treated by ultrasound (US), γ-irradiation (IR), and ultrasound-assisted γ-irradiation (US-

IR) were subjected to analyses in comparison with the untreated samples (control). They were analyzed at room temperature in the range of 4000 to 650 cm^{-1} using an attenuated total reflectance (ATR) mode of operation. A number of 64 scans were accumulated at a resolution of 4 cm^{-1}. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units. The secondary structure of proteins was also studied within the Amide I region (1700–1600 cm^{-1}) using the method of second derivative spectra according to Byler and Susi (1988). The different Amide I components (α-helix, β-sheet, β-turn, unfolded/random coils) were identified and conformation changes were estimated by calculating sample/control ratios, based on the intensity of the negative bands of derivative spectra.

2.10. *In Vitro* Digestibility

The *in vitro* digestibility of fermented and non-fermented beverages, non-enriched and enriched with treated and non-treated cricket proteins, was performed according to Wang et al. (2008) with minor modifications. Sample digestion test was carried out using pepsin and trypsin sequential digestion treatment. A quantity of 0.5 g of protein sample was dissolved in 9.5 mL of 0.1 M HCl and digested at 37 °C with 10 mg of pepsin previously dissolved in 0.5 mL of 0.1 M HCl at 1:50 (w/v) E:S ratio and a stirring speed of 100 rpm. Sample aliquots (2 mL) were taken at 0, 30, 60, 90 and 120 min, respectively. After pepsin digestion, the mixture was adjusted to pH 8.0 using 1 M NaOH. Then, 10 mg of trypsin was added at a 1:50 E:S ratio, at 37 °C and 100 rpm. Samples of 2 mL were taken at various digestion times during an additional period of 120 min. The soluble nitrogen release during the digestion process was determined using the trichloroacetic acid (TCA) precipitation method at a final concentration of 10% (Lacroix et al., 1983). For each aliquot taken during pepsin and trypsin digestion, a volume of 2 mL of 20% TCA was added to the pepsin and/or trypsin hydrolysates. The precipitate was then centrifuged at 10,000× g for 20 min, and the supernatant was assayed for nitrogen content using the Kjeldahl method as described above. The percentage of nitrogen released was defined as follows:

% Nitrogen release = (Nitrogen content in the supernatant/Nitrogen content in the sample) × 100 (5)

2.11. Peptide Profile (SEC-HPLC)

A volume of 1 mL of each sample was taken at various time of digestion, heated at 95 °C for 5 min to inactivate the digestive enzymes, centrifuged at 10,000× g for 20 min and the supernatant was then recovered and filtered through a 0.2 µm filter and analyzed by SEC-HPLC. The molecular

weight distribution of soluble fractions of peptides in fermented and non-fermented beverages was carried out using an Agilent 1260 Infinity HPLC System (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a size-exclusion column Biosep 5 µm SEC-s2000 145 Å (particle size 5 µm, pore size 145 Å, 300 × 7.8 mm; Phenomenex Inc., Torrance, CA, USA) and a guard column (SecurityGuard cartridge for GFC 2000; 4 × 3 mm; Phenomenex Inc.). A 100 mM sodium phosphate buffer solution (pH 6.8) was used as the mobile phase. The volume of sample was 20 µL and the analysis was carried out at ambient temperature using a flow rate of 1 mL/min for 20 min. Detection was performed at 280 nm using a 1260 diode array detector (DAD). The peptides were identified by comparing the retention times with standard proteins such as bovine thyroglobulin (670 kDa), IgA (300 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and uridine (244.2 Da). Chromatograms were collected by using OpenLAB ChemStation software (Rev.C.01.07 SR2 [255], Beijing, China). The baseline was corrected manually, and the total surface area of chromatograms was integrated and split into 3 groups of molecular weights (>3000 Da; 3000-260 Da; <260 Da) using the function split manually integrated peaks and expressed as a percentage of the total area. Analysis of peptide profile was performed on fermented and non-fermented control, Cr, US-IR, US-E, US-EWC and US-IRE beverages before and during in vitro digestion.

The relative difference of M_W distribution between the end and the beginning of the in vitro digestion (Δ value) was determined as follows:

$$\Delta = \%E - \%B/\%F \quad (6)$$

2.12. Statistical Analysis

All treatments and analyses were performed in triplicate (n = 3). The results were reported as mean values ± standard deviation. Statistical analysis was carried out using SPSS software version 22 (IBM Corporation, Somers, NY, USA) and the significant differences ($p \leq 0.05$) between the means were evaluated using one-way analysis of variance (ANOVA). Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis.

3. Results and Discussion

3.1. Protein Solubility

Measurement of protein solubility was used to characterize the fragmentation or agglomeration of are (Amiri et al., 2018; Jambrak et al., 2009; Morel et al., 2000) presented in Figure 1a and show

that the US treatment significantly increased the protein solubility ($p \leq 0.05$) as compared to the untreated samples. However, an increase of US time (from 10 to 40 min) did not increase the solubility ($p > 0.05$), meaning that a time of 10 min was sufficient to reach a high solubility of proteins (>20%). This effect could be due to a linkage break between chitin and proteins, resulting from a modification of electrostatic links, which facilitates the redirection of hydrophilic amino acids to the aqueous phase (Kjartansson et al., 2006; Xiong et al., 2018). Other observations explain that an increase of protein solubility could be due to the capacity of US treatment to lead changes in the three-dimensional structure of proteins, associated with interactions between the hydrophobic and hydrophilic surface, and resulting in higher electrostatic forces that increase protein–water interactions, and hence solubility (Amiri et al., 2018; Jambrak et al., 2009; Morel et al., 2000).

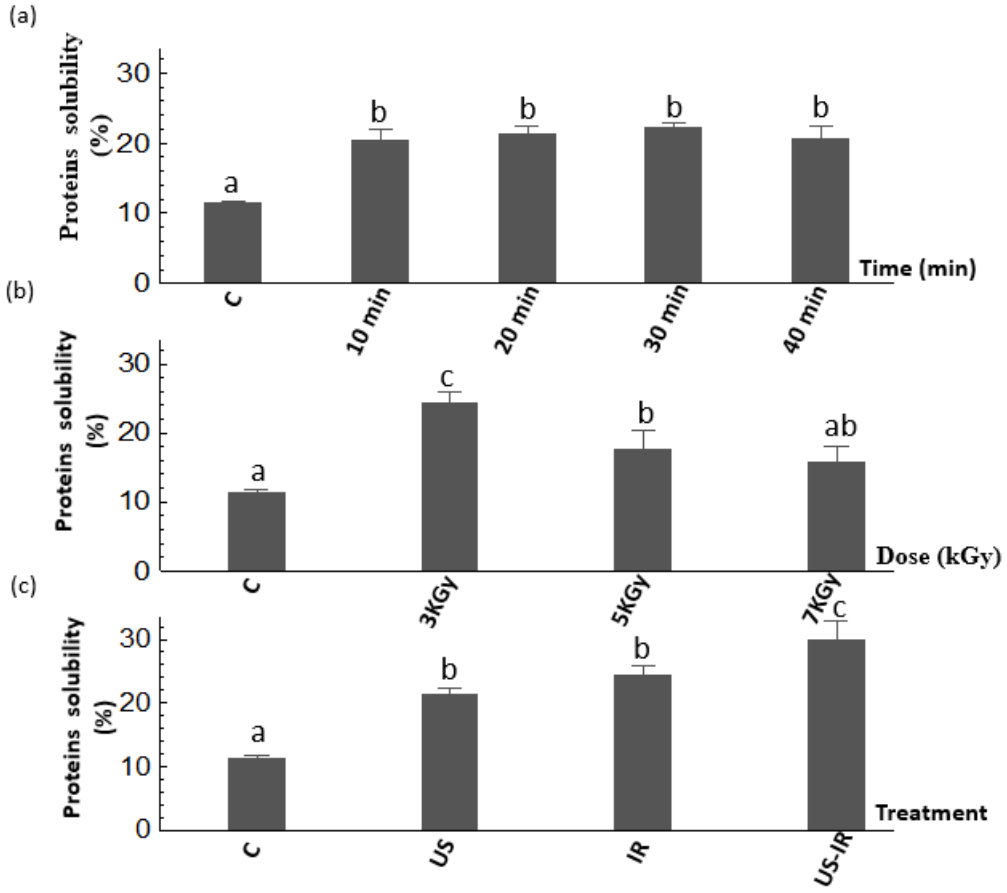


Figure 1. Solubility of cricket proteins in untreated samples (C), samples treated by ultrasound (US) at increasing times (10, 20, 30 and 40 min) (a), by γ-irradiation (IR) at increasing doses (3, 5 and 7 kGy) (b), and by combined ultrasound-assisted γ-irradiation (US-IR; 20 min; 3 kGy) (c). Different letters above the bars indicate significant differences among the mean values of the samples ($p \leq 0.05$). Data shown is the mean ± SD (n = 3).

The effect of γ-irradiation treatment is shown in Figure 1b. A significant increase ($p \leq 0.05$) in protein solubility was observed at an optimal dose of 3 kGy compared to untreated samples and

other treatments. Indeed, the protein solubility increased from 11.6 to 24.4% (~2-fold) after irradiation treatment of 3 kGy. Similarly, Ahmed et al. (2018) found that doses higher than 0.5 kGy significantly enhanced the protein solubility for sesame protein with an optimal dose of 1 kGy. In addition, Dogbevi et al. (2000) reported that the increase of protein solubility was due to the unfolding of proteins through their deamination during irradiation, which can change the protein statutes from water anti-binding to water binding by the conversion of amide groups into acid groups (Shih and Kalmar, 1987). In the current study, the increase in protein solubility could be attributed to the destruction of chitin-protein binding by the application of γ-irradiation which further favors the deamination of protein molecules (Dogbevi et al., 1999). On the other hand, the decrease of protein solubility observed at doses > 3 kGy could be explained by their denaturation due to the aggregation of unfolded proteins to form a high molecular weight protein network (Afify et al., 2011). In addition, Maity et al. (2009) reported a decrease in the solubility of vegetable proteins from Oryza sativa L. after a treatment of 6 kGy. The combined treatment US-IR (Figure 1c) was realized using the irradiation dose (3 kGy) and ultrasound duration (t_{US} = 20 min). Results show that this treatment was able to improve the protein solubility from 11.6 to 29.9% as compared to the untreated sample. The energy delivered by the combination of ultrasound and γ-irradiation has further improved the fragmentation of proteins and consequently led to more soluble proteins following the degradation of small protein aggregates.

3.2. Sulfhydryl Groups and Surface Hydrophobicity

The effect of US treatment at increasing durations (0, 10, 20, 30 and 40 min) on the content of total free sulfhydryl (SH) groups, surface free SH groups and on the surface hydrophobicity of cricket proteins is presented in Figure 2a. The results of the content of surface and total SH groups significantly increased ($p \leq 0.05$) after the application of US treatment. However, no significant difference ($p > 0.05$) of total SH groups was observed between all treatments with a range of 2.48–3.22 μmol/g. Only slight differences ($p \leq 0.05$) of surface SH groups were observed between treatments, with a maximum of 0.54 μmol/g obtained after 20 min of treatment. The surface hydrophobicity (Ho) was also significantly increased after treatments ($p \leq 0.05$), with maximum values observed after 20 min and 30 min showing a hydrophobicity of 5210 and 5844, respectively, and no significant difference between these two treatments was observed ($p > 0.05$). These observations are probably due to the unfolding of proteins, following the application of ultrasound, and may have led to the exposure of hydrophobic amino acids or clusters and the SH-containing amino groups to the external surface of protein molecules (Arzeni et al., 2012; Hou et al., 2017). Similar results have been reported by Higuera-Barraza et al. (2017), who applied ultrasound

treatment at different times in squid (Dosidicus gigas) mantle proteins, finding a significant increase in Ho according to the time and amplitude used compared to the untreated samples. The effect of IR treatment on SH groups and surface hydrophobicity is shown in Figure 2b. The content of free SH increased with increasing irradiation doses, from 0.69 to 3.79 µmol/g at 7 kGy, the maximum irradiation conditions for the release of SH groups. In addition, the maximum content of surface SH was obtained at 3 kGy, showing a significantly higher value of 0.64 µmol/g ($p \leq 0.05$) compared to 0.22 µmol/g for untreated proteins. The surface hydrophobicity increased significantly from 2601 to 5097 after a treatment of 5 kGy and then decreased to a value of 3881 at 7 kGy. No significant difference ($p > 0.05$) was observed between the treatments of 3 kGy (4394.6) and 5 kGy (5097) which are considered as optimal treatments to increase the Ho of proteins. This observation is in contradiction with the results obtained for proteins solubility, which showed a decrease in the solubility with an increase in the irradiation dose. We suggest that a high irradiation dose might have the potential to break disulfide and hydrogen bonds, ionic and hydrophobic interactions and lead to an increase in total SH content (Fombang, 2005). The decrease in solubility with the dose of 7 kGy could be associated with a Maillard reaction, when the amino groups of lysine react with the carbonyl groups of reducing sugars, as reported by Hooshmand and Klopfenstein (Hooshmand and Klopfenstein, 1995) after irradiation of maize and wheat flours at 7.5 kGy. It could also be explained by a high irradiation dose effect on non disulfide covalent bonds that form and cause the closing of protein structures. Consequently, some peptide bonds were masked or smaller peptides polymerized to form a highly cross-linked protein network (Fombang, 2005). The combined treatment US-IR (Figure 2c) generated a significant increase in SH content ($p \leq 0.05$) compared to the untreated sample by reaching 2.87 µmol/g, but no significant difference ($p > 0.05$) was noted compared to US alone (2.67 µmol/g) and IR alone (2.58 µmol/g). In counterpart, US-IR treatment showed the highest content of surface SH (1.13 µmol/g), significantly higher ($p \leq 0.05$) than control (0.22 µmol/g) and individual treatments (0.54 µmol/g for US and 0.64 µmol/g for IR). Similarly, the surface hydrophobicity increased significantly ($p \leq 0.05$) with the combined treatment compared to the untreated and IR samples, but no significant difference ($p > 0.05$) was observed between US-IR and US alone treated samples. From these observations, although US-IR treatment contributed to a significant increase of protein solubility, it did not trigger a major change in total SH bonds compared to individual treatments. However, US-IR treatment induced a significantly higher content of surface SH groups ($p \leq 0.05$) compared to individual treatments, and a higher Ho ($p \leq 0.05$) compared to IR alone without affecting the Ho level of proteins treated by US alone. A possible explanation is that the combined treatment led to protein unfolding, thereby exposing SH groups to the outer surface of the proteins, which may

have a functional or structural role in proteins. Overall, these trends observed in Figure 2 are in accordance with solubility results (Figure 1), as protein solubility increased with the SH content and the Ho of treated proteins (Azagoh et al., 2016).

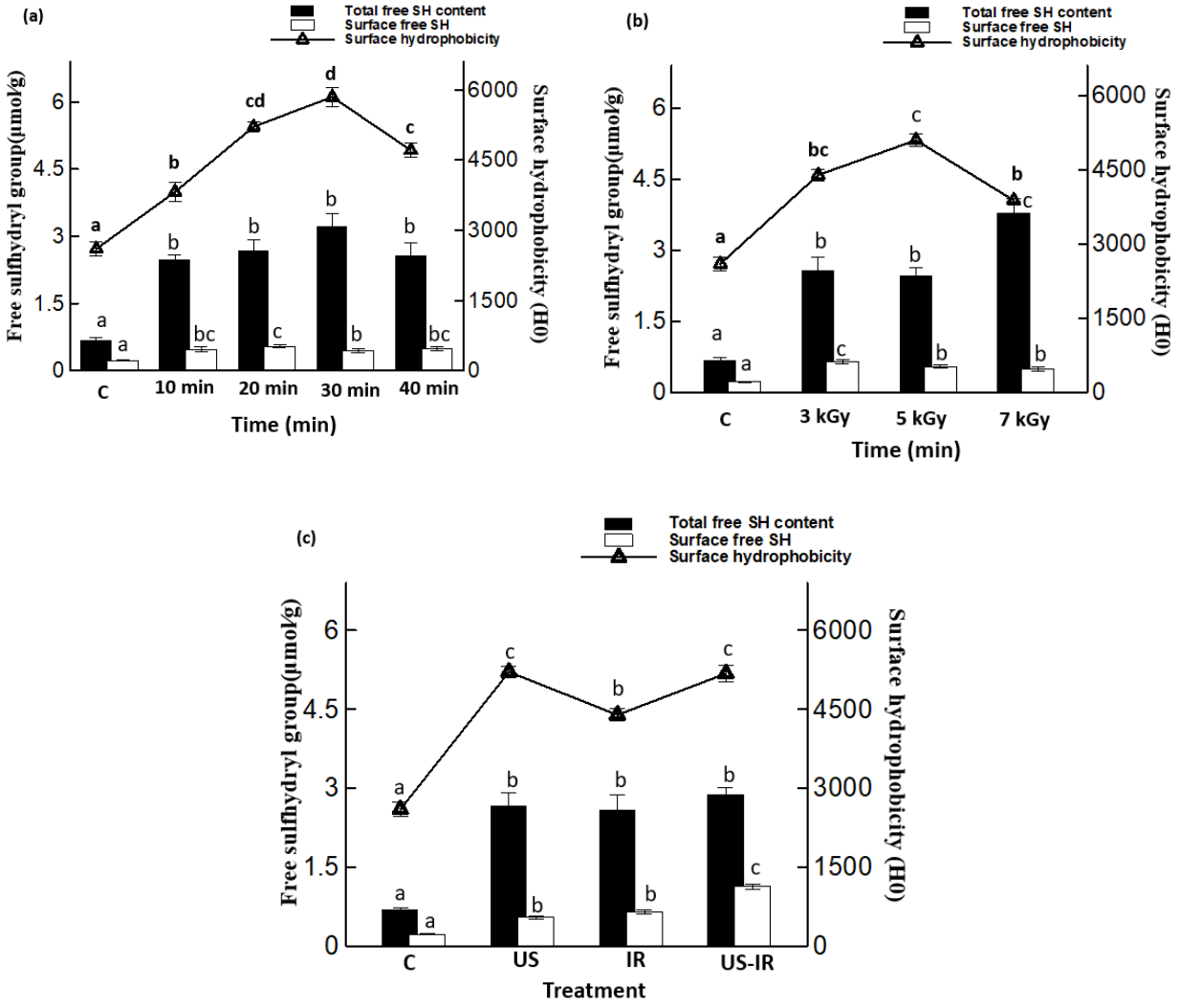
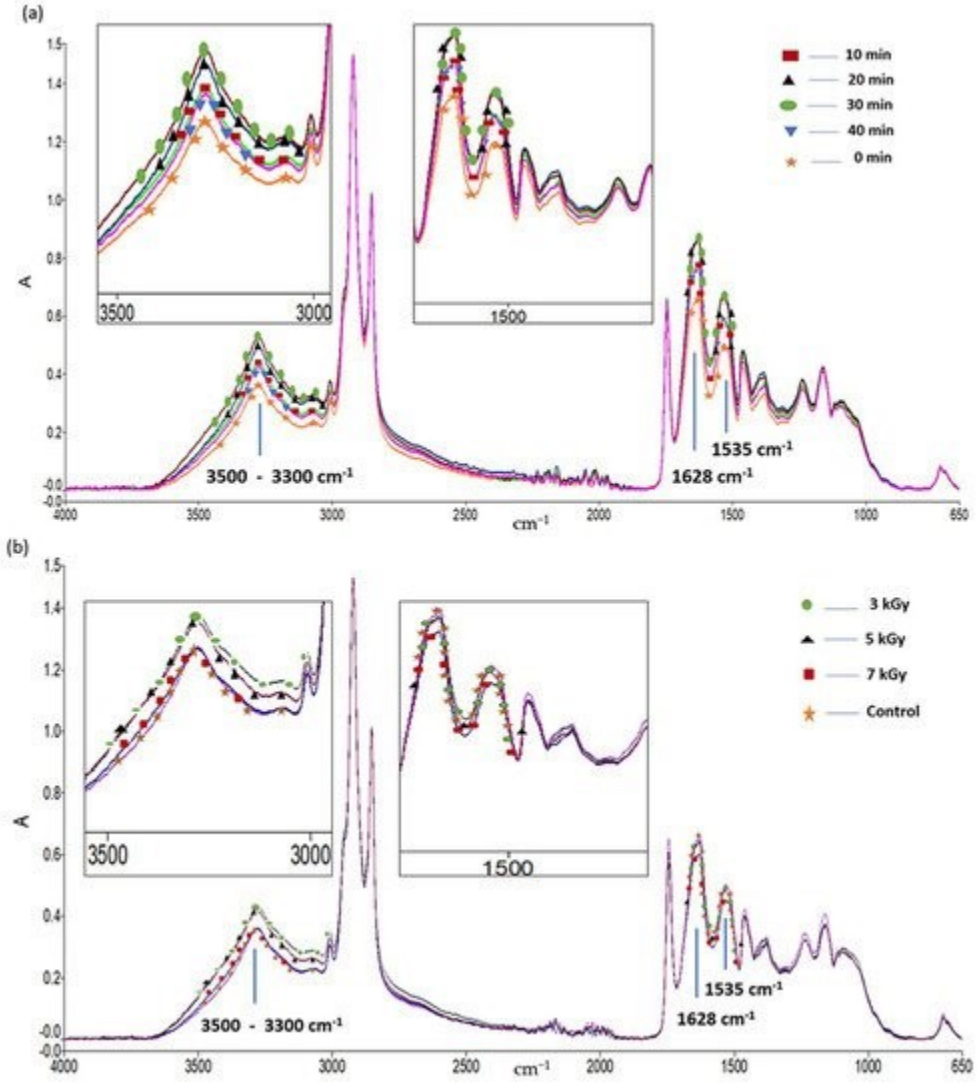


Figure 2. Sulfhydryl group content in cricket proteins for untreated samples (C), samples treated by ultrasound (US) at different times (10, 20, 30 and 40 min) (a), by γ-irradiation at different doses (3, 5 and 7 kGy) (b), and by combined ultrasound- assisted γ-irradiation (US-IR; 20 min; 3 kGy) (c). Different letters above the bars indicate significant differences among the mean values of the samples ($p \leq 0.05$). Data shown is the mean ± (n=3).

3.3. ATR-FTIR Analysis

ATR-FTIR spectroscopy of cricket proteins was performed to verify the effect of US, IR and combined US-IR treatments on the intermolecular interactions associated with the presumed hydrolysis of cricket proteins. Primary spectra of samples treated by the different treatments are presented in Figure 3. The results show that the intensities of some bands were affected by the

type of treatment. In the quantitative region (3600–3000 cm^{-1}), it is clear that the O-H and N-H stretching vibrations—comprising Amide A band at 3300 cm^{-1} (O-H stretching) and Amide B band at 3100 cm^{-1} (N-H stretching)—increased after treatments, mainly due to the presence of more free amino and hydroxyl groups, and most likely related to partial hydrolysis. However, no evidence of change in hydrogen bonding was systematically observed between all treated samples and control as no significant shift of the O-H stretching mode to lower frequencies was observed, most likely due to the fact these doses did not influence the hydrogen bonding of solid proteins at the different doses of US and IR. Furthermore, the bands related to the secondary structure of proteins also increased in intensity after treatment compared to the untreated samples. Indeed, Figure 3 also shows that the Amide I band at 1628 cm^{-1} (C=O stretching coupled to C-N stretching and N-H bending) and the Amide II band at 1535 cm^{-1} (referred to C-N stretching coupled to N-H bending), generally showed higher intensities after treatments of proteins. These structural changes of protein configuration have a noticeable influence on the protein stability, as previously reported by Vargas et al. (2021). Within the region 1700–1600 cm^{-1} (Amide I), the main band has been identified as being sensitive to particular secondary structural changes, as previously reported (Gallagher, 2021; Garidel and Schott, 2006; Kong and Yu, 2007). Therefore, the estimated secondary structure of cricket proteins subjected to the different treatments was determined in this infrared region and listed in Table 1.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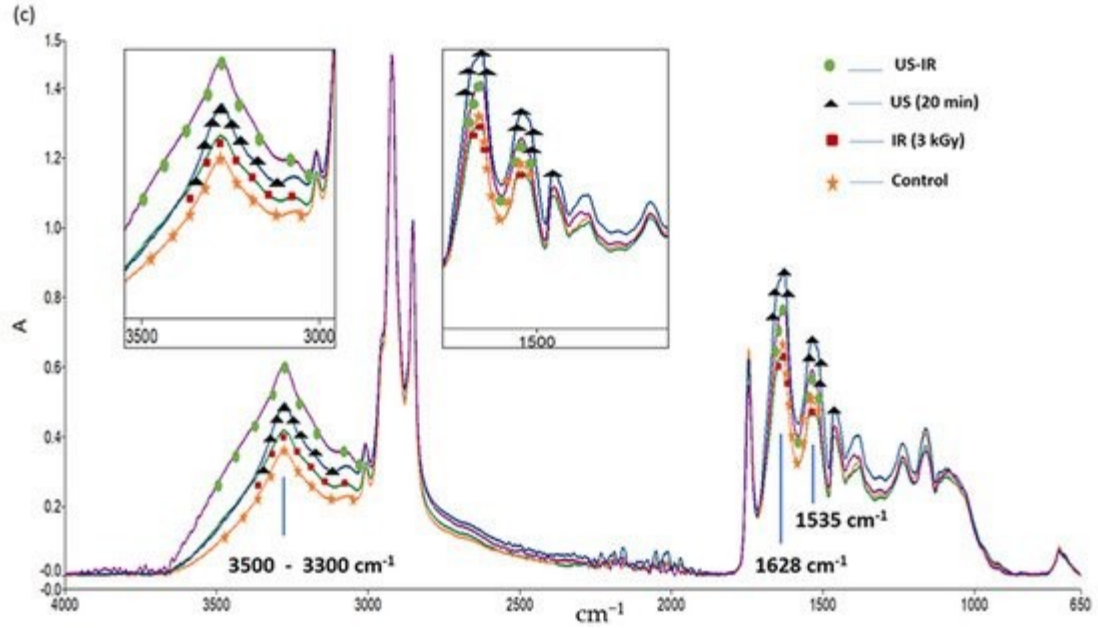

Figure 3. FTIR spectra of cricket proteins before treatments (control), after ultrasound (US) treatment (10, 20, 30 and 40 min) (a), γ -irradiation (IR) treatment (3, 5 and 7 kGy) (b), and after single and combined treatments US, IR and IR assisted with US (US-IR) (c). For each global spectrum, focused areas in the regions 3500-3300 cm^{-1} and 1628-1200 cm^{-1} indicate the main vibration changes related to the secondary structure of proteins.

Table 1. Effect of ultrasound treatment on the secondary structure of cricket protein. Data were collected from the amplitude of negative bands of second derivative spectra in the Amide I region (1700–1600 cm−1) and the ratio of treatment/control was calculated.

Treatment effect (ratio treatment/control)	α-Helix 1653 cm^{-1}	β-Sheets 1623 cm^{-1}	β-Sheets 1637 cm^{-1}	Random coils 1645 cm^{-1}	β-Turns 1663 cm^{-1}	β-Turns 1694 cm^{-1}
US effect						
10 min	1.32	1.08	0.79	1.19	1.38	1.00
20 min	1.17	1.29	1.20	1.74	3.11	1.21
30 min	0.88	1.18	1.01	1.83	1.55	1.40
40 min	0.96	1.18	1.19	1.36	1.77	1.22
IR effect						
3 kGy	0.20	0.74	0.70	1.68	1.59	0.74
5 kGy	0.96	0.70	0.48	1.35	1.35	0.42
7 kGy	0.18	0.67	0.55	1.66	1.08	0.50
US-IR effect						
US-IR	1.02	1.07	1.00	3.47	1.70	0.55

US: ultrasound treatment (10, 20, 30 and 40 min), IR: γ -irradiation treatment (3, 5 and 7 kGy), US-IR: IR assisted with US.

