UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Marjeta MENCIN

IZBOLJŠANJE DOSTOPNOSTI IN ANTIOKSIDATIVNE AKTIVNOSTI VEZANIH FENOLNIH SPOJIN IZ SEMEN PIRE (*Triticum spelta* L.) Z IZBRANIMI BIOTEHNOLOŠKIMI PROCESI

DOKTORSKA DISERTACIJA

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IMPROVEMENT OF ACCESSIBILITY AND ANTIOXIDANT ACTIVITY OF BOUND PHENOLIC COMPOUNDS OF SPELT (*Triticum spelta* L.) SEEDS BY SELECTED BIOTECHNOLOGICAL PROCESSES

DOCTORAL DISSERTATION

Ljubljana, 2022

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Komisije za doktorski študij Univerze v Ljubljani z dne 18. 5. 2021 je bilo potrjeno, da kandidatka izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje živilstvo. Za mentorico je bila imenovana doc. dr. Petra Terpinc.

Doktorska disertacija je zaključek interdisciplinarnega doktorskega študijskega programa Bioznanosti. Raziskovalno delo je bilo opravljeno na Katedri za tehnologije rastlinskih živil in vino, Katedri za biotehnologijo, mikrobiologijo in varnost živil in Katedri za biokemijo in kemijo živil, Oddelka za živilstvo, ter na Katedri za sadjarstvo, vinogradništvo in vrtnarstvo, Oddelka za agronomijo, Biotehniške fakultete, Univerze v Ljubljani.

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Datum zagovora:

Doktorandka:
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KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK UDK 633.111.5:581.142:631.531:547.56(043)=163.6
KG pira, vezane in ekstraktibilne fenolne spojine, antioksidativna aktivnost, kaljenje, fermentacija, encimska obdelava, abiotski stres, biološka dostopnost, znotrajcelična oksidacija, *in vitro* gastrointestinalna prebava
AV MENCIN, Marjeta, mag. inž. živ.
SA TERPINC, Petra (mentorica)
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ZA Univerza v Ljubljani, Biotehniška fakulteta, Interdisciplinarni doktorski študijski program Bioznanosti, znanstveno področje živilstvo
LI 2022
IN IZBOLJŠANJE DOSTOPNOSTI IN ANTIOKSIDATIVNE AKTIVNOSTI VEZANIH FENOLNIH SPOJIN IZ SEMEN PIRE (*Triticum spelta* L.) Z IZBRANIMI BIOTEHNOLOŠKIMI PROCESI
TD Doktorska disertacija
OP X, 125 str., 2 sl., 3 pril., 138 vir.
IJ sl
JI sl/en
AI V doktorski disertaciji smo preučili vpliv različnih biotehnoloških procesov (kaljenja, fermentacije, encimske obdelave) na vsebnost in dostopnost ekstraktibilnih in vezanih fenolnih spojin v semenih pire in na njihovo *in vitro* ter *in vivo* antioksidativno aktivnost. Glavne identificirane fenolne spojine v pirinih semenih so bile ferulna, *p*-kumarna, kavna in *p*-hidroksibenzojska kislina. Pri iskanju optimalnih pogojev abiotskega stresa med kaljenjem semen za čim večji prirast fenolov, je kaljenje pri povečani slanosti in osmolarnosti brez aplicirane mehanske poškodbe najbolj povečalo vsebnost fenolnih spojin in izboljšalo njihovo antioksidativno aktivnost. Kombiniranje različnih biotehnoloških procesov predstavlja bistveno učinkovitejši način za povečanje vsebnosti fenolnih spojin kot posamezen proces. Največjo absolutno vsebnost ekstraktibilnih in vezanih posameznih fenolnih kislin smo določili v kaljenih semenih, fermentiranih s *S. cerevisiae*. Nadalje je obdelava kaljenih in fermentiranih semen s petimi hidrolitičnimi encimi znatno povečala vsebnost ekstraktibilnih fenolnih spojin, hkrati pa je imela negativen vpliv na vsebnost vezanih fenolnih spojin. Fenolne spojine iz kaljenih semen niso zaščitile modelnega organizma *S. cerevisiae* pred oksidacijo, medtem ko je večina ekstraktibilnih fenolnih spojin iz fermentiranih in encimsko obdelanih semen znižala znotrajcelično oksidacijo. Večji celični privzem fenolnih spojin ne pomeni nujno tudi večje antioksidativne aktivnosti *in vivo*, poleg količine je pomembna tudi oblika fenolnih spojin, ki vstopajo v celico. Rezultati kažejo na spremembe v sestavi ekstraktov med simulirano *in vitro* prebavo, saj so antioksidanti istega ekstrakta pokazali drugačen odziv proti različnim prostim radikalom v primerjavi z neobdelanimi semeni. Največjo vsebnost biološko dostopnih fenolnih spojin smo ugotovili v kaljenih semenih, fermentiranih s *S. cerevisiae*. Izgube fenolnih spojin po prebavi so bile velike, kljub temu pa so biološko obdelana semena imela še vedno znatno večjo vsebnost fenolnih spojin v primerjavi z neobdelanimi semeni.

KEY WORD DOCUMENTATION

ND Dd
DC UDC 633.111.5:581.142:631.531:547.56(043)=163.6
CX spelt, bound and extractable phenolic compounds, antioxidant activity, germination, fermentation, enzymatic treatment, abiotic stress, bioaccessibility, intracellular oxidation, *in vitro* gastrointestinal digestion
AU MENCIN, Marjeta
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PB University of Ljubljana, Biotechnical Faculty, Interdisciplinary Doctoral Programme in Biosciences, Scientific Field Food Science
PY 2022
TI IMPROVEMENT OF ACCESSIBILITY AND ANTIOXIDANT ACTIVITY OF BOUND PHENOLIC COMPOUNDS OF SPELT (*Triticum spelta* L.) SEEDS BY SELECTED BIOTECHNOLOGICAL PROCESSES
DT Doctoral dissertation
NO X, 125 p., 2 fig., 3 ann., 138 ref.
LA sl
AL sl/en
AB Within the scope of the doctoral dissertation, we investigated the influence of different biotechnological processes (germination, fermentation, enzymatic treatment) on the content and accessibility of extractable and bound phenolic compounds in spelt seeds, as well as on their antioxidant activity *in vitro* and *in vivo*. The major phenolic compounds identified in spelt seeds were ferulic, *p*-coumaric, caffeic and *p*-hydroxybenzoic acids. When searching for the optimal conditions of abiotic stress during seed germination to produce the greatest increase in phenolics, germination at increased salinity and osmolarity without applied mechanical damage showed the highest increase in phenolic compounds content and antioxidant activity. Combining different biotechnological processes represents the more effective way to increase the content of phenolic compounds than a single process. The highest absolute content of extractable and bound individual phenolic acids was found in germinated seeds fermented with *S. cerevisiae*. Moreover, treatment of germinated and fermented seeds with five hydrolytic enzymes significantly increased the content of extractable phenolic compounds, but at the same time had a negative effect on the content of bound phenolic compounds. Phenolic compounds of germinated seeds could not protect the model organism *S. cerevisiae* from oxidation, whereas most extractable phenolic compounds from fermented and enzymatic treated seeds reduced intracellular oxidation. Greater cellular uptake of phenolic compounds does not necessarily mean greater antioxidant activity *in vivo*, in addition to the quantity, the form of phenolic compounds entering the cell is also important. The results suggest changes in the composition of the extracts during simulated *in vitro* digestion, since antioxidants of the same extract showed a different response against various free radicals compared to the raw seeds. The highest content of bioaccessible phenolic compounds was found in germinated seeds fermented with *S. cerevisiae.* Losses of phenolic compounds after digestion were high, however, the bioprocessed seeds still contained higher content of phenolic compounds compared to raw seeds.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

ABTS	2,2'-azino-bis-3-etilbenzotiazolin-6-sulfonska kislina (ang. *2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid*)
C4H	cinamat-4-hidroksilaza (ang. *cinnamate 4-hydroxylase*)
C4L	*p*-kumarat-CoA ligaza (ang. *p-coumarate-CoA ligase*)
DNK	deoksiribonukleinska kislina (ang. d*eoxyribonucleic acid*)
DPPH	2,2-difenil-1-pikrilhidrazil (ang. *2,2-diphenyl-1-picrylhydrazyl*)
GI prebava	gastrointestinalna prebava (ang. *gastrointestinal digestion*)
HPLC-MS/MS	tekočinska kromatografija visoke ločljivosti (ang. *high-performance liquid chromatography*) sklopljena s tandemsko masno spektrometrijo (ang. *mass spectrometry*)
ICO	znotrajcelična oksidacija (ang. *intracellular oxidation*)
MKB	mlečnokislinske bakterije (ang. *lactic acid bacteria)*
$O_2^{\bullet -}$	superoksidni anionski radikal (ang. *superoxide anion radical*)
PAL	fenilalanin amonijak liaza (ang. *phenylalanine ammonia-lyase*)
$ROO^{\bullet}$	peroksilni radikal (ang. *peroxyl radical*)
ROS	reaktivne kisikove zvrsti (ang. *reactive oxygen species*)
SPE	ekstrakcija na trdno fazo (ang. *solid phase extraction)*
SS	suha snov (ang. *dry weight*)
TE	trolox ekvivalent (ang. *trolox equivalent*)
TPC	skupne fenolne spojine (ang. *total phenolic content*)

