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Astaksantyna i postbiotyki – dwa obiecujące czynniki zwiększające wrażliwość na insulinę w zespole metabolicznym koni

Astaxanthin and postbiotics – two promising agents to increase insulin sensitivity in equine metabolic syndrome

Praca doktorska wykonana pod kierunkiem:

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(2) **Mularczyk, M**.; Bourebaba, N.; Marycz, K.; Bourebaba, L. Astaxanthin Carotenoid Modulates Oxidative Stress in Adipose-Derived Stromal Cells Isolated from Equine Metabolic Syndrome Affected Horses by Targeting Mitochondrial Biogenesis. *Biomolecules* **2022**, *12* (8), 1039. https://doi.org/10.3390/biom12081039. **(IF=6.064)**

(3) Bourebaba, Y.; Marycz, K.; **Mularczyk, M**.; Bourebaba, L. Postbiotics as Potential New Therapeutic Agents for Metabolic Disorders Management. Biomed. Pharmacother. 2022, 153, 113138. https://doi.org/10.1016/j.biopha.2022.113138. **(IF=7.419)**

(4) **Mularczyk, M**.; Bourebaba, Y.; Kowalczuk, A.; Marycz, K.; Bourebaba, L. Probiotics-Rich Emulsion Improves Insulin Signalling in Palmitate/Oleate-Challenged Human Hepatocarcinoma Cells through the Modulation of Fetuin-A/TLR4-JNK-NF-KB Pathway. Biomed. Pharmacother. 2021, 139, 111560. https://doi.org/10.1016/j.biopha.2021.111560. **(IF= 4.545)**

Spis treści

WYKAZ SKRÓTÓW

ALT – (ang. *alanino aminotransferase*) – aminotransferaza alaninowa

AMPK – (ang. *AMP-activated protein kinase*) – kinaza białkowa aktywowaną przez AMP

AST – (ang. *Aspartate aminotransferase*) – aminotransferaza asparaginianowa

AT – (ang. *Adipose tissue*) – tkanka tłuszczowa

BCS – (ang. *Body condition scoring*) – skala oceny kondycji

CAGR – (ang. *Compound Annual Growth Rate*) – skumulowany roczny wskaźnik wzrostu

CMA – (ang. *Chaperone-mediated autophagy*) – autofagia zależna od białek opiekuńczych

EFSA – (ang. *European Food Safety Authority*) – Europejski Urząd ds. Bezpieczeństwa Żywności

EMS – (ang. *Equine metabolic syndrome*) – syndrom metaboliczny koni

EPS – (ang. Exopolysaccharides) – pozakomórkowe polisacharydy

ER – (ang. *Endoplasmic reticulum*) – reticulum endoplazmatyczne

ERK – (ang. *Extracellular signal-regulated kinases*) – kinaza białkowa regulowana pozakomórkowo

EV- (ang. *Extracellular vesicles*) – pęcherzyki zewnątrzkomórkowe

FDA- (ang. *Food and Drug Administration*) – Amerykańska Agencja Żywności i Leków

FFA – (*ang.Free fatty acid)* – wolne kwasy tłuszczowe

GGT – (ang. *Gamma-glutamyl transferase*) – gamma-glutamylotransferaza

GPR43 – (ang. *G-protein-coupled receptor 43*) – receptor 43 sprzężony z białkiem G

GRAS – (ang. *Generally Recognized As Safe*) – ogólnie uznawane za bezpieczne

HepG2 – (ang. *Human liver cancer line*) – linia komórkowa ludzkiego raka wątroby

HFD – (ang. *High fat diet*)- dieta wysokotłuszczowa

HOMA-IR – *(ang. Homeostatic Model Assesment – Insulin Resistance)* – wskaźnik insulinooporności

IL-1 – (ang. *Interleukin 1*) – interleukina 1

IL-6 – (ang. *Interleukin 6*) – interleukina 6

IR – (ang. *Insulin resistance*) – insulinooporność

IRF4 – (ang. *Interferon Regulatory Factor 4*) – czynnik regulujący interferon 4

LAB – (ang. *Lactic acid bacteria*) – bakterie kwasu mlekowego

MetS – (ang. *Metabolic syndrome*) – syndrom metaboliczny

NAFLD – (ang. *Non-alcoholic fatty liver disease)* – niealkoholowe stłuszczenie wątroby

NF-κB – (ang. *Nuclear factor kappa-light-chain-enhancer of activated B cells*) – jądrowy czynnik transkrypcyjny kappaB

NOD2 – (ang. *Nucleotide-binding oligomerization domain-containing protein 2*) – białko 2 zawierające domenę oligomeryzacji wiążącą nukleotydy

NOD21 – (ang. *Nucleotide-binding oligomerization domain-containing protein 1*) – białko 1 zawierające domenę oligomeryzacji wiążącą nukleotydy

NOS – (ang.*nitric oxide synthase*) – syntaza tlenku azotu

NSC – (ang. *Nonstructural carbohydrates*) – niestrukturalne węglowodany

PUFA – (ang. *Polyunsaturated fatty acids*) – wielonienasycone kwasy tłuszczowe

RNS – (ang. *Reactive nitrogen species*) – reaktywne formy azotu

ROS – (ang. *Reactive oxygen species*) – reaktywne formy tlenu

SCFA – (ang. *Short – chain fatty acids*) – krótkołańcuchowe kwasy tłuszczowe

SGLT2 – (ang. *Sodium-glucose co-transport 2*) – kotransporter sodowo-glukozowy 2

SO – (ang. *Oxidative stress*) – stress oksydacyjny

T1R2/3 – (ang. *Sweet taste receptor*) – receptor słodkiego smaku

TLR2 – (ang. *Toll-like receptor 2*) – receptor toll-podobny 2

TLR4 – (ang. *Toll-like receptor 4*) – receptor toll-podobny 4

TNF-α – (ang. *Tumor necrosis factor α*) – czynnik martwicy nowotworów

STRESZCZENIE

Syndrom metaboliczny koni (EMS) to coraz częstsze schorzenie endokrynologiczne powodowane przekarmianiem koni paszami wysokoenergetycznymi, co prowadzi do otyłości oraz insulinooporności. EMS definiowany jest jako konstelacja symptomów klinicznych takich jak: (i) oporność na insulinę, (ii) w większości przypadków otyłość, (iii) przebyty lub chroniczny ochwat, (iv) systemowy i lokalny odczyn zapalny oraz podwyższony stres oksydacyjny i zapalenie wątroby. Wcześniejsze badania wykazały, że wątroba koni cierpiących na EMS charakteryzuje się stłuszczeniem oraz nadekspresją markerów modulujących odpowiedź na niepofałdowane białka, co w konsekwencji prowadzi do apoptozy zależnej od stresu siateczki śródplazmatycznej.

Celem pracy doktorskiej była ocena wykorzystania astaksantyny, jako czynnika terapeutycznego w przywracaniu równowagi metabolicznej w komórkach macierzystych zrębu pochodzących z tkanki tłuszczowej koni cierpiących na EMS, poprzez celowanie w stres oksydacyjny i leżącą u podstaw dysfunkcję mitochondriów. W tym celu oceniono wpływ astaksantyny na żywotność komórek macierzystych pochodzących z tkanki tłuszczowej (ASC), ich apoptozę, poziom reaktywnych form tlenu (ROS/NO), dynamikę mitochondriów i metabolizm, a także zdolność antyoksydacyjną astaksantyny. Druga analizowana hipoteza badawcza zakładała, że preinkubacja hodowli komórek wątrobowych (HepG2) z wywołaną insulinoopornością *in vitro,* wraz z emulsją pro- i postbiotyczną, będzie chronić komórki w warunkach normalnych i IR przed apoptozą, łagodzić stres oksydacyjny, a także poprawiać metabolizm i dynamikę mitochondriów oraz zmniejszać aktywację szlaku *Fetuin-A/TLR4/JNK/NF-κB*. Wyniki badań sugerują, że astaksantyna poprawia stan metaboliczny końskich ASC dotkniętego zespołem metabolicznym, natomiast emulsja pro- i postbiotyczna wykazała działanie ochronne przed zapaleniem, otyłością oraz insulinoopornością związaną z wątrobą.

Podsumowując, przeciwutleniacze w połączeniu ze stymulatorami mikrobiomu mogą stanowić nowe podejście dieto-terapeutyczne, a uzyskane wyniki stanowią podstawę do stworzenia potencjalnych biofarmaceutyków pozyskiwanych z naturalnych źródeł do prewencji syndromu metabolicznego u koni.

ABSTRACT

Equine Metabolic Syndrome (EMS) is an increasingly common endocrine disease caused by overfeeding horses with high-energy feeds, leading to obesity and insulin resistance. EMS is defined as a constellation of clinical symptoms such as: (i) insulin resistance, (ii) obesity in most cases, (iii) past or chronic laminitis, (iv) systemic and local inflammatory reaction and increased oxidative stress and hepatitis. Previous studies have shown that the liver of horses suffering from EMS is characterized by steatosis, overexpression of markers modulating the response to unfolded proteins, which lead to stress-dependent apoptosis of the endoplasmic reticulum.

This dissertation aimed to evaluate the use of astaxanthin as a therapeutic agent in restoring metabolic balance in EMS stromal stem cells by targeting abnormal oxidative stress and mitochondrial dysfunction. Effects of astaxanthin on cell viability, apoptosis, reactive oxygen levels, mitochondrial dynamics and metabolism, as well as its antioxidant capacity were assessed. The second analyzed research hypothesis assumed that the pre-incubation of insulin resistance induced HepG2 cell with a pro and postbiotic emulsion, would protect cells under normal and IR conditions from apoptosis, alleviate oxidative stress, as well as improve metabolism and dynamics of mitochondria, and reduce pathway activation Fetuin A / TLR4 / JNK / NF-κB. The results suggested that astaxanthin improves the metabolic status of equine ASC affected by metabolic syndrome. At the same time, the pro-and post-biotic emulsion is protective against inflammation, obesity and hepatic insulin resistance.

In conclusion, antioxidants in combination with microbiome stimulators may constitute a new dietary and therapeutic approach. The obtained results are the basis for the creation of potential biopharmaceuticals for the prevention of metabolic syndrome in horses.

1. WSTĘP

1.1 Syndrom metaboliczny koni

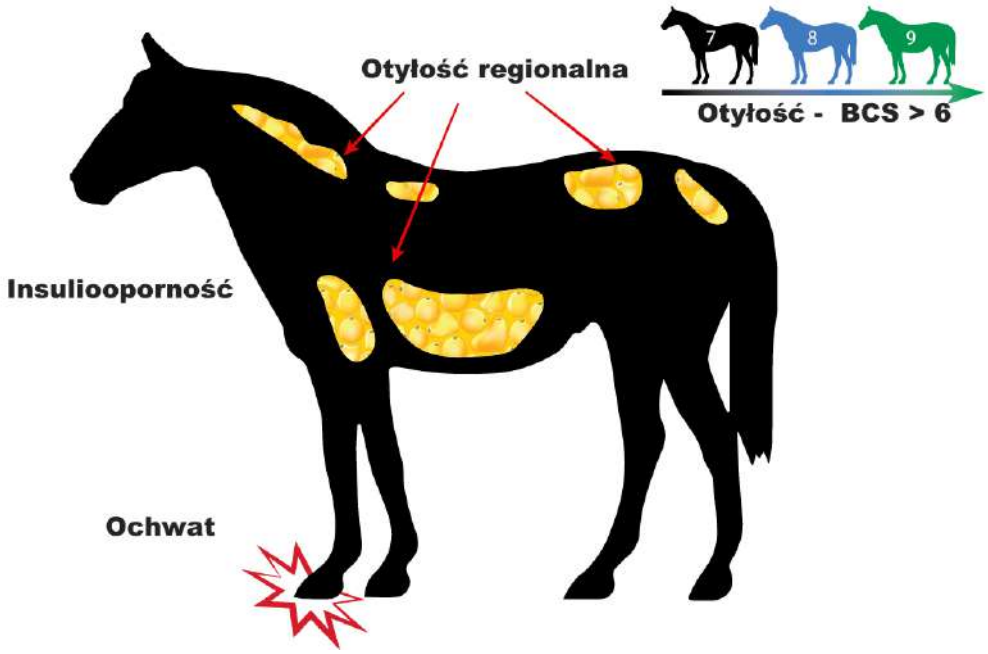
Syndrom metaboliczny koni (EMS) jest coraz częściej diagnozowanym zaburzeniem endokrynologicznym, które dotyka koni na całym świecie. EMS charakteryzuje się zbiorem następujących czynników: (i) miejscową otyłością, (ii) insulinoopornością oraz (iii) ochwatem, zarówno przebytym lub przewlekłym [1]. Chociaż ostatnio otyłość została wykluczona jako konieczne kryterium diagnostyczne, nadal pozostaje pierwszym, zauważalnym elementem diagnostyki różnicowej koni dotkniętych EMS.

Fizyczne cechy EMS obejmują otyłość uogólnioną, regionalną lub obie te cechy. Wzrost otyłości w określonych lokalizacjach (otyłość regionalna), objawia się rozrostem podskórnej tkanki tłuszczowej otaczającej więzadło karkowe szyi, rozwój poduszek tłuszczowych w pobliżu głowy, ogona oraz nagromadzenie tłuszczu w przestrzeni nad łopatką (ryc. 1) [2]. Dodatkowo, konie dotknięte EMS charakteryzują się podwyższonym indeksem oceny kondycji ciała (ang. Body condition scoring; BCS), gdzie wskaźnik BSC jest wyższy niż 6. Ponadto EMS charakteryzuje się: (i) hiperinsulinemią lub nieprawidłową odpowiedzią insuliny na doustną glukozę, (ii) hiperleptynemią wynikającą ze zwiększonego wydzielania hormonu leptyny przez adipocyty, (iii) nadciśnieniem tętniczym, które jest uznawane za kluczowy składnik ludzkiego zespołu metabolicznego (MetS), (iv) zwiększonym poziomem ogólnoustrojowych markerów stanu zapalnego, (v) hipertriglicerydemią lub łagodną trójglicerydemią i dyslipidemią [3].

Dotychczasowe badania wskazały na upośledzenie czynności wątroby u koni cierpiących na EMS na poziomie molekularnym, charakteryzujące się zwiększoną apoptozą i podwyższonym stresem oksydacyjnym, nadmierną akumulacją lipidów i nasileniem stanu zapalnego [4]. W przypadku koni dotkniętych EMS obserwuje się zwiększone stłuszczenie wątroby oraz występowanie stanu zapalnego, co wiąże się ze wzrostem stężenia aminotransferazy asparaginianowej (AST), aminotransferazy alaninowej (ALT) oraz gamma-glutamylotransferazy (GGT) we krwi [4]. Co więcej, komórki wątroby charakteryzuje insulinooporność, stan zapalny, apoptoza i zmniejszony poziom autofagii chaperonowej (CMA), co wywołuje zmiany w metabolizmie wątroby. W obrazie histologicznym wątroby obserwuje się znaczącą infiltrację przez komórki układu immunologicznego oraz odkładanie nadmiernej ilości kropel tłuszczu w hepatocytach. Ponadto, wykazano, że tkanka tłuszczowa (AT) koni cierpiących na EMS charakteryzuje się hiperplazją i hipertrofią [5]. Coraz więcej

uwagi poświęca się badaniu wpływu AT na przebieg EMS, ponieważ jest ona uważana nie tylko za tkankę magazynującą energię, ale także za bardzo aktywny narząd dokrewny. AT obficie wytwarza i uwalnia adipokiny, w tym leptynę, rezystynę, białko wiążące retinol 4 i wisfatynę. Ponadto nadmierne wydzielanie cytokin prozapalnych, takich jak interleukina 1 (IL-1), interleukina 6 (IL-6) oraz czynnik martwicy nowotworu alfa (TNF α) prowadzi do miejscowego i ogólnoustrojowego zapalenia u koni dotkniętych EMS [6].

EMS wiąże się również z rozwojem ochwatu, choroby metabolicznej, która powoduje poważne zmiany patologiczne w kopytach i prowadzi do rotacji kości kopytowej. Co więcej EMS prowadzi do poważnych zaburzeń wrażliwości na insulinę [7].



Rycina 1. Czynniki charakteryzujące konie obarczone syndromem metabolicznym (EMS). Źródło grafiki: https://www.kindredbio.com/pipeline/equine/kind-015/

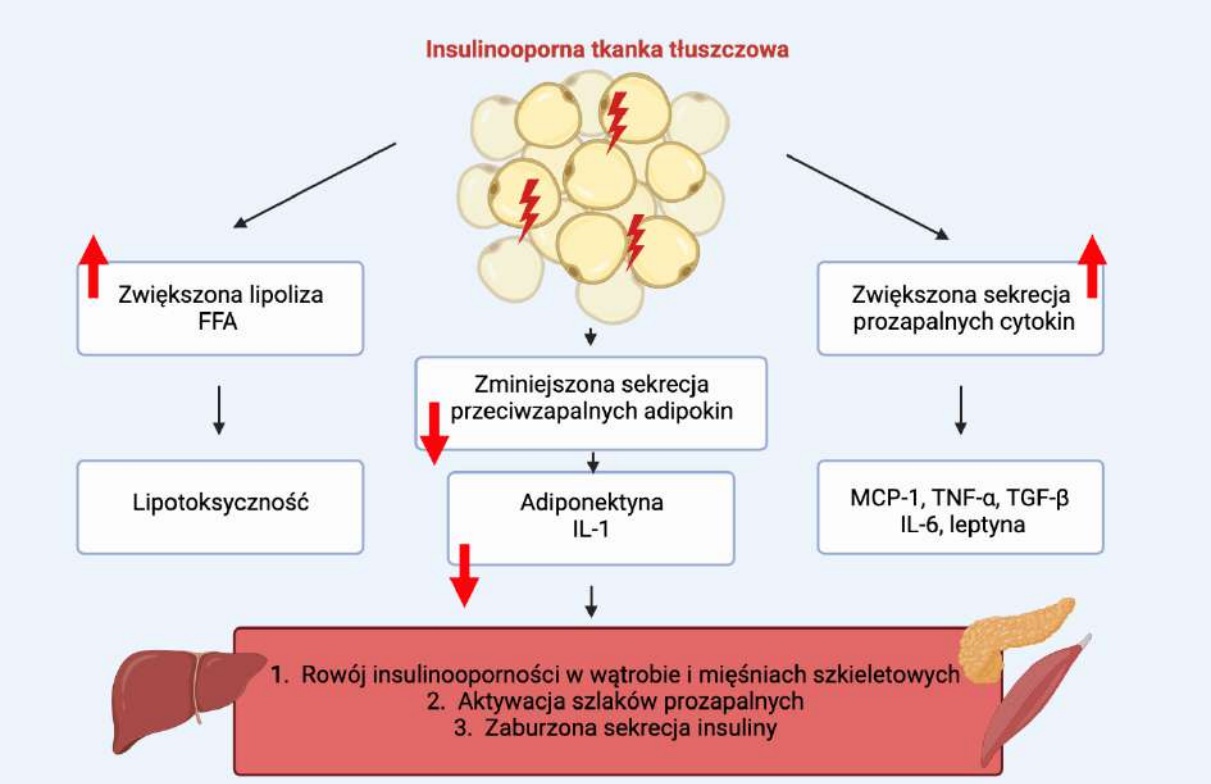
1.2 Udział stresu oksydacyjnego w przebiegu EMS

Stres oksydacyjny (ang. Oxidative stress OS) odgrywa szczególną rolę w progresji zespołu metabolicznego u koni. Wykazano, że nadmierna akumulacja reaktywnych form tlenu (ROS) zarówno w tkance tłuszczowej jak i w krwi obwodowej zakłóca szlak sygnałowy insuliny, przyczyniając się w ten sposób do rozwoju insulinooporności (IR). Dowiedziono, że dyslipidemią, hiperglikemia, stres retikulum endoplazmatycznego

(ER) oraz syntaza tlenku azotu (NOS) są odpowiedzialne za nadprodukcję ROS [8]. Co więcej, dysfunkcja mitochondriów jest najczęściej związana ze zwiększonym wydzielaniem ROS przez organelle lub pośrednio poprzez wysyłanie sygnałów na powierzchnię ER, która z kolei wytwarza jeszcze więcej ROS. Ponadto może wywołać ogólnoustrojowy stan zapalny charakterystyczny dla zespołu metabolicznego i cukrzycy [9]. Badania na zwierzętach wykazały pozytywną korelację między akumulacją tkanki tłuszczowej a stresem oksydacyjnym, który spowodował zwiększoną produkcję reaktywnych form tlenu (ROS) i nadekspresję oksydazy NADPH, z jednoczesnym obniżeniem ekspresji enzymów antyoksydacyjnych [10].

Stres oksydacyjny i stan zapalny rozwijają się przy zaburzonej podaży energii, która przekracza pojemność adipocytów. W rezultacie dochodzi do zwiększonego uwalniania adipokin w tym: cytokin prozapalnych, takich jak interleukina-1 (IL-1), interleukina-6 (IL-6) i czynnika martwicy nowotworu alfa (TNF-α) [11]. Skutkuje to przewlekłym stanem zapalnym niskiego stopnia, który z poziomu tkanki tłuszczowej dociera do układu krążenia i innych narządów. W konsekwencji stanu zapalnego rozwija się insulinooporność, gdyż TNF-α zapobiega fosforylacji receptorów insuliny, ingerując w ich kaskadowe działanie i uniemożliwiając ich funkcjonowanie. Kolejną przyczyną powstawania zapalenia jest stres oksydacyjny wywołany wadliwym metabolizmem adipocytów. W trakcie, gdy objętość tkanki tłuszczowej wzrasta, niewystarczające nawadnianie może prowadzić do braku tlenu, a tym samym do martwicy komórek adipocytarnych [12]. Proces fagocytozy, w celu wyeliminowania apoptycznych komórek, powoduje nasilenie nacieku zapalnego, a także stresu oksydacyjnego poprzez uwolnienie wolnych rodników, takich jak tlenek azotu i nadtlenek wodoru, co może może nasilać objawy kliniczne EMS [13].

Wywołana w ten sposób dysfunkcja tkanki tłuszczowej charakteryzuje się opornością na antylipolityczne działanie insuliny, zwiększoną lipolizą i uwalnianiem wolnych kwasów tłuszczowych oraz glicerolu, które z kolei są odpowiedzialne za akumulację trójglicerydów i lipotoksyczność w wątrobie, mięśniach i trzustce, zaburzając wydzielanie insuliny. Ponadto uwalnia adipokiny, które aktywują szlaki prozapalne w tych narządach (ryc.2) [14].



Rycina 2. Zależność między insulinoopornością i dysfunkcją tkanki tłuszczowej a insulinoopornością wątroby i mięśni [źródło własne na podstawie [14]].

1.3 Kluczowe organy i szlaki metaboliczne w rozwoju syndromu metabolicznego

Tkanka tłuszczowa (AT) jest kluczowym organem w rozwoju chorób metabolicznych [15]. Jest w niej magazynowana nadwyżka energii w postaci obojętnych trójglicerydów, co prowadzi do zwiększenia objętości kropel lipidów, a w efekcie, do rozrostu tkanki tłuszczowej i późniejszej otyłości [16]. AT wydziela także hormony, w tym: adiponektyne, wisfatyne i angiotentyne, które modulują insulinooporność i oś zapalną. Koordynują one także metabolizm ogólnoustrojowy, regulując aktywność narządów, takich jak: mięśnie, wątroba, trzustka i mózg [17,18]. Nadmierne nagromadzenie tkanki tłuszczowej u koni wiąże się z deregulacją mitochondriów, zmianami w sygnalizacji insulinowej, zwiększonym metabolizmem glikokortykosteroidów, ale także ze zmianami zawartości lipidów i podwyższonym poziomem leptyny w osoczu [19]. Tkanka tłuszczowa może stać się oporna na insulinę, ale poprzez uwalnianie wolnych kwasów tłuszczowych, cytokin prozapalnych i adipokin odgrywa również kluczową rolę w rozwoju insulinooporności w całym organizmie [20].

U koni dotkniętych EMS, AT wydziela różne mediatory prozapalne, w tym czynnik martwicy nowotworu α (TNF-α) i interleukiny [21]. Co więcej, adipocyty nie tylko syntetyzują triglicerydy, ale są również zdolne do uwalniania wolnych kwasów tłuszczowych (FFA) i glicerolu jako produktów hydrolizy triglicerydów [22]. Wysoki poziom FFA we krwi jest silnie skorelowany z otyłością i insulinoopornością [23], natomiast wzrost tkanki tłuszczowej prowadzący do otyłości związany jest z dysfunkcją endokrynnych adipocytów. W efekcie nadmierna podaż kalorii wyzwala odpowiedź zapalną w adipocytach i ich dysfunkcję poprzez działanie cytokin, takich jak TNFα, które zmniejszają sygnalizację insuliny i hamują adipogenezę [24]. Udowodniono, że komórki ASC izolowane od koni EMS mają ograniczony potencjał proliferacyjny, zwiększone tempo starzenia się, zwiększoną apoptozę, nadmierną akumulację ROS i pogorszenie stanu mitochondriów [25]. Należy zauważyć, że dysfunkcja ASC prowadzi do nieprawidłowej przebudowy tkanki tłuszczowej, co wiąże się z większym ryzykiem zaburzeń metabolicznych [26]. Ponadto, akumulacja reaktywnych form tlenu (ROS) w połączeniu ze zwiększonym stanem zapalnym są ważnymi składnikami utrudniającymi różnicowanie ASC do adipocytów i ograniczają ich multipotencję [27,28]. Istotnym elementem w różnicowaniu adipocytów jest kompleks genów zaangażowanych w maszynerię szlaku fosforylacji oksydacyjnej OXPHOS w mitochondriach [29]. Od tego szlaku zależy udział mitochondriów adipocytów w metabolizmie energetycznym całego ciała lub ich plastyczności [30]. Dodatkowo, szlak OXPHOS mitochondriów w adipocytach może zmieniać ich wrażliwość na insulinę i/lub funkcję ze względu na wysokie wymagania energetyczne związane z magazynowaniem kwasów tłuszczowych, wydzielaniem adipokin, sygnalizacją insulinową i wychwytem glukozy [31]. Wyniki badań przeprowadzonych przez Rong i in., wykazują, że u myszy z otyłością indukowanych wysokoenergetyczną dietą ekspresja genów OXPHOS była znacznie zmniejszona w porównaniu z myszami karmionymi dietą o standardowej zawartości tłuszczu [32].

Kolejnym kluczowym organem uczestniczącym w rozwoju syndromu metabolicznego jest wątroba, która odgrywa zasadniczą rolę w homeostazie metabolicznej. Jako narząd dokrewny jest częścią sieci, która może wpływać na zdrowie metaboliczne całego organizmu. Po spożyciu pokarmu wątroba przechodzi z konwersji zapasów glikogenu na wychwyt oraz przechowywanie glukozy z krwi. Ta zmiana jest stymulowana przez hormon anaboliczny, insulinę, który jest uwalniany przez komórki beta trzustki w odpowiedzi na wysoki poziom glukozy we krwi po posiłku. Insulina jest niezbędna do utrzymania poziomu glukozy we krwi, w ściśle kontrolowanym zakresie [33]. Hormon ten aktywuje transporter glukozy, dzięki czemu przenika ona do wnętrza komórki. Co więcej, przewlekłe zapalenie

związane z otyłością trzewną – charakteryzujące się produkcją nieprawidłowych adipokin i cytokin, takich jak TNF-α, FFA , IL-1, IL-6, leptyna i rezystyna – wywołuje w wątrobie insulinooporność, poprzez hamowanie sygnalizacji insulinowej w hepatocytach [34]. W przebiegu chorób metabolicznych takich, jak otyłość, insulinooporność, cukrzyca typu 2 czy NAFLD, zauważono podwyższony poziom białka fetiuna-A czyli alfa-2-HS-glikoproteiny wydzielanej przez tkankę tłuszczową i wątrobę [35]. Wykazano, że fetuina-A moduluje receptor insulinowej kinazy tyrozynowej w wątrobie poprzez wiązanie się z jego podjednostką β, zapobiegając w ten sposób jego autofosforylacji, która bezpośrednio hamuje aktywność kinazy tyrozynowej [36]. Fetuina-A może również pośredniczyć w przewlekłym zapaleniu poprzez szlak receptora TLR4, prowadząc do zaostrzenia IR i późniejszego rozwoju NAFLD [37]. Badania wykazały, że nadekspresja fetuiny-A indukuje lipotoksyczność i dalszą insulinooporność w komórkach β, poprzez nasilenie aktywacji receptora TLR4, w którym udział biorą wolne kwasy tłuszczowe; a także pośredniczy w uwalnianiu mediatorów prozapalnych poprzez indukcję jądrowego czynnika κB (NF-κB), który dereguluje sygnalizację insulinową [38,39].

Biorąc pod uwagę stopień zaangażowania tkanki tłuszczowej i wątroby w rozwój chorób metabolicznych, do badań w ramach realizowanej pracy doktorskiej wykorzystano komórki ASC izolowane od koni ze zdiagnozowanym syndromem metabolicznym. Jako alternatywę dla pierwotnych hodowli komórkowych hepatocytów, wykorzystano hodowle linii komórkowych pochodzących z ludzkiego raka wątroby (HepG2), które wykazują typowe cechy morfologiczne hepatocytów oraz wyrażają specyficzne dla nich markery genetyczne. w badaniach wykorzystano model linii HepG2 z indukowaną insulinoopornością.

1.4 Prewencja i leczenie syndromu metabolicznego u koni

Choroby metaboliczne to rosnące zagrożenie wśród ludzi i zwierząt. Dotychczas jedyną metodą prewencji jest odpowiednia dieta i wysiłek fizyczny. W związku z tym, postępowanie dietetyczne jest obecnie interwencją pierwszego wyboru w leczeniu koni cierpiących na tę chorobę. Protokół żywieniowy dla koni z syndromem metabolicznym powinien obejmować niski indeks glikemiczny i wysoką zawartość błonnika oraz niską zawartość węglowodanów niestrukturalnych (NSC). Dieta koni EMS powinna być oparta na paszy o niskiej lub średniej zawartości węglowodanów niestrukturalnych o zawartości poniżej 10% [40]. Przeciążenie NCS może przyczynić się do poważnych powikłań, jak ochwat,

IR czy kolka. Węglowodany w żywieniu koni można podzielić na trzy grupy: (i) cukry proste, (ii) sacharoza, skrobia, fruktany oraz (iii) węglowodany strukturalne (pektyny, celulozy i hemicelulozy). Węglowodany mogą być trawione w jelicie cienkim jako monosacharydy lub mogą być fermentowane w jelicie grubym, co prowadzi do produkcji lotnych kwasów tłuszczowych lub kwasu mlekowego. Ponadto wykazano, że obfita produkcja mleczanu przyczynia się do rozwoju ochwatu [41].

Wysoki poziom krążącej glukozy ze strawionych NCS stymuluje trzustkę do wydzielania dużej ilości insuliny, prowadząc w konsekwencji do rozwoju IR i/lub ochwatu. W ten sposób obniżenie poziomu NSC w diecie konia pomaga w utrzymaniu równowagi energetycznej. Utrzymanie rygoru kalorycznego jest czasochłonne i wymaga od właściciela dużego zaangażowania w przygotowywanie posiłków oraz kontrolę czasu wypasu na pastwisku.

Równocześnie zalecana jest regularna aktywność ruchowa, która nie tylko przyczynia się do redukcji masy ciała, ale także poprawia wrażliwość na insulinę [42]. Wykazano, że ograniczenia dietetyczne w połączeniu z ćwiczeniami również zwiększają tę wrażliwość u otyłych kucyków opornych na insulinę, jednak treningi o niskiej intensywności i bez ograniczeń dietetycznych mogą nie być wystarczające [43,44].

W leczeniu EMS coraz częściej wykorzystuje się leki stosowane w medycynie ludzkiej. Najczęstszym przykładem jest chlorowodorek metforminy stosowany jako doustny lek przeciwcukrzycowy w leczeniu cukrzycy typu 2, szczególnie, jeśli towarzyszy jej nadwaga lub otyłość. Badania przeprowadzone przez Durhama potwierdziły, że podstawowe stężenie insuliny zmniejszało się z czasem, po podaniu doustnym chlorowodorku metforminy w dawce 15 mg/kg co 12 godzin [3]. Natomiast, biodostępność metforminy po podaniu doustnym jest ograniczona u koni i nie poprawia wrażliwości na insulinę [45]. Obecnie prowadzone są również badania nad zastosowaniem takich leków jak: lewotyroksyna, która przyspiesza utratę masy ciała, pioglitazon stosowany w leczeniu cukrzycy typu 2 u ludzi, inhibitory kotransportera sodowo-glukozowego 2 (SGLT2) czy inhibitora receptora słodkiego smaku (T1R2/3). W większości rezultaty prowadzonych badań nie są zadowalające, a farmakoterapia EMS nie może być stosowana jako substytut terapii dietetycznej i ćwiczeń fizycznych [3].

Analizując skomplikowany schemat leczenia EMS, powinno zwrócić się szczególną uwagę na konieczność stosowania prewencyjnych rozwiązań, które mogłyby uchronić konie będące w grupie ryzyka. Pod uwagę należy wziąć fakt, iż insulinooporność jest jedną z głównych składowych syndromu metabolicznego [46]. Natomiast stres oksydacyjny został

uznany za kluczowy mechanizm w insulinooporności [47]. Stres oksydacyjny jest definiowany przez nadmiar endogennych form oksydacyjnych, które zarówno uszkadzają komórki, jak i manipulują szlakiem sygnałowym.

Naturalny okazuje się fakt, iż coraz więcej badań poświęconych prewencji syndromu metabolicznego u ludzi i koni skupia się nad wykorzystaniem bioaktywnych substancji łagodzących lub zapobiegających insulinooporności, poprzez modulowanie stresu oksydacyjnego w komórce.

1.5 Bioaktywne substancje pozyskiwane z mikroorganizmów

Mikroorganizmy, takie jak bakterie, grzyby, ale także rośliny, wytwarzają metabolity wtórne, które charakteryzują się szerokim spektrum aktywności biologicznej. Rośliny i drobnoustroje są głównym źródłem naturalnie występujących związków bioaktywnych w wielu zastosowaniach biotechnologicznych. Najwcześniejsza dokumentacja, potwierdzająca stosowanie naturalnych metabolitów w celu poprawy zdrowia u ludzi, pochodzi z Mezopotamii z okresu od 2900 do 2600 p.n.e., natomiast na początku XX wieku około 80% wszystkich leków pozyskiwano ze źródeł roślinnych [48,49]. Dopiero odkrycie penicyliny z *Penicillium notatum* przez Alexandra Fleminga w 1928 roku zmieniło podejście do pozyskiwania bioaktywnych substancji i zapoczątkowało erę wykorzystywania na skalę przemysłową mikroorganizmów, do produkcji substancji dla sektora medycznego, rolniczego, spożywczego oraz badań naukowych [50].

Wykorzystanie mikroorganizmów, jako producentów bioaktywnych substancji, wypiera tradycyjne metody syntezy chemicznej. W porównaniu z metodami chemii syntetycznej, biosynteza drobnoustrojów ma wiele zalet. Po pierwsze, unika stosowania metali ciężkich, rozpuszczalników organicznych oraz silnych kwasów i zasad, umożliwiając w ten sposób proces syntezy przebiegający w sposób przyjazny dla środowiska oraz pozwalający uniknąć stosowania wysokich temperatur, wymaganych w wybranych procesach chemicznych. Takie podejście jest nie tylko korzystne ze względów ekologicznych, ale również ekonomicznych. Po drugie, enzymy mają zwykle stosunkowo wysoką specyficzność substratową, co pomaga ograniczyć tworzenie się produktów ubocznych. Po trzecie, niektóre związki o złożonej strukturze mają już naturalne ścieżki syntezy, podczas gdy ustalenie chemicznych ścieżek syntezy tych złożonych związków jest bardzo trudne. Wreszcie inżynieria metaboliczna oferuje sposoby dalszej poprawy wydajności i produktywności docelowego związku [51]. Różnorodność mikrobiologiczna

tworzy ogromną pulę nowych substancji, stanowiących cenne źródło dla innowacyjnej biotechnologii. Istnieje ponad 23 000 znanych metabolitów wtórnych drobnoustrojów, z których 42% są wytwarzane wyłącznie przez promieniowce, podczas gdy 42% jest wytwarzana przez grzyby, a pozostałe 16% przez eubakterie [52]. Metabolity drobnoustrojów stały się już znaczącym źródłem leków ratujących życie, w tym głównie infekcji bakteryjnych i grzybiczych (amfoterycyna, erytromycyna, penicylina, streptomycyna, tetracykliny i wankomycyna), leków przeciwnowotworowych (bleomycyna, doksorubicyna, daunorubicyna i mitomycyna), zmniejszających ryzyko odrzucenia przeszczepu (cyklosporyna i rapamycyna) oraz regulujących poziom cholesterolu (lowastatyna i mewastatyna) [53]. Niektóre z pierwotnych i wtórnych metabolitów mają również specyficzny potencjał przeciwutleniający. Utlenianie to reakcja chemiczna, w wyniku której powstają wolne rodniki (ROS), prowadząc w ten sposób do reakcji łańcuchowych, które w konsekwencji mogą uszkadzać komórki organizmu [54]. Przeciwutleniacze można zdefiniować jako cząsteczki, które w niskich stężeniach opóźniają lub zapobiegają utlenianiu, działając na błony biologiczne lub na poziomie wewnątrzkomórkowym, chroniąc w ten sposób komórki różnych narządów i różnych układów biologicznych. Przeciwutleniaczem jest substancja zdolna do eliminowania ROS i ich pochodnych bezpośrednio lub pośrednio, działająca jako regulator obrony antyoksydacyjnej lub inhibitor wytwarzania reaktywnych form molekularnych [55]. Wolne rodniki to reaktywne formy zawierające jeden lub więcej niesparowanych elektronów. Reaktywne formy tlenu (ROS) i reaktywne formy azotu (RNS) to wolne rodniki, które są związane z atomem tlenu $(O)_2$. Rodniki te są generowane jako produkty uboczne metabolizmu komórkowego i promieniowania jonizującego i należą do nich: anion ponadtlenkowy (O_2^-), nadtlenek wodoru (H_2O_2), rodnik hydroksylowy (OH) i tlen singletowy ($^1O_2$). Co więcej, istnieją inne ważne biologicznie wolne rodniki: wodoronadtlenek lipidów (ROOH), rodnik peroksylowy (ROO) i rodnik alkoksylowy (RO), które są związane z lipidami błonowymi; tlenek azotu (NO), dwutlenek azotu (NO_2) i nadtlenoazotyny (ONOO-), które są reaktywnymi formami azotu; oraz rodnik tiolowy (RS), który ma niesparowany elektron na atomie siarki [56]. Dotychczas zaproponowano dwa podstawowe mechanizmy działania przeciwutleniaczy: pierwszy jako mechanizm zrywania łańcucha oksydacyjnego, dzięki któremu główny przeciwutleniacz przekazuje elektron wolnemu rodnikowi. Drugi mechanizm obejmuje usuwanie inicjatorów ROS/RNS (drugorzędowych przeciwutleniaczy) przez wygaszanie katalizatora inicjującego łańcuch [57]. Przeciwutleniacze mogą wywierać wpływ na systemy biologiczne poprzez różne mechanizmy, w tym oddawanie elektronów, chelatację jonów

metali, współprzeciwutleniacze lub regulację ekspresji genów [58]. Do fizjologicznych funkcji organizmu potrzebna jest niewielka ilość reaktywnych form tlenu (ROS), natomiast ich nadmiar powoduje uszkodzenia oksydacyjne, negatywnie wpływając na DNA i struktury białkowe komórki oraz powodując peroksydację lipidów błon komórkowych [59]. Brak równowagi pomiędzy ilością wolnych rodników i antyoksydantów prowadzi do rozwoju stresu oksydacyjnego w komórce. U zwierząt gospodarskich istnieje dobrze zdefiniowana korelacja między wystąpieniem zatrzymania łożyska, ochwatu czy zapalenia macicy, a obniżeniem stanu antyoksydacyjnego. Stres oksydacyjny jest również powiązany z wieloma zaburzeniami patologicznymi, które negatywnie wpływają na stan zdrowia, dobrostan i parametry produkcyjne zwierząt [60].

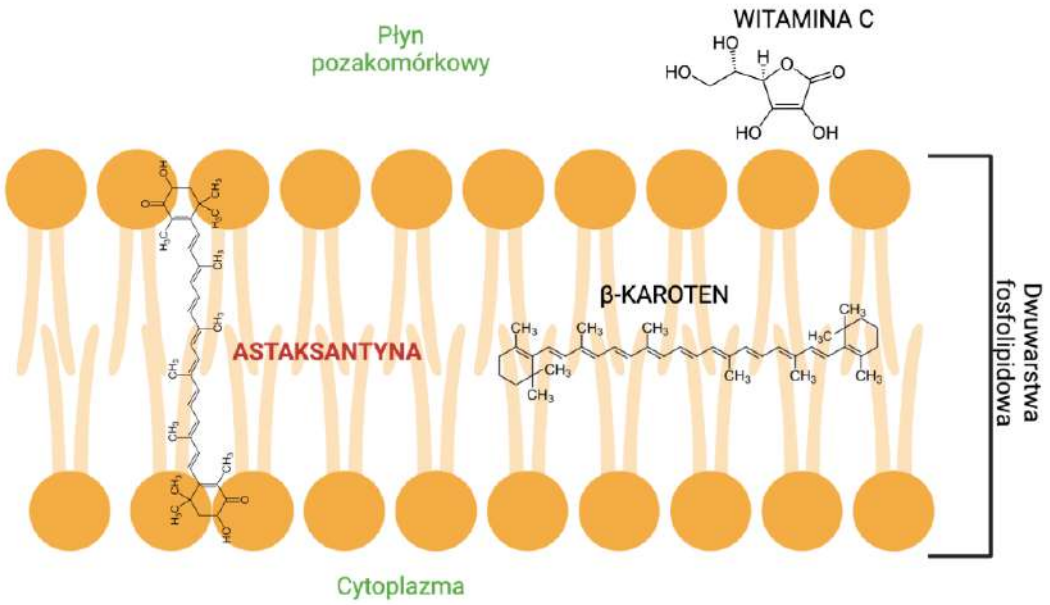
W związku z obecnym trendem w formułowaniu diet ze składnikami bogatymi w wielonienasycone kwasy tłuszczowe (PUFA), które są wrażliwe na peroksydację lipidów, wzrosło zapotrzebowanie na stosowanie antyoksydantów w żywieniu zwierząt. Doskonałym przykładem wykorzystania mikroorganizmów do syntezy antyoksydantów są drożdże *Phaffia rhodozyma,* produkujące jeden z najsilniejszych naturalnych przeciwutleniaczy – astaksantynę.

1.6 Astaksantyna

Astaksantyna jest karotenoiem ksantofilowym, metabolitem wtórnym naturalnie syntetyzowanym głownie przez mikroalgi i drożdże. Cieszy się dużym zainteresowaniem ze względu na jej potencjalne działanie farmakologiczne, w tym działanie przeciwnowotworowe, przeciwcukrzycowe, przeciwzapalne i antyoksydacyjne, a także działanie ochronne względem systemu nerwowego, sercowo-naczyniowego, wzroku i skóry [61]. Naturalnym źródłem astaksantyny są algi *Haematococcus pluvialis*, drożdże *Phaffia rhodozyma*, łosoś, pstrąg, kryl, krewetki i raki [62]. Karotenoidy są wchłaniane przez organizm podobnie jak lipidy i transportowane przez układ limfatyczny do wątroby. Ich wchłanianie jest uzależnione od towarzyszących składników diety. To właśnie dieta wysokocholesterolowa może zwiększać wchłanianie karotenoidów, podczas gdy dieta niskotłuszczowa zmniejsza ich dostępność. Astaksantyna po spożyciu z kwasem żółciowym tworzy micele w jelitach, które częściowo są wchłaniane przez komórki śluzówki jelit. Komórki błony śluzowej jelit włączają astaksantynę do chylomikronów, które są trawione przez lipazę lipoproteinową po uwolnieniu do limfy w krążeniu ogólnoustrojowym, następnie chylomikrony są usuwane przez wątrobę i inne tkanki,

natomiast astaksantyna jest asymilowana z lipoproteinami i transportowana do tkanek [63,64]. Spośród kilku naturalnie występujących karotenoidów astaksantyna jest uważana za jeden z najsilniejszych karotenoidów zdolnych do ochrony komórek, lipidów i lipoprotein błonowych przed uszkodzeniem oksydacyjnym [65].

Astaksantyna (3,3′-dihydroksy-β,β′-karoten-4,4′-dion) w swojej strukturze zawiera dwa pierścienie β-jononowe, połączone łańcuchem polienowym oraz utlenione ugrupowania ketonowe i hydroksylowe [66]. Astaksantyna charakteryzuje się czerwonym kolorem, który wynika z obecności podwójnych sprzężonych wiązań w centrum związku. Ten rodzaj wiązania działa jako silny przeciwutleniacz, oddając elektrony i reagując z wolnymi rodnikami, w celu przekształcenia ich w bardziej stabilny produkt i zakończenia reakcji łańcuchowej wolnych rodników. Astaksantyna może również wychwytywać wolne rodniki w jej końcowym ugrupowaniu pierścieniowym. Zasugerowano, że atom wodoru przy C3 jest miejscem wychwytywania rodników. Ponieważ astaksantyna wykazuje zarówno właściwości lipofilowe, jak i hydrofilowe, cząsteczka ta może być umiejscowiona zarówno wewnątrz jak i na zewnątrz komórki, gdzie może zmiatać rodniki z powierzchni komórki i wnętrza błony fosfolipidowej. Ta cecha sprawia, że astaksantyna jest wyjątkowa w porównaniu do innych przeciwutleniaczy, takich jak β-karoten czy witamina C, które mogą znajdować się odpowiednio tylko wewnątrz lub na zewnątrz dwuwarstwowej błony lipidowej (ryc.3) [62,67].



Rycina 3. Lokalizacja astaksantyny, β-karotenu i witaminy C w błonie komórkowej [źródło własne na podstawie [68]].

Błony komórkowe to struktury szczególnie wrażliwe na działanie wolnych rodników ze względu na wysoką zawartość wielonienasyconych kwasów tłuszczowych (PUFA) oraz ich aktywność metaboliczną, która endogennie generuje inne metabolity utleniające [69]. Astaksantyna chroni błony komórkowe przed ROS i uszkodzeniami oksydacyjnymi. Ze względu na swoją strukturę chemiczną, jej grupy polarne zachodzą na obszary polarne błony komórkowej, podczas gdy centralny obszar niepolarny cząsteczki wpasowuje się w wewnętrzny obszar niepolarny błony. Astaksantyna zmiata wolne rodniki tlenowe i inne reaktywne formy (siarka i węgiel), zarówno poprzez oddawanie elektronów, jak i wiązanie się z wolnym rodnikiem, tworząc produkt niereaktywny. Ponadto obecność szeregu wiązań sprzężonych w centralnym niepolarnym regionie astaksantyny umożliwia cząsteczce usuwanie wolnych rodników (elektronów o wysokiej energii) z wnętrza komórki poprzez ich transport wzdłuż własnego łańcucha węglowego [70]. Co ciekawe, astaksantyna skuteczniej niż β-karoten zapobiega peroksydacji lipidów oraz wykazała działanie dwukrotnie skuteczniejsze niż β-karoten w hamowaniu peroksydacji liposomów indukowanej przez ADP i Fe^{2+}.