Regarding US treatment (Figure 3a), the highest intensities in typical peaks related to proteins (Amide A, B, I and II) correspond to the treatments of 20 and 30 min whereas lower intensities were observed at 10 and 40 min. The increase of the intensity in Amide I and Amide II bands observed at 20 and 30 min may be due to the fragmentation of cricket proteins that could lead to the unfolding of the compact protein structure and which is possibly attributed to more intensive interactions between water and protein (Qu et al., 2018). Similarly, the effect of the US duration on the proteins structure was evaluated by other authors. Hence, it was found that the application of US treatment on β-lactoglobulin (Ma et al., 2018b) and on chicken bones (Dong et al., 2019) could lead to secondary structural changes, suggesting that US treatment could disrupt

intermolecular interactions and decrease hydrogen bonding, resulting in more peptide release. Results of Table 1 indicate an increase mainly in α-helix, β-sheet, random coil and β-turn structures of cricket proteins, and these changes were altogether observed at 20 min. The increase in α-helix and β-sheet under the effect of US treatment was also reported by Chandrapala et al. (2012) and Ma et al. (2018). These spectral changes reflect the disruption on the interactions between different parts of the protein molecules induced by US treatment which led to conformation changes (Aouzelleg et al., 2004). It is to be noted that, although a lower absorbance of the Amide I band was observed at 40 min of US treatment compared to 20 and 30 min, the second derivative function indicates no significant variation of α-helix (ratio treatment/control of 0.96 at 1653 cm^{-1}) compared to a higher level of β-sheets and β-turns, hence suggesting a highly stabilized protein network at this dose. This observation coincides with the results of surface hydrophobicity analysis which showed a higher Ho after 20 min of the ultrasound treatment. This suggests that US treatment for 20 min may result in conformational changes relative to a more stable, ordered structure.

Figure 3b shows the FTIR profile of samples subjected to IR treatment. Results show an increase in the peak intensity of Amide A and Amide B bands but no change in the Amide I mode, after application of γ-irradiation. Moreover, it is interesting to note that the absorbances of Amide A and B decrease with the irradiation dose (from 3 to 7 kGy). This result suggests that γ-irradiation changed the protein structure in such a way that was attributed to the release of peptides due to protein degradation. Similar observations were reported by Maity et al. (2009) for sunflower protein isolate. The influence of irradiation on the secondary structure of cricket proteins is shown in Table 1. The results indicate that, compared to the control, an important decrease of α-helix structure—and to a lesser degree of β-sheets—was observed towards a substantial increment of random coils and β-turns (at 1663 cm^{-1}) in irradiated samples. These changes are particularly observed at 3 kGy. Thus, it can be inferred that under γ-irradiation proteins might unfold with weakened intermolecular hydrogen bonds, causing the disruption of β-sheets and favoring the formation of unordered structures. It was observed by Malik et al. (2017) that γ-irradiation decreased α-helix content and increased the β-sheets, but also the β-turn and random coil contents of sunflower protein isolate. Lee and Song, (2002) explained the change in the secondary structure of myoglobin by the cleavage of covalent bonds and the formation of aggregated products. Similar results were found by Le Tien et al. (2000) who observed a decrease of α-helical structure content of whey protein-based films due to the ability of γ-irradiation to alter protein conformation.

Finally, as shown in Figure 3c, cricket proteins treated with US-IR combined process showed the highest intensity of –OH and –NH stretching bonds, but also high intensities of Amide I and Amide II bonds (intermediate between single treatments) compared to the untreated and treated samples with US (20 min) and IR (3 kGy) alone. This observation is in accordance with the important protein solubility of samples treated by US-IR (Figure 1c), suggesting a potential higher degree of hydrolysis or a higher exposure of hydroxyl and amino groups of the proteins to solvation after the combined treatment. Moreover, the combined treatment affected more significantly the random coil secondary structure of proteins, as shown in Table 1 Indeed, US-IR treatment generally presented a similar effect to that of IR single treatment at 3 kGy, but a synergic effect on random coils was measured with a high ratio of 3.47 compared to untreated proteins. Thus, US-IR treatment could form a synergistic potentialization for structure modification of proteins through the destruction of covalent bonds, molecular interactions or conformational ordering. Therefore, these results suggest that infrared absorbances of primary as well as derivative spectra of treated proteins were greatly affected by the local environment of the different structural groups related to protein conformation. Sonication and irradiation caused microstructural changes at the molecular level. These changes may be attributed to fragmentation, cross-linking and aggregation, as previously observed by other authors (Dogan et al., 2007; Gaber, 2005; Karra et al., 2020; Tian et al., 2020; Vargas et al., 2021).

3.4. *In Vitro* Digestibility

Different effects of US, IR and enzymatic hydrolysis of the cricket proteins on the protein digestibility in the enriched beverages were evaluated and results are presented in Figure 4. The in vitro digestibility test was monitored during 2 h of pepsin treatment followed by 2 h of trypsin treatment. As illustrated in Figure 4a, the percentage of digestibility (nitrogen release) of non-fermented (C NF) and fermented (C F) non-enriched beverage (control) is presented all along the in vitro digestion time. Figure 4b presents the effect of US-IR combined treatment, i.e., the digestibility of non-fermented vs. fermented enriched beverages containing cricket proteins (Cr NF and Cr F, respectively), and non-fermented vs. fermented beverages containing cricket proteins treated by US-IR process (US-IR NF and US-IR F, respectively). Figure 4c illustrates the effect of an additional enzymatic treatment, i.e., the digestibility of non-fermented vs. fermented beverages containing cricket proteins: (i) treated by combined US and enzymatic hydrolysis, the soluble part (US-E NF and US-E F, respectively), (ii) treated by combined US and enzymatic hydrolysis using the whole product of hydrolysis (US-EWC NF and US-EWC F), and (iii) treated by combined US, IR and enzymatic hydrolysis (US-IRE NF and U-IRE F, respectively).

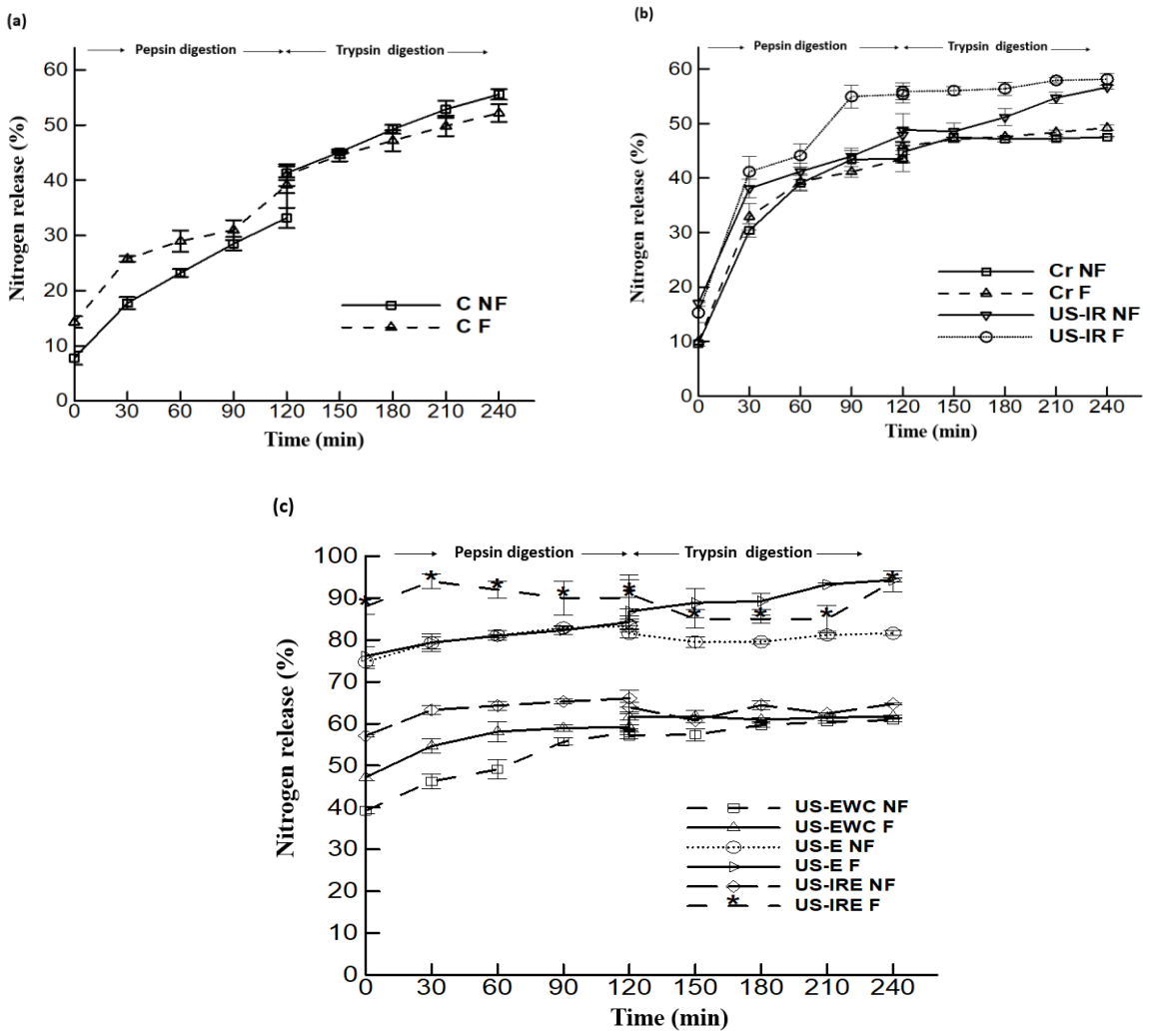

Figure 4. Percentage of nitrogen released from fermented (F) and non-fermented (NF) non-enriched (control C) (a), untreated cricket protein-enriched (Cr) beverages, treated with γ-irradiation assisted with ultrasound (US-IR) (b), and treated cricket protein-enriched beverages subjected to enzymatic hydrolysis assisted with ultrasound (US-E), treated cricket protein-enriched beverages subjected to enzymatic hydrolysis assisted with ultrasound the whole product of hydrolysis (US-EWC), to enzymatic hydrolysis assisted with γ-irradiation and ultrasound (US-IRE) (c) during pepsin (120 min) and trypsin (120 min) simulated digestion. Data shown is the mean ± SD, n = 3.

Results show that the nitrogen release increased during the digestion in all samples, but the profile of nitrogen release is different depending the treatment applied on proteins. In addition, an improved digestibility was observed in all fermented samples (assignation F) as compared to their respective non-fermented counterparts. In particular, the digestibility of enriched beverages was reached more rapidly in products containing treated cricket proteins (Figure 4b,c) compared to non-enriched ones (Figure 4a). Indeed, the region of 30–40% nitrogen was reached at 120 min

for non-enriched control samples (Figure 4a) and measurements evolved linearly up to the region of 50–55% nitrogen at 240 min, with very similar curves in the portion of 120–240 min. In comparison, for enriched beverages with or without US-IR treatment (Figure 4b), the region of 30–40% nitrogen was reached after only 30 min and a plateau at 45% nitrogen was observed in untreated samples after 120 min, whereas a continuous slight increase was measured in treated samples (US-IR NF and US-IS F) up to 55% of nitrogen at 120 min. This improvement of nitrogen release was expectedly more noticeable in beverages containing cricket proteins treated with enzymatic hydrolysis (Figure 4c)—with much higher percentages at 0 min comprised between 40 and 90%, compared to 10–15% in other treatments presented in Figure 4a,b—and the higher digestion of fermented products (F) was evidenced compared to non-fermented (NF) ones. Indeed, non-fermented and fermented samples submitted to combined US and enzymatic hydrolysis before centrifugation (US-EWC NF and US-EWC F) showed the lowest percentages of nitrogen throughout all digestion. Although a high percentage of 40 and 47% was found at 0 min, respectively, a low final value of 57% was found after 240 min, which is equivalent to US-IR NF and US-IR F samples (without enzymatic treatment) in Figure 4b. However, the soluble part (supernatant) obtained from combined US and enzyme treatment after centrifugation (US-E NF and US-E F) induced an increase of nitrogen release during digestion, starting at 74–76% at 0 min and increasing to 81 and 94% at 240 min for US-E NF and US-E F samples, respectively. Regarding the treatment combining ultrasound, enzyme and irradiation (US-IRE NF and US-IRE F), nitrogen release started from 57 and 90% at 0 min, and a release of 66 and 94% was reached at 120 min after pepsin simulated digestion, followed by a plateau from 120 to 240 min (trypsin digestion). Overall, the most digestible products were found to be US-E F and US-IRE F beverages. These results are mainly due to the role of the enzymatic pre-treatment which ensures a pre-digestion of proteins by link breakage between proteins and other constituents, which prevent the good digestibility of cricket proteins, in particular chitin, as already reported by other studies (Gildberg and Stenberg, 2001; Marono et al., 2015; Yi et al., 2016). Furthermore, the dissociated state of the proteins resulting from enzymatic hydrolysis promotes an easier digestion (Hall et al., 2017a). In addition, this finding was consistent with the result of Lacroix et al. (1983), which showed that the isolation of the by-products of molecular weight lower than 5000 Da using the ultrafiltration procedure has allowed a significant increase on the digestibility and the net protein ratio of rapeseed proteins hydrolysate. In addition, considering the proteins without enzymatic treatment, the US-IR beverage is associated with improved digestibility ($p \leq 0.05$) compared to the non-enriched control beverage (C) and the enriched beverage (Cr). Previous works found that the application of US pre-treatment ensures the degradation of protein structure,

enhances peptide cleavage and improves protein digestibility (Shin et al., 2011), but also US could extract the bioactive peptides from the biological matrix (Jia et al., 2010). Likewise, γ-irradiation has been shown to break down proteins in a random way to produce peptides and improve digestibility (Zhang et al., 2020; Zuleta et al., 2006). These observations are in accordance with the results obtained in this study treated with these processes and which have succeeded in an improved solubility, the release of total and surface SH bonds, and changes in the secondary structure of proteins. Furthermore, US-IR-assisted enzymatic hydrolysis (US-IRE) resulted in a faster digestion than the US-E process. This observation may be due to the structural changes and exposure of more easily digestible peptides and amino acids with digestive enzymes (T. Li et al., 2019), associated with irradiation that probably promoted the digestion and utilization of hydrolysates primarily by ferments and secondly by digestive enzymes (Ahmed et al., 2018; Fombang et al., 2005a). Thus, this proposed mechanism could explain the pronounced difference of digestibility observed between the US-IRE NF and US-IRE F beverages.

3.5. Molecular Weight (M_w) Distribution by SEC Analysis

The molecular weight (M_w) distribution of protein hydrolysates obtained during the digestion of the different NF and F beverages (Control, Cr, US-IR, US-E, US-EWC and US-IRE) is presented in Table 2. The results show that the control beverages (NF and F) had a high percentage of medium M_w peptides (MMW between 3000–260 Da) with values of 63–64% at the end of the digestion period, a low percentage of low M_w peptides (LMW < 260 Da) at 34% and a very low fraction of high Mw peptides (HMW > 3000 Da) at 2% of peptides. Conversely, enriched beverages with proteins (Cr, US-IR, US-E, US-EWC and US-IRE) were characterized by a high proportion of LMW peptides with percentages around 51–53% for Cr samples, 48–50% for US-IR, 57–58% for US-E, 65–69% for US-EWC and approximately 81% for US-IRE samples, at the end of digestion. This higher percentage of LMW peptides was observed at the expense of a significant decrease of MMW peptides, with values around 43–45% for Cr samples, 46–49% for US-IR, 41–42% for US-E, 30–35% for US-EWC and 18–19% for US-IRE samples, at the end of digestion. The percentage of HMW peptides remained very low regardless of the treatment, with higher values of 3.0–3.5% in Cr and US-IR samples compared to control, and lower values ranging from 0.3 to 0.9% under enzymatic treatments (US-E, US-EWC and US-IRE), at the end of digestion.

Table 2. Percentage of molecular weight (MW) distribution during digestion (0 to 240 min) of non-fermented (NF) and fermented (F) beverages non-enriched (Control), untreated cricket protein-enriched beverages (Cr) and those treated with different processes (US-IR, US-E, US-EWC and US-IRE). Data shown are means ± SD (n = 3).

Percentage of MW distribution, (%)									
Samples	**MW (Da)**	**NF[1] (0 min)**	**NFP[2] (120 min)**	**NFPT[3] (240 min)**	**Δ_{NF}[7]**	**F[4] (0 min)**	**FP[5] (120 min)**	**FPT[6] (240 min)**	**Δ_{F}[7]**
Control	>3000	6.7±0.3	3.9±0.1	2.2±0.0	-67.1	6.4±0.0	4.6±0.2	2.3±0.1	-62.5
	3000-260	60.0±0.5	61.6±0.5	63.6±0.8	6.0	59.1±1.1	60.0±0.4	63.4±0.6	7.6
	<260	33.3±0.9	34.5±0.4	34.2±0.7	2.7	34.5±1.0	35.4±0.1	34.3±0.6	-0.6
Cr	>3000	5.0±0.61	3.8±0.41	3.2±0.35	-36.0	4.9±0.7	3.8±0.4	3.0±0.4	-38.7
	3000-260	37.7±2.9	38.8±0.2	43.3±0.1	17.9	41.4±0.8	43.3±0.6	45.5±0.5	10
	<260	57.3±3.2	57.4±0.5	53.5±0.3	-6.6	53.7±0.0	52.9±0.2	51.5±0.0	-4.1
US-IR	>3000	4.5±0.1	3.0±0.1	3.3±0.4	-26.6	4.5±0.1	3.7±0.2	3.5±0.1	-22.2
	3000-260	37.1±0.7	39.3±0.3	49.1±0.3	32.3	44.7±1.61	43.1±1.6	46.0±0.4	2.9
	<260	58.4±0.6	57.7±0.2	47.6±0.4	-18.4	50.8±1.4	53.2±1.5	50.5±0.5	-0.6
US-E	>3000	1.8±0.0	1.7±0.1	0.9±0.2	-50.0	2.2±0.7	1.3±0.0	0.9±0.7	-59.0
	3000-260	38.7±0.3	39.4±0.0	41.7±0.4	7.7	39.1±0.4	40.5±0.3	41.4±1.2	5.9
	<260	59.5±0.3	58.9±0.1	57.4±0.4	-3.5	58.7±0.3	58.2±0.2	57.7±0.1	-1.7
US-EWC	>3000	1.3±0.6	0.8±0.0	0.5±0.0	-61.5	1.8±0.3	0.7±0.0	0.5±1.0	-72.2
	3000-260	29.4±0.3	30.0±0.0	30.5±0.3	3.7	30.1±0.3	31.1±0.0	34.7±2.5	15.3
	<260	69.3±0.4	69.1±0.5	69.0±0.4	-0.5	68.2±0.4	68.2±0.2	64.8±2.8	-4.9
US-IRE	>3000	1.3±0.2	0.8±0.5	0.7±0.6	-85.7	1.3±0.1	0.7±0.4	0.3±1.0	-76.9
	3000-260	18.9±0.3	19.3±0.4	18.5±0.1	-2.1	20.4±0.4	19.4±0.0	19.0±2.5	-6.8
	<260	79.8±0.3	79.8±1.0	80.7±0.7	1.1	78.3±0.4	79.8±0.3	80.7±2.8	3.1

[1] NF: non-fermented, at the beginning of the digestion. [2] NFP: non-fermented, after the pepsin digestion. [3] NFPT: non-fermented, after the pepsin + trypsin digestion. [4] F: fermented, at the beginning of the digestion. [5] FP: fermented, after the pepsin digestion. [6] FPT: fermented, after the pepsin + trypsin digestion. [7] Δ: relative difference of MW distribution between the end and the beginning of the in vitro digestion of non-fermented beverages (Δ_{NF}) and fermented beverages (Δ_{F}). US-IR: treated with γ-irradiation assisted with ultrasound. US-E: treated cricket protein-enriched beverages subjected to enzymatic hydrolysis assisted with ultrasound. US-EWC: treated cricket protein-enriched beverages subjected to enzymatic hydrolysis assisted with ultrasound, whole product of hydrolysis. US-IRE: enzymatic hydrolysis assisted with γ-irradiation and ultrasound.

Otherwise, should be noted that a decrease in the percentage of HMW peptides was observed all along the pepsin-trypsin digestion in all samples, as this fraction may be more sensitive to the different treatments. Furthermore, the fermented samples US-E F, US-EWC F and US-IRE F are characterized by a very weak or negligible percentage of HMW peptides accompanied by the highest percentage of LMW peptides compared to the other treatments without enzyme (control, Cr, US-IR). These results reflect the high degree of protein degradation in these products, and some authors have suggested that the enrichment of beverages with proteins under hydrolysates form allows enhanced protein digestibility and tends to increase the level of functional and bioactive peptides (Calbet and Holst, 2004; Koopman et al., 2009). Additionally, the pre-digestion of cricket protein with the US-E and US-IRE treatments could facilitate the role of pepsin and trypsin enzymes during the digestion but could also mask the proteolytic activity of ferments during fermentation. In the same context, Li et al. (2013) found that suitable enzymatic hydrolysis was an optimal approach to improve some physical properties, immunoreactivity and in vitro protein digestibility of soy protein isolate for infant formula. The profile change over the US-IRE treatment may be explained by the proteolysis of large insoluble proteins into soluble peptides. Hence, hydrolysis into smaller peptides confirmed the fast digestion when the enzymatic hydrolysis was combined with γ-irradiation, ensuring the breakdown of polypeptide chains and a strong formation of small peptides (Gaber, 2005).

In order to better assess the efficiency of peptide hydrolysis with and without fermentation, the relative difference of M_W distribution between the end and the beginning of the in vitro digestion (Δ value) was determined. The analysis of the peptide profile by SEC-HPLC did not show a critical change caused by fermentation, except for the US-IR treatment as related to MMW and LMW peptides. In general, the Δ values in other treatments were slightly or not at all influenced by the fermentation. However, for US-IR treatment, the fermentation impacted more the conversion of MMW and LMW peptides, with Δ_{NF} = 32.3% and Δ_F = 2.9% for MMW peptides and Δ_{NF} = −18.4% and Δ_F = −0.6% for LMW peptides. These data suggest that the fermentation process generated a much less sensitive product to US-IR treatment as it reduced considerably the conversion of peptides into lower Mw during digestion. This observation can be explained by the fact that for fermented products, a pre-digestion started during fermentation which conducted to a faster hydrolysis by digestive enzymes under the combined effect of ultrasound and γ-irradiation. Furthermore, this could be corroborated by the occurrence that pepsin and trypsin cleaved additional peptide bonds that were not cleaved previously by ferments. According to Ogodo et al. (2018) and Pranoto et al. (2013), the degradation of high M_w peptides into smaller ones, produced during fermentation and digestion, has a significant effect on the digestibility of proteins.

4. Conclusions

Industries that manufacture functional beverages are looking for alternatives to improve the nutritional quality of their products as well as their shelf life. Insect protein is a rich source of amino acids, and in this work the effect of ultrasound (US), γ-irradiation (IR), and enzymatic hydrolysis (E) of cricket proteins and their combined processes on the physicochemical, structural properties and in vitro digestibility on fermented beverages enriched with such cricket protein was analyzed. Combined treatment of ultrasound-assisted γ-irradiation (US-IR; dose = 3 kGy, t_{US} = 20 min) improved the solubility of cricket proteins and increased their surface and total sulfhydryl group content. This also ensured a higher level of hydroxyl/amino groups susceptible to solvation associated with a reduction of random coils in protein conformation possibly associated with aggregation, as shown by FTIR analysis, as well as a high level of medium and low Mw peptides after in vitro digestion, as shown by HPLC/SEC analysis. This treatment showed a high digestibility compared to the non-enriched and enriched beverages, but the treatments combined with enzymatic hydrolysis US-E and US-IRE induced a much higher positive effect in improving the digestibility of fermented (F) beverages and higher formation of low M_w peptides after digestion (up to 81%). Therefore, it is reasonable to assume that a combined treatment of cricket proteins is very promising to ensure a high digestibility of protein-enriched beverages with very satisfying functional and nutritional properties. The results obtained from this study offer a new, promising perspective for the valorization of cricket proteins in the application of functional foods.

Author Contributions

Conceptualization, M.L.; methodology, C.D. and J.M.; software, C.D. and S.S.; validation, C.D., M.M. and M.L.; formal analysis, C.D.; investigation, C.D.; resources, C.D.; data curation, C.D., M.M., S.S. and M.L.; writing—original draft preparation, C.D.; writing—review and editing, C.D., M.M., B.A., S.S. and M.L.; visualization, C.D; supervision, M.L.; project administration, M.L.; funding acquisition, M.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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Conflicts of Interest

M.M. is an employee of Bio-K Plus, a Kerry company. This company provided Bio-K+ beverages and supplied cricket powder. The authors declare no conflict of interest.

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Lien entre les chapitres 2 et 3

Le chapitre 2 « **Effet de prétraitement physique et enzymatique sur les propriétés nutritionnelles et fonctionnelles des boissons fermentées enrichies en protéines de criquet** » a permis de sélectionner le procédé pertinent pour prétraiter la poudre de criquet et obtenir une protéine avec des propriétés fonctionnelles et structurelles améliorées avec une digestibilité élevée. Le traitement combiné d'hydrolyse enzymatique assisté par ultrasons a été choisi pour continuer la suite du projet.

Pour confirmer les résultats du test *in vitro* et mieux évaluer la qualité protéique de boisson, une étude *in vivo* a été réalisée sur un modèle animal en utilisant des rats Wistar. Ainsi, la digestibilité *in vivo*, les paramètres nutritionnels à savoir le CEP et l'effet de cette boisson sur le microbiote intestinal ont été déterminés et présentés dans le chapitre 3.

CHAPITRE 3: EVALUATION OF THE NUTRITIONAL QUALITY AND *IN VIVO* DIGESTIBILITY OF PROBIOTIC BEVERAGES ENRICHED WITH CRICKET PROTEINS

Évaluation de la qualité nutritionnelle et de la digestibilité *in vivo* de boisson probiotique enrichie en protéines de criquet

Chaima Dridi, Mathieu Millette, Blanca Aguilar, Sebastien Lacroix, Tommaso Venneri, Stephane Salmieri, Zahra Allahdad, Vincenzo Di Marzo, Cristoforo Silvestri, Monique Lacroix.

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Le nombre de figures et de tableaux et le style des références ont été présentés selon le guide des auteurs du journal

Contributions des auteurs

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Chaima Dridi avec l'aide de Dr. Sebastien Lacroix et Dr. Tommaso Venneri pour le séquençage et les analyses bio-informatiques. Les discussions sur les résultats et les protocoles ont été assistés par Dr. Mathieu Millette et Stéphane Salmieri. L'article a été écrit par Chaima Dridi, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri, Dr. Mathieu Millette, Dr. Zahra Allahdad, Pr. Blanca Aguilar, Pr. Vincenzo Di Marzo, Pr. Cristoforo Silvestri et Pr. Monique Lacroix.