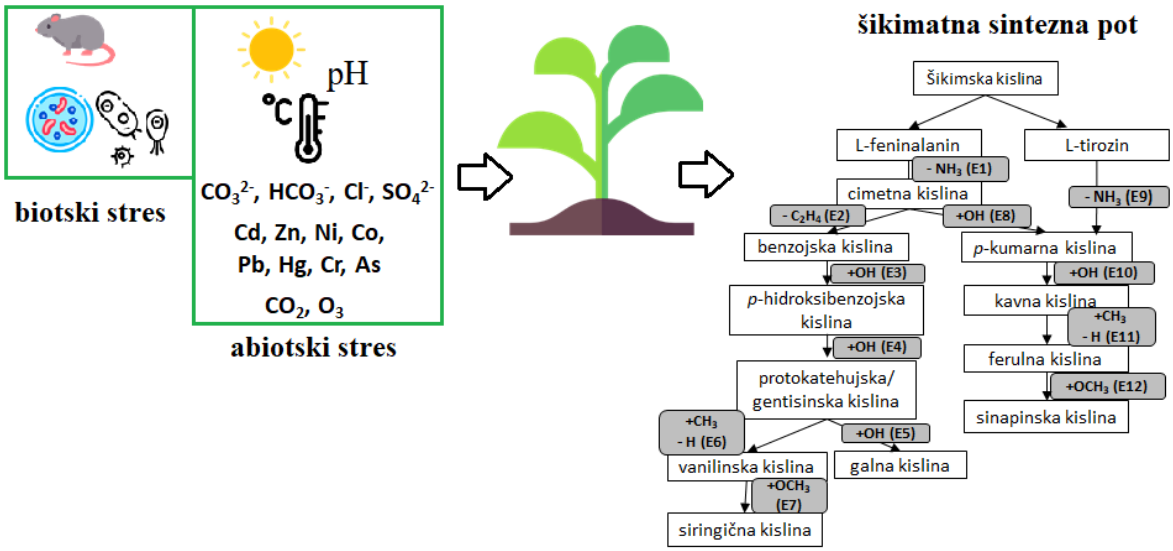
1 UVOD S PREDSTAVITVIJO PROBLEMATIKE, CILJEV IN HIPOTEZ

Reaktivne kisikove zvrsti (ROS) so kemijsko reaktivne zvrsti (superoksidni anion, vodikov peroksid, hidroksilni radikal, singletni kisik), ki imajo sposobnost, da s svojim delovanjem škodujejo vitalnim celičnim komponentam, vključno z lipidi, proteini in DNK, kar lahko povzroča številne bolezni. Naravni antioksidanti (fenolne spojine, vitamini in karotenoidi) so učinkoviti pri preprečevanju bolezni, povezanih z oksidativnim stresom (Van Hung, 2016). Predvideva se, da fenolne spojine z ohranjanjem ravnovesja med oksidanti in antioksidanti igrajo pomembno vlogo v boju proti oksidativnemu stresu v človeškem telesu (Babbar in sod., 2014). Antioksidanti delujejo na različne načine, kot reducenti, lovilci prostih radikalov in/ali kot kelatorji kovin. Na dva različna prosta radikala se lahko isti antioksidant različno odzove (Prior in sod., 2005). Fenolne spojine lahko reagirajo z radikali po dveh glavnih mehanizmih, in sicer preko prenosa vodikovega atoma, kjer fenolna spojina odda vodikov atom radikalu, pri tem se cepi vez med kisikom in vodikom v hidroksilni skupini fenolne spojine, posledično nastane fenolni radikal. Lahko pa fenolne spojine reagirajo z radikali preko prenosa elektrona, kjer fenoksidni anion, ki nastane pri deprotonaciji hidroksilne skupine fenolne spojine, odda elektron (Huang in sod., 2005).

Fenolne spojine so spojine z vsaj enim aromatskim obročem, na katerega je vezana ena ali več hidroksilnih skupin. Običajno se fenolne spojine delijo na fenolne kisline (C6-C1 in C6-C3), flavonoide (C6-C3-C6), kumarine (C6-C3), tanine (hidrolizabilni in kondenzirani), stilbene (C6-C2-C6), lignane $(C6\text{-}C3)_2$ in lignine $(C6\text{-}C3)_n$. Fenolne kisline delimo na hidroksibenzojske kisline (C6-C1): vanilinska, siringična, salicilna, protokatehujska, *p*-hidroksibenzojska kislina; in hidroksicimetne kisline (C6-C3): *p*-kumarna, ferulna, kavna, sinapinska kislina, itd. Večje vsebnosti hidroksicimetnih kislin najdemo v različnih semenih, kjer so hkrati tudi prekurzorji za nastanek molekul, kot so stilbeni, halkoni, lignini, lignani, kumarini in flavonoidi (Alam in sod., 2013). Funkcionalne skupine, kot sta hidroksilna in metoksi skupina, narekujejo molekularno strukturo fenolnih spojin, kar vpliva na njihovo bioaktivnost. V žitih so bile določene predvsem vezane oblike *p*-kumarne, ferulne, protokatehujske, *p*-hidroksibenzojske in vanilinske kisline (Alves in sod., 2016). Za pirina semena so značilne relativno nizke vrednosti ekstraktibilne siringične, galne, kavne (Gawlik-Dziki in sod., 2012), protokatehujske, sinapinske in ferulne kisline (Starzyńska-Janiszewska in sod., 2019). Vendar pa so vezane fenolne spojine v piri zastopane v bistveno večjih količinah, med katerimi izstopajo *p*-kumarna, ferulna, *p*-hidroksibenzojska, kavna (Mencin in sod., 2021a), sinapinska in klorogenska kislina (Gawlik-Dziki in sod., 2012). Poleg fenolnih kislin sodi med fenolne spojine tudi obsežna skupina flavonoidov (C6-C3-C6). Osnovno strukturo flavonoidov sestavljata dva aromatska obroča in med njima heterociklični obroč s kisikovim atomom. Njihova struktura je odvisna od vezave in orientacije različnih funkcionalnih spojin na aromatskem in heterocikličnem obroču. Flavonoidi so lahko prisotni tako v topni prosti kot netopni vezani obliki. Žita so glavni naravni vir vezanih flavonoidov (Shahidi in Yeo, 2016).

Fenolne spojine so sekundarni metaboliti, katerih največja vloga je zaščita rastline pred biotskim in abiotskim stresom (slika 1) (Santos-Sánchez in sod., 2019). Dejavniki, kot so UV sevanje, temperatura, suša, poškodbe lahko vplivajo na sintezo in akumulacijo fenolnih spojin. Poleg abiotskega stresa pa rastlini predstavljajo stres še biotski dejavniki, to so škodljivi organizmi (Liu, 2007). Zaradi funkcij, ki jih imajo fenolne spojine, njihova sinteza in akumulacija poteka v rastlinskih celicah tkiv, ki so bolj izpostavljena biotskemu in abiotskemu stresu (celice povrhnjice). Procesi za nastanek sekundarnih metabolitev se ponavadi začnejo takrat, ko v rastlinskih celicah nastaja premalo primarnih antioksidantov za detoksifikacijo ROS, lahko pa se začnejo tudi zaradi izpolnjevanja fizioloških vlog (privabljanje opraševalcev, očvrstitev celične stene, itn). Rastline se proti abiotskemu stresu borijo z različnimi mehanizmi, delimo jih na encimatske in neencimatske. Encimatski mehanizmi vključujejo antioksidativne encime (superoksid dismutaza, glutation peroksidaza, katalaza, gvajakol peroksidaza, itd.) in encime askorbatno-glutationskega cikla (Caverzan in sod., 2016). Med neencimske antioksidante pa prištevamo askorbinsko kislino, glutation, kot tudi tokoferole, karotenoide in fenolne spojine (Caverzan in sod., 2016).