Przeprowadzono wiele badań nad bezpieczeństwem stosowania astaksantyny u ludzi i zwierząt, w efekcie których wykazano wysokie bezpieczeństwo jej stosowania oraz brak skutków ubocznych lub toksyczności podczas kumulacji w tkankach [71]. Niemniej jednak, nadmierne spożycie astaksantyny może prowadzić do zmiany pigmentacji skóry zwierząt. Nadmierna suplementacja astaksantyną skutkowała zwiększeniem enzymów antyoksydacyjnych i obniżonym ciśnieniem krwi u szczurów z nadciśnieniem [72]. Jako dodatek do pasz, Amerykańska Agencja ds. Żywności i Leków (FDA) zatwierdziła dawkę astaksantyny do 80 mg/kg, podczas gdy Europejski Urząd ds. Bezpieczeństwa Żywności (EFSA) do 100 mg/kg [73].

Ostatnie badania kliniczne wykazały jednak korzystny wpływ na organizm przy dawkach do 8 mg na dobę lub nawet wyższych. W raporcie dotyczącym bezpieczeństwa stosowania badacze ocenili ponad 80 badań klinicznych w celu wykrycia skutków ubocznych i obaw związanych z bezpieczeństwem astaksantyny. Ich raport wykazał, że w żadnym z ocenianych badań nie odnotowano poważnych skutków niepożądanych, nawet w badaniach, w których podawano wysokie dawki astaksantyny (do 45 mg). Zgłoszono tylko pewne łagodne zdarzenia niepożądane. Ponadto, w żadnym z badań nie stwierdzono wykrywalnej zmiany parametrów wątrobowych [73].

Według Grand View Research, wielkość światowego rynku astaksantyny w 2020 roku została wyceniona na 1371,24 mln USD i oczekuje się, że w latach 2021-2028 będzie rosła w średnim rocznym tempie wzrostu (CAGR) 16,8%. Kluczowym czynnikiem napędzającym ten wzrost jest duże zapotrzebowanie na nutraceutyki, czyli substancje biologicznie czynne, które korzystnie wpływają na organizm. Wynika to z ich właściwości przeciwutleniających oraz zwiększonego zapotrzebowania w akwakulturze i sektorze produkcji pasz dla zwierząt. Szacuje się, że segment nutraceutyków odnotuje najszybszy wzrost w prognozowanym okresie ze względu na lepsze właściwości przeciwutleniające astaksantyny w porównaniu z innymi suplementami diety, łatwiejszą dostępność i szerszy zakres zastosowań w przemyśle nutraceutyków. Segment akwakultury i pasz dla zwierząt zdominował rynek w 2020 r. z udziałem w przychodach wynoszącym ponad 47% i przewiduje się, że w okresie prognozy będzie rosnąć na stałym poziomie CAGR, ze względu na jego szerokie zastosowanie jako dodatku paszowego [74].

Karotenoidy, w tym astaksantyna, zyskały szczególne zainteresowanie w ciągu ostatnich dziesięcioleci ze względu na ich silne działanie przeciwutleniające, naprawcze, przeciwzapalne i potencjalne działanie przeciwstarzeniowe. Mogą być stosowane do zapobiegania chorobom związanym ze stresem oksydacyjnym i przewlekłym stanem zapalnym, a astaksantyna jest obecnie jednym z najsilniejszych karotenoidów dostępnych komercyjnie na rynku. Badania przeprowadzone przez Nishida i in. wykazały, że suplementacja astaksantyną skutecznie stymulowała biogenezę mitochondriów i znacząco poprawiała insulinooporność poprzez aktywację szlaku AMPK w komórkach mięśni szkieletowych [75]. Kolejne badania wykazały iż, suplementacja astaksantyny u szczurów (w dawce 50 mg/kg/dzień) przez 22 tygodni wywołała znaczące obniżenie ciśnienia tętniczego oraz poziomu glukozy we krwi na czczo, wskaźnika homeostazy insulinooporności (HOMA-IR) jak również poprawiła wrażliwość na insulinę [76]. Kolejno, badania przeprowadzone przez Zhuge i in. wykazały, że suplementacja astaksantyną (w dawce 30 mg/kg/dzień) przez 3 tygodnie znacząco obniżyła poziom glukozy we krwi i cholesterolu całkowitego oraz podwyższyła poziom cholesterolu lipoprotein o wysokiej gęstości we krwi u szczurów z cukrzycą [77].

1.7 Postbiotyki

Najnowsze badania z udziałem ludzi sugerują, że zmiany w mikrobiomie jelitowym – społeczności mikroorganizmów występujących w jelicie grubym – mogą przyczyniać się do rozwoju wielu zaburzeń ogólnoustrojowych.

Obecnie, jako główne sposoby modulowania mikroflory jelitowej uznaje się stosowanie: prebiotyków, probiotyków, synbiotyków lub postbiotyków. Prebiotyki są wykorzystywane przez mikroorganizmy jako pokarm, a jednocześnie mogą wywierać korzystny wpływ na zdrowie gospodarza [78]. Jako źródła prebiotyków można wymienić wybrane owoce i warzywa, natomiast sztucznie produkowane prebiotyki to m.in.: laktuloza, galaktooligosacharydy, fruktooligosacharydy, maltooligosacharydy, cyklodekstryny, laktosacharoza. Laktuloza stanowi znaczną część produkowanych oligosacharydów (aż 40%). Uważa się, że fruktany, takie jak inulina i oligofruktoza, są najczęściej stosowane i skuteczne w odniesieniu do wielu gatunków probiotyków [79]. Probiotyki to selektywnie dostarczane do przewodu pokarmowego pożyteczne mikroorganizmy, które według Organizacji ds. Wyżywienia i Rolnictwa/Światowej Organizacji Zdrowia to „Żywe mikroorganizmy, które podawane w odpowiednich ilościach, przynoszą korzyści zdrowotne gospodarzowi” [80]. Ponadto, mikroorganizmy probiotyczne to głownie bakterie kwasu mlekowego (LAB), które są ujęte w kategorii „ogólnie uznane za bezpieczne (GRAS)” przez amerykańską Agencję ds. Żywności i Leków (FDA) [81]. Pomimo kilku metaanaliz potwierdzających kliniczną skuteczność probiotyków w różnych chorobach (m.in. ostrej infekcji przewodu pokarmowego i nieswoistych zapaleniach jelit), poszczególne doniesienia coraz częściej podważają ich skuteczność i bezpieczeństwo, zwłaszcza u pacjentów wysokiego ryzyka. W związku z tym rośnie zainteresowanie zastępczą grupą preparatów: postbiotykami [78].

Postbiotyki, to każda substancja uwalniana lub wytwarzana w wyniku aktywności metabolicznej mikroorganizmu, która wywiera korzystny (bezpośredni lub pośredni) wpływ na gospodarza. Mogą zawierać one wiele różnych składników, w tym metabolity, krótkołańcuchowe kwasy tłuszczowe (SCFA), frakcje komórek drobnoustrojów, białka funkcjonalne, pozakomórkowe polisacharydy (EPS) czy enzymy. Postbiotyki to grupa substancji obejmująca wszystkie substancje pochodzenia bakteryjnego/grzybicznego o korzystnym wpływie na żywiciela [82].

Zdolność postbiotyku, który może być niejednorodną mieszaniną składników, do wywoływania skutków zdrowotnych u docelowego gospodarza może być napędzana przez

wiele różnych mechanizmów. W niektórych przypadkach mechanizmy te mogą być podobne do tych opisanych w przypadku probiotyków. Ponieważ postbiotyki są nieożywione, te bioaktywne cząsteczki muszą zostać zsyntetyzowane przez mikroorganizmy progenitorowe przed inaktywacją i w ilościach wystarczających do wywołania korzystnego efektu zdrowotnego. Postuluje się pięć mechanizmów działania postbiotyków (ryc.4):

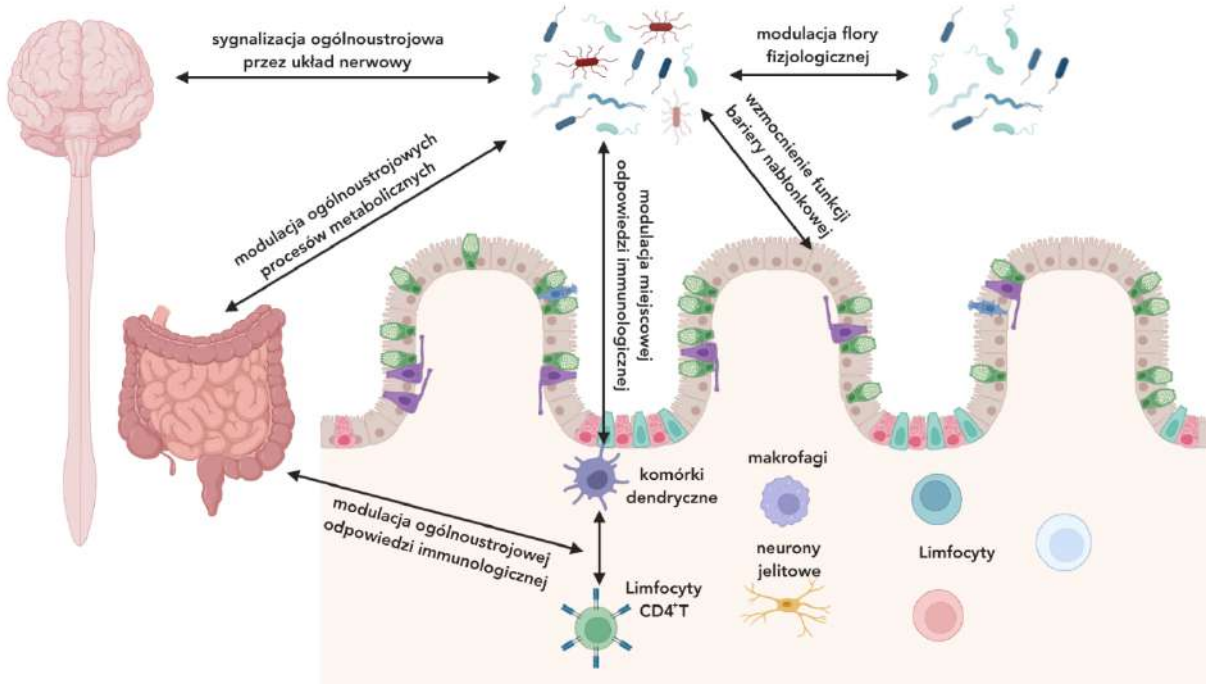
(1) modulacja rezydentnej mikroflory,

(2) wzmocnienie funkcji bariery nabłonkowej,

(3) modulacja lokalnych i ogólnoustrojowych odpowiedzi immunologicznych,

(4) modulacja ogólnoustrojowych odpowiedzi metabolicznych,

(5) ogólnoustrojowa sygnalizacja przez układ nerwowy [83].

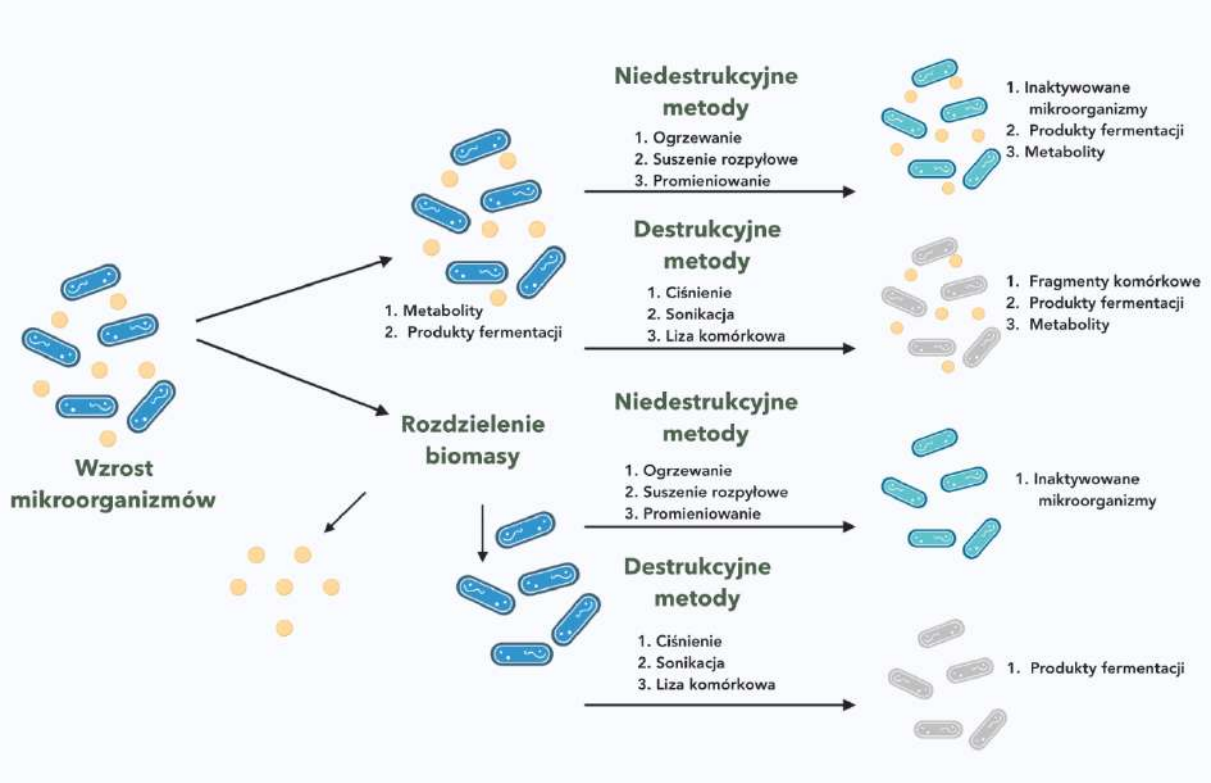


Rycina 4. Mechanizmy oddziaływania postbiotyków [źródło własne na podstawie [83]].

Postbiotyki można podzielić na trzy główne podgrupy, do pierwszej z nich należą: metabolity oraz substancje pochodzące z drobnoustrojów jak enzymy, białka, peptydy, kwasy organiczne, witaminy, minerały, bakteriocyny, peptydy przeciwdrobnoustrojowe i pęcherzyki zewnątrzkomórkowe (EV) czy egzopolisacharydy. Do drugiej podgrupy należą cząsteczki, które kształtują strukturę komórki, w tym składniki ściany komórkowej, takie jak polimery kwasu, peptydoglikany, muropeptydy pochodzące z peptydoglikanów, frakcje

powierzchniowe komórek. Trzecia grupa to bezkomórkowe ekstrakty i lizaty, supernatanty z hodowli lub biosurfaktanty (związane ze ścianą komórkową) [84].

Do ekstrakcji i oczyszczania postbiotyków wykorzystuje się liczne i zróżnicowane techniki (ryc. 5). Muszą one prowadzić do dezintegracji błony komórkowej i zakłócać integralność komórki, aby odzyskać zawartość wewnątrzkomórkową. Wśród stosowanych technik znajdują się: ekstrakcja rozpuszczalnikiem, odbiałczanie i wytrącanie, rozdzielanie przez elektroforezę i analiza za pomocą chromatografii cieczowej lub sonikacji oraz hydrofobowa chromatografia gradientowa. W odniesieniu do inaktywacji drobnoustrojów można zastosować różne procedury lub techniki, w tym podwyższoną temperaturę, wysokie ciśnienie, napromienianie lub sonikację. W przypadku postbiotyków stanowiących elementy strukturalne komórki utrata żywotności następuje w efekcie ekspozycji na czynniki, które zmieniają strukturę komórki drobnoustrojów (np. pęknięcie DNA, komórka uszkodzenie otoczki) i/lub zmiana ich funkcji fizjologicznych (np. inaktywacja kluczowych enzymów lub dezaktywacja selektywności błony) [85].



Rycina 5. Techniki wykorzystywane do ekstrakcji i oczyszczania postbiotyków [źródło własne na podstawie [86]].

Mikrobiota jelitowa, czyli rozległy i złożony zbiór mikroorganizmów bytujących w przewodzie pokarmowym, może przyczyniać się do rozwoju stanu zapalnego, ale czynniki pochodzące z mikroflory mogą również mieć potencjał przeciwzapalny oraz chroniący m.in. przed insulinoopornością. Dobrym przykładem są wyniki badań uzyskanych w trakcie immunizacji ekstraktami pochodzącymi z flory jelitowej, które sprzyjają tolerancji immunologicznej i zmniejszają insulinooporność wywołaną dietą wysokotłuszczową (HFD) u myszy [87,88]. Postbiotyki, takie jak egzopolisacharyd z *Lactobacillus plantarum* L-14, mogą hamować różnicowanie niedojrzałych komórek w dojrzałe adipocyty, a także kontrolować przyrost masy ciała i profile lipidowe u myszy poprzez regulację szlaku sygnałowego TLR2-AMPK [89]. Poza tym długołańcuchowy polifosforan z *Lactobacillus brevis* może poprawić stan zapalny jelit i funkcję bariery jelitowej poprzez aktywację szlaku sygnałowego kinaz białkowych regulowanych pozakomórkowo (ERK) [90]. Dodatkowo, dipeptyd muramylowy ze ściany komórkowej bakterii był korzystnym postbiotykiem, który mógł łagodzić oporność na insulinę wywołaną otyłością poprzez celowanie w białko 2 zawierające domenę oligomeryzacji wiążącą nukleotydy (NOD2) i czynnik regulujący interferon 4 (IRF4) [87].

Podobnie jak żywe komórki probiotyków, postbiotyki regulują schorzenia metaboliczne poprzez promowanie wytwarzania korzystnych metabolitów bakteryjnych, takich jak octan, propionian, maślan, izowalerianian, kwas mlekowy i palmitoiloetanoloamid. Postbiotyki prowadzą do zwiększenia ekspresji genów związanych ze szlakami sygnałowymi, w których pośredniczą AMPK, ERK, GPR43, IRF4, NOD2 i TLR2, a także do poprawy szlaków metabolicznych tryptofanu-serotoniny przy jednoczesnym obniżeniu ekspresji szlaków sygnałowych związanych z TLR4, NF-κB, NOD1 i TNF-α [91].

Tkanka tłuszczowa (AT) koni EMS charakteryzuje się hiperplazją i hipertrofią. AT jest uważana nie tylko za tkankę magazynującą energię, ale również za bardzo aktywny narząd dokrewny. U koni dotkniętych EMS AT wydziela różne mediatory prozapalne, w tym czynnik martwicy nowotworu α (TNF-α) i interleukiny. Adipocyty nie tylko syntetyzują i montują triglicerydy, ale są również zdolne do uwalniania wolnych kwasów tłuszczowych i glicerolu jako produktów hydrolizy triglicerydów. Wysoki poziom FFA we krwi jest silnie skorelowany z otyłością i insulinoopornością. Rozrost tkanki tłuszczowej prowadzący do otyłości związany jest z dysfunkcją endokrynnych adipocytów, a nadmierna podaż kalorii wyzwala odpowiedź zapalną w adipocytach i ich dysfunkcję poprzez działanie cytokin, takich jak TNFα, który zmniejsza sygnalizację insulinową i hamuje adipogenezę. Komórki ASC wyizolowane z koni EMS mają ograniczony potencjał proliferacyjny, zwiększone

tempo starzenia się, apoptozę, nadmierną akumulację ROS i pogorszenie stanu mitochondriów. Dysfunkcja ASC prowadzi do nieprawidłowej przebudowy tkanki tłuszczowej, co wiąże się z większym ryzykiem zaburzeń metabolicznych.

Syndrom metaboliczny koni to coraz częstsze schorzenie endokrynologiczne, które swoją kulminację kliniczną osiąga w rozwoju ochwatu - choroby często wymagającej eutanazji zwierzęcia. U ludzi cierpiących na insulinooporność, wątroba stanowi kluczowy organ inicjujący systemowy stan zapalny, podczas gdy u koni zaburzenia metabolizmu wątroby są wciąż mało poznanym procesem. Zaburzenia metabolizmu wątroby u koni związane z EMS charakteryzują się niespecyficznym nagromadzeniem się lipidów w hepatocytach, zwiększoną ekspresją aminotransferazy aspartazy (AST) oraz gamma-glutamylotranspeptydaza (GGTP) prowadząc do rozwoju stresu siateczki śródplazmatycznej, podwyższonego stresu oksydacyjnego oraz zapalenia co w konsekwencji skutkuje rozwojem insulinooporności.

Obecnie, poza zaleceniami restrykcji kalorycznych oraz wzmożoną aktywnością ruchową, nie istnieją skuteczne farmakologiczne metody leczenia EMS. Skuteczna prewencja EMS ma kluczowe znaczenie, a dotychczasowe doniesienia potwierdzają znaczący wpływ silnych przeciwutleniaczy oraz stymulacji mikrobiomu jelitowego jako kluczowych czynników w prewencji syndromu metabolicznego. Konieczne jest poznanie mechanizmów molekularnych zaangażowanych niwelowanie rozwoju tych jednostek chorobowych.

1.8 Cel pracy

Celem badań realizowanych w ramach przedstawionej pracy doktorskiej była ocena wpływu bioaktywnych substancji pochodzenia mikrobiologicznego: ekstraktów astaksantyny z biomasy drożdży *Phaffia rhodozyma* oraz emulsji pro- i postbiotycznych, które wykazują działanie uwrażliwiające na insulinę komórki progenitorowe tkanki tłuszczowej (ASC) izolowane od koni cierpiących na syndrom metaboliczny oraz komórki HepG2 z indukowaną insulinoopornością.

Wśród celów szczegółowych przedstawionej pracy należy wymienić:

1. Optymalizację wzrostu drożdży *Phaffia rhodozyma* oraz pozyskiwania i izolacji astaksantyny,
2. Optymalizację wzrostu szczepów probiotycznych i formulacja emulsji micelarnej,

3. Ocenę wpływu astaksantyny na funkcje komórkowe i dynamikę mitochondriów, poprzez zmniejszenie stresu oksydacyjnego w ASC (EMS),
4. Ocenę wpływu emulsji bogatej w probiotyki na zmniejszenie insulinoopornosci indukowanej przez akumulację wolnych kwasów tłuszczowych w linii komórkowej HepG2 oraz wyjaśnienie powiązanych szlaków molekularnych, ze szczególnym uwzględnieniem szlaku Fetuiny-A.

1.9.1 Hipoteza badawcza

Hipoteza badawcza niniejszej pracy zakłada, że preinkubacja komórek ASC (izolowanych od koni cierpiących na EMS) z astaksantyną, obniżać będzie apoptozę, łagodzić stres oksydacyjny oraz usprawniać mitochondrialny system fosforylacji oksydacyjnej (OXPHOS), a tym samym kontrolować i niwelować stres oksydacyjny, poprawiając w ten sposób stan metaboliczny ASCs. Natomiast preinkubacja komórek HepG2 z indukowaną insulinoopornością *in vitro,* wraz z emulsją pro- i postbiotyczną, w warunkach IR, będzie chronić komórki przed apoptozą, łagodzić stres oksydacyjny, a także poprawiać metabolizm i dynamikę mitochondriów oraz zmniejszać aktywację szlaku *Fetuin-A/TLR4/JNK/NF-κB* . Zakłada się, że zastosowana emulsja pro- i postbiotyczna będzie wykazywała działanie ochronne przed zapaleniem, otyłością oraz insulinoopornością związaną z funkcją wątroby.

1.9.2 Zadania badawcze

Przeprowadzenie badań *in vitro* z użyciem komórek ASCs izolowanych od koni obarczonych syndromem metabolicznym, na liniach komórkowych HepG2 z indukowaną insulinoopornością, poprzedzały badania polegające na pozyskiwaniu bioaktywnych substancji z biomasy drożdżowej oraz bakteryjnej, które obejmowały:

1. Optymalizację wzrostu drożdży *Phaffia rhodozyma* oraz akumulacji i izolacji astaksantyny,
2. Optymalizację wzrostu bakterii kwasu mlekowego oraz procesu technologicznego pozyskiwania pro- i postbiotycznej emulsji,
3. Ocenę wpływu astaksantyny na funkcje komórkowe i dynamikę mitochondriów, poprzez zmniejszenie stresu oksydacyjnego w EMS ASCs,

4. Ocenę wpływu emulsji bogatej w probiotyki na zmniejszenie IR indukowanej przez akumulację wolnych kwasów tłuszczowych w ludzkiej linii komórkowej raka wątroby oraz wyjaśnienie powiązanych szlaków molekularnych, ze szczególnym uwzględnieniem szlaku Fetuiny-A.

2. ARTYKUŁ NAUKOWY I PRZEGLĄDOWY – ASTAKSANTYNA

Astaksantyna jest silnym przeciwutleniaczem, suplementem odżywczym, a także obiecującym związkiem terapeutycznym, który wykazuje działanie przeciwko różnym wyniszczającym chorobom i zaburzeniom. Naukowcy potwierdzili korzystne działanie astaksantyny przeciwko wybranym niezakaźnym chorobom przewlekłym, takim jak choroby sercowo-naczyniowe, nowotworowe, cukrzyca, zaburzenia neurodegeneracyjne i immunologiczne. Astaksantyna wykazuje aktywność antyoksydacyjną około 100-500 razy wyższą niż inne antyoksydanty, takie jak α-tokoferol i β-karoten. Stres oksydacyjny jest głównym czynnikiem przyczyniającym się do patogenezy różnych chorób. Zwiększony stres oksydacyjny może uszkodzić mitochondria, a późniejsza dysfunkcja mitochondriów generuje nadmiar mitochondrialnych reaktywnych form tlenu, które powodują uszkodzenia komórek. Dysfunkcja mitochondriów aktywuje również mitochondrialny szlak apoptotyczny, powodując śmierć komórki. Astaksantyna jako jeden z najsilniejszych naturalnych antyoksydantów wywiera działanie przeciwutleniające i przeciwzapalne na różne linie komórkowe. W ten sposób astaksantyna utrzymuje integralność mitochondriów w różnych stanach patologicznych.

W ramach przygotowań do prowadzonych badań nad wpływem astaksantyny na metabolizm komórek przygotowany został artykuł przeglądowy: „Astaxanthin and other Nutrients from Haematococcus pluvialis-Multifunctional Applications". Praca nad tym artykułem stanowiła inspirację do oceny astaksantyny jako czynnika modulującego zaburzony metabolizm komórek ASC izolowanych od koni obarczonych syndromem metabolicznym. W rezultacie opublikowany został artykuł naukowy: „Astaxanthin Carotenoid Modulates Oxidative Stress in Adipose-Derived Stromal Cells Isolated from Equine Metabolic Syndrome Affected Horses by Targeting Mitochondrial Biogenesis". Uzyskane wyniki, potwierdziły założoną hipotezę badawczą i wykazały obiecujące zdolności astaksantyny do poprawy zaburzonego metabolizmu komórkowego ASC, zapobiegniu apoptozy, zmniejszenia stresu oksydacyjnego i odwrócenia dysfunkcji mitochondriów, które są kluczowym elementem w rozwoju EMS i związane są z zaburzeniami właściwości regeneracyjnych komórek ASC. Ponadto, wyniki badań dotyczące modulacji dynamiki mitochondrialnej i OXPHOS poprzez traktowanie ASC astaksantyną, dostarczają nowych informacji na temat leczenia insulinooporności i otyłości. Zebrane w artykule przeglądowym dane oraz przeprowadzone badania, których wyniki ujęto w poniższym artykule naukowym wskazują na korzystne działanie modulujące

metabolizm komórek z insulinoopornością i stresem oksydacyjnym. Co więcej, rosnące zapotrzebowanie rynku na naturalne dodatki żywieniowe, które stanowią prewencję chorób metabolicznych wśród zwierząt gospodarskich i towarzyszących stale rośnie. Biorąc to pod uwagę, uzyskane wyniki stanowią podstawę do prowadzenia dalszych badań, a w efekcie komercjalizacji suplementu dla zwierząt.

marine drugs

MDPI

Review

Astaxanthin and other Nutrients from *Haematococcus pluvialis*—Multifunctional Applications

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Abstract: Bioactive compounds of natural origin are gaining increasing popularity. High biological activity and bioavailability, beneficial effects on health and safety of use are some of their most desirable features. Low production and processing costs render them even more attractive. Microorganisms have been used in the food, medicinal, cosmetic and energy industries for years. Among them, microalgae have proved to be an invaluable source of beneficial compounds. *Haematococcus pluvialis* is known as the richest source of natural carotenoid called astaxanthin. In this paper, we focus on the cultivation methods of this green microalga, its chemical composition, extraction of astaxanthin and analysis of its antioxidant, anti-inflammatory, anti–diabetic and anticancer activities. *H. pluvialis*, as well as astaxanthin can be used not only for the treatment of human and animal diseases, but also as a valuable component of diet and feed.

Keywords: *Haematococcus pluvialis*; microalgae; astaxanthin; applications

1. Introduction

Nowadays microalgae are gaining in popularity not only because of their high nutrient content, but also because of the promotion of a healthy diet [1]. The employment of microalgae on an industrial scale began in 1950, when Burlew proposed the use of microalgae as an alternative source of protein for plants and animals. Since then, algae cultivation has become more common, not only for the application in the food industry, but also for animal and aquaculture feed purposes [2–5]. Microalgae, as a source of active biomolecules, are used in the pharmaceutical and cosmetics industry [2,3]. Algae are gaining also a lot of attention in terms of the production of biodegradable plastics and biofuels [2,6,7].

The application of microalgae on an industrial scale is facilitated by the rapid growth of biomass, the conversion of CO_2 in the process of photosynthesis, not requiring environmental conditions, and huge demand in food and energy production [8]. Microalgae can occur in oceans, rivers or lakes [9]. *Haematococcus pluvialis* can grow in particularly difficult conditions, such as the arctic waters of the White Sea [6].

Researchers are continuously confirming the beneficial effects of microalgae components and their biological properties (e.g., antioxidant and anti-inflammatory) [10]. Among those components, starch, cellulose and β-1,3 glucan are considered as the most important polysaccharides. These substances are used in the pharmaceutical and cosmetics industries as well as in dietary supplements [3,5,11]. The same goes for lipids such as hydrocarbons and polyunsaturated fatty acids. Many of the bioactive compounds which have strong antibacterial, antifungal and antiviral properties have found application in the production of vaccines, antibiotics, agrochemicals and cosmetics [12]. Proteins such as enzymes,

amino acids and polypeptides are used in the food and pharmaceutical industries, whereas pigments including carotenoids or chlorophylls are widely employed in the food technology, chemical and pharmaceutical industries [13].

Among many species of microalgae, one of the most important is *Haematococcus pluvialis*, which is a source of one of the strongest natural antioxidants, which is astaxanthin [14]. This microalga has very wide applications, which are shown in Figure 1a. This figure summarizes the number of publications describing the potential use of *H. pluvialis* (Figure 1a), as well as its high-value molecule-astaxanthin (Figure 1b), according to the Web of Science database.

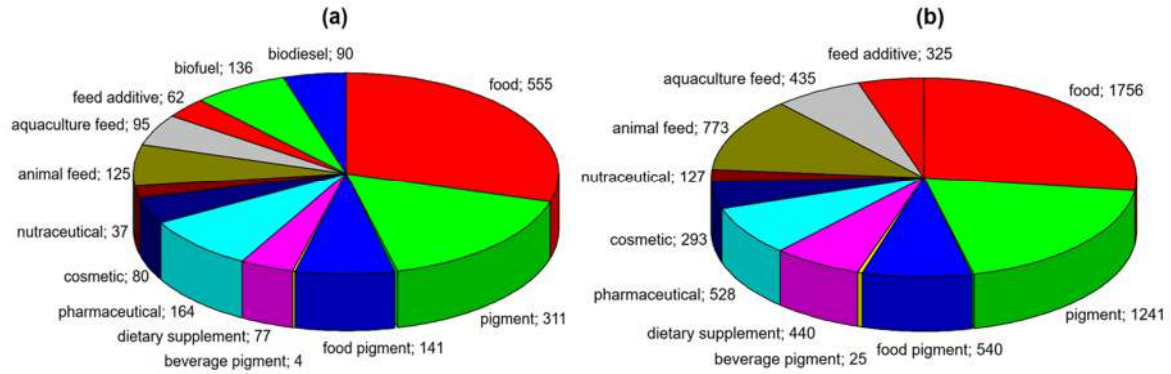


Figure 1. Potential applications of (**a**) *Haematococcus pluvialis* and (**b**) astaxanthin, extracted from this microalga (according to the Web of Science, accessed on 3 August, 2020).

Most applications of both microalga and astaxanthin are related to human nutrition (food, pigments for food and beverages) and health (pharmaceuticals, nutraceuticals and dietary supplements). Much fewer publications are devoted to the use of microalga and extracted astaxanthin in animal nutrition. Therefore, in the presented review, we focused on the biological properties of astaxanthin (antioxidant, anti-inflammatory and anti–diabetic, anticancer) and its application in the nutrition and treatment of humans and animals. These descriptions were preceded by information on the cultivation of *Haematococcus pluvialis*, its biochemical composition and isolation of astaxanthin, also by means of the innovative extraction techniques. This review presents a comprehensive approach to astaxanthin. The general outline of this review is presented in Figure 2.

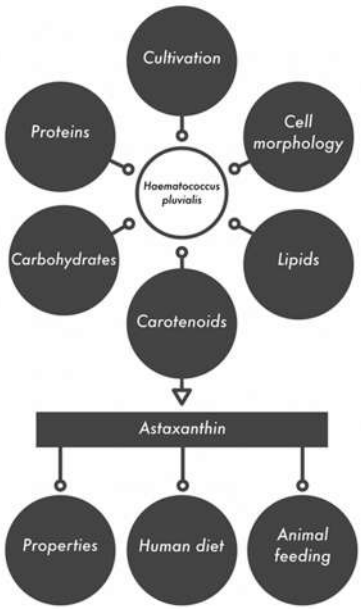
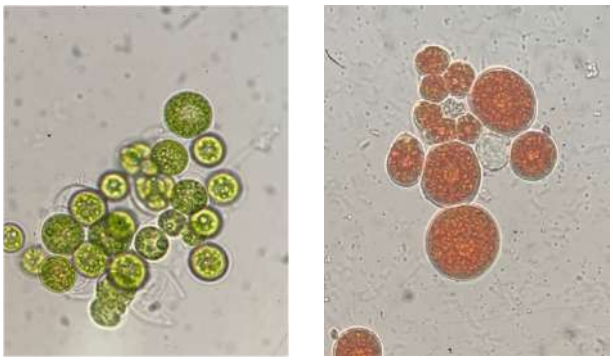


Figure 2. *Haematococcus pluvialis* as a source of active compounds and their applications.

2. Cell Morphology of Haematococcus Pluvialis

Haematoccocus pluvialis is a unicellular, spherical, green biflagellate oleaginous cell with a diameter of ~30 µm [15,16]. Based on their life cycle, morphology and physiology, *H. pluvialis* can either exist as vegetative green cells (Figure 3a)—capable of swimming due to their two flagellas, closely connected with green stage and biomass accumulation—or as red cysts (Figure 3b), which accumulate astaxanthin in response to stressful environmental conditions. The cells observed during the vegetative phase are: macrozooids (zoospores), microzooids and palmella, while the cells of the astaxanthin accumulation phase are asexual aplanospores [17].



(**a**) (**b**)

Figure 3. Cell morphology of *Haematococcus pluvialis* on (**a**) green stage (**b**) red stage (own source).

Macrozooids are biflagelled, spherical, ellipsoidal or pear-shaped cells with dimensions of 8 to 20 µm. Cells at this stage can produce 2–32 daughter cells by asexual reproduction, through the formation of a sporangium [18]. Unfavourable conditions can cause an increase in cell size and loss of both flagellas. During that process, within 1–2 days, macrozoids become an amorphous, multilayered, non-motile form called palmella. Persisting unfavourable conditions lead to the transformation into the asexual aplanospore stage [19]. A thick and rigid trilaminar sheath and the secondary cell wall protects aplanospores against acetolysis, high light irradiance and high salinity. Accumulation of astaxanthin usually occurs in aplanospores and is induced by nutrient deprivation or high light exposure [20]. Numerous studies have been performed in order to assess the effect of nitrogen limitation or light intensity on cell morphology. The obtained collective information enables a more accurate optimization of the *H. pluvialis* culture process. More detailed studies on the correlation between cell structure in each growth phase and the mechanisms of astaxanthin accumulation could further improve the production of expected metabolites.

3. Cultivation of Haematococcus Pluvialis

Optimized environmental parameters allow for achieving high biomass growth and, consequently, astaxanthin accumulation. Cultivation of *Haematococcus pluvias* can be divided into two stages: the first one, called green stage, and the second one–red stage [21]. At the beginning, microalgae divide into daughter cells under favorable temperature, pH, amounts of nitrates, metals or a light wave length and intensity. In response to adverse conditions, cells typically stop dividing. Stress conditions cause accumulation of carotenoids, mostly astaxanthin. The temperature during the green phase should be within a range of 25–30 °C [22]. The light intensity below 150 µmoL photons m^{-2} s^{-1} enables cell division. The typical irradiation is 40–50 µmoL photons m^{-2} s^{-1} and pH should be kept at 7 [23]. Cycles of alternating light and darkness are 12:12 or 16:8 [24]. The most commonly used media for *H. pluvialis* growth are KM1, BBM, Z8, BG- 11, OHM, and their modified versions. BBM and Z8 are used for autotrophic cultures while KM1 medium is suitable for heterotrophic cultures. A mixture of BBM medium and an organic carbon source, e.g., sodium acetate, can be used in a mixotrophic

culture [25,26]. There are several growth modes of microalgae. In a photoautotrophic mode, microalgae use light as a source of energy and an inorganic compound as a source of carbon; in that case, light is obligatory. For the photoheterotrophic mode, however, the source of carbon is organic and light is not obligatory but it might be used aa a source of energy. In a heterotrophic mode, a source of energy and carbon are both organic. The mixotrophic way of microalgae cultivation uses organic and inorganic sources of carbon and energy [27]. After reaching a high cell concentration, the red phase (the astaxanthin accumulation phase) occurs. For this purpose, stress factors are used: hunger for nitrogen, high light intensity, high temperature or the presence of metals [28].

For mass production of *H. pluvialis*, bioreactors or production ponds are used, it is also possible to combine both systems. The main factors in selecting a cultivation method are: capital and operational costs, cultivation area, climatic conditions (light, temperature, rain), the possibility of contamination, water availability, level of automation and system efficiency [29]. Many producers of natural astaxanthin belong to the association of producers of natural astaxanthin derived from *Haematococcus pluvialis*: the Natural Algae Astaxanthin Association (NAXA) [30]. Cyanotech Corporation in Kailua-Kona, Hawaii, is one of the producers of natural astaxanthin from *Haematococcus*. The large 500-cubic-meter ponds of algal culture have an annual production capacity of more than 70 metric tons of *Haematococcus* algae meal with a minimum of 1.5% of astaxanthin (NatuRose®) [31]. Mera Pharmaceuticals, Cyanotech Inc., Algatechnologies Ltd., Biogenic Co. Ltd. are some of the more important producers of *H. pluvialis*-derived astaxanthin. Over 95% of astaxanthin available on the market is chemically synthesized, only < 1% is obtained from *H. pluvialis* [7].

To this date, many laboratory scale *H. pluvialis* cultivation models were considered. In the Web of Science database (accessed 3 August 2020), over 392 publications concern methods of *H. pluvialis* cultivation. However, the industrial scale is of greater interest. Microbial infection of the culture and uncompetitive costs of obtaining astaxanthin, relative to chemical synthesis are the main obstacles to overcome. The optimization of *H. pluvialis* cultivation could contribute to the creation of local microalgae crops for astaxanthin production. The use of municipal and industrial waste substrates for industrial cultivation would increase the attractiveness of *H. pluvialis*, while reducing the costs of the process. However, *H. pluvilais* has the ability to absorb metal ions in bioremediation processes and poses a risk of heavy metal contamination when cultivated in wastewater. Therefore, it is necessary to either control the quality of waste or thoroughly purify astaxanthin and other metabolites before introducing them into the human or animal diet [32].

4. Biochemical Composition of *Haematococcus pluvialis*

4.1. Proteins and Carbohydrates

The maturation of cells and the passage through successive stages of life cycles results in an altered biochemical profile of the cell. Most of *H. pluvialis* green stage cells are characterized by a high protein content of 29–45% per dry weight (d.w.) [33]. Protein content in palmella decreases to 36% d.w., while in the red stage cells, this content is within a range of 21–23% d.w. Carbohydrates as starch allow the cell to survive during a prolonged stress. The content increases from 15–17% d.w. in the green stage to 60–74% d.w. in the red cyst. As Recht et al. (2012) demonstrated in their research, total carbohydrates can increase rapidly, by up to 63% d.w, during the first day of stress exposure, decrease to 41% d.w. on the following day, and remain at this level until the end of the cultivation [34,35].

4.2. Lipids

In the green stage, total lipid content varies from 20% to 25% (Table 1), with approximately 10% of the lipids being composed predominantly of polyunsaturated fatty acids (PUFAs) deposited in chloroplasts [14]. The accumulation of lipids ensues from unfavorable environmental conditions. These include limiting nitrogen and phosphorus content, high salinity, high light intensity and extreme temperatures. According to Hagen et al. (2002), the most effective factor causing the accumulation

of lipids in the cell is nitrogen limitation [18]. Studies performed by Boussiba and Vonshak (1991) showed a simultaneous increase in the content of oleic acid (C18:1 monounsaturated fatty acid) and astaxanthin esters during nitrogen limitation 0.15 g/L and high light amounting 170 μmol photons $m^{-2}s^{-1}$ [7]. Furthermore, the results obtained by Damiani et al. (2010) indicate a correlation between the stress conditions of algae growth and the increase in the lipid content in the cell [36]. In this case, the use of unfavorable conditions caused a significant increase in the total lipid when compared to the control culture. In the first variant of stress-conditions, a culture full medium without aeration and continuous light with an intensity of 300 µmoL photons $m^{-2}s^{-1}$ were used. In the second variant, a medium without nitrogen and aeration, and the same intensity of light as in the first case were applied. The total lipids content in dry weight was respectively 34.85% and 32.99%. For the control culture with a full medium, continuous aeration with the mixture of air (500–700 mL/min) and CO_2 (0.3 mL/min) and 12 h of illumination with a white lamp 90 µmoL photons $m^{-2}s^{-1}$, the total content of lipids was 15.61%. This study also showed a significant increase in the phospholipid content within cultures maintained in unfavorable conditions. The highest content of neutral lipids and glycolipids was obtained in the first variant of stress conditions and it was 19.80% and 7.85%, respectively, while for the second variant, these values were 16.60% and 6.67%. Only the content of phospholipids was higher in the second variant and was equal to 9.80%, while for the first variant it was 9.5%. In the control culture, the following contents were obtained: neutral lipids 9.20%, glycolipids 3.70% and phospholipids 1.87%. The most common fatty acids in *H. pluvialis* cells are palmitic, linoleic and linolenic acid (Table 1) [19]. The fatty acid profile is dependent on the strain. The *H. pluvialis* KORDI03 profile consists of a low 15.0% content of saturated fatty acids (SFAs) and 6.0% monounsaturated fatty acids (MUFAs), while the content of polyunsaturated fatty acids is 79% [15]. Strain CCALA 1081 isolated from rainwater in Baha Blanca, Argentina, represents a higher SFAs content which ranged from 27.81% to 30.36%, while that of MUFA from 18.96% to 20.07% and that of PUFAs from 43.15% to 47.23% [20]. Cerón et al. (2007) noticed that the nitrogen reduction has a significant impact on the production of fatty acids. Lowering the amount of nitrogen in the culture medium to 1.7 mM led to obtaining 7.60% of fatty acids in dry weight, while the culture maintained in a medium with a higher nitrogen content of 4.7 mM presented a lower amount of fatty acids: 2.1% d.w. In the aforementioned study, it was also demonstrated that the nitrogen content has an effect on the fatty acid profile. With a reduction of nitrogen content to 2 mM, the oleic acid level increases, which is 50% of the total fatty acid content [37]. As for Liang et al. (2015) research, the culture of microalga under control conditions that promote the multiplication of the biomass resulted in a low total lipid content of 13.6%. This research also proved the influence of stress factors on lipid accumulation in the cell. Biomass cultivation in a medium without nitrogen resulted in the accumulation of lipids at the level of 46.71% d.w. In the second variant, an increase in the light intensity from 50 to 350 µmoL photons $m^{-2}s^{-1}$ and the use of medium without nitrogen caused an increase in the lipid content to 46.87% d.w. The best results—56.92% d.w.—were obtained for the full medium and a high light intensity of 350 µmoL photons m^{-2} s^{-1} [38].

Table 1. Comparison of fatty acid compositions (%) of different *H. pluvialis* strains.

Fatty Acids	Kim et al., 2015 [19]	Lorenz, 1999 [39]	Scodelaro Bilbao et al., 2016 [40]	Lei et al. 2012 [41]
C12:0 lauric	N/A	0.1	N/A	0.28
C14:0 myristic	0.1	0.5	1.99	0.65
C15:0 pentadecanoic acid	0.1	N/A	N/A	0.25
C16:0 palmitic	13.7	29	22.9	12.7
C16:1 palmitoleic	0.5	0.6	0.35	0.7
C16:2	0.4	N/A	N/A	N/A
C16:3	3.5	N/A	N/A	N/A
C16:4	3.3	N/A	N/A	N/A

Table 1. *Cont.*

Fatty Acids	Kim et al., 2015 [19]	Lorenz, 1999 [39]	Scodelaro Bilbao et al., 2016 [40]	Lei et al. 2012 [41]
C17:0 margaric	N/A	0.2	N/A	0.23
C17:1 margaroleic	N/A	1.3	N/A	0.0
C18:0 stearic	0.7	2.1	1.15	4.79
C18:1 oleic	4.9	25.9	16.3	11.2
C18:2 linoleic	24.9	20.8	23.9	13.0
C18:3 linolenic	39.7	12.8	12.5	2.84
C18:4 octadecatetraenoic	5.8	1.4	N/A	N/A
C20:0 arachidic	N/A	0.6	N/A	0.35
C20:1 gadoleic	0.5	0.3	N/A	1.3
C20:2 eicosadenoic	N/A	1.2	2.21	0.87
C20:3 eicosatrienoic gamma	N/A	0.5	N/A	0.18
C20:4 arachidonic	0.9	1.4	1.92	1.77
C20:5 eicosapentaenoic	0.6	0.6	0.66	0.99
C22:0 behenic	N/A	0.4	N/A	0.16
C24:0 lignoceric	0.3	0.2	0.33	0.4
C24:1 nervonic acid	0.1	0.1	N/A	0.14
Σ SFAs	15	33.2	25.3	19.8
Σ MUFAs	6	28.1	16.6	12.97
Σ PUFAs	79.1	38.7	41.2	19.65

N/A–not available.

4.3. Carotenoids

The carotenoid content also changes during cell transformation. It increases in *H. pluvialis* cell from 0.5% d.w. in the green phase to 2–5% d.w. in the red phase. Lutein with a content of 70–80% is the main carotenoid in green cells. The second component with the highest content is β-carotene (16.70% d.w.), the amounts of violaxanthin and neoxanthin are, respectively, 12.5% and 8.3% d.w. The aforementioned compounds are not found in red phase cells or are only present in small amounts. The next pigment, present only in green cells, is chlorophyll, the content of which is 1.5–2% d.w. [14]. Astaxanthin is the most important carotenoid obtained from *H. pluvialis* and it is accumulated inside the cell only during the red phase. Its content can reach up to 80–99% of the total carotenoids [42,43]. According to Web of Science (accessed 3 August 2020), 850 publications were closely related to the topic of the carotenoids, 319 to lipids and 248 to proteins from *H. pluvialis*. Studies on the effects of external factors are mainly based on the accumulation of astaxanthin. However, it is equally important to carry out more thorough research to optimize the culture process in order to increase the content of lipids and proteins. *Haematococcus pluvialis* is a promising cell factory for biofuels and animal feed. Unfortunately, there is not much research concerning the optimization of production and obtaining of triglycerides and astaxanthin simultaneously; obtaining two metabolites of *H. pluvialis* from one culture would make the process more profitable.