Résumé

Les boissons riches en protéines sont parmi les produits les plus consommés, mais peu offrent des ingrédients nutritionnels ou actifs au-delà de leur contenu nutritionnel de base. Les insectes constituent une source exceptionnelle de protéines, qui peuvent soutenir la croissance. Le but de cette étude était de déterminer la qualité protéique d'une boisson fermentée enrichie d'hydrolysats de protéines de criquet (CP.H) ou de protéines entières de criquet (CP) et de déterminer leurs effets sur le microbiome intestinal. La qualité des protéines a été évaluée en tant que le Coefficient d'Efficacité Protéique (CEP), Coefficient Protéique Net (CEPN), digestibilité apparente (AD) et réelle (TD) réalisée sur un modèle animal. Les résultats ont montré que l'incorporation de CP.H, en plus de l'amélioration de la digestibilité *in vivo*, a augmenté significativement le CEP et le CEPN ($P \leq 0.05$) par rapport au CP, de 1,7 à 2,0 et de 0,4 à 1,0, respectivement. AD de CP.H était de 94%, ce qui était proche du groupe caséine (96%) et significativement ($P \leq 0.05$) plus élevé que le groupe CP (85%). Les résultats ont également montré que la consommation d'un régime à base de protéines de criquet a maintenu une composition équilibrée du microbiome fécal et a réduit la proportion de bactéries pathogènes dans l'intestin. Par conséquent, les deux groupes de protéines de criquet (CP.H et CP) peuvent être considérés comme des protéines de bonne qualité ainsi qu'une bonne alternative pour la consommation humaine.

Mots clés : Protéines de criquet ; Hydrolysats de protéines de criquet ; Probiotiques ; Qualité nutritionnelle ; Digestibilité ; Microbiome intestinal.

Evaluation of the nutritional quality and *in vivo* digestibility of probiotic beverages enriched with cricket proteins

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Abstract

High protein beverages are among the most consumed, but few offer nutritional or active ingredients beyond their basic nutritional content. Insects provide an exceptional source of protein, which can support growth. The purpose of this study was to determine the protein quality of fermented beverage enriched with cricket protein hydrolysates (CP.H) or whole cricket proteins (CP) and to determine their effects on the gut microbiome. The protein quality was evaluated as the Protein Efficiency Ratio (PER), Net Protein Ratio (NPR), the apparent (AD) and the true (TD) digestibility realised in an animal model. Results showed that the incorporation of CP.H, in addition to *in vivo* digestibility enhancement, increased the PER and the NPR significantly ($P \leq 0.05$) as compared to CP, from 1.7 to 2.0 and from 0.4 to 1.0, respectively. AD of CP.H was 94% which was close to the casein group (96%) and significantly ($P \leq 0.05$) higher than the CP group (85%). Results also showed that, the intake of a cricket protein-based diet maintained a balanced composition of fecal microbiome and reduced the proportion pathogenic bacteria in the gut. Therefore, the two groups of cricket protein (CP.H and CP) can be considered as good quality protein as well as a good alternative for human consumption.

Keywords: Cricket proteins; Cricket protein hydrolysates; Probiotics; Nutritional quality; Digestibility; Gut microbiome

1. Introduction

With the emergence of diet-related illnesses such as cardiovascular disease, diabetes, intestinal infections and other illnesses, there is a growing trend towards functional food consumption positively affecting various targets in the body to promote health. Functional foods are associated with the concept of probiotics. Indeed, probiotics included in different food systems bring about various health benefits mainly by maintaining normal intestinal microbiota, protection against gastrointestinal pathogens (Plummer et al., 2004), improving the immune system (Chabot et al., 2001), reducing serum cholesterol and blood pressure and prevention of childhood diarrhea (McFarland, 2007). Moreover, protein enriched products are considered as functional foods that are in increasing demand and are considered an integral part of food development to fill deficiencies in essential amino acids, especially for children and the elderly who are malnourished and suffer from loss of muscle mass due to deficiency of protein for energy generation (Douglas et al., 2013). Indeed, some studies showed that dietary proteins, next to exercise, are thought to play a role in preventing and managing sarcopenia (Bauer et al., 2013; Nieuwenhuizen et al., 2010; Song et al., 2019). Recently, several unconventional protein resources were introduced as sustainable alternatives. This is due to the increase of needs in proteins synchronized with the growth of global populations and limitations in the availability of fresh water and arable land (Plaza et al., 2008). Insects are considered as one of the most interesting protein sources for the future. They are known for many favorable attributes, such as the lower environmental impact and the higher nutritional value. More importantly, they meet the WHO essential amino acids requirement (Rumpold and Schlüter, 2013) with a high protein content. They were also demonstrated to be a good source of fatty acids, vitamins and minerals such as zinc, iron, potassium, calcium and magnesium (Durst and FAO, 2010).

Digestibility and amino acid composition required for growth and body functions, are the determining factors of the nutritional quality of proteins (Florence et al., 2014). Insect proteins are more digestible than plant-based proteins such as peanuts and lentils, but less digestible than animal-based proteins such as beef and egg white (REF). However, the presence of chitin in the exoskeletons of insects, as long polymer with a structure similar to cellulose, limit significantly their absorption (Oibiokpa et al., 2018 ; Marono et al., 2015). Physical and biological processes applied as a pre-treatment on proteins have been demonstrated to strongly improve the nutritional properties and the digestibility of the vegetables proteins and insect proteins (Körzendörfer et al., 2019; Kuznetsova et al., 2014). Among these methods, fermentation was used to decrease the levels of anti-nutrient in food grains, increase the protein availability, digestibility and nutritive

value (Ali et al., 2003; Beausoleil et al., 2007; 2018; Afify et al., 2012). Furthermore, protein hydrolysates obtained after protease enzymes treatment were used to substitute native proteins for infant food and beverage formulations to decrease allergenicity, increase adsorption, accelerate protein digestion and improve functional properties (Sinha et al., 2007 ; Calbet and Holst, 2004). In the study conducted by Hall et al. (2017), the hydrolysis of whole cricket proteins using the alcalase enzyme could yield protein hydrolysates with high functional properties that are suitable for food applications. In addition, an improvement of the biological properties such as the antioxidant activity and the inhibition of angiotensin-converting enzyme (ACE) activity was observed for insect protein hydrolysates (Vercruysse et al., 2009).

Actually, few studies are available on the nutritional quality of insects, as there are few studies related to enzymatic hydrolysis, the protein efficiency index, in vivo digestibility and the effect that the consumption of the insect protein can have on the gut microbiome(Nakagaki et al., 1987; Oibiokpa et al., 2018; Poelaert et al., 2018).

This study was conducted to evaluate the effect of fermentation with a specific probiotic formulation comprised of *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R and *Lacticaseibacillus rhamnosus* CLR2 on the nutritional quality of beverages enriched with whole cricket protein (CP) and cricket protein hydrolysates (CP.H) by determining the protein efficiency ratio (PER), the net protein efficiency ratio (NPR), the true digestibility (TD) and the apparent digestibility (AD) using *in vivo* tests which were carried out on an animal model. Finally, the effect of cricket proteins on the composition of the gut microbiome was investigated.

2. Materials and Methods

2.1. Materials

Organic Cricket flour (60% protein content) was produced by Nexxus Foods (Montreal, QC, Canada). The fermented and the non-fermented beverages used in this study were produced by Bio-K Plus a Kerry company (Laval, QC, Canada). The pellets of the experimental diets given to rats during this study were manufactured by the company Research Diets, Inc (New Brunswick, NJ, USA). Alcalase enzyme (from *Bacillus licheniformis* ≥ 2.4 Units/g of protein) was supplied by Sigma-Aldrich (Oakville, ON, Canada). All other reagents were of analytical grade.

2.2. Ultrasound pretreatment and enzymatic hydrolysis

Ultrasound pre-treatment (US) was done using a sonicator QSonica Q500 (model FB-505; Fisher Scientific, Ottawa, ON, Canada). US probe (model CL-334) was plunged into a flask, containing 25 g of cricket powder diluted in 200 mL of distilled water and the operation was conducted in batch mode. The sonicator operated at a maximal power of 500 W and frequency of 20 kHz. Samples were treated for 15 min (pulsed mode: on-time 5 s and off-time 2 s) and the amplitude was fixed at 60%. The flask containing the sample was immersed into a cooling bath to avoid heating induced by the US treatment. Then, the suspension was used for the enzymatic hydrolysis using alcalase enzyme, according to an optimum hydrolysis condition ensured based on a previous study carried out in our laboratory (Dridi et al., 2021): the enzyme/substrate (E:S) ratio 1:10 (w/w), reaction time 180 min, hydrolysis temperature 55°C and pH 8. At the end of the reaction, the mixture was heated at 95°C for 10 min to inactivate the enzyme. The mixture was then cooled down at room temperature in an ice bath, followed by centrifugation at 13,000 x g for 20 min. The supernatant was collected and freeze-dried (Labconco Freezone® 2.5 L, model 7670521, Fisher Scientific) to produce the protein hydrolysates used for the enrichment of fermented beverages.

2.3. Preparation of beverages

Beverages have been manufactured at Bio-K Plus, a Kerry company (Laval, QC, Canada). The ingredients were weighed and hydrated with filtered water. The mixture was then pasteurized at 90 ± 2°C for 60 sec, packaged in 98 g plastic bottles, aluminium sealed, and then cooled to 37°C. Beverages were inoculated with three probiotic strains (*L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2), at 10^8 CFU/mL and stored at 4 °C. The whole cricket protein (CP) and the cricket protein hydrolysates (CP.H) were used for the enrichment of fermented beverages, for a total protein amount of 13% (w/w). Beverages were freeze-dried and then grounded for the preparation of diet in pellets form to be administrated to the rats.

2.4. Amino Acid composition and score

The amino acid composition of different protein sources has been provided by the supplier (Nexxus Foods for the cricket protein and Research diet for the casein). The amino acid score (AAS) was expressed according to the standard method (FAO, 2007) as follows:

$$\text{Amino acid score} = \left[\frac{\text{Protein essential amino acids EAA contents}}{\text{Recommended essential amino acids}}\right] \quad \text{Eq (1)}$$

The protein quality depends on the proportion of the amino acid so that it is qualified as a limiting amino acid when its value is inferior to 1. The proportion of essential amino acids (E) to the total amino acids (T) of the protein was also calculated as follows:

$$\frac{E}{T}\% = \left[\frac{\text{Ile+Leu+Lys+Met+Cys+Phe+Tyr+Thr+Val+His}}{Ala+Asp+Arg+Gly+Glu+Ile+Leu+Lys+Met+Cys+Phe+Tyr+Pro+Ser+Thr+Val+His} \times 100\right] \quad \text{Eq (2)}$$

2.5. Preparation of experimental diets in rats

Four diets were evaluated in this study. The composition of each diet is presented in Table 1. The formulation was prepared according to the AOAC 960.48 method. The percentage of proteins was adjusted to 10% for all the formulations except the negative control (protein free diet). Casein was used as a reference protein. The freeze-dried beverages CP and CP.H were used as a protein source. Soybean oil, vitamin, mineral, cellulose, sucrose and starch were also added to the formulations and their amounts were calculated to have equal calorie counts for all diets. The diets were fed to the rats in the form of pellets.

Table.1. Formulation of experiment diets (g/100g) offered to rats during the animal studies.

Ingredients	Casein	Protein free	[1]CP.H	[2]CP
Casein	11.4	-	-	-
CP.H	-	-	20.6	-
CP	-	-	-	19.1
Mineral mix	4.5	4.5	4.5	4.5
Vitamin mix	1.2	1.2	1.2	1.2
Soybean oil	7.9	7.9	7.6	4.1
Cellulose	5	5	4.7	3.4
Sucrose	10	10	10	10
Starch	60	71.4	51.4	57.7
Protein %	10	0	10	10

[1]CP.H, Cricket hydrolysates enriched fermented beverage; [2]CP, Whole cricket powder enriched fermented beverage;

2.6. Animal study design

A prior approval of the National Experimental Biology Centre and the Institutional Animal Care Committee of the INRS Armand-Frappier Health and Biotechnology Research Centre, in accordance with the principles of the Canadian Council on Animal Care, was obtained (Protocol CIPA no 1809-04) before conducting the *in vivo* study. This experiment was carried out using growing male Wistar rats of 20-23 days old. During the entire experimentation, rats were housed in separated cages. They were distributed in 4 groups of 7 rats. Cycles of 12 hours of light-dark and temperature of 20 ± 0.5 °C was fixed throughout the experiment period. For the first 5 days, the rats received a standard diet for the acclimation. In the subsequent 14 days, rats fed *ad libitum* with the experimental diet for each group.

2.7. Protein Efficiency Ratio (PER)

The daily food intake and feed waste were daily determined every day and body weight of each group was recorded every 2 days. The rats weight gain was determined as follows:

Body weight gain (W_g) = Final body weight – Initial body weight Eq (3)

Protein Efficiency Ratio (PER) was performed according to the official procedures of the AOAC Official Method 960.48, using the following equation,

$$\mathrm{PER} = \left[\frac{W_g}{P_i}\right] \quad \text{Eq (4)}$$

where W_g stands for the body weight gain and P_i is the consumed protein

The Adjusted PER was calculated according to the equation below:

$$\mathrm{APER} = \left[\frac{2.5}{\text{Experimental PER value of the control casein}}\right] * \text{PER value of respective treatment} \quad \text{Eq (5)}$$

The Net Protein Efficiency Ratio (NPR) was determined based on the à weight gain of experimental group, the weight loss (W_l) of the group fed with no protein diet and the consumed protein (P_i):

$$\mathrm{NPR} = \frac{W_g + W_l}{P_i} \quad \text{Eq (6)}$$

2.8. Protein digestibility

Complete feces were collected daily during the 14 days and stored at 4 °C. Feces were dried in an oven (Thermo scientific, Germany) at 105°C for 24 h, weighed and ground then analysed for

the total nitrogen (N) by Kjeld Hal method according to AOAC: Official Methods 991.20 (2000). The apparent protein digestibility and the true digestibility were determined from the equation 7 and 8 as follows:

$$\text{Apparent protein digestibility} = \left[\frac{\text{Ni}-\text{Ne}}{\text{Ni}}\right]*100 \quad \text{Eq (7)}$$

$$\text{True protein digestibility} = \left[\frac{\text{Ni}-(\text{Ne}-\text{Nepf})}{\text{Nepf}}\right]*100 \quad \text{Eq (8)}$$

Where N_i corresponds to the amount of nitrogen ingested in the diet, N_e stands for the quantity of nitrogen excreted in feces and N_{epf} is the fecal nitrogen in the protein free group, corresponding to the metabolic loss in feces. The protein digestibility corrected amino acid score (PDCAAS) for each protein source was also determined from the amino acid chemical score corrected by the protein digestibility (Boye et al., 2012) and expressed as follows :

$$\text{PDCAAS} = \text{Amino acid score} \times \text{True digestibility (\%)} \times 100 \quad \text{Eq (9)}$$

2.9. 16S rDNA Sequencing and Metagenomics Analysis

At the end of 14 day of experiment, feces were collected from the colon of rats and stored in a sterile plastic tube. DNA from fecal samples were extracted using Qiagen DNeasy PowerSoil Kit. The DNA concentrations of the extracts were measured fluorometrically with the Quant-iT PicoGreen dsDNA kit (Thermo Fisher Scientific, MA, USA), and the DNAs were stored at −20°C until 16S rDNA library preparation according to the Illumina “Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System” protocol. Briefly, 15 ng of DNA was used as template, and the V3-V4 region of the 16S rRNA gene was amplified by PCR using the following primers: 16S Amplicon PCR Forward Primer = 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’, 16S Amplicon PCR Reverse Primer = 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’ followed by a second PCR reaction to introduce indexes (Nextera XT Index; Illumina). The 16S metagenomic libraries were eluted in 30 µl of nuclease-free water and 1 µl was qualified with a Bioanalyser DNA 1000 Chip (Agilent Technologies, CA, USA) to verify the amplicon size (expected size ~600 bp) and quantified with a Qubit (Thermo Fisher Scientific, MA, USA). Libraries were then normalized and pooled to 2 nM, denatured, and diluted to a final concentration of 10 pM and supplemented with 5% PhiX control (Illumina). Sequencing (2 × 300 bp paired-end) was performed using the MiSeq Reagent Kit V3 (600 cycles) on an Illumina MiSeq system (Illumina, CA, USA). Sequencing reads were generated in less than 65 h. Image analysis and

base calling were carried out directly on the MiSeq. Preprocessing of obtained sequences and bacterial taxa assignation was performed according to the Dada2 pipeline (Version 1.10.1) using the Ribosomal Database Project (RDP release 11) reference database (Sah et al., 2016). Analyses were then conducted on sequence counts normalized by Cumulative Sum Scaling (CSS) (MetagenomeSeq R package) (Ripley, 2001). CSS divides sequence counts by the median sequence counts of each sample (Paulson et al., 2013). CSS-normalized counts are thus expressed in relation to the entire bacterial composition of each sample and are viewed as being more appropriate than Total Sum Scaling (Weiss et al., 2017).

2.10. Statistical analysis

For the *in vivo* analysis, seven rats were used in each group, the results were reported as mean values $\pm$ standard deviation. For the nutritional evaluation, statistical analysis was carried out using SPSS software version 22 (IBM, NY, USA) and the significant differences ($P \leq 0.05$) among the means were evaluated using one-way analysis of variance (ANOVA). For the gut microbiota characteristics, the potential relationship between the diets groups and microbial community structure was examined through Principal Component Analysis (PCA) and Principal Coordinate Analysis (PCoA), performed using Bray–Curtis dissimilarity indexes of beta-diversity and Permutational multivariate analysis of variance (PERMANOVA) (VeganR package) (Bray and Curtis, 1957). Two-way ANOVA (taxa abundance ~ Genotype*Diet) followed by Tukey HSD post-hoc were conducted to evaluate the influence of diet group and protein type on gut microbial taxa. Results were considered statistically significant at $P \leq 0.05$ or FDR-adjusted $P \leq 0.1$. Analyses were performed with R software version 3.4.3. (Ripley, 2001).

3. Results and discussion

3.1. Amino Acid composition and score

The amino acid profile of casein and cricket protein is presented in Table 2. Both casein and cricket are characterized by a complete amino acid profile. Indeed, they contain both essential and non-essential amino acids. Similar results were found by Oibiokpa et al. (2018) who noted that the cricket protein could be a good source of essential amino acid such as Sulphur-containing amino acids (methionine and cystine), which play an important role in body growth. Also, cricket protein has a high content of lysine and methionine, which were found to be low in plant-based protein and in most diets such as rice, hemp and pea (Mokrane et al., 2010; Zhao et al., 2020). Threonine and cystine were the least abundant (0.93 and 0.84 g/100 g respectively). Cricket

protein was also characterized by significant levels of the essential amino acid histidine and the conditionally essential amino acid arginine, which must be provided by the diet in sufficient amounts for the growth for children and in pregnant and lactating women (Chatterjea and Shinde, 2008). The amino acid score determination showed that the cricket protein had an adequate amino acid balance as compared to the Food and Agriculture Organization (FAO, 2007) amino acid requirements. Indeed, all the amino acids presented a score above 1. The determination of essential amino acid to total amino acid ratios (E/T) showed a percentage of 44.5% and 53.7 % for cricket and casein, respectively. These values are above 36%, which are adequate to consider the protein as an ideal protein (FAO, 2007).

Table.2. Amino acid composition and AAS of casein and cricket proteins.

	Amino Acid composition			**AAS**	
Amino acid	**Casein**	**Cricket**	**FAO/WHO Required**	**Casein**	**Cricket**
His	4.5	2.39	**1.5**	3.0	1.60
Ileu	7.5	4.31	**3.0**	2.55	1.44
Leu	15.7	7.67	**5.9**	2.66	1.30
Lys	13.0	6.00	**4.5**	2.88	1.33
Met	5.0	1.81	**1.6**	3.12	1.13
Cys	1.2	0.84	**0.6**	2	1.41
Cys + Met	6.2	2.66	**2.2**	2.81	1.21
Phe + Tyr	10.4	9.69	**3.8**	2.73	2.55
Thr	7.1	0.93	**2.3**	3.08	1.71
Trp	2.1	6.09	**0.6**	3.5	1.55
Val	9.2	6.15	**3.9**	2.35	1.58
EAA/T (%)	53.7	44.5	**36.0**	-	-

AAS: Amino Acid score, EAA: Essential Amino Acid. [1] FAO/WHO required in g/100g of proteins for adult for an ideal protein

3.2. Animal growth, food and protein intake

The results related to the growth, food and protein intake of animals fed with diets containing casein, CP.H- and CP-enriched fermented beverages are shown in Table 3. All the groups had

an initial weight ranged between 100 and 109g. However, at the end of the experiment, the group fed with casein showed the highest weight (188.7g±12.4 g) ($P \leq 0.05$) compared to the groups fed with CP.H (164.6± 7.8 g) and fed with CP (137.7±11.6g). Consistently, the group fed with casein had the highest daily weight gain ($P \leq 0.05$) (6.3±0.8 g/day) followed by rats fed the CP.H based-diet (3.9 ±0.5 g/day), which is also significantly ($P \leq 0.05$), higher than CP based-diet (2.7 ±0.8 g/day). Weight reduction was observed for the group fed with the protein-free diet. Protein intake is related to the food intake. The food intake value of animals fed with CP.H (19.2±1.5 g/day) was higher than in animals fed CP-based diets (15.5 ±2.6 g/day($P \leq 0.05$), thus the protein intake of animals fed the CP.H-based diet was significantly higher than that for the CP group ($P \leq 0.05$). Our results showed a positive effect of both the treated (protein hydrolysates) and non-treated (whole protein) cricket protein-based diets, which ensured the growth of rats. This could be explained by the fact that cricket protein constitutes a very rich source of essential amino acids involved in growth (Dias et al., 1997). The study of Oibiokpa et al. (2018) demonstrated that cricket was a good source of amino acids specially the sulphur containing amino acids which are considered growth promoting factors. For the food intake, the difference between CP.H and CP based-diet could be due to the presence of chitin which is considered an antinutritional factor and which could reduce the appetite of rats, decrease fat absorption and the absorption of food through the intestine (Mang et al., 2016; Ngatchic et al., 2013 ; Marono et al., 2015). Indeed, the pre-treatment of cricket protein by enzymatic hydrolysis assisted by ultrasound, in addition to the fermentation, has diminished the chitin content following the breakdown of chitin-protein bonds during the process (Synowiecki and Al-Khateeb, 2000; Xie et al., 2021). As a consequence, the cricket-based diet became more tolerable by rats as suggested by the increased daily food intake.

Table.3. Effect of different diets on food intake, protein intake and weight gain of rats.

Group	Food intake (g/day)	Protein intake (g/day)	Initial weight (g)	Final weight (g)	Average daily gain (g/day)
Casein	20.8±1.5^{c}	2.0±0.1^{c}	100.5±2.9^{a}	188.7±12.4^{d}	6.3±0.8^{d}
Protein-free	9.4±0.5^{a}	0.0±0.0^{a}	106.3±3.1^{b}	78.8±1.9^{a}	-1.9±0.1^{a}
CP.H	19.2±1.5^{c}	1.9±0.1^{cb}	109.0±2.4^{b}	164.6±7.8^{c}	3.9±0.5^{c}
CP	15.5±2.6^{b}	1.6±0.3^{b}	100.6±2.2^{a}	137.7±11.6^{b}	2.7±0.8^{b}

CP.H, Cricket hydrolysates enriched fermented beverage; CP, Whole cricket powder enriched fermented beverage; Within each column, means with the same letter are not significantly different ($p > 0.05$).

3.3. Protein Efficiency Ratio

PER, APER and NPR values are presented in Table 4. PER is a measure of the ability of a protein to support the growth of young growing rats (Boye et al., 2012) which is a reflection of protein digestibility and amino acid bioavailability. In our study, the PER values obtained were 3.2, 2.0 and 1.7 for casein, CP.H and CP fed rats groups, respectively.

Table. 4. Protein Efficiency Ratio (PER), Adjusted Protein Ratio (APER) and Net Protein Ratio (NPR) of casein, CP.H- and CP-enriched fermented beverages

Group	PER	APER	NPR
Casein	3.2±0.2^{c}	2.5±0.0^{c}	2.2±0.2^{c}
CP.H	2.0±0.2^{b}	1.5±0.1^{b}	1.0±0.2^{b}
CP	1.7±0.1^{a}	1.3±0.1^{a}	0.4±0.3^{a}

CP.H, Cricket hydrolysates enriched fermented beverage; CP, Whole cricket powder enriched fermented beverage; Within each column, means with the same letter are not significantly different ($P > 0.05$).

PER of casein was significantly ($P \leq 0.05$) higher as compared to the CP.H and CP-based diets, and the PER of CP.H was significantly than that of CP. According to Friedman (1996), protein sources with a PER less than 1.5 are of low quality. Hence, cricket protein, whether as native or hydrolyzed form, is of a good quality. The higher PER value of CP.H (PER=2±0.2) reflected its good absorption as compared to CP (PER=1.7±0.1), which may be due to the increase of the of essential amino acid amounts and the reduction of the allergenicity under the effect of the enzymatic hydrolysis (Kechaou et al., 2009). In addition, fermentation has been proven to release peptides, increase amino acid amount and improve digestibility (Espinosa-Páez et al., 2017; Pranoto et al., 2013). The values obtained are in agreement with Piccolo *et al.* (2017) who found that PER of insect meal was between 2.26 and 1.79 depending on the amount of chitin, comparable to soybeans (Carvalho et al., 2013) and higher than wheat and corn meal (Pires et al., 2006). The APER of CP.H was significantly ($P \leq 0.05$) higher than that of CP-based diet (1.5 and 1.3, respectively), but not as high as that of Casein. The NPR takes into consideration the weight loss of rats fed a protein-free diet to measure how well a protein is digested and used (Nielsen, 2017). The NPR value of the CP.H-based diet was significantly ($P \leq 0.05$) higher than that of the CP-based diet with values of 1.0 and 0.4, respectively. Therefore, the NPR value of the CP.H beverage represented about 50 % of that of casein while for the NPR for the CP beverage was 18.2% of that of casein. Taken together, our results highlight the impact of enzymatic

hydrolysis and fermentation of the enriched beverage with crickets' proteins on the bioavailability of amino acids, making cricket protein more utilisable.

3.4. Protein digestibility

Apparent and true protein digestibility of different diets are shown in Table 5. Apparent digestibility (AD) of the casein-based diet (95%) was significantly ($P \leq 0.05$) higher than corresponding value of the CP.H-based diet (92.2%) which is in turn significantly higher than that of CP-based diet (83.7%) ($P \leq 0.05$). The low true digestibility (TD) value observed with rats fed with CP could be attributed to the proteins linkage to chitin, adversely affecting its digestibility (Belluco et al., 2013). Similarly, Bovera et al. (2016) found that the use of *Tenebrio molitor* larvae in broiler chickens reduced protein digestibility due to the presence of chitin. Several authors (Hill et al., 2005; Pieper et al., 2008) observed that the indigestible portion of diets remains in the intestinal tract and is used as a substrate by intestinal bacteria subsequently affecting growth. Dietary fibers like chitin are able to make a complex with gut epithelium and mucus, preventing access of digestive enzymes and absorption of nutrients, hence reducing protein digestion rate (Devi et al., 2014). In this context, some scientists proposed to use an upstream process for insect to partially or totally remove chitin through disruption of its binding to protein with the aim of improving the quality of proteins (Rumpold and Schlüter, 2013). Thus, the treatments applied in this study led to a better digestion of insect proteins in rats. Results of TD values were higher than AD for all the diets reflecting the high absorption of nitrogen in protein-based diets. Nevertheless, AD and TD of casein-based diet were greater than those of the CP.H and CP-based diets indicating that the nitrogen absorbed from casein was high when compared to nitrogen from cricket proteins. However, an improvement in AD and TD occurred after the ultrasound assisted enzymatic hydrolysis pre-treatment and fermentation via probiotic bacteria. The results obtained from the CP-based diet are in accordance with those reported by Oibiokpa et al. (2018), and other studies found that TD values of insects were in the range of 76-98% (Yang et al., 2014).