Biosinteza fenolnih spojin lahko poteka po večih poteh, med katerimi je najpomembnejša šikimatna pot, predstavljena na sliki 1, kjer kot produkt pretvorbe šikimske kisline nastaneta dve aromatski aminokislini fenilalanin in tirozin (Shahidi in Yeo, 2016). Aminokislina fenilalanin predstavlja glavni prekurzor sinteze fenilpropanoidov (fenilpropanoidna pot) in fenolnih spojin, ki nastanejo iz njih. Eden najpomembnejših encimov pri biosintezi fenolnih spojin je fenilalanin amonijak liaza (PAL), ki katalizira pretvorbo fenilalanina v *trans*-cimetno kislino in hkrati predstavlja vezni člen med primarnim in sekundarnim metabolizmom. *trans*-Cimetna kislina se s hidroksilacijo pretvori v *p*-kumarno kislino, reakcijo katalizira encim cinamat-4-hidroksilaza (C4H) (Petersen in sod., 2010). *p*-Kumarna kislina predstavlja prekurzor za hidroksicimetne kisline (kavno, klorogensko, ferulno kislino). Kavna kislina se sintetizira z vezavo hidroksilne skupine na mesto 3, nadalje se ferulna kislina sintetizira z metilacijo kavne kisline. Fenolne kisline nastanejo predvsem z reakcijami deaminiranja, hidroksiliranja in metiliranja (Heleno in sod., 2015). Hidroksicimetne kisline so metabolno bolj ali manj nereaktivne, zato je za nastanek njihovih derivatov potrebno aktivirati karboksilne skupine z encimom *p*-kumarat-CoA ligaza (C4L). *p*-Kumarna, ferulna in kavna kislina se smatrajo za dobre substrate omenjenemu encimu (Petersen in sod., 2010). Tvorba benzojske kisline, ki je prekurzor hidroksibenzojskih kislin, je lahko posledica razgradnje – skrajšanja stranske verige cimetne kisline za dva ogljikova atoma (slika 1). Tako kot pri cimetni in *p*-kumarni kislini, lahko iste reakcije hidroksiliranja in metiliranja potekajo na aromatskem obroču benzojske kisline, pri čemer nastajajo ustrezni derivati (protokatehujska kislina, *p*-hidroksibenzojska kislina, itd.) (Heleno in sod., 2015). Biosinteza fenolnih spojin je kompleksen proces, ki poteka predvsem na citoplazemski površini endoplazmatskega retikuluma, iz katerega se nastale fenolne kisline transportirajo do vakuole ali do matrice celične stene s pomočjo citoplazemskih veziklov ali transmembranskih prenašalnih proteinov (Shahidi in Yeo, 2016).



Slika 1: Enostaven shematski prikaz biosintezne poti fenolnih kislin, ki izhaja iz šikimske kisline. Encimi: (E1) fenilalanin amonijak liaza, (E2) oksidaza, (E3) benzojska kislina-4-hidroksilaza, (E4) *p*-hidroksibenzojska kislina-3-hidroksilaza, (E5) protokatehujska kislina-5-hidroksilaza, (E6) protokatehujska kislina-3-*O*-metiltransferaza, (E7) vanilinska kislina-5-hidroksilaza in vanilinska kislina-5-*O*-metiltransferaza, (E8) cimetna kislina-4-hidroksilaza, (E9) tirozin amonijak liaza, (E10) *p*-kumarna kislina-3-hidroksilaza, (E11) kavna kislina-3-*O*-metiltransferaza, (E12) ferulna kislina-5-hidroksilaza in kavna kislina/5-hidroksiferulna kislina-*O*-metiltransferaza (povzeto po Heleno in sod., 2015).

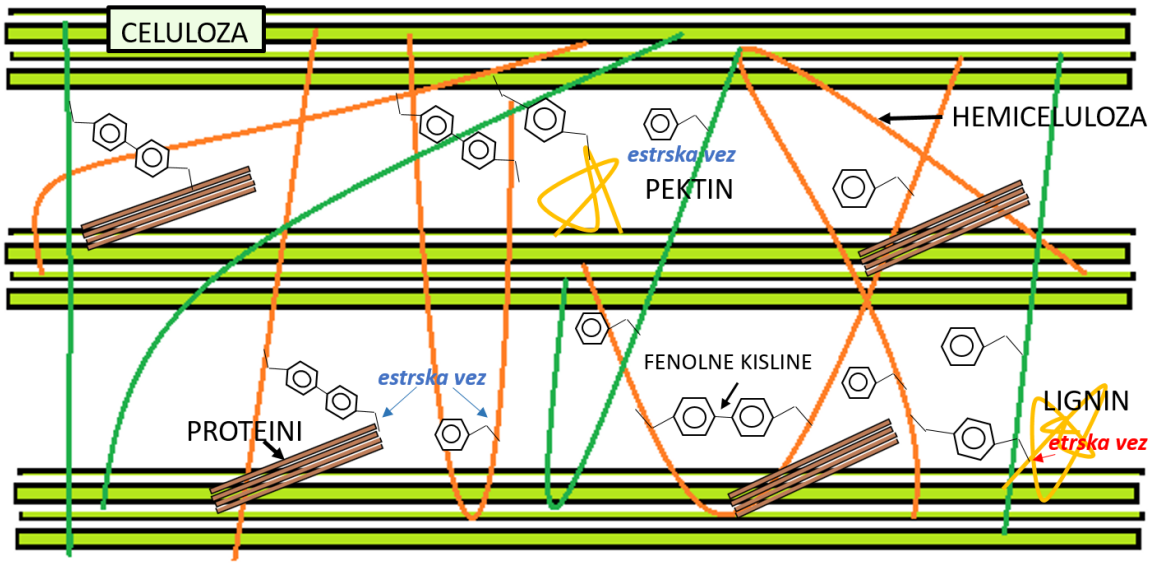
Na kemijske in biološke lastnosti fenolnih spojin vpliva tako število kot tudi vrsta vezanih skupin. S povečanjem števila hidroksilnih skupin in sladkornih enot narašča topnost fenolnih spojin v vodi, medtem ko metilacija hidroksilnih skupin povzroča padec topnosti. Proste hidroksilne skupine so bolj reaktivne kot glikozilirane in metilirane hidroksilne skupine, kar pomembno vpliva na njihovo antioksidativno aktivnost (Halbwirth, 2010).

Piro (*Triticum spelta* L.) taksonomsko uvrščamo v družino trav (*Poaceae*), rod pšenice (*Triticum*) in predstavlja najstarejšo obliko heksaploidne pšenice. Pira izvira iz Bližnjega vzhoda, v Evropi pa so jo gojili predvsem v višje ležečih predelih, saj velja za nezahtevno in prilagodljivo poljščino. Pridelava pire se je v začetku 20. stoletja močno zmanjšala, v zadnjih časih pa postaja pomembna alternativa navadni pšenici in ostalim žitom, zaradi večjega povpraševanja po ekološko pridelani hrani in pozitivnih učinkov na zdravje ljudi (Bojňanská in Frančáková, 2011). Prednosti pire so nizki stroški pridelave, primerna je za gojenje brez pesticidov, saj ima dobro odpornost proti boleznim in škodljivcem, raste tudi v težkih pridelovalnih razmerah, vendar pa je hektarski pridelek nižji kot pri pšenici. Pira v primerjavi s pšenico vsebuje več beljakovin (Pruska-Kedzior in sod., 2008), prostih sladkorjev (Zörb in sod., 2007), maščobnih kislin in lipidov (Ruibal-Mendieta in sod., 2005). Pri vsebnosti bioaktivnih spojin nekateri avtorji navajajo, da večjih razlik med piro in pšenico ni mogoče opaziti (Bonafaccia in sod., 2000), medtem ko drugi avtorji pripisujejo pirini moki večjo vsebnost antioksidantov kot navadni pšenični moki (Wang in sod., 2020). Bioaktivne spojine se v žitnih zrnih večinoma nahajajo v zunanjih plasteh zrna, zato med

bolj zdrave živilske izdelke sodijo polnozrnati izdelki. V žitnih semenih so najpogostejše bioaktivne spojine: prehranske vlakinine (β-glukani, arabinoksilani, rezistentni škrob), fenolne spojine (fenolne kisline, flavonoidi), antociani, karotenoidi, izoflavoni, steroli, fitati, itd. (Gani in sod., 2012).

Fenolne spojine žitnih semen glede na način ekstrakcije delimo na ekstraktibilne, ki jih lahko ekstrahiramo z mešanico vodnega in organskega topila, in vezane, ki med ekstrakcijo ostanejo v celičnih stenah, njihova sprostitev iz matrice pa je mogoča s kislinsko ali alkalno hidrolizo. Tako kislinska kot alkalna hidroliza nista specifični in lahko privedeta do strukturnih sprememb fenolnih spojin, zato težko sklepamo, kakšna je bila izhodna struktura spojin v vzorcu. Bolj specifična in učinkovita metoda je encimska hidroliza, kjer z uporabo različnih encimov (hidrolaz) cepimo glikozidne vezi in razgradimo celično steno (Carboni Martins in sod., 2022). Obe obliki fenolnih spojin (ekstraktibilno in vezano) je potrebno upoštevati, če želimo dobiti čim bolj reprezentativne in pravilne rezultate (Madhujith in Shahidi, 2009).