5. Astaxanthin as a Valuable Biologically Active Compound

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) belongs to the group of carotenoids naturally occurring in such organisms as microalgae, crustaceans, fish and some birds [44,45]. This red carotenoid pigment is classified as xanthophyll due to its powerful antioxidant ability. Astaxanthin is made of two β-ionone ring systems within its structure that are linked by a polyene chain and contain the oxygenated keto and hydroxyl moieties [46]. Due to its structure, astaxanthin is a

promising factor in the prevention of diseases associated with oxidative stress, including diseases of the vascular and cardiac system, diabetes and cancers [47]. The construction of astaxanthin enables it to combine biological membranes and to reduce and stabilize free radicals. Most often people supply astaxanthin from foods such as seafood. Ambati et al. (2014) noted 6 mg of astaxanthin per kg of flesh of European trout, 25 mg/kg in flesh of Japanese trout and 6–8 mg/kg in flesh of farmed Atlantic salmon [48]. In nature, the highest content of astaxanthin is in microalga *Haematococcus pluvialis* which can accumulate up to 5% d.w. Synthetic astaxanthin dominates commercially because of lower costs of production [49]. *H. pluvialis* is perceived by many researchers as a primary source of astaxanthin for the food industry because of 3*S*, 3*S*′ stereoisomer, which is the most effective isomer for human application, compared to such isomers as 3*R*, 3*S*′ and 3*R*, 3*R*′. This spatial arrangement of atoms increases the bioavailability of astaxanthin [50]. Astaxanthin has a 10 times stronger antioxidant activity than that of β-carotene, and 100 times stronger than that of α-tocopherol [51].

5.1. Astaxanthin Accumulation

The life cycle of *H. pluvialis* is influenced by inductive factors that cause astaxanthin accumulation and non-inductive factors necessary to maintain cell growth during the green phase [50]. The synthesis of astaxanthin in *H. pluvialis* cell occurs in the red phase. To enter this phase, unfavorable environmental conditions must occur, such as nitrogen reduction, high light intensity, salinity, pH change or extreme temperatures. Ethanol regulates the expression of carotenogenesis genes and significantly increases the accumulation of astaxanthin in cells [52]. Ota and Kawano (2019) have shown a protective activity of astaxanthin on the cell. During short-term exposure of cells to high light intensity (10–15 min), astaxanthin migrated from the inside of the cell to its wall. After reducing the intensity of light, astaxanthin returned to the centre of the cell. This phenomenon indicates that the red pigment contained in the cell is used as a protective factor against high light intensity [16]. Astaxanthin and triacylglycerols (TAGs) accumulate together in the lipid bodies during the red phases. Astaxanthin is synthesized from isoprene units, which are the basic units for carotenoid synthesis [14]. Isopentenyl pyrophosphate (IPP) is the precursor for carotenoid synthesis; isopentenyl is converted to pyrophosphate using isopentenyl pyrophosphate isomerase. Then phytoene synthase catalyzes the phytoene synthesis reaction. The conversion of phytotene to lycopene takes place with the participation of the following enzymes: phytoene desaturase and carotene desaturase. Lycopene β-cyclase is responsible for the cyclization of lycopene to β-carotene. The last step involves the conversion of β-carotene to astaxanthin using the enzymes β-carotene ketolase, β-carotene oxidase and β-carotene hydroxylase [21].

5.2. Astaxanthin Recovery

Recovery of astaxanthin from *Haematococcus pluvialis* consists mainly of the following stages: (I) cell breakage, (II) alkaline treatment, (III) solvent extraction, (IV) solvent removal, (V) purification, (VI) resuspension in oil and (VII) single step-alkaline extraction [33,53,54]. Due to the thick cell wall, microalgae require mechanical disruption of the cells before applying the solvent [55]. Mechanical methods consisting of grinding, compression or pressing under high pressure are the most effective. To obtain the highest durability, it is necessary to dry the pigment or all biomass [54]. The use of traditional solvent extraction has many disadvantages, such as large volumes of organic solvents, high extraction temperature, the risk of the thermal degradation of extracted molecules or the presence of solvent residues in the extracts [55]. Therefore, innovative extraction techniques are becoming more and more popular [56–58]. Among them, we can distinguish microwave-assisted extraction (MAE) [56], ultrasound-assisted extraction (UAE) [56,59], supercritical fluid extraction (SFE) [55,60,61] and enzyme-assisted extraction (EAE). The main advantages of those methods are reduced solvent usage, short extraction time and higher extraction yield [58].

In the case of MAE, the extraction induces changes in the cell wall caused by electromagnetic waves. The MAE extraction rate can be correlated with heat and mass transfer gradients working in the same direction [62]. In the study published by Ruen-ngam et al. (2011), a 74% recovery

rate of astaxanthin was reached using the MAE method with acetone, for a duration of 5 min at 75 °C [56]. Ultrasound assisted extraction is recognized as an alternative to traditional astaxanthin extraction methods. In addition, ultrasound procedures are much faster than traditional methods [63]. Ruen-ngam et al. (2011) obtained the highest astaxanthin recovery (73%) after 60 min of extraction with acetone at a temperature of 45 °C [56]. Di Sanzo et al. (2018) showed that SFE was very efficient in the recovery of astaxanthin; as with optimal extraction conditions (50 °C and 550 bars), a 98.6% recovery rate was achieved [61]. Wang et al. (2012) showed similar results with the astaxanthin yield being 87.4% under optimal experimental conditions (65 °C, 435 bar, co-solvent 2.3 mL/g) [60], whereas Molino et al. (2018) observed 92% for 65 °C and 550 bar [55]. A promising technique is enzyme-assisted extraction, where optimal experimental conditions (pH, temperature and time) and properly selected enzymes (mainly pectinase, cellulase) and their doses allow for efficient astaxanthin release from *Haematococcus pluvialis*. In the case of cellulase used in a 3% concentration, the extraction yield was about 60% (pH 5, 3 h, 65 °C) [64]. It shows that innovative extraction techniques provide a high extraction yield of this pigment. Obtaining astaxanthin from microalgae is an expensive process due to the cost of biomass cultivation, which accounts for 20%–30% of the total production costs [55].

According to the Web of Science database (accessed 3 August, 2020), 268 publications on extraction of astaxanthin from *Haematococcus pluvialis* have been published so far. A total of 98 publications concerned the traditional solvent extraction, 52–SFE, 12–UAE, 11–MAE and 3–EAE. These data also show a recent growing interest in modern extraction techniques: 43 publications on SFE have been published in the recent 10 years.

6. Biological Properties of Astaxanthin

Antioxidants are chemical compounds that prevent oxidation in small concentrations or delay the oxidation of substrates [33]. The term "antioxidants" also includes some semi-synthetic analogues of plant substances, natural plant extracts, synthetic food additives and medicines. Antioxidative compounds are divided into (I) enzymes (superoxide dismutase, catalase and glutathione peroxidase) and (II) non-enzymatic substances (vitamins A, C, E, carotenoids, polyphenols and glutathione). Oxidative damage is caused by reactive oxygen species (ROS) and free radicals [52]. Free radicals are molecules containing at least one unpaired electron on the outer electron shell. As a consequence, free radicals seek to pair electrons either by taking them away or giving them to other molecules. In the body, around 90% of free radicals are generated by the respiratory chain, while the remaining 10% originates from physiological reactions of the cell [65].

The formation of free radicals can also be caused by ultraviolet, ionizing radiation, ultrasound or elevated temperature and in the metabolism processes of various exogenous chemical compounds [65,66]. Importantly, free radicals are necessary for the proper course of many life processes. They partake in the regulation of gene expression, protein phosphorylation and calcium concentration in cells, activate control of proteins cell divisions, and participate in the elimination of microorganisms. However, the occurrence of excessive free radicals can lead to structure damage and the disturbances of vital cell functions, disruption of homeostasis and even death as a result of apoptosis or necrosis [66,67]. Carotenoids have a polyene chain and long conjugated double bonds. The structure of these compounds is responsible for antioxidant activity, such as the hardening of singlet oxygen and the removal of radicals to complete the chain reactions [49]. The structure of astaxanthin consists of a conjugated polyene chain in the center and hydroxyl and ketone moieties on each ion ring. Astaxanthin is characterized by higher biological activity than other antioxidants because it can bind to the cell membrane from the inside to the outside [68]. The astaxanthin end ring captures radicals on the surface and inside the cell membrane, while the polyene chain does so only in the cell membrane [69]. Astaxanthin, after the quenching of singlet oxygen, dissipates energy through interaction with the solvent; then, the carotenoid structure returns to its original state [46,70]. According to the reviewed studies, astaxanthin exhibits higher antioxidant activity compared to various carotenoids, such as α-carotene, β-carotene, lycopene and lutein; additionally, it induces paroxidanase,

an enzyme with antioxidant activity [70,71]. Miki (1991) found out that astaxanthin had 10 times higher antioxidative activity than lutein, zeaxanthin and β-carotene canthaxanthin, and 100 times higher than that of α-tocopherol [51].

6.1. Anti-Lipid Peroxidation Properties

Lipid peroxidation involves the oxidation of lipids and in consequence formation of lipid peroxides. The reaction consists of three stages: initiation, propagation and termination. Lipid peroxidation yields aldehydes (e.g., malondialdehyde-MDA), hydroxyaldehydes (e.g., 4-hydroxynonenal) and hydrocarbons (e.g., ethane) [72]. These compounds can modify the physical properties of cell membranes, including reduction in the hydrophobicity of the lipid interior of the membranes, disturbance in the lipid asymmetry of the membranes, inhibition of the activity of transporting proteins, depolarization of membranes and inhibition of the activity of membrane enzymes. These changes may result in the loss of intracellular membrane and plasma membrane integrity [73]. The antioxidant activity of astaxanthin is pH-dependent. Mano et al. (2018) showed that astaxanthin strongly inhibits the formation of by-products of lipid peroxidation (thiobarbituric acid reactive substances) in zwitterionic phosphatidylcholine liposomes at pH 7.4 (80%) and at pH 8 (65%). Furthermore, it also slightly inhibits the process of lipid peroxidation at pH 6.2 (20%) and 6.8 (30%) [74]. In the case of ulcerated rats, astaxanthin supplementation decreased the anti-lipid peroxidation effect. The level of thiobarbituric acid reactive substances in serum (as nmoL malondialdehyde per mg serum) decreased from 3.76 nmoL MDA/mg (control) to 2.04 nmoL MDA/mg, 1.94 nmoL MDA/mg and 1.56 nmoL MDA/mg, respectively, for a dose of astaxanthin 100, 250 and 500 μg/kg b.w per day [75].

Interestingly, astaxanthin is more effective than β-carotene in the prevention of lipid peroxidation. As Goto et al. (2001) found out, astaxanthin was twice as effective as β-carotene in inhibiting ADP and Fe^{2+} induced liposomal peroxidation. The supposed mechanism of astaxanthin activity in the prevention of lipid peroxidation results from the interaction of its ending rings of with hydrophilic polar sites of phospholipids membrane and forming an intramolecular hydrogen-bonded five-membered ring which increases the hydrophobicity of astaxanthin [45]. The effect of astaxanthin on the lipid peroxidation was assessed in LDL studies in ex vivo conditions [76]. For two weeks, volunteers were given different doses of astaxanthin (1.8, 3.6, 14.4 and 21.6 mg/day). Samples of LDL from the group of people who received astaxanthin were characterized by lower susceptibility to oxidation when compared to the control group (LDL from group not consuming astaxanthin) [77].

6.2. Anti-Inflammatory Effects

Chronic inflammation is the main pathophysiological factor in many diseases, such as diabetes or many neurodegenerative diseases. Due to the high percentage of polyunsaturated fatty acids in the plasma membranes, immune cells are particularly sensitive to oxidative stress, overproduction of reactive oxygen species disturbs the antioxidant balance [78]. Astaxanthin is a powerful antioxidant that inhibits inflammation in biological systems. This carotenoid is able to regulate the immune response or reduce inflammation associated with peripheral diseases [79,80]. Studies have shown that astaxanthin can regulate microglial cells, which are non-neuronal cells of the central nervous system. Microglial cells are tissue-specific resident macrophages which control homeostasis and are involved in the immune response. These cells after recognizing the threat, such as the presence of pathogens or cell damage, release pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and NO. In the initial phase, this response is effective in danger neutralization; however, due to the toxic nature of proinflammatory molecules, prolonged action of microglia can destroy the central nervous system [81,82]. In studies of Choi et al. (2008) and Kim et al. (2010), the use of astaxanthin resulted in decreased secretion of IL-6, Cox-2 and iNos/nitric oxide in microglia during the presence of bacteria [83,84]. Park et al. (2009) reported a reduction of κB and neurodegeneration in the frontal cortex and hippocampus. This research described the performance of mice treated with astaxanthin in the Morris water maze [85]. Astaxanthin plays important roles in the amelioration of inflammatory

diseases including arteriosclerosis, inflammatory bowel disease, sepsis, rheumatoid arthritis, gastric inflammation and brain inflammatory diseases.

6.3. Anti–Diabetic Activity

Insulin resistance is a serious disorder of glucose homeostasis, which is characterized by decreased insulin sensitivity of various tissues, such as skeletal muscle, adipose tissue or liver. Insulin resistance is one of the causes of type 2 diabetes and gestational diabetes; usually, it is also a factor in the course of type 1 diabetes. Insulin resistance is often accompanied by hyperinsulinaemia [86]. Astaxanthin improves the whole body's insulin sensitivity and insulin stimulated glucose uptake in the muscle of insulin-resistant animals. Astaxanthin reduces the level of oxidative stress caused by hyperglycemia in pancreatic β cells and has a positive effect on serum glucose and insulin [87]. Studies in astaxanthin-fed mice have reported increased insulin sensitivity in both hypertensive rats and mice fed with high fat and high fructose diets, while the level of albumin in the urine of diabetic mice was significantly lower than in the control group (without astaxanthin in diet) [87,88]. Bhuvaneswari et al. (2012) confirmed that astaxanthin stimulates the signaling pathway of the insulin receptor substrate (IRS) -PI3K-AKT, due to the reduction of the serine phosphorylation of IRS proteins, and increases glucose metabolism by regulating metabolic enzymes [89]. Astaxanthin is also responsible for lowering the level of cholesterol in blood as well as the level of triglycerol in the liver and stimulating the expression of antioxidant genes. In addition, astaxanthin reduces the expression of CYP2E1 and, as a result, increases the sensitivity of cells to insulin and inhibits liver damage [90]. In the early stages of diabetes, astaxanthin protects pancreatic β cells, increases insulin sensitivity and improves glucose metabolism. As a consequence, insulin resistance and blood glucose levels decrease. Supplementation with astaxanthin reduces oxidative stress, inflammation and lipid peroxidation, therefore it prevents such complications of diabetes as: retinopathy, neuropathy, nephropathy and cardiovascular complications [91]. Mechanisms underlying antidiabetic effects of astaxanthin (Figure 4) are as follows: (I) activation of IRS-PI3K-Akt signals and increased glucose metabolism in the liver; (II) normalization of hexokinase activity, pyruvate kinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase and glycogen phosphorylase; (III) protection against oxidative stress and cytotoxicity in pancreatic cells; (IV) reduction of serine kinases activity; and (V) reduction of MDA [47,89,92–94].

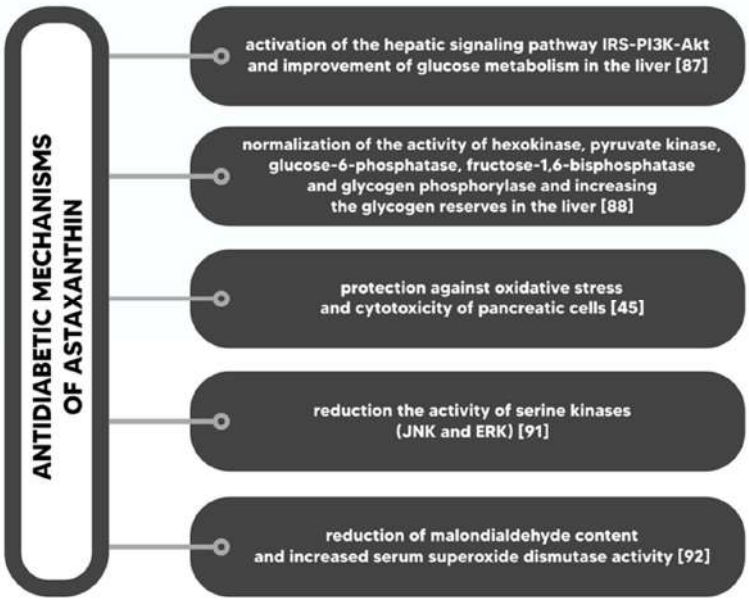


Figure 4. Antidiabetic mechanisms of astaxanthin.

6.4. Anticancer Activity

Astaxanthin has the ability to inhibit cancer cell growth. Antioxidant compounds decrease mutagenesis and carcinogenesis by inhibiting oxidative damage to cells. Studies have reported that astaxanthin not only inhibits the proliferation of colon cancer cells but can also cause their apoptosis. Palloza et al. (2009) used an extract from *H. pluvialis*, which inhibited the growth of human colon cancer cells. Astaxanthin was included in the extract and was responsible for stopping the progression of the cell cycle and promoting the apoptosis [95]. Astaxanthin showed higher antitumor activity than other carotenoids, including canthaxanthin and β-carotene [96,97]. Jyonouchi et al. (2008) and Nakano et al. (2008) proved increased levels of immune cells, natural killer cells and plasma γ interferon in mice after astaxanthin treatment [98,99]. The oral administration of astaxanthin inhibited carcinogenesis in the urinary bladder of mice and in the colon and oral cavity of rats. This effect has been partially assigned to the suppression of cell proliferation. Research performed by Song et al. (2011) showed high anti-proliferative activity of astaxanthin against tumor cells like: SHZ-88 breast cancer cells, hepatoma CBRH-7919 cells and Lewis cells. A significant correlation was observed between astaxanthin concentrations and anti-proliferative activity. The most sensitive cell line to astaxanthin with half a maximal inhibitory concentration of 39 μM was the CBRH-7919 line [100].

According to Web of Science (accessed on 3 August 2020), 269 publications refer to anti-inflammatory effect of astaxanthin and 238 publications refer to anti-lipid peroxidation effect. Studies on the effects of astaxanthin on human health mainly cover such disorders as metabolic diseases, cancer and inflammatory diseases, as well as skin and eye conditions. Studies on the effects of astaxanthin on animal organisms are promising, mainly due to the antioxidant properties of the compound. Research has demonstrated an astaxanthin-induced reduction in blood glucose and insulin levels; astaxanthin has also sensitized cellular receptors to insulin. Correlation of excess free radicals and the development of metabolic diseases, such as type 2 diabetes and insulin resistance, have been confirmed by many studies. However, not all of them prove the antioxidant effect of carotenoids, which necessitates the use of new models and pathways.

6.5. Other Potential Applications of Astaxanthin

Astaxanthin has a beneficial effect, due to its high antioxidant and anti-inflammatory potential. As a result, more and more research is being conducted on the biological activities of astaxanthin in the context of the nervous, visual and cardiovascular systems.

Hypertension is a major risk factor for cardiovascular disease. Overproduction of reactive oxygen and nitrogen species results in diseases, such as hypertension, atherosclerosis, endothelial dysfunction or arrhythmias [68]. Oral administration of astaxanthin to hypertensive rats decreased nitric oxide products and lowered blood pressure [101,102]. In vivo studies have shown that astaxanthin supplementation decreased the level of TG, TC, LDL-C, IL-6, CRP and LPO; as a result, this improved the antioxidant defense capacity and choroidal blood flow velocity. Astaxanthin also increased SOD activity and decreased PG-E2, LT-B4, NO, IL-8 and IFN- γ production [103–105]. Astaxanthin has a cardiovascular protective effect in animals, but there is a lack of research supporting the therapeutic benefit of astaxanthin in atherosclerotic cardiovascular disease in humans.

Astaxanthin has a beneficial effect in the prevention and treatment of eye diseases, such as age-related macular degeneration, glaucoma, cataract or keratopathy. Astaxanthin crosses the barrier of the circulatory system and the retina of eye. As the only antioxidant, it builds into the eye's cell membrane, protecting it against damage and free radicals. Astaxanthin due to antioxidant activity inhibited ischemia induced retinal cell death [106]. Supplementation of rats with astaxanthin at a dose of 5 mg/kg/day for 8 weeks resulted in a decrease in retinal apoptosis, a decrease in the production of protein carbonyl and NOS-2, which brought an increase in retinoprotective properties [107]. Astaxanthin counteracts many eye diseases because of its anti-inflammatory, antioxidant properties and regulation of metabolism [108].

Fakhri et al. (2019) showed that astaxanthin blocks neurodegenerative pathways, such as oxidative stress, inflammation and apoptosis, and can pass through the blood brain barrier [109]. Supplementation lowers the expression of Bax and Cleaved-caspase-3, which inhibits and reduces neuronal apoptosis and pathological tissue damage. Oral supplementation with astaxanthin in rats after surgery decreased the expression of NF-KB and TNF-α, which resulted in a reduction of cerebral edema and neurological dysfunction [110,111]. Astaxanthin is considered as a multi-target pharmacological agent against neurological disorders including Parkinson's disease, Alzheimer's disease, brain and spinal cord injuries, neuropathic pain, aging, depression and autism [112].

7. Astaxanthin in the Human and Animal Diet

Astaxanthin is commercially available mainly in the form of dietary supplements, oils or dried aplanospores. The cell walls of aplanspor must be broken because they undergo partial digestion. Oils containing astaxanthin are not organoleptically attractive, due to the smell and taste of algae [113]. Satisfactorily, in recent years, there has been more and more research into the incorporation of astaxanthin into food and animal feed.

7.1. Human Diet

Good results have been obtained for whole grain cakes. In vitro digestion studies showed a decrease in glucose release proportional to the rise in astaxanthin concentrations in the cake formulation [114]. Studies performed by Mercke et al. (2003) confirmed the beneficial effect of lipids on the absorption of astaxanthin. Patients took 40 mg of astaxanthin in various oil-based formulations. All formulation carriers increased the absorption of astaxanthin. The best effect was demonstrated by formulations with the highest content of the hydrophilic synthetic surfactant [115]. Furthermore, consumption of *H. pluvialis* biomass as a dietary supplement in combination with olive increases the antioxidant properties and bioavailability of astaxanthin [116]. In the stomach, astaxanthin accumulates in drops of lipids and is incorporated into micelles that diffuse into plasma membranes of enterocytes and are transported in the circulation by low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [117]. Astaxanthin in combination with fish oils increases hypolipidemic/hypocholesterolemic effect in plasma. The combination showed increased phagocytic activity of activated neutrophils [118]. Diet and smoking have a big influence on the absorption of astaxanthin. The bioavailability of astaxanthin in smokers is reduced by 40% [119]. Carotenoids are absorbed into the bloodstream in a similar manner as lipids; subsequently, they are transported via the lymphatic system to the liver. The absorption process highly depends on the accompanying dietary components. The intake of carotenoids can be increased by a high cholesterol diet, while a low-fat diet reduces their absorption [48]. Carotenoid absorption involves the following steps: (I) release of carotenoids from food matrix, (II) solubilization of carotenoids into mixed lipid micelles; (II) cellular uptake of carotenoids by intestinal absorptive cells (enterocytes); (IV) incorporation of carotenoids into chylomicrons; (V) secretion of carotenoids and their metabolites associated with chylomicrons into the lymph within the systemic circulation; and (VI) tissue distribution, metabolism and recycling of carotenoids [76,120–123]. Astaxanthin is well tolerated by the human organism and numerous studies have not revealed any toxic effects [124,125]. The European Food Safety Authority (EFSA) on Additives and Products or Substances used in Animal Feed (FEEDAP) recommended the use of astaxanthin in a dose of 0.034 mg/kg of body weight, 2.38 mg per day in a 70 kg human [126,127]. According to Spiller and Dewell (2003), a daily dose of astaxanthin equal to 2–4 mg is safe. They did not report toxic effects when adults consumed up to 6 mg/day. Satisfactory astaxanthin bioavailability results were obtained with a daily astaxanthin dose of 40 mg/day. This dose was well tolerated by the human body [115]. Commercial astaxanthin-containing products are available in the form of both daily capsules, soft gels, energy drinks and powders [48]. Research into patients suffering from functional dyspepsia indicates that safety doses are from 16 to 40 mg per day [128]. The presence of dietary fat enhances the assimilation of astaxanthin in the small intestine [119]. Excessive consumption of astaxanthin leads to the accumulation of pigment in

tissues and skin, which is desirable in the breeding of some animals, e.g., salmon. Studies on rats showed an increase in the content of antioxidant enzymes, such as catalase, superoxide dismutase and glutathione peroxidase after cyclic dosing of astaxanthin [116,124]. Astaxanthin has the status of pure antioxidant, which means that it does not have any pro-oxidative properties [129]. The main obstacle to the industrial use of astaxanthin is its low chemical stability during storage. To maintain its high stability, it is necessary to provide protection against adverse effects of temperature, pH and light exposure [48]. The best solution for achieving high bioavailability and stability is combining astaxanthin with edible oils. According to Ranga Rao et al. (2007), astaxanthin was fairly stable in rice bran, coconut, groundnut, mustard, gingelly, palm oils, sunflower and olive, when stored at room temperature for four months [130]. Ambati et al. (2014) showed the stability of astaxanthin at 70–90 °C in ricebran, palm oils and gingelly with an 84%–90% retention of the astaxanthin content. Compounds such as polyphenols, tocopherols or flavonoids have a positive impact on improving the stability of carotenoids [48]. According to Peng et al. (2010), encapsulation of astaxanthin within liposomes improved stability and bioavailability [118]. Similar effects were observed using microencapsulation with polymeric nanospheres, b-cyclodextrin, hydroxypropyl-b-cyclodextrin and sulfobutyl ether b-cyclodextrin, as documented by various researchers [48,131,132]. Satisfactory results have been obtained for the spray drying of microcapsules with the addition of maltodextrin and gelatin in a ratio of 2.1:1. The microcapsules showed good spherical shapes with a smooth surface and good solubility. The use of cheap carriers makes the process economically profitable [129].

7.2. Animal Feed

Astaxanthin has been used in feeding animals due to its strong antioxidant activity and safety of use and also because this pigment improves the organoleptic properties of animal products. Animals cannot synthesize carotenoids on their own, which is why they must ultimately obtain this pigment from the diet, in which plants and algae are rich [133,134].

Astaxanthin from *Haematococcus pluvialis* is mainly used in the aquaculture industry for the pigmentation of fish [14]. This interest in natural pigments results from the growing awareness of consumers and their demand for natural products [31,123]. Astaxanthin improves not only the coloration of many aquatic animal species, but also increases the survival of animals, their stress tolerance, disease resistance and growth performance [123]. The intensity of the pink-orange color of animals such as lobsters, aquarium fish, shrimps and salmon increased after astaxanthin supplementation. In the literature, there are examples of a beneficial effect of this natural pigment on animal health and quality of animal products. Astaxanthin supplemented to the diet of Atlantic cod (*Gadus morhua* L.) increased the fertilization, improved survival of larvae and reduced the embryonic mortality [135]. Dore and Cysewski (2003) found that natural astaxanthin from *Haematococcus* was equally effective with synthetic astaxanthin at pigmenting rainbow trout, when used at a dose of 50 mg/kg of feed. What is important it that the *Haematococcus* algae meal as a source of astaxanthin is safe and non-toxic [31]. It was found that *Haematococcus pluvialis* supplemented to the diet of rainbow trout (3 g/kg of feed) effectively enhanced the antioxidant activity and some biochemical parameters (e.g., decrease in serum glucose levels, decrease in triglyceride and cholesterol levels) [136]. Astaxanthin is of great interest in crustacean farming, such as the giant tiger prawn (*Penaeus monodon*). This carotenoid reverses the blue color syndrome found in farmed prawns with pigment deficiency. Food containing 50–100 g of astaxanthin per 1 kg of feed restored and harmonized pigmentation of shrimps within 4 weeks [137]. Ju et al. (2012) additionally found that the defatted *Haematococcus pluvialis* meal can serve as not only an alternative pigmentation ingredient in Pacific white shrimp feed, but also as a source of protein. This additive caused additionally a higher growth rate [138].

Haematococcus pluvialis and its pigment—astaxanthin—can also be used in livestock feeding. Astaxanthin used as a feed additive in poultry farming strengthened muscles, enhanced yolk color and prevented fat oxidation. The addition of this carotenoid did not, however, affect egg weight or size [139]. Astaxanthin improved yellow pigmentation of the feet and beaks of poultry. Chickens fed

with astaxanthin-containing feed not only increased their weight faster and had a greater increase in the muscle mass, but also showed increased fertility [140]. Supplementation with astaxanthin reduced chicken mortality associated with yolk sac inflammation and further increased resistance to *Salmonella* infection [141]. Waldenstedt et al. (2003) showed that astaxanthin-rich algal meal (7, 36, or 179 mg astaxanthin/kg feed) reduced caecal colonization of *Clostridium perfringens* of broiler chickens. The concentrations of this pigment increased in the kidney, intestine and breast muscle when compared to the control birds [142].

One of the most important features of pork for the consumer is its color. Oxymyoglobin oxidation is the main cause of meat color deterioration. Smith et al. (2003) proved that vitamin E added to the feed has an antioxidant effect and extends the usefulness of pork [143]. Astaxanthin accumulated in muscle tissue as a result of feeding has a better antioxidant effect than that added during meat processing. This is a very promising result, so much so that astaxanthin's antioxidant activity is four times higher than that of Vitamin E. Antioxidants not only affect the quality of meat, but also the health and condition of pigs [144]. Yang et al. (2006) demonstrated a tenfold reduction in back fat content and an increase in muscle mass after 14 days of feeding with 3 mg/kg of astaxanthin. Further reports refer to the improvement of pork carcass quality after using 48 mg/kg of astaxanthin for 3 months and 66.7 mg/kg for 42 days [139,145]. The supplementation of astaxanthin to the pig diet increased also the color of meat. Loin chops from pigs supplemented with dietary astaxanthin were darker and less yellow than loin chops from control pigs [146]. Furthermore, Bergstrom et al. (2009) found out that the loin muscle of pigs fed with astaxanthin (5 and 10 mg/kg) had a darker color, which can contribute to improved consumer acceptance of fresh pork [141].

Serwotka-Suszczak et al. (2019) showed that the extract from *Haematococcus pluvialis* additionally enriched with Mg(II) ions during cultivation improved insulin resistance in equine adipose-derived stromal cells. Therefore, this microalga can be used in the treatment of metabolic disorders [147]. Astaxanthin supplemented to the diet of dogs exerted its antioxidant properties, increased cell-mediated and humoral immune response and reduced DNA damage and inflammation [80].

There are several commercially available products based on *H. pluvialis*-derived astaxanthin, such as AstaEquus® (astaxanthin extract feed supplement for horses) and Novaasta® (astaxanthin extract feed supplement for animals): both produced by BioReaI, Sweden; Asta powder™ (powder for animal feed supplement, Atacama Bio Natural, Chile); Naturose™ (Algae meal; pigmentation source for ornamental fish and animals, produced by Cyanotech Corporation, USA) [13,14,31]; and astraZanthin™ (astaxanthin for dogs, LaHaye Laboratories, Redmond, WA, USA) [80].

8. Conclusions

Microalga *H. pluvialis* is the most attractive natural source of astaxanthin because it has the highest content in dry weight. The production of *H. pluvialis* on an industrial scale still faces difficulties related to the sensitivity of the strain to changes in growth conditions. Improving astaxanthin accumulation by *H. pluvialis* would reduce production costs and increase the availability of this carotenoid. The properties of astaxanthin, such as antioxidative, anti-inflammatory, antineoplastic, immunomodulatory activity and safety of its use, resulted in the increased interest in astaxanthin on the part of many scientific centers and food producers. Many scientific reports indicate that astaxanthin has a beneficial effect on the human body. There is a need to deepen the knowledge about astaxanthin. Some tests still need validating before it is possible to explain exactly the processes behind the effects of astaxanthin.

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Astaxanthin Carotenoid Modulates Oxidative Stress in Adipose-Derived Stromal Cells Isolated from Equine Metabolic Syndrome Affected Horses by Targeting Mitochondrial Biogenesis

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Abstract: Astaxanthin is gaining recognition as a natural bioactive component. This study aimed to test whether astaxanthin could protect adipose-derived stromal stem cells (ASCs) from apoptosis, mitochondrial dysfunction and oxidative stress. *Phaffia rhodozyma* was used to extract astaxanthin, whose biocompatibility was tested after 24, 48 and 72 h of incubation with the cells; no harmful impact was found. ASCs were treated with optimal concentrations of astaxanthin. Several parameters were examined: cell viability, apoptosis, reactive oxygen levels, mitochondrial dynamics and metabolism, superoxide dismutase activity, and astaxanthin's antioxidant capacity. A RT PCR analysis was performed after each test. The astaxanthin treatment significantly reduced apoptosis by modifying the normalized caspase activity of pro-apoptotic pathways (p21, p53, and Bax). Furthermore, by regulating the expression of related master factors SOD1, SOD2, PARKIN, PINK 1, and MFN 1, astaxanthin alleviated the oxidative stress and mitochondrial dynamics failure caused by EMS. Astaxanthin restored mitochondrial oxidative phosphorylation by stimulating markers associated with the OXPHOS machinery: COX4I1, COX4I2, UQCRC2, NDUFA9, and TFAM. Our results suggest that astaxanthin has the potential to open new possibilities for potential bio-drugs to control and suppress oxidative stress, thereby improving the overall metabolic status of equine ASCs suffering from metabolic syndrome.

Keywords: astaxanthin; antioxidant; equine metabolic syndrome; ASCs; mitochondria; OXPHOS

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1. Introduction

Metabolic syndrome consists of various endocrine disorders encompassing insulin resistance, diabetes, obesity, and inflammation, all of which are known to substantially increase the risk of developing atherosclerotic cardiovascular disease as well as vascular and neurological complications [1]. Those diseases can develop independently, but in some cases, they may form a cluster that leads to an overall metabolic syndrome, affecting both humans and animals [2,3]. Equine metabolic syndrome (EMS) poses a particular risk to horses; it is characterized by the following factors: regional adiposity in the neck, tail head, and above the eye, insulin resistance, and laminitis, both chronic and/or acute [4]. So far, no efficient pharmacological strategy for EMS treatment has been introduced, and existing protocols are essentially based on prevention, mainly through dietary restrictions and increased physical activity [5]. Interestingly, despite regular training and a proper diet, EMS can also affect sport horses, which may indicate a more complex pathophysiological pathway [6].

One of the significant hallmarks of EMS is insulin resistance (IR) [7]. Insulin resistance is defined as a diminished biological response to insulin, a peptide hormone that regulates the anabolic response to nutrient availability by binding to receptors anchored in the plasma membrane of target cells in peripheral tissues [8,9]. IR underlines several conditions related to the metabolic syndrome, including hypertension, type 2 diabetes, and circulatory and heart diseases [10]. The metabolic role of insulin is to stimulate glucose uptake in skeletal muscle and adipocytes, promote glycogen synthesis in skeletal muscle, inhibit hepatic glucose production, and inhibit lipolysis in adipocytes [11,12].

Adipose tissue is a highly active, endocrine organ that secretes hormones and cytokines and stores energy [13]. Several studies have demonstrated that adipose tissue (AT) homeostasis is highly affected by EMS [6,14,15]. It is also considered as a key factor in overall insulin resistance and obesity development [16]. Adipose tissue is recognized as one of the main components of systemic metabolic regulation, controlling lipid mobilization and maintaining body temperature [17]. A surplus of dietary energy is stored as neutral triglycerides in adipose tissue, leading to an increase in the volume of lipid droplets and, as a result, the expansion of adipose tissue and subsequent obesity [18]. Adipose tissue, as an endocrine organ, is responsible for the synthesis and secretion of several hormones including leptin, adiponectin, visfatin, and angiotentin, which modulate insulin resistance and the inflammatory axis [6,19]. These hormones orchestrate systemic metabolism by regulating metabolically active organs such as muscles, liver, pancreas, and brain [18]. Excessive adipose tissue in horses is associated with mitochondrial dysregulation, changes in insulin signaling, increased glucocorticoid metabolism, but also changes in plasma lipid content and elevated plasma leptin levels [20]. Adipose tissue may become resistant to insulin, but due to the release of free fatty acids, pro-inflammatory cytokines, and adipokines, it also plays a key role in insulin resistance development in the whole body [21].

In EMS affected horses, AT secretes a variety of pro-inflammatory mediators, including tumor necrosis factor α (TNF-α) and interleukins [22]. Adipocytes not only synthesize and assemble triglycerides but are also able to release free fatty acids (FFA) and glycerol as triglyceride hydrolysis products [23]. High levels of FFA in the blood are strongly correlated with obesity and insulin resistance [24]. The increase in body fat leading to obesity is connected with endocrine adipocyte dysfunction [20]. Excessive caloric intake triggers an inflammatory response in adipocytes and their dysfunction through the action of cytokines such as TNFα, which reduces insulin signaling and inhibits adipogenesis [25]. Kornicka et al. demonstrated that ASC cells isolated from EMS horses have limited proliferation potential, increased senescence, apoptosis, excessive accumulation of ROS, and deterioration of the mitochondria [26]. ASC dysfunction leads to abnormal remodeling of adipose tissue, which is associated with a higher risk of metabolic disorders [27].

Recent data demonstrated that the accumulation of reactive oxygen species (ROS) paired with increased inflammation are important components that hinder the ability of ASCs to differentiate into adipocytes and limit their multipotency [28,29]. ROS are highly reactive molecules that are mainly derived from the mitochondrial electron transport chain (ETC) [30]. Cells convert molecular oxygen into superoxide anions through the monovalent reduction of molecular oxygen in ETC, respiratory burst in phagocytes resulting from the ionization of cell membrane components, and as by-products of several cellular enzymatic reactions [31]. ROS regulate some of the signaling pathways as well as cytokine secretion, proliferation, differentiation, and gene expression, and play an important role in adipogenesis [32]. However, elevated ROS levels can lead to adipocyte overgrowth and, consequently, the formation of hypertrophic adipocytes [33].

Oxidative stress is defined as the imbalance between increased levels of reactive oxygen species (ROS) and low activity of antioxidant mechanisms [34]. Increased oxidative stress damages cellular structures and may lead to acute tissue injury [35]. According to Perez-Torres et al., many natural extracts and compounds have shown valuable therapeutic potential due to their ability to efficiently scavenge various reactive oxygen species (ROS)

and to prevent the activation of NF-κB and subsequent overexpression of its underlying target genes, including those involved in inflammation [36].

Carotenoids, a class of tetraterpenoids and naturally occurring pigments, have attracted great interest in the last few decades due to their potent biological activities that include antioxidant, antiproliferative, anti-inflammatory, and anti-ageing properties [37]. Astaxanthin, a red pigment usually obtained from various microorganisms and marine animals such as *Haematococcus pluvialis* algae or *Phaffia rhodozyma* yeast, emerged as a promising novel antioxidant that could be beneficial in preventing and/or reducing the risk of developing certain chronic diseases that are associated with oxidative stress-induced cellular and tissular damages [38]. Indeed, astaxanthin is able to quench and scavenge ROS and free radicals (hydrogen peroxide, superoxide anion, singlet oxygen, etc.) in both the inner and outer layers of cellular membranes, which shows its unique potential compared to most common antioxidants, which act either in the outer (vitamin C) or inner (e.g., vitamin E and β-carotene) layer of the membrane [37].

Many reports have already evidenced the beneficial pharmacological properties of astaxanthin in terms of its anti-inflammatory, immune-stimulating, anticancer, antidiabetic and antioxidant activities [39–43]. Astaxanthin has been demonstrated to decrease hyperglycemia-induced oxidative stress in pancreatic β-cells and to improve glucose and serum insulin levels in diabetics patients [44]. Another study reported the preventive effects of astaxanthin on a model of high glucose-induced inflammation and apoptosis in proximal tubular epithelial cells, which encourages its use for the development of new therapeutic formulations that could be applied to different pathologies and conditions [45]. At the request of the European Commission, the Panel on Nutrition, Novel Food and Food Allergens (NDA) issued statement on the safety of astaxanthin when used as a novel nutrient in food supplements at a maximum concentration of 8 mg/day [46]. In clinical studies of astaxanthin supplementation (4 mg daily) in a group of professional cyclists, an improvement of 5% in the 20 km time trial after 28 days was observed [47]. The effect of astaxanthin on skin condition was assessed in a group of 12 women receiving oral astaxanthin supplementation at a dose of 12 mg/day for 16 weeks; it was found that the treatment significantly improved skin hydration and reduced wrinkle parameters compared to the placebo group. Additionally, the levels of interleukin-1α in the stratum corneum increased significantly in the placebo group [48]. Subsequent studies have shown that oral administration of astaxanthin at a dose of 6 mg/day for 30 days alleviates the symptoms of dry eye disease in elderly patients [49]. The effect of astaxanthin supplementation at a dose of 200 mg/kg/day on cardiac diseases was assessed on a group of rats. Its administration reduced cardiomyocyte damage, inhibited inflammatory cell infiltration, preserved cardiac fiber structure, and prevented collagen deposition and stabilized levels of TGF-β1 protein in the left ventricle of high-fat rats [50]. On the other hand, dietary supplementation of astaxanthin at 0.3 mg/kg/day in healthy and obese dogs for 6 and 8 weeks, respectively, effectively activated antioxidant function and improved liver metabolic function and subsequent lipid metabolism in obese animals [51]. Collectively, these data suggest that astaxanthin may represent a novel drug candidate for the proper management of metabolic disturbances not only in humans but also in veterinary medicine. In the present study, we hypothesize that astaxanthin could be a beneficial therapeutic lead for restoring the metabolic balance in EMS adipose-derived stromal stem cells by targeting aberrant oxidative stress and the underlying mitochondrial dysfunction. For this purpose, we assessed the effects of astaxanthin on cell viability, apoptosis, reactive oxygen levels, mitochondrial dynamics, and metabolism, as well as its antioxidant capacity. The obtained results suggest that astaxanthin improves the metabolic status of equine ASC affected by metabolic syndrome. This finding opens up new possibilities for creating potential biopharmaceuticals.

2. Materials and Methods

2.1. Yeast Biomass and Astaxanthin Extraction

Phaffia rhodozyma NCYC 874 was obtained from the National Collection of Yeast Culture (UK) and cultured in Yeast Extract-Peptone-Dextrose medium (Sigma Aldrich; Poznań, Poland) for 72 h at 21 °C in a shaker at 160 rpm (LS 500 POL-EKO Aparatura; Wodzisław Śląski, Poland). Cells were afterwards collected by centrifugation at 3200× *g* for 5 min, 4 °C.

The biomass (5 g) was extracted with 100 mL of acetone (Sigma Aldrich; Poznań, Poland) in a shaker at 160 rpm at 30 °C for 2 h. After pelleting the yeast cells by centrifugation at 3200× *g* for 5 min, 4 °C the solvent was evaporated in a ventilated incubator completely from the sample at 35 °C in the dark and stored at 4 °C in the dark until use.

2.2. Equine ASCs Cell Culture

Equine ASCs were obtained from the cell collection of the Department of Experimental Biology, University of Environmental and Life Sciences, Wroclaw, Poland. Stem cells population expressed both CD90 and CD105 markers and were negative for CD45 and CD34 which excluded their hematopoietic origin [52].

ASCs cultures were maintained in 75 cm^2 flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1000 mg/L glucose, supplemented with 5% of fetal bovine serum (FBS), and 1% of a penicillin and streptomycin (PS) solution, and incubated at 37 °C in a humidified 5% CO_2 incubator. Cultured cells were harvested every three days (80–90% of confluence) and detached from the flasks with a trypsin-EDTA solution (TrypLE Express, Life Technologies; Carlsbad, CA, USA). Cells were multipotent and able to differentiate into adipocytes, chondrocytes and osteoblast in vitro as in previous study [53].

2.3. Determination of Cell Viability and Proliferative Activity by TOX8 Assay

The effect of astaxanthin on the viability and proliferation of equine ASC cells was assessed using a Resazurin-based assay kit (TOX8). Briefly, cells were seeded onto 96-well plate at a density of 8 × 10^3 cells/well in a final volume of 100 µL of DMEM complete culture medium. Next, cells were treated with 1, 5, 10, 20, 50 and 100 µg/mL of astaxanthin for 24, 48 and 72 h. After each incubation, the remaining medium was removed, and 100 µL of a 10% resazurin solution were added to each and incubated for 2 h at 37 °C in a humidified 5% CO_2 incubator. Absorbance was measured spectrophotometrically (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany) at a wavelength of 690 nm as a reference wavelength and 600 nm for resazurin. Two optimal concentrations of astaxanthin, i.e., 10 and 20 µg/mL, were selected for further experiments.

2.4. Bromodeoxyuridine (BrdU) Assay

The effects of astaxanthin on the DNA synthesis and proliferation were assessed using the 5-bromo-2-deoxyuridine (BrdU) Cell Proliferation ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, after 24 h treatment of EqASC cells with astaxanthin at concentrations of 10 and 20 µg/mL, BrdU reagent was added to all culture media and incubated at 37 °C during 72 h. After fixation of the cell, the detection of the incorporated BrdU was performed using anti-BrdU primary monoclonal antibody and secondary goat anti-mouse IgG conjugated with horseradish peroxidase (HRP). HRP substrate degradation was measured using a plate reader spectrophotometer (Spectrostar Nano; BMG Labtech, Ortenberg, Germany) at a 450 nm wavelength.

2.5. Colony-Forming Unit-Fibroblast (CFU-fs) Assay

The effect of astaxanthin on the ability of cells to form colonies was assessed by seeding 100 EqASC cells (HE and EMS) per well in six-well plates. Astaxanthin was subsequently added to the culture medium at different concentrations of 10 and 20 µL/mL and incubated for 7 days at 37 °C and 5% CO_2. The cells colonies were fixed in 4% cold paraformaldehyde and stained with pararosaniline solution. Colonies containing more than

50 cells were counted using an inverted microscope (AxioObserverA1; Zeiss, Oberkochen, Germany). The calculation of the efficiency of colony forming (CFU) was based on the following formula:

$$\text{CFU-fs (\%)} = (\text{Number of colonies}/\text{Initial cell number}) \times 100. \quad (1)$$

2.6. Flow Cytometric Analysis of Cell Viability and Apoptosis

The percentage of viable and apoptotic cells were determined using the MUSE™ Annexin V & Dead Cell Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, after treatment with astaxanthin, all treated and untreated EqASC cells were collected by trypsinization and suspended in Hanks' Balanced Salt Solution (HBSS) containing 1% FBS. Then, cells were stained with the Annexin V & Dead Cell reagent for 20 min at room temperature and analyzed using the Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany). The cell survival and apoptosis ratio were calculated by the identification of four distinct populations: (i) non-apoptotic cells, not undergoing detectable apoptosis, i.e., Annexin V (−) and 7-AAD (−); (ii) early apoptotic cells, Annexin V (+) and 7-AAD (−); (iii) late apoptotic cells, Annexin V (+) and 7-AAD (+); and (iv) cells that had died through non-apoptotic pathway, i.e., Annexin V (−) and 7-AAD (+).

2.7. Intracellular Reactive Oxygen Species Determination

The accumulation of intercellular reactive oxygen species (ROS) was evaluated using the Muse® Oxidative Stress Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, untreated and treated EqASC cells were collected and washed with HBSS. The cells were resuspended in the Muse Oxidative Stress working solution and incubated for 30 min at 37 °C in the dark. The measurement of ROS+ versus ROS− populations was performed by a Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany).