The PDCAAS calculated for casein-, CP.H- and CP-based diets was 100, 100 and 94.3 %, respectively (table 5). This parameter was recommended by FAO and WHO as an indicator of protein quality in food. These results showed that cricket-based diet has a high nutritional value which was found to be higher than those of plant-based proteins such as pea, rice (Rutherfurd *et al.,* 2015; Mathai *et al.,* 2017) and faba bean (Nosworthy et al., 2018), which are lacking some essential amino acids namely the lysine and methionine (Manus et al., 2021). The CP-based diet showed the lowest PDCAAS value which can be explained by its lower true digestibility as

compared to the casein and CP.H-based diet (Marinangeli and House, 2017). The value of 100% observed for the CP.H-based diet reflected the improvement of the nutritional quality of cricket protein following the pre-treatment process. Thus, treatments applied in this study led to a better digestion of insect proteins in rats suggesting that enzymatic hydrolysis followed by fermentation could be an effective way of rendering cricket protein more nutritious.

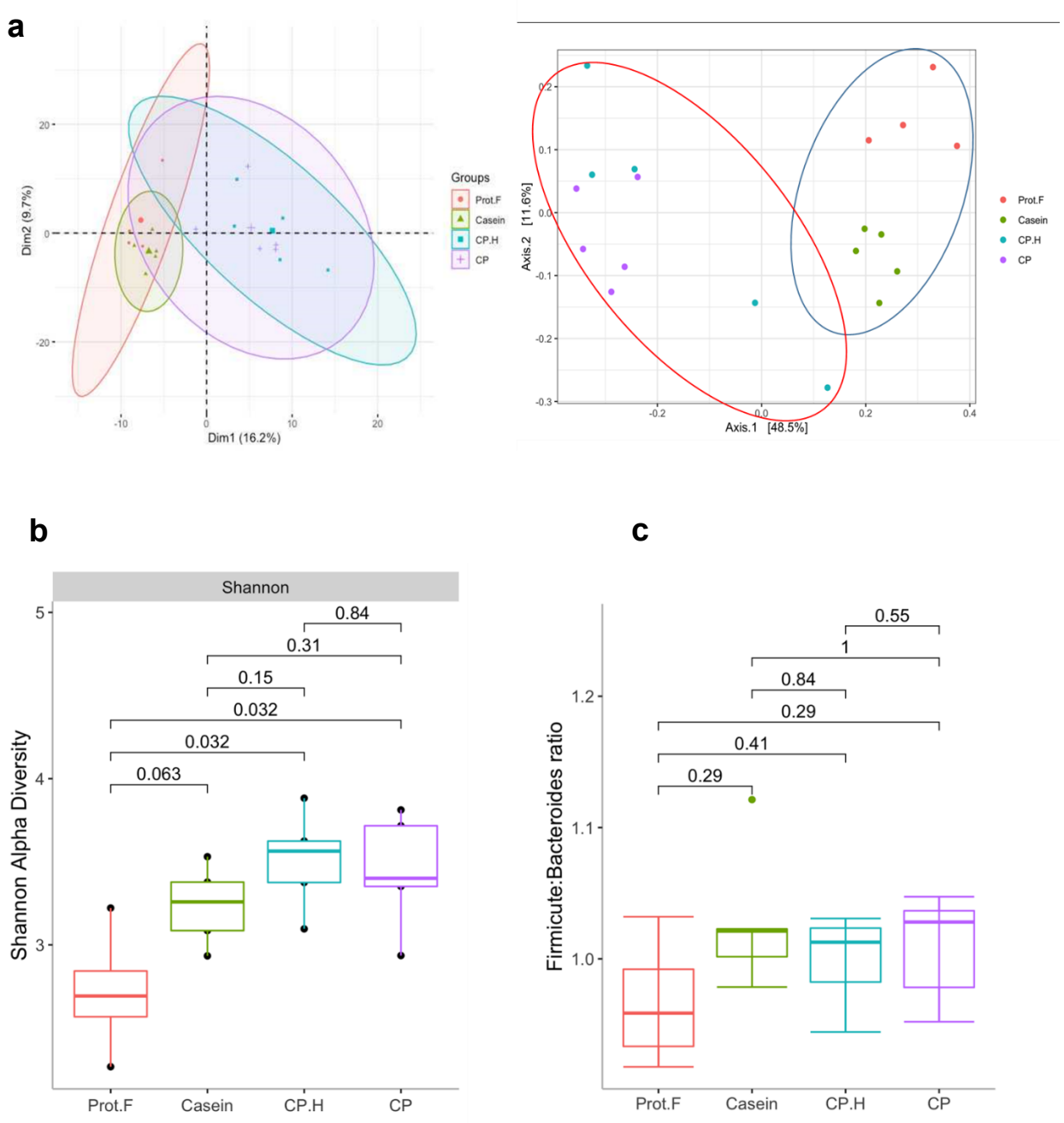
Table.5. The apparent and the true digestibility of casein, CP.H and CP enriched fermented beverages.

Group	AD (%)	TD (%)	PDCAAS (%)
Casein	95.0±0.2^{c}	96.6±0.3^{c}	100
CP.H	92.2±0.8^{b}	94.0±0.8^{b}	100
CP	83.7±1.0^{a}	85.8±1.5^{a}	94.3

CP.H, Cricket hydrolysates enriched fermented beverage; CP, Whole cricket powder enriched fermented beverage; Within each column, means with the same letter are not significantly different (*P* > 0.05).

3.5. Gut microbiota: diet effect

We analyzed whether the different diets modified the gut microbiome of rats. Principle Component Analysis (PCA) suggested that the casein-based diet did not significantly alter the gut microbiome as compared to the protein-free-based diet (Fig. 1a, left). Subsequent Principal Coordinate Analysis (PCoA) followed by ANCOVA analysis revealed that the CP.H and CP diets were statistically different from the casein (*P*=0.024 and *P*=0.024, respectively) as well as the protein-free diet (*P*=0.049 and *P*=0.024, respectively) (Fig. 1a, right). The CP.H- and CP-based diets showed a similar overall gut bacteria architecture however. The effect of diet group on microbial diversity within the gastrointestinal tract was determined using the Shannon Alpha Diversity index (Fig. 1b).


a
Dim2 (9.7%)
Dim1 (16.2%)
Groups
Prot.F
Casein
CP.H
CP
Axis.2 [11.6%]
Axis.1 [48.5%]
b
Shannon
Shannon Alpha Diversity
0.84
0.31
0.15
0.032
0.032
0.063
Prot.F
Casein
CP.H
CP
c
Firmicute:Bacteroides ratio
0.55
1
0.84
0.29
0.41
0.29
Prot.F
Casein
CP.H
CP

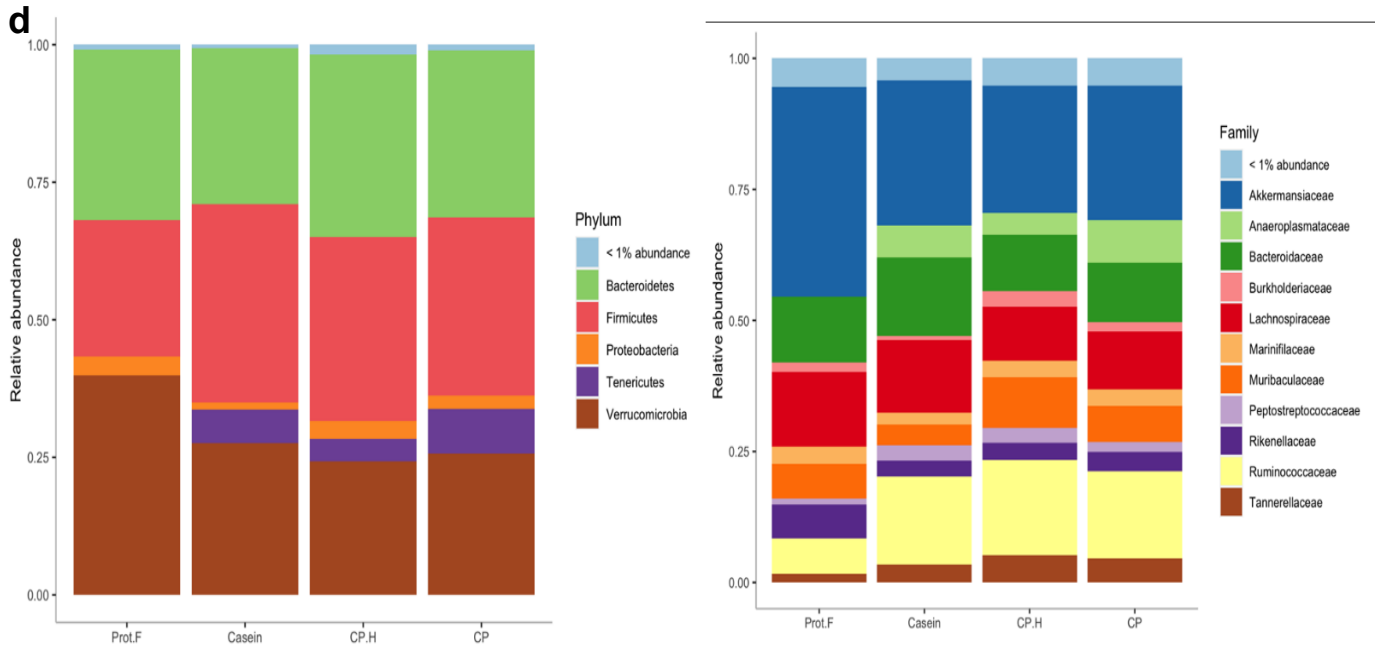


Fig.1. Comparison between the gut microbiota of rats according to the diet and protein type feeding. (A) Principal Component Analysis (PCA) (left side) and Principal Coordinate Analysis (PCoA) (right side) of fecal microbiota from feed with different diets. Permutational multivariate analysis of variance (PERMANOVA) p values are indicated for each diet type. Each point represents one biological sample. (B,C) Shannon alpha-diversity index (B) evaluating gut microbiota richness and evenness and *Firmicutes* to *Bacteroidetes* ratio (C). (D) Relative abundance of gut microbiota at the phylum (left side) and family level (right side). *P* <0.1 or *P* <0.05, significant differences between different dietary protein.

Prot.F, Protein free-based diet; Casein, casein-based control diet; CP.H, Cricket hydrolysates enriched fermented beverage; CP, Whole cricket powder enriched fermented beverage

Supplementation with casein showed a trend for increased alpha diversity (*P*=0.063), however, both the CP.H- and CP-based diets had significantly increased alpha diversity at the end of the protocol. No difference in alpha diversity was observed between casein, CP.H and C.P-based diets, indicating that the alpha diversity of gut microbiota increased due to the diverse nutrients such as amino acids provided by protein-based diets serves to provide a variation of substrates essential in the gut for taxa proliferation (Andreotti et al., 2011; Laparra and Sanz, 2010). None of the diets showed any differences in the Firmicutes to Bacteroides ratio (F:B ratio) (Fig. 1c). However, we did note a non-statistically significant trend for decreased F:B ratio in the group fed the protein free-based diet compared to the protein-based diets (casein, CP.H and CP). While F:B ratio has been though to correlate with obesity and other diseases (Barlow et al., 2015; Krajmalnik-

Brown et al., 2012), though recent evidence has cast doubt on this with respect to obesity (Karlsson et al., 2013; Li et al., 2017), the protein-based diet used in this study, did not cause obesity, as the rats in our study were all of normal weight for their age, growing. However, while the animals fed with protein, did not show obese characteristics, animals fed the protein free-based diet showed a loss of weight after 14 days (from 103.3 to 78.8 g). The results of this research is in a good agreement with those of Stull et al. (2018) for the human gut microbiota and Jarett et al. (2019) for the dogs gut microbiota in which cricket consumption didn't alter alpha and beta diversity. The relative abundance of rats fed with different diets is presented in Fig. 1d, left. At the phyla level, the most predominant phyla for the four groups were *Verrucomicrobia*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Further, an additional phylum (*Tenericuteria*) was significantly increased in all protein-containing groups as compared to the protein-free group (Casein vs. Protein Free; *P*=6.7e-08, CP.H vs. Protein Free; *P*= 7.5e-07, CP vs. Protein Free; *P*= 1.9e-07).

At the family level (Fig. 1d, right), significant differences in abundance of several taxa were observed between groups fed with protein-based diets, independently of the protein type, and the group fed the protein free-based diet. Indeed, *Anaeroplasmataceae* was only detected in the gut of animals fed with protein when compared to the group fed without protein and, *Peptostreptococcaceae*, *Eggerthellaceae*, *Ruminococcaceae*, *Tannerellaceae*, *Christensenellaceae* and *Clostridiales_vadinBB60_group* were all increased in response to protein-supplemented diets (see supplementary table 1). The increase of the proportion of *Tenericutes*/*Anaeroplasmataceae* is known to be associated with positive energy balance (Pedersen et al., 2013). Based on these results, casein, CP.H and CP could be considered as a diet for the maintain of the energy and the support of growth. As shown in the fig. 2a, the taxa *Family XIII* and *Clostridaceae* who may have a role in the metabolism of protein in the intestinal tract and associated with the butyrate kinase butyrate-synthesis pathway, which allows the production of butyrate from protein (Pilla and Suchodolski, 2020), were higher in the protein-based diets and increased especially for rats fed with CP and CP.H. Chai et al. (2019) found that several genera within these families are potential butyrate-producers. In contrast to the number of families that were observed to be increased similarly by all protein-supplemented diets, only *Enterococcaceae* was found to be decreased in a statistically significant manner, and then, only by CP.H, (see supplementary table 2). Interestingly, our analysis did identify a subset of bacterial families that were specifically modified by the individual protein supplemented diets (Fig 2b). *Deferribacteraceae*, *Barnesiellaceae* and *Lactobacillaceae* were only increased by CP.H (though trends for increases were observed in the CP-fed rats for

the latter two as well), while *Erysipelotrichaceae* and *Streptococcaceae* were increased by casein alone. In contrast, *Family_XIII* was strongly increased with the cricket protein-based diets independently of protein form (hydrolysates or whole protein).

When we went on to examine the effects of the different diets at the genus level, several interesting observations were made. *Anaeroplasma*, *Roseburia*, *Romoutsia* and *Ruminiclostridium_9* were all significantly increased in protein-containing diets as compared to the protein-free control (Data not shown). These bacteria are associated with the fermentation of fiber into metabolites as short-chain fatty acids, including acetate, propionate, and butyrate which acts as an anti-inflammatory constitutive (Tomova et al., 2019). In contrast, other genera showed protein-specific alterations in relative abundance. Rats fed with casein, showed strong trends for increased abundance of UCG-014 and USG-005 (belonging to *Ruminococcaceae*), *Asteroleplasma* and *Lactococcus* (though only the latter showed a statistically significant higher levels as compared to every other diets), while *Ruminococcus_2* was decreased (data not shown).

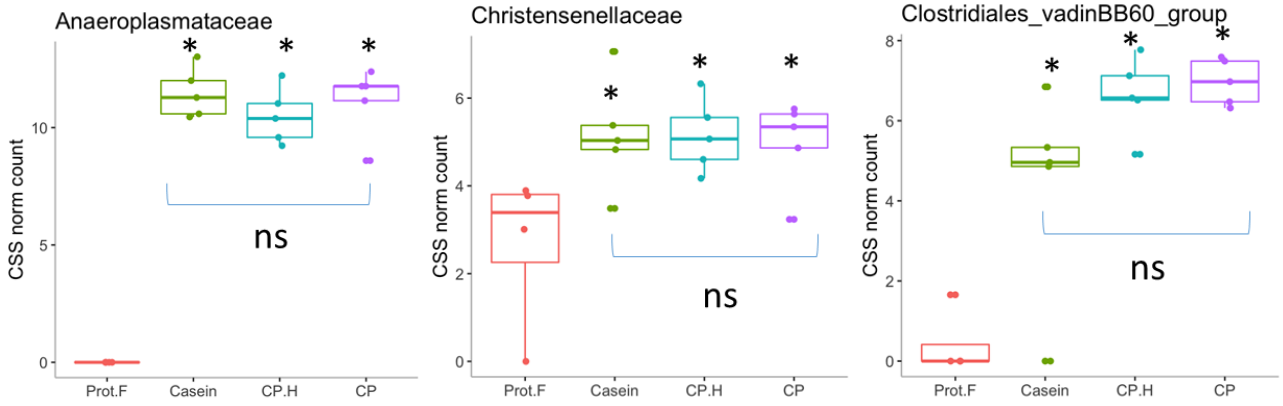
Conversely, CP and/or CP.H specifically increased the abundances of *Parabacteroides*, *Tyzzerella*, *Intestinimonas* and *Caproiciproducen,* associated with short chain fatty acids production (Zhang et al., 2021), in comparison to the protein-free diet, and of *Anaerovorax*, the *Ruminococcaceae* genera *UCG-007*, *UCG-009*, *UCG-013* and *UBA1819* in comparison to the casein diet. This suggests that these latter genera are specifically responsive to the fermented protein diets and not just to protein-rich diets in general. Interestingly, we identified several genera that specifically were increased by either CP (*GCA-900066755*, *Faecalitalea*, *Ruminococcaceae*_NK4A214_group and *Negativibacillus*) or CP.H (*GCA-900066575*, *Candidatus_Soleaferrea*, *Family_XIII_AD3011_group* and *Butyrivibrio*). These latter genera therefor appear to be very sensitive to whether the cricket protein is hydrolysed or not. The *Lactobacillus* genus was found to be higher for rats fed with CP.H (P=0.056), while the protein-free group had the lowest abundance of this genus. *Lactobacillus* spp. plays a key role in the maintenance of the metabolic balance, hence It will have a beneficial effect when it finds in greater abundance. It may reduce the antigen load from gut microbiota to the host and performs an anti-inflammatory response (Zhang et al., 2010). The presence of *Lactobacillus* spp. in high abundance for rats fed with CP.H, could be explained by the survival of *Lactobacillus* spp. from fermented milk, during digestion. Indeed, the presence of proteins in the form of hydrolysates is meant a substrate more assimilable by bacteria which has an effect on the viable count of these bacteria as well as their ability to resist to the stressful gastrointestinal conditions. The CP-based diet did not have such an effect on the *Lactobacillus* spp. abundance, despite the presence of these

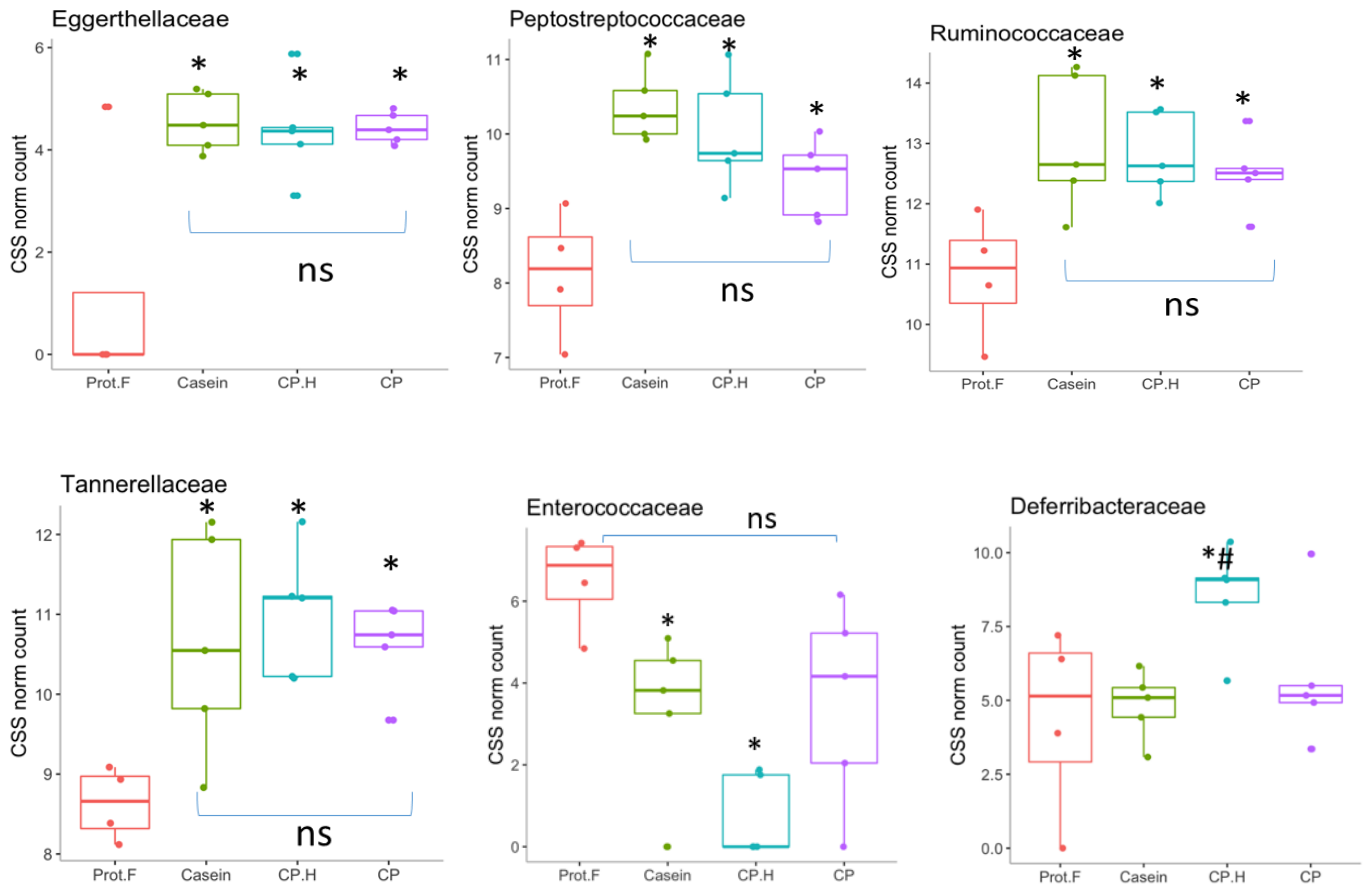
bacteria in the diet. Our results suggested that cricket protein hydrolysates may be more beneficial for the protection and for the proliferation of *Lactobacillus* genus as compared to the whole cricket protein.

Streptococcaceae/Lactococcus has been reported to be associated to the inflammation and metabolic syndrome (Jiao et al., 2018). Other studies (Li et al., 2019; Schots et al., 2020) found that the decrease of *Streptococcaceae/Lactococcus* might play an important role in the prevention of metabolic syndrome. In our study, the family of *Streptococcaceae* showed a decrease particularly for rats fed with CP, while *Lactococcus* genera was lower for both of CP.H and CP-based diet.

Previous studies (Jarett et al., 2019; Yang et al., 2013) showed that the abundance of *Ruminococcaceae* in the gut is thought to have roles in the fermentation of fiber. This indicates the positive correlation with chitin in the cricket protein as a fermentable and indigestible fiber. So that, the whole cricket protein may favor the colonization of gut bacteria that have the capacity to degrade chitin. In addition, Nicholson et al. (2012) showed that gut bacteria can utilize indigestible carbohydrates to produce short-chain fatty acids for colonocytes. Furthermore, short-chain fatty acids have shown to be correlated with health benefits such as glucose homeostasis, lipid metabolism and reduced colon cancer risk (Byrne et al., 2015). These results confirmed that the whole cricket protein and cricket protein hydrolysates did not disrupt the healthy microbiota, on the contrary, they could be potential health promoting ingredients.

a




Eggerthellaceae
Peptostreptococcaceae
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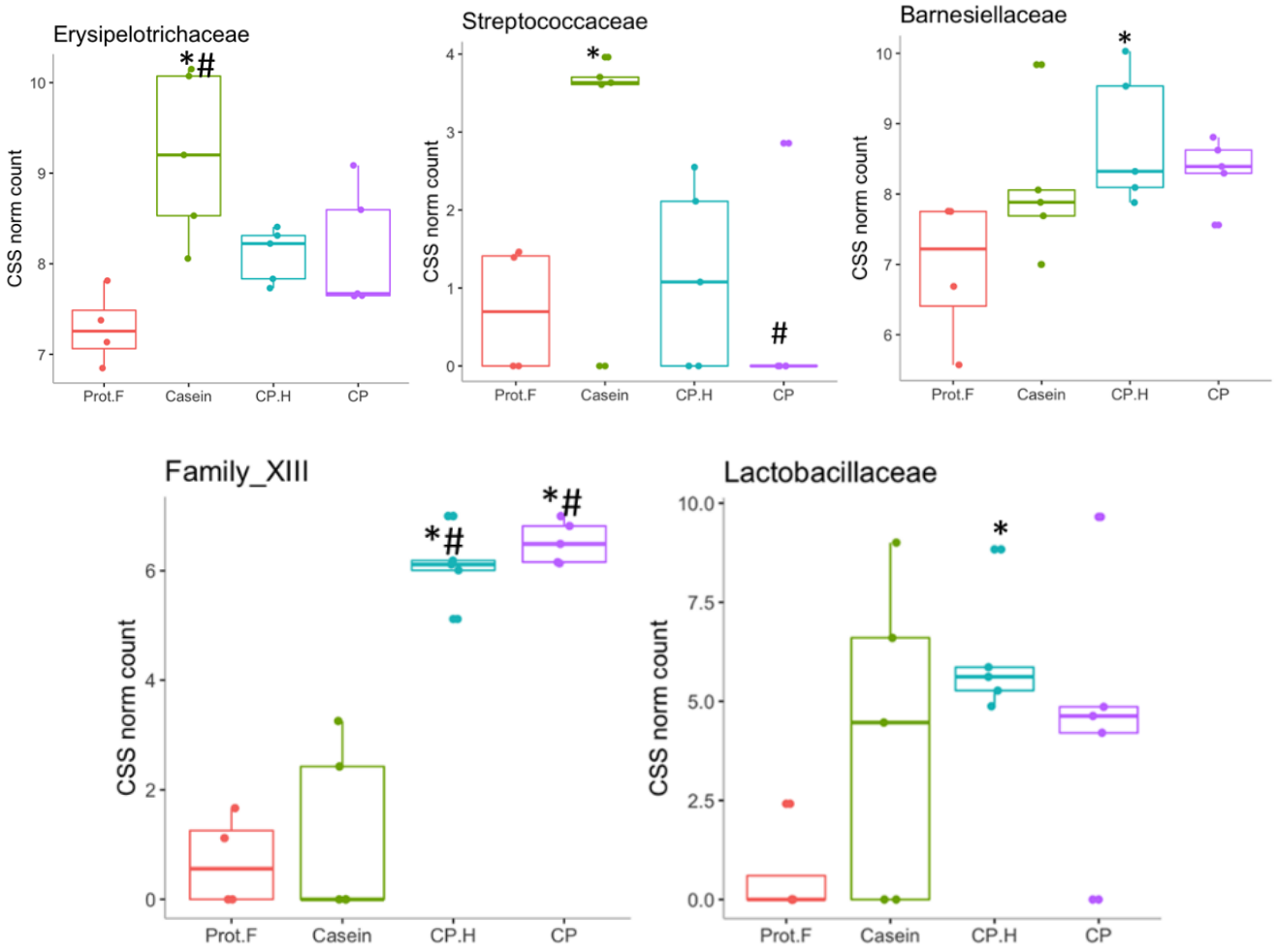
Erysipelotrichaceae
Streptococcaceae
Barnesiellaceae
Family_XIII
Lactobacillaceae
CSS norm count
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Casein
CP.H
CP