Fenolne spojine se v rastlinski celici nahajajo v topni obliki, kamor spadajo proste in konjugirane fenolne spojine, ter v netopni vezani obliki. Proste fenolne spojine so v nevezani obliki, konjugirane pa so vezane na topne molekule z majhno molekulsko maso (sladkorji, maščobne kisline), običajno pride med njimi do tvorbe kovalentnih vezi, lahko pa tudi do nekovalentnih vezi, vodikovih vezi ali hidrofobnih interakcij med različnimi molekulami. Večina topnih fenolnih spojin se nahaja v vakuolah rastlinskih celic (Shahidi in Yeo, 2016; Xu in sod., 2020). Polisaharidi, ki spadajo med topne prehranske vlaknine, predstavljajo sterično oviro, ki lahko zaščiti fenolne spojine pred oksidacijo. Topni polisaharidi, naj bi po mnenju nekaterih raziskovalcev imeli sposobnost, da iz svojega aktiviranega reducirajočega konca donirajo vodik oksidiranim fenolnim spojinam in tako sinergistično izboljšajo antioksidativno aktivnost slednjih (López-Perea in sod., 2019; Xu in sod., 2020). Proste in konjugirane fenolne spojine tako uvrščamo med ekstraktibilne fenolne spojine, netopne vezane fenolne spojine pa med neekstraktibilne (Xu in sod., 2020). Količinsko najbolj zastopana oblika pa so netopne vezane fenolne spojine (slika 2). Karboksilna skupina fenolnih kislin (benzojske in cimetne kisline) lahko tvori estrske vezi s hidroksilno skupino komponent celične stene (strukturni ogljikovi hidrati, proteini). Med hidroksilnimi skupinami fenolnih spojin in komponentami celične stene (lignin) se lahko tvorijo etrske vezi (Acosta-Estrada in sod., 2014). Nadalje se lahko med ogljikovim atomom fenolnih spojin in ogljikovim atomom komponent celičnih sten tvorijo tudi C-C vezi (C-glikozidi) ali pa fenolne spojine sodelujejo pri tvorbi vodikovih in elektrostatskih vezi (Shahidi in Yeo, 2016). Vzpostavljene kovalentne vezi med fenolnimi spojinami in komponentami celične stene imajo pomembno vlogo pri krepitvi rigidne strukture matrice celične stene. Prav tako vezane fenolne spojine ščitijo pred vdorom številnih patogenih mikroorganizmov, s trpkostjo odvračajo insekte in ostale živali ter ščitijo pred UV-poškodbami (Shahidi in Yeo, 2016).



Slika 2: Poenostavljena predstavitev zamreženja med strukturnimi komponentami rastlinske celične stene in fenolnimi spojinami. Prikaz kovalentnih vezi: etrske vezi med fenolnimi spojinami in ligninom; estrske vezi med fenolnimi spojinami in strukturnimi ogljikovimi hidrati, proteini (povzeto po Acosta-Estrada in sod., 2014).

Različne raziskave so v žitih potrdile prevlado vezane oblike fenolnih spojin nad ekstraktibilno obliko. Vezane fenolne spojine predstavljajo glede na skupno količino fenolnih spojin: 92 % v piri (Mencin in sod., 2022a), 69 % v koruzi (Van Hung, 2016), 75 % v pšenici in ovsu, 74 % v rižu (Adom in Liu, 2002), v ječmenu pa med 73 % in 82 %, odvisno od sorte (Madhujith in Shahidi, 2009). Vezane fenolne spojine se nahajajo v otrobih žitnih semen, zato je pomembno poudariti, da uživanje žitnih izdelkov pripomore k večjemu vnosu fenolnih spojin le pri uživanju polnozrnatih izdelkov. Nadalje so Wang in sod. (2020) ugotovili, da polnozrnata pšenična moka vsebuje 2-5-krat večjo vsebnost antioksidantov kot bela pšenična moka.

Ferulna kislina je najbolj pogosta hidroksicimetna kislina v rastlinskem svetu, hkrati je tudi prevladujoča fenolna kislina v žitnih zrnih (Boz, 2016; Coghe in sod., 2004; Gani in sod., 2012). Ferulna kislina obstaja v prosti, konjugirani in vezani obliki (Fazary in Ju, 2007). Na splošno je ferulna kislina nakopičena v zunanjem sloju žitnih semen (otrobi), najmanjšo vsebnost ferulne kisline pa so določili v endospermu (Ndolo in sod., 2013). Znano je, da se delež proste ferulne kisline v žitnih zrnih giblje od 0,2 do 0,5 %, večina pa je vezane na komponentne celične stene. Pšenični otrobi vsebujejo približno 0,8–2 g ferulne kisline/kg suhe snovi (SS), kar predstavlja 90 % vseh fenolnih spojin v pšenici (Oghbaei in Prakash, 2016). Podoben trend smo opazili tudi pri piri, kjer je ferulna kislina predstavljala 92 % vseh identificiranih fenolnih kislin (Mencin in sod., 2022a). V naši raziskavi (Mencin in sod., 2022a) smo poročali, da neobdelana pirina semena vsebujejo 0,74 g skupne ferulne kisline/kg SS, od tega 99 % v vezani obliki. Do podobnih rezultatov so prišli tudi Gawlik-Dziki in sod. (2012), saj so ugotovili, da pirina semena vsebujejo 0,2–0,8 g skupne ferulne

kisline/kg SS, od tega 84–99 % vezane oblike. Ferulna kislina se lahko z etrskimi vezmi veže z ligninom in z estrskimi vezmi s polisaharidi in strukturnimi proteini in tako deluje kot povezovalec med polimeri celične stene (de Oliveira Silva in sod., 2015). Nadalje je možen nastanek dimera ferulnih kislin dveh sosednjih arabinoksilanov, ki prispeva k zamreženosti in tako ovira delovanje hidrolaz, kar posledično zmanjša učinkovitost encimske razgradnje in prebavljivosti polisaharidov celične stene. Zamreženost komponent celične stene s ferulno kislino in njenimi dimeri vpliva na lastnosti celične stene, kot so adherenca, raztegljivost, dostopnost in biološka razgradljivost (de Oliveira Silva in sod., 2015).

Struktura matrice otrobov in način, kako so fenolne spojine vključene v matrico, močno vplivata na njihovo biološko dostopnost. Zato so se razvile različne tehnologije, s katerimi olajšamo cepitev vezi, povečamo količino sproščenih vezanih fenolnih spojin in posledično izboljšamo njihovo biološko dostopnost (Wang in sod., 2014). Biotehnološki procesi, kamor spadajo kaljenje, fermentacija in encimska obdelava, trenutno predstavljajo eno izmed najbolj aktualnih tem s področja izboljšanja dostopnosti in razpoložljivosti fenolnih spojin.

Kaljenje predstavlja ekonomično in učinkovito naravno metodo, ki zmehča strukturo in izboljša prehransko vrednost semena - poveča se vsebnost bioaktivnih spojin, hkrati pa se zmanjša vsebnost antinutritivnih komponent (Singh in Sharma, 2017). Kaljenje poveča aktivnost hidrolitičnih encimov (amilaz, proteaz, itd.), spodbudi nastanek novih in aktivacijo tistih, ki so v nekaljenem semenu v neaktivnem stanju. V začetni fazi kaljenja pride do razgradnje makrohranil (ogljikovih hidratov, proteinov, lipidov), kar poveča količino njihovih presnovnih produktov (enostavnih sladkorjev, prostih aminokislin, organskih kislin), istočasno pa se sproži sinteza sekundarnih metabolitov. Z razgradnjo makrohranil se posledično sproščajo tudi vezane fenolne spojine, kar v začetni fazi kaljenja pomeni upad vezane frakcije in porast proste (Terpinc, 2019). Na omenjene spremembe lahko dodatno vpliva abiotski stres med kaljenjem. Rastline se na stresne pogoje odzivajo tako, da inducirajo sintezo različnih zaščitnih spojin, tudi številnih fenolnih spojin, ki omogočajo preživetje in nadaljnjo rast v takšnih pogojih (AL-Quraan in sod., 2013; Chen in sod., 2019; Falcinelli in sod., 2017; Ma in sod., 2019; Randhir in sod., 2004). Različne raziskave so pokazale, da je količina fenolnih spojin in razmerje med ekstraktibilnimi in vezanimi fenolnimi spojinami odvisno od obdelave semen pred kaljenjem - pogojev namakanja (Xu in sod., 2009; Yang, 2001) in od pogojev, v katerih seme kali: temperature (Chavarín-Martínez in sod., 2019; Mencin in sod., 2021a; Paucar-Menacho in sod., 2017), časa kaljenja (Kim in sod., 2018; Paucar-Menacho in sod., 2017; Terpinc in sod., 2016), načina osvetljevanja (Xiang in sod., 2017), relativne vlage (Yang, 2001).