2.8. Endogenous Antioxidant Activities Assays

The activity of endogenous antioxidant enzymes was evaluated after the collection of lysed cells by sample assay buffer and centrifugation at 1200× *g* for 10 min. The antioxidant capacity was measured using the Cayman's Antioxidant Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. Briefly, samples were mixed with 10 µL of metmyoglobin and 150 µL of chromogen in a 96-well plate and compared to 10 µL of Trolox standard at different concentrations. Then 40 µL of hydrogen peroxide was added to each plate and incubated for 5 min at room temperature. Data analysis was performed using a spectrophotometer plate reader (Spectrostar Nano; BMG Labtech, Ortenberg, Germany) at 750 nm. The activity of superoxide dismutase (SOD) was measured using the Cayman Superoxidase Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the instruction in the user's guide. Briefly, 200 µL of radical detector was mixed with 10 µL of each sample on standard concentration in a 96-well plate. Then 20 µL of xanthine oxidase was added to each well and incubated for 30 min in room temperature and read immediately at 460 nm using spectrophotometer plate reader (Spectrostar Nano; BMG Labtech, Ortenberg, Germany).

Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in mM and activity percent for SOD.

2.9. Mitochondrial Membrane Potential Assay (MMP)

Measurement of changes in mitochondrial membrane potential ($\Delta\Psi m$) was performed with the MUSE™ MitoPotential Assay kit (Merck Millipore, Darmstadt, Germany). After treatment of the cells with astaxanthin, the culture medium was removed and EqASC cells were washed twice with HBSS, then incubated with the MitoPotential fluorescent dye for

30 min at 37 °C. The percentage of depolarized cells (depolarized alive + depolarized dead) was determined by Muse™ Cell Analyzer (Merck Millipore, Darmstadt, Germany).

2.10. Mitochondrial Network Fluorescent Staining

To visualize the morphology of the mitochondria, cells were stained with MitoRed (Sigma Aldrich, Poznan, Poland) fluorescent dye (1:1000 in culture medium), following each related treatment, and incubated for 30 min at 37 °C, prior to fixation in 4% paraformaldehyde at room temperature for 45 min. ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen™, Warsaw, Poland) was used for nuclei staining. The stained cells were observed using a confocal microscope (Observer Z1 Confocal Spinning Disc V.2 Zeiss with live imaging chamber) and captured with a Canon PowerShot camera. Obtained photomicrographs were merged and analyzed using ImageJ software (Bethesda, Rockville, MD, USA). Differences in mitochondria morphology were further evaluated using the Imaris software (Imaris®, Bitplane AG, Oxford Instruments, Zürich, Switzerland).

2.11. Mitochondria Isolation for Transcriptomic Analysis

To investigate if the treatment of astaxanthin influenced mitochondrial metabolic changes, total mitochondria were isolated from EqASC cells using the commercial Thermo Scientific™ Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, Warsaw, Poland), according to the manufacturer's instructions. Briefly, all treated and untreated cells were collected from culture flasks and washed three times with cold HBSS, then centrifuged at 300× *g* for 4 min, at 4 °C. remaining cells pellets were sequentially lysed using the provided lysis reagents containing a protease inhibitors cocktail (1:1000) on ice. The remaining cellular derbies and cytosolic fractions were discarded by centrifuging cell lysates at 700× *g* for 10 min at 4 °C. The obtained total mitochondria-rich supernatants were subsequently centrifuged at 12,000× *g* for 15 min at 4 °C. The final mitochondria pellets were then resuspended in TRIzol reagent for mtRNA isolation.

2.12. RNA Extraction and Real-Time Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from EqASC cells using TRIzol reagent according to the manufacturer's instructions. RNA purity and concentration were measured using a nanospectrophotometer (WPA, Biowave II, Cambridge, UK). cDNA was prepared from total isolated RNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Gdańsk, Poland) by the mean of a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Mt-cDNA samples were preamplified prior to quantitative RT-PCR analysis using a pool of primers based on targeted mitochondrial-related genes as in the cycling conditions: 95 °C for 2 min, followed by 18 cycles at 95 °C for 3 s, annealing for 3 min and 72 °C for 3 s.

The gene expression levels were evaluated by real-time reverse transcription polymerase chain reaction (RT-qPCR) using SensiFAST SYBR Green Kit (Bioline, London, UK) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Briefly, 10 µL total volume of each reaction consisted of 5 µL of SensiFAST SYBR Master mix, 2.5 µL of targeted primer and 2.5 µL of tested cDNA. Thermal cycle conditions were as follows: 95 °C for 2 min, then 40 cycles at 95 °C for 15 s, annealing for 15 s in temperature specified for tested primers, and elongation at 72 °C for 15 s. The results were reported regarding the expression of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative gene expression was calculated for all the tested groups, i.e., healthy, EMS, and EMS treated with astaxanthin using the $2^{-\Delta\Delta CQ}$ method. The sequences for all used primers are listed in Table 1.

Table 1. Gene expression.

Gene	Primer	Sequence 5′–3′	Amplicon Length (bp)	Accession No.
Parkin	F: R:	GTGCAGAGACCGTGGAGAAA GCTGCACTGTACCCTGAGTT	294	NM_013987.3
Sod1 (Cu/Zn SOD)	F: R:	CATTCCATCATTGGCCGCAC GAGCGATCCCAATCACACCA	130	NW_001867397.1
Sod2 (Mn SOD)	F: R:	GGACAAACCTGAGCCCCAAT TTGGACACCAGCCGATACAG	125	NW_001867408.1
Pink1	F: R:	GCTTGGGACCTCTCTTGGAT CGAAGCCATCTTGAACACAA	142	NM_032409.3
Casp9	F: R:	CAGGCCCCATATGATCGAGG CTGGCCTGTGTCCTCTAAGC	142	NM_032996.3
Casp3	F: R:	GGCAGACTTCCTGTATGCGT CCATGGCTACCTTGCGGTTA	167	XM_023630401.1
Bcl-2	F: R:	ATCGCCCTGTGGATGACTGAG CAGCCAGGAGAAATCAAACAGAGG	129	NM_000633.2
p21	F: R:	AGAAGAGGCTGGTGGCTATTT CCCGCCATTAGCGCATCAC	169	NM_001220777.1
p53	F: R:	AGATAGCGATGGTCTGGC TTGGGCAGTGCTCGCTTAGT	381	NM_001126118.1
Casp8	F: R:	ACTGTGATGTTGCTGGGACT CTTTCTCCTGGTGCATCTATCG	177	XM_001496753.4
Bax	F: R:	ACCAAGAAGCTGAGCGAGTGTC ACAAAGATGGTCACGGTCTGCC	356	XM_011527191.1
Mfn1	F: R:	GTTGCCGGGTGATAGTTGGA TGCCACCTTCATGTGTCTCC	146	NM_033540.3
OPA1	F:R:	CTTCTCTTGTTAGGTTCACCTGG TGTAAGAGAATGAGCTCACCAAG	110	XM_003363363.4
GAPDH	F: R:	GTCAGTGGTGGACCTGACCT CACCACCCTGTTGCTGTAGC	256	NM_001357943.2
Wnt3	F: R:	CACCTGCAAGTAGGGAGCCA GCTTCCCAGAGGACTTCGGT	80	XM_014739584.2
NDUFA9	F: R:	TTGGTATTCAGGCCACACCC GCTGGCTTCACGTCTTCAAC	103	XM_001494601.4
UQCRC2	F: R:	TGCTTCGTCTTGCATCCAGT AACTCCGGTGACGTGGTAAC	193	XM_001494381.5
COX4I1	F: R:	GAATAGGGGCACGAACGAGT GCCACCCACTCCTCTTCAAA	138	XM_023637444.1
COX4I2	F: R:	CCCCACCCCAGATGTTCT CGTGGTAGTTGGTGTAGGG	135	XM_005604417.3
OXA1L	F: R:	GACCTAGAAACCGTGGGACG GGAAGATCACTTGGCTCCCC	105	XM_008528958.1
MRPL24	F: R:	ATGATCCCTAGCGAAGCACC TGTAGAGACTCGTACCCGCT	123	XM_001500466.4
MTERF4	F: R:	CGCCACCTCCGTGCTATG CCCAAATGAGGGGCATCAGG	147	XM_023644068.1
PUSL1	F: R:	TCAGCCACTTCCAGGACCTA AGCCACATCCAAGCTGTCTG	120	XM_023636046.1
TFAM	F: R:	ATGATGGCTTTGAGTCCAGG CTAGATGATGGCGGGAGACTT	154	XM_023643450.1

Parkin Parkin RBR E3 ubiquitin protein ligase PARK2, Sod1 (Cu/Zn SOD) copper-zinc-dependant superoxide dismutase (CuZnSOD), Sod2 (Mn SOD) manganese-dependent superoxide dismutase (MnSOD), Pink1 PTENinduced putative kinase 1, Casp-9 caspase 9, Casp3 Caspase 3, Bcl-2 B cell lymphoma 2, p21 cyclin-dependent kinase inhibitor 1, p53 tumor suppressor p53, Casp-8 caspase 8, Bax BCl-2 associated X protein, Mfn-1 mitofusin 1, OPA-1 OPA1 Mitochondrial Dynamin Like GTPase, GAPDH glyceraldehyde 3-phosphate dehydrogenase, Wnt3 Wnt Family Member 3, NADH ubiquinone oxidoreductase subunit A9, UQCRC2 Ubiquinol-Cytochrome C Reductase Core Protein 2, COX4I1 Cytochrome c oxidase subunit 4 isoform 1, COX4I2 Cytochrome c oxidase subunit 4 isoform 2. MRPL24 Mitochondrial Ribosomal Protein L24, Mterf4 Mitochondrial Transcription Termination Factor 4, Tfam Mitochondrial transcription factor A, Pusl1 Pseudouridine Synthase Like 1, OXA1L mitochondrial inner membrane protein.

2.13. Western Blot Analysis

The EqASC cells were collected from each culture flask and homogenized in a mixture of phosphatase, protease inhibitor, and lysis buffer (Tris at 50 mmol/L pH 7.4, NaCl at

150 mmol/L, 0.1% SDS, 0.5% sodium deoxycholate, protease cocktail, 1 mmol/L PMSF, 10 mmol/L sodium ascorbate, 1% Triton X-100, 10 mmol/L of sodium azide, and Trolox at 5 mmol/L) on ice, in order to perform protein profiling. Proteins were collected by centrifugation of cell lysates for 20 min at 4 °C and 6000× *g* to remove insoluble materials, and subsequently transferred to new 1.5 mL Eppendorf tubes. Protein concentration was determined using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit. SDS-polyacrylamide gel electrophoresis was performed for 90 min in Tris/glycine/SDS 100 V buffer using a Mini-PROTEAN Tetra vertical electrophoresis cell (Bio-Rad, Hercules, CA, USA), on samples diluted in 4 × Laemmli Loading Buffer (Bio-Rad, Hercules, CA, USA) and denatured at 95 °C for 5 min. Then protein transfer was performed using polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) with a Mini Trans-Blot®Cell transfer apparatus (Bio-Rad, Hercules, CA, USA) in Tris/glycine/methanol buffer with 100 V, 250 mA at 4 °C for 45 min. Protein membranes were blocked in 5% skim milk solution in TBST for 1 h at room temperature. Protein detection was performed by incubation overnight at 4 °C in primary antibodies (Table 2), and secondary antibodies conjugated to HRP, dilution 1: 2500 in TBST, for 1 h at room temperature. Chemiluminescent signals were acquired using the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA) and quantified by Image Lab software (Bio-Rad, Hercules, CA, USA).

Table 2. List of antibodies employed for protein profiling using western blot analysis.

Antibody	Dilution	Catalog No.
PINK 1	1:1000	Biorbyt, orb331223
MFF	1:1000	Biorbyt, orb325479
β-Actin	1:1000	Sigma Aldrich, a2066

2.14. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 8.0 (San Diego, CA, USA). Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunett's post hoc multiple comparison test. Asterisk (*) signs indicated statistical significance of EqASCs EMS control versus EqASCs healthy cells and EqASCs EMS control versus EqASCs EMS-Astaxanthin treated groups respectively. The p values lower than 0.05 ($p < 0.05$) were summarized with one asterisk (*), $p < 0.01$ with two asterisks (**), and $p < 0.001$ with three asterisks (***).

3. Results

3.1. Astaxanthin Improves Viability and Proliferation in EMS ASCs Affected Cells

The impact of astaxanthin on cell proliferation was evaluated in terms of metabolic activity, DNA synthesis, and colony forming assays. As illustrated in Figure 1a, EMS affected cells were characterized by considerably lowered metabolic activity, as evidenced by the reduced ability to efficiently metabolize the Resazurin blue dye, in comparison to healthy cells after 24, 48, and 72 h of incubation. The obtained results also revealed that astaxanthin had no cytotoxic effect on EqASC cells at all tested concentrations (1, 5, 10, 20, 50, and 100 µg/mL). What is more, after 48 and 72 h of incubation, astaxanthin significantly increased the proliferation capacity of EMS EqASC cells in comparison to untreated cells ($p < 0.001$) (Figure 1a). Similar trends were observed after BrdU incorporation analysis, which revealed that EMS-affected ASCs had decreased newly synthetized DNA and thus reduced proliferation potential, by contrast to healthy untreated cells (Figure 1b). Treatment of EMS affected cells with astaxanthin at 10 and 20 g/mL resulted in a significant improvement in cell proliferation and division, as demonstrated by the increased amount of newly synthesized DNA in opposition to untreated EMS cells control group ($p < 0.01$). Analogously, the clonogenic fibroblast precursor (CFU-F) assay revealed a significant increase in the number of cells colonies formed by EMS EqASC cells after astaxanthin treatment at the two optimal tested concentrations (Figure 1c), whereas untreated cells had a lower

ability to form colonies when compared to healthy control cells ($p < 0.001$). Astaxanthin promoted cell proliferation and division, and the proportion of CFU-Fs produced in the astaxanthin-treated groups was equivalent to that observed in normal ASC cells.

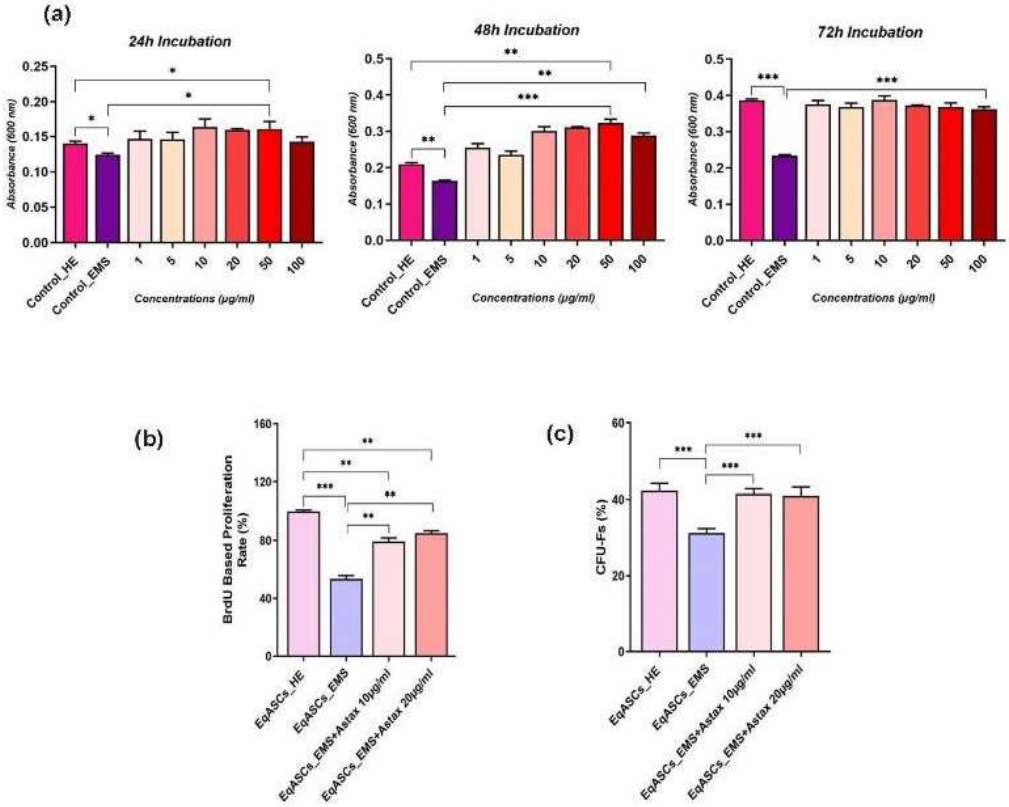

Figure 1. Impact of astaxanthin on cell proliferation. (**a**) The average absorbance at 600 nm after 24 h, 48 h, and 72 h of metabolized resazurin dye by healthy, treated, and untreated cells are shown by histograms. (**b**) Percentage of incorporated BrdU in newly synthetized DNA. (**c**) Cell proliferation assay using the clonogenic fibroblast precursor (CFU-F) assay. A comparison of EMS, treatment groups and untreated healthy cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2. Astaxanthin Reduced Cell Apoptosis in Equine ASC Cells Suffering from EMS

The proportion of viable cells versus cells undergoing apoptosis following astaxanthin treatment was determined using the Muse® Annexin V & Dead Cell test, as well as by measuring the gene expression of apoptosis master regulators. In contrast to healthy, non-affected cells, EMS cells were prone to increased apoptosis, as demonstrated by a drop in the percentage of live cells, and a resulting increase in the proportion of apoptotic and dead cells (Figure 2a). EMS ASC cells treated with two different concentrations of astaxanthin (10 and 20 µg/mL), displayed a significantly lower number of dead cells when compared to EMS untreated group (Figure 2b), suggesting that astaxanthin has a beneficial effect on cell viability. Furthermore, treated cells had low to moderate percentages of total apoptotic and dead cells, indicating that the carotenoid exerts an anti-apoptotic impact. Similarly, the relative expression of pro- and anti-apoptotic markers was assessed at mRNA level using the RT-qPCR approach. Obtained data clearly demonstrated that EMS cells were characterized by a considerable elevation of key pro-apoptotic factors expression, including *p21*, *p53*, *Bax*, *Casp-3*, *Casp-8*, and *Casp-9* in comparison to healthy control cells (Figure 2c), while apoptosis inhibitor *Bcl-2* appeared to be markedly downregulated in EMS cells ($p < 0.05$).

Treatment of EMS ASC cells with astaxanthin enabled the significant reduction of the excessive apoptosis by suppressing *p21*, *p53*, *Bax*, *Casp-3*, *Casp-8*, and *Casp-9* overexpression while simultaneously improving *Bcl-2* pro-survival transcript expression (Figure 2c), imply-

ing that astaxanthin has a strong anti-apoptotic and pro-survival effect on EMS ASC cells.

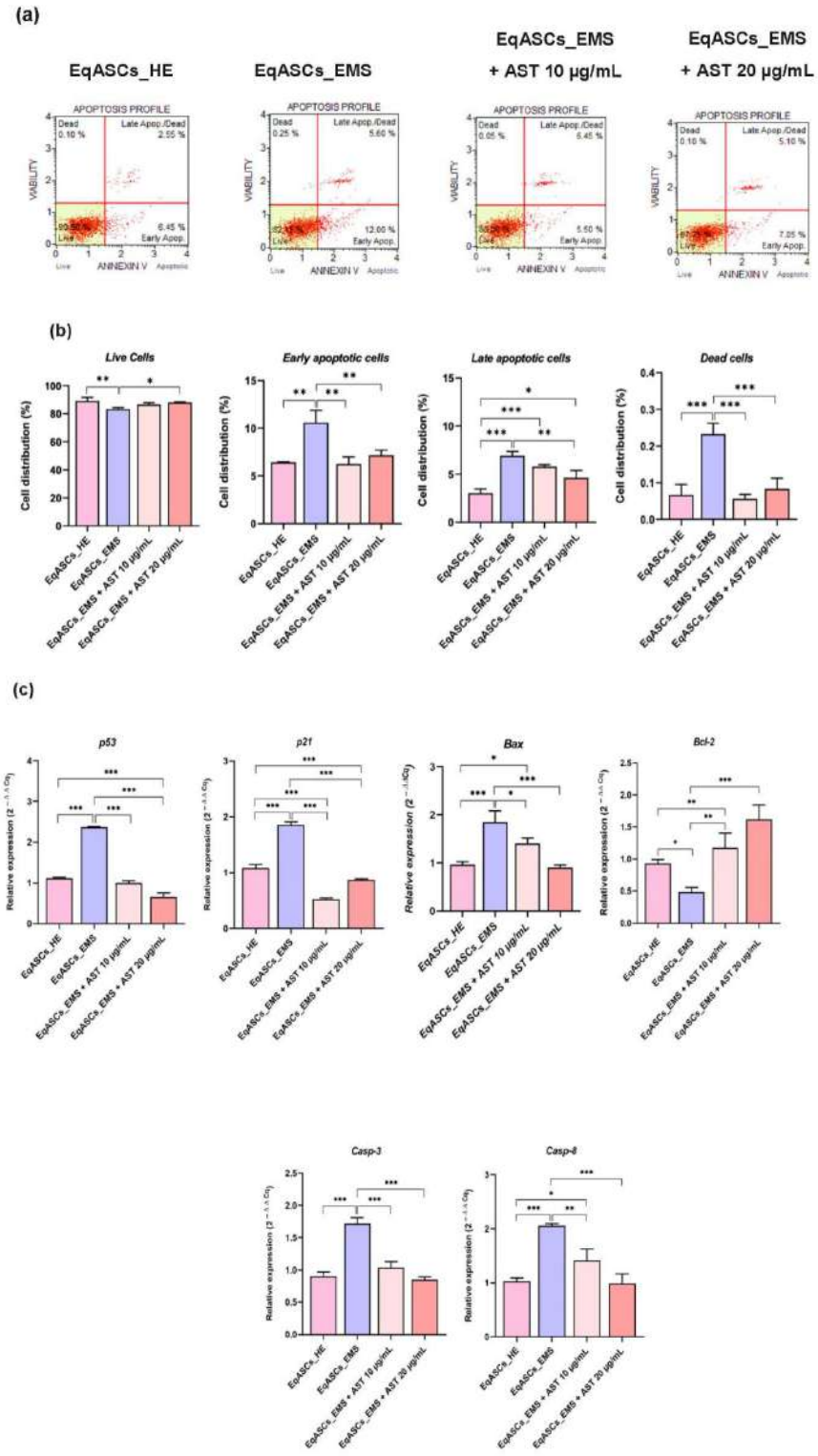

Figure 2. Antiapoptotic effect of Astaxanthin on EMS ASC cells. (**a**) The Muse® Annexin V & Dead Cell assay was used to assess live cells, early and late apoptotic cells, and dead cells. (**b**) According to the Muse® Annexin V & Dead Cell assay, histograms reflect the ratio of live, early apoptotic, late apoptotic, and dead cells. (**c**) Bar charts illustrating the relative expression of major apoptotic markers. A comparison of EMS, treatment groups and untreated healthy cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Astaxanthin Decreases Oxidative Stress in Equine EMS ASCCells

Oxidative stress represents one of the most prominent hallmarks of EMS in ASC cells, leading to global metabolic failure, and ultimately, to cellular death. In this study, the Muse Oxidative Stress Assay was used to assess intracellular ROS accumulation in untreated and astaxanthin-treated cells (Figure 3a). A significant rise in intracellular ROS in EMS ASC cells was found (Figure 3b), confirming the initiation of oxidative stress during EMS. The treatment with astaxanthin over a period of 24 h effectively abolished the relevant EMS-associated oxidative stress, as demonstrated by the decrease in the number of ROS positive cells in comparison to untreated EMS cells ($p < 0.001$). The antioxidant effect of astaxanthin was further investigated by measuring SOD enzyme activity and total cellular antioxidant capacity (Figure 3c). In ASC cells affected by EMS, the collected data revealed a substantial decrease in SOD activity and total endogenous antioxidant capacity ($p < 0.001$). Treatment with astaxanthin resulted in a substantial restoration and stimulation of the cellular antioxidant capacity, together with improved enzymatic efficacy of SOD under EMS condition. This effect was further confirmed by RT-qPCR results, which showed that EMS affected cells had dysregulated *Sod1* and *Sod2* gene expression (Figure 3d), whereas EMS ASC cells treated with astaxanthin displayed an upregulation and recovery of the same transcripts, indicating that astaxanthin improved the antioxidant status of affected cells, resulting in oxidative stress mitigation.

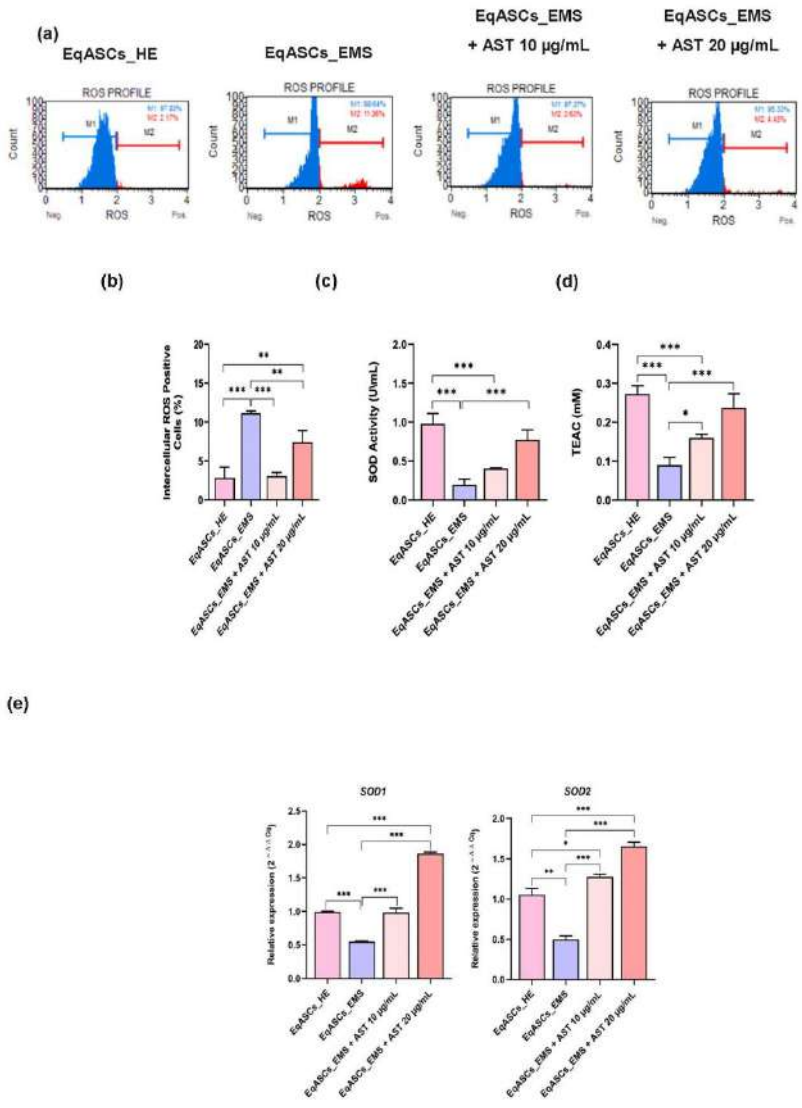

Figure 3. Effect of Astaxanthin on oxidative stress in EMS affected ASC cells. (**a**) Dot-Plots for

intracellular ROS production detected by dihydroethidium (DHE) fluorescence staining. (**b**) Average percentages of total ROS+ cells in each experimental group. (**c**) Measurement of SOD Activity performed with Cayman Superoxidase Dismutase Assay Kit. (**d**) antioxidant capacity by Cayman's Antioxidant Assay Kit. (**e**) Relative gene expression of *SOD1* and *SOD2* transcripts. A comparison of EMS, treatment groups and untreated healthy cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. Astaxanthin Enhances Mitochondrial Dynamics in EMS Affected ASC cells

Mitochondrial metabolism disruption is a key player in oxidative stress, cellular ROS overproduction, and resulting pro-apoptotic pathways activation, which are characteristic of EMS. The significant increase in total living and dead cells exhibiting depolarized mitochondrial membrane potential in EMS untreated ASC cells (Figure 4a,b), in contrast to metabolically normal cells ($p < 0.05$), revealed that the EMS condition caused mitochondrial activity collapse. In addition, there was evidence of a significant breakdown in mitochondrial dynamics. An analysis of the morphology of the mitochondria highlighted an evident mitochondrial tubular network disruption, as EMS untreated cells exhibited poor and fragmented mitochondrial networks, characterized by reduced branched tubular and globular structures and critical loss in mitochondrial loops (Figure 4c–e), suggesting the depletion of fusion capacity in favor of sustained fission. In fact, EMS untreated cells showed significant downregulation of mitochondrial fusion related factors, such as *MFN-1* and *OPA-1* (Figure 4f), while the expression of fission associated markers, including *Parkin* and *Fis-1* ($p < 0.01$), appeared to be significantly higher than that of healthy control cells. Western blot analysis further showed increased MFF and Pink1 protein expression, two major regulators of mitochondrial fission in EMS untreated ASC group (Figure 4g) compared to control cells ($p < 0.01$), confirming the aberrant and persistent mitochondrial fragmentation and division under EMS condition. Twenty-four hours of astaxanthin conditioning resulted in a substantial improvement of overall mitochondrial function (Figure 4). In fact, EMS ASC cells treated with astaxanthin were characterized by enhanced membrane potential, as evidenced by the decrease in the percentage of cells with depolarized mitochondria (Figure 4b).

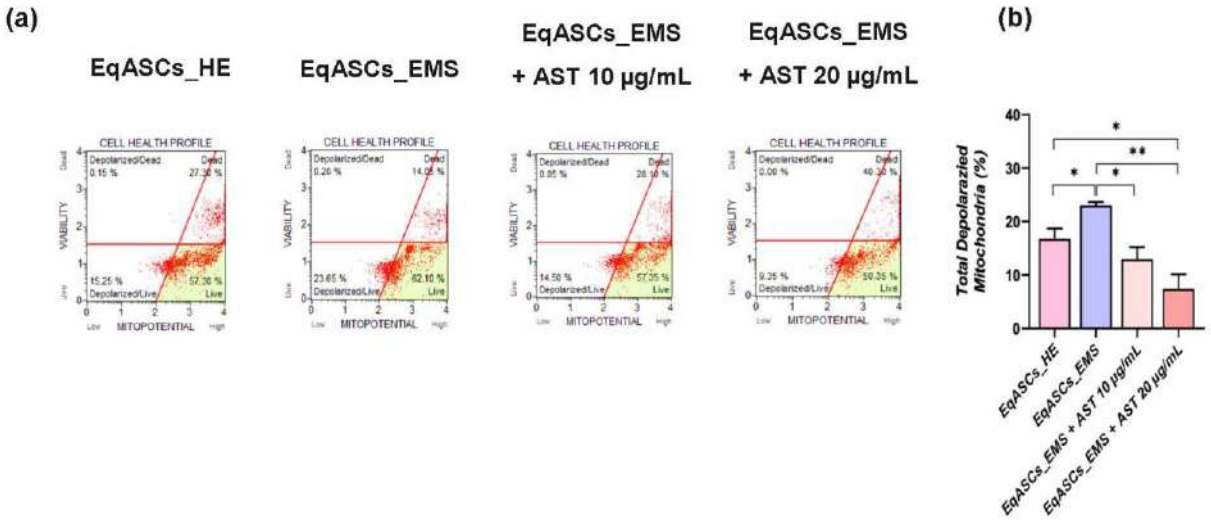

Figure 4. *Cont.*

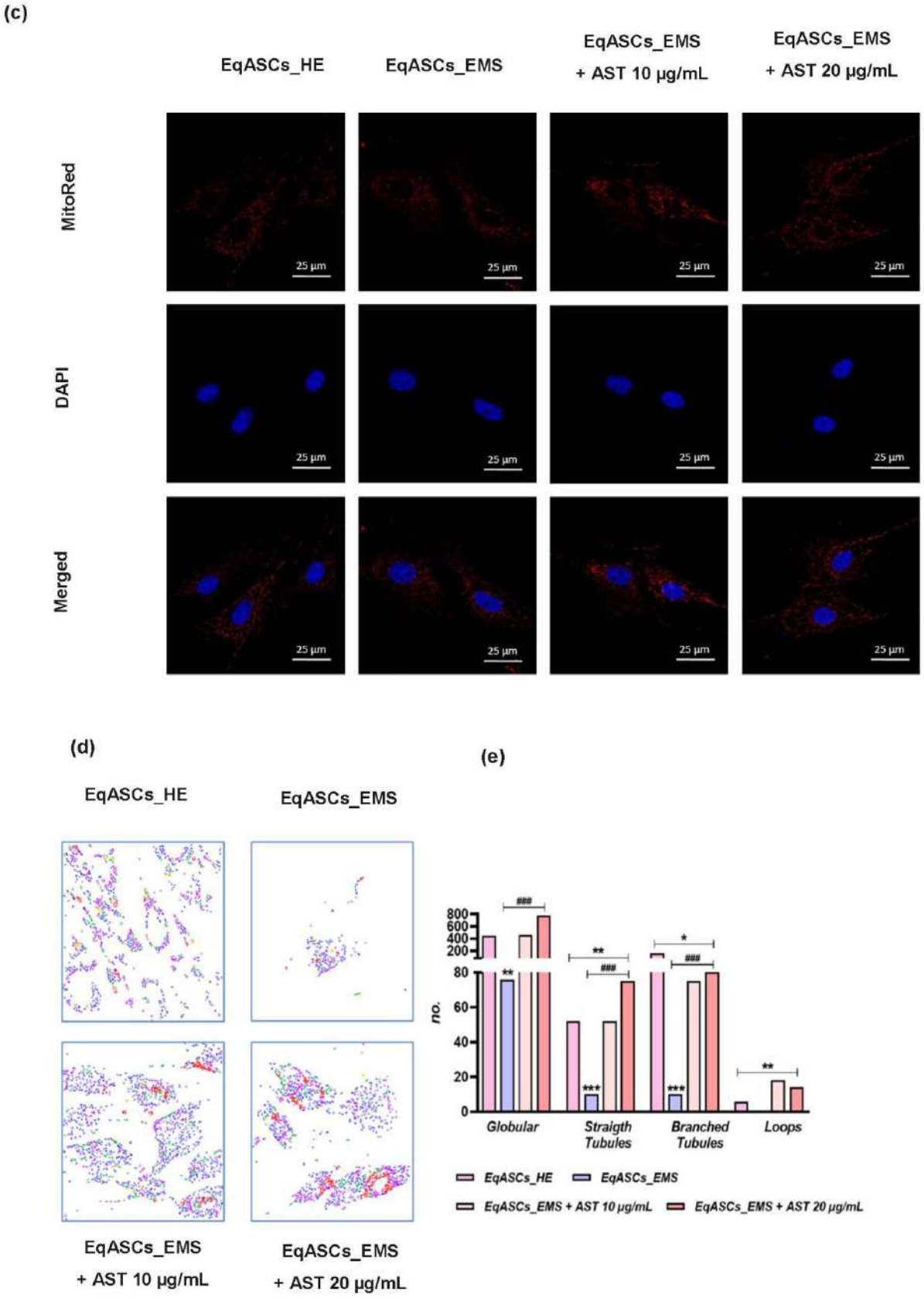

Figure 4. *Cont.*

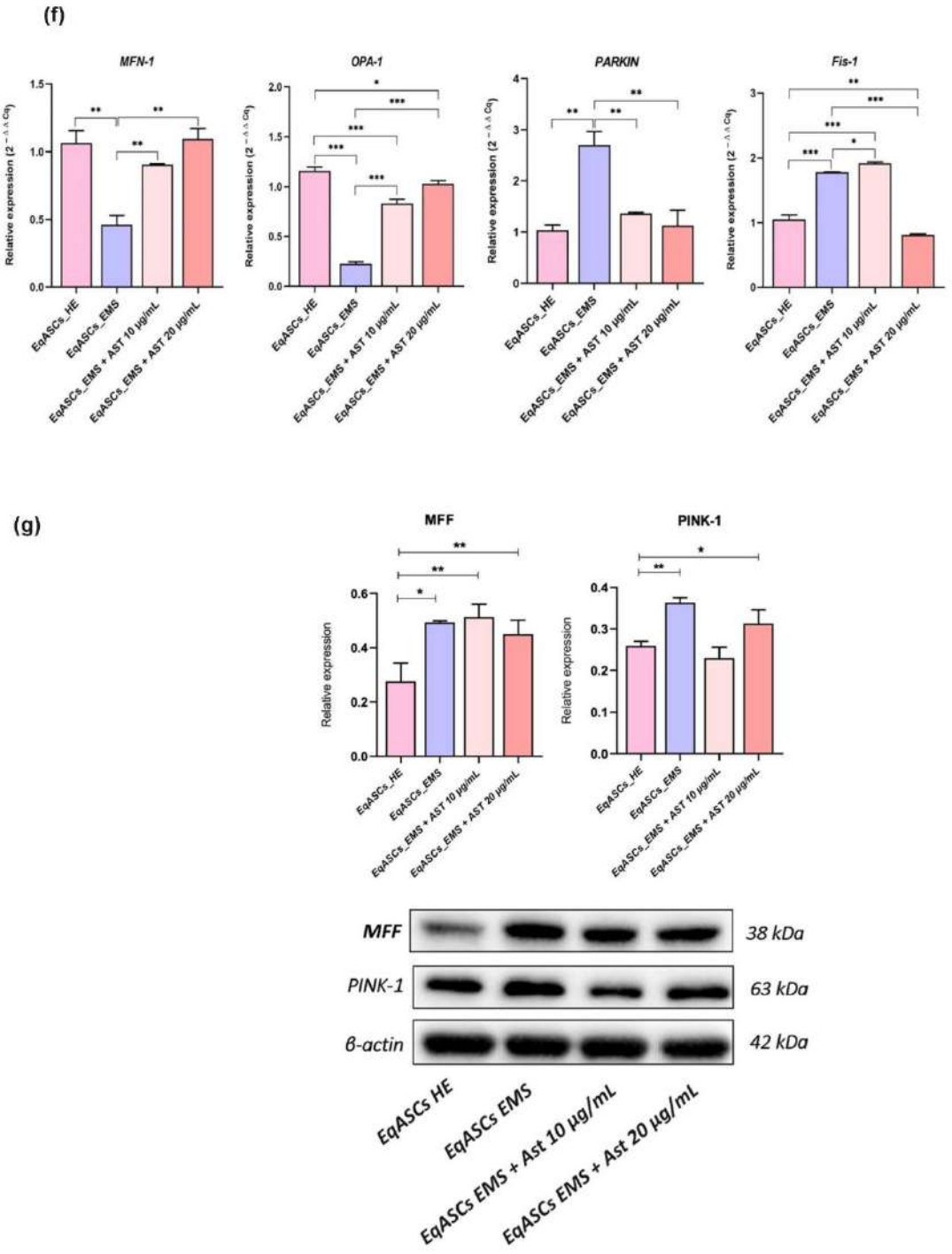

Figure 4. The effect of astaxanthin on mitochondrial dynamics in EMS ASC cells (**a**) Flow cytometric analysis of Mitochondrial membrane potential (MMP). (**b**) Percentages of total depolarized mitochondrial membrane potential. (**c**) Epi-fluorescent confocal microscope images of MitoRed stained cells; scale bar size 25 µm. (**d**) Imaris mitochondrial morphology analysis micrographs Mitochondrial morphology analysis. (**e**) Mitochondrial morphology analysis. (**f**) Representative Bar-Charts of the relative expression of mitochondrial fusion and mitophagy markers. (**g**) Levels of MFF, and Pink-1 were estimated with the western blot method. Relative expression was estimated using Image Lab software after normalization with β-actin (loading control). A comparison of EMS, treatment groups, and untreated healthy cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A hashtag (#) refers to a comparison of the EMS and astaxanthin treated groups. ### $p < 0.001$.

Compared to EMS-untreated EqASC cells, astaxanthin-treated cells further showed mild mitochondrial dynamics perturbations, as demonstrated by the positive regulation of fusion/fission balance and the consequent suppression of excessive *Parkin* and *Fis-1* transcripts expression, the downregulation of MFF and Pink1 proteins levels, as well as the restoration of *MFN-1* and *OPA-1* transcription (Figure 4f). As a result, a restoration of the mitochondrial network architecture with promoted globular structures, branched tubular shape, and triple-stranded loop distribution in carotenoid-treated cells was observed (Figure 4c–e), all of which suggest that astaxanthin may lower oxidative stress and apoptosis by rebalancing mitochondrial dynamics.

3.5. Astaxanthin Supports the Transcription of Mitochondrial Metabolism Related Effectors

Mitochondria are considered central metabolic hubs that maintain overall energetic homeostasis through respiration and electron transfer reactions. To further support the evidence that astaxanthin may exert its antioxidant effect through the amelioration of mitochondrial activity and metabolism, an analysis of the expression of genes linked to mitoribosomes and mitochondrial oxidative phosphorylation machinery (OXPHOS) in all experimental groups of cells was carried out using RT-qPCR. The obtained data (Figure 5a) showed that EMS cells displayed diminished *Uqcrc2*, *Ndufa9*, *Cox4l1*, and *Cox4l2* gene expression as compared to non-stressed cells. Furthermore, master regulators of mitochondrial transcription machinery as well as mitochondrial ribosomal biogenesis and translation, namely *Pusl1*, *Mrpl24*, and *Tfam*, were profoundly downregulated under EMS condition, which indicated a severe failure in mitochondrial biogenesis and oxidative phosphorylation (Figure 5b). Moreover, the *Wnt3* transcript, which is involved in the upregulation of the OXPHOS complex, was found to be significantly downregulated in EMS affected cells, suggesting a disruption of the mitochondrial metabolic regulation pathways. The addition of astaxanthin to EMS ASCs cell cultures for a period of 24 h significantly improved overall mitochondrial metabolism, as evidenced by a visible recovery of the expression of OXPHOS associated complexes units, including *Uqcrc2*, *Ndufa9*, *Cox4l1*, and *Cox4l2*, together with the restoration of factors involved in mitochondrial translation processes, including *Pusl1*, *Mrpl24*, and *Tfam*, as well as a member of the *Wnt/β-catenin Wnt3* signaling pathways, which exert various regulatory actions in cellular energy metabolism (Figure 5). These results indicate the likely specific antioxidant effect of astaxanthin, i.e., it may target mitochondrial metabolism and its associated oxidative phosphorylation machinery.

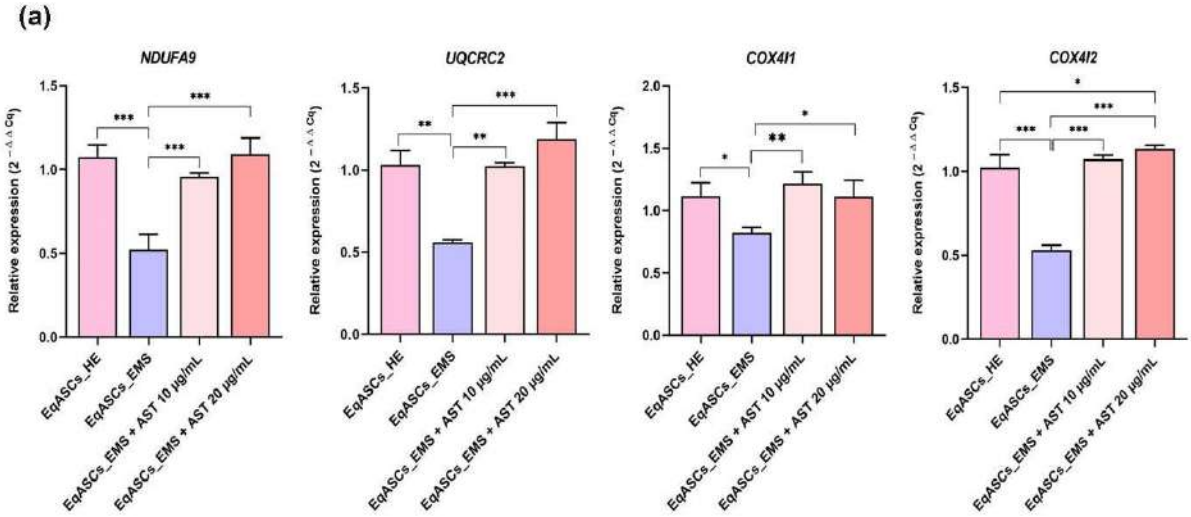

Figure 5. *Cont.*

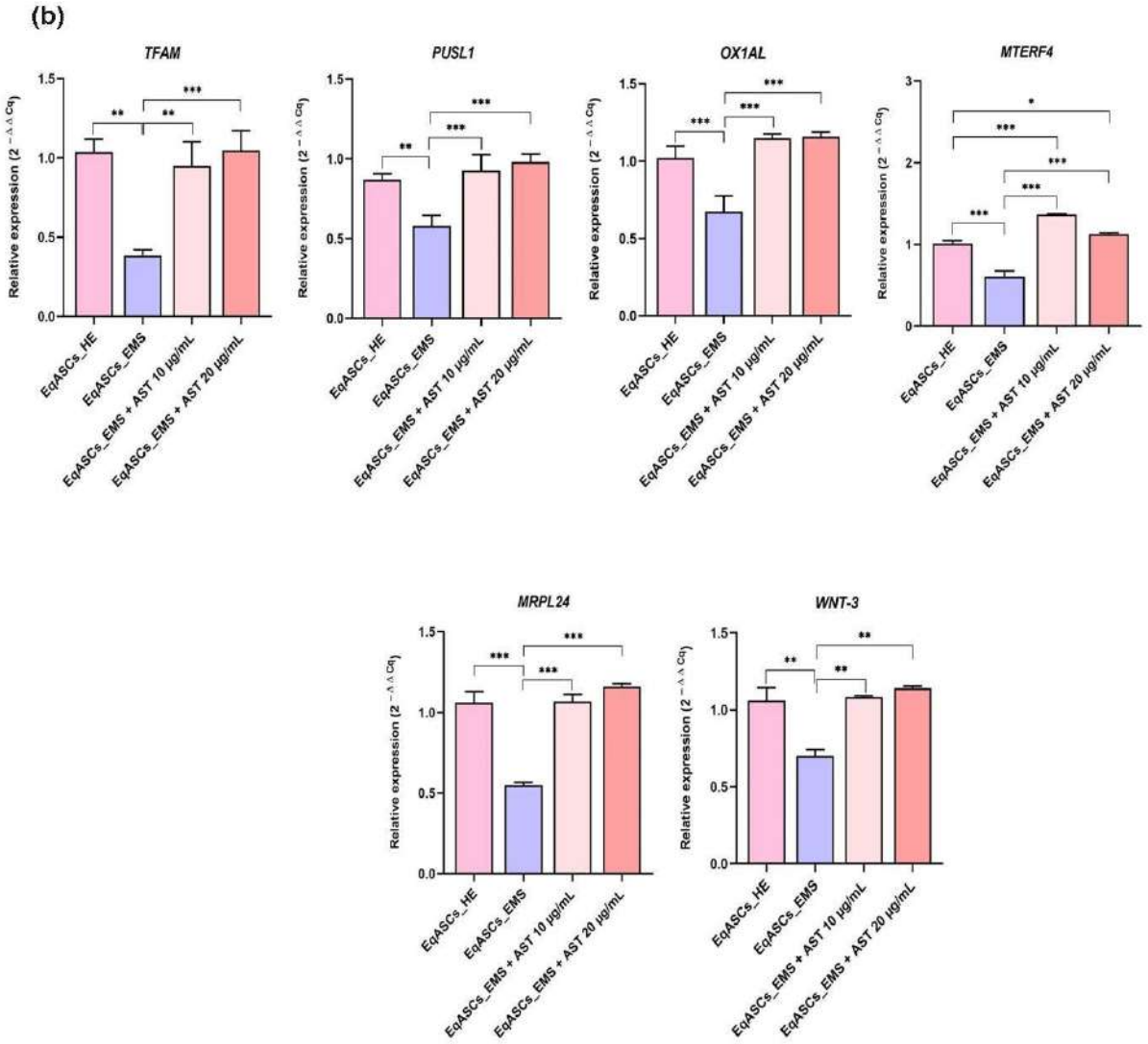

Figure 5. Effect of Astaxanthin on Mitochondrial metabolism. Expression of (**a**) genes encoding for the OXPHOS complexes (**b**) genes encoding of mitochondrial translational machinery regulators. A comparison of EMS, treatment groups and untreated healthy cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

In the present study, we investigated the effects of astaxanthin on preventing apoptosis, mitochondrial dysfunction and oxidative stress of adipose-derived stromal stem cells affected by EMS. Astaxanthin is a promising bioactive compound for the prevention of several human diseases as well as for the maintenance of good health [41]. Astaxanthin exhibits a wide range of biological functions, most of which are associated with its antioxidant and anti-inflammatory effects [54]. The growing popularity of astaxanthin is supported by its high ability to absorb oxygen radicals, i.e., 100 to 500 times higher than that of α-tocopherol, and 10 times more active inhibition of free radicals than related antioxidants (α-tocopherol, α-carotene, β-carotene, lutein and lycopene) [55]. Astaxanthin reduces cell apoptosis, ameliorates oxidative stress and mitochondrial dysfunction and modulates mitochondrial dynamics [56,57]. This study provides strong evidence for the role of astaxanthin in equine metabolic syndrome prevention and treatment.