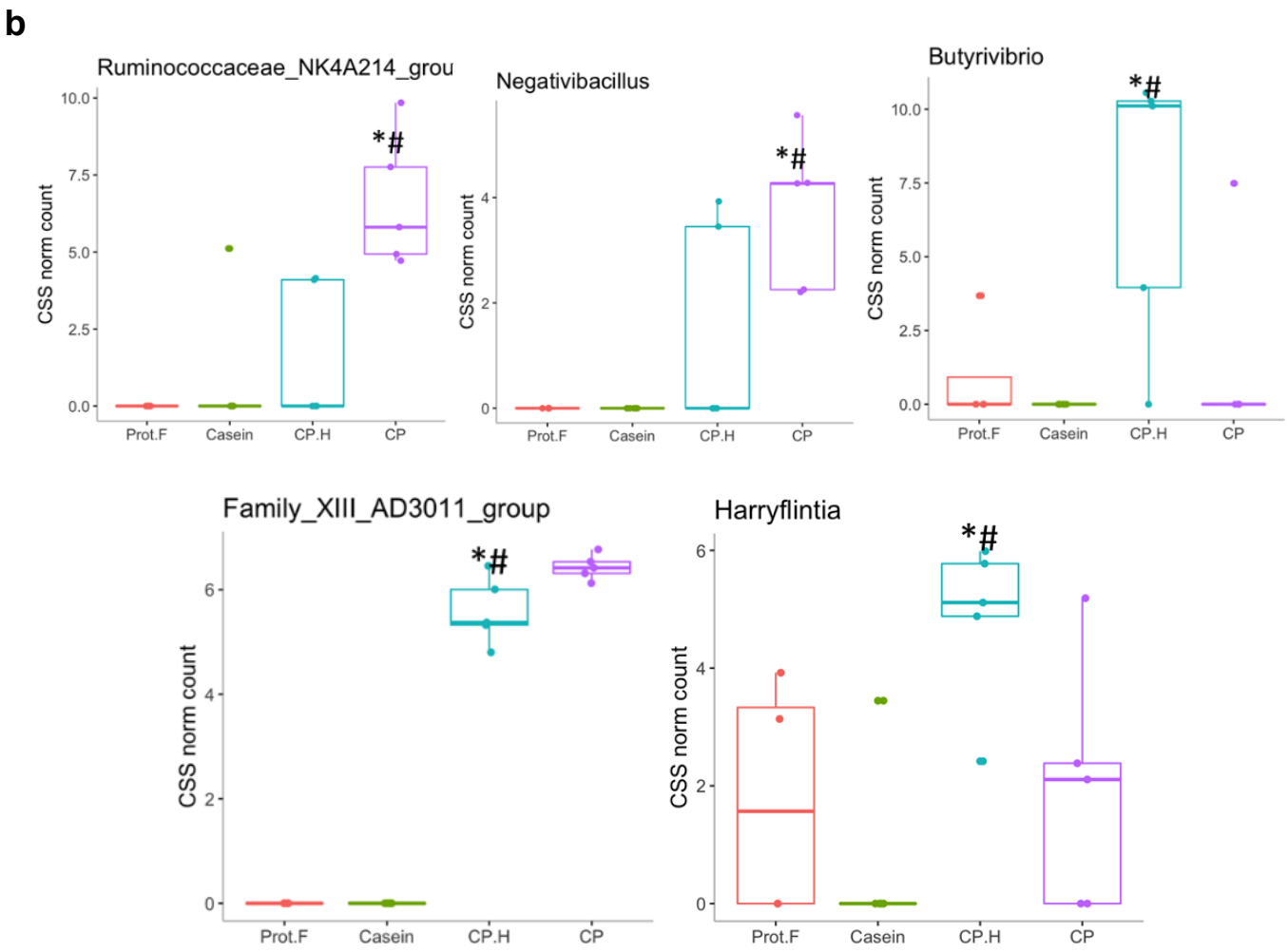


Fig.2. Assessment of differences in the family level (A) and in the genera level (B) of the gut microbiota of rats in receipt of different diets. Prot-F (red)-, Casein (green)-, CP.H (blue)-, and CP (purple)-fed animals. *P<0.1 denote statistically different groups compared to the Prot-F, #P<0.1 denote statistically Protein-specific changes. Prot.F, Protein free-based diet; Casein, casein-based control diet; CP.H, Cricket hydrolysates enriched fermented beverage; CP, Whole cricket powder enriched fermented beverage.

3.6. The Correlation between bacterial taxa and nutritional parameters

The association of gut microbiota with the nutritional parameters was determined and results are shown in Fig. 3. At the family level (Fig.3a), *Anaeroplasmataceae*, *Bacteroidaceae, Christensenellaceae,* and *Ruminococcaceae* were positively correlated with the weight gain (rho = 0.47, 0.42, 0.40 and 0.50 respectively), food intake (rho = 0.39, 0.41, 0.42 and 0.53 respectively), protein intake (rho = 0.38, 0.27, 0.38 and 0.54 respectively), and PER (rho = 0.47, 0.39, 0.43 and0.47 respectively). While, *Erysipelotrichaceae, Peptostreptococcaceae* and *Streptococcaceae*

were positively correlated with all the nutritional parameters as well as digestibility (rho = 0.45, 0.68, and 0.50 respectively), with the latter having a statistically insignificant correlation with protein intake. *Clostridiales*_vadinBB60_group showed a positive correlation with the protein intake (rho = 0.43) and a negative correlation with the apparent digestibility (rho = -0.43) while *Enterococcaceae* was negatively correlated with the protein intake (rho = -0.43). Conversely, Family_XIII, which was particularly changed with CP.H and CP-base diet, showed a decrease when the digestibility increased (rho = -0.60). The similar negative correlation was observed for *Bacillaceae* and *Clostridiales* vadinBB60 group with digestibility (rho = -0.44 and 0.43 respectively). Other bacteria did not show any correlation with nutritional parameters. Pozuelo et al. (2015) observed a high proportion of *Erysipelotrichaceae* in healthy and lean individuals. It was found to be related to the availability of butyrate. This suggests that a high protein diet increases the abundance of *Erysipelotrichaceae* which may increase the availability of butyrate. Hence the positive correlation between *Erysipelotrichaceae* and the food and protein intake.

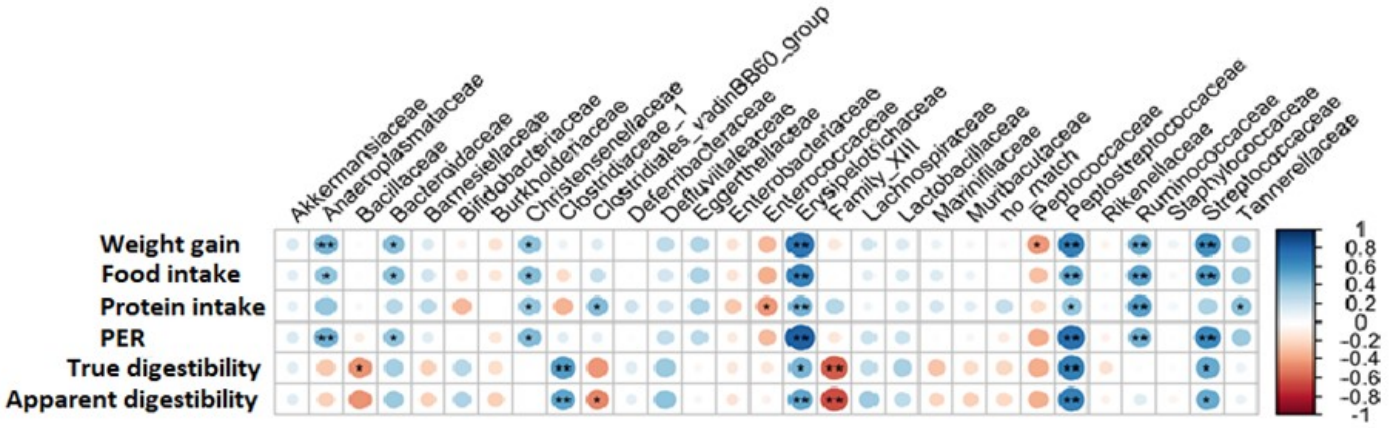
A study in humans (Säemann et al., 2000) showed an anti-inflammatory property of butyrate, which could be implicated in the regulation of immune responses. In addition, butyrate has been shown to be effective in suppressing cancer and treating mucosal inflammation in both human and animal models (Khempaka et al., 2011; McIntyre et al., 1993). Furthermore, Van der Wielen et al. (2000) reported that the increase of butyric acid could be related to the decrease of the amount of *Enterobacteriaceae*.

The positive correlation between *Rumincoccaceae* and the nutritional parameters reflected the importance of a protein-enriched diet for the protection of the intestinal microbiome. On the other hand, no correlation was observed between *Lactobacillaceae* abundance and the nutritional parameters mentioned above, despite the presence of these bacteria in the CP.H and CP-based diet.

At the genus level (Fig. 3B), we detected a dichotomy between genera with respect to their correlations with various parameters; while *Asteroleplasma*, *Erysipelatoclostridium*, *Lactococcus*, *Roseburia*, and the *Ruminococcaceae* genera *UCG-005* and *UCG-014* were all positively correlated with weight gain, food intake, protein intake and PER, but not digestibility, *Aldecreutzia*, *Anaerovorax*, *Faecalitalea*, *Family_XII_AD3011_group*, *GCA900066755*, *Negativibacillus*, *Peptococcus*, *Ruminoclostridium_9* and *Ruminococcaceae_NK4A214_group* were all negatively correlated with digestibility intake (rho = -0.58, -0.25, -0.59, -0.75, -0.43, -0.53, -0.54, -0.50 and -0.73 respectively). While we found that *Bifidobacterium* was negatively correlated with the protein intake (rho = -0.39). According to Stull et al. (2018), cricket-based diet could increase the

Bifidobacterium for humans. However, in our study the abundance did not differ between diets. Similar observations were observed by Jarett et al. (2019) who found that cricket-based diet did not affect this genera in the gut microbiome of dogs. The presence of chitin characterized by a similar structure to cellulose (Takegawa et al., 2010), could explain the high abundance of *Ruminococcaceae_NK4A214_group* in rats fed with the CP-based diet as well as the negative correlation with the digestibility as the CP showed a low digestibility compared to the casein and CP.H. *Erysipelatoclostridium*, is considered as an opportunistic pathogens (Auch et al., 2010; Gouret et al., 2009). It was observed to be lower for animals fed with CP.H and CP compared to those fed with casein and protein free-based diet. These results confirmed that the whole cricket protein or cricket protein hydrolysates may maintain a more balanced composition and reduce the pathogens within the gut bacteria. Other pathogens such as *Enterococcaceae*, showed a decrease when the weight gain, food intake, protein intake and digestibility increased (rho = -0.38, -0.39, -0.45, and -0.35 respectively) indicating the importance of a diet rich in protein for the protection of the gut microbiota against pathogens.

a



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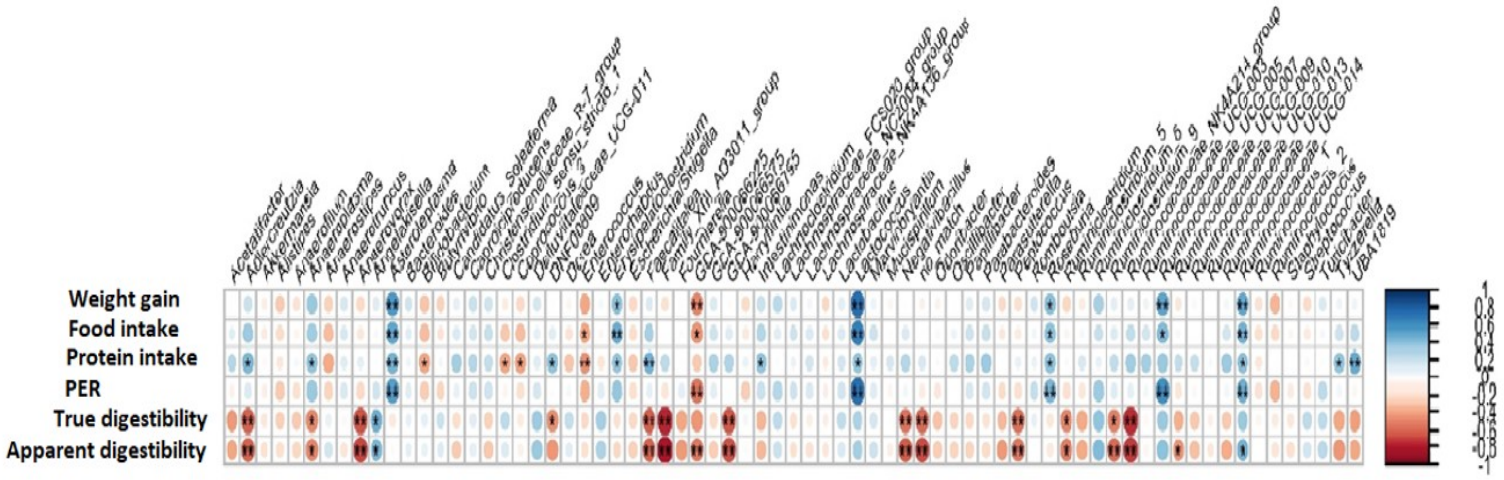

Fig.3. Associations between bacterial families (A), genera (B) and the nutritional quality. *Family /Genera showing statistically significant differences (P<0.1).

4. Conclusion

This study examined the nutritional quality of probiotic beverage enriched with cricket protein as native or pre-treated with enzymatic hydrolysis following ultrasound. The analysis of amino acid content showed that cricket protein benefits from a complete amino acid profile implicated in growth. An improvement of the growth parameters, food intake and PER was observed for the rats fed with cricket protein hydrolysates (CP.H) as compared to those fed with the whole cricket protein (CP). Overall, CP.H showed a high digestibility (94%) which was closed to the casein digestibility. Although improvement of the nutritional quality was observed, enrichment of fermented beverage with cricket protein hydrolysates did not bring a significant change in the gut microbiome when compared to CP-based diet, although some taxa specific differences between the cricket proteins were identified. Nonetheless, cricket protein could be an ingredient-based diet for the maintenance of a healthy and balanced gut microbiome and the reduction of the proportion of pathogenic bacteria. More research is however needed to further understand the beneficial effects of cricket protein hydrolysates with probiotics on other nutrition aspects.

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Conflicts of interest

The authors declare that there is no conflicts of interest in this study.

Ethical Approval

The protocol for the animal study was approved on December 20, 2018, by the National Experimental Biology Centre and the Institutional Animal Care Committee of the INRS Armand-Frappier Health and Biotechnology Research Centre, in accordance with the principles of the Canadian Council on Animal Care, (Protocol CIPA no 1809-04).

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Lien entre les chapitres 3 et 4

Le chapitre 3 a permis d'évaluer la qualité protéique des boissons CPH et CP, ainsi que leur effet sur le microbiote intestinal. Le chapitre 4 « **La stabilité au cours de l'entreposage d'une boisson probiotique fermentée enrichie en hydrolysats de protéines de criquet** » a pour objectif d'étudier les caractéristiques physicochimiques, nutritionnelles et microbiologiques du produit au cours de l'entreposage. Il a également permis de déterminer l'acceptabilité du produit par l'évaluation des propriétés sensorielles. Une évaluation de la résistance des probiotiques dans des conditions gastrointestinales *in vitro* a été effectuée afin de comprendre si la présence des protéines de criquet dans la boisson permet d'assurer la survie bactérienne au cours d'une simulation gastrointestinale.

CHAPITRE 4: STORAGE STABILITY OF A FERMENTED PROBIOTIC BEVERAGE ENRICHED WITH CRICKET PROTEIN HYDROLYSATES

Stabilité au cours de l'entreposage d'une boisson probiotique fermentée enrichie en hydrolysats des protéines de criquet

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Contribution des auteurs

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Chaima Dridi et les discussions sur les résultats et les protocoles ont été assistés par Mathieu Millette et Stéphane Salmieri. L'article a été écrit par Chaima Dridi, tandis que les corrections et révisions ont été effectuées par Mathieu Millette, Stéphane Salmieri, Blanca Aguilar et Monique Lacroix.

Résumé

Les protéines de criquet ont été hydrolysées et fermentées avec des bactéries probiotiques pour le développement d'une boisson nutraceutique enrichie à haute valeur nutritionnelle d'hydrolysats de protéines de criquet (CPH) et de probiotiques. La qualité physico-chimique, nutritionnelle et microbiologique de la boisson à base de CPH a été évaluée pendant 5 mois de stockage à 4°C. De plus, l'effet de l'enrichissement et de la fermentation de la boisson a été comparé à celui des boissons non enrichies et non fermentées. Les résultats ont montré que l'enrichissement en CPH augmentait la viscosité, réduisait le pH et augmentait l'acidité titrable et était accompagné du maintien d'une concentration élevée de probiotiques viables par rapport à la boisson non enrichie avec 8,45 log/mL après 5 mois. La fermentation a légèrement affecté les paramètres de couleur au cours du temps. De plus, le CPH fermenté a montré une teneur élevée en peptides de bas poids moléculaire (LMW) < 260 Da qui se sont formés en continu pendant le stockage pour atteindre un pourcentage de 74,7% contre 59,9% pour les non enrichis. De plus, les probiotiques ont présenté une grande résistance aux conditions gastro-intestinales *in vitro* principalement pour la boisson enrichie en CPH qui a montré un taux de survie de 83 % des probiotiques viables après 5 mois. L'analyse sensorielle des boissons a montré que la boisson à base de CPH était particulièrement appréciée pour sa texture et sa couleur, par rapport à son homologue non enrichi.

Mots-clés : Fermentation ; Période de stockage ; Hydrolysats de protéines de criquet ; Probiotiques ; Évaluation de la qualité ; Stabilité ;

Storage stability of a fermented probiotic beverage enriched with cricket protein hydrolysates

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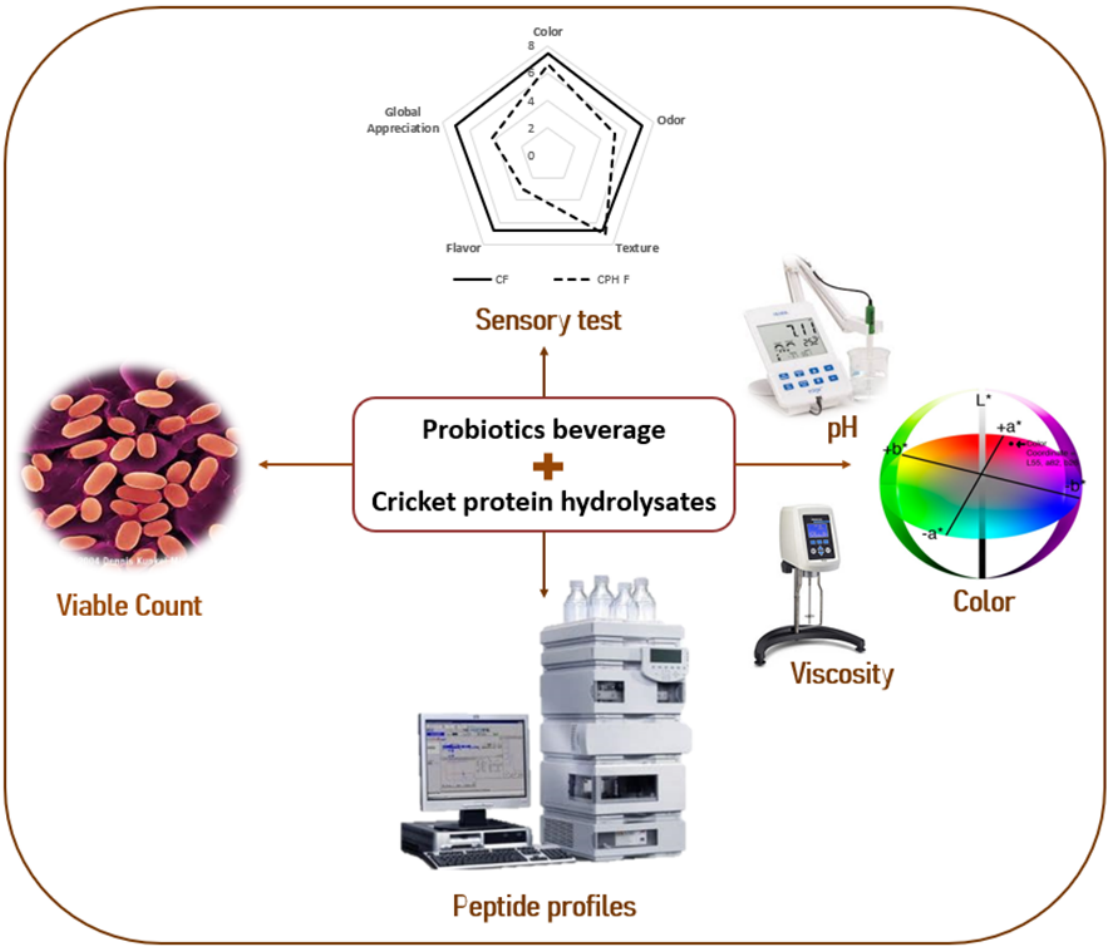

Graphical abstract

Abstract

Cricket proteins were hydrolyzed and fermented with probiotic bacteria for the development of a nutraceutical beverage enriched with high nutritional value of cricket protein hydrolysates (CPH) and probiotics. Physicochemical, nutritional and microbiological quality of the CPH-based beverage was evaluated during 5 months of storage at 4°C. Also, the effect of the beverage enrichment and fermentation was compared to non-enriched and non-fermented beverages. Results showed that the enrichment with CPH increased the viscosity, reduced the pH and increased the titratable acidity accompanied by the maintenance of a high concentration of viable probiotics com- pared to the non-enriched beverage with 8.45 log/mL after 5 months. Fermentation affected slightly the color parameters over time. Also, the fermented CPH showed a high content of low molecular weight (LMW) peptides < 260 Da which were continuously formed during storage to reach a percentage of 74.7% compared to 59.9% for the non-enriched ones. Moreover, probiotics presented a high resistance to the *in vitro* gastrointestinal conditions mainly for the CPH-enriched beverage which showed a survival rate of 83% of viable probiotics after 5 months. The sensory analysis of beverages showed that the CPH-based beverage was particularly appreciated for its texture and color, compared to its non-enriched counterpart.

Keywords: Fermentation; Storage period; Cricket protein hydrolysates; Probiotics; Quality evaluation; Stability;

1. Introduction

Functional foods are at the crossroad of foods which provide basic physiological functions, and drugs to treat diseases. They include bioactive peptides, oligosaccharides, organic acids, prebiotics, phytonutrients and other biologically active components (Cencic and Chingwaru, 2010). Probiotics are a part of the emerging generation of active ingredients. They are defined as living microorganisms, primarily bacteria which are safe for human consumption and which have health benefits beyond traditional nutritional effects, when ingested in adequate amounts (Hill et al., 2014). In addition to the health benefits (Didari, 2015; Michail and Kenche, 2011; Rafter et al., 2007), probiotics can produce enzymes which have been shown to improve protein bioavailability by increasing the production of free amino acids and may enhance the nutritional status of the host, especially if the host is deficient in endogenous protease production (Arora et al., 2010). In the other hand, fermentation using lactic acid bacteria (LAB) was demonstrated as an efficient method of improving food appearance, flavors and aroma (Sharma et al., 2020). Also, it could provide a significant improvement of the physical and chemical properties, enhance nutritional quality while increasing the shelf life of products (Şanlier et al., 2019).

The enrichment of probiotic-based products with proteins constitutes a new challenge to further improve the nutritional value and the effect of these products on health. Indeed, the addition of proteins could fill the deficiencies in certain essential nutrients especially for children and elderly who are malnourished or suffer from a lack of energy and muscle problem (Douglas et al., 2013). Furthermore, the enrichment of probiotics products was shown to enhance the growth of probiotics but it could also be considered as a procedure to improve the technological and sensory properties. For example, whey protein isolate and concentrate are the most used proteins for dairy product enrichment, they have a significant influence on the texture and fermentation process of the product (Morell et al., 2017; Nastaj et al., 2019). Other studies mentioned that the incorporation of proteins (Isanga and Zhang, 2009; Menéndez et al., 2006) could bring changes on the physicochemical properties of formulated products, impacting both their rheological and organoleptic characteristics and improve their functional properties. Due to the current state of the environment and in response to the growing consumer interest in a sustainable system with more economical and environmentally friendly food, plant proteins and other unconventional animal proteins have become more popular (Ainis et al., 2018; Alves and Tavares, 2019). Insect proteins are considered as one of the most interesting protein source for the future. It has many favorable attributes, such as a positive effect on the environment, high production yields at a very low cost compared to traditional sources (Huis, 2013), and a high protein and amino acids content (Makkar

et al., 2014). Indeed, insect proteins meet the World Health Organization (WHO) essential amino acid content requirements (Rumpold and Schlüter, 2013). However, the valorization of such proteins is still limited as they are characterized by a poor digestibility compared to other animal-based proteins due to the presence of chitin (Marono et al., 2015; Oibiokpa et al., 2018). Several processes applied on protein before incorporation can strongly affect their technological and nutritional properties, and their digestibility (Körzendörfer et al., 2019; Kuznetsova et al., n.d.). Among these procedures, enzymatic hydrolysis using proteases was used for the treatment of cricket proteins. It resulted in an increased yield of protein hydrolysates with high functional properties and suitable for food applications (Hall et al., 2017). Certainly, enzymatically manufactured protein hydrolysates have decreased allergenicity, increased adsorption due to their peptide size, and improved functional properties and *in vitro* digestibility (Li et al., 2013). Whereas, processing such as fermentation may increase amino acids amount, peptides release and the digestibility (Afify et al., 2012; Raveschot et al., 2018). On the other hand, the enrichment of probiotic products could have effects on several parameters related to the stability of the product, their acceptability from a sensory point of view and the improvement and maintenance of nutritional value, in particular the benefits provided by probiotics throughout the storage period.

Thus, the aim of this study was to develop a fermented enriched beverage with cricket protein hydrolysates (CPH) while evaluating physicochemical properties (pH, titratable acidity, viscosity and color), sensory preference (hedonic test), nutritional quality (peptide profiles), probiotics viability and resistance in gastrointestinal conditions during refrigerated storage. These are the most important parameters in fermented products via the analyses of fermented enriched beverage vs fermented non-enriched beverage counterparts.

2. Materials and Methods

2.1. Materials

The fermented beverage, contained *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus* (*Lactobacillus casei*) LBC80R and *Lacticaseibacillus* (*Lactobacillus*) *rhamnosus* CLR2, and the non-fermented beverage used within the framework were prepared by Bio-K+ (a Kerry company; Laval, QC, Canada). Organic Cricket flour (60% protein content) was produced by Nexxus Foods (Montreal, QC, Canada). Alcalase enzyme FG (from *Bacillus licheniformis* ≥ 2.4 Units/g of protein) was from Novozymes and supplied by IMCD (Quebec, QC, Canada).