Drugi segment povečanja biološke dostopnosti in razpoložljivosti bioaktivnih spojin predstavljajo različni tipi fermentacije (alkoholna, mlečnokislinska, spontana, itd.) (Angelino in sod., 2017). Prednost tega procesa je, da poleg encimov iz žit sodelujejo tudi encimi mikroorganizmov, ki pripomorejo k razgradnji celične stene. Prav tako pa

fermentacija spodbuja sintezo novih spojin kot tudi encimsko transformacijo že prisotnih bioaktivnih spojin. Dosedanje študije o vplivu fermentacije na vezane fenolne spojine v različnih žitih so potrdile povečanje njihove biološke dostopnosti in razpoložljivosti (Wang in sod., 2014). Moore in sod. (2006) so potrdili, da obdelava s kvasovkami (*Saccharomyces cerevisiae)* znatno poveča skupno vsebnost prostih fenolnih spojin in antioksidativno aktivnost pšeničnih otrobov. Avtorji študije so izpostavili različno zmožnost kvasovk za presnavljanje posameznih fenolnih spojin. Do podobnih ugotovitev so pri fermentaciji ječmena in ovsa s probiotičnimi bakterijami prišli tudi Hole in sod. (2012), kjer se je znatno povečala vsebnost prostih fenolnih kislin. Spaggiari in sod. (2020) so v fermentiranih pšeničnih otrobih ugotovili relativno veliko vsebnost kavne kisline, kar nakazuje na metabolno aktivnost mikroorganizmov. Že Žilić in sod. (2015) so v svoji študiji na pšenici izpostavili, da lahko bakterije *Lactobacillus* spp. proizvajajo kavno kislino iz klorogenske kisline. Vpliv fermentacije na fenolne spojine je v glavnem odvisen od vrste semen (Đorđević in sod., 2010), vrste mikroorganizmov (Đorđević in sod., 2010; Katina in sod., 2007b; Mencin in sod., 2022a) in pogojev fermentacije (temperatura, pH, čas) (Boskov Hansen in sod., 2002; Katina in sod., 2007b). Fermentacija z mlečnokislinskimi bakterijami (MKB) povzroči znižanje pH vrednosti, nižja pH vrednost pa spodbudi aktivnost hidrolaz in tako prispeva k razpadu arabinoksilanov in k hidrolizi estrov in glikozidov fenolnih spojin (Gänzle, 2014).

Naslednja učinkovita metoda za sproščanje vezanih fenolnih spojin iz komponent celične stene je encimska obdelava. Pri encimski obdelavi semena in otrobe obdelamo neposredno s hidrolitičnimi encimi, s čimer izboljšamo biološko dostopnost in razpoložljivost fenolnih spojin. Encimi, ki hidrolizirajo celično steno (celulaze, ksilanaze, esteraze itd.), so bili že večkrat uporabljeni za razgradnjo matrice žitnih otrobov (Acosta-Estrada in sod., 2014; Bei in sod., 2018; Mencin in sod., 2022b; Moore in sod., 2006; Sørensen in sod., 2003). Feruloil esteraze spadajo v skupino esteraz, ki hidrolizirajo estrsko vez med hidroksicimetnimi kislinami (npr. ferulno kislino) in hemicelulozo, prisotno v celični steni rastlin. Prav tako je feruloil esteraza sposobna katalizirati cepitev kovalentne vezi med dvema ferulnima kislinama, ki sta pritrjeni na sosednja arabinoksilana. Encim postane aktiven tudi med kaljenjem, njegova aktivnost pa med kaljenjem počasi pada. Prisotnost encima so našli tudi pri laktobacilih, prisotnih v našem črevesju (Faulds in sod., 2004; Gänzle, 2014). Znanstveni viri (Mathew in Abraham, 2004; Moore in sod., 2006; Sancho in sod., 2001; Sørensen in sod., 2003) opisujejo sinergistične interakcije med ksilanazo, ki katalizira razgradnjo polisaharidov v fragmente z nižjo molekulsko maso, in med feruloil esterazo, ki sprošča fenolne spojine, pri čemer slednja olajša dostopnost ksilanaze do komponent celične stene. V povezavi z razgradnjo celične stene se omenjajo tudi α-amilaze in proteaze. Proteini v pirinih otrobih predstavljajo okoli 15–17 %, zato ne preseneča, da je bila proteaza del teh raziskav (Singh in sod., 2016). Ker lahko med karboksilnimi in hidroksilnimi skupinami fenolnih kislin in škrobom ali drugimi polisaharidi pride do tvorbe vodikovih vezi, kovalentnih vezi ali kelacije, lahko hidroliza škroba in polisaharidov z uporabo α-amilaze

sprosti pomemben del fenolnih spojin. Uporaba omenjenega encima je smotrna, ko so v vzorcu prisotni otrobi žitnih semen, ki vsebujejo veliko fenolnih spojin. Endosperm, ki je sestavljen predvsem iz škrobnih zrnc, vsebuje relativno malo fenolnih spojin, zato razgradnja škroba z α-amilazami sprosti malo fenolnih spojin (Yu in sod., 2001). Za encimsko obdelavo velja, da je okolju prijazna, energetsko učinkovita in sprošča le specifične spojine, ne da bi poškodovala ostale spojine (Ferri in sod., 2020).

Narejenih je bilo tudi nekaj študij na področju kombiniranja fermentacije in kaljenja ter fermentacije in encimske obdelave različnih žit. Kaljenje prispeva k večji količini fermentacijskih virov (sladkor/dušik), hkrati pa kaljenje, fermentacija, dodatek ustreznih encimov pripomorejo k večji koncentraciji in aktivnosti hidrolitičnih encimov. Literaturni viri kažejo na sinergističen učinek pri sočasni uporabi različnih biotehnoloških procesov. Katina in sod. (2007b) so poročali, da je fermentacija kaljenih rženih semen z uporabo kvasovke *S. cerevisiae* znatno povečala vsebnost skupnih fenolnih spojin v primerjavi z zgolj kaljenimi semeni. Raziskava, ki so jo naredili Montemurro in sod. (2019), je pokazala, da fermentacija kaljenih zrn pšenice, ječmena in kvinoje s kislim testom ne vpliva zgolj na povečanje fenolnih spojin, ampak tudi na povečanje vsebnosti peptidov, prostih aminokislin, γ-aminomaslene kisline ter zmanjša koncentracijo fitinske kisline, kondenziranih taninov, rafinoze in inhibitorjev tripsina. V drugi raziskavi so Anson in sod. (2009) raziskovali vpliv kombiniranja kvasne fermentacije in obdelave s hidrolitičnimi encimi na biološko dostopnost fenolnih spojin iz pšeničnih otrobov. Ugotovili so, da je bila vsebnost prostih fenolnih kislin v pšeničnih otrobih večja za 1,6-krat, kot če so jih podvrgli zgolj fermentaciji. Razlago za tako izboljšavo so pripisali hidrolitičnim encimom, ki omogočajo hidrolizo različnih polimerov, kar privede do povečane razgradnje celične stene. Zaradi omenjenega razloga so obdelani pšenični otrobi pokazali znatno izboljšanje biološke dostopnosti in/ali razpoložljivosti fenolnih spojin, določenih z *in vitro* testi (Anson in sod., 2009) in s kliničnimi študijami (Anson in sod., 2011).

Uživanje bioaktivnih komponent je ključno predvsem zaradi njihovih pozitivnih učinkov na naše zdravje. Preden lahko posamezni spojini pripišemo pozitivno delovanje, je potrebno poznati njeno biološko dostopnost in razpoložljivost v človeškem organizmu. V kolikšnem obsegu bodo spojine učinkovite v našem telesu, je odvisno od vrste in količine zaužite snovi, njihove zmožnosti sprostitve iz hrane, sprememb, ki se zgodijo med prebavo, metabolizma in prenosa snovi po krvnem obtoku. Biološka dostopnost (ang. *bioaccessibility*) je definirana kot količina snovi, ki se lahko sprosti iz matrice hrane v prebavni trakt in je na voljo za absorpcijo v tankem črevesu. Biološka razpoložljivost (ang. *bioavailability*) pa je definirana kot količina snovi, ki se je prebavila in je sposobna prehajati skozi črevesni epitelij in sčasoma doseči tarčno tkivo, kjer izvaja svoje biološke aktivnosti. Proces biološke razpoložljivosti je sestavljen iz biološke dostopnosti in aktivnosti. Biološka aktivnost je proces, ko bioaktivne spojine dosežejo krvno-žilni obtok, preko katerega se transportirajo do tarčnih tkiv, kjer potekajo interakcije z ostalimi molekulami (Cilla in sod., 2018).

Razgradnja hrane, ki je ključna za biološko dostopnost in razpoložljivost, se prične v ustih, nadaljuje pa se v želodcu in v črevesu. Kako učinkovit bo proces prebave, je odvisno od različnih dejavnikov: sestave in količine matrice, sinergizma oz. antagonizma snovi v njem, od fizikalno-kemijskih parametrov, kot so pH, temperatura in tekstura matrice. Biološka dostopnost neke spojine se lahko močno razlikuje od njene biološke razpoložljivosti, saj prevladujoča bioaktivna komponenta v zaužitem živilu ni nujno tudi najbolj zastopan metabolit v tarčnem tkivu (Liu, 2007). Poznavanje bioloških učinkov bioaktivnih spojin v človeškem organizmu je še vedno precej okrnjeno (Heleno in sod., 2015). Približno 5–10 % celotnega prehranskega vnosa fenolnih spojin se lahko absorbira v tankem črevesu (Vitaglione in sod., 2008). Sproščene topne fenolne spojine so v tankem črevesu podrvržene konjugaciji, ki povzroči spremembe v njihovi strukturi. Reakcije glukuronidacije, metilacije ali sulfatacije privedejo do nastanka različnih derivatov fenolnih spojin. Strukturne spremembe, ki nastanejo, lahko izboljšajo ali zmanjšajo biološko aktivnost zaužitih fenolnih spojin (Heleno in sod., 2015). Vezane fenolne spojine niso dostopne encimom gastrointestinalnega (GI) trakta. Več kot 90 % fenolnih spojin v žitnih semenih ima tako nizko biološko dostopnost (Vitaglione in sod., 2008). Neabsorbirane fenolne spojine se transportirajo v debelo črevo, kjer njihovo razgradnjo deloma katalizirajo encimi črevesne mikrobiote, zlasti ksilanaze in esteraze. Številne raziskave, ki se ukvarjajo z biološko učinkovitostjo fenolnih spojin, spregledajo vprašanje njihove dostopne koncentracije in presnovne oblike, v kateri fenolne spojine vstopajo v krvni obtok.