Our research has shown that EMS affects ASC viability and correlates with oxidative stress. EMS contributes to insulin resistance and the secretion of various adipokines, which worsens the metabolic state of the body [58]. Our studies on the effect of astaxanthin on

ASC (healthy and EMS controls) showed an improvement in cell viability without cytotoxic effect, mainly at concentrations of 10 and 20 µg/mL. Moreover, the results showed that treating EMS ASCs with two different concentrations of astaxanthin significantly increased their proliferation rates compared to untreated EMS cells, which had reduced proliferation and colony formation capacity and a high apoptotic tendency. Research by Weiss et al. has shown an over-activation of apoptotic pathways in ASC cells affected by EMS, with a consequent reduction in proliferation rate and an increase in cell death rate [59]. These results correlate with our studies, which showed an increased number of apoptotic cells accompanied by critical upregulation of pro-apoptotic players including *p53*, *p21*, *Bax*, *Casp-3*, *Casp-9* and *Casp-8* transcripts, in parallel with a loss of expression of the *Bcl-2* cell survival gene. According to Cui et al., astaxanthin is a powerful anti-apoptotic agent which prevents ochratoxin A-induced heart damage and cardiomyocyte apoptosis in mice, mainly by regulating the expression of *Keap1*, *Nrf2*, *Bax*, *Bcl-2*, *Casp-3* and *Casp-9* both at the mRNA and protein level [60]. Guo et al. reported that astaxanthin significantly stimulated phosphorylation in an experimental model of acute kidney injury induced by burns in rat, which then allowed the inhibition of activation of further pro-apoptotic factors, including cytochrome c and *caspase-3/9* axis [61]. The above reports support our results, where the use of astaxanthin in EMS ASC cultures for 24 h resulted in a clear stimulation of the metabolic activity and proliferation rate of cells, along with the promotion of their ability to create colonies, while preventing increased apoptosis by suppressing *p53*, *p21*, *Bax*, *Casp-3*, *Casp-9* and *Casp-8* expression and simultaneously restoring *Bcl-2* survival gene expression.

Oxidative stress disturbs cell homeostasis because of an excess of free radicals in relation to the number of antioxidant molecules [62]. An excess of generated ROS can have a negative impact on cell development, lipid metabolism, nucleic function, cellular communication and control, genetic mutations and biological activity, immune activation and inflammation [63]. Oxidative stress is involved in the development of insulin resistance and coexisting inflammation [64]. Additionally, it has been shown that oxidative stress in obese patients with insulin resistance and hyperglycemia increases the risk of aggressive cardiovascular diseases and pro-inflammatory changes and reduces the bioavailability of nitric oxide [65]. In this study, we confirmed that EMS ASC was characterized by increased intracellular accumulation of ROS due to the onset of severe oxidative stress and decreased expression of the antioxidant enzymes SOD1 and SOD2. Simultaneously, EMS ASCs showed a significant decrease in total endogenous antioxidant capacity and an associated decreased SOD enzymatic activity. EMS is strongly linked to insulin resistance; it affects an increasing number of horses and has already been reported to exhibit strong and excessive oxidative stress, which strongly impairs metabolic functions and accelerates cellular senescence and ageing, leading to multipotency limitation [66]. ROS play a significant role in diseases related to metabolic dysregulation and inflammation; therefore, EMS ASC cells affected by prolonged oxidative stress are characterized by an overproduction of harmful ROS, accompanied by the breakdown of cellular antioxidant defense mechanisms, including a decrease in the activity of antioxidant enzymes and their expression at the gene level [6,67]. According to our results, astaxanthin modulates oxidative stress within ASC cells; this was evidenced by the observed drop in the number of ROS positive cells and the restoration of the expression of the two antioxidant enzymes (SOD1 and SOD2). Astaxanthin has a strong antioxidant effect due to the presence of two oxidized groups on each ring, so it can regulate key signaling pathways by regulating or activating various molecules and pathways, such as *PI3K/AKT* and *JAK/STAT-3* (signal transducers and transcription activators), *Nrf2* (NF-E2 related factor 2), *NF-κB* (kappa nuclear factor-activated B-cell light chain enhancer), MAPK (mitogen-activated protein kinases) and PPARγ (peroxisome proliferator-activated gamma receptor) [68]. Astaxanthin showed remarkable protective effects on cellular membranes and associated lipid peroxidation due to its polar/non-polar chemical structure [69]. In addition, it has been proven that astaxanthin has a strong effect on the reduction of lipid damage in liposomes treated with H_2O_2, tert-butyl hydroperoxide (t-ButOOH) or ascorbate and Fe^{2+}: EDTA [70]. Research by Cui et al. and Xue et al. showed

that astaxanthin reduces the levels of ROS which are directly related to LDL oxidation and overall lipid peroxidation. The observed effects were related to the stimulation of expression and mobilization of the nuclear factor 2-related transcription factor 2 (*Nrf2*), followed by upregulation of its target antioxidant genes, including phase II biotransformation enzymes [60,71].

Our research has shown changes in mitochondrial membrane potential, which is a key marker of mitochondrial dysfunction and is considered an early indicator of cellular stress and apoptosis. In addition, a morphological analysis showed a loss of mitochondrial network integrity, as evidenced by excessive cleavage and decreased fusion, which was further confirmed by the observed dysregulation of the axis of fusion/cleavage markers, including *Parkin*, *Fis-1*, *MFN-1* and *OPA-1*. The performed analysis of the integrity of mitochondrial biogenesis showed a breakdown in mRNA expression of *Mrpl24*, *Mterf4*, *Tfam* and *Pusl1*, major mtDNA regulators and key players in the biogenesis of mitochondrial ribosomes and translation machines. Our report correlates with the research of Marycz et al., where ASC EMS was shown to suppress gene expression related to selective mitophagy and mitochondrial dynamics and biogenesis, including *Pink*, *Parkin*, *PGC1-α* and *PDK4* [72]. Initially, astaxanthin has an antioxidant effect, eliminating reactive oxygen and nitrogen species and other free radicals [73]. However, there are more and more reports on the stimulation of the action and function of mitochondria in cells treated with astaxanthin [71,74–77]. According to our research, cells treated with astaxanthin showed increased mitochondrial dynamics and biogenesis, as demonstrated by restoring mitochondrial transmembrane potential, restoring proper mitochondrial morphology, and regulating fission events and fusion dynamics. Research of Nishid et al. also confirmed the beneficial effect of astaxanthin on the maintenance and enhancement of mitochondrial activity through a direct impact on the *AMPK/Sirtuin/PGC-1α* pathway and other pathways [56]. Nawaz et al. confirmed the ability of astaxanthin to stimulate mitochondrial function, where astaxanthin was shown to reduce insulin resistance in diet or myotubule-induced obesity in vitro by modulating insulin signaling in an antioxidant and antioxidant-independent manner and by activating mitochondrial energy metabolism via the activation of the AMP-activated protein kinase pathway (AMP) coactivator γ coactivator-1α (PGC-1α) peroxisome proliferator activated receptor in skeletal muscle [56]. Yu et al. proved that astaxanthin maintained mitochondrial integrity by increasing *PGC-1α* expression and maintained normal tubular structure and heat-induced oxidative stress in C2C12 myoblasts [78]. Additionally, Jiang et al. showed that astaxanthin induced the expression of genes encoded in mitochondria, increased the number of viable copies of mitochondria and stimulated the activity of mitochondrial respiratory chain complex enzymes in BPA-damaged rat kidneys [79].

Cellular energy mainly results from the production of ATP via oxidative phosphorylation (OXPHOS) [80]. The tightly folded inner membranes of mitochondria called cristae contain OXPHOS (I-IV) complexes, including ATP synthase (V complex), while complexes I-IV are multi-subunit enzymes that work together to form an electrochemical proton gradient in the mitochondrial inner membrane that is used for the production of ATP by oxidative phosphorylation [81]. The OXPHOS system may trigger the development of several cellular disorders, including inflammation, oxidative stress and apoptosis [82]. Here, we showed for the first time that equine ASC cells affected by EMS presented a serious failure in the OXPHOS machinery, as evidenced by the substantial downregulation of the *Ndufa9, Uqcrc2, Cox4i1* and *Cox4i2* transcripts, suggesting that one of the molecular mechanisms leading to oxidative stress and mitochondrial failure during EMS lies in the affliction of the OXPHOS system and downstream disruption of mitochondrial metabolism and cellular energy homeostasis. Moreover, *Wnt3* gene expression, which has been implicated in the regulation and activation of the OXPHOS action, appeared to be significantly compromised, indicating a concomitant loss of regulatory pathways. Our outcomes established that astaxanthin also targets mitochondrial OXPHOS complexes by upregulating the expression levels of each related complex mRNA together with *Wnt3* regulator, supporting

the hypothesis that astaxanthin's antioxidant properties may involve a restoration of proper oxidative phosphorylation.

Currently, to the best of our knowledge, there are no published clinical studies on astaxanthin supplementation in horses suffering from EMS. Nevertheless, studies by Sato et al. showed that supplementation of astaxanthin and L-carnitine during training of Thoroughbred horses reduced the incidence of exercise-induced muscle damage due to their mutual antioxidant properties [83]. Research on the use of natural extracts against EMS was conducted by Nawrocka et al. Those authors showed that a 3-month supplementation of *Spirulina plantesis*, rich in phycocyanin and β-carotene, reduced body mass, as well as improving serum insulin levels and sensitivity in the group of horses with EMS [84]. In a study on a rat model by Hussein et al., supplementation with astaxanthin at a dose of 50 mg/kg/day for 22 weeks lowered blood pressure and fasting blood glucose levels, as well as insulin resistance index, which subsequently restored insulin sensitivity and overall metabolic homeostasis [85]. Zhuge et al. also investigated the anti-diabetic effects of astaxanthin in male diabetic rats who were fed a diet containing 15 mg/kg/day of astaxanthin for 3 weeks. Supplementation with astaxanthin significantly lowered the level of blood glucose and total cholesterol (TC) and increased the level of high-density lipoprotein (HDL-C) cholesterol [86]. In view of the above reports, it would be reasonable to conduct clinical trials on astaxanthin supplementation in horses with EMS in order to confirm whether Astaxanthin exhibits similar in vivo antioxidant and antidiabetic effects.

5. Conclusions

According to the presented results, astaxanthin showed promising abilities for the treatment of metabolically afflicted ASC cells, and prevented the occurrence of apoptosis, reduced oxidative stress, and reversed mitochondrial dysfunctions, which are key players in the development of EMS condition and are known to seriously impair the regenerative properties of ASC cells. Furthermore, through the modulation of mitochondrial dynamics and OXPHOS, astaxanthin is providing new insights in the treatment of insulin resistance and obesity. Additional indepth study would provide more precisions regarding the detailed mechanisms by which Astaxanthin improves mitochondrial biogenesis and thus ameliorates the fate of malfunctioning EMS ASCs.

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3. ARTYKUŁ NAUKOWY I PRZEGLĄDOWY – PROBIOTYKI I POSTBIOTYKI

Choroby metaboliczne to jedna z najszybciej rozwijających się jednostek chorobowych zarówno wśród ludzi i zwierząt. Co ciekawe, coraz więcej doniesień naukowych podkreśla ścisłą korelację między zaburzeniami metabolicznymi i mikrobiotą jelitową. Probiotyki są selektywnie dostarczanie do przewodu pokarmowego, natomiast postbiotki stanowią zespół metabolitów probiotycznych o korzystnym działaniu na organizm gospodarza. Zarówno probiotyki i postbiotyki są silnymi regulatorami mikrobioty jelitowej oraz zapobiegają chorobom metabolicznym w szczególności otyłości i cukrzycy typu 2. Podstawowe mechanizmy związane z drobnoustrojami jelitowymi to głównie modulacja składu mikroflory jelitowej, regulacja metabolitów drobnoustrojów jelitowych i poprawa funkcji bariery jelitowej.

W celu oceny kolejnej grupy naturalnych substancji jako czynników prewencyjnych dla rozwoju chorób metabolicznych opublikowany został artykuł przeglądowy na temat postbiotyków: „Postbiotics as potential new therapeutic agents for metabolic disorders management". Ponownie, artykuł ten stanowił podstawę do zaplanowania i przeprowadzenia badań nad wpływem probiotyków i postbiotyków na komórki HepG2 z indukowaną insulinoopornością. W efekcie opublikowany został artykuł naukowy: „Probiotics-rich emulsion improves insulin signalling in Palmitate/Oleate-challenged human hepatocarcinoma cells through the modulation of Fetuin-A/TLR4-JNK-NF-κB pathway", który potwierdził drugą założoną hipotezę badawczą dotyczącą ochronnego wpływu pro i postbiotycznej emulsji względem komórek HepG2 z indukowaną insulinoopornością. Uzyskane wyniki dowiodły, iż emulsja pro- i postbiotyczna wykazuje działanie ochronne względem komórek HepG2 z indukowaną insulinoopornością poprzez ochronę przed apoptozą związaną z lipotoksycznością, a także poprawę metabolizmu i dynamiki mitochondriów oraz zmniejszenie stresu oksydacyjnego. Ponadto zastosowanie emulsji spowodowało istotne zmniejszenie aktywacji szlaku *Fetuina-A/TLR4/JNK/NF-κB* przy jednoczesnym przywróceniu kaskad sygnalizacyjnych insuliny, co sugeruje ich ochronny wpływ na stany zapalne, otyłość oraz insulinooporność wątroby.

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Review

Postbiotics as potential new therapeutic agents for metabolic disorders management

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ABSTRACT

The prevalence of obesity, diabetes, non-alcoholic fatty liver disease, and related metabolic disorders has been steadily increasing in the past few decades. Apart from the establishment of caloric restrictions in combination with improved physical activity, there are no effective pharmacological treatments for most metabolic disorders. Many scientific-studies have described various beneficial effects of probiotics in regulating metabolism but others questioned their effectiveness and safety. Postbiotics are defined as preparation of inanimate microorganisms, and/or their components, which determine their safety of use and confers a health benefit to the host. Additionally, unlike probiotics postbiotics do not require stringent production/storage conditions. Recently, many lines of evidence demonstrated that postbiotics may be beneficial in metabolic disorders management via several potential effects including anti-inflammatory, antibacterial, immunomodulatory, anti-carcinogenic, antioxidant, antihypertensive, anti-proliferative, and hypocholesterolaemia properties that enhance both the immune system and intestinal barrier functions by acting directly on specific tissues of the intestinal epithelium, but also on various organs or tissues. In view of the many reports that demonstrated the high biological activity and safety of postbiotics, we summarized in the present review the current findings reporting the beneficial effects of various probiotics derivatives for the management of metabolic disorders and related alterations.

1. Introduction

Metabolic disorders are among the most common and challenging diseases in modern medicine. Obesity, dyslipidemia, diabetes mellitus, osteoporosis, and metabolic syndrome are the most widespread pathologies characterized by metabolic failure [1]. The development of metabolic disorders occurs when physiological metabolic pathways are disrupted, due to inadequate diet, sedentary work, and lack of physical activity [2]. Genome polymorphism related to lipid metabolism, glucose absorption, insulin signaling, or appetite control contribute to the development of metabolic diseases [3].

Metabolic syndrome (MetS) can be defined as a clusters of interconnected biochemical, physiological, metabolic, and clinical factors including central obesity, visceral adiposity, impaired glucose tolerance, hyperglycemia, insulin resistance (IR), high triglyceride, atherogenic dyslipidemia, low high-density lipoprotein (HDL) cholesterol levels, and/or hypertension [4,5], which collectively increase the risk of type 2 diabetes mellitus (T2DM), atherosclerotic cardiovascular disease (ASCVD), as well as vascular and neurological consequences like cerebrovascular accident [6]. Insulin resistance and obesity appear to be the most relevant factors in the MetS pathogenesis, in addition to the proinflammatory state that likely participate to the condition's development [7]. Furthermore, most of the pathways involved in MetS have been shown to be triggered by visceral adiposity, emphasizing the importance of a high caloric intake as a major causative factor [8]. Chronic inflammation, IR, and neurohormonal activation appear to be the primary actors in the onset, development, and transition of MetS to CVD, among all the hypothesized pathways [9].

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MetS treatment mainly involves lifestyle improvement, diet, and increasing physical activity as well as targeted pharmacological treatment to specific affection, that currently faile to efficiently control the disease progression, due to the lack of a single etiological factor or central pathophysiological alteration [10,11]. Weight loss represents the first-line treatment for obesity management; indeed, a fat mass loss of about 7% has a significant effect on lowering blood pressure glucose levels, triglycerides, and total cholesterol, and improving adipokines and inflammatory markers (adiponectin and tumor necrosis factor-alpha) expression [12–14]. Weight loss is also considered as a strong predictor of improved insulin sensitivity as a 5–10% body weight reduction, has been reported to increase insulin sensitivity in peripheral tissues by 30–60%, whereas weight regain significantly predicted a reduced insulin sensitivity [15,16]. Currently, research on the correlation between diet and metabolic syndrome focuses not only on caloric restriction and macronutrient supplementation but also on the effect of bioactive ingredients issued from natural sources, in a view of maintaining body weight and restoring insulin sensitivity [17].

Recently, several studies have been undertaken to highlight the influence of gut microflora (GM) on metabolic syndrome development, due to the close interaction of the GM with his host, which is critical for gut homeostasis [18]. The gut microbiota is a key element in regulating the metabolic pathways leading to the conversion of ingested nutrients into energy; in fact, crosstalk between the GM and host is mediated by metabolites (postbiotics), which are produced in the colon by the fermentation of non-digestible substances [19]. In addition, many lines of evidence indicate that an imbalance in the composition of gastrointestinal microflora may contribute to the development of insulin resistance associated with obesity [20]. Comparative studies of obese versus lean patients-derived gut microbiota showed differences in the overall composition; 50% fewer *Bacteroidetes* proportion and higher amount of intestinal *Firmicutes* in obese; which hypothesizes that manipulating the gut microbiota may facilitate weight loss or prevent obesity in humans [21,22]. Disruption of the natural balance between the gut microbiome and the host's immune system may lead to the translocation of bacterial fragments in the gut and, as a result, metabolic endotoxemia (caused by bacteria or bacterial fragments). This pathological condition further triggers to low-grade systemic inflammation, which can contribute to the development of insulin resistance, diabetes, and obesity [23].

Probiotic microbes affect the gut microbiota in the large intestine, but they also have an effect on other organs, either by modulating immune parameters, intestinal permeability, and allowing the movement of bacteria from the gastrointestinal tract to extra-intestinal tissues, or by supplying biologically active metabolites [22,24]. Despite the many beneficial effects of probiotics on human health, some reports questioned their effectiveness and safety, especially in high-risk patients. Therefore, there is a growing interest in the use of probiotic bacteria-derived metabolites, also known as postbiotics instead of probiotics pools [25,26]. Postbiotics are defined as soluble non-viable metabolites (vitamins, peptidoglycan, polysaccharids, biosurfactants,…) produced by a bacterial or probiotic metabolic process that display direct or indirect beneficial effects on the host[27,28]. Postbiotics can directly affect metabolism, insulin secretion, and insulin sensitivity, such as postbiotic muramyl dipeptide reduces inflammation and promotes insulin signaling in a state of metabolic endotoxemia, glycemia, and obesity along a pathway that includes NOD2 [29,30].

Due to the many beneficial effects of postbiotics, as well as concerns related to supplementation with live probiotic microorganisms, we analyzed current reports on the use of individual types of postbiotics in the prevention, mitigation, and treatment of metabolic disorders.

2. Gut microbiota – multifunctional role

In recent years, there has been more and more research in the field of microbiome-host interactions due to the wide spectrum of metabolic functions and correlation with metabolic diseases. The intestinal microbiota is a community consisting mainly of bacteria distributed in the intestinal lumen, with approximately 10^{11}–10^{12} microorganisms/gram of intestinal content, most of which are anaerobic bacteria (about 95%) [31,32]. The gut microbiome is dominated by *Bacteroidetes* and *Firmicutes* and accounts for approximately 90% of all gut microbiota. *Firmicutes* are over 200 different types, the most numerous are: *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus*, over 95% predominance of *Clostridium* genera. In contrast, the main representatives of *Bacteroidetes* are *Bacteroides* and *Prevotella* [33,34].

The composition of the healthy human gut microbiota is constantly changing throughout life. New-born's intestines are colonized immediately after birth. The type of birth affects the nature and degree of colonization. Vaginal delivery increases new-born intestinal colonization in *Lactobacillus, Prevotella,* and *Sneathia* species. However, after cesarean section, the majority of the recorded genera are *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* [35–37]. Up to the age of 5, the child's intestinal microflora is enriched with *Bacteroidetes*, followed with *Lachnospiraceae*, *Anaerovorax*, *Bifidobacterium*, and *Faecalibacterium* [35]. In contrast, people over 65 years of age exhibit a decrease in *Bacteroidetes* and *Firmicutes* compared to healthy adults [38].

The basic substrate for harvesting energy by the intestinal microbiota are carbohydrates supplied with food, which are converted into short-chain fatty acids (SCFA), mainly propionate, butyrate, and acetate in the fermentation process [39]. Over 70% of the oxygen used up in the energy processes of colonocytes is produced by the oxidation of butyrate. As signaling molecules, SCFAs modulate the GPR43 and GPR41 receptors of mammalian G protein-coupled receptors (GPCR) [40]. Previous studies have shown that a mechanism involving GPR43 regulates adipose tissue homeostasis and increases its metabolic activity [41]. SCFA regulates gene expression and accelerates the proliferation of epithelial cells, while inhibiting the proliferation of cancer cells by regulating apoptosis. The intestinal microbiota is involved in the digestion of lipids and their conversion into lipoproteins, which facilitate the transport of lipids from the intestine to the liver [42]. The type of lipids supplied with the diet has a significant impact on the profile of the gut microbiota. Studies on the effects of various sources of dietary lipids in mice resulted in physiological gut microbiota changes. The introduction of animal fats (lard) to the diet reduced the amount of *Lactobacillus* and *Bifidobacterium*, while milk fat increased the availability of sulfur, necessary for the growth of *Bilophila wadsworthia* that is associated with increased pro-inflammatory immune responses [43,44]. Undigested proteins in the small intestine pass to the colon where they are metabolized by among others *Klebsiella, Streptococcus, Mitsuokella,* and *Succinivibrio dextrinosolvens, Escherichia coli,* and *Anaerovibrio lipolytica* species [45]. To do this, bacteria activate the proteinase and peptidase enzyme pathways, including L-histidine to histamine or glutamate to γ-aminobutyric acid [46,47]. Some of the crucial vitamins are not synthesized in mammalian cells, but rather produced by plants, yeasts, and bacteria, mainly *Bacteroides, Bifidobacterium*, and *Enterococcus* [48]. The vitamins synthesized by the intestinal microflora include vitamin K and B vitamins, biotin, cobalamin, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamin [49]. The main element of host metabolism regulation by the intestinal microflora lies in the deconjugation of primary bile acids and their biotransformation to secondary bile acids [50]. Major biotransformations include hydrolysis of conjugated bile acids to free bile acids and glycine or taurine by bile salt hydrolase (BSH) [51].

Another important function of the gut microflora, apart from the metabolic function, is the immune function. The strong connection between host immunity and the gut microflora has been described by many studies using germ-free animal models (GF). The lack of colonization of the GF animals intestines by physiological microflora is associated with intestinal defects of the lymphoid tissue as well as immune functions [52]. The intestinal microflora regulates immune homeostasis by modulating innate and adaptive immunity. Delayed microbial colonization in the host gastrointestinal tract leads to pathological changes in

the development of the gut-associated lymphoid tissue (GALT), leading to persistent immune dysregulation [53]. The gut microflora regulates the development of antigen-presenting cells (APCs). As a result of this cooperation, APCs limit immune responses against the physiological intestinal flora, while protecting against the invasion of intestinal pathogens [54]. Natural killer (NK) cells regulate responses to bacterial infections by stimulating the production of IFNγ. NK are able to recognize and respond to molecules induced on the cell surface by stress signals and viral infections [55]. The commensal microflora contributes to the differentiation of the lymphocytic population co-expressing stimulating NK cell receptors and the transcription factor RORγt, which mediates the production of interleukin-22 (IL-22) [56]. Many components of the gut microflora regulate the differentiation and function of T cells. Proper Th1/Th2 cells balance is essential for the regulation of host immunity. Intestinal commensal *Bacteroides fragilis* produces polysaccharide A, which activates CD4 + T cells, causing a Th1 response to correct the deflection of Th2 cells in GF mice [57,58].

Scientific reports show the connection between gut microbiota and brain in the case of anxiety, depression, cognitive disorders, and autism spectrum disorders (ASD) [59]. GF mice without accompanying microflora exhibit impaired memory, recognition disorders, and emotional disturbances, as opposed to conventionally bred mice. Differences in important neurotransmitters (5-HT, NMDA, and BDNF) were noted in GF mice [60,61]. The gut microbiota has a significant influence on the gut-brain axis (GBA), cooperating not only locally with intestinal cells, but also directly with the central nervous system through neuroendocrine and metabolic pathways. The disturbance of the physiological balance of the intestinal microflora leads to cellular changes in the gut-endocrine and immune systems, which affect anxiety behavior, the development of depression, and autism [62].

The gut microbiome plays a constant, key role in maintaining host-microbial homeostasis. Disturbances in this balance lead to inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), diabetes, obesity, cancer, cardiovascular and central nervous system diseases [63]. Dysbiosis is a disorder of the digestive tract bacterial flora in relation to the endobiogenic needs of the body. Dysbiosis can be caused by insufficiency or loss of diversity in commensal flora or pathogenic flora competing with the commensal microbiome [64]. In addition to the pathogenic flora, the gut commensal flora is influenced by several key factors related to the host's environment, type of diet and antibiotics intake. Age is a critical factor influencing the gut microflora. The composition of the flora of new-borns is influenced by the type of delivery as well as the presence or the amount of breast milk in the child's diet. By consuming mother's milk, an infant receives an average of $\sim 10^6$ bacterial cells/ml of milk [65,66]. Antibiotics influence the composition of the intestinal microflora in two direct and indirect ways. During antibiotic therapy, not only the population of pathogenic bacteria is reduced, but also the bacteria of the natural intestinal flora. Continuous ampicillin therapy disrupted microbial homeostasis resulting in increased NF-κB production in the colon tissues of a mouse model [67,68]. Moreover, treatment-induced dysbiosis with a mixture of ampicillin, metronidazole, vancomycin, and neomycin resulted in a decrease in the number of T cells and uveitis as a consequence of an increase in Treg levels [69]. A stable intestinal microflora is a key factor in maintaining homeostasis throughout the body.

It has been established that gut microbiota's greatest positive effects are mostly mediated by the production of a range of bioactive metabolites named postbiotics [70]. These components are of unlimited interest and can be particularly useful in preventing certain diseases. As a matter of fact, several studies have related the emergence of metabolic diseases with imbalanced or modified GM as well as reduced bacteria diversity and its components [70,71]. As a result, boosting the microbiota becomes a valuable approach for the management of these physiopathology.

3. Postbiotics

It is well documented that the microbiological, physicochemical, and sensory properties of an inoculated food can be modified by microorganisms. Probiotics are the most interesting organisms commonly used in industrial foods due to their healthy advantage in secreting several substances called postbiotics that ensure food safety [72,73].

Postbiotics is a newly emerged term that refers to a wide range of bioactive molecules including non-viable/inactivated microbial cells, cell compounds, and any soluble products or metabolic byproducts resulting from microorganisms (probiotics), which directly or indirectly mediat positive biological activity when administered to consumers (Fig.01) [27,73–76]. In view of that, the current accepted definition of this concept was "preparation of inanimate microorganisms and/or their components that confers a health benefit to the host" [77]. In order to identify postbiotics, the most commonly used term, various terminologies have been suggested in the literature, such as non-viable probiotics, non-biotics, proteobiotics, pharmabiotics, metabiotics, parapsychobiotics, paraprobiotics, inactivated probiotics, tyndallized probiotics, or ghost probiotics [77–86]. Accordingly, postbiotics have been recognized to mimic probiotics functions and activities [87], and do not require stringent manufacturing or storage conditions, making them ideal for developing nations (Fig. 1).

3.1. Probiotics producing postbiotics

High effective postbiotics including cytoplasmic extracts and cell wall components were identified in several species belonging to *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Bacillus*, *Streptococcus*, or *Faecalibacterium* genera [88–91]. *Lactobacilli* species which include *L. rhamnosus*, *L. bulgaricus, L. acidophilus*, *L. reuteri*, *L. casei*, *L. fermentum*, *L. paracasei*, *L. helveticus*, *L. johnsonni*, and *L. gasseri* [92–98], as well as *Bifidobacterium* such as *B. bifidum*, *B. longum* subspecies *infantus*, *B. breve*, and *B. longum* are the most common probiotics producers of postbiotics. In fact, it has been documented that when *B. longum* has been administered to mice, a significant antibody response has been observed for the cytoplasmic fraction, while cell-wall fraction did not induce any immune response [99]. Moreover, *L. plantarum* species, that produce metabolites containing a high amount of combined organic acids and bacteriocin, have a potential effect as food biopreservative and gut health in animals [100]. In addition to that, lipoteichoic acid extracted from *L. plantarum* exerted a significant attenuation of flexPGN-induced pro-inflammatory signals in human monocytic THP-1 cells [95]. Oral administration of *Faecalibacterium prausnitzii* supernatant, the only one species within the genus, sensibly decreased the severity of 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis in mice and tended to correct the dysbiosis associated with TNBS colitis, highlighting its potent anti-inflammatory effects [89]. Another potential probiotic, *Saccharomyces cerevisiae* yeast, is usually used for bioproduction of metabolites having postbiotics potentialities as anti-inflammatory components [101]. Cell wall fragments and metabolites obtained from *Bacillus coagulans* presented in human polymorphonuclear (PMN) cells a spontaneous inhibition and oxidative stress-induced reactive oxygen species (ROS) formation [90].

3.2. Classification of postbiotics and its therapeutic benefits

Currently, scientists have identified various forms of postbiotics molecules resulting from extracellular and intracellular probiotic cells. These components include peptidoglycans-derived muropeptides, exopolysaccharide (EPS), teichoic acids, and surface protruding molecules like fimbriae, pili or flagella that constitute cell wall components, secreted proteins/peptides, bacteriocins such as acidophilin, reuterin, and bifidin, cell-free supernatant, organic acids such as lactic acid and acetic acid, vitamins, short-chain fatty acids like butyric acid and propionate, neurotransmitters, biosurfactants, etc [86,93,102]. Hence, due

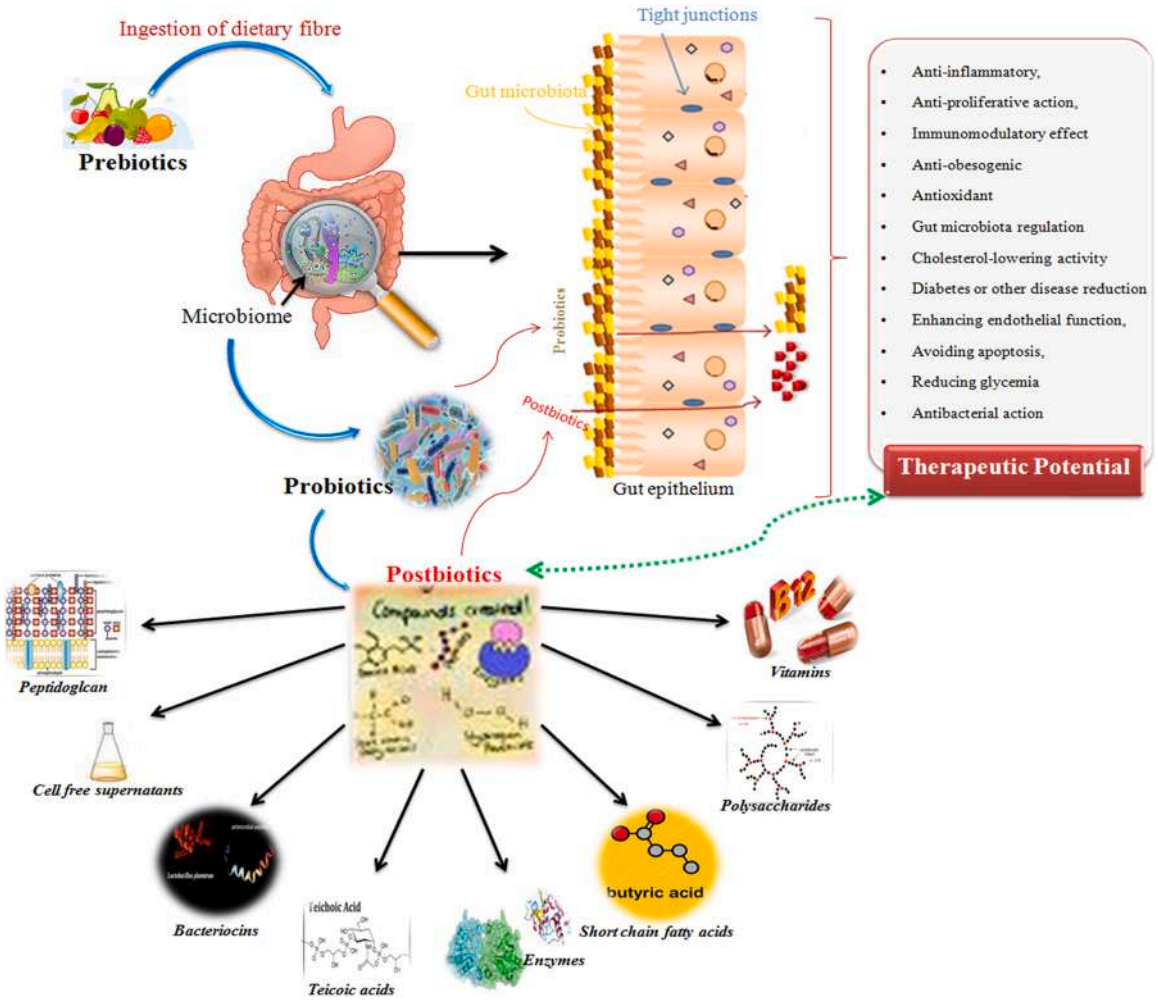

Fig. 1. Overview of relation between gut microbiota, probiotics, and different postbiotics, that could have health-promoting effects via (in)direct metabolic pathways.

to their established chemical structures, long storage stability, postbiotics have been documented to have health advantages by exhibiting local effects on specific tissues of the gut epithelium, but also by affecting multiple other organs and tissues (Table 1). They aim to mimic the therapeutic effect of probiotics, thereby avoiding the risk of live microorganisms administration to altered intestinal barriers or compromised immune defenses, in addition, to being more stable and having a longer shelf life [87,103]. Many potential activities of postbiotics have been reported including anti-inflammatory and antibacterial properties, immunomodulatory, antiobesogenic, anticarcinogenic, antihypertensive, hypocholesterolemic, antiproliferative, and antioxidant activities [81,103], boosting the immune system and strengthening the function of the gut barrier. The diversity of these postbiotics components requires prior purification in order to study their precise therapeutic effect targeting a particular disease and underlying the possible molecular mechanism of each molecule [86].

3.2.1. *Peptidoglycan*

The peptidoglycan also called murein, mucopeptide, or mucocomplex is an indispensable component of the bacterial wall that maintains the shape of cells and provides mechanical protection against osmotic pressure. This element forms a thin layer in Gram-negative bacteria and an abundant layer among Gram-positive bacteria, that contain glycan strands cross-linked with peptide chains by covalent N-terminus bouds [104]. Previous investigations established the in vitro and in vivo anti-cancer, anti-inflammatory, anti-proliferative, and immunomodulatory effect of peptidoglycan obtained from probiotic bacteria [86,105–107].

Treatment of lipopolysaccharides (LPS)-stimulated RAW 264.7 cells with 200 μg/ml of *Lactobacillus acidophilus* peptidoglycan significantly diminished cyclooxygenaes-2 (COX-2) and induced nitric oxide synthase (iNOS) levels, enzymes that play important role in the inflammatory response [108]. It has been demonstrated that peptidoglycan produced by *Lactobacillus* are responsible for inhibiting interleukin-12 (IL-12) production, a heterodimeric pro-inflammatory cytokine, which is implicated in the regulation of T-cells and stimulate the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). However, peptidoglycans can also stimulate pro-inflammatory cytokines production in macrophages such as TNF-α or IL-12 and the IL-12p35 mRNA expression, which suggest the dual pro- and anti-inflammatory properties of peptidoglycan [105]. It is admitted that nucleotide-binding oligomerization domain (NOD) proteins can detect bacterial pepdidoglycan muropeptides with a specific detection of meso-diaminopimelic (mesoDAP) by NOD1 and muramyl dipeptide (MDP) by NOD2 [109,110]. Peptidoglycan containing MDP was showed as an insulin-sensitizing postbiotic by activating NOD2, and can decrease adipose tissue inflammation and glucose intolerance in obese mice without affecting body weight or changing the microbiota composition [30]. In fact, NOD2 protects against inflammatory colitis by reducing inflammation generated by other bacterial products [111] and insulin resistance [112]. NOD2 activates via receptor-interacting serine/threonine-protein kinase 2 (RIPK2), NF-κB [113]. According to

Table 1
Biological activity of postbiotics in metabolic disorder.

Components	Microorganisms	Therapeutic benefits	Mecanisms involved	References
Peptidoglycan	*L. acidophilus*	Anti-inflammatory effect Insulin-sensitizing and glucose intolerance	- Level reduction of COX-2 and iNOS induction. - IL-12 production inhibition - NOD2 and IRF4 intervention	[82,100–103]
Teichoic acids	*L. plantarum* *L. paracasei* *L. rhamnosus*	Immunomodulatory action Anti-obesogenic, and anti-inflammatory effect	- Inhibition of JNK, ERK, and p38 kinase phosphorylation. - Improvement of phosphor-p38-AMPK levels and a reduction in NF-κB. - Reduction in IL-6, TNF-α, and IL-1β expression.	[104–107]
Cell-Free Supernatants	*Lactobacillus* strains like *L. acidophilus* *L. casei,* *L. reuteri,* *L. lactis* *B. longum* *Saccharomyces* species like *S. boulardii*	Antioxidant activity, Anti-inflammatory effect Anti-obesogenic and IR reduction	- ROS and RNS scavenging properties. - Scavenging free-radical DPPH. - Inhibition of linoleic acid peroxidation. - Decrease in TNF-α secretion and risie in IL-10 discharge. - Reduction in the production of NO, COX-2, and Hsp70. - Hepatic FGF21 up-regulation. - FGF21–adiponectin signaling.	[108–115, 115,116]
Exopolysaccharides	*Bacillus* sp. *L. delbrueckii* *L. plantarum*	Antioxidant effect, Insulin resistance and type 2 diabetes regulation, Anti-adipogenesis activity, Hyperglycemia, and dyslipidemia improvment	- ROS and RNS scavenging properties. - AS160-mediated pathway - AMPK/ PI3K/Akt pathway. - Regulation of SCD1, ACC, SREBP-1c, and FAS. - Reduction in VLDL, LDL, and triglycerides levels, and increase in HDL.	[82,117–121]
Extracellular Vesicles	*A. muciniphila* *Propionibacterium freudenreichii*	Anti-obesogenic, and anti-inflammatory effect	- Fat accumulation reduction. - NF-κB pathway modulation.	[178–181] [175]
Short chain fatty acids (butyric, propionic, and acetic acids)	*Lactobacillus* spp.	Reduce inflammatory risk diseases, obesity, diabetes or other disease	- Rise in energy consumption and fatty acid oxidation. - PGC-1α modulation via AMPK activation and HDACi inhibition. - Down-regulation of PPARγ.	[122–124]
Enzymes	*Lactococcus* sp. *Lactococcus lactis* *S. thermophilus* *L. casei* *L. fermentum* *B. adolescentis*, *B. longum*, *B. infantis*, *B. breve*	Antioxidant, Reducing intestinal inflammatory	- Scavenge free radicals via CAT, GPx, NADH-oxidase and SOD that dismutate free radicals into O_2 and H_2O_2.	[109, 125–129]
Bacteriocins	*L. plantarum*	Anti-bacterial, Anti-inflammatory, Anti-obesogenic, Reduce diabetes	- Function on cytoplasmic membranes via pores creation. - Reduction in TNF-α and IL-6 concentration. - Stimulate reductions in weight gain and food intake. - Decrease in PAI-1.	[130,131]
Vitamins	*B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum*, *B. infantis* *B. animalis* subsp. *lactis*	Antioxidant, enhancing endothelial function, avoiding apoptosis, reducing glycemia	- Decrese in SOD and CAT activity. - Superoxide radicals production reduced by NOS. - Direct ROS scavenging.	[132–136]

A.: Akkermansia; L.: Lactobacillus; B.: Bifidobacterium; S.: Saccharomyces; P.: Pediococcus; ROS: reactive oxygen species; RNS: reactive nitrogen species; COX-2: cyclooxygenaes-2; iNOS: nitric oxide synthase; IL-12: interleukin-122; NOD2: nucleotide-binding oligomerization domain 2; IRF4: interferon regulatory factor 4; JNK: c-Jun N-terminal kinase; ERK: extracellular signal-regulated kinase; NF-κB: nuclear-factor κB protein; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; IL-1β: Interleukin 1 Beta; AMPK: AMP-activated kinase; SCD1: stearoyl-CoA desaturase 1; ACC: acetylCoA carboxylase; SREBP-1c: sterol regulatory element-binding protein 1c; FAS: fatty acid synthase; VLDL: very-low-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein-cholesterol; PI3K:; Akt: protein kinase; DPPH: α,α-Diphenyl-b-Picrylhydrazyl; IL-10: interleukin-10; NO: nitric oxide; COX-2: cyclooxygenase 2; Hsp70: heat shock proteins 70; HDACi: histone deacetylases; PPARγ: peroxisome proliferator-activated gamma receptor; CAT: catalase; GPx: glutathione peroxidase; SOD: peroxide dismutase; H2O2: hydrogen peroxide; PAI-1: plasminogen activator inhibitor-1; NOS: nitric oxide synthase.

Watanabe et al. (2014), MDP's anti-inflammatory effect can be linked to NOD2 activation and enhancing interferon regulatory factor 4 (IRF4) expression, this, by tumor necrosis factor receptor associated factor 6 (TRAF6) and RICK (receptor interacting serine–threonine kinase) binding [114].

Only the immunomodulatory, anti-proliferative or anti-tumor activity of peptidoglycan isolated from probiotics have been studied up to now, and only few information are related to the effect of peptidoglycans on different metabolic syndrome associated dysfunctions (diabetes, obesity, insulin resistance, etc). For that reason, further investigations must be undetaken in order to elucidate all the possible signaling pathways related to component for metabolic syndrome treatment.

3.2.2. *Teichoic acids*

Among cell wall compounds of Gram-positive bacteria, teichoic acids (TA) forms the anionic glycopolymers (ribitol) containing phosphodiester-linked polyol repeated units of the peptidoglycans, and plays a crucial function in cell structure, cell division control, give pathogenesis, and in antibiotic resistance, contribute to host-bacterial interaction and other key biochemical and metabolic aspects of cell

physiology [86,115–117]. TA can be divided in two types, lipoteichoic acids (LTAs) with glycolipid fixed in the bacterial membrane which can be spontaneously released into the microenvironment [26] and wall teichoic acids (WTAs) covalently linked to the peptidoglycan that comprises a major chain polymer composed of phosphodiester-linked polyol repeated units and a disaccharide linkage unit [118]. LTA obtained from *Bifidobacterium animalis* subsp. *lactis* BPL1 was identified as a novel lipid modulator with fat-reducing capabilities through the insulin-like signaling pathway (IGF-1) in a *Caenorhabditis elegans* pre-clinical model, suggesting a potent therapeutic and/or preventive application of this component in metabolic syndrome and diabetes-related disorders [119].

Several studies showed the immunomodulatory effect of LTA [120]. In fact, LTA extracted from *Lactobacillus plantarum* inhibited the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase phosphorylation, the degradation of inhibitor-of-kappaB isoforms (IκBα and IκBβ), as well as the activation of the LPS-induced TNF-α production, in mice with endotoxin shock [120]. Likewise, LTA obtained from *L. paracasei* strain D3–5 can improve high-fat in metabolic dysfunctions, physical and cognitive functions and reduce inflammation [121]. LTA administration improved levels of phospho-p38-AMPK, which contributes to TLR2 and p38-AMPK signaling activation. The same study indicates a considerable reduction in nuclear-factor κB protein (NF-κB), that causes a decrease in pro-inflammatory cytokines expression, including IL-6, TNF-α, and IL-1β [121]. The study of Claes et al. demonstrated that LTA of *L. rhamnosus* GG strain has NF-κB dose-dependent activation in intestinal cell line HEK293T and Caco-2 cells after association with toll-like receptor (TLR2/6) [122]. In another study, inhibition of TNF-α; via the blockage of p65 and p38 phosphorylation; or IFN-γ; with inhibiting signal transducer and activator of transcription (STAT1/2) and Janus kinase 2 (JAK2) phosphorylation; mediated complement component 3 (C3) mRNA and protein expression in HaCaT cells treated with lipoteichoic acid obtained from *L. plantarum* K8 have been observed. It is widely accepted that C3 plays an important function in three distinct pathways implicated in the immune system which is activated by cytokines like IL-1, IL-2, IL-6, TNF-α, and IFN-γ [123]. Although several studies demonstrated the anti-inflammatory potential of LTA, others affirm that LTA does not relieve inflammatory processes and contributes to the intestinal tissues deterioration [124–126]. Taking into account the potential activities of teichoic acids as anti-inflammatory, immunomodulatory, and anti-obesogenic molecule, and the discrepancies in the outcomes of different studies showing side effects causing excessive inflammation, further analyzes are required in order to determine the safety of LTA and thus rule out possible specific results.