2.2. Protein treatment

Combined treatment of ultrasound (US) assisted enzymatic hydrolysis was used for the preparation of cricket protein hydrolysates. The condition of the US pre-treatment and enzymatic hydrolysis were set according to a previous study carried out in our laboratory (Dridi et al., 2021). Briefly, US was carried out using a sonicator QSonica Q500 (model FB-505; Fisher Scientific, Ottawa, ON, Canada) and the operation was conducted in a batch mode. The optimized conditions were set up as follows: time of 15 min (pulsed mode: on-time 5 s and off-time 2 s), power of 500 W, frequency of 20 kHz and amplitude of 60%. Enzymatic hydrolysis was done after US pre-treatment, using alcalase enzyme at 55°C for 3 h under constant stirring with an enzyme/substrate (E:S) ratio of 1:10 (w/w). Following hydrolysis, the enzyme was inactivated at 95°C for 10 min, cooled down to room temperature and then centrifuged at 13,000× g for 20 min (Kechaou et al., 2009). The supernatant was collected and freeze-dried (Labconco Freezone® 2.5 L, model 7670521, Fisher Scientific) to obtain the enzymatic hydrolysate sample used subsequently for the enrichment of the beverages.

2.3. Beverage preparation

Beverages studied were: fermented and non-fermented non-enriched beverage (C F and C NF, respectively) as a control (commercial product Bio-K+™ Blueberry, 3% of rice proteins) and fermented and non-fermented enriched beverage with CPH (CPH F and CPH NF) (total protein content: 13%). Ingredients in powder form of Bio-K+™ Blueberry were hydrated with filtered water. The mixture was pasteurized at 90 ± 2°C for 60 s, packaged in 98 g pots, sealed, and then cooled to 37°C. Fermentation was carried out using the combination of three probiotics bacteria (*L. acidophilus* CL1285. *L. casei* LBC80R and *L. rhamnosus* CLR2) that were inoculated at 10^8 CFU/mL, incubated at 37 ± 1°C for 14 ± 2 h and cooled to 4°C.

2.4. Physicochemical Properties

pH and titratable acidity (TA). The pH of F and NF beverages was measured using a pH meter (Accumet®Basic AB15, Fisher Scientific, Ottawa, ON, Canada). The titratable acidity (TA) was determined by direct titration with NaOH 0.1 M using phenolphthalein indicator according to AOAC Official Method 942.15 (2000) and was expressed on 1.0 mL of 0.1 M NaOH being equivalent to 0.009 g lactic acid.

Color. The color evaluation of the F and NF enriched (CPH) and non-enriched beverages (C) were carried out using a Konica Minolta CR10 Plus Color reader CR 10 (Konica Minolta Sensing, Inc, Mahwah, NJ, USA). The measured parameters were: lightness L^* (from black = 0 to white = 100), the color saturation or chroma C^* (+: high color intensity; -: low color intensity), the hue angle h° (from red = 0° to green = 180°)—indicating color changes between a^* (green color) and the intersection of a^* and b^*, and the total color difference (ΔE) of the products from the beginning to the end of the storage.

Viscosity. The viscosity was measured using a Brookfield LVDV-II+ programmable viscometer (AMETEK Brookfield Engineering, Middleboro, MA, USA). Analyses were performed with samples of 16 mL by using a UL Adapter equipped with a YULA-15 spindle (entry code: 00) and mounted in "Closed tube" mode, at 4°C. Samples of non-enriched beverages were analyzed at 100 rpm CPH enriched beverages were analyzed at 50, 20, 5, 2.5 and 2 rpm during storage. Viscosity measurements were taken under laminar flow conditions within a satisfying range from 10 to 100% of torque. The measured absolute viscosity was expressed in centipoise (cP).

2.5. Molecular weight distribution by Size Exclusion-High Performance Liquid Chromatography (SEC-HPLC)

To determine the molecular weight (Mw) distribution during the storage period, all fermented and non-fermented beverages were centrifuged at 10,000 x *g* for 20 min. The supernatant was then recovered and filtered with a 0.2 µm filter and analyzed by SEC-HPLC. SEC-HPLC was performed using a HPLC System (Agilent Technologies 1260, Saint-Laurent, QC, Canada). The system was equipped with a C_{18} guard column and a size exclusion column (Biosep-SEC 2000 Phenomenex ®. 5 mm particle size, pore size 145 Å. 300 x 7.8 mm; Torrance, CA, USA). The volume of injection was 10 µL. The analysis was carried out at ambient temperature using a flow rate of 1 mL/min for 20 min. Detection was performed at 280 nm using a diode array detector (DAD). The Mw calibration curve was performed using standard proteins: Bovine thyroglobulin (670 kDa), IgA (300 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and uridine (244.2 Da). A 100 mM sodium phosphate buffer solution (pH 6.8) was used as the mobile phase. The chromatograms of peptides profile were separated into 3 ranges of Mw (> 3000 Da, HMW; 3000-260 Da, MMW; < 260 Da, LMW). Chromatograms were collected using OpenLAB ChemStation software (Rev.C.01.07 SR2 [255]), the baseline was manually corrected, and the total surface area of chromatograms was integrated and split into the three groups of Mw (HMW, MMW, LMW) using the function "Split manually integrated peaks" and expressed as a percentage of the total area

(Dridi et al., 2021). Analysis of peptide profiles were done on fermented and non-fermented control and CPH-based beverages every 21 days during storage at 4°C for 5 months (147 days).

2.6. Viable probiotics count during storage

The total viable count of probiotics contained in the enriched (CPH) and the non-enriched (C) beverages was evaluated. A quantity of 25 g of beverages was added to 225 mL of sterile peptone water. A serial dilution was carried out and then the required dilutions were inoculated into MRS agar (Alpha Biosciences Inc., Baltimore, MD, USA) and incubated 72 h at 37°C under anaerobic conditions (pour plating). The colonies were then enumerated, and the results were expressed as log CFU/mL of beverages.

2.7. *In vitro* gastrointestinal tolerance assessment

The viability of probiotics was carried out under *in vitro* simulated gastrointestinal conditions. The simulated gastric fluid (SGF) was made by dissolving 2.0 g of NaCl and 3.2 g of pepsin (356 U / mg) in 900 mL of distilled water and the pH was adjusted at 2.0 using HCl 1N according to Millette et al. (2013). The SGF solution was filtered through a 0.22 µm filter for sterilization. The simulated intestinal fluid (SIF) was prepared by supplementing the phosphate buffer solution with trypsin (1511 U / mg). The pH of the solution was adjusted to 8.0 and filtered through a 0.22 µm filter for sterilization (Guo et al., 2009).

A volume of 1 mL of probiotic beverages (C and CPH) was added to 24 mL of the SGF and then incubated at 37°C as recommend by USP Pharmacopeia (Moreton, 2015) for 120 min under stirring (200 rpm). At the end of the reaction time, 1 mL of SGF was transferred to 24 mL of the SIF and the mixture was incubated at 37°C for 3 h under stirring (200 rpm). A volume of 1 mL of SGF and or SIF was withdrawn then diluted in sterile peptone water, plated, incubated and enumerated as described above. The survival rate of probiotics was calculated based on the cell counts (log CFU/mL) at t = 0 (before GI passage), t = 2 h (after 2 h in SGF) and t = 5 h (after 3 h in SIF), according to the following formula:

$$\mathrm{SR}\ \% = \frac{\mathrm{Log\ CFU\ N}}{\mathrm{Log\ CFU\ N0}} * 100 \qquad (1)$$

where N_0 and N are the population numbers before and after the tests, respectively.

2.8. Sensory analysis

Sensory analysis of the fermented non-enriched beverage (control C F) vs the fermented CPH-enriched beverage (CPH F) was carried out by 15 panelists using a 9-point hedonic scale (1 = dislike extremely; 5 = neither like nor dislike; 9 = like extremely) according to a procedure by Ben Fadhel et al. (2016). For each panelist, 2 cups of beverages were served to evaluate color/appearance, smell, texture, flavor and global appreciation.

2.9. Statistical analysis

All treatments and analyzes were performed in triplicate (n = 3). The results were reported as mean values ± standard deviation. Statistical analysis was carried out using SPSS software version 22 (IBM Corp., Somers, NY, USA) and the significant differences ($P \leq 0.05$) between the means were evaluated using one-way analysis of variance (ANOVA). Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were applied for comparisons.

3. Results and discussion

3.1. pH and titratable acidity (TA)

The variations of pH and TA in fermented beverages during storage are presented in **Figure 1**. Results show that the pH values decreased significantly ($P \leq 0.05$) whereas the TA increased significantly ($P \leq 0.05$) throughout the storage period. However, measurements indicated low variations for both variables (variations < 1 unit of pH and < 0.5% of TA). Indeed, the pH of the non-enriched beverage (C F) varied from 4.35 to 3.73 after 21 days and became stable until the end (3.42 after 147 days). As for the CPH F- based beverage, the pH changed slightly with significant differences ($P \leq 0.05$), varying from 4.76 to 4.04 at the end of the storage time. The decrease in pH was more pronounced for the beverage C F, suggesting a buffering effect of the proteins (Playne and McDonald, 1966). A decrease of the pH was also reported by other authors for probiotic yogurt and fermented milk after 35 days of storage at 5°C (Mani-López et al., 2014a). A greater increase of TA was recorded for the CPH beverage. In fact, TA varied from 0.84 to 1.27% at the end of storage, while that of C F beverage varied from 0.13 to 0.30. From this, it can be assessed that fermented beverages underwent post-fermentation acidification, as evidenced by a decrease of pH during refrigerated storage. The post-fermentation acidification can be explained by the persistent metabolic activity of lactobacilli during storage at 4°C (Beal et al., 1999). In this regard, Akin and Ozcan, (2017) suggested that the fortification of fermented beverages with plant-based proteins such as gluten from wheat, peas and soybeans, made it

possible to increase the TA compared to the non-enriched fermented beverage. Also, Dabija et al. (2018) found that the addition of 4% of hemp proteins enhanced the acidity of yogurt compared to the non-enriched product. Although it was observed that acidity increased in all cases, with similar rates of pH decrease, the rate of TA increase in C F beverage was lower than in CPH F beverage. According to Wang et al. (2009), the pH and the TA are not necessarily related to each other, which could be explained by the fact that TA represents the total quantity of non-dissociated acids, while the pH corresponds to the proton concentration generated in the product (Tutu and Ciornea, 2011).

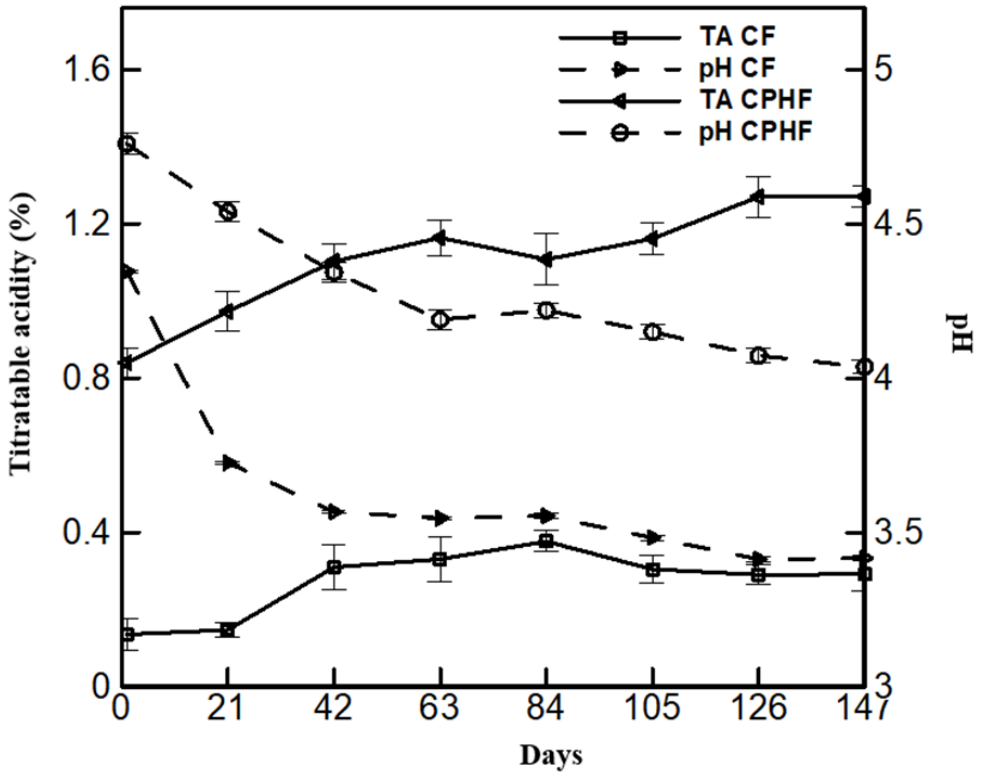

Figure 1. pH and titratable acidity (%) of fermented non-enriched beverage (control C F) and fermented beverage enriched with cricket protein hydrolysates (CPH F) during storage at 4°C for 5 months.

3.2. Viscosity

The effect of storage time on the viscosity of the non-fermented and fermented control beverages (C NF and C F respectively) and the non-fermented and fermented enriched beverages (CPH NF and CPH F respectively), was evaluated and results are presented in **Table 1**. A significant higher viscosity ($P \leq 0.05$) was recorded in CPH beverages compared to C beverages, with values of 2.3-2.9 cP for C beverages and values of 9.7-10.7 cP in CPH beverages, on day 0. Throughout

all storage, the viscosity of the C NF beverage increased significantly ($P \leq 0.05$) from 2.9 on day 0 to 4.5 cP on day 147. Similarly, the viscosity of the C F beverage increased from 2.3 to 3.3 cP. However, these variations of viscosity are not perceptible. On the other hand, the variations of viscosity in CPH beverages were much higher and corresponded to perceptible changes in terms of liquid texture. Indeed, the viscosity of the CPH NF beverage increased significantly ($P \leq 0.05$) from 10.7 on day 0 to 81.7 cP on day 147 while that of the CPH F beverage increased significantly ($P \leq 0.05$) from 9.7 to 19.5 cP. These higher increases of the viscosity during storage—especially for CPH NF—could be due to the interactions between some constituents such as protein-polysaccharide interactions (e.g. starch from rice and/or chitin from cricket) or protein-protein interactions (e.g. cricket and rice proteins) favored by the decrease in pH which could increase the water retention capacity (Soukoulis et al., 2007; Sun-Waterhouse et al., 2012). Thus, the weakening of the coagulum during storage could cause the release of water from the structure and the separation of the serum, which increased crosslinking between protein particles and structural resistance (Ozcan, 2013). Also, Hassan et al. (2012) reported that an increase in viscosity is generally related to an increase in the acidity. The increased viscosity is linked to an increase of dry matter (DM) content following the fortification of beverage with proteins. Indeed, beverage contained 15% DM when it was composed of 3% proteins (non-enriched C) and contained 27% DM when composed of 13% proteins (data not shown). In this context, Yadav et al. (2016) found that the apparent viscosity of a whey protein concentrate (WPC)-fortified beverage increased significantly with an increase of WPC content. On day 0, CPH F and CPH NF beverages had a very similar viscosity (9.7 and 10 cP, respectively). This observation could be explained by the effect of the enzymatic hydrolysis as a pre-treatment which reduced the particle size and improved the functional properties of cricket proteins, namely solubility, water-holding capacity, gelling capacity and other properties which have an effect on the viscosity of the product (León-López et al., 2020). However, the viscosity varied between CPH F and CPH NF beverages and became more pronounced at the end of storage (day 147) with a higher significant difference ($P \leq 0.05$) of 19.5 cP for CPH F compared to 81.7 cP for CPH NF beverage. This finding could be explained by the effect of the lactic acid bacteria (LAB) after fermentation, which play an important role in the hydrolysis of high Mw peptides into low Mw peptides, therefore resulting in a lower viscosity of F beverage compared to NF counterpart. Same observations were reported by Manus et al. (2021) for a fermented Bio-K+ beverage enriched with rice and pea proteins. Additionally, Gotcheva et al. (2001) observed that the viscosity of Boza, a traditional cereal-based Turkish beverage was decreased under the effect of the fermentation with LAB. We can also note, that after a considerable increase for the enriched beverages CPH F and CPH NF (271.9 and 396.3

cP, respectively) on day 84, the viscosity started to decline. This observation could be due to the fact that the protein gels are acid-sensitive. It is also important to mention that the acidity of NF beverage was increased during the storage (data not shown).

The acidification of the product could affect interactions between the polypeptide chains and lead successively to a water uptake inside the gel, a structure weakening and a decrease in viscosity (Bulatović et al., 2014). Furthermore, it is to be mentioned that the correlation between the viscosity and the syneresis is among the important negative aspects of a beverage selection by consumers (Folkenberg et al., 2006). Indeed, the increase in viscosity was found to be correlated with a strong protein gel that loses the ability to hold the water which is drained from proteins and appears on the surface of enriched beverages. Thus, the CPH-enriched beverage could have a less stable protein gel compared to non-enriched Bio-K+ beverage.

Table 1. Effect of the storage at 4°C on the viscosity (cP) of C NF, C F, CPH NF and CPH F beverages.

	Viscosity (cP)			
Days	C NF	C F	CPH NF	CPH F
0	2.95 ± 0.0^{aA}	2.32 ± 0.0^{aB}	10.7 ± 0.0^{aC}	9.70 ± 0.0^{aD}
21	3.21 ± 0.2^{bA}	3.82 ± 0.2^{dB}	22.2 ± 0.5^{bC}	19.6 ± 0.7^{bD}
42	2.18 ± 0.0^{cA}	3.53 ± 0.0^{cB}	117.4 ± 2.1^{cC}	50.0 ± 1.0^{dD}
63	3.93 ± 0.0^{dA}	3.40 ± 0.0^{bB}	113.4 ± 0.7^{cC}	81.4 ± 0.2^{eD}
84	4.95 ± 0.0^{eA}	4.23 ± 0.0^{fB}	396.3 ± 0.8^{dC}	271.9 ± 4.1^{fD}
105	5.40 ± 0.3^{fA}	4.04 ± 0.0^{eB}	193.6 ± 2.5^{eC}	19.7 ± 0.6^{bD}
126	5.27 ± 0.0^{fA}	3.95 ± 0.0^{eB}	181.0 ± 0.4^{fC}	42.3 ± 1.2^{cD}
147	4.46 ± 0.0^{gA}	3.30 ± 0.0^{bB}	81.7 ± 0.1^{cC}	19.5 ± 0.6^{bD}

Mean values with different lowercase letters within the same column are significantly different ($P \leq 0.05$). Mean values with different uppercase letters within the same row for the same base product are significantly different ($P \leq 0.05$). **C NF**: Non-fermented non-enriched beverage control. **C F**: Fermented non-enriched beverage control. **CPH NF**: Non-fermented enriched beverage with cricket protein hydrolysates. **CPH F**: Fermented enriched beverage with cricket protein hydrolysates.

3.3. Color

Color is an important attribute in food. It is the first characteristic perceived by consumers and therefore, it often influences consumer preference. The impact of protein enrichment and fermentation on the color of the tested beverages is shown in **Table 2**.

Lightness (L^*). The results show a decrease in the L* value (lightness) after the addition of proteins. Indeed, L^* decreased significantly ($P \leq 0.05$) from 64.4-68.2 for non-enriched beverages (C NF and C F) to 39.5-44.8 for enriched ones (CPH NF and CPH F) on day 0. This darkening result can be explained by the presence of particles—possibly agglomerated due to protein-polysaccharide and protein-protein interactions—obstructing the diffusion of light after the enrichment of beverage with protein hydrolysates. Inversely, the lightness of beverages was significantly increased ($P \leq 0.05$) by fermentation with L^* values on day 0 increasing in non-enriched beverages from 64.4 (C NF) to 68.2 (C F) and in enriched beverages, from 39.5 (CPH NF) to 44.8 (CPH F). This difference could be due to the role of LAB which ensure the degradation of particles during fermentation (Barbut, 2010). Regarding the effect of storage, lightness increased significantly ($P \leq 0.05$) from 64.4 to 67.4 in C NF beverage and from 44.8 to 47.4 in beverage CPH F, between days 0 and 147. On the other hand, no significant difference ($P > 0.05$) was observed in C F beverage (from 68.2 to 68.0) and a very slight reduction was observed with values decreasing from 39.5 to 38.2 in CPH NF beverage. Overall, these changes during storage may not be perceptible due to low variations of L^*.

Chroma (C^*). Chroma (color intensity) is the chromatic strength of a product color i.e. the perceived amount of difference from a grey of the same lightness. On day 0, the chroma of CPH NF was significantly ($P \leq 0.05$) lower than CPH F, whereas no significant difference ($P > 0.05$) of chroma was observed between the other sample groups (C NF and C F). Indeed, the color intensity of the CPH product was 17.9 and 11.9 (for CPH F and CPH NF, respectively) compared to a range 17.9-19.9 in the other groups. Similar findings were also obtained by da Costa et al. (2018) on the a kefir beverage produced from yam (*Colocasia esculenta L.*) and sesame seed (*Sesamum indicum L.*). The authors explained the increase of C^* by the synthesis of metabolites during the fermentation process. Regarding the effect of storage, no significant difference ($P > 0.05$) of chroma was observed overtime in each non-enriched beverage (C NF and C F). However, a more important and significant variation of chroma ($P \leq 0.05$) was determined in enriched beverages (CPH NF and CPH F), with values decreasing from 11.9 to 10.9 in CPH NF and increasing from 17.9 to 20.5 in CPH F, between days 0 and 147. Even if these variations are not perceptible, these results indicate the positive effect of fermentation on the color of enriched beverage during

storage, as the color intensity of the product was enhanced. A study of Pereira et al. (2013) also showed a positive effect of the fermentation of cashew apple juice on the color during storage when compared to the non-fermented one.

Hue angle (h°). The protein enrichment of the beverage induced a significant reduction of hue angle ($P \leq 0.05$) in the beverage. Indeed, on day 0, the hue angle was 112.9-113.4° in C NF and C F beverages whereas this value range was decreased to 93.4°in CPH NF and 82.6° in CPH F. In other words, not only the protein enrichment of the product had an effect on the hue, but also the fermentation process associated to enrichment strengthened an additional reduction of the hue in the beverage. As a result, in terms of hue interpretation into its 2 components a^* and b^*, the color of the control fermented beverage (C F) tended to yellow-green characteristic—mostly due to the color of the rice—whereas the fermented, enriched beverage (CPH F) was associated with an orange-khaki color—mostly due to a combination of the color of rice mixed with cricket proteins, but also the influence of fermentation. This variation of h° was mainly due to a larger variation of a^* (data not shown) that evolved from a slight green aspect to a slight red color component. The storage time did not induce any significant effect ($P > 0.05$) on the hue of each beverage group, suggesting a good stability of the products over time.

Total color difference (ΔE). The total change of color (ΔE) of beverages throughout storage was determined (data not shown) to evaluate the impact of the treatments (protein enrichment and fermentation) in comparison to control sample (ΔE = 0), on each day of analysis. The results showed a ΔE > 5, indicating very noticeable visual changes in the Bio-K + beverage following the enrichment with CPH.

The total color difference ΔE was also measured for a same beverage between day 0 and day 147 (**Table 2**). Low variations of ΔE (< 3) did not implicate any noticeable visual changes of color during time in each group, such conditions being supported by other authors (Adekunte et al., 2010; Vichi et al., 2004). This result of total color stability throughout storage may be considered a good result, which can guarantee the acceptability of the product by the consumer during the storage period whether it is the Bio-K+ beverage or the CPH-enriched beverage.

Table 2. Effect of the storage at 4°C on the color of C NF, C F, CPH NF and CPH F beverages.

Days		C NF	C F	CPH NF	CPH F
0	L*	64.4 ± 0.6^{aA}	68.2 ± 0.6^{aB}	39.5 ± 0.3^{aC}	44.8 ± 0.2^{aD}
	C*	18.7 ± 0.2^{aA}	19.9 ± 0.5^{abA}	11.9 ± 0.1^{aC}	17.9 ± 0.3^{aAB}
	h°	112.9 ± 1.1^{abA}	113.4 ± 0.4^{abA}	93.4 ± 0.4^{aB}	82.6 ± 0.7^{aC}
21	L*	63.9 ± 0.0^{aA}	67.6 ± 0.1^{aB}	39.5 ± 0.3^{aC}	44.9 ± 0.2^{aD}
	C*	19.4 ± 0.0^{abA}	21.0 ± 0.0^{aA}	11.6 ± 0.1^{aC}	19.1 ± 0.2^{bAB}
	h°	111.6 ± 0.3^{aA}	108.9 ± 0.7^{aB}	94.8 ± 0.1^{abC}	82.6 ± 0.7^{aD}
42	L*	63.7 ± 0.8^{bA}	67.7 ± 0.1^{aB}	38.9 ± 0.0^{abC}	46.0 ± 0.0^{bD}
	C*	19.2 ± 0.6^{abA}	19.4 ± 0.7^{bB}	11.2 ± 1.7^{bC}	19.4 ± 0.1^{bcB}
	h°	123.8 ± 2.4^{cA}	116.1 ± 2.1^{bB}	95.2 ± 0.1^{abc}	81.9 ± 0.0^{abD}
63	L*	64.9 ± 0.6^{aA}	68.1 ± 0.1^{aB}	38.4 ± 0.1^{bcC}	46.1 ± 0.0^{bcD}
	C*	19.5 ± 0.2^{abA}	20.1 ± 0.0^{abB}	10.9 ± 0.1^{bcC}	19.5 ± 0.0^{bcA}
	h°	110.8 ± 0.6^{aA}	113.2 ± 0.6^{abB}	96.2 ± 0.3 ^{aC}	81.9 ± 0.2 ^{abD}
84	L*	66.9 ± 0.0^{dA}	68.0 ± 0.5^{aB}	38.2 ± 0.0^{cC}	46.8 ± 0.1^{cD}
	C*	20.2 ± 0.3^{abA}	19.4 ± 0.6^{bAB}	10.6 ± 0.1^{cC}	19.6 ± 0.0^{bcA}
	h°	111.7 ± 1.3^{aA}	116.2 ± 2.0^{bB}	96.7 ± 0.5^{bC}	81.9 ± 0.2^{aD}
105	L*	66.3 ± 0.1^{cA}	68.0 ± 0.4^{aA}	38.4 ± 0.0^{bcB}	46.4 ± 0.3^{bcC}
	C*	21.1 ± 0.1^{bA}	19.0 ± 0.8^{bB}	10.9 ± 0.0^{bcC}	20.1 ± 0.2^{cD}
	h°	105.9 ± 0.3^{abA}	116.5 ± 0.6^{bB}	96.0 ± 0.0^{bC}	80.4 ± 0.3^{abD}
126	L*	67.4 ± 0.4^{cA}	68.9 ± 0.8^{aA}	38.2 ± 0.0^{cB}	47.0 ± 0.1^{cC}
	C*	19.4 ± 0.8^{aA}	19.9 ± 0.0^{abA}	10.7 ± 0.0^{cB}	20.1 ± 0.1^{cA}
	h°	114.8 ± 0.7^{abA}	115.1 ± 0.6^{bA}	96.1 ± 0.2^{bB}	81.7 ± 0.1^{abC}
147	L*	67.4 ± 0.2^{cA}	68.0 ± 0.3^{aB}	38.2 ± 0.1^{cC}	47.4 ± 0.0^{cD}
	C*	19.4 ± 0.3^{abA}	19.6 ± 0.8^{abAB}	10.9 ± 0.2^{cC}	20.5 ± 0.0^{cB}
	h°	116.7 ± 0.8^{bA}	115.9 ± 2.2^{bA}	95.4 ± 0.2 ^{bB}	81.6 ± 0.2^{abC}
ΔE		2.5± 1.0^{A}	2.1± 0.5 ^{A}	1.7± 0.1 ^{B}	2.7± 0.0 ^{A}

Means with different lowercase letters in the same column are significantly different ($P \leq 0.05$). The means with different capital letters in the same row for the same commodity are significantly different ($P \leq 0.05$). C NF: Non-fermented non-enriched beverage control. C F: Fermented non-enriched beverage control. CPH NF: Non-fermented enriched beverage with cricket protein hydrolysates. CPH F: Fermented enriched beverage with cricket protein hydrolysates. L*: lightness, C*: chroma, h°: hue angle.