Za določanje biološke dostopnosti se najpogosteje uporabljajo *in vitro* prebavni modeli, s katerimi posnemamo prebavo v ustih, želodcu in tankem črevesu, včasih pa tudi prebavo v debelem črevesu (fermentacijo) (Rocchetti in sod., 2018). *In vitro* statični prebavni modeli so razmeroma poceni in preprosti za uporabo, so hitri in nimajo etičnih omejitev, pogoje prebave je mogoče nadzorovati, vzorčenje je preprosto in rezultati so ponovljivi. Bili so že uporabljeni za posnemanje fiziološkega sproščanja fenolnih spojin iz nekaterih žit in žitnih izdelkov (Gawlik-Dziki in sod., 2009; Mencin in sod., 2022c; Saura-Calixto in sod., 2007). Te raziskave so pokazale, da se fenolne spojine lahko sprostijo iz matrice hrane med simulirano *in vitro* GI prebavo, zaradi česar postanejo biološko dostopne. V ustnem delu prebave se doda encim α-amilazo pri pH 7, prebava v ustih ponavadi traja okoli 2 min, sledi želodčna faza prebave, kjer se doda encim pepsin, hrana je izpostavljena pH 2 za 2 h, na koncu sledi še simulacija pogojev tankega črevesa, kjer se pH dvigne nazaj na 7, doda se pankreatin in žolčne soli (Brodkorb in sod., 2019; Minekus in sod., 2014). Na ta način se določi biološka dostopnost določene snovi, ki se lahko absorbira, vendar pa te raziskave ne prikažejo, koliko snovi dejansko vstopi v naš krvni obtok. Biološko razpoložljivost se nadalje lahko določi z *in vivo* poskusi na živalih ali ljudeh, kjer pa je potrebno pridobiti dovoljenje etične komisije. Biološka razpoložljivost je najbolj odvisna od same strukture bioaktivne snovi, saj se fenolne spojine najpogosteje nahajajo v glikozilirani obliki kot estri oz. polimeri, ki se morajo najprej hidrolizirati na mono- in dimere, da se lahko absorbirajo skozi celice tankega črevesa. Med prebavo pa lahko fenolne spojine pridejo v stik tudi z

drugimi snovmi, kot so ogljikovi hidrati, proteini, lipidi in vlaknine. Fenolne spojine se lahko povežejo tudi s prehranskimi vlakninami, kar pa preprečuje stik encimov s substratom in posledično fenolne spojine prehajajo v vezani, nerazgrajeni obliki do debelega črevesa. Nadalje so črevesni mikroorganizmi sposobni pretvoriti nekatere fenolne spojine v aktivnejše spojine, ki se lažje absorbirajo (Călinoiu in Vodnar, 2018; Gong in sod., 2018). Metabolizem fenolnih spojin s strani MKB je povezan z delovanjem dekarboksilaz, ki omogočajo dekarboksiliranje hidroksibenzojskih ali hidroksicimetnih kislin in nastanek ustreznih 4-vinil derivatov, in z delovanjem reduktaz, ki hidrogenirajo dvojne vezi. Različni sevi lahko omogočijo nastanek različnih metabolitev iz istega substrata (Călinoiu in Vodnar, 2018). Potrebno je poudariti, da absorpcija fenolnih spojin še vedno ni povsem pojasnjena, zato je potreba po raziskavah na tem področju velika.

Fenolne spojine zaradi svoje strukture izražajo antioksidativno aktivnost. Prostim radikalom oddajo elektron ali vodikov atom in jih na ta način stabilizirajo (Terpinc in Abramovič, 2010). Novejše raziskave ugotavljajo, da imajo fenolne spojine pozitiven vpliv na sestavo črevesne mikrobiote, kar ima velik pomen za pravilno delovanje prebave in imunskega sistema, hkrati pa zavirajo rast patogenih mikroorganizmov (Espín in sod., 2017). Različne študije poročajo, da imajo hidroksicimetne kisline antioksidativne in protivnetne lastnosti ter sposobnost zaviranja razvoja različnih kroničnih bolezni (Alam in sod., 2013). Kot že omenjeno, je ferulna kislina po kvantiteti gotovo najpomembnejši antioksidant iz pire, številne raziskave pa so podkrepile tudi njeno učinkovitost. Antioksidativna aktivnost ferulne kisline je močno povezana s hidroksilno skupino, ki je vezana nanjo, saj je ta, preko mehanizma lovljenja radikalov, sposobna ustaviti verižno reakcijo oksidacije (de Oliveira Silva in Batista, 2017; Nenadis in Tsimidou, 2002; Shahidi in Chandrasekara, 2010). Drug zanimiv antioksidativni mehanizem ferulne kisline je, da deluje tudi kot UV absorber (de Oliveira Silva in Batista, 2017). Ferulna kislina ima veliko sposobnost lovljenja prostih radikalov, ki sicer povzročijo oksidativne poškodbe celične membrane in DNK. Pripisujejo ji tudi zmanjšanje tveganja za nastanek kroničnih bolezni, kot so diabetes, srčno-žilne bolezni, rak (Boz, 2016), hkrati naj bi ferulna kislina delovala protimikrobno in protivnetno (de Oliveira Silva in Batista, 2017). Prav tako pa na naše zdravje pozitivno vplivajo tudi presnovni produkti fenolnih spojin. Terpinc in sod. (2011) so v svoji raziskavi ugotovili, da se je presnovni produkt ferulne kisline, 4-vinil gvajakol, izkazal kot uspešnejši antioksidant pri lovljenju alkilperoksilnega radikala v emulziji od ferulne kisline. Glede na različne študije, kjer so z *in vitro* testi ugotavljali antioksidativno aktivnost, so ugotovili, da vezane fenolne spojine izkazujejo znatno večjo antioksidativno aktivnost v primerjavi s prosto in konjugirano obliko (Acosta-Estrada in sod., 2014; Chen in sod., 2017; Pang in sod., 2018). V *in vivo* raziskavi, ki so jo naredili Costabile in sod. (2008) na 32 zdravih prostovoljcih, starih med 20 in 42 let, so ugotovili, da uživanje polnozrnate pšenice prispeva k povečani koncentraciji ferulne kisline v krvi, hkrati pa so ugotovili, da so pripravki za zajtrk iz polnozrnate pšenice pozitivno vplivali na črevesno mikrobioto, saj se je povečala populacija bifidobakterij in MKB.

1.1 NAMEN DELA

V okviru raziskave smo želeli ugotoviti vpliv različnih biotehnoloških procesov na sproščanje vezanih fenolnih spojin iz pirinih semen in posledično na njihovo vsebnost, dostopnost in *in vitro* ter *in vivo* antioksidativno aktivnost. Glavni poudarek je bil na povečanju vsebnosti zdravju koristnih bioaktivnih spojin, predvsem hidroksicimetnih in hidroksibenzojskih kislin, ki so v žitnih semenih najbolj zastopane. Želeli smo preveriti možnost uporabe posameznih biotehnoloških procesov ali kombinacije le-teh z namenom izboljšanja biološke dostopnosti fenolnih spojin v pirinih semenih. Tako obdelana semena bi nadalje lahko dodajali v živila, kot so kruh in drugi pekovski izdelki, saj bi z zauživanjem teh izdelkov povečali vnos antioksidantov in posledično pozitivno vplivali na zdravje ljudi.

Kar nekaj raziskav je bilo že narejenih na področju vpliva posameznih biotehnoloških procesov na sproščanje vezanih fenolnih spojin iz drugih žitaric, medtem ko so podatki za piro skopi. Kombiniranje različnih biotehnoloških procesov med seboj in njihov vpliv, ne samo na *in vitro* antioksidativno aktivnost, ampak tudi na antioksidativno aktivnost v živi kvasni celici, ki nam da vpogled v *in vivo* okolje, do sedaj še ni bilo podrobno raziskano in je v literaturi samo omenjeno.

Z optimiziranjem biotehnoloških procesov lahko povečamo sproščanje vezanih fenolnih spojin iz celične stene, zato smo si zadali naslednje cilje:

- preučiti vpliv abiotskega stresa (temperatura, voda, svetloba, mehanska poškodba, raztopina NaCl in raztopina sorbitola) med kaljenjem pirinih semen na razmerje med vsebnostjo ekstraktibilnih in vezanih fenolnih spojin in na njihovo antioksidativno aktivnost;
- preveriti vpliv posameznih mikroorganizmov (kvasovke *S. cerevisiae*, MKB *L. plantarum*, avtohtona mikrobiota) na učinek fermentacije pirinih semen za sprostitev fenolnih spojin in njihovo antioksidativno aktivnost;
- raziskati, kakšen vpliv (sinergističen, antagonističen) ima kombiniranje postopkov kaljenja in fermentacije oz. kaljenja in encimske obdelave pirinih semen na sprostitev fenolnih spojin in njihovo antioksidativno aktivnost;
- na modelnem organizmu kvasovke *S. cerevisiae* preučiti vpliv ekstraktov iz pirinih semen na celični privzem posameznih fenolnih spojin;
- z *in vitro* oralno, gastro in intestinalno prebavo ugotoviti dostopnost fenolnih spojin.