3.2.3. Polysaccharides

Polysaccharides, also known as glycans, are large abundant macromolecules, containing intracellular and structural polysaccharides, which are main components of the cell wall (lipopolysaccharides (LPS), peptidoglycan, and capsular polysaccharides (CPS) secreted as exopolysaccharides (EPS) throughout bacterial growth, present on the cell surface or secreted into the surrounding environment). It exhibits different chemical properties, physical characteristics, and biological functions, typically associated with structure or storage [127,128], these depending on different factors namely, composition of the medium, temperature, pH, as well as the stage of cell growth [129]. Immense diversity among polysaccharides structures produced by lactic acid bacteria with a single, two, or more monosaccharides, that can be unbranched or branched, neutral or charged, classified into homo-polysaccharides or hetero-polysaccharides has been reported [128,130]. Several probiotics have the ability to produce EPS such as *Streptococcus thermophilus*, *Lactobacillus rhamnosus*, *L. amylovorus*, *L. fermentum*, *Pediococcus pentosaceus*, *Leuconostoc* sp., etc. [131–133].

In preclinical research, the lactic acid bacteria-EPS has been recognized to have many biofunctional characteristics such as anti-oxidative effect by scavenging a broad variety of free radicals, immunomodulatory effect, gut microbiota regulation, and cholesterol-lowering activity by attaching to free cholesterol [86,134–136]. According to Lee and colleagues in 2021, obesity and metabolic disorders can be prevented and treated with EPS. Indeed, EPS from *Lactobacillus plantarum* L-14 can improve adipocyte glucose absorption via the AS160-mediated pathway, suggesting that it could be utilized to treat insulin resistance and type 2 diabetes. At the early stage of adipogenic differentiation, EPS extracted from L-14 strain can decrease lipid accumulation via regulating AMPK's downstream targets, such as stearoyl-CoA desaturase 1 (SCD1), acetylCoA carboxylase (ACC), sterol regulatory element-binding protein 1c (SREBP-1c), and fatty acid synthase (FAS), which explains the anti-adipogenic activity of EPS without affecting cell viability [137]. In another study, EPS from *Bacillus subtilis* sp. *suppress* (BSEPS) was beneficial in the control of hyperglycemia and dyslipidemia in experimentally induced diabetes [138]. BSEPS raised insulin levels while decreasing glucose and troponin blood concentrations. Total blood cholesterol, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and triglycerides were all lowered by BSEPS, but high-density lipoprotein-cholesterol was increased (HDL) in diabetic rats, which suggests that BSEPS of *B. subtilis* improves hyperglycemia, and dyslipidemia [138]. In adult rats, the polysaccharide levan obtained from *Bacillus licheniformis* was effective in inhibiting hyperglycemia and oxidative stress caused by diabetes, suggesting that supplementing the diet with levan might help in restraining diabetes-associated molecular disturbances. In fact, when compared to untreated diabetic rats, the blood glucose level was reduced by − 110%. Levan's ability to lower blood glucose levels can be related to improved peripheral sensitivity to leftover insulin, a stimulation of Langerhans islets, and levan's potent antioxidant characteristics [139]. In another investigation, it was found that the concentration of glucose in the supernatant of insulin-resistant HepG2 cells was reduced after treatment with 10^{-8} M of EPS H31-2 from *Lactobacillus plantarum* H31, indicating that EPS H31-2 could aid glucose uptake via AMPK/ PI3K/Akt pathway in insulin-resistant HepG2 cells. Furthermore, EPS H31-2 increased the expression of the glycometabolism-related genes glucose transporter 4 (GLUT-4), protein kinase B (Akt-2), and AMP-activated kinase (AMPK). These findings revealed that *Lactobacillus plantarum* EPS H31-2 might effectively suppress pancreatic α-amylase activity, which results in a decrease in blood glucose level in T2DM patients, suggesting that it could be used to prevent and treat diabetes mellitus [140].

It has been documented that the *Bacillus* sp. strain LBP32 extracellular polysaccharides (EPS), a potent antioxidant, can stop lipopolysaccharides (LPS)-induced inflammation in macrophages by preventing ROS accumulation and NFκB activation [141]. Indeed, through inflammation, reactive oxygen species (ROS) can stimulate the redox-sensitive transcription factor-like NFκB, and therefore cytokines expression [142]. Macrophages produce and release inflammatory cytokines like TNF-α, IL-6, NO during inflammatory responses [143], that participate to the development of the disease. It has been found that EPS exerts an inhibitory effect on the TNF-α, IL-6, and NO production induced by LPS in addition to decreasing the levels of iNOS expression at mRNA and protein levels. Furthermore, EPS obtained from *Bacillus* sp. LBP32 strain can stop the activation of NFκB signaling, via the reduction of IKK phosphorylation, [141]. In another study, the biological functions of *Lactobacillus delbureckii* EPS have been investigated, showing strong antioxidant effect in a dose-dependent manner (0.5–10 mg/ml), as compared to ascorbic acid used as a control [136]. It has been additionally shown that EPS from *Lactobacillus* exerts outstanding antioxidant functions in vitro and in vivo [144]. Zhang et al. have reported that EPS from *L. plantarum* strain C88 exhibits antioxidant properties that can include reactive oxygen species (ROS) scavenging, a restoration of intracellular SOD and T-AOC capacities, and diminution of lipid peroxidation in H_2O_2-challenged Caco-2 cells [145].

3.2.4. Cell-Free Supernatants

Cell-free supernatants (CFS) encompass biomolecular and active metabolites with low or high molecular weight (organic acids, diacetylene, carbon dioxide, bacteriocins-like substances, etc.) usually secreted by lactic acid bacteria and yeasts that potentially promote health homeostasis [146,147]. Moreover, the variability in CFS composition depends on the medium growth nutrients [148]. Several biological activities inter alia oxidative stress reduction, anti-inflammatory, anti-tumoral, antibacterial effects as well as biofilms formation suppression have been attributed to CFS, which can be helpful in clinical practice [149–154]. The antioxidant potential of various *Lactobacillus* strains-derived intracellular fractions has been proposed to mediate the increase of glutathione cellular content, an important non-enzymatic antioxidant that plays a pivotal role in sustaining the intracellular redox state [155]. On the other hand, the antioxidant character of such non-enzymatic postbiotics might also be related to a scavenging effect toward ROS and reactive nitrogen species [150,156].

Cell-free extracts produced from lactic acid bacteria appear to have a significant antioxidant capacity when compared to intact whole cell cultures, which can be attributed to enzymatic or non-enzymatic intracellular antioxidants [150,157,158]. One of the oxidation reactions most widely discussed is lipid peroxidation pathways, that have been postulated along with free radical chain reaction as the main actors of cellular and tissular damages in the course of aging and related degenerative diseases [157,159–161]. The funding of Lin et Chang, [157]; demonstrated the ability of *B. longum* and *L. acidophilus* CFS to strongly scavenge the α,α-Diphenyl-b-Picrylhydrazyl (DPPH) free radical and inhibit linoleic acid peroxidation. Similarly, reports from the literature imply that species from *Saccharomyces* genus generate some metabolites that are potent antioxidant. The extracts of *Saccharomyces boulardii*, a yeast probiotic known to possess favorable effects on gut health, include antioxidants mixture that have multiple antioxidant activities comparable to those of flavonoids, or phenolic compounds by effectively quenching intracellular ROS and other free radical. such preparation can therefore substantially reduce oxidative stress and help in modulating various disorders associated with mitochondrial failure and Redox imbalance [162].

Besides, anti-inflammatory and antioxidant effects on intestinal epithelial cells, neutrophils, and macrophages have been formerly attributed to *Lactobacillus casei* and *L. acidophilus* that have shown ability to decrease the pro-inflammatory tumor necrosis factor-α (TNF-α) cytokine secretion albeit rising interleukin-10 (IL-10) discharge, which is a master anti-inflammatory mediator [154]. In fact, according to De Marco et al. study, human intestinal epithelial cell line (HT-29) stimulated with lipopolysaccharides (LPS), overexpressed interleukin-8 (IL-8) cytokine and prostaglandin E2 (PGE-2) [163,164], which was markedly reversed following treatment of inflamed cells with *Lactobacillus reuteri*, *L. lactis* and *Saccharomyces boulardii* supernatants, as observed by the strong decrease in PGE-2 levels. Interestingly, only *L. lactis* derivatives were able to suppress IL-8 production. Soluble factors obtained from *L. reuteri* strain CRL1098 have been shown to considerably decrease pro-inflammatory mediators such as nitric oxide (NO), cyclooxygenase 2 (COX-2), heat shock proteins 70 (Hsp70), TNF-α and IL-6 release; in an in vitro model of inflammation induced in macrophages; in addition to pro-using LPS [165]. *S. boulardii* demonstrated similar anti-inflammatory properties attributable to excreted low molecular weight soluble factors that are involved in the suppression of NF-κB activation and NF-κB-mediated IL-8 gene expression in monocytes and intestinal epithelial cells [166].

Furthermore, *Lactobacillus rhamnosus* GG supernatant (LGGs)-based therapy substantially decreased body weight gain, body fat mass, hepatic fat deposition, adiposity, insulin resistance, and liver damage by reducing *de novo* lipogenesis and augmenting lipid β-oxidation as well as increasing energy expenditure in High-fat/high-fructose diet plus intermittent hypoxia (HFDIH)-exposed mice; these effects being associated to a regulation of the FGF21–adiponectin axis. LGGs treated HFDIH-induced metabolic dysfunction in mice further reduced adipocyte size via lipolysis enhancement and adipose tissue inflammation by lowering pro-inflammatory cytokines levels and improving hepatic FGF21 expression [167]. Interestingly, a multi-strain death (*L. brevis* CCFM648, *L. casei* CCFM419, *L. rhamnosus* Y37, *L. plantarum* X1, and *L. plantarum* CCFM36), showed positive outcomes in reducing fasting blood glucose (FBG), hemoglobin A1c (HbA1C), and leptin levels as well as enhancing the glucagon-like peptide-1 (GLP-1) levels in a high-fat diet and streptozotocin-induced type 2 diabetes in mice [168]. However, in this same study, it was also found that living probiotics relieved hypoglycemia symptoms in the host more effectively than death multi-strains probiotic by lowering insulin resistance.

3.2.5. Extracellular vesicles (EVs)

Extracellular vesicles are considered as spherical structures released by microorganisms that cargo various bioactive components including, proteins, enzymes, polysaccharides, toxins, etc. [169]. Survival, competition, pathogenesis, and immunomodulation are among mechanisms that bacterial EVs are able to regulate [170], in addition to being capable to easily cross the mucus layer and interact with the host, avoiding sepsis risk [171].

The decreases of barrier integrity and obesity have been linked in previous research. Increased permeability of the intestinal barrier causes metabolic endotoxemia, which is considered as a primary contributor to obesity-related metabolic diseases [172]. It has been established by several investigations that EVs can ameliorate obesity and its underlying pathways. As a matter of fact, pasteurized *Akkermansia muciniphila*, a probiotic species, and its derived extracellular vesicles (EVs) exerted a reducing effect on fat accumulation, body weight gain, and pathological abnormalities in HFD-fed mice when compared to other conventional treatments; the tested EVs had the greatest impact on adipocytes size, eWAT weight, lipids balance, expression of inflammatory cytokines in the adipose tissue [173–176], in addition to a substantial enhancement of glucose tolerance in diabetic mice [177]. It has been also demonstrated that anti-inflammatory action of *Propionibacterium freudenreichii* EVs is essentially associated to a modulation of the NF-κB pathway, which was dependent on their concentration [170].

3.2.6. Short chain fatty acids

Short-chain fatty acids (SCFAs), primarily butyric, propionic, and acetic acids, are the major volatile fatty acids metabolites produced mainly by anaerobic microbial fermentation in the gastrointestinal tract [26,178,179]. SCFAs production tends to participate in preserving the gut barrier function [180], contribute to the metabolism of carbohydrates and lipids, and act in distinct processes in other tissues, such as adipose tissue remodeling and immune system. Due to their involvement in energy and lipids metabolism, these molecules can contribute to the reduction of inflammatory risk diseases, obesity, diabetes, and other metabolic failures [180]. In fact, it has been reported that butyrate supplementation in high fat diet-induced obesity prevents the development of insulin resistance (IR) and obesity in experimental mice model [181]. With increased butyrate concentration, cholesterol, total fatty acids as well as triglycerides were reduced, and a consequent decrease in adiposity led to insulin sensitivity amelioration. The butyrate anti-obesity activity may be due to the rise in energy consumption and fatty acid oxidation, thus, its mechanism of action is linked to the promotion of energy expenditure and the induction of mitochondrial metabolisms (PGC-1α modulating) via AMP kinase (AMPK) activation and histone deacetylases (HDACi) inhibition [181].

The potential SCFA modulatory effect can be based on peroxisome proliferator-activated gamma receptor (PPARγ) down-regulation in high-fat diet-induced metabolic disruptions, which increased mitochondrial uncoupling protein 2 expression and increased the AMP-to-ATP ratio, stimulating oxidative metabolism via AMPK in the liver and adipose tissue. SCFA treatment conduced to liver PPARγ decrease, resulting in a sharp reduction in triglyceride concentrations, in both

liver and adipose tissue, a decrease in body weight and a restoration of insulin sensitivity [182]. This potentiality is a promising alternative for treating obesity and diabetes since, PPARγ, a nuclear receptor, is implicated in adipocytes growth and differentiation genes transcription, in addition to its role as a receptor for insulin-sensitizing thiazolidinediones that are usually used for type 2 diabetes treatment [183]. De Vadder et al. demonstrated metabolic benefits of SCFA by promoting body weight loss and glucose control through intestinal gluconeogenesis pathway, glucose, and energy homeostasis regulation. Short-chain fatty acids propionate and butyrate trigger gluconeogenesis with complementary mechanisms, while propionate, a gluconeogenesis substrate, triggers intestinal gluconeogenesis gene expression through neutral gut-brain circuit that includes the fatty acid receptor FFAR3, butyrate via a cAMP-dependent mechanism; stimulate gluconeogenesis gene expression [184]. In 2012, Lin et al. reported the protective ability of SCFA (butyrate, propionate, and acetate) on diet-induced obesity in mice. An inhibited food intake has been observed with propionate and butyrate treatment, which regulates body weight through anorexigenic gut hormones modulation [185]. Decreases in food intake and body weight appear to be mediated by SCFA acetate via the promotion of glutamate-glutamine transcellular cycle, contributing to a rise in lactate and γ-aminobutyric acid (GABA) production, which mediate an anorectic signal induction in hypothalamic arcuate nucleus (ARC).

An oral acetate administration to obesity-linked type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats demonstrated a significant protective effect against fat-liver accumulation resulting from a decrease in adipose tissue lipids accumulation together with an improved glucose tolerance and insulin resistance. Indeed, the transcripts of many lipogenic genes were reduced by acetate in the liver. Tissues absorption of acetate was activated by the catalytic activity of acetyl-CoA synthetase (AceCS) in the cytosol in acetyl-CoA with the simultaneous formation of AMP. The rise in AMP concentration resulted in an enhanced AMP/ATP ratio, and an increased AMPK phosphorylation in the liver [186]. The expression of genes involved in gluconeogenesis such as glucose-6-phosphatase and sterol regulatory element-binding protein-1 and lipogenesis, partly regulated by 5'-AMP-activated protein kinase (AMPK) in the liver, was also decreased by acetate administration in diabetic KK-A(y) mice, which ameliorated hyperglycemia in this model [187].

3.2.7. Enzymes

Enzymes are currently used in various applications such as food, paper industry for kraft pulp prebleaching, agriculture, feed, leather sectors and tend to be used in new applications regarding therapeutic strategies due to their substantial low cost [188]. Probiotics can produce a variety of enzymes that can be implicated in various biological processes such as peptidases lipases, amylases, proteases, ureases, phenoloxidases, etc. and among this immense diversity, several enzymes are known as antioxidant proteins.

It is accepted that lactic acid bacteria can scavenge free radicals via their own antioxidant enzymes systems such as catalase (CAT), glutathione peroxidase (GPx), NADH-oxidase and peroxide dismutase (SOD) that dismutate free radicals into O_2 and hydrogen peroxide (H_2O_2), thus, playing an important role in combating ROS and oxidative stress [26,189]. In cell-free extracts of strains belonging to *Lactococcus* and *S. thermophilus*, superoxide dismutase (SOD) activity has been documented, as an antioxidant enzyme that helps in preventing free radical accumulation in the host, with *Lactococcus* showing greater activity than *S. thermophilus* [150]. In mice treated with 1,2-dimethylhydrazine (DMH), catalase (CAT)-produced by *Lactococcus lactis* strain was able to enhance CAT activity, which increased antioxidant ability in reducing H_2O_2 levels, avoiding or mitigating the severity of intestinal pathologies caused by ROS [190]. It has been reported in another study that *Lactobacillus casei* strain BL23 can produce CAT or SOD promoting initial weight loss recovery in mice with Crohn's disease caused by trinitrobenzene sulfonic acid, furthermore, and improving enzymatic activities and reducing intestinal inflammation in the gut [191]. Moreover, several lactic acid bacteria such as *Bifidobacterium adolescentis*, *B. longum*, *B. infantis*, or *B. breve* are able to degrade hydrogen peroxide through the release of NADH peroxidase, [192]. It has been demonstrated that *Lactobacillus plantarum* plays an important antioxidant role via GPx concentration increase [193]. The highest catalase activity was observed in *L. plantarum* 30B, a human strain, while the greatest superoxide anion dismutation activity was found in the human strain *L. acidophilus* 900 among 25 different lactobacilli. Furthermore, *L. acidophilus* strain 900 was more effective in suppressing the inflammatory process than *L. plantarum* 30B strain in an animal model of inflammatory bowel diseases (IBD), suggesting that H_2O_2 is less toxic than superoxide anion radical, and ROS [194]. This study highlighted that *Lactobacillus* strains anti-inflammatory potency is based on each strain's profile of antioxidant enzymes expression. Two strains of *L. fermentum* E-3 and E-18 obtained from intestinal microflora of a healthy child were found to exhibit antioxidant activity by expressing high content of GPx and Mn-SOD, essential for lipid peroxidation prevention as well as hydrogen peroxide elimination [195].

3.2.8. Bacteriocins

Bacteriocins are extracellular antimicrobial peptides that are synthesized in ribosomes as primary metabolites by phylogenetically varied bacteria and archaea [196], and are considered as significant contributors to the intestinal microbiota biodiversity [197]. Probiotics can produce a number of bacteriocins that inhibit both pathogenic closely related species and microorganisms-causing damage, which attract large attention as natural bio-preservatives due to their wide applications in food processing and fermentation [86,198,199]. Lactic acid bacteria-produced bacteriocins are usually small cationic peptides (of 30–60 amino acids) that function on cytoplasmic membranes via pores creation, which causes intracellular vital constitutive components leakage [198]. In addition to their potential applications in food preservations, bacteriocins secreted by probiotics have a clinical field as they showed an inhibitory potential against several antibiotic-resistant pathogens like *Mycobacterium tuberculosis*, *Listeria monocytogenes* infections [200–202]. Moreover, it has been reported that bacteriocins contribute to the anti-inflammatory capacities of probiotics [197]. In fact, plantaricin EF (PlnEFI), a bacteriocin class II, produced by *Lactobaccillus plantarum* showed potent anti-inflammatory activity against 2, 4,6-trinitrobenzene sulfonic acid (TNBS)-induced inflammatory bowel disease in mouse model. Non-significant amounts of colonic tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were recorded in mice consuming *L. plantarum* NCIMB8826 compared to PlnEFI-deficient mutant *L. plantarum* LM0419 [197].

In obese humans as well as diet-induced obese mice, *L. plantarum* has been shown to minimize weight gain and inflammation in liver and adipose tissue [203–206]. Heeney et al. established the substantial *L. plantarum* bacteriocin plantaricin EF activity as a probiotic effector in mediating body weight and food intake reduction in absence of observable changes in microbiota or metabolome composition along with high-fat diet (HFD) mice [207]. Furthermore, plasminogen activator inhibitor-1 (PAI-1), which is implicated in the development of type 2 diabetes and obesity [208], was significantly decreased after *L. plantarum* treatment in opposition to the mutant. These findings supported that PlnEF peptides can avoid cytokine-induced disturbances to the stability of the epithelial barrier, which indicates the direct modulatory effect of plantaricin on intestinal epithelium [207].

3.2.9. Vitamins

Vitamins consist on thermosensitive organic compounds implicated in different biological processes in the organisms. These molecules are required in small quantities via natural diet exogenic supplementation due to the incapacity of mammalian cells to biosynthesize vitamins like vitamin A, D, E, etc., by commensally gut bacteria and some probiotics [86,209,210]. Several vitamins in the B-complex group are specific

coenzymes in crucial metabolic reactions, vitamin K, on the other hand, is the only fat-soluble vitamin that functions as a coenzyme [86,211]. It has been documented that certain probiotics have the ability to synthesize folates, B-complex vitamins family acting in the 1-carbon metabolism to facilitate *de novo* amino acids and nucleoside synthesis [212]. It is widely admitted that altered food habits, unbalanced diets, or malnutrition conduce to a considerable deficiency in vitamins, which can alter the different biological processes.

The excessive accumulation of reactive oxygen species can alter the lipids and proteins nature causing eventual cellular dysfunctions, conducing to irreversible malfunctions including diabetes and its complications, microvascular and cardiovascular affections [213,214]. In order to quench reactive oxygen species (ROS), living organisms can use enzymatic defenses or non-enzymatic defenses such as vitamin C, E natural antioxidants [215,216]. In adults with T2DM, oxidative stress can be promoted by the deficiency in folate and vitamin B12 [217]. Folate or vitamin B9 corresponds to an essential dietary component in human nutrition where the daily recommended folate intake for adults is 400 μg/day [218] and is one of the multiple metabolites produced by probiotics, which is implicated in several metabolic pathways [219]. It has been documented that folate plays a crucial role in reducing oxidative stress (OS), enhancing endothelial function, and avoiding apoptosis through decreasing the levels of plasma homocysteine [220,221]. In fact, it has been demonstrated that folic acid administration can reduce glycemia and ameliorate the activity of certain enzymes like superoxide dismutase (SOD) and catalase (CAT) in experimentally induced diabetes mellitus (DM) [222]. The decrease in SOD and CAT in diabetic rats after folate treatment could be the result of superoxide radical diminution due to folate effect. Indeed, the folic acid was previously verified for its antioxidant effect by reducing the production of superoxide radicals catalyzed by nitric oxide synthase (NOS), [223]. Positive folate effects are believed to be regulated by the major circulating metabolite, 5-methyltetrahydrofolate (5-MTHF). 5-MTHF enhances BH_4 bioavailability, interacts directly with NOS, and eliminates ROS, particularly superoxide radicals, and indirectly stimulates NO synthesis and avoids the development of superoxide by raising BH_4 availability [222,224]. Rossi et al. reported the potential folate production in many probiotic strains [212]. An important difference in the accumulation of vitamins was established among *B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum* and *B. infantis* when cultured in a low-folate semi-synthetic medium with *B. infantis* and *B. bifidum* designed as elevated folate accumulators [225]. Moreover, it has been reported the antioxidant activity of intracellular folate cell-free obtained from *L. heveticus* CD6 in protecting from free radicals [226].

Vitamin B12 (B12), also called cobalamin, an important water-soluble vitamin that can be obtained from fish, meat, dairy products, fortified cereals as well as supplements consumption [227,228] is essential for haematopoiesis and neuronal health maintenance [229]. Vit B12 has been demonstrated to directly scavenge ROS and to regulate glutathione (GSH) conservation. Additionally, vitamin B12 and GSH deficiency have been closely related to several oxidative stress-related diseases including T2DM [230]. Several investigations showed an elevation in tumor necrosis factor-alpha (TNF-α) activity and epidermal growth factor reduction in B12-deficient rats or diabetic patients compared to controls, suggesting the potential protective role of vitamin B12 against inflammation-induced OS by regulating cytokine as well as growth factors expression [231]. Moreover, it is assumed that homocysteine mediates ROS increase by several pathways. Therewith, B12 decreases in the course of OS the production of homocysteine, and finally reduces OS triggered by advanced end products of glycation [232]. Recently, genes encoding enzymes implicated in the biosynthesis of cobalamin (B12) have been identified in probiotics like *L. reuteri* [233,234], *L. sanfranciscensis* [235], *L. coryniformis* [236], *L. plantarum* [237], *L. rossiae* [238], *L. fermentum* [239], that were shown to produce vitamin B12, that could serve as potential alternatives in industrial production of this vitamin. *B. animalis* subsp. *lactis* HNO19 supplementation demonstrated an important enhancement in vitamin B6 and B12 blood concentrations in pregnant women [240]. However, little information regarding the implication of vitamins produced by probiotics and their implication in metabolic syndrome are available and further investigations must be carried out.

Our review highlights that the discovery and repurposing of microbial-derived natural compounds should be further investigated for their effects on various metabolic disorders and the underlying molecular pathways, and that postbiotics may provide an underappreciated avenue of prospective therapeutic alternatives. However, supplementary studies and efforts are required to evaluate and describe the new postbiotic metabolites that may help in the understanding of signaling pathway modulation in metabolic disorders and related risk factors.

4. Perspectives for clinical practice

In the past few decades, obesity, diabetes, non-alcoholic-fatty liver disease (NAFLD), and related metabolic disorders prevalence experienced a dramatic increased incidence. The physiopathology of metabolic disorders is still not fully established and subjected to controversy; however, considering the significant rise in the research and clinical emphasis over the last two decades, it is accepted to consider a dynamic relationship between the environment, hereditary sensitivity, tolerance to insulin, and pathological function of adipose tissue. It is then, necessary to highlight the different strategies involved in the management of this syndrome by promoting an integrated therapeutic strategy emphasizing much more on natural products such as probiotics, prebiotics, and postbiotics.

Through its associations with environmental, nutritional, and host genetic factors, the gut microbiota has appeared as a significant contributor in obesity and metabolic syndrome improvement. Among gut microbiota, probiotics are the most important members, and it is now well admitted that probiotics have an immense potential in treating several diseases and among them risk factors of metabolic disorders. However, some disadvantages in using probiotics in therapeutics remain unsolved, in particular maintaining the viability of probiotics during the manufacture and storage that can be affected by several factors like temperature, pH, nutrient availability, process procedures, etc., in addition to the presence of antibiotics resistance genes in certain probiotics strains, which can be transmitted to commensal microflora or opportunistic pathogens via horizontal gene transfer due to a long-term use, which conduce to hazardous clinical consequences. Furthermore, some isolated cases of probiotics systemic and local infections like *Lactobacillus* bacteremia or fungemia with *Saccharomyces cerevisiae* and *S. boulardii* have been reported. In fact, *Lactobacillus* GG caused sepsis, liver abscess, and endocarditis in patients with severe disease like diabetes mellitus, cancer, inflammatory bowel disease, etc.

All these elements cause a certain probiotic instability undermining the expected benefits when used in therapeutic approaches. On the other hand, postbioticsare expected to be more stable than the live bacteria they originate from. Till now, the implication of probiotics and particularly its postbiotics in the progression of metabolic disorders in humans or animals have not yet been completely elucidated, nevertheless, following many studies; postbiotics components can modulate anti-inflammatory effect on high-fat diet induction through different pathways. In addition, postbiotics may exert antioxidant activity by direct ROS scavenging, making postbiotics potential components that could have an important role in medical applications.

However, extensive clarification on postbiotics-classes effects on the initiation and development of metabolic disorders requires further exhaustive studies. For that reason, fundamental and clinical investigations are needed to recognize the cellular and molecular signaling pathways of the metabolism pathophysiology, but also the early identification of cardiovascular risk factors by assessing the exact clinical consequences and inference of probiotics and their metabolites concentrations.

5. Conclusion

Metabolic disorders refer to a cluster of endocrine abnormalities ranging from insulin resistance, diabetes mellitus and obesity that poses a real public health issue due to their expanding prevalence worldwide. In this review, we summarized the potential effect of postbiotics, cell-wall components, and/or metabolites of probiotics, on the host in terms of antioxidant, anti-obesogenic, immunomodulatory, hypo-cholesterolemic, antidiabetic, and anti-inflammatory potentials. In view of the available information and the research carried out on probiotics and their metabolites and/or cell-wall components called postbiotics, the potential beneficial effects of postbiotics are undeniable and should be explored as biotherapeutic approaches in order to manage inflammatory, obesity, type 2 diabetes and oxidative stress-associated disorders, providing a safe substitute option to chemical drug treatment, however supplementary in-depth investigations are requested for a better understanding of the implicated signaling pathways governing the observed effects.

CRediT authorship contribution statement

Yasmina Bourebaba: Conceptualization, Methodology, Writing-Original draft preparation, Writing- Reviewing and Editing. **Malwina Mularczyk:** Writing- Original draft preparation. **Lynda Bourebaba**: Supervision, Visualization, Writing- Reviewing and Editing. **Krzysztof Marycz:** Conceptualization, Writing- Reviewing and Editing.

Declaration of conflicting interest

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The authors declare no conflict of interest.

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Data availability

No data was used for the research described in the article.

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Original article

Probiotics-rich emulsion improves insulin signalling in Palmitate/Oleate-challenged human hepatocarcinoma cells through the modulation of *Fetuin-A/TLR4-JNK-NF-κB* pathway

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ABSTRACT

Background: Fetuin-A, also known as α2-Heremans-Schmid glycoprotein (*AHSG*), is an abundant plasmatic serum protein synthesized predominantly in liver and adipose tissue. This glycoprotein is known to negatively regulate insulin signaling through the inhibition of insulin receptor (IR) autophosphorylation and tyrosine kinase activity, which participates in insulin resistance (IR) and metabolic syndrome development. Recent studies demonstrated that IR and associated metabolic disorders, are closely related to the gut microbiota and modulating it by probiotics could be effective in metabolic diseases management.
Objective: In this present work we aimed to evaluate the effects of a probiotics-rich emulsion on reducing the IR induced by free fatty acids accumulation in human hepatocarcinoma cell line, and to elucidate the implicated molecular pathways, with a specific emphasis on the hepatokin Fetuin-A-related axis.
Results: Here we showed, that probiotics improve HepG2 viability, protect against apoptosis under normal and IR conditions. Moreover, the emulsion was successful in attenuating oxidative stress as well as improving mitochondrial metabolism and dynamics. Interestingly, application of the probiotics to lipotoxic HepG2 cells resulted in significant reduction of *Fetuin-A/TLR4/JNK/NF-κB* pathway activation, which suggests a protective effect against inflammation, obesity as well as liver related insulin resistant.
Conclusion: Overall, the presented data reports clearly on the potent potential of probiotics formulated in an emulsion vehicle to enhance metabolic functions of affected IR HepG2 cells, and suggest the possibility of using such preparations as insulin sensitizing therapy, playing at the same time protective role for the development of liver related insulin resistant.

1. Introduction

Insulin resistance (IR), obesity and hypertension are widely recognized as being the most important risk factors for metabolic syndrome (MetS). These encompassed components of MetS, particularly IR, clearly contribute to the development of other pathologies such as type 2 diabetes mellitus (T2DM), atherosclerotic cardiovascular disease (ASCVD), and non-alcoholic fatty liver disease (NAFLD) [1]. Insulin resistance is associated with impaired insulin metabolism or tolerance resulting in abnormal response to a glucose challenge, elevated fasting glucose levels and/or persistent hyperglycemia, as well as insulin desensitization of peripheral tissues, which can lead also to suppressed glucose production in the liver [2]. The development of peripheral and hepatic IR is considered as a pivotal feature in the NAFLD progression [3,4], which can trigger, in some patients, steatohepatitis development [5,6].

Fetuin-A (Fet-A), an α2-Heremans-Schmid glycoprotein (AHSG) is a 64 kDa glycoprotein currently found in abundant concentrations in human serum and secreted mainly from adipose tissue and liver [7,8].

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Fetuin-A has an ectopic calcium deposition inhibitory action as well as a protective activity against vascular calcification [9] and considered as one of the hepatokines associated to metabolic diseases [10,11]. In fact, epidemiologic investigations reported on the systematically elevated Fet-A level in the course of metabolic syndrome and its related risk factors such as insulin resistance, dyslipidemia [12–15], obesity [16] and its associated complications such as type 2 diabetes mellitus (T2DM) [17], and finally NAFLD [8,18]. Indeed, it has been demonstrated that endogenous Fet-A protein naturally modulates the activity of the insulin tyrosine kinase receptor in the liver by binding to its β subunit, thus preventing its auto-phosphorylation, which directly inhibits its tyrosine kinase activity [19,20] and the forward recruitment of glucose transporter 4 (GLUT4); taken together with liver saturated fatty acids accumulation, Fet-A may also mediate chronic inflammation through the Toll-like receptor 4 (TLR4) pathway, leading to an exacerbation of IR and subsequent development of NAFLD [21,22]. Many lines of evidence have indicated that Fet-A exerts its modulatory effects mainly through the TLR4-JNK-NF-κB pathway; as a matter of fact, previous studies showed that overexpression of Fet-A induces lipotoxicity and downstream insulin resistance in β cells, by potentiating the free fatty acids (FFA)-mediated toll-like receptor 4 (TLR4) activation, resulting in triggering the c-Jun NH_2-terminal kinase (JNK), which in turn mediates the release of pro-inflammatory mediators by inducing the nuclear factor-κB (NF-κB), that negatively crosstalk with insulin signaling [23–25]. Since Fet-A plays a major role in the development of many disorders, recent attention has been payed to take this protein as a new potential target in the treatment of these disorders, especially for IR and T2DM.

Interestingly, it has been documented that several natural components like probiotics, prebiotics, and synbiotics used as dietary supplements, can have a beneficial effect on hepatic insulin resistance through restoring the signalization of hepatic T cells receptors and decreasing insulin resistance and fatty liver without any obvious toxicity to the organism. Currently, more and more investigations are directed towards the development of new therapeutic strategies via probiotic targeting the gut-liver axis, in the prevention of the appearance and/or progression of IR, diabetes, MetS, and NAFLD [26–28]. Evidence has shown that, the gut-microbiome has been associated to several diseases, particularly with acid metabolism, obesity, type 2 diabetes, inflammatory bowel disease, cardiovascular disease, and intestinal infection [29–31]. However, probiotics enable the regulation of the intestinal flora imbalance and the reduction of harmful and toxic metabolites, which makes them of significant value for the treatment of IR, T2DM, NAFLD as well as others metabolic disorders [32].

Bacteria and yeasts are the major microbiota of human gut have a multiple functional benefit by helping intestinal epithelium growth and survival, providing a barrier for pathogens colonization, promoting anti-inflammatory microenvironment, modulating host defences and immune system, and protecting against gastrointestinal infections [33–35]. Due to its multiple benefits, an expansion use of probiotic health-based as functional foods and pharmaceutical products was observed, however, to provide these benefits, probiotics should be in a viable form and at an appropriate amount [36]. Many reports indicated that the probiotic survivals are uncertain in the gastrointestinal tract [37,38]. Therefore, microencapsulation technology of probiotic bacterial cells, a physical barrier, allows protecting them from the unfavorable environmental conditions and having a deep viability improvement of these microbes in the gastrointestinal tract [39].

Lactobacillus, *Bifidobacterium*, and yeasts (*Saccharomyces*) are the most used probiotics [40,41]. Among these available probiotic lactic acid bacteria, several strains of *Lactobacillus* genus have been demonstrated to have positive influence on glucose metabolism and improve insulin sensitivity by promoting glucose transporter 4 (GLUT-4) transcription [31], in addition to a protective properties on NAFLD damage evolution [42,43]. According to Lee et al. in 2006 [44] L. *rhamnosus* PL60 can provide an anti-obesity effect and reduce liver steatosis after eight week of oral administration to diet of obese mice. The administration of a mixture containing several probiotic strains (*Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus bulgaricus*) to ob/ob mice for four weeks under high-fat diet, showed a decreasing of total fatty acids content in liver and amino-transferase plasma levels reduces [45]. This same mixture of lactic acid bacteria can improve insulin resistance and hepatic steatosis in mice [46]. Furthermore, other investigations have demonstrated several effects of these probiotics in mouse model like anti-oxidant, anti-inflammatory, and anti-fibrogenetic [47]. A mixture of 2.5×10^9 CFU/g probiotic (*Lactobacillus casei* W56, L. *salivarius* W24, L. *acidophilus* W37, L. *brevis* W63, *Bifidobacterium bifidum* W23, *B. lactis* W52, *Lactococcus lactis* W19, and *Lactococcus lactis* W58) oral administration in T2DM patients for 12 weeks twice a day reduced insulin resistance and diminished moderately abdominal adiposity among medication naïve T2DM patients [49]. Moreover, in a [48], it has been demonstrated an IR decrease in women with gestational diabetes (GDM) without alteration of fasting glucose or lipid profiles [49]. Also, co-supplementation each two weeks for 12 weeks of 8×10^9 CFU/g probiotic mix (with 2×10^9 for each of *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, L. *fermentum*, and L. *reuteri*) and vitamin D (50,000 IU) in T2DM subjects with CHD showed a beneficial effect on total antioxidant capacity, glycemic control, inflammatory markers, and HDL-cholesterol.

Because of its relative novelty in the context of metabolic disorders treatment strategies, only few clinical experiments have been assessed on the evaluation of the real role that encapsulated probiotics can have in a preventive NAFLD and NASH therapeutic agents in human. For this reason, this study aimed to investigate a probiotic emulsion formulation mimicking natural micellar structures that are known to confer protection and increase significantly bacterial survival in acidic gastric tract, in order to highlight the relevant beneficial effects of probiotics microorganisms potentiated by microencapsulation in reducing the insulin resistance induced by free fatty acids accumulation in human hepatocarcinoma cell line, and to elucidate the implicated molecular pathways, with a specific emphasis on the hepatokin Fetuin-A and related *TLR4-JNK-NF-κB* pathway.

2. Materials and methods

All chemicals and reagents were obtained from Sigma Aldrich (Poznań, Poland), unless otherwise stated. Cell culture reagents were purchased from BioWest (VWR International, Gdańsk, Poland).

2.1. Cell culturing and maintenance

2.1.1. HepG2 cells

The culture of human hepatocellular carcinoma HepG2 cells obtained from the ATCC® HB-8065™ line provided by the American Type Culture Collection (Manassas, VA, USA) was performed in culture flasks containing Dulbecco's modified Eagle's medium (DMEM, Gibco Carlsbad, CA) with low-glucose concentration, and 10% (v/v) fetal bovine serum (FBS) inactivated by heating (FBS, Gibco Carlsbad, CA), in addition to glutamine at 2 mM (Gibco Carlsbad, CA). The cell cultures were maintained under humidified atmosphere (95%), with 5% CO_2 at 37 °C. Cells were harvested using Trypsin/EDTA solution (Gibco Carlsbad, CA) every 72 h, when they reached confluency of 70–80%.

2.1.2. Bacterial cells

Two lactic acid bacteria strains namely, *Lactobacillus rhamnosus* NCIMB 8010 and *Pediococcus acidilactici* NCIMB 8018, obtained from National Collection of Industrial, Food and Marine Bacteria, UK, were used in this study. Lyophilized strains were suspended in 1 ml De Mann–Rogosa–Sharpe (MRS) broth (Biomaxima; Poland), and then grown at 37 °C for 48 h in 25 ml of the same medium under 160 rpm shaking condition (LS 500 POL-EKO Aparatura; Poland). After

cultivation, bacterial suspension was added to 250 ml MRS broth and grown to an optical density (OD) of 1.0 at 600 nm under the same conditions. Cells were harvested by centrifugation at 3500 × *g* for 15 min at 4 °C (Mpw 380-R, Mpw Med. Instrument; Poland). The cell pellet was washed and resuspended directly into 150 ml cultivation medium. This inoculum was then added to 1500 ml MRS broth in 5000 ml flask [50].

2.2. Bacterial strains emulsion

For preparing probiotics-rich emulsion, 0.5% (w/w) methylcellulose (Certech; Poland) was dispersed in 16.6% (w/w) rapeseed oil (Kruszwica; Poland) using a stirrer at 500 rpm for 5 min (MS 11, Wigo; Poland). Bacteria biomass with cultivation medium at 83.4% (w/w) was added to the mixture, while continuing to stir. 50, 100, 200, 300 and 400 bar homogenization pressure were used to obtain a stable emulsion in high pressure homogenizer (NS1001L2K, Niro – Savi; Italy) [51]. Emulsion stability was assessed visually and samples disintegrated into water and oil phases after 1 month of storage were discarded. Survival rate of probiotic bacteria before and after pressure homogenization process was determined in the MRS agar. 0.9% saline was used to prepare the serial dilutions and culture was plated by the pour plate technique and incubated at 37 °C for 48 h. The plating procedures were carried out in triplicates (Fig. 1) [52].

2.3. Fluorescent detection of probiotic bacteria in formulated emulsion

Bacterial DNA-staining fluorochrome 4'6-diamidino-2-phenylindole (DAPI) was used for the visualization of probiotic bacteria in the prepared emulsion. For that, 10 µl of probiotics emulsion containing $1{,}7 \times 10^{12}$ cfu/ml were fixed for 20 min at room temperature with 4% PFA, then washed three times with MiliQ H_2O. Thereafter, DNA was stained using the 4′, 6-diamidino-2-phenylindole (DAPI) for confocal imaging. A confocal microscope (Observer Z1 Confocal Spinning Disc V.2 Zeiss with a live imaging chamber) was employed for capturing the photomicrographs that were subsequently processed by ImageJ software (Bethesda, MD, USA).

2.4. Biocompatibility assessment of probiotics-rich emulsion

Safety evaluation of formulated probiotics-rich emulsion was determined in terms of cell growth and viability. HepG2 cells were seeded at the same density in 96-well plates for cytotoxicity assay, and in 6-well plates for cell viability assessment, and left to attach in culture incubator for 24 h. Probiotics-rich emulsion was afterwards added to the cells at different concentrations and incubated for 24 h, 48 h and 72 h under control conditions. A set of HepG2 cells treated with only clear bacterial culture medium under the same conditions was included in the test. Growth rate was then estimated using the resazurin-based (TOX-8) kit test solution (Sigma Aldrich, Poznań, Poland); and cellular viability was investigated by the means of an Annexin V & Cell Death kit as previously described [53]. Resazurin reduction was measured using a spectrometer (BMG Labtech, Germany) for resazurin at a specific 600 nm wavelengths and 690 nm reference wavelengths, and results were normalized to a negative control. Total living and apoptotic cells were monitored using the Muse Cell analyzer.

2.5. Experimental model establishment and related treatments

To establish the beneficial effects of probiotics-rich emulsion application on FFA-induced lipotoxicity, HepG2 cells were seeded in 6-well plates and left to adhere overnight. Optimal concentration of probiotics-rich emulsion has been subsequently added to the cells 24 h prior to FFAs treatment.

Preconditioned and untreated FBS-starved HepG2 cells were afterwards exposed to a long-chain FFAs (oleic acid/palmitic acid, 2:1) mixture at 2 mM concentration for 24 h in order to induce excessive FFAs accumulation [54]. Fatty acids were complexed with bovine serum albumin (BSA, SERVA Electrophoresis GmbH, Heidelberg, Germany) at a molar ratio of 2:1 preceding to their FBS-free culture medium addition. BSA supplemented medium was used for all control groups. All treatments lasted for 24 h, and then cells were collected for further analysis.

2.6. Oil Red O staining

The accumulation of FFAs within the treated and untreated HepG2 cells was established by Oil Red O staining. Briefly, after three HBSS washes, the cells were fixed in 4% PFA for 45 min at room temperature followed by incubation with 60% isopropanol for 5 min. The fixed cells were then stained with 0.5 g/ml Oil Red O in 60% aqueous isopropanol solution for 15 min at room temperature and then rinsed with 60% aqueous isopropanol and PBS. The nuclei were counterstained with haematoxylin for 1 min. The cells were acquired under an inverted microscope (AxioObserverA1, Zeiss), and pictures were captured using a Canon PowerShot digital camera. For quantitative analysis, internalized Oil Red O dye was eluted using 100% 2-propanol to each well, and incubated for 10 min at room temperature. Eluates were then transferred to a clear 96-well microtiter plate and 2-propanol was used as a blank [55]. Absorption was measured in triplicate at 510 nm on a microtiter plate reader (BMG Labtech, Germany).

2.7. Flow cytometric analysis of early apoptosis

The early apoptotic stages of cultured human HepG2 cells after treatment with probiotics-rich emulsion and FFAs was assessed using the Annexin-V and Dead Cell Assay kit™ (Cat. No. MCH100105, Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol, through the detection of the labelled phosphatidylserine (PS) on

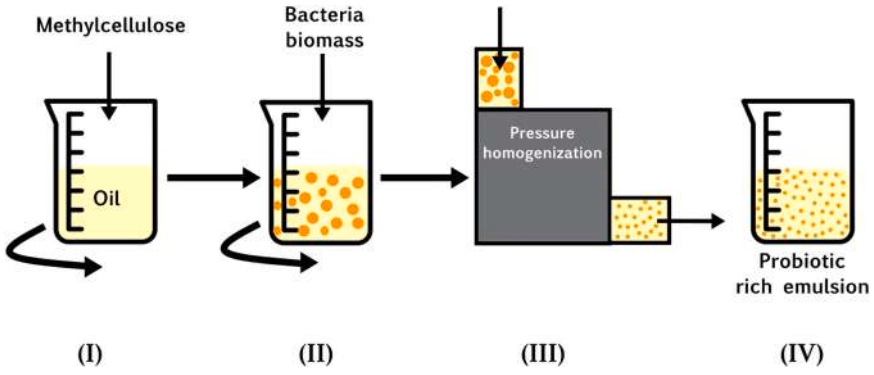

Fig. 1. Scheme of preparing probiotic rich emulsion: (I) mixing methylcellulose with oil; (II) adding bacterial biomass and cultivation medium; (III) pressure homogenization; (IV) probiotic rich emulsion, (own source).

the membrane cell [56]. All treated and untreated cells were harvested with trypsinization, rinsed with HBSS, and stained with the Annexin V & Dead Cell working reagent for 20 min at room temperature. The apoptotic ratio was considered across four populations: (i) non-apoptotic cells, not undergoing detectable apoptosis: Annexin V (−) and 7-AAD (−); (ii) early apoptotic cells, Annexin V (+) and 7-AAD (−); (iii) late apoptotic cells, Annexin V (+) and 7-AAD (+); and (iv) cells that have died through non-apoptotic pathway: Annexin V (−) and 7-AAD (+), and established using the Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany).

2.8. Quantification of multicaspase activity

Detection of multiplecaspase activation was done by Muse Multi-Caspase assay kit (Cat. No. MCH100109, Merck Millipore, Darmstadt, Germany), following the manufacturer's instructions. HepG2 cells were cultured in the presence of probiotics-rich emulsions prior to undergoing FAAs-induced lipotoxicity. The activation of cellular caspases was evaluated by incubating all experimental groups with fluorescent detection staining for 30 min [54]. The percentage of caspases-positive cells was then monitored using the Muse Cell analyzer (Merck Millipore, Darmstadt, Germany).

2.9. Mitochondrial transmembrane potential assay

Changes in mitochondrial transmembrane potential (ΔΨ) were assessed with a Muse™ MitoPotential Assay kit (Cat. No. MCH100110, Merck Millipore, Darmstadt, Germany). Based on the fact that cationic and lipophilic fluorescent probe that accumulates in high potential membranes under high fluorescence, and decreases proportionally with mitochondrial membrane depolarization [57]. For that, after HepG2 cells treatment with the probiotics-rich emulsion during 24 h, and following exposure to FFAs, samples were collected, washed with HBSS and incubated with the working fluorescent dyes solutions according to the manufacturer's instructions. The total depolarized cells percentage (depolarized live + depolarized dead cells) was determined using the Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany).