3.4. Variation of peptides profiles

The variation of the peptide profiles of (C NF), (C F), (CPH NF) and (CPH F) beverages during refrigerated storage was studied. Results are given by groups of Mw (**Figure 2**): peptides of high Mw (HMW > 3000 Da), medium Mw (3000 Da < MMW < 260 Da) and low Mw (LMW < 260 Da). The protein enriched beverages (CPH NF and CPH F) showed a significant higher increase ($P \leq 0.05$) in the percentage of LMW peptides after 5 months of storage compared to non-enriched products. The levels of LMW varied from 62.0 to 73.5 % in CPH NF and from 65.1 to 74.7% in CPH F. This continuous formation of LMW peptides occurred to the detriment of a continuous decrease MMW peptides as the level of HMW remained constant (low levels of 3.9-3.1 % in CPH NF and 1.5-1.0% in CPH F). The level of MMW peptides decreased from 34.0 to 23.4 % in CPH NF and from 33.4 to 24.2 % in CPH F beverage. Therefore, the enriched products exhibited similar profiles of peptide conversion. This could be explained by the persistence of the proteolytic activity of LAB during storage which allowed the release of peptides, amino acids and other important metabolites (Donkor et al., 2007). Donkor et al. (2006) also found that milk proteins fermented with *L. acidophilus* L10, *B. lactis* B94, *L. paracasei* L26 were hydrolyzed by extracellular proteases produced by LAB, resulting in an increase of free amino acid during storage. Another study by Undhad et al. (2021) showed an enhancement of *Angiotensin converting enzyme* ACE inhibitory, antioxidative activities of a fermented soy-based beverage after 15 days of storage. Also, fermentation exerted a significant additional effect ($P \leq 0.05$) on the reduction of HMW peptides effect in CPH beverage compared to non-enriched C beverage. This additional effect made it possible to obtain a higher % of LMW peptides, more assimilable i.e more absorbable by the intestines and therefore a more digestible product (Ogodo et al., 2018; Pranoto et al., 2013).

Contrary to the enriched (CPH) beverage, the non-enriched one (C) did not generate as many LMW peptides during storage. Moreover, the C NF beverage contained a low % of MMW and HMW peptides due to a continuous significant increase ($P \leq 0.05$) of HMW peptides (from 2.7 to 15.3%) after 5 months. This observation could be explained by a susceptibility to the agglomeration of peptides at 4°C which promotes peptide polymerization and gelation. In comparison, a more stable profile of LMW peptides was observed in all other beverage groups with low levels of LMW peptides in C NF, (53 %) when compared to C F (60 %). Here again, fermentation was mainly responsible for the conversion of HMW peptides into MMW and LMW counterparts.

Furthermore, the release of peptides is related to the viability of LAB that maintain a proteolytic activity during storage, which was tested in this study (section 3.5). This could explain the difference of release percentage of LMW peptides between C F and CPH F. Hence, the results indicate that the protein fortification with CPH of a beverage fermented in the presence of Bio-K+ probiotic bacteria, has the potential to produce health-promoting bioactive MMW and LMW peptides which could contribute to antioxidant, antimicrobial and antihypertensive activities. Thereby, the characterization of these peptides and the determination of their biological activities should be carried out in future studies.

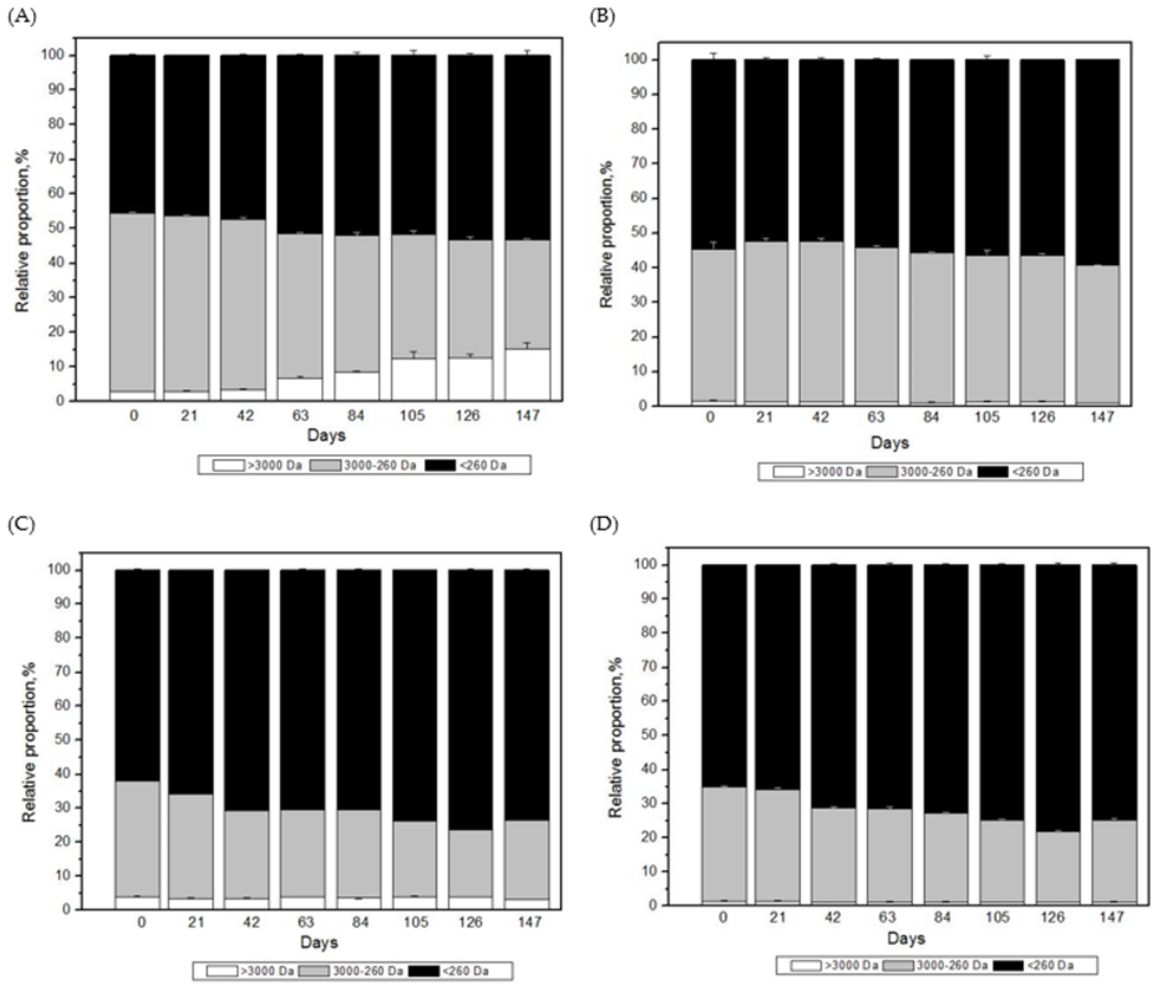

Figure 2. Molecular weight distribution of non-fermented non-enriched (C NF) (A), fermented non-enriched (C F) (B), non-fermented cricket protein hydrolysates-enriched (CPH NF) (C) and fermented cricket protein hydrolysates-enriched (CPH F) (D) beverages during storage at 4°C for 5 months.

3.5. Microbial viability of probiotics during storage

The variation in LAB concentration during storage for the fermented, non-enriched beverage (C F) and enriched beverage (CPH F) is presented in **Figure 3**. Results show a significant decrease ($P \leq 0.05$) in viable cells for C F (from 8.8 Log CFU/mL on day 0 to 7.3 Log CFU/mL, i.e. 1.5-Log reduction) after 5 months. In comparison, a similar significant decrease ($P \leq 0.05$) of cells was observed in CPH F beverage (from 9.5 to 8.5 Log CFU/mL, i.e. 1-Log reduction), but with a significant higher absolute number of LAB population. This observation could be due to the role of the added proteins which ensure the bacterial growth and maintain a favorable and adequate environment of LAB throughout the storage time (Felix da Silva et al., 2017). Similarly, Zhao et al. (2006) reported that the incorporation of casein hydrolysates increased the probiotic counts during the initial stage of fermentation and delayed the decline of probiotic organism counts during storage. Other studies (Dave and Shah, 1998; Sodini et al., 2002) found that milk supplementation with peptides and amino acids may also increase the viability of probiotic organisms. CPH constitute a source of essential amino acids, namely tryptophan and cysteine for bacteria growth (Dave and Shah, 1998). In this sense, it is evidenced that the supplementation of Bio-K+ beverage with CPH affected positively the survival of the LAB strains and their acidifying activity during storage.

These results are in accordance with those of pH and TA measured in **Figure 1**. Indeed, the LAB viability of the 2 beverages decreased slowly at the beginning of storage as pH decreased and TA increased. This may be due to the cessation of LAB metabolic activity after a long time of storage or could be explained by the secretion of inhibitory metabolites—such as bacteriocins—produced by ferments and which could affect their viability (Mani-López et al., 2014). Similar reports observed a loss of probiotics counts (*L. acidophilus*) in yogurt after 28 days of storage (Damin et al., 2008). Despite this LAB declining, C F and CPH F beverages remained at the level recommended by FAO/WHO (2002) (counts $\geq 10^7$ CFU/mL) to be considered as functional products with a beneficial effect from probiotics.

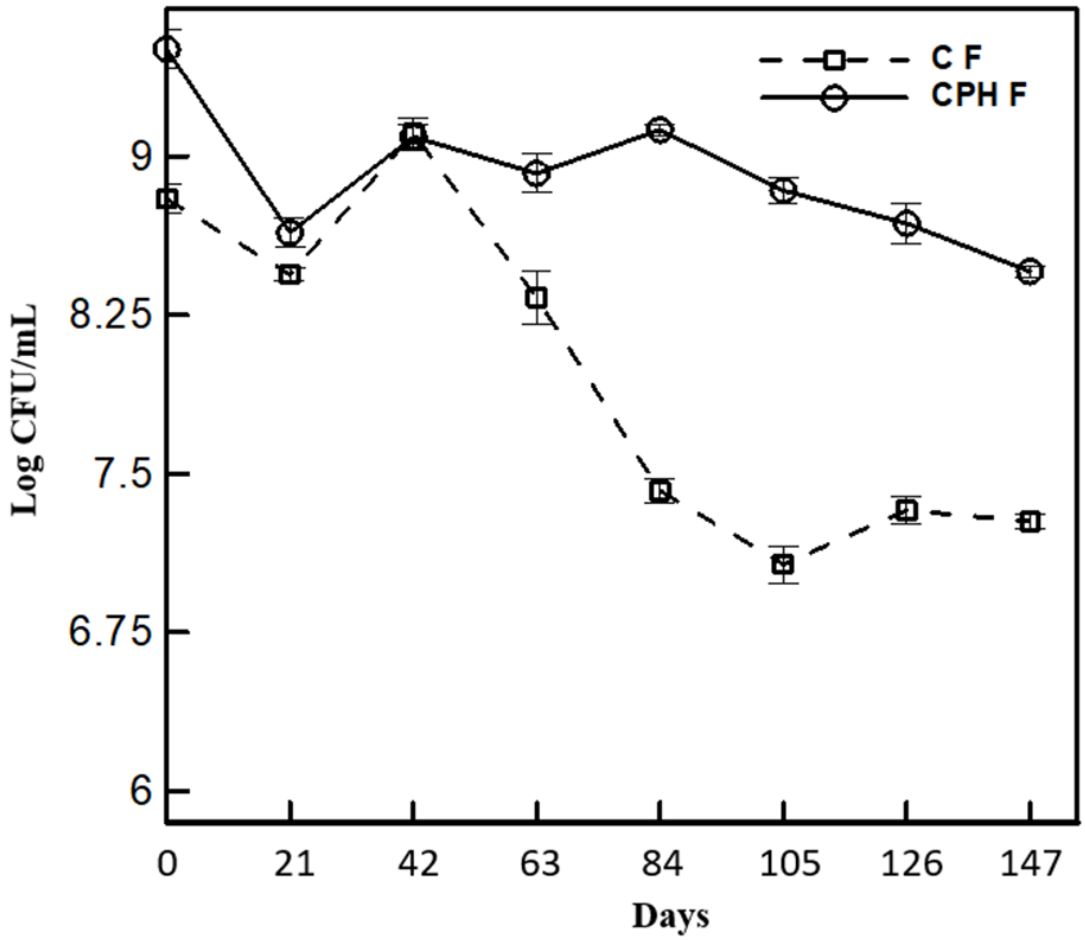

Figure 3. Concentration of Lactobacillus sp. of fermented non-enriched (CF) and fermented cricket protein hydrolysates-enriched (CRH F) beverages during storage at 4°C for 5 months.

3.6. *In vitro* resistance of probiotics under simulated gastrointestinal conditions

The results of the assay on the survival of LAB under simulated gastrointestinal conditions was carried out over storage (from 0 to 147 days) are displayed in **Table 3**.

Gastric phase. Data highlight no significant decrease ($P > 0.05$) of bacterial survival rate (SR, %) in the fermented, control (C F) and fortified (CPH) beverages after gastric phase and all along storage. Otherwise, it is interesting to note that SR values increased at mid-storage before decreasing slowly in both beverages, with averages of 89.7% in C F product on day 63 and 108.5% in CPH F on day 84. Hence, a more important increase of SR in gastric fluid was observed in CPH F beverage compared to C F. This higher resistance could be due to the intrinsic characteristics of LAB which develop mechanisms of resistance—and adaptation—to acidic environments such as the maintenance of intracellular pH and cell membrane functionality (de Souza et al., 2019).

Hence, these results reported the protective effect of the CPH in a fermented beverage. The presence of a structured protein matrix like CPH could positively affect the probiotic survival through the storage duration as compared to the non-enriched product.

Intestinal phase. Regarding the intestinal conditions, significant differences ($P \leq 0.05$) were noted after intestinal phase and during storage for the 2 beverages. The SR of probiotics under these intestinal conditions was > 90% in the beverages from day 0 to day 21 and then, this percentage decreased more significantly ($P \leq 0.05$) to reach values of 50.9 and 83.4% for C F and CPH F respectively, on day 147. Besides, SR values decreased at mid-storage before increasing slowly in both beverages, with averages of 29.4% in C F product on day 84 and 74.0% in CPH F on day 63. Hence, a more severe decrease of SR in intestinal fluid was observed in CF beverage compared to CPH. This finding maybe explained by a division or weakening of bacterial cell wall after mid-storage followed by a recovery of bacteria cell, resulting in an increased viability and resistance capacity thereafter. As observed in the gastric phase, the effect of the intestinal conditions was more intense in the C F beverage than in the CPH-based beverage all along storage.

Gastric conditions constitute a defense mechanism against pathogenic microorganisms, also becoming the first barrier to be overcome by probiotic cultures (Martins et al., 2018). Our results show a good resistance of the probiotics, under gastric conditions, when present in the non-enriched (C F) beverage. The decrease in SR during the intestinal phase could be due to a weakening of the defense mechanisms of LAB during storage. Indeed, a strain showing a SR value < 50% indicates a high sensitivity to gastric juice (HCl, pepsin) (Martins et al., 2018). Therefore, it can be assumed that the protein fortification of the beverage was able to provide protection to LAB in gastric fluid to maintain a high SR under the total gastrointestinal transit throughout the whole storage period. Millette et al. (2013) demonstrated that the Bio-K+ probiotic formulation can resist under gastric conditions at pH 2.0, which is in agreement with our results. To improve the SR of LAB under the gastrointestinal transit in long-term storage, the development of an adequate technology is recommended in various research studies. Encapsulation is one of the effective solutions (And and Kailasapathy, 2005; Millette et al.,2013) but our results show that protein fortification may be a new alternative to ensure the survival of probiotics under stressful gastrointestinal tract conditions, even after 5 months of storage.

Table 3. Viability of probiotics during exposure to simulate gastric and intestinal fluid.

Days	Gastric phase (pH=2.0)		Intestinal phase (pH=8.0)	
	C F	CPH F	C F	CPH F
	Survival rate (SR) %			
0	74.8 ± 0.3^{cB}	97.9 ± 1.2^{aA}	93.6 ± 1.4^{aB}	97.4 ± 1.0^{aA}
21	84.9 ± 1.4^{bA}	91.1 ± 1.0^{bA}	90.6 ± 0.3^{aA}	93.1 ± 1.2^{bA}
42	69.2 ± 0.6^{dB}	98.0 ± 0.3^{aA}	58.2 ± 0.3^{cB}	79.9 ± 0.6^{cA}
63	89.7 ± 0.6^{aB}	99.4 ± 1.3^{aA}	65.0 ± 0.7^{bA}	74.0 ± 1.8^{dA}
84	62.0 ± 1.1^{eB}	108.5 ± 0.2^{cB}	29.4 ± 0.8^{eB}	75.8 ± 0.1^{dA}
126	71.4 ± 1.9^{cdB}	96.0 ± 1.1^{aA}	55.3 ± 0.3^{cdB}	82.3 ± 1.7^{cA}
147	72.0 ± 0.9^{cdB}	97.4 ± 2.3^{aA}	50.9 ± 1.8^{dB}	83.4 ± 1.8^{cA}

Means with different lowercase letters in the same column are significantly different ($P \leq 0.05$). The means with different capital letters in the same row for the same commodity are significantly different ($P \leq 0.05$). C F: Fermented non-enriched beverage control. CPH F: Fermented enriched beverage with cricket protein hydrolysates.

3.6. Sensory attributes

Results of sensory evaluation related to the color, odor, texture, flavor and global appreciation of C F and CPH F beverages are presented in **Figure 4**. Samples were ranked from extremely like (9) to extremely dislike (1) hedonic evaluations. Overall, the results showed that the enriched beverage CPH F was comparable to the control beverage C F and accepted in terms of color (6.7 and 7.4 respectively) and texture (7.1 and 6.7 respectively) with no significant difference ($P > 0.05$). However, significant differences ($P \leq 0.05$) were noticed when comparing odor, flavor and global appreciation. These attributes were ranked as significantly less acceptable ($P \leq 0.05$) for CPH F in comparison with C F, with respective values of 5.1 and 7.1 in flavor, 3.0 and 6.7 in flavor; 4.3 and 7.1 in global appreciation. The CPH F beverage was characterized by an intense acidity flavor, a very pronounced bitterness and a cooking taste which reduced the product score. The intense acidity may be due to the protein-probiotics interaction. Indeed, the addition of proteins was reported to increase the growth of probiotics, which in turn increased the acid production

(Felix da Silva et al., 2017). In fact, the protein enrichment of a fermented product increases the productivity of bacteria in organic acids (Lucas et al., 2004). As for the cooking taste, the cricket powder was obtained by roasting process which led to the development of additional aromas. For the bitterness, other searchers found that enzymatic hydrolysis resulted in strongly hydrophobic bitter LMW peptides and generated more aromatic amino acids (Kukman et al., 1995; Molina et al., 1999). Thus, the presence of different flavors in a same product can give it an unpleasant, pungent taste.

These results show that the protein enrichment processing had a detrimental effect on the organoleptic quality of the beverage, in particular odor and flavor. From that, an improvement in odor/flavor will be necessary to make the product more acceptable to the consumer, for example by using aroma or flavor masking agents. Likewise, an optimization of treatment processes (hydrolysis parameters) and/or of the recipe (protein content in the beverage) may be required for better sensory properties.

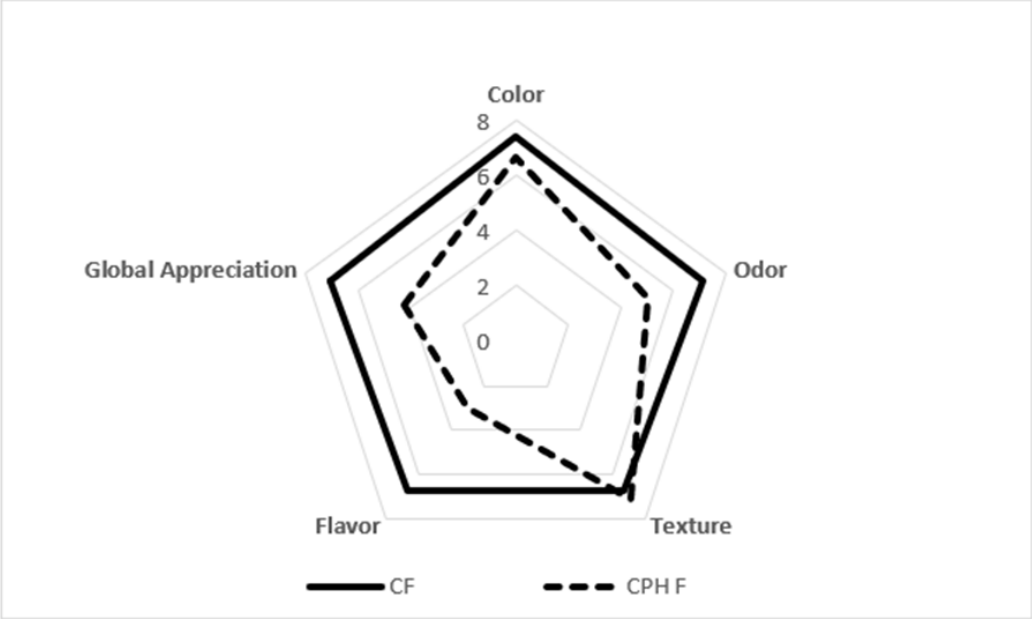

Figure 4. Sensory profile of fermented non-enriched (CF) and fermented, enriched beverage with cricket protein hydrolysates (CPH F) beverages.

4. Conclusion

This study demonstrated that CPH combined with probiotics, can improve the physicochemical properties and nutritional value of the beverage while guaranteeing a high rate of survival of probiotics. Our results showed that the addition of CPH increased the lactic acid concentration and decreased the pH—meaning a high productivity of acid by probiotics, due to the presence of CPH when compared to C F-beverage. The viscosity of beverage was affected with the storage period and increased especially for the enriched beverage. In addition, the fermentation showed a positive impact by the reducing of particles resulting in lower viscosity. The results of colorimetry suggested that storage duration had no effect on color parameters (L*, C*, hue, and ΔE) of enriched and non-enriched beverages as ΔE < 3 did not indicate noticeable visual changes in beverages. Therefore, the fermentation provided improvements to parameters such as increased lightness (L^*) and color intensity (C^*). The evaluation of peptide profiles during storage showed a greater release of LMW peptides after 5 months, especially for the CPH F beverage. Ferments used for the beverage's fermentation (*L. acidophilus* CL1285. *L. casei* LBC80R and *L. rhamnosus* CLR2) showed a good survival during 5 months of storage, especially after the CPH enrichment. The CPH F beverage was a better growth stimulant of probiotics and improved the resistance of LAB under simulated gastrointestinal conditions compared to the non-enriched beverage (C F). However, further improvement of odor/flavor is mandatory to make the product more acceptable for commercialization.

Author Contributions

Conceptualization, M.L.; methodology, C.D.; software, C.D. and S.S.; validation, C.D., M.M. and M.L.; formal analysis, C.D.; investigation, C.D.; resources, C.D.; data curation, C.D., M.M., S.S. and M.L.; writing—original draft preparation, C.D.; writing—review and editing, C.D., M.M., B.A., S.S. and M.L.; visualization, C.D; supervision, M.L.; project administration, M.L.; funding acquisition, M.L.

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Conflicts of Interest: The authors declare no conflicts of interest.

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CHAPITRE 5 : DISCUSSION GÉNÉRALE

1. Sélection de procédés

Le but de cette étude est d'élaborer une boisson nutraceutique qui regroupe les bienfaits des probiotiques et des protéines.

Dans la perspective de mettre en place un système alimentaire durable, les protéines de criquet ont été sélectionnées parce qu'elle sont une source protéique non-conventionnelle, facile à produire, bénéfique pour l'environnement et de bonne qualité nutritionnelle (Nongonierma and FitzGerald, 2017). Toutefois, la présence de chitine limite sa digestibilité.

L'objectif du chapitre 2 était de sélectionner un procédé pertinent pour améliorer les propriétés fonctionnelles et structurelles des protéines de criquet avant incorporation dans la boisson probiotique pour améliorer la disponibilité et la digestibilité de ses protéines et donc la valeur nutritive de la boisson.

Les traitements de la poudre de criquet avec IR (3 kGy), US (20 min) et US-IR ont permis d'augmenter la solubilité des protéines et d'entrainer des modifications de la structure primaire et secondaire tout en augmentant la teneur en liaison et en surface sulfhydryle (SH). Ainsi, le traitement combiné US-IR a montré que le pourcentage de solubilité est plus élevé (~30%) par rapport à l'échantillon non traité (11%). Cette observation pourrait être due à la destruction de la liaison chitine-protéine et/ou à des modifications de la structure tridimensionnelle des protéines, associées à des interactions entre la surface hydrophobe et hydrophile tout en entraînant des forces électrostatiques plus élevées qui augmentent les interactions protéine-eau, et donc la solubilité (Amiri et al., 2018; Yu et al., 2020). Ainsi, l'exposition des groupes SH à la surface externe des protéines pourrait être expliquée par le dépliement des protéines et la rupture des liaisons disulfure et hydrogène sous l'effet des procédés de prétraitement, conduisant à une augmentation de la teneur totale en SH (Fombang et al., 2005). Également, l'analyse via le spectre FT-IR de la structure primaire et secondaire des protéines de criquet après traitement a montré que US-IR a modifié la conformation des protéines par la diminution d'hélice- α et des feuilles- β et une augmentation de pelote aléatoire d'une manière attribuée à la libération des peptides suite à la dégradation des protéines.

D’autre part, les protéines de criquet traitées avec US-IR ont engendré la plus forte intensité au niveau des liaisons -OH et -NH, mais aussi des intensités élevées au niveau des bandes amide I et amide II par rapport aux échantillons traités par US seul (20 min) et IR seul (3 kGy). Ces résultats coïncident avec les résultats de l’analyse de la solubilité et l’analyse de la teneur en SH, suggérant un potentiel d'hydrolyse plus élevé ou une exposition plus élevée des groupes hydroxyle et amine des protéines à la solvatation après le traitement combiné.

Le traitement combiné US-IR a été sélectionné pour prétraiter la poudre de criquet avant incorporation dans la boisson fermentée. D’autres procédés combinés ont été utilisés : l’hydrolyse enzymatique assistée par ultrasons suivie d’une centrifugation ou non (US-E et US-EWC, respectivement) et hydrolyse enzymatique assistée par irradiation gamma et ultrason (US-IRE).

2. Digestibilité *in vitro*

Des boissons non-enrichies (contrôle C), enrichies en protéines de criquet non traitée (Cr) et enrichies en protéines de criquet traitées avec les procédés cités ci-dessus fermentées et non fermentées, ont été élaborées et l’effet sur la digestibilité *in vitro* et le profil peptidique a été étudié. Les résultats ont montré une digestibilité maximale de 94% pour les boissons fermentées enrichies en protéines prétraitées par US-E et US-IRE. Ceci pourrait être dû au rôle du prétraitement enzymatique qui assure une prédigestion des protéines par rupture des liens entre les protéines et d’autres constituants qui empêchent la bonne digestibilité des protéines de criquet, en particulier la chitine (Hall et al., 2017b; Yi et al., 2016). Cependant, le traitement US-IRE a permis d’avoir une digestibilité plus rapide par rapport à US-E (94% après 30 min de la digestion à la pepsine pour US-IRE comparativement à 94% après 240 min de la digestion à la pepsine et à la trypsine pour US-E). Cette observation peut être due aux changements structurels et à l'exposition des peptides et d'acides aminés plus facilement digestibles par les enzymes digestives, associée à une irradiation qui a probablement favorisé la digestion et l'utilisation des hydrolysats d'une part par les ferments et d'autre part par les enzymes digestives (Fombang et al., 2005b; Hassan et al., 2018).