Raziskava predstavlja velik doprinos tako za znanost kot tudi za tehnologijo in proizvodnjo izdelave funkcionalnih živil, ki bi vsebovala biološko obdelana semena pire z izboljšano biološko dostopnostjo fenolnih spojin.

<u>Poskus 1: Vpliv različnih pogojev abiotskega stresa med kaljenjem pirinih semen na vsebnost ekstraktibilnih in vezanih skupnih in individualnih fenolnih spojin ter na njihovo antioksidativno aktivnost</u>

Dosedanja literatura poroča, da kaljenje povzroča povečano aktivnost hidrolitičnih encimov, posledično pride do razgradnje makrohranil, istočasno pa se sproži sinteza sekundarnih metabolitov. Na obseg opisanih sprememb lahko vplivamo z aplikacijo abiotskega stresa, saj se rastline na stresne pogoje odzivajo tako, da inducirajo sintezo različnih zaščitnih spojin, tudi številnih fenolnih spojin. Za kaljenje pod različnimi stresnimi pogoji smo izbrali oluščena pirina semena sorte Ostro. Cilj raziskave je bil preučiti, kateri pogoji abiotskega stresa najbolje vplivajo na povečanje vsebnosti fenolnih spojin in njihove antioksidativne aktivnosti med kaljenjem, zato smo za kontrolo namesto nekaljenih semen izbrali semena, kaljena pri optimalnih pogojih. S predposkusi smo ugotovljali pogoje za čim večji prirast fenolnih spojin. Po koncu kaljenja smo pirinim semenom določili ekstraktibilne in vezane skupne fenolne spojine (TPC), fenolni profil s HPLC-MS/MS metodo ter *in vitro* in *in vivo* antioksidativno aktivnost. Pri tem poskusu smo dali poudarek na interakcijo pirinih antioksidantov z različnimi prostimi radikali ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $ROO^{\bullet}$), kot tudi njihovi reaktivnosti v homogenem in heterogenem mediju, zato smo uporabili štiri različne *in vitro* antioksidativne teste. Rezultate smo obdelali z multivariatno analizo.

Poskus 2: Vpliv različnih tipov fermentacije na sprostitev fenolnih spojin in njihovo antioksidativno aktivnost v neobdelanih, kaljenih in encimsko obdelanih pirinih semenih

Dosedanje študije o vplivu fermentacije na vezane fenolne spojine v različnih žitih so potrdile povečanje biološke dostopnosti fenolnih spojin, saj so med postopkom fermentacije vključeni tako encimi iz žitnih semen kot tudi mikroorganizmov. Vpliv fermentacije na fenolne spojine je v glavnem odvisen od vrste mikroorganizmov (MKB, kvasovke, avtohtona mikrobiota) in pogojev fermentacije (temperatura, pH, čas), zato smo te parametre v predposkusih optimizirali. Fermentacija je potekala na neobdelanih (semena, ki niso bila obdelana z nobenim biotehnološkim procesom), kaljenih in encimsko obdelanih pirinih semenih sorte Ostro. Cilj poskusa je bil ugotoviti učinek različnih tipov fermentacije pirinih semen na sprostitev fenolnih spojin in njihovo antioksidativno aktivnost ter nadalje raziskati, kakšen vpliv ima kombiniranje postopkov kaljenja in fermentacije oz. encimske obdelave in fermentacije pirinih semen na sprostitev fenolnih spojin. Določili smo ekstraktibilne in vezane TPC, fenolni profil s HPLC-MS/MS metodo ter *in vitro* in *in vivo* antioksidativno aktivnost. Rezultate smo obdelali z multivariatno analizo.

Poskus 3: Vpliv encimske obdelave na sprostitev fenolnih spojin in njihovo antioksidativno aktivnost v neobdelanih, kaljenih in fermentiranih pirinih semenih

Pri encimski obdelavi semena obdelamo neposredno s hidrolitičnimi encimi. Encimski pristop k razgradnji osnovnih elementov celične stene se je pokazal kot učinkovit z uporabo ksilanaz, β-glukanaz in celulaz v kombinaciji s feruloil esterazami. S poskusom smo preverili, kako različne kombinacije encimov vplivajo na sproščanje vezanih fenolnih spojin iz celične stene pirinih semen ter ali kombiniranje omenjenih biotehnoloških procesov vpliva sinergistično ali antagonistično na vsebnost fenolnih spojin in na njihovo antioksidativno aktivnost. Pirina semena smo obdelali s petimi različnimi encimi: s celulazami, ksilanazami,

feruloil esterazami, proteazami in α-amilazami. Čas, razmerje med vzorcem in pufrom in aktivnost posameznih encimov smo določili na podlagi predposkusov. Po 4 h encimske obdelave pirinih semen smo vzorce liofilizirali in ekstrahirali ter jim določili ekstraktibilne in vezane TPC, fenolni profil s HPLC-MS/MS metodo ter *in vitro* in *in vivo* antioksidativno aktivnost. Rezultate smo obdelali z multivariatno analizo.

Poskus 4: Vpliv biološke obdelave pirinih semen na biološko dostopnost fenolnih spojin sproščenih med *in vitro* prebavo

Z metodo *in vitro* prebave poskušamo predvideti biološko dostopnost fenolnih spojin, tako da posnemamo *in vivo* fiziološke pogoje, z upoštevanjem prisotnosti in koncentracije prebavnih encimov in soli, pH vrednosti, časa in temperature prebave. Vzorce, ki so bili izpostavljeni GI prebavi, smo izbrali na podlagi naših treh predhodnih raziskav, kjer smo ugotavljali, s katerimi biotehnološkimi procesi in njihovimi kombinacijami najbolj povečamo vsebnost fenolnih spojin iz semen pire. Simulacija prebave je vključevala oralno (2 min), gastro (2 h) in intestinalno (2 h) fazo. Po končani prebavi je supernatant (ekstraktibilne fenolne spojine) predstavljal biološko dostopne fenolne spojine, medtem ko je hidrolizat (vezane fenolne spojine) predstavljal biološko nedostopne, ampak stabilne fenolne spojine. Prebavljenim vzorcem smo določili vsebnost ekstrabilnih in vezanih TPC, fenolni profil s HPLC-MS/MS metodo ter *in vitro* antioksidativno aktivnost, izračunali smo tudi odstotek biološko dostopnih in stabilnih fenolnih spojin v posameznem vzorcu. Določali smo korelacijo med neprebavljenimi in prebavljenimi ekstraktibilnimi fenolnimi spojinami ter korelacijo med deležem ekstraktibilnih fenolnih spojin in njihovo biološko dostopnostjo. Rezultate smo obdelali z multivariatno analizo.

1.2 RAZISKOVALNE HIPOTEZE

Postavili smo naslednje raziskovalne hipoteze:

- H1: Kaljenje pirinih semen pod kontroliranim abiotskim stresom pripomore k izboljšanju antioksidativne aktivnosti fenolnih spojin.
- H2: Učinkovitost fermentacije z namenom povečanja fenolnih antioksidantov je večja z uporabo izbranih mikroorganizmov (kvasovk, mlečnokislinske bakterije) kot v primeru spontane fermentacije.
- H3: Kaljenje in fermentacija učinkujeta sinergistično na povečanje dostopnosti fenolnih spojin.
- H4: Vsebnosti posameznih fenolnih spojin so odvisne od pogojev fermentacije in kaljenja.
- H5: Antioksidativna aktivnost, določena v celicah kvasovke S*accharomyces cerevisiae,* ni v korelaciji z antioksidativno aktivnostjo, določeno z različnimi *in vitro* testi.

2 ZNANSTVENA DELA

2.1 KOMBINIRAN ABIOTSKI STRES IZBOLJŠA FENOLNI PROFIL IN AKTIVNOSTI EKSTRAKTIBILNIH IN VEZANIH ANTIOKSIDANTOV IZ SEMEN KALJENE PIRE (*Triticum spelta* L.)

Mencin M., Abramovič H., Jamnik P., Mikulič Petkovšek M., Veberič R., Terpinc P. 2021a. Abiotic stress combinations improve the phenolics profiles and activities of extractable and bound antioxidants from germinated spelt (*Triticum spelta* L.) seeds. Food Chemistry, 344: 128704, doi:10.1016/j.foodchem.2020.128704: 12 str.