2.10. Intracellular reactive oxygen species measurement

To analyze and measure the oxidative stress degree (OS), a Muse® Oxidative Stress Kit (Cat. No. MCH100111, Merck Millipore, Darmstadt, Germany) was used in order to determine the percentage of cells undergoing OS as defined by the overproduction of ROS (namely, superoxides) [58]. HepG2 cultures were washed with HBSS, suspended in a 1X assay buffer (Muse® Oxidative Stress Kit) and incubated for 30 min at 37 °C with the provided detection reagent. The ROS positive cells were monitored by the Muse® Cell Analyzer Merck Millipore, Darmstadt, Germany.

2.11. Antioxidant enzymes activities assessment

Superoxide dismutase enzyme activity was measured using the SOD assay kit-WST (ScienCell Research Laboratories, San Diego, USA) and Catalase enzyme activity was evaluated using the Catalase (CAT), Colorimetric assay Kit (MyBioSource, San Diego, USA), following both manufacturers' instructions. Briefly, 1×10^6 HepG2 cells were incubated with probiotics-rich emulsion for 24 h, and subsequently subjected to FFAs treatment (2 mM PA/OA) for additional 24 h. Cells were then washed with cold-HBSS and suspended in cold-lysis assay buffer. The supernatants containing proteins were used for determination of SOD and CAT enzyme activities. These were added to each well in microtiter plates with the appropriate working solutions (according to the manufacturer's instructions). The color changes were measured at 438 nm for SOD and 240 nm for CAT, using a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). Results were expressed as percentage for SOD and nmol of decomposed H2O2 / min / mg protein for CAT.

2.12. Glucose uptake assay

Glucose uptake assay was investigated using the fluorescent 2-NBDG glucose analogue. After 24 h pretreatment of HepG2 cells with probiotics emulsion, 2 mM of PA/OA were given to the cultures for another 24 h. Cells were washed with PBS three times and then, incubated at 37 °C in a fresh culture medium in presence or absence of 100 nM insulin during 30 min. The 2-NBDG (100 μM), was added then and incubated for additional 30 min prior to cell isolation at 37 °C [59]. The uptake of 2-NBDG was stopped by removing the culture medium, cells were subsequently rinsed with cold HBSS, and fixed for 15 min at room temperature with 4% PFA. HepG2 cells were mounted in ProLong Gold Antifade containing DAPI (Life Technologies, Warsaw, Poland) and were visualized and imaged using confocal microscopy (Zeiss Cell Observer SD).

2.13. Gene expression analysis

Total RNA contained within treated and untreated HepG2 cells was isolated using the TRIzol method as recommended by the supplier (Sigma, St. Louis, MO, USA), then RNA extract was quantified by a nanospectrophotometer (WPA, Biowave II, Germany) for concentration and purity determining. Genomic DNA (gDNA) digestion and cDNA synthesis were performed through a reverse transcription reaction with oligo (dT) primers using a Tetro cDNA Strand cDNA Synthesis Kit (Bioline, London, UK) in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) following the kit instructions. Gene expression levels of targeted genes (Table 1) were analyzed by Real-time Quantitative Reverse Transcription PCR, in triplicate, using the SensiFAST SYBR Green Kit (Bioline, London, UK) for target mRNA detection in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Amplified reactions were carried out in a 10 μl total volume containing a SYBR-Green Master Mix, forward and reverse primers, and tested samples, then subjected to the following cycles conditions: 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s, annealing for 15 s, and elongation at 72 °C for 15 s. All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) house-keeping gene expression [60].

2.14. Western blot analysis

In order to analyze effect of probiotics-rich emulsion on insulin signaling related proteins expression and phosphorylation patterns, treated and untreated HepG2 cells were exposed to 100 nM of human insulin infusion for 30 min and 100 μM glucose for additional 30 min in order to trigger phosphorylation cascades of proteins of interest. For proteins profiling analysis, cells were detached from culture dishes and homogenized in RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, protease cocktail, 1 mmol/L PMSF, 10 mmol/L sodium azide, 10 mmol/L sodium ascorbate, and 5 mmol/L Trolox) with protease and phosphatase inhibitor cocktail on ice; After centrifugation at 20 min, 6000g, 4 °C to remove insoluble materials, supernatants were transferred to fresh tubes and stored at − 80 °C until further use. The protein concentration was determined using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Life Technologies, USA). For proteins separation, cell lysates were firstly diluted with 4 × Laemmli loading buffer (Bio-Rad, USA) and denatured for 5 min at 95 °C. Samples were then subjected to SDS-polyacrylamide gel electrophoresis at 100 V for 90 min in Tris/glycine/SDS buffer using Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) using a transfer apparatus Mini Trans-Blot® Cell (Bio-Rad, USA) at 100 V, 250 mA for 45 min at 4 °C in Tris/glycine buffer/methanol. The membranes of non-phosphorylated

Table 1
Sequences of primers used in qPCR.

Gene	Primer	Sequence 5'–3'	Amplicon length (bp)	Accession No.
Mfn1	F:	GTTGCCGGGTGATAGTTGGA	146	[41] NM_033540.3
	R:	TGCCACCTTCATGTGTCTCC		
Mfn2	F:	AATCTGAGGCGACTGGTGAC	126	XM_024451299.1
	R:	GGACATTGCGCTTCACCTTC		
Parkin	F:	GTGCAGAGACCGTGGAGAAA	294	NM_013987.3
	R:	GCTGCACTGTACCCTGAGTT		
Sod1 (cu/zn sod)	F:	CATTCCATCATTGGCCGCAC	130	NW_001867397.1
	R:	GAGCGATCCCAATCACACCA		
Sod2 (mn sod)	F:	GGACAAACCTGAGCCCCAAT	125	NW_001867408.1
	R:	TTGGACACCAGCCGATACAG		
Cat	F:	ACCAAGGTTTGGCCTCACAA	112	XM_014851065.1
	R:	TTGGGTCAAAGGCCAACTGT		
GPx	F:	TCCGGGACTACACCCAGATG	108	NM_000581.4
	R:	TCTTGGCGTTCTCCTGATGC		
AHSG	F:	AGAGGCAGCCAAGTGTAACC	110	NM_001354572.2
	R:	GGAACACCATGCAGGTCACT		
TLR4	F:	GACGGTGATAGCGAGCCAC	173	NM_138554.5
	R:	TTAGGAACCACCTCCACGCAG		
Pink1	F:	GCTTGGGACCTCTCTTGGAT	142	NM_032409.3
	R:	CGAAGCCATCTTGAACACAA		
NF-kB-p65	F:	CTGTTCCCCCTCATCTTCCC	113	L19067.1
	R:	GTATCTGTGCTCCTCTCGCC		
MAPK8	F:	TCAGAAGTTGCCTAGGATCAAAGA	100	XM_024448079.1
	R:	TTGTCACGCTTGCTTCTGCT		
MAPK9	F:	TTGCATCATGGGAGAGCTGG	106	NM_001364611.2
	R:	CTCTGCTGATGGTGTTCCCA		
MAPK14	F:	AACAGGATGCCAAGCCATGAG	164	NM_001315.3
	R:	GCTTGGGCCGCTGTAATTCT		
p53	F:	AGATAGCGATGGTCTGGC	381	NM_001126118.1
	R:	TTGGGCAGTGCTCGCTTAGT		
Insr	F:	TAGACGTCCCGTCAAATATTGC	244	AH002851.2
	R:	GAAGAAGCGTAAAGCGGTCC		
Irs2	F:	GAGCTGTGGCGTTTCACATC	234	NM_003749.3
	R:	AGCTTCGGGCTGAAACAGT		
Akt1	F:	CTGTCATCGAACGCACCT	178	NM_005163.2
	R:	GTCTGGATGGCGGTTGTC		
Akt2	F:	TCAAAGAAGGCTGGCTCCAC	205	M95936.1
	R:	TGTACCCAATGAAGGAGCCG		
Pi3k	F:	TTTAATCTGCCAGGCGGAGG	151	NM_006218.4
	R:	CCAGAATTCCATGGGGCAGT		
Bax	F:	ACCAAGAAGCTGAGCGAGTGTC	356	XM_011527191.1
	R:	ACAAAGATGGTCACGGTCTGCC		
Bcl-2	F:	ATCGCCCTGTGGATGACTGAG	129	NM_000633.2
	R:	CAGCCAGGAGAAATCAAACAGAGG		
p21	F:	AGAAGAGGCTGGTGGCTATTT	169	NM_001220777.1
	R:	CCCGCCATTAGCGCATCAC		
Casp3	F:	CTCTGGTTTTCGGTGGGTGT	136	NM_004346.4
	R:	CTTCCATGTATGATCTTTGGTTCC		
Casp9	F:	CAGGCCCCATATGATCGAGG	142	NM_032996.3
	R:	CTGGCCTGTGTCCTCTAAGC		
GAPDH	F:	GTCAGTGGTGGACCTGACCT	256	NM_001289746.1
	R:	CACCACCCTGTTGCTGTAGC		

Mfn1: Mitofusin 1; ***Mfn2***: Mitofusin 2; ***Parkin***: Parkin RBR E3 ubiquitin protein ligase (PARK2); ***Pink1***: PTEN-induced putative kinase 1; ***Sod1 (Cu/Zn SOD)***: Copper-zinc-dependant superoxide dismutase *(CuZnSOD*; ***Sod2 (Mn SOD)***: Manganese-dependent superoxide dismutase (MnSOD*)*; ***CAT***: *Catalase;* ***AHSG***: *alpha 2-HS glycoprotein;* ***GPx***: *Glutathione Peroxidase;* ***TLR4***: Toll like receptor 4; ***Pink1***: PTEN-induced putative kinase 1; ***NF-kB-p65***: Nuclear Factor-kappa-B transcription factor p65; ***MAPK8***: Mitogen-activated protein kinase 8; ***MAPK9***: Mitogen-activated protein kinase 8; ***MAPK14***: Mitogen-activated protein kinase 14; ***P53***: tumor suppressor p53; ***Insr:*** Insulin Receptor***; Irs2:*** Insulin Receptor Substrate 2***; Akt1:*** Serine/threonine 308 Kinase 1***; Akt2:*** serine/threonine kinase 2; ***Pi3k:*** Phosphoinositide 3-Kinase***; Bcl-2***: B-cell lymphoma 2; ***Bax***: BCl-2 associated X protein; ***p21***: Cyclin-dependent kinase inhibitor 1; ***Casp3***: Caspase 3; ***Casp9***: Caspase 9; ***GADPH***: Glyceraldehyde-3-phosphate dehydrogenase.

proteins were blocked using 5% non-fat milk solution prepared in TBST, whereas phosphorylated proteins membranes were blocked in 5% BSA in TBST. Each protein was detected by overnight incubation at 4 °C with primary antibodies listed in Table 2 and HRP-conjugated secondary antibodies (dilution 1:2500 in TBST, 1 h incubation at room temperature). Chemiluminescent signals were detected using ChemiDoc MP Imaging System (Bio-Rad, USA) and quantified with Image Lab Software (Bio-Rad, USA) [61].

2.15. Statistical analysis

The obtained results from biological assays were statistically analyzed with a one-way variance analysis (ANOVA) using GraphPad Prism software version 5.0 (San Diego, CA, USA) according to Dunnett's post-hoc multiple comparison test. The statistical significance between the mean values of the independent groups (FFA-induced versus healthy control or FFA-induced control versus the probiotics-rich emulsion treated) were indicated with Asterisk (*) and Hash (#) signs respectively. All statistical comparisons were performed two-sided in the sense of an exploratory data analysis using $p < 0.05$ (*/#), $p < 0.01$ (**/##),

Table 2
List of antibodies employed for proteins profiling using western blot analysis.

Antibody	Dilution	Catalog No.
p-Insr(Y1361)	1:500	Biorbyt, orb393084
p-Akt(Ser473)	1:500	Biorbyt, orb304681
p-Pi3k-P85α/γ/β(Y467/Y199/Y464)	1:500	Biorbyt, orb544410
Insr	1:1000	Invitrogen, MA1-10865
Akt Pan	1:1000	Invitrogen, 44-609G
Pi3kCD	1:1000	Invitrogen, PA5-83748
Fetuin-A	1:1000	Biorbyt, orb574837
NF-kappaB p65(phospho-T254)	1:1000	Biorbyt, orb304547
JNK1/JNK2(phospho-Thr183/Tyr185)	1:1000	Biorbyt, orb15028
GAPDH	1:2000	Sigma Aldrich, A5441

p-Insr(Y1361): Phosphorylated Insulin Receptor (Y1361); ***p-Akt(Ser473)***: Phosphorylated Protein Kinase B (Serine 473); ***p-Pi3k-P85α/γ/β(Y467/Y199/Y464)***: Phosphorylated Phosphatidylinositol 3-Kinase P85alpha/gamma/beta (Y467/Y199/Y464); ***Insr***: Insulin Receptor; ***Akt***: Protein Kinase B; ***Pi3kCD***: Phosphatidylinositol 3-Kinase; ***NF-kappaB p65(phospho-T254)***: Phosphorylated Nuclear Factor-kappa-B transcription factor p65; ***JNK1/JNK2(phospho-Thr183/Tyr185)***: Phosphorylated c-Jun N-terminal kinase.

and $p < 0.001$ (***/###) as level of significance. Data are represented as means ± standard deviation (SD) of at least three independent experiments ($N \geq 3$).

3. Results

3.1. Probiotics-rich emulsion stability and probiotic cells viability

To select the process parameters for the preparation of a rich probiotic emulsion, the survival rate of probiotic bacteria after pressure homogenization and the stability of the rich probiotic emulsion during 1-month storage were correlated. The highest survival rate was obtained for the pressure of 50, 100 and 200 bar and it was respectively: 82.6%, 89.5% and 85.7% (Table 3). However, only the 100-bar homogenization sample was stable after 1 month of storage (Fig. 2, II). DAPI staining confocal fluorescence imagines of control emulsion confirm the absence of bacteria (Fig. 3, I). While the imagines of probiotic rich emulsion show both *Lactobacillus rhamnosus* and *Pediococcus acidilactici* (Fig. 3, II). Imagine of rich probiotic emulsion shows the chains of short rods formed by L. *rhamnosus* typical for this species, as well as cocci of *P. acidilactici* in pairs and tetrads form.

3.2. Probiotics-rich emulsion biocompatibility to HepG2 cell line

To evaluate the relative safety of the proposed probiotics-riche emulsion (PRE), the HepG2 cells' health status was evaluated in terms of viability and cytotoxicity, after exposure to different concentrations of PRE as well as control growth medium (CGM), using TOX8 assay as well as Annexin V test. Resazurin-based assay (TOX-8) results demonstrated that PRE positively affect cell proliferation at a concentration of 10%, after both 24 and 48 h incubation (Fig. 4.a) as compared to untreated cells ($p < 0.01$). Nevertheless, 100% of PRE appeared to reduce significantly the proliferative rate of HepG2 after 24, 48 and 72 h incubation. Interestingly, clear CGM significantly reduced HepG2 metabolic activity under the same conditions, following 24, and 48 of incubation at the same 10% concentration; indicating that growth medium maybe toxic for cells when no probiotic bacteria are cultured (Fig. 4.b). Moreover, 72 h incubation with the clear culture medium resulted in strong inhibition of cellular proliferation, while the presence of probiotics bacteria ensured proper metabolic activity and viability (Fig. 4.a). These results were further supported by the Annexin V & Dead Cells data that demonstrated an increase in total apoptotic cells after CGM exposure; while fewer apoptotic HepG2 cells were monitored in the PRE-treated group, in regards to untreated group of cells (Fig. 4.c). Based on the obtained results, a 10% PRE treatment was chosen as an effective concentration for the following experiments.

Table 3
Viable cell count before and after pressure homogenization process.

Pressure [bar]	Viable cell count before homogenization process [cfu/ml]	Viable cell count after homogenization process [cfu/ml]	Survival rate [%]
50	$2{,}3 \times 10^{12}$	$1{,}9 \times 10^{12}$	82,6
100	$1{,}9 \times 10^{12}$	$1{,}7 \times 10^{12}$	89,5
200	$2{,}1 \times 10^{12}$	$1{,}8 \times 10^{12}$	85,7
300	$2{,}0 \times 10^{12}$	$1{,}3 \times 10^{12}$	65,0
500	$2{,}1 \times 10^{12}$	$1{,}4 \times 10^{12}$	66,7

cfu: colony forming unit.

3.3. Probiotics-rich emulsion attenuates FFAs-induced steatosis in HepG2 cell line

In order to observe hepatic lipid accumulation within PRE-treated and untreated HepG2 cells after exposure to the mixture of two fatty acids, which co-incubation can lead to steatogenesis and apoptosis simultaneously in hepatocytes, experimental cells were stained with Oil Red O, and visually observed by microscope. Following 24 h incubation in the presence of FFAs formulation, HepG2 cells exhibited visible higher amount of accumulated intracellular lipid droplets in comparison to HepG2 cells cultured in basal conditions (Fig. 5.a). By contrast, PRE supplemented cultures were characterized by lower amount of stained intracellular neutral lipid droplets when compared to untreated cells (Fig. 5.a). to enable the better assessment of the differences in lipids accumulation degree in the different experimental groups, a quantitative approach for eluted Oil Red O dye was undertaken. As demonstrated in Fig. 5.b. FFA-challenged HepG2 cells displayed significant lipid incretion versus healthy cells ($p < 0.001$). Moreover, in this FFAs-induced steatosis in HepG2 cells, the high build-up of lipid droplets was obviously mitigated after PRE supplementation, by contrast to PA/OA-treated cells ($p < 0.001$).

3.4. Probiotics-rich emulsion reduces FFAs-induced Lipoapoptosis in HepG2 cells

To investigate the protective effect of PRE on Palmitate/Oleate (PA/OA)-induced lipotoxicity, changes in cell viability and apoptosis induced by FFAs mixture in the presence or absence of PRE were analysed using the Muse Annexin V & Dead Cells kit. As illustrated in Fig. 6.b, exposure of HepG2 cells to 2 mM FFAs significantly reduced the number of living cells, and markedly induced cellular apoptosis after 24 h incubation, as evidenced by higher percentage of early and late apoptotic cells when compared to untreated healthy cells ($p < 0.001$). In the presence of 10% PRE, the inhibiting effect of PA/OA mixture on cell viability was significantly ameliorated (Fig. 6.b). Indeed, pre-treatment of HepG2 cells with PRE during 24 h enabled the significant attenuation of apoptosis triggered by FFAs overaccumulation, especially through the reduction of late apoptotic cells percent in comparison to untreated control cells ($p < 0.001$); while considerably diminishing the total number of dead cells in opposition to both healthy and FFAs-challenged cells ($p < 0.001$; $p < 0.001$). To further elucidate whether protective effect of PRE on cellular lipoapoptosis is linked to the regulation of the main pro- and anti-apoptotic markers, the expression of their mRNA levels was evaluated using RT-qPCR. As shown in Fig. 6.c., PA/OA-overloaded HepG2 cells were characterized by overexpression of key pro-apoptotic genes, namely *p53*, *p21* and *Bax*, concomitantly to significant *Bcl-2* transcript expression in comparison to healthy cells ($p < 0.01$; $p < 0.001$). Anti-apoptotic effect of PRE was confirmed through the downregulation of *p53*, *p21* and *Bax* transcripts upon exposure to FFAs mixture (Fig. 6.c). However, PRE supplementation appeared to not be successful in restoring the suppressed *Bcl-2* expression as compared to both healthy and FFAs-control cells ($p < 0.001$).

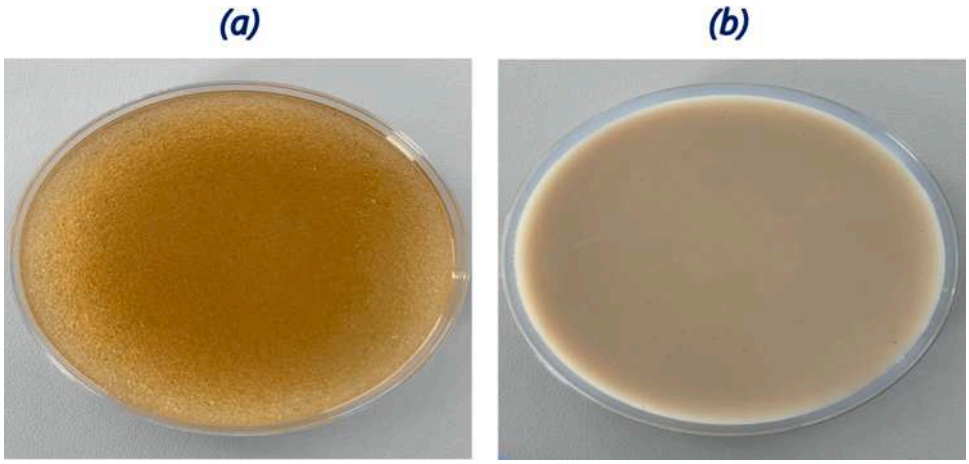

Fig. 2. (a) emulsion before pressure homogenization and (b) emulsion after pressure homogenization and 1-month storage.

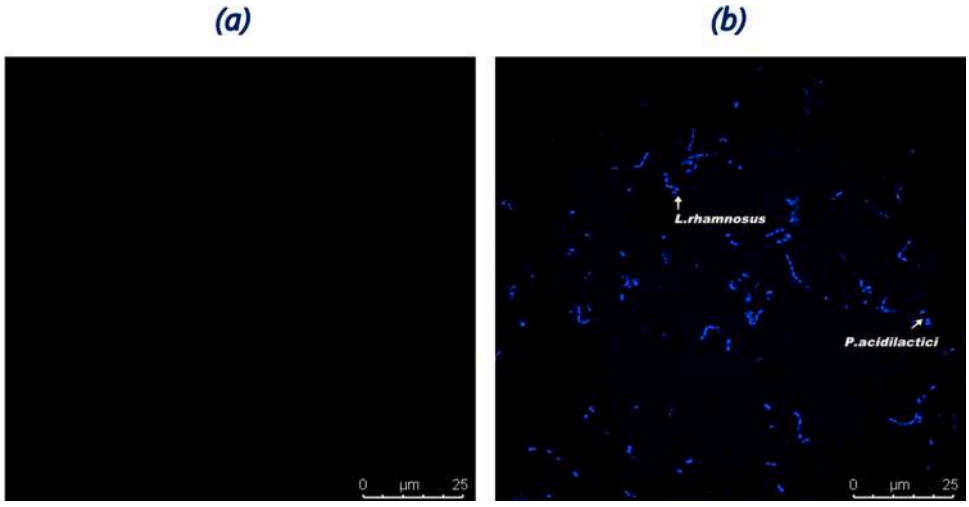

Fig. 3. DAPI staining of (a) control emulsion and (b) probiotic rich emulsion.

3.5. Probiotics-rich emulsion inhibits caspases activation in lipotoxic HepG2 cells

To further establish the involvement of PRE in FAAs-activated caspases, the Muse™ multicaspase assay kit was used to detect initiator as well as executioner caspases activation rates (caspase-1, 3, 4, 5, 6, 7, 8, and 9). Obtained data (Fig. 7.b) evidenced a considerable activation of cellular caspases in response to PA/OA challenging ($p < 0.001$). Moreover, pre-treatment of HepG2 cultures with 10% PRE resulted in significant lowering of total activated caspases positive cells in comparison to untreated cells ($p < 0.001$). What is more, the PRE treatment allowed a relative normalization of caspases-mediated apoptosis, to the basal activity of healthy population (Fig. 7.b). Likewise, activation of caspases 3 and 9 within FFAs-conditioned cells was confirmed through the significant induction of *Casp-3* and *Casp-9* relative genes expression ($p < 0.01; p < 0.001$). Similarly, addition of PRE to HepG2 cells prior to FFAs treatment led to a positive regulation of the two genes expression patterns (Fig. 7.c), and resulted in a significant downregulation of that transcripts when compared to both healthy and FFAs-treated cells ($p < 0.05; p < 0.001$).

3.6. Probiotics-rich emulsion improves mitochondrial dynamics in FFAs-overloaded HepG2 cells

To subsequently establish whether PRE reduces FFAs-induced apoptosis in HepG2 cells through mitochondria-mediated pathway, mitochondrial transmembrane destabilization was analysed by means of a Muse MitoPotential assay. Obtained data showed that PA/OA challenging induced significant damage to mitochondria after 24 h incubation (Fig. 8.b). Indeed, the FFAs treatment increased the depolarized mitochondrial membrane and decreased the polarized membranes in HepG2 cells, while no change in membrane potential was detected in control cells ($p < 0.001$). Pre-treatment of HepG2 cells with 10% PRE consistently prevented the loss of MMP and improved the polarization status of mitochondrial membranes in regards to both FFA-positive and negative cells ($p < 0.001; p < 0.05$). To substantiate the protective effects of PRE against mitochondrial dysfunction, the changes in expression levels of main mitochondrial dynamics genes were measured using RT-qPCR. As illustrated in Fig. 8.c, PA/OA exposure resulted in significant failure in *Mfn1*, *Mfn2*, *Pink1* and *Parkin* transcripts, as a result of mitochondrial impairment upon FFAs accumulation within HepG2 cells ($p < 0.001$). The application of PRE to deficient HepG2 cells engendered a notable restoration of both *Mnf1* and *Mfn2* mRNA relative expression, that are two essential effectors of mitochondrial fusion (Fig. 8.c). Furthermore, PRE appeared to successfully regulate the expression patterns of the two mitophagy-related markers namely, *Pink1* and *Parkin*, in regards to control cells ($p < 0.05; p < 0.001$).

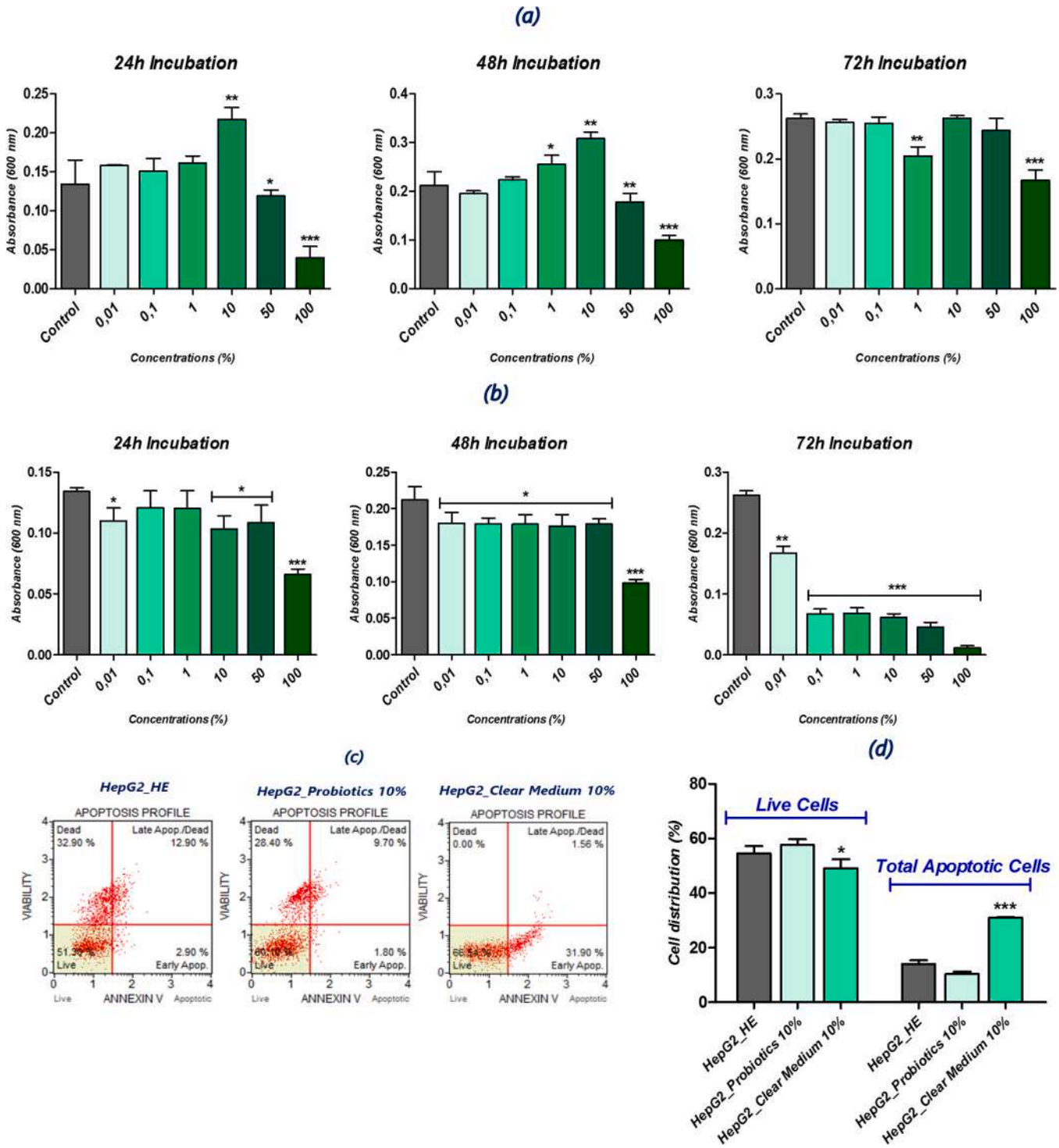

Fig. 4. Probiotics-rich emulsion is biocompatible with HepG2 cells but not clear culture medium. (a) *TOX8* viability assay for Probiotics-rich emulsion. (b) *TOX8* viability assay for clear culture medium. (c) Muse Annexin V & Dead Cells dot plots representation. (d) Average percentages of living cells and total apoptotic cells within treated and untreated HepG2 cell population. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_Probiotics 10%: HepG2 cells treated with 10% of the Probiotics-Rich emulsion for 24 h; HepG2_Clear Medium 10%: HepG2 cells treated with 10% of the clear culture medium for 24 h.

3.7. Probiotics-rich emulsion attenuates oxidative stress in affected HepG2 cells

With a view of additionally examine the connexion between mitochondrial dysfunction improvement and oxidative stress alleviation, which is known to play important roles in FFAs-induced lipotoxicity. As compared to healthy HepG2 cells, exposure to PA/OA mixture triggered to significant accumulation of intracellular ROS (Fig. 9.b), which features an activated oxidative stress ($p < 0.001$). PM treatment excreted substantial antioxidant effect as evidenced by the sharp lowering or total ROS-positive cells ($p < 0.001$). More interesting, probiotics were able to decrease the levels of ROS below the basal threshold in regards to

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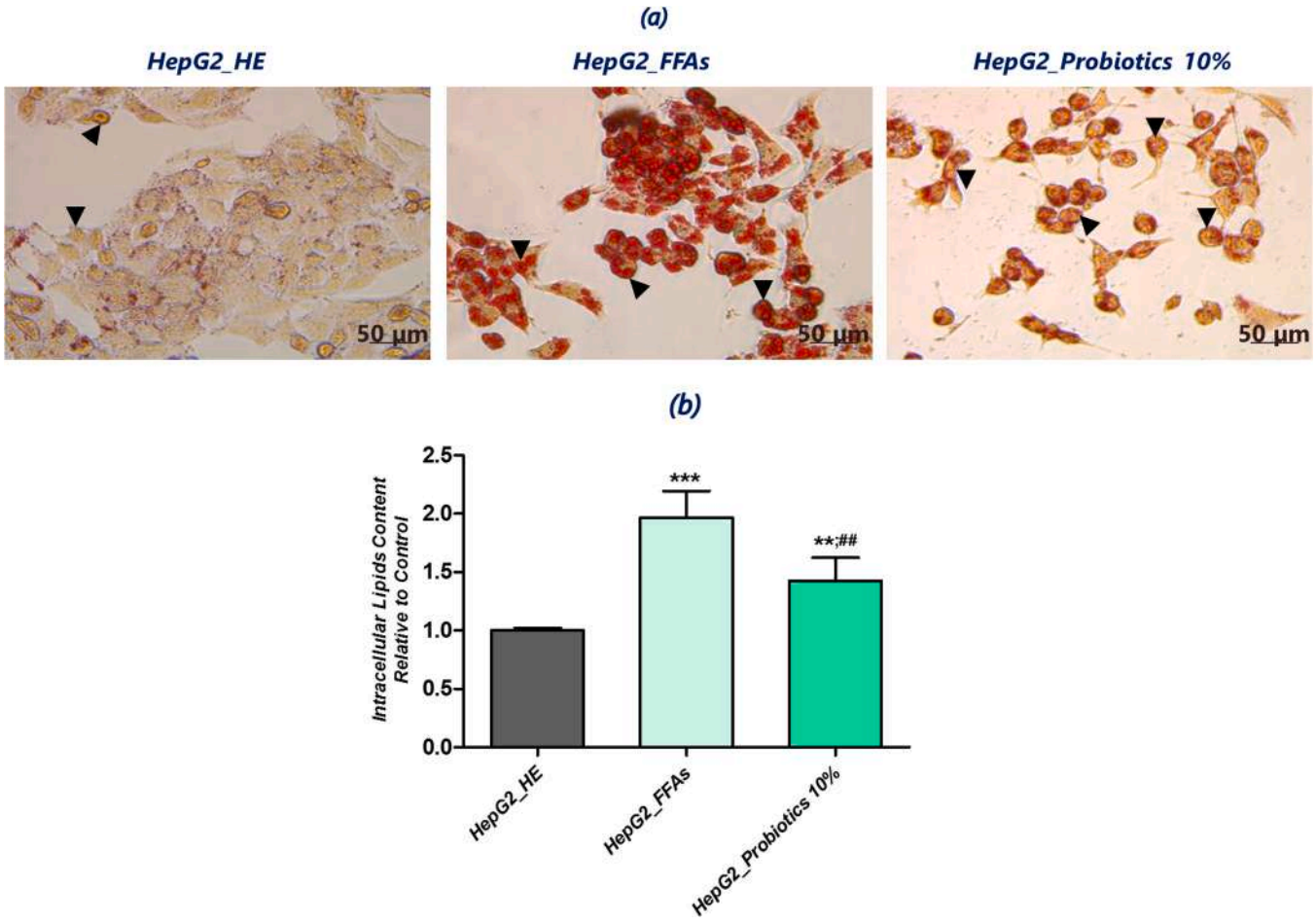

Fig. 5. Probiotics-Rich Emulsion attenuates lipid accumulation in HepG2 cells. (a) Representative photomicrographs of HepG2 cells treated with FFAs and PRE stained with Oil Red O staining at 40-fold magnification. (b) The histogram summarizes the mean percentage ± SD of three independent quantitative analysis of cellular steatosis measured through the elution of deposited Oil Red O in the cell. An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

healthy cells ($p < 0.001$). To provide more insights on the antioxidant support conferred by the PRE supplementation, expression of the main endogenous antioxidant enzymes was also evaluated. As depicted in Fig. 9.c, injured HepG2 cells were characterized by a marked decline in the relative expression of the mitochondrial *Sod2* gene ($p < 0.01$), in parallel to up-regulation of the cytosolic *Sod1* and peroxisomal *Cat* enzymes-related genes in comparison to untreated group of cells ($p < 0.001$). Moreover, intrinsic activities of both Catalse and Superoxide dismutase (Fig. 9.d) enzymes were severely impaired under lipotoxic condition in regards to normal control cells ($p < 0.001$). As expected, PRE application prior to FFAs-elicitation remarkably suppressed the loss of main antioxidant enzymes genes transcription balance, and enhanced the relative expression of all *Sod1*, *Sod2* and *Cat* transcripts when compared two both experimental control groups ($p < 0.05$: $p < 0.001$); additionally, the PRE application resulted in significant restoration of SOD and CAT enzymes activities as compared to FFAs-treated cells (Fig. 9.d).

3.8. Probiotics-rich emulsion restores impaired insulin signalling pathway in insulin-resistant HepG2 cells

To evaluate the potential protective effect of PRE pre-treatment against the alterations caused on insulin signalling related effectors by FFAs overaccumulation, the genes and proteins expression levels of key regulators were analysed, as well as glucose absorption extend using fluorescence microscopy. The glucose uptake was decreased markedly after treatment with PA/OA mixture, indicating the establishment of IR upon accumulation of FFAs (Fig. 10.a). Furthermore, PRE treatments significantly increased the insulin-stimulated glucose uptake, as evidenced by the increase in fluorescent signal, in comparison to untreated cells (Fig. 10.a).

To another extend, the measurement of genes relative expression highlighted a substantial suppression of *Insr*, *Irs1*, *Akt1*, *Akt2* and *Pi3K* transcripts (Fig. 10.b) for FFAs-induced insulin resistant HepG2 cells in opposition to healthy control ($p < 0.001$). By contrast, *Irs2* analysis showed significant upregulation of its mRNAs following FFAs elicitation ($p < 0.01$). Conversely, pre-treatment of HepG2 cells with 10% PRE prevented the dysregulated expression of insulin related markers *Inrs*, *Akt1*, *Pi3K*, *Irs1* and *Irs2* comparatively to PRE-untreated IR HepG2 cells, while no changes in *Akt2* gene expression were reached after PRE addition (Fig. 10.b). To further elucidate the implication of PRE in restoring insulin signalling transduction while a sharp disruption in genes expression was observed, we assessed insulin-stimulated signalling cascades by profiling proteins phosphorylation patterns (Fig. 10.d). As expected, PA/OA exposure markedly impaired the phosphorylation status of *phospho-Insr(Y1361)*, *phospho-Akt(Ser473)* and *phospho-Pi3k-P85alpha/gamma/beta (Y467/Y199/Y464)* as a consequence of IR development and impeded insulin signalling (Fig. 8.d). Consecutively,

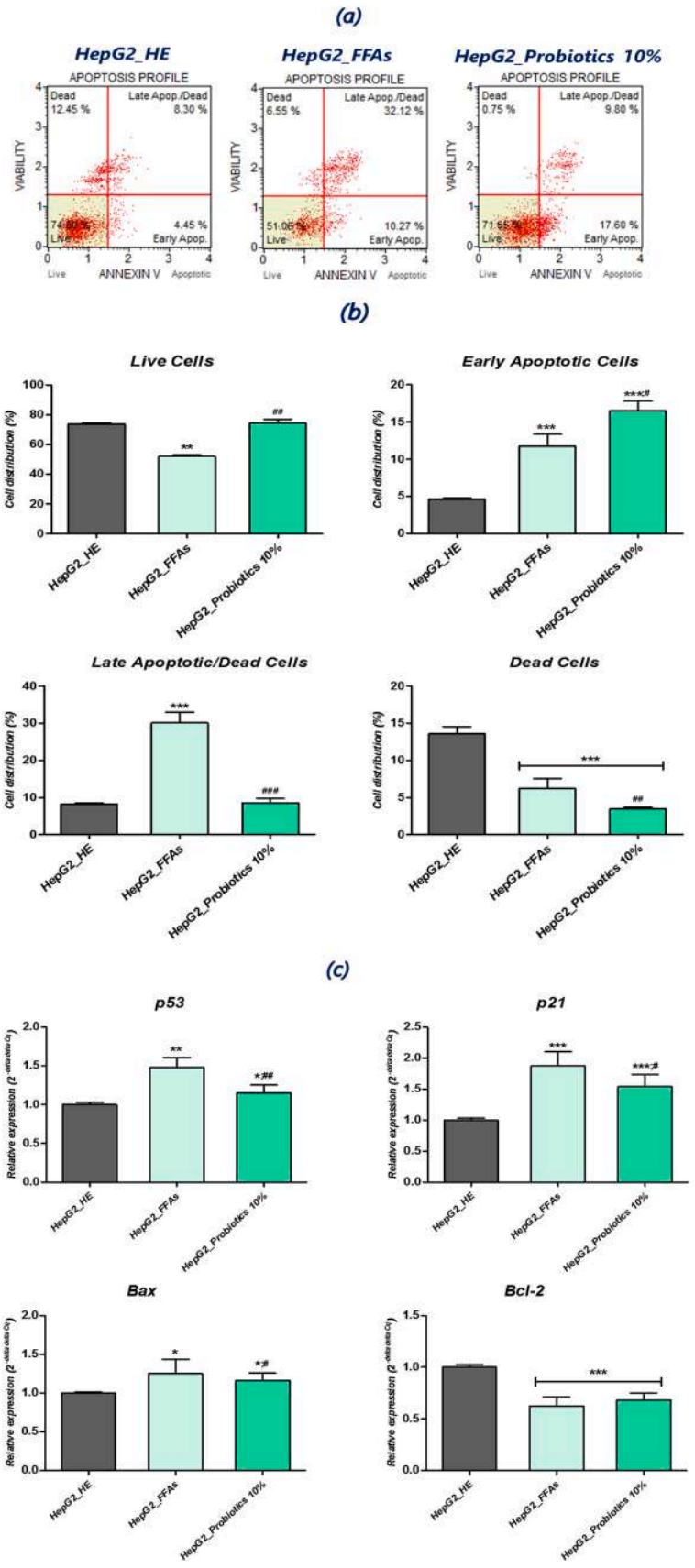

Fig. 6. Probiotics-Rich Emulsion reduces lipoapoptosis in HepG2 cells following intracellular FFA accumulation. (a) Apoptosis profile plots. Each plot is a representative figure of the three replicates of each determination. (b) Bar charts depicting the percentage of live, early and late apoptotic cells, as well as total dead cells. (c) Representative bar charts of the relative expression of apoptotic key markers. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

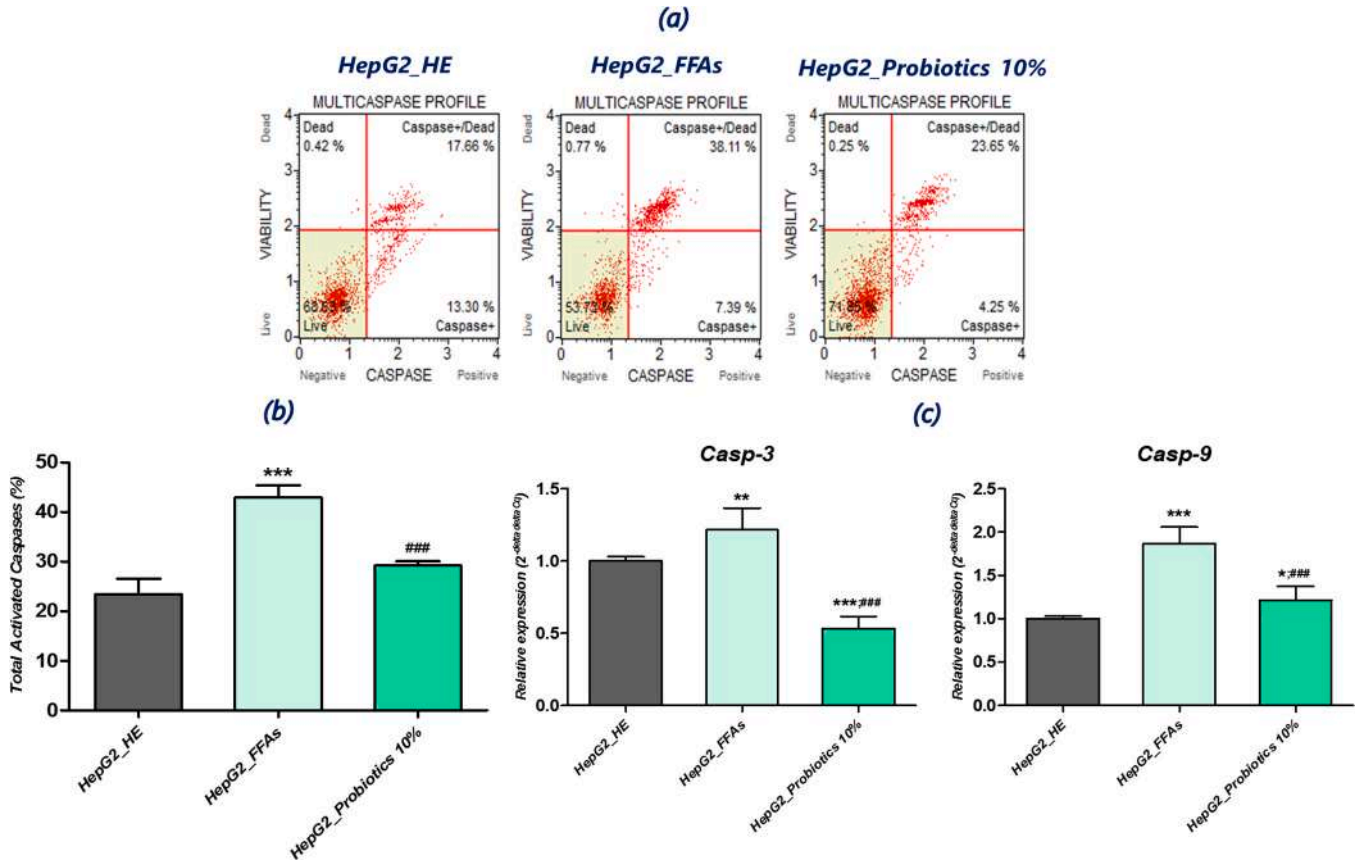

Fig. 7. Probiotics-Rich emulsion decreases multicaspase enzyme activation (a) Multicaspase profile plots. Each plot is a representative figure of the three replicates. (b) Bar charts depicting the average of the total activated multicaspase in treated and untreated HepG2 cells. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

HepG2 preconditioning with PRE contributed to the restoration of the diminished *phospho-Insr(Y1361)*, *phospho-Akt(Ser473)* and *phospho-Pi3kP85alpha/gamma/beta (Y467/Y199/Y464)* levels after insulin stimulation in relation to FFAs-treated and untreated control cells (Fig. 10.d). What is more, probiotics-rich emulsion enabled the increase in total *Insr*, *Akt* and *Pi3kCD* proteins abundance, which confirms the stimulatory effect of tested formulation of corresponding genes expression.

3.9. Probiotics-rich emulsion prevents insulin resistance onset through the regulation of Fetuin-A/TLR4/JNK/NF-κB axis in FFAs-challenged HepG2 cells

To additionally bring more consistency regarding the PRE-mediated molecular mechanisms in enhancing insulin sensitivity, the examination of FFAs-stimulated *Fetuin-A/TLR4/JNK/NF-κB* pathway has been considered as probable target for probiotics, since its activation in the liver highly depends on lipids overaccumulation. As demonstrated in Fig. 11.a, PA/OA treatment strongly promoted the expression of Mitogen-activated protein kinase genes, namely *MAKP8* that encodes for *JNK1* protein, *MAPK9* encoding *JNK2* protein and *MAPK14* that codify for *p38-α* protein, as well as *TLR4* and *NF-κB* mRNA in a significant manner over healthy cells ($p < 0.001$). These results being in correlation with an observed up-regulation of *AHSG* gene expression, which is encoding Fetuin-A protein ($p < 0.001$). Moreover, FFAs exposure stimulated not only genes expression, but increased also the phosphorylation levels (Fig. 11.c) of *p-NF-kappaB p65(T254)* and *p-JNK1/JNK2 (Thr183/Tyr185)*, and obviously triggered to an increase in Fetuin-A protein abundance ($p < 0.001$). Obtained outcomes demonstrated also that PRE supplementation may exerts its protective effects against FFAs-triggered insulin resistance through the modulation of *Fetuin-A/TLR4/JNK/NF-κB* pathway, as evidenced by the suppressed overexpression of both *MAPK8*, *MAPK9*, *MAPK14*, *TLR4*, *NF-κB and AHSG transcripts* versus insulin resistant cells ($p < 0.001$). Moreover, the probiotics-rich emulsion enabled a relative normalization and attenuation of the phosphorylation cascades involving *p-NF-kappaB p65(T254)* and *p-JNK1/JNK2 (Thr183/Tyr185)*, while sensibly reducing the expression of Fetuin-A protein ($p < 0.001$) in the same time (Fig. 11.c).