Au niveau des profils peptidiques, les boissons fermentées US-E F, US-EWC F et US-IRE F sont caractérisées par un pourcentage très faible ou négligeable (de 2.2 à 1.3 %) de peptides haut poids moléculaire (>3000 Da) accompagnés d’un pourcentage plus élevé de peptides de bas poids moléculaire (<260 Da) (de 58.7 à 78.3 %) par rapport aux autres traitements (contrôle, Cr, US-IR). Ces résultats reflètent le degré élevé de dégradation des protéines dans ces produits. Plusieurs auteurs ont suggéré que l'enrichissement des boissons avec des protéines sous forme

d'hydrolysats permet une meilleure digestibilité des protéines et tend à augmenter le niveau de peptides fonctionnels et bioactifs (Calbet and Holst, 2004). Le changement de profil par rapport au traitement US-IRE peut s'expliquer par la protéolyse de grosses protéines insolubles en peptides solubles. Par conséquent, l'hydrolyse en peptides plus petits a confirmé la digestion rapide lorsque l'hydrolyse enzymatique était combinée à une irradiation, assurant la rupture des chaînes polypeptidiques et une forte formation de petits peptides (Gaber, 2005).

3. Qualité protéique et digestibilité *in vivo*

Dans le but d'évaluer la qualité protéique de boissons probiotiques enrichies en protéines et de confirmer les résultats du test *in vitro*, une expérience *in vivo* a été effectuée sur un modèle animal.

Le profil d'acides aminés de la protéine de criquet a été analysé et les résultats ont montré que le criquet est caractérisé par un profil d'acides aminés complet. En effet, il contient à la fois des acides aminés essentiels et non essentiels. Oibiokpa et al. (2018) ont noté que la protéine de criquet pourrait être une bonne source d'acides aminés essentiels tels que les acides aminés soufrés (méthionine et cystéine) qui jouent un rôle important pour la croissance du corps. En outre, la protéine de criquet possède une teneur élevée en lysine et en méthionine, qui se sont avérées faibles dans les protéines d'origine végétales et dans la plupart des régimes alimentaires tels que le riz, le chanvre et les pois (Mokrane et al., 2010; Zhao et al., 2020; House et al., 2010).

La détermination du score en acides aminés a montré que la protéine de criquet ne présente aucun déficit en acides aminés essentiels (l'indice chimique supérieur à 1) Il est caractérisé par un profil complet et équilibré comparées aux exigences de la FAO et de l'OMS. Le rapport des acides aminés essentiels et des acides aminés totaux (E / T) a également été calculé. Les résultats ont montré un pourcentage de 44,5%. Cette valeur est au-dessus de 36%, donc le criquet pourrait être considéré comme une protéine idéale (FAO/WHO, 1990).

Après 14 jours de suivi, les résultats de la prise de nourriture et des protéines, le poids initial, le poids final et le gain moyen journalier ont été déterminés pour les groupes nourris avec la caséine, la boisson enrichie en hydrolysats protéiques de criquet (CPH), la boisson enrichie en protéine de criquet non traitée (CP) et nourris avec un régime sans protéine. Le groupe nourri avec la caséine présente le poids le plus élevé ($P \leq 0.05$) suivi des groupes nourris avec CPH puis celui nourri avec CP. Par conséquent, le groupe nourri avec de la caséine a le gain de poids quotidien le plus élevé suivi par le régime à base de CPH qui est significativement ($P \leq 0.05$) plus élevé que le régime à base de CP. Tandis qu'une perte de poids a été observée pour le groupe nourri avec un régime sans protéines. Ceci reflète le rôle des protéines dans la croissance du corps et le maintien

du métabolisme (Gilbert et al., 2011). D'autre part, ces résultats ont montré l'effet positif du régime alimentaire à base des hydrolysats de protéines de criquet et de protéines de criquet non traité (protéine entière) qui ont assuré la croissance des rats. Des observations similaires ont été notées par d'autres chercheurs (Piccolo et al., 2017 ; Bovera et al., 2016) qui ont trouvé que l'utilisation de la farine de larves de *Tenebrio molitor* pour nourrir la daurade et les poulets avait un effet positif sur la croissance de ces animaux.

Le CEP reflète la capacité de la protéine de soutenir la croissance de jeunes rats en croissance (Boye et al., 2012) tout en prenant en compte l'effet de la digestibilité des protéines et la biodisponibilité des acides aminés. Le CEP de la caséine était plus élevé (3.2) par rapport aux régimes à base de CPH (2.0) et de CP (1.7). La valeur la plus faible a été notée pour le groupe des rats nourris avec CP avec une différence significative (($P \leq 0.05$) par rapport au groupe des rats nourris avec CPH (1.7 et 2.0, respectivement). D'après Friedman (1996), les sources de protéines avec un CEP inférieur à 1,5 sont de faible qualité. Pour cela, nos résultats montrent que la protéine de criquet présente une bonne qualité nutritive. La valeur élevée du CEP pour CPH reflète leur bonne absorption par rapport au régime alimentaire à base de protéines de criquet entière, ce qui peut être dû à l'augmentation de la quantité d'acides aminés essentiels et à la diminution de l'allergénicité causée par l'hydrolyse enzymatique (Kechaou et al., 2009). La fermentation pourrait également avoir un rôle dans l'amélioration de la qualité nutritive des protéines par leur capacité à assurer la libération des peptides, l'augmentation de la quantité d'acides aminés et l'amélioration de la digestibilité (Manus et al., 2021; Pranoto et al., 2013).

Le CEPN (Coefficient d'Efficacité Protéique Net) prend en compte la perte de poids des rats nourris avec un régime sans protéines afin de déterminer à quelle niveau une protéine est digérée et utilisée (Nielsen, 2002). La valeur CEPN d'un régime à base de CPH était significativement ($P \leq 0.05$) plus élevée que celle de CP avec des valeurs de 1.0 et 0.4, respectivement. Par conséquent, le CEPN de la boisson CPH a représenté d'environ 50% de la valeur CEPN de la caséine tandis que la boisson CP a représenté 18.2% du CEPN de la caséine.

Ces résultats soulignent l'impact du traitement notamment de l'hydrolyse enzymatique et de la fermentation sur la biodisponibilité des acides aminés qui ont permis d'améliorer la qualité protéique de la protéine de criquet. De même, la digestibilité de CPH (92.2%) est plus élevée que celle de CP (83.7%). Cette différence pourrait être due à la présence de la chitine qui est un polymère non digestible qui pourrait se complexer avec le contenu intestinal et empêche l'accès des enzymes digestives et l'absorption des nutriments (Belluco et al., 2013 ; Devi et al., 2014).

Le PDCAAS est un paramètre recommandé par la FAO et l'OMS comme un indicateur de la qualité des protéines dans les aliments. Le régime à base de CP avait la valeur de PDAACS la plus faible (94.3 %), ce qui pourrait donc être dû à une digestibilité réelle inférieure par rapport au régime à base de caséine et de CPH qui ont des valeurs PDCAAS de 100 %. En effet, le PDCAAS inférieur généré par les aliments est lié à des niveaux inférieurs d'acides aminés indispensables et / ou à une digestibilité réelle plus faible, de sorte que les aliments sont considérés comme des protéines de qualité inférieure (Marinangeli and House, 2017). La valeur de 100% observée pour le régime à base de CPH reflète l'amélioration de la qualité nutritionnelle de la protéine de criquet suite au processus d'hydrolyse enzymatique assistée par ultrasons.

4. Microbiote intestinal

L'effet de régime alimentaire sur la diversité microbienne dans le tractus gastro-intestinal a été étudié à l'aide de l'indice de diversité alpha de Shannon. La faible diversité a été observée pour le groupe nourri avec un régime sans protéines par rapport aux régimes à base de protéines ($P \leq 0,05$) tandis qu'aucune différence significative ($P > 0,05$) n'a été observée entre la caséine, CPH et CP. Ce résultat indique que la diversité alpha du microbiote intestinal a été augmentée en raison des divers nutriments tels que les acides aminés fournis par les régimes à base de protéines et sert à fournir une variation de substrats essentiels dans l'intestin pour la prolifération des taxons (Andreotti et al., 2011; Laparra and Sanz, 2010). Le ratio *Firmicutes*/*Bacteroidetes* (ratio F:B) est un paramètre associé à l'obésité et à d'autres maladies (Barlow et al., 2015; Krajmalnik-Brown et al., 2012). Les résultats ont montré que le changement de régime n'a pas affecté le rapport F:B ($P >0,05$). Cependant, le groupe nourri avec un régime sans protéines avait un ratio (F:B) inférieur à celui des groupes nourris avec un régime à base de protéine (caséine, CPH et CP). Par conséquent, ces résultats sont similaires à ceux de Stull et al. (2018) pour le microbiote intestinal humain et Jarett et al. (2019) pour le microbiote intestinal des chiens où la consommation de criquet n'a pas modifié la diversité alpha et bêta par rapport à un régime contenant une autre source de protéines.

L'abondance relative des bactéries intestinales des rats nourris avec des régimes alimentaires différents a été déterminée. Au niveau des phylums, un phylum supplémentaire (*Tenericuteria*) a été observé pour les groupes nourris avec des protéines (caséine, CPH et CP) par rapport au groupe nourri avec un régime sans protéines. Au niveau des familles, *Anaeroplasmataceae* n'a été détecté que dans l'intestin des animaux nourris avec des protéines par rapport au groupe sans protéines. Cependant, les groupes nourris avec la caséine, CPH et CP sont différents en termes de proportion de chaque famille bactérienne. Sur la base de ces résultats, la caséine, CPH et CP

pourraient être considérés comme des régimes pour le maintien de l'énergie et le soutien de la croissance par le fait que l'augmentation de la proportion de *Tenericutes/Anaeroplasmatacea* connue pour être associée à un bilan énergétique positif (Pedersen et al., 2013).

La comparaison des abondances bactériennes pour le régime à base de CPH et le régime à base de CP montre une différence non significative (P>0,1) au niveau des genres, sauf pour, *Adlercreutzia*, *GCA-900066755*, *Ruminococcaceae_NK4A214_group* et *Negativibacillus* qui ont augmenté de manière significative ($P\leq$0,1) avec un régime à base de CP. Le genre *Lactobacillus* spp. s'est avéré être plus élevé chez les rats nourris avec CPH, tandis que le groupe sans protéine a la plus faible abondance de ce genre.

Les lactobacilles jouent un rôle clé dans le maintien de l'équilibre métabolique. Il a eu un effet bénéfique comme la réduction de charge d'antigène et la prévention des inflammations, lorsqu'il a été trouvé en plus grande abondance (Zhang et al., 2010). La présence de *Lactobacillus* spp. en forte abondance chez les rats nourris avec CPH, pourrait s'expliquer par la survie des lactobacilles issus du lait fermenté, lors de la digestion. En effet, la présence de protéines sous forme d'hydrolysats se traduit par un substrat plus assimilable par les bactéries ce qui a un effet sur la viabilité de ces bactéries ainsi que leur capacité à résister aux conditions gastro-intestinales stressantes. En outre, *Ruminococcaceae* et *Clostridiales* étaient plus élevées dans le régime à base de protéines et ont augmenté en particulier pour les rats nourris avec CP. Des études précédentes, (Jarett et al., 2019; Yang et al., 2013) ont montré que l'abondance de ces bactéries dans l'intestin aurait un rôle dans la fermentation des fibres. Cela indique la corrélation positive avec la chitine présente dans la protéine de criquet en tant que fibre non digestible. Ainsi, la protéine de criquet peut favoriser la colonisation de bactéries intestinales à savoir *Ruminococcaceae,* qui ont la capacité de dégrader la chitine pour produire les acides gras à chaîne courte (Gomes et al., 2020).

La corrélation entre l'abondance des bactéries et les paramètres nutritionnels a montré que certaines bactéries pathogènes à savoir *Erysipelatoclostridium* et *Enterococcaceae* (Auch, 2010; Gouret et al., 2009) ont une faible abondance pour les animaux nourris avec un régime protéique particulièrement avec CPH et CP. Ces résultats ont confirmé que la protéine de criquet entière ou les hydrolysats de protéine de criquet peuvent maintenir une composition plus équilibrée et réduire les bactéries à potentiel pathogène dans le microbiote intestinal ainsi que la prévention de développement du cancer colorectal par le maintien d'une production élevée en butyrate et en acides gras à chaîne courte (Gonçalves et al., 2016; Wang et al., 2019). Cette production est

favorisée par une forte abondance de certains genres producteurs de butyrate à savoir *Erysipelotrichaceae, Ruminococcaceae, Clostridaceae* and Family XIII (Chai et al., 2019).

5. Analyses au cours de l'entreposage

L'évaluation des paramètres physicochimiques, microbiologiques, nutritionnels et sensoriels des boissons fermentées enrichies en hydrolysats des protéines d'insectes (CPH) et non enrichis (C), a été effectuée pendant 147 jours à 4 °C.

5.1. pH et acidité titrable

Une diminution du pH a été observée au cours du stockage. Cependant, cette diminution est plus prononcée pour la boisson C par rapport à CPH, suggérant l'effet tampon des protéines. Quant à l'acidité titrable, une augmentation plus importante a été enregistrée pour le breuvage CPH par rapport au breuvage C. Ces résultats laissent suggérer que les boissons fermentées ont subi une acidification post-fermentation pendant le stockage réfrigéré. Ceci pourrait être expliqué par la persistance de l'activité métabolique des lactobacilles pendant la conservation à 4°C (Beal et al., 1999). Ainsi, la supplémentation en protéine de boissons fermentées a entraîné un degré d'acidification plus élevé. Dans ce contexte, Akin and Ozcan (2017) ont observé une augmentation de l'acidité titrable pour les breuvages enrichis en protéines végétales comme le gluten du blé, le pois et le soja, par rapport au breuvage fermenté non enrichi.

5.2. Viscosité

La variation de la viscosité au cours de l'entreposage a été étudiée. Une augmentation de la viscosité a été enregistrée surtout pour les boissons enrichies en protéines. L'augmentation de la viscosité pendant la période de stockage, pourrait être due aux interactions entre les différents constituants. Ces interactions peuvent être de type protéine-polysaccharide (peut-être de l'amidon de riz provenant de la formulation de base de la boisson à 3% des protéines de riz et/ou de la chitine de criquet) et/ou de la protéine-protéine (protéine de criquet et de riz). Elles sont favorisées par la baisse du pH et qui pourrait augmenter la capacité de rétention d'eau (Soukoulis et al., 2007; Sun-Waterhouse et al., 2012). Ainsi, l'affaiblissement du coagulum pendant le stockage pourrait provoquer la libération d'eau de la structure et la séparation du sérum, ce qui a augmenté la réticulation entre les particules de protéines et la résistance structurelle. Au jour 0 de stockage, la boisson CPH fermentée et la boisson CPH non-fermentée avaient une viscosité très similaire ($P > 0.05$) (9.7 et 10 cP, respectivement). Cette observation pourrait s'expliquer par l'effet de

l'hydrolyse enzymatique en tant que prétraitement qui a réduit la taille des particules et a amélioré les propriétés fonctionnelles des protéines de criquet, à savoir la solubilité, la capacité de rétention d'eau, la capacité de gélification et d'autres propriétés qui ont un effet sur la viscosité du produit (León-López et al., 2020). Cependant, la différence entre les boissons CPH F et CPH NF devient plus prononcée pendant le stockage. Ce résultat pourrait s'expliquer par l'effet des probiotiques après fermentation, qui jouent un rôle important dans l'hydrolyse des peptides de poids moléculaire élevés en peptides de faible poids moléculaire résultant en une viscosité inférieure par rapport à la même boisson non fermentée. La même observation a été rapportée par Manus et al. (2021) pour la boisson fermentée enrichie en protéines de riz et de pois.

5.3. Analyse de la couleur

Les résultats ont montré une augmentation de la valeur L* après fermentation dans tous les échantillons et une diminution de ce paramètre suite à l'ajout des protéines. Ceci est expliqué par la présence des particules obstruant la diffusion de la lumière suite à l'enrichissement des boissons en hydrolysats protéiques. En revanche, la luminosité des produits fermentés était très élevée par rapport aux produits non fermentés ($P \leq 0.05$), ce qui reflète le rôle des probiotiques dans la dégradation des particules au cours de la fermentation (Barbut, 2010). Cependant, L* a augmenté significativement ($P \leq 0.05$) au cours de stockage pour passer de 43.9 au jours 0 à 47.4 au jours 147 pour CPH F alors qu'une variation faible pour la même boisson non fermentée a été notée. Ces résultats ont révélé l'effet des bactéries qui ont permis la diffusion de lumière à travers le gel formé après fermentation. Quant au paramètre C* les valeurs obtenues sont positives pour toutes les boissons évaluées. Également, une légère variation au cours de stockage a été observée en particulier pour les boissons fermentées. De ce fait, l'intensité de couleur pour les produits fermentés est plus élevée par rapport aux produits non fermentés ($P \leq 0.05$). Le temps de stockage n'a pas montré d'effet sur l'angle h* et ceci pour tous les échantillons. En outre, l'enrichissement de boisson en protéine a affecté la valeur h*. En effet, h* est significativement plus élevé pour la boisson contrôle ($P \leq 0.05$) par rapport aux boissons enrichies CPH avec des valeurs comprises entre 112 et 113 et entre 94 et 82 respectivement. Par conséquence, la couleur de la boisson contrôle est plus proche au jaune-verdâtre caractéristique de la couleur de riz alors que la boisson enrichie CPH est proche de rouge-orange. Le calcul de la variation de la couleur ΔE entre le jour 0 et le jour 147 a montré que les valeurs obtenues sont < 3. Donc la différence de couleur au cours de stockage est non détectable à l'œil humain (Adekunte et al., 2010; Vichi et al., 2004).

5.4. Analyse de profils peptidiques

L'analyse des profils peptidiques au cours de stockage a montré une augmentation de pourcentage des peptides de haut poids moléculaire accompagnée d'une diminution de pourcentage des peptides de faible et de moyen poids moléculaire pour la boisson contrôle C NF. Pendant ce temps, un profil stable tout au long de la période de stockage réfrigéré, a été noté pour la boisson fermentée (C F). Une légère variation de différentes proportions des peptides a été observée pour la boisson enrichie CPH, caractérisée par un pourcentage très élevé en peptides de bas poids moléculaires (<260 Da) et de moyens poids moléculaires (entre 3000 et 260 Da) et un pourcentage faible en peptide de haute poids moléculaires (>3000) par rapport au même breuvage non fermenté et par rapport aux breuvages non enrichis. La fermentation aurait donc un meilleur effet sur CPH que sur C qui a permis d'obtenir des peptides de faible poids moléculaire, plus assimilable et plus absorbable par les intestins et donc d'obtenir un produit plus digestible (Ogodo et al., 2018 ; Pranoto et al., 2013).

5.5. Analyse microbiologique

La variation de la concentration en probiotiques au cours de stockage durant 147 jours pour les boissons non enrichies CF et enrichies CPH F a été déterminée. Les résultats ont montré une diminution rapide des cellules viables pour la boisson non enrichie (passe de 8.79 Log UFC/mL au jour 0 à 7.27 Log UFC/mL au jour 147). Tandis que, la boisson enrichie en CPH a montré un taux stable jusqu'à 84 jours suivi d'une diminution lente et d'une variation faible au cours de la conservation réfrigérée (réduction de 1 log). L'enrichissement des breuvages en protéines peut aider au maintien de la viabilité des probiotiques (Felix da Silva et al., 2017). En effet, la présence des protéines peut assurer la croissance des bactéries et garde un milieu favorable et adéquat pour ces bactéries tout au long de la période de conservation. En effet, les hydrolysats de protéines de criquet constitue une source d'acides aminés essentiels à savoir le tryptophane et la cystéine (Dave and Shah, 1998) pour la croissance des bactéries. Lucas et al. (2004) ont trouvé que l'ajout d'hydrolysats a amélioré la croissance de *S. thermophilus* et l'acidification, réduisant ainsi le temps de fermentation.

La survie des probiotiques dans le tube gastro-intestinal simulé au cours de stockage a été évaluée. Aucune diminution significative n'a été notée pour les probiotiques présents dans la boisson enrichie CPH au cours de la simulation gastrique tout au long de la période de stockage ($P > 0,05$) avec un taux de survie (SR) de 97% après 147 jours de stockage. Le taux de survie des probiotiques dans la phase intestinale pour la même boisson est de plus de 90% au cours

des premiers 21 jours d'entreposage et ce pourcentage a diminué légèrement pour atteindre une valeur de 83% au jour 147. Tandis que, la boisson non-enrichie (C) a montré une sévère diminution pour atteindre des valeurs ≤ 50%. Ces résultats montrent que l'enrichissement de boissons en protéines a permis de fournir une protection aux bactéries envers les conditions de tube gastrique pour garder un taux de survie élevé dans les phases gastriques et intestinales *in vitro* tout au long de la période de conservation. Millette et al. (2013) ont démontré que la formulation Bio-K+ pouvait résister aux conditions gastriques à un pH 2.0 ce qui est en accord avec les résultats de cette étude.

5.6. Analyse sensorielle

Les résultats de l'analyse sensorielle ont montré que la boisson enrichie CPH a été comparativement à la boisson témoin, acceptée en termes de couleur et de texture. Tandis que la même boisson a été classée comme moins acceptable par rapport au C F ($P \leq 0.05$) en termes de saveur et d'appréciation globale. La boisson à base d'hydrolysats de protéines de criquet se caractérise par une acidité intense, une amertume très prononcée et un goût de cuisson qui ont diminué le score du produit en termes de goût et d'appréciation globale. L'acidité intense est due à l'interaction protéine-probiotique. En effet, l'enrichissement d'un produit fermenté en protéines a augmenté la productivité des bactéries en acides organiques (Lucas et al., 2004). Quant au goût de cuisson, la poudre de criquet a été obtenue par grillage qui conduit au développement des arômes. Pour l'amertume, il a été montré que l'hydrolyse enzymatique peut entraîner la production des peptides de faible poids moléculaire amers fortement hydrophobes et génère davantage des acides aminés aromatiques (Kukman et al., 1995; Molina et al., 1999). L'utilisation de protéines d'insectes ayant suivi des traitements de chaleur moins intense pourrait réduire le goût de cuisson. D'autre part, l'amertume peut aussi être réduite par l'ajout de produits masquants.

CHAPITRE 6 : CONCLUSION GÉNÉRALE

L'objectif principal du projet était de mettre au point une boisson probiotique fermentée à base de riz et enrichie en protéines de criquet de haute valeur nutritive et de bonne digestibilité. Afin d'y arriver, il a fallu mettre en place un procédé de prétraitement qui permet de modifier les propriétés fonctionnelles et structurales des protéines tout en limitant les pertes en nutriments essentiels. Notre choix s'est porté sur le traitement combiné d'hydrolyse enzymatique assistée par ultrasons. Ainsi, l'effet de la fermentation sur les propriétés physicochimiques, organoleptiques et la digestibilité de boisson enrichie *in vitro* et *in vivo*, a été étudié.

L'effet de la fermentation lactique notamment via les probiotiques *L. acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2 sur les propriétés nutritionnelles et la digestibilité des protéines d'insectes n'est pas décrit dans la littérature. Ainsi, le traitement des protéines d'insectes avec un procédé combiné à savoir l'ultrason suivi par hydrolyse enzymatique n'est pas encore abordé dans les travaux de recherche.

C'est dans cette optique, l'étude a montré que le prétraitement des protéines de criquet permet d'améliorer les propriétés fonctionnelles et structurelles. Une augmentation de la solubilité et une augmentation de la teneur en liaison et en surface sulfhydryles ont été observées par rapport à la poudre de criquet non traitée. En plus, des modifications de la structure primaire et secondaire ont été notées sous l'effet de prétraitement.

L'évaluation de la digestibilité *in vitro* a montré que la fermentation a pu améliorer et accélérer la cinétique de la libération d'azote au cours de la digestion des boissons enrichies en protéines prétraitées. De plus, cette boisson a révélé une augmentation de la teneur en peptides de faibles poids moléculaires accompagnée d'une diminution de la teneur en peptides de haut poids moléculaires au cours de la digestion ce qui reflète une amélioration de la qualité nutritive de boisson suite à la libération des peptides qui pourrait être des peptides bioactifs.

L'analyse de la teneur en acides aminés a montré que la protéine de criquet est caractérisée par un profil complet en acides aminés impliqués dans la croissance. Une amélioration des paramètres de croissance, de la prise alimentaire et de Coefficient d'Efficacité Protéique a été observée chez les rats. Dans l'ensemble, les hydrolysats des protéines de criquet résultant du prétraitement, a montré une digestibilité élevée qui était proche de la digestibilité de la caséine avec une valeur maximale de PDCAAS. Par ailleurs, une diète à base des protéines de criquet et en présence des probiotiques, a maintenu une composition équilibrée du microbiote intestinal et

a pu réduire les bactéries pathogènes de l'intestin. Par conséquent, les protéines de criquet peuvent être considérées comme une protéine de bonne qualité nutritive et une bonne alternative pour la consommation humaine.

La stabilité de boisson fermentée enrichie en hydrolysats des protéines de criquet a été effectuée durant 147 jours. Cette boisson a conservé un grand nombre des probiotiques viables tout au long de stockage avec une meilleure résistance aux conditions gastro-intestinales stressantes *in vitro.* Ainsi, le prétraitement des protéines de criquet couplé avec la fermentation lactique a permis d'améliorer les propriétés physico-chimiques de boissons. Une diminution de la viscosité et une augmentation de l'acidité ont été observées par rapport aux boissons fermentées non enrichies. Ceci a mis en évidence la synergie entre les protéines et les probiotiques mais aussi le rôle de prétraitement dans l'amélioration de critères d'acceptabilité de produits notamment les propriétés technologiques, physicochimiques et texturales. Au niveau sensoriel, la boisson a été appréciée pour sa texture et son apparence, moyennement appréciée pour son odeur et moins appréciée pour sa saveur.

À l'issue de ces résultats, la boisson développée pourrait être considérée comme une bonne source protéique, complète et équilibrée. Elle répond au désir des consommateurs en un produit sain, salubre, de bonne qualité nutritive et de haute digestibilité tout en garantissant un bon équilibre du microbiote intestinal. Les personnes âgées, les enfants et les sportifs peuvent être considérés comme des cibles pour cette boisson qui renforce l'établissement d'un système alimentaire durable.

Comme perspectives, il sera intéressant d'optimiser les paramètres du procédé testé (hydrolyse enzymatique assisté avec ultrasons) en relation avec la fermentation et/ou de tester la combinaison d'autres procédés pour réduire le coût, l'impact du procédé sur la qualité organoleptique et augmenter davantage la fonctionnalité et la qualité nutritive des protéines. Il sera également important de faire des analyses complémentaires pour mieux caractériser et mettre en valeur la bioactivité des peptides résultant de la fermentation via les probiotiques (par exemple l'activité antioxydante, effet sur la réduction de la pression sanguine et l'activité antimicrobienne) et donc mieux classer le produit comme un aliment fonctionnel qui regroupe les bienfaits des probiotiques et les protéines.

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