4. Discussion

Liver related insulin resistance becomes a common hallmark factor that leads to obesity, insulin dysregulation and metabolic syndrome development in both human being as well as animals [54]. Metabolic deterioration of liver is associated with excessive accumulation of free fatty acids, exhaustive oxidative stress, cellular apoptosis and inflammation, impairment of insulin singling pathways, which trigger to lipotoxicity and as recently showed, to abnormal elevated Fetuin-A levels in affected subjects [14]. Here we showed, that probiotics-rich emulsion (PRE) in dose depend manner improves HepG2 viability and protects against apoptosis under the both normal as well as insulin resistance condition. Interestingly, we noted that the poor emulsion exerted a strong cytotoxic effect on HepG2 cells which was abolished, when PRE were incorporated to the emulsion. This clearly indicates on beneficial

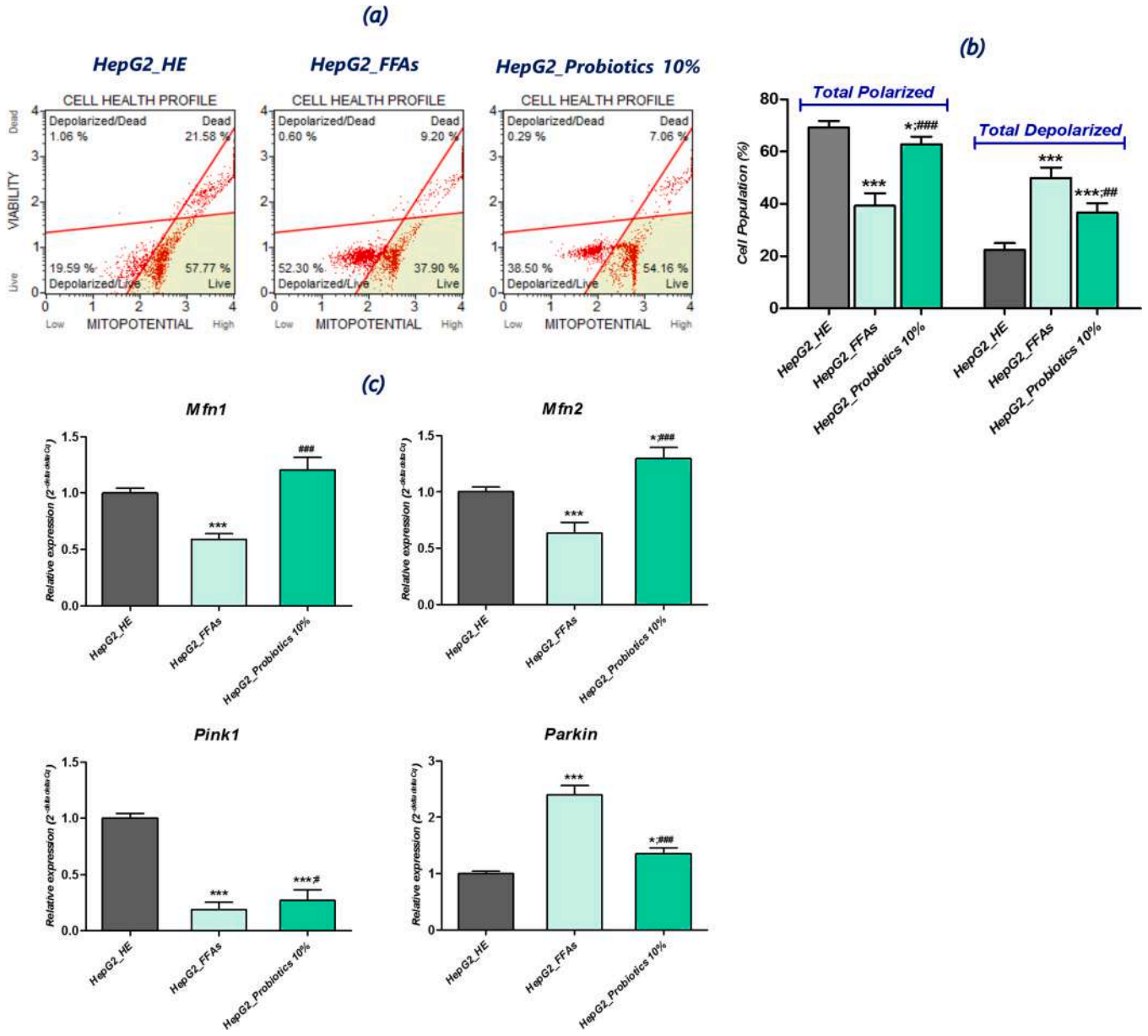

Fig. 8. Probiotics-rich emulsion prevents FFAs-induced mitochondrial dysfunction. (a) Mitochondrial membrane potential (MMP) evaluation using the flow cytometric analysis. (b) Histograms depicting percentages of polarized and depolarized mitochondrial membrane potential. (c) Representative Bar-Charts of the relative expression of mitochondrial fusion and mitophagy regulators. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

effect of PRE on hepatocytes viability as well as proliferative activity. Here we demonstrated, that PRE significantly reduces expression of proapoptotic related markers i.e. *p21* and *p53* and at the same time inhibits expression of *Bax* - common transcript inducing cellular apoptosis. Obtained data are strongly correlated with observed reduced activity of *Casp-3* and *Casp-9*, as well as total caspase activity in insulin resistant HepG2 cells treated with PRE. It stands in a good agreement with Sharma and colleges findings, which showed that probiotics protects primary hepatocytes against apoptosis through the modulation of both *Bax/Bcl-2* and caspases axis. The protective effect of *Enterococcus lactis* IITRHR1 (El(SN)) and *Lactobacillus acidophilus* MTCC447 (La(SN)) lysates has been explained by improvement of hepatocytes viability mediated by the reduced oxidative stress [56]. Recently, *Ren and colleagues* showed, that administration of probiotics in NAFLD affected rats improves liver metabolism as well as protects hepatocytes against its pathophysiological deterioration due to its protective effect against oxidative stress and improvement of mitochondrial function [62]. These stands in a good agreement with our findings, since we evidenced, that PRE in insulin resistant HepG2 reduces ROS level together with improving activity of *Sod2*, which protects the cells against mitochondrial related apoptosis. Similarly, *Wu and Colleagues*, highlighted in their study that rat treatment with a *Bacillus SC06* suspension can attenuate oxidative stress-induced intestinal disorders and apoptosis, via the increase in both *GSSG/GSH* and *Sod1/Sod2/GSH-Px* activities and p38-mediated autophagy [63]. Moreover, we noted that PRE modulates mitochondrial dynamics in insulin resistant HepG2 cell through activation of Mitofusin-1 and Mitofusin-2 transcripts - the master regulators of mitochondrial fusion. *Mfn2* is a dynamin-like protein anchored in the

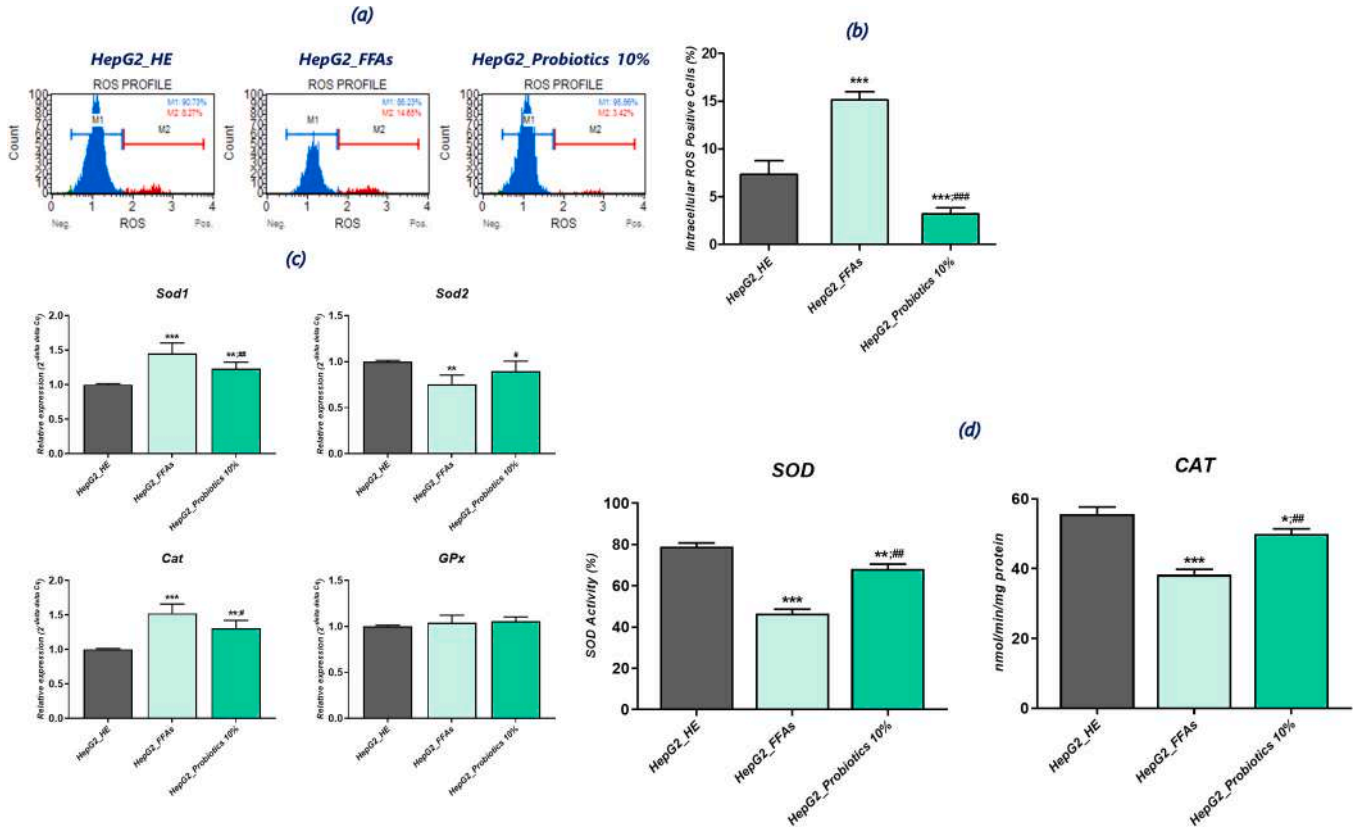

Fig. 9. Probiotics-Rich Emulsion moderates oxidative stress in PA/OA-treated HepG2 cells. (a) Dot-Plots for intracellular ROS production detected by dihydroethidium (DHE) fluorescence staining. (b) Average percentages of total ROS^{+} cells in each experimental group. (c) Relative expression of the main endogenous antioxidant enzymes-related genes. (d) Representative data for superoxide dismutase (SOD) and catalase (CAT) enzymatic activities. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

outer mitochondrial membrane, which modulates mitochondrial morphological homeostasis. Numerous studies reported on its reduced expression in the course of insulin resistance; however, the exact underling mechanism is still elusive [64,65]. Recently, *Gan* et al. claimed that *Mfn-2* ameliorates high-fat diet-induced insulin resistance in liver of rats as well as improves expression of *INSR*, *IRS2*, *Glut2*, *Pi3K-p85* and *Akt2* restoring thus sensitivity of peripherical tissues to insulin [59]. In turn, *Nie* ascertained, that *Mfn-2* reduces oxidative stress and therefore improves insulin sensitivity in rat muscles [66]. Moreover, our outcomes showed, that PRE enhanced expression of PTEN-induced putative kinase 1 (*Pink1*) and Parkin in insulin resistant hepatocytes. Recently, it was reported that hepatic lipotoxicity as well as insulin resistant lead to significant mitochondrial damage, which in turn deteriorates mitochondrial respiratory chain function and fatty acid oxidative degradation [67,68]. Surprisingly, the enhanced expression of both *Pink1/Parkin* observed in the current study might be explained by a probable protective effect against insulin resistant related mitochondrial alterations through the initiation of mitophagy-mediated suppression of damages mitochondria [67,69–72]. Observed effect in this investigation strongly suggests that PRE by reducing the accumulation of reactive oxygen species (ROS) and the decline in antioxidant enzymes in the course of installed oxidative stress protects mitochondria against Palmitate/Oleate-induced insulin resistant and lipotoxicity across modulating mitochondrial metabolism and dynamics.

Numerous investigations already reported on the beneficial effects of probiotics supplementation in the course of liver insulin resistance, non-alcoholic fatty liver (NAFLD) as well as cirrhosist [73,74]. The liver sensitivity for insulin in mentioned above diseases plays a critical role in protection against accumulation of ROS and other oxidative stress-related free radicals, improving hepatocytes metabolic functions as well as protecting against lipotoxicity induced insulin resistance [75]. In this study we showed, that application of PRE for palmitate/oleate-induced insulin resistant HepG2 cells substantially improves insulin sensitivity. We found, that exposure of insulin resistant hepatocytes to PRE resulted in elevated activation of *Akt/Pi3K* pathway. Moreover, we demonstrated the enhanced expression of insulin receptor (*Insr*), Insulin receptor substrate 1 and 2 (*Irs1* and *Irs2*) in HepG2 cell treated with PRE. We also evidenced the supported phosphorylation of both *phospho-Insr(Y1361)*, *phospho-Akt(Ser473)* and *phospho--Pi3kP85alpha/gamma/beta(Y467/Y199/Y464)* upon PRE supplementation, which is known to be a critical event during insulin signalling cascades. Obtained results are strongly correlated with recently published data of *Kobyliak* et al. [76] who showed, that treatment of insulin resistance new born rats with probiotics protects against obesity development, decreases proinflammatory cytokines levels including IL-1β, IL-12Bp40, and increases adiponectin and TGF-β levels. What is more, enhanced sensitivity for insulin through elevated expression of *Insr* was found. Our and other research groups findings indicates on insulin sensitizing effect of PRE in insulin resistance hepatocytes, what shed a promising light for its future application in metabolic related diseases. Finally, our finding emphasized that PRE represses the Fetuin-A mediated *TLR4/JNK/NF-κB* signalling pathway mobilization leading to improvement of insulin sensitivity in palmitate/oleate insulin resistant HepG2 cells. We found that pre-treatment of those cells with PRE results in significant reduction of Fetuin-A at the gene' and protein' level, probably due to a minimization in FFAs accumulation within the

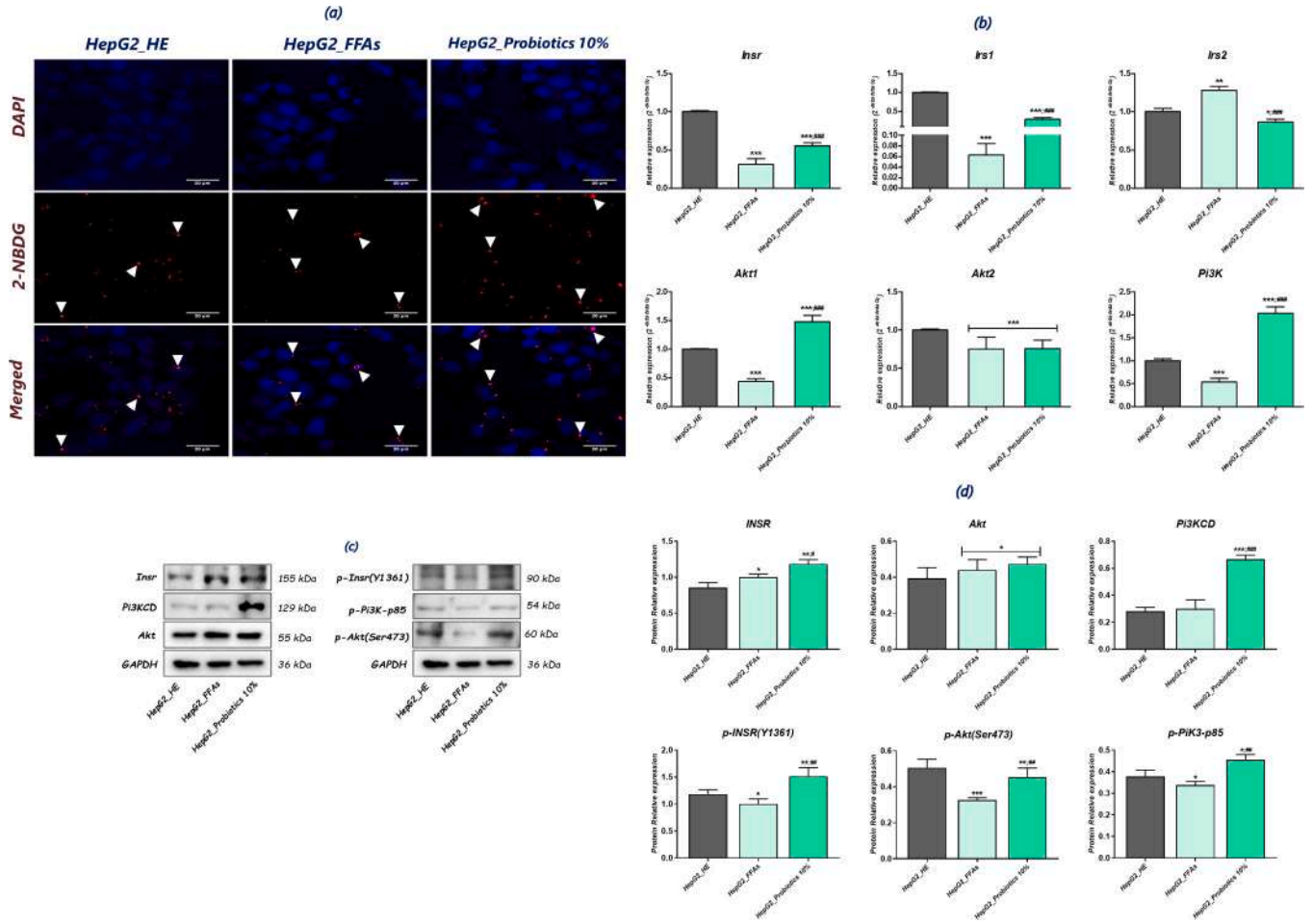

Fig. 10. Probiotics-rich emulsion reverses the FFAs-mediated defective insulin signalling in IR HepG2 cells. (a) Representative confocal micrographs of 2-NBDG uptake (magnification x63). (b) The mRNA levels of key genes involved in insulin downstream signalling execution. (c) Representative blots of Western blotting analysis of total and phospho-related proteins abundance in experimental HepG2 cells. (d) Densitometric analysis of immunoblot data for relative protein expression levels of total and phosphorylated insulin-responsive proteins normalized to GAPDH (Loading control). Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

cells. Presently obtained data support the outcomes from *Khalili* et al. [77] research, which outlined significant reduced serum Fetuin-A levels after *Lactobacillus casei* probiotics consumption patients with T2DM, while increasing SIRT1 levels, that exhibited strong improved glycaemic response following eight weeks treatment, indicating that probiotics treatments may be successful in regulation Fetuin-A metabolic action in the course of obesity and insulin resistance.

Fetuin-A, a secreted glycoprotein, has been proven to inhibit insulin-induced insulin receptor (*Insr*) autophosphorylation and tyrosine kinase activity and stimulate inflammatory responses in both cellular and animal's models [54]. The fetuin gene (AHSG) is located on human chromosome 3q27, which was identified as a susceptibility locus for syndrome X, metabolic syndrome as well as type 2 diabetes [78,79]. Furthermore, Fetuin-A knockout mice were characterized by enhanced glucose sensitivity, resistance to weight gain, lower serum free fatty acid levels and higher insulin sensitivity [20]. Thus, it was speculated that Fetuin-A may play a critical role in the regulation of insulin sensitivity, weight gain, and fat accumulation and might become a novel therapeutic target in the treatment of insulin resistance related treatment. In this study we were also interested in exploring the influence of PRM in reversing Fetuin-A triggered *TLR4/JNK/NF-κB* pathway activation, which is known to play pivotal role in the exacerbation of obesity-associated low-grad inflammation, that extremely contributes to insulin resistance maintenance [80]. As a matter of fact, previous experiments determined that Fetuin-A is a central factor in FFAs-induced lipotoxicity, which mediates downstream *FFAs–TLR4* crosstalk, resulting in lipotoxicity-mediated inflammatory damage in β-cells. Subsequently, the same study elucidated an enhanced expression of *p-JNK* and *NF-κB subunit P65*, which was abolished upon *TLR4* silencing. Furthermore, inhibition of *p-JNK* or *NF-κB* activity was found to significantly attenuate the lipotoxic effect of Fetuin-A associated to FFAs such as palmitate, suggesting the tight relationship between *FFAs–Fetuin-A–TLR4* complexes and *JNK/NF-κB* activity [24]. In addition, there seems to be an interesting crosstalk between Fetuin-A and *NF-κB*, since it has been reported that its p65 subunit, when activated through hight FFAs levels, translocate to the nucleus and combines with DNA to promote transcription activity of AHSG promoter increasing its expression in hepatocytes [81]. Here, we established that PRE application significantly prevented the expression of *TLR4/JNK/NF-κB* axis - the master regulators of Fetuin-A protein, through the repression of *MAPK8*, *MAPK9*, *MAPK14*, *TLR4*, *NF-κB and AHCG* related genes expression, as well as inhibition of phosphorylation-dependent activation of the *p-NF-kappaB p65(T254)* and *p-JNK1/JNK2(Thr183/Tyr185)* proteins. These findings are in accordance with previous researches, that

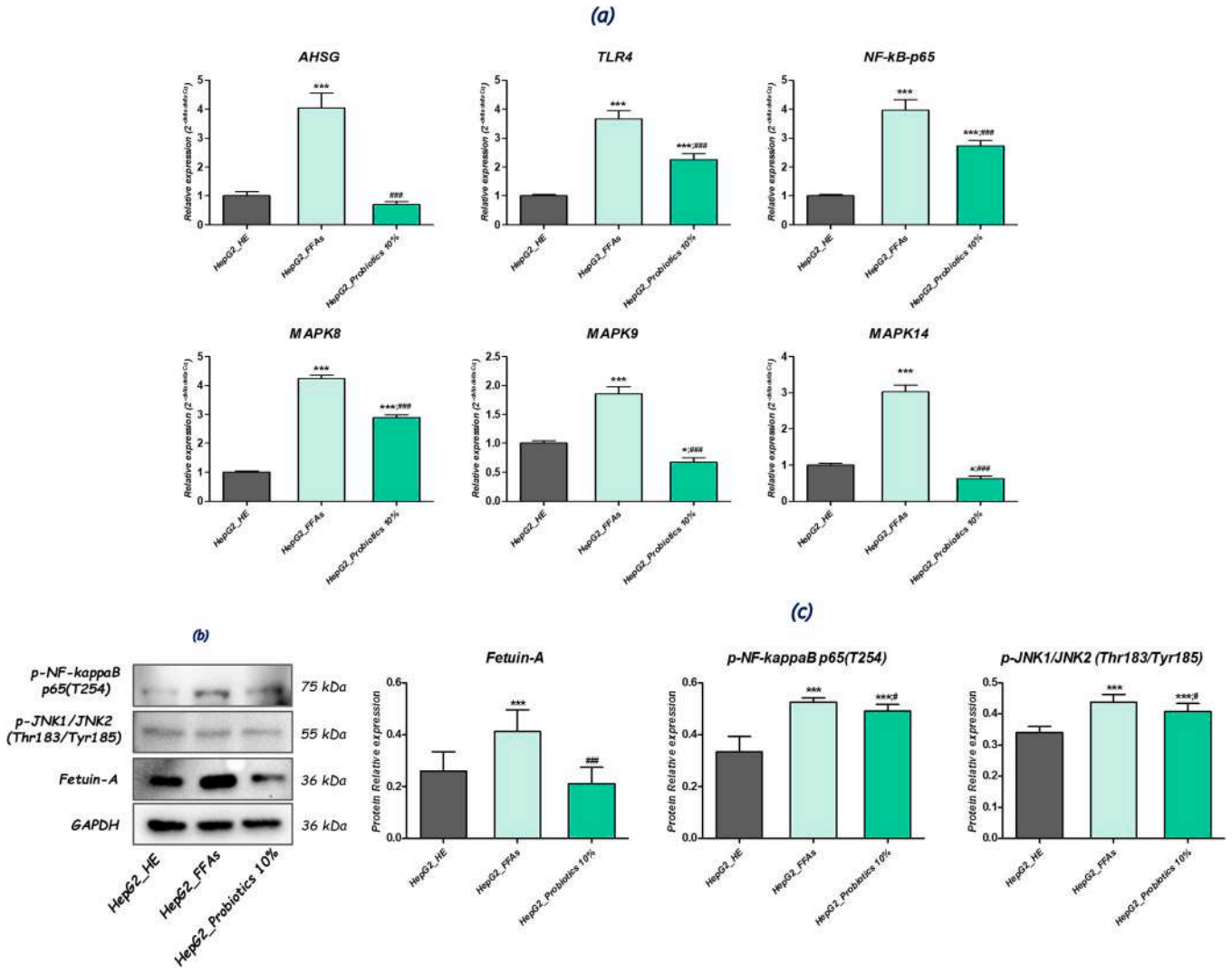

Fig. 11. Probiotics-Rich Emulsion abrogates Fetuin-A mediated *TLR4/JNK/NF-κB* activation in lipotoxic HepG2 cells. (a) Graphical summarizing of relative gene expression data for Fetuin-A activated *TLR4/MAPK/NF-κB* mediators. (b) Western blots of HepG2 cell lysates probed with *Fetuin-A*, *p-NF-kappaB p65(T254)* and *p-JNK1/JNK2 (Thr183/Tyr185)* antibodies. (c) Relative protein levels quantified in regards to *GAPDH* endogenous reference. Representative data from three independent experiments are shown ± SD (n = 3). An asterisk (*) indicates a comparison of all treated groups to untreated healthy cells. A hashtag (#) refers to a comparison of the PRE-treated groups to FFA-challenged cells. */# $p < 0.05$, **/## $p < 0.01$, *** /### $p < 0.001$. HepG2_HE: HepG2 healthy untreated cells; HepG2_FFAs: HepG2 cells exposed to the Palmitate/Oleate combination; HepG2_Probiotics 10%: HepG2 cells pre-treated with 10% of the Probiotics-Rich emulsion and exposed to the Palmitate/Oleate combination.

reported on the potent regulatory effects of probiotics in the initiation of *TLR4/NF-κB* pathways. In fact, *Lactobacillus reuteri and Lactobacillus delbrueckii* subsp. *bulgaricus* strains were shown to efficiently prevent mRNA expression of *IL-6*, *TNF-α*, *TLR4* and *NF-κB,* as well as diminish the *TLR4*, *IL-1* and *TNF-α* proteins levels via the inhibition of the *NF-κB* signalling pathway in rat intestinal tissue [82].

According to the Web of Science, there are only five manuscripts related to the effects of probiotic bacteria on fetuin-A protein (accessed 12 March, 2021). Styriak et al. described the effect of different culture media of *Lactobacillus fermentum* L 670, *Lactobacillus plantarum* L 5 and *Lactobacillus casei* subsp. *pseudoplantarum* L.*c* on the binding of these bacteria to fetuin-A, as also rated the binding of 11 strains of *Lactobacillus* spp., one *E. coli* and two *Enterococcus faecium* strains to fetuin-A from foetal calf serum [83,84]. While Roos et al. demonstrated that fetuin-A reduces the binding of *Lactobacillus reuteri* 1063 surface protein to the mucosa of the gastrointestinal tract of pigs and chickens [85]. Finally, only two of these manuscripts were closely related to our report. Hallajzadeh et al. [86] showed that the supplementation of mice with *Lactobacillus delbrueckii* subsp. *lactis* PTCC1057 lowered serum Fetuin-A levels. In their reports, they also confirmed higher concentrations of Fetuin-A in the serum of diabetic mice than in the non-diabetic group [86]. Also, the above-mentioned team of Khalili et al. demonstrated decreased levels of Fetuin-A in the serum after supplementation with *Lactobacillus casei* in patients with T2DM [77]. However, in both cases of supplementation with probiotic bacteria in humans and mice, their impact on Fetuin-A levels was determined by biochemical tests using blood samples. Thus, the conducted studies confirmed the beneficial effect of probiotics on the reduction of Fetuin-A serum levels, but did not explain the mechanisms of this action. In our research, we have demonstrated for the first-time possible mechanisms of the influence of probiotics bacteria on the Fetuin-A at the molecular level. Overall, presented data clearly indicates, that PRE may modulate activity of Fetuin-A and its downstream mediators, and therefore becomes a valuable tool for improving liver insulin sensitivity in the course of insulin resistance, metabolic syndrome and obesity.

5. Conclusion

In this study for the first time, we showed, that probiotics emulsion protects palmitate/oleate induced insulin resistance in HepG-2 cells by protection against lipotoxicity related apoptosis, as well as improving mitochondrial metabolism and dynamics, and reducing oxidative stress. Moreover, application of PRE resulted in significant reduction of *Fetuin-A/TLR4/JNK/NF-κB* axis, while restoring insulin signalling cascades, which suggests their protective effect on inflammation, obesity as well as liver related insulin resistant. Obtained data shed a promising light for PRE as an insulin sensitizing agent playing at the same time protective role for the development of liver related insulin resistant.

Conflict of interest statement

The authors declare that they have no competing interests.

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CRediT authorship contribution statement

M. Mularczyk prepared all the bacterial related materials and results, participated in data collection, data analysis, data interpretation and manuscript writing. Y. Bourebaba participated in literature search and writing manuscript. A. Kowalczuk participated in data analysis, manuscript edition and financial support. K. Marycz- designed and coordinated the study, participated in writing the manuscript and provided funding. L. Bourebaba participated in designing and coordinating the study, experiment planning, cell culture and data analysis, in the interpretation of obtained result, validation, methodology, data curation, manuscript writing and provided a part of funding.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not applicable.

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4. DYSKUSJA

Syndrom metaboliczny koni (EMS), podobnie jak ludzki syndrom metaboliczny (MetS), obejmuje zbiór objawów klinicznych związanych z otyłością, dysregulacją insuliny i podatnością na wtórną chorobę zapalną. Chociaż wtórne stany zapalne wynikające z EMS mogą zagrażać życiu, diagnoza nie jest prosta i często komplikuje obecność innych współistniejących jednostek chorobowych [93]. Tkanka tłuszczowa pochodząca od koni cierpiących na EMS charakteryzuje się wyraźną hipertrofią adipocytów oraz zwiększoną ekspresja adipokin (leptyna) i cytokin zapalnych (TNFα, IL1β i CCL2) w porównaniu z tkanką koni zdrowych [19]. Podobnie jak w przypadku tkanki tłuszczowej, u koni cierpiących na EMS obserwuje się upośledzenie czynności wątroby na poziomie molekularnym, co charakteryzuje się zwiększoną apoptozą i zwiększonym stresem oksydacyjnym retikulum endoplazmatycznego oraz nadmierną akumulacją lipidów i nasileniem stanu zapalnego [94].

Wyniki uzyskane w ramach prowadzonych badań potwierdziły założenia pierwszej hipotezy badawczej, zakładającej, że preinkubacja komórek ASC izolowanych od koni cierpiących na EMS z astaksantyną obniżać będzie apoptozę, łagodzić stres oksydacyjny oraz usprawniać mitochondrialny system fosforylacji oksydacyjnej (OXPHOS), a tym samym kontrolować i niwelować stres oksydacyjny, poprawiając stan metaboliczny ASC. Traktowanie astaksantyną komórek ASC izolowanych od koni z syndromem metabolicznym w dawce 10 i 20 µg/ml znacząco zmniejszyło apoptozę poprzez modyfikację znormalizowanej aktywności kaspazy szlaków proapoptotycznych (p21, p53 i Bax). Ponadto, regulując ekspresję powiązanych głównych czynników SOD1, SOD2, PARKIN, PINK 1 i MFN 1, astaksantyna łagodziła stres oksydacyjny i zaburzenia dynamiki mitochondriów. Astaksantyna przywróciła mitochondrialną fosforylację oksydacyjną poprzez stymulację markerów związanych z maszynerią OXPHOS: COX4I1, COX4I2, UQCRC2, NDUFA9 i TFAM. Uzyskane wyniki pozostają w korelacji z doniesieniami innych autorów, co zostało opisane w dyskusji załączonego artykułu naukowego „Astaxanthin Carotenoid Modulates Oxidative Stress in Adipose-Derived Stromal Cells Isolated from Equine Metabolic Syndrome Affected Horses by Targeting Mitochondrial Biogenesis". Niemniej, warto zwrócić uwagę na rosnące zainteresowanie badaczy wpływem astaksantyny na zaburzenia metaboliczne różnych typów komórek. Wyniki badań przeprowadzonych przez Tsai i in. sugerują, że astaksantyna w dawce 25 µg/ml skutecznie hamowała akumulację lipidów w adipocytach 3T3-L1, a jej działanie jest skorelowane z regulacją genów związanych z lipogenezą i akumulacją triglicerydów w adipocytach 3T3-

L1 [95]. Badania prowadzone przez Wang i in. Sugerują, że astaksantyna może także zapobiegać rozwojowi stłuszczenia wątroby i stresu oksydacyjnego związanego z ryzykiem choroby metabolicznej, dzięki właściwościom modulującym oś jelitowo-wątrobową [96]. Natomiast, badania Yinhua i in. Wykazały, że astaksantyna, w dawce 0,02% w codziennej diecie, hamowała i odwracała insulinooporność wywołaną lipotoksycznością i stłuszczeniowe zapalenie wątroby u myszy, poprzez osłabienie akumulacji lipidów w wątrobie [97]. Co ciekawe, Kim i in. wykazali, że astaksantyna w dawce 0,035% hamuje stany zapalne i zwłóknienie wątroby i tkance tłuszczowej oraz wzmacnia mięśnie szkieletowe u myszy [98]. Również badania prowadzone przez Urakaze i in. Potwierdzają skuteczność stosowania astaksantyny w walce z cukrzycą i miażdżycą. Ustna suplementacja astaksantyny w dawce 12 mg przez 12 tygodni w grupie 53 osób istotnie obniżyła poziom glukozy po 120 minutach, poprawiając wrażliwość na insulinę [99].

Kolejny etap badań również potwierdził słuszność hipotezy, która zakładała, iż preinkubacja komórek HepG2 wraz z emulsją pro- i postbiotyczną, w warunkach IR, będzie chronić komórki przed indukowaną insulinoopornością *in vitro.* Wyniki uzyskane w ramach prowadzonych badań wskazują, że emulsja pro- i postbiotyczna poprawia żywotność HepG2, chroni przed apoptozą w warunkach normalnych i IR. Ponadto emulsja skutecznie łagodziła stres oksydacyjny oraz poprawiała metabolizm i dynamikę mitochondriów. Komórki wątroby poddane działaniu 10% emulsji pro- i postbiotycznej znacznie zmniejszyły aktywację szlaku *Fetuina-A/TLR4/JNK/NF-κB*, co sugeruje działanie ochronne przed zapaleniem, otyłością oraz insulinoopornością związaną z upośledzoną funkcją wątroby. Porównanie uzyskanych wyników z doniesieniami pozostałych autorów przedstawiono w dyskusji artykułu: „Probiotics-rich emulsion improves insulin signalling in Palmitate/Oleate-challenged human hepatocarcinoma cells through the modulation of Fetuin-A/TLR4-JNK-NF-κB pathway". Badania prowadzone przez Lee i in. wskazują, że probiotyki zapewniają równowagę mikrobiologiczną w przewodzie pokarmowym oraz wykazują znaczną zdolność antyoksydacyjną. W hodowlach komórek HepG2 probiotyki łagodziły stres oksydacyjny wywołany przez H2O2, pośrednicząc w peroksydacji lipidów i regulacji poziomu glutationu oraz zwiększały syntezę enzymów antyoksydacyjnych, w tym katalazę, dysmutazę ponadtlenkową i peroksydazę glutationową [100]. Również wyniki Won i in. potwierdzają skuteczność stosowania produktów probiotycznych w prewencji insulinooporności. Zastosowanie ekstraktów probiotycznych w hodowlach IR HepG2 zwiększyło wychwyt glukozy i podniosło zawartość glikogenu w komórkach oraz znacząco zmniejszyło przyrost masy ciała, stłuszczenie wątroby i znacznie poprawiło profil surowicy,

w tym poziomy ALT, HBA1c, TG i LDL/HDL. Wykazano również, że ekstrakty probiotyczne zwiększyły tolerancję glukozy poprzez szlak sygnałowy PI3K/Akt [101]. Badanie, przeprowadzone na grupie 23 osób ze zdiagnozowaną insulinooporności i cukrzycą, wykazało, że suplementacja probiotyczna przez 4 tygodnie wywierała korzystny wpływ na poziom glukozy na czczo i markery insulinooporności bez żadnych działań niepożądanych [102].

Powyższe doniesienia korelują z wynikami uzyskanymi w trakcie realizacji niniejszej pracy doktorskiej. Skuteczność i bezpieczeństwo astaksantyny jak i probiotyków oraz postbiotyków została potwierdzona na modelu komórkowym *in vitro* oraz przez innych badaczy *in vivo* na modelach zwierzęcych oraz ludziach. Przeprowadzone badania stanowią silną podstawę do dalszych badań i prewencyjnych metod zapobiegania syndromu metabolicznego u koni.

5. PODSUMOWANIE I WNIOSKI KOŃCOWE

Analiza najnowszych doniesień naukowych oraz wyniki przeprowadzonych badań wskazują na zasadność i skuteczność stosowania bioaktywnych substancji pochodzenia mikrobiologicznego w walce z chorobami metabolicznymi. Wyniki uzyskane w trakcie realizacji pracy doktorskiej wykazały potencjał ochronny względem linii komórkowych HepG2 z indukowaną insulinoopornością oraz komórek ASC izolowanych od koni z syndromem metabolicznym. W ostatnich latach, niepokojącym staje się gwałtowny wzrost zachorowań na schorzenia o podłożu endokrynologicznym, takich jak cukrzyca czy syndrom metaboliczny koni. Co ważniejsze, poza zaleceniami restrykcji kalorycznych oraz wzmożoną aktywnością ruchową, nie istnieją skuteczne farmakologiczne metody leczenia EMS. Uzyskane wyniki w badaniach z wykorzystaniem astaksantyny oraz probiotyków i postbiotyków mogą przyczynić się do opracowania innowacyjnych strategii terapeutycznych mających na celu uwrażliwienie organizmu na insulinę oraz ochronę przed stresem oksydacyjnym i zaburzeniami metabolizmu mitochondriów. W rezultacie zarówno probiotyki, postbiotyki i astaksantyna mogą stanowić naturalny czynnik zapobiegający chorobom metabolicznym nie tylko u koni.

<u>Najważniejsze wyniki uzyskane w trakcie prac badawczych wykonanych podczas realizacji pracy doktorskiej:</u>

1. Optymalizacja wzrostu i akumulacji astaksantyny w hodowli drożdży *Phaffia rhodozyma,*
2. Optymalizacja wzrostu szczepów probiotycznych do produkcji metabolitów o korzystnym działaniu terapeutycznym,
3. Optymalizacja procesu technologicznego produkcji emulsji probiotycznej chroniącej związki aktywne oraz komórki bakterii przed niekorzystnym wpływem środowiska górnego odcinka przewodu pokarmowego,
4. Ocena zdolności astaksantyny do zmniejszenia apoptozy poprzez modyfikację aktywności kaspazy szlaków proapoptotycznych (p21, p53 i Bax), poprawy żywotności i proliferacji, zmniejszenia stresu oksydacyjnego, poprawy dynamiki mitochondriów w populacji EMS ASC,

5. Przywrócenie przez astaksantynę mitochondrialnej fosforylacji oksydacyjnej poprzez stymulację markerów związanych z OXPHOS: COX4I1, COX4I2, UQCRC2, NDUFA9 i TFAM,
6. Wykazanie, że emulsja pro- i postbiotyczna uwrażliwia na insulinę komórki HepG2, chroni przed opornością na insulinę wywołaną palmitynianem/oleinianem poprzez ochronę przed apoptozą związaną z lipotoksycznością, a także poprawę metabolizmu i dynamiki mitochondriów oraz zmniejszenie stresu oksydacyjnego,
7. Ustalenie, że zmniejszenie aktywacji *osi Fetuina-A/TLR4/JNK/NF-κB* zachodzi przy jednoczesnym przywróceniu kaskad sygnalizacyjnych insuliny, co sugeruje ochronny wpływ probiotyków i postbiotyków na stany zapalne, otyłość oraz insulinooporność wątroby.

Najważniejsze wnioski wyciągnięte na podstawie uzyskanych wyników:

1. Astaksantyny moduluje stres oksydacyjny i metabolizm mitochondrialny w komórkach zrębowych pochodzących z tkanki tłuszczowej izolowanych od koni dotkniętych zespołem metabolicznym.
2. Probiotyki i postbiotyki poprawiają sygnalizację insulinową w komórkach HepG2 narażonych na działanie palmitynianu/oleinianu poprzez modulację *szlaku Fetuina-A/TLR4-JNK-NF-* κB.

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Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

Malwina Katarzyna Mularczyk Wrocław, 22.08.23

imię i nazwisko

Katedra Biologii Eksperymentalnej

afiliacja

OŚWIADCZENIE

Oświadczam, że w pracy Mularczyk M., Michalak I., Marycz K. 2020, Astaxanthin and other Nutrients from Haematococcus pluvialis—Multifunctional Applications, Marine Drugs, 18, 459; mój udział polegał na:

- przygotowaniu koncepcji pracy,
- przygotowaniu wstępnego szkicu pracy,
- przygotowaniu wszystkich rozdziałów pracy: (roz. 1. Introduction, 2. Cell Morphology of Haematococcus pluvialis, 3. Cultivation of Haematococcus pluvialis, 4. Biochemical Composition of Haematococcus pluvialis, 5. Astaxanthin as a Valuable Biologically Active Compound, 6. Biological Properties of Astaxanthin, 7. Astaxanthin in the Human and Animal Diet, 8. Conclusions)
- przygotowaniu rycin (fig.1., fig. 2., fig. 3., fig. 4.) oraz tabel (tab.1)
- kontakcie z wydawcą i recenzentami – w szczególności przygotowanie i naniesienie poprawek odnoszących się do uwag recenzentów

PODPIS ZAUFANY
MALWINA
MULARCZYK
30.08.2023 11:30:50 [GMT+2]
Dokument podpisany elektronicznie podpisem zaufanym

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data i podpis

Potwierdzam treść oświadczenia.

Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-08-30.

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Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

Malwina Katarzyna Mularczyk
imię i nazwisko
Katedra Biologii Eksperymentalnej
afiliacja

Wrocław, 22.08.23

OŚWIADCZENIE

Oświadczam, że w pracy Mularczyk M., Bourebaba N., Marycz K., Bourebaba L. 2022, Astaxanthin Carotenoid Modulates Oxidative Stress in Adipose-Derived Stromal Cells Isolated from Equine Metabolic Syndrome Affected Horses by Targeting Mitochondrial Biogenesis, Biomolecules, 12, 1039; mój udział polegał na:

- przygotowaniu koncepcji pracy,
- opracowaniu metodologii pozyskiwania astaksantyny (podrozdział 2.1. Yeast Biomass and Astaxanthin Extraction),
- prowadzeniu testów na liniach komórkowych (podrozdział 2.2. Equine ASCs Cell Culture 2.3. Determination of Cell Viability and Proliferative Activity by TOX8 Assay, 2.4. Bromodeoxyuridine (BrdU) Assay, 2.5. Colony-Forming Unit-Fibroblast (CFU-fs) Assay),
- przeprowadzeniu na liniach komórkowych: oceny akumulacji reaktywnych form tlenu (podrozdział 2.7. Intracellular Reactive Oxygen Species Determination), ocenie aktywności antyoksydacyjnej (podrozdział 2.8. Endogenous Antioxidant Activities Assays) oraz ocenie metabolizmu mitochondrialnego (podrozdział 2.9. Mitochondrial Membrane Potential Assay (MMP)),
- analizie wyników (podrozdział 3.1. Astaxanthin Improves Viability and Proliferation in EMS ASCs Affected Cells, 3.2. Astaxanthin Reduced Cell Apoptosis in Equine ASC Cells Suffering from EMS, 3.3. Astaxanthin Decreases Oxidative Stress in Equine EMS ASCCells, 3.4. Astaxanthin Enhances Mitochondrial Dynamics in EMS Affected ASC cells) oraz przygotowaniu wstępu (1. Introduction) oraz dyskusji (4. Discussion),
- nadzorze i przygotowaniu odpowiedzi dla recenzentów.

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PODPIS ZAUFANY
MALWINA
MULARCZYK
30.08.2023 11:32:08 [GMT+2]
Dokument podpisany elektronicznie
podpisem zaufanym

Potwierdzam treść oświadczenia.

Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-08-30.

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data i podpis promotora

Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

Malwina Katarzyna Mularczyk Wrocław, 22.08.23

imię i nazwisko

Katedra Biologii Eksperymentalnej

afiliacja

OŚWIADCZENIE

Oświadczam, że w pracy Bourebaba Y., Marycz K., Mularczyk M., Bourebaba L. 2022, Postbiotics as potential new therapeutic agents for metabolic disorders management, Biomedicine & Pharmacotherapy, 153, 113138; mój udział polegał na:

- przygotowaniu streszczenia oraz abstraktu graficznego,
- przygotowaniu wstępu (opis zaburzeń metabolicznych, czynników wpływających na to zaburzenie oraz przedstawiłam cel pracy),
- przygotowaniu opisu mikrobiomu - definicja, rola, znaczenie, (2. Gut microbiota – multifunctional role),
- przygotowaniu opisu części dotyczącej biosurfaktantów -opis i wpływ postbiotyków na stan zapalny organizmu, aktywność przeciwutleniającą oraz scharakteryzowałam mechanizmy ich działania, (4. Perspectives for clinical practice).

PODPIS ZAUFANY
MALWINA
MULARCZYK
30.08.2023 11:33:08 (GMT+2)
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podpisem zaufanym

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data i podpis

Potwierdzam treść oświadczenia.

Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-08-30

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Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

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Wrocław, 22.08.23

OŚWIADCZENIE

Oświadczam, że w pracy Mularczyk M., Bourebaba Y., Kowalczuk A., Marycz K., Bourebaba L. 2021, Probiotics-rich emulsion improves insulin signalling in Palmitate/ Oleate-challenged human hepatocarcinoma cells through the modulation of Fetuin-A/TLR4-JNK-NF-κB pathway, Biomedicine & Pharmacotherapy, 139, 111560; mój udział polegał na:

- przygotowaniu wszystkich materiałów i wyników związanych z hodowlą bakteryjną (2.1.2. Bacterial cells, 2.2. Bacterial strains emulsion, 2.4. Biocompatibility assessment of probiotics-rich emulsion, 3.1. Probiotics-rich emulsion stability and probiotic cells viability, 3.2. Probiotics-rich emulsion biocompatibility to HepG2 cell line),
- przeprowadzeniu na liniach komórkowych: testów cytotoksyczności, ocenie akumulacji reaktywnych form tlenu, ocenie aktywności antyoksydacyjnej oraz ocenie metabolizmu mitochondrialnego (2.8. Quantification of multicaspase activity, 2.9. Mitochondrial transmembrane potential assay, 2.10. Intracellular reactive oxygen species measurement, 2.11. Antioxidant enzymes activities assessment, 2.12. Glucose uptake assay, 3.4. Probiotics-rich emulsion reduces FFAs-induced Lipoapoptosis in HepG2 cells, 3.6. Probiotics-rich emulsion improves mitochondrial dynamics in FFAsoverloaded HepG2 cells, 3.7. Probiotics-rich emulsion attenuates oxidative stress in affected HepG2 cells, 3.8. Probiotics-rich emulsion restores impaired insulin signalling pathway in insulin-resistant HepG2 cells),
- przygotowaniu wstępu (1. Introduction) oraz dyskusji (4. Discussion),
- udziale w przygotowaniu odpowiedzi dla recenzentów.

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Potwierdzam treść oświadczenia.

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Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-08-30.