Namen študije je bil raziskati vpliv kaljenja pirinih semen pod različnimi stresnimi pogoji na antioksidativne lastnosti njihovih ekstraktibilnih in vezanih fenolnih spojin. V kombinaciji z ustreznimi pogoji abiotskega stresa smo ugotovili, da kaljenje pirinih semen pri 25 °C ob dodatku 25 mM NaCl in 50 mM sorbitola brez aplicirane mehanske poškodbe znatno pripomore k povečanju vsebnosti skupnih fenolov in omogoča učinkovitejše lovljenje prostih radikalov ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $ROO^{\bullet}$). Alkalna hidroliza netopnega ostanka kaljenih pirinih semen je omogočila določitev večine prisotnih fenolnih kislin, med katerimi sta bili ferulna in *p*-kumarna kislina najbolj zastopani. Vezane fenolne spojine so izražale večjo antioksidativno aktivnost kot ekstraktibilne fenolne spojine. Med ekstraktibilnimi fenolnimi spojinami se je najbolj povečala vsebnost heksozida *p*-kumarne kisline (146 %), medtem ko je bilo med identificiranimi vezanimi fenolnimi spojinami največje relativno povečanje ugotovljeno pri *p*-kumarni kislini (171 %). Ekstraktibilne in vezane fenolne spojine iz kaljenih semen niso znižale znotrajcelične oksidacije v celicah kvasovk *Saccharomyces cerevisiae*.

Food Chemistry 344 (2021) 128704

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Abiotic stress combinations improve the phenolics profiles and activities of extractable and bound antioxidants from germinated spelt (*Triticum spelta* L.) seeds

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ARTICLE INFO

Keywords:
Germination
Abiotic stress
Phenolics
Spelt
In vivo antioxidant activity

ABSTRACT

The aim of this study was to investigate the effects of germination of spelt seeds under different stress conditions on the antioxidant characteristics of their extractable and bound phenolics. Germination under combined stress of 25 mM NaCl and 50 mM sorbitol without subsequent mechanical stress had considerable impact on total phenolics contents and scavenging activities against different free radicals (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, $O_2^{\bullet-}$, ROO$^{\bullet}$). Alkaline hydrolysis of extracts from germinated seeds provided the majority of their phenolic acids, where ferulic and *p*-coumaric acids were the most representative. The phenolics liberated from their bound form also had greater antioxidant activities. For the extractable phenolics, *p*-coumaric hexoside increased the most (146%), while among the bound phenolics identified, the highest relative increase was for *p*-coumaric acid (171%). The germinated seeds showed no effects on intracellular oxidation in cells of the yeast *Saccharomyces cerevisiae*.

1. Introduction

Spelt (*Triticum spelta* L.) is an ancient cousin of modern wheat (*Triticum aestivum* L.). It requires modest soil conditions with little nitrogen fertilisation and adapts well to cool and humid climatic conditions. Spelt cultivar "Ostro" is a Swiss variety sown in October and November. It grows slightly higher than wheat up to 170 cm. The yield of spelt ranges from 2.5 to 4 tons of dehulled seeds/ha. Some studies have shown that spelt contains higher levels of minerals, proteins and soluble dietary fibre than wheat (Munoz-Insa, Gastl, & Becker, 2016).

Current studies indicated that germinated cereal grains show higher total phenolics content (TPC) and antioxidant activity (AA) than ungerminated buckwheat (Terpinc, Cigić, Polak, Hribar, & Požrl, 2016), rice, barley, wheat and oats (Singh & Sharma, 2017). During germination, the endogenous enzymes of the cereal seeds are activated, and some of their important substances are broken down into smaller molecules, such as protein and starch (Chen, Yu, Wang, Gu, & Beta, 2016). From the moment the dormancy of seeds is interrupted, their synthesis of phenolics and other biochemical compounds increases. Biochemical compounds may have health benefits through multifactorial physiological mechanisms, which include their AAs. The biosynthesis of phenolics is a complex pathway that involves a number of different chemical reactions, and this is endogenously regulated during plant growth and can be stimulated by exogenous factors (e.g., abiotic stress), such as salinity, temperature, mechanical damage and water (Chen et al., 2016). Increased TPC of seeds during germination under salinity stress has been shown in a study by Lim, Park, Kim, Jeong, and Kim (2012), which reported that TPC of germinated buckwheat seeds treated with different salinity stress (10, 50, 100 mM NaCl) was increased (57%, 121%, 153%) compared to the control (0 mM). Falcinelli et al. (2017) also reported that moderate salinity (25–50 mM NaCl) compared to the control (0 mM) resulted in the highest relative increase (up to 35%) in TPC during rapeseed germination. As the main function of plant secondary metabolites is to protect the plant from biotic and abiotic stress, the same strategies for production of metabolites were applied in the present study to potentially increase the yield of phenolic antioxidants. Different responses under various adverse conditions were expected subsequent to triggering of different mechanisms to cope with stress situations (Gawlik-Dziki, Dziki, Pietrzak, & Nowak, 2017).

Ferulic acid, which is the predominant phenolic acid in spelt, has

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https://doi.org/10.1016/j.foodchem.2020.128704
Received 1 September 2020; Received in revised form 9 November 2020; Accepted 18 November 2020
Available online 23 November 2020

been extensively studied. It has been confirmed that this phenolic acid has various bioactivities that can have positive effects on human health once it is transformed in the body or degraded into bioavailable metabolites by the gut microbiota (Heleno, Martins, Queiroz, & Ferreira, 2015). Phenolics from plant sources can be 'extractable' (free, soluble conjugates) or 'insoluble', in a bound form. The soluble conjugates are in the cytoplasmic vacuoles and are bound to various carbohydrates or organic acids. However, the majority of phenolics in spelt seeds are insoluble, as they are bound by ester and ether bonds to cell wall materials, such as lignin, cellulose and arabinoxylan (Nayak, Liu, & Tang, 2015). These bound phenolics can be released by acid, alkali or enzymatic hydrolysis of samples before extraction (Liyana-Pathirana & Shahidi, 2006). There is, however, a lack of information on how the transformation of extractable and bound phenolics and their AAs vary during germination of spelt seeds. Chen et al. (2017) found that free and bound phenolics contents significantly increased in germinated wheat and the AA was increased as a result. Gawlik-Dziki, Świeca, and Dziki (2012) reported that hydroxybenzoic acid derivatives were dominant in the free phenolics fraction, while hydroxycinnamic acid derivatives (mainly ferulic acid) were predominant in the bound phenolics fraction of spelt extracts. Bound phenolic acids also showed higher AAs than free ones.

Also, to the best of our knowledge, none of the *in vivo* studies has investigated the connections between the AAs and the phenolics of germinated spelt seeds. Cigut, Polak, Gašperlin, Raspor, and Jamnik (2011) tested the cellular uptake of caffeic acid, *p*-coumaric acid, ferulic acid and their mixture and reported that tested phenolics did not enter the cells.

Therefore, the aims of the present study were: (i) to determine the stress conditions under which the germination of spelt seeds results in an increased TPC and increased AA; (ii) to establish the relationships between the AAs determined in different *in vivo* and *in vitro* antioxidant assays; and (iii) to determine the profiles of the extractable and bound fractions of the phenolics from spelt seeds germinated under various combinations of abiotic stress.

2. Materials and methods

2.1. Materials

Spelt (*Triticum spelta* L. cv. Ostro) seeds were cultivated in the Dolenjska region, Slovenia in integrated agricultural production system, and mechanically harvested in July 2018. Dehulled spelt seeds with a moisture content of 11.2% were stored in the dark at 1 °C until analysis for a maximum of 20 weeks. Chloroform, methanol (99.9%), formic acid, sodium hydroxide, sodium carbonate, potassium dihydrogen phosphate and disodium hydrogen phosphate were from Merck (Darmstadt, Germany). 2,2-Azino-bis-3-etilbenzotiazolin-6-sulfonic acid (ABTS) reagent, β-carotene, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) reagent, linoleic acid (99%), Tween 20, Folin–Ciocalteu reagent, Trolox, sodium dihydrogen phosphate dehydrate, 2′,7′-dichlorodihydrofluorescein diacetate, butylated hydroxytoluene, nitroblue tetrazolium, β-nicotinamide adenine dinucleotide, phenazine methosulphate, *p*-hydroxybenzoic acid, ferulic acid, *p*-coumaric acid and caffeic acid were from Sigma-Aldrich (Steinheim, Germany). Phosphate-buffered saline was from Oxoid (Hampshire, England). All of the chemicals and reagents used for the present study were of analytical quality. Water (Milli-Q; Millipore) was used to prepare the working solutions.

2.2. Seed germination

After disinfection of the seeds with hot water (47 °C, 5 min), they were soaked in distilled water at room temperature for 2 h. The seed germination conditions were designed in analogy with the Aubry method (MEBAK, 2011). In detail, the soaked seeds (15.8 g) were placed in a petri dish (diameter, 11 cm) on a moistened layer of cotton wool and filter paper and covered with a second layer of filter paper. The petri dishes were placed in the dark in the germination chamber for a total of 144 h under all conditions tested. During seed germination, water was added manually with a pipette by applying it evenly over the entire surface of the seeds. The spelt seeds were then germinated under the various stress conditions (with the aim of covering a wide range of stress conditions found in the literature), as also detailed in Supplementary Materials Table S1.

The control sample had 24 mL water added, and was germinated at 20 °C. This water volume represented four-fold the weight of the two filter paper sheets and the layer of cotton. For the individual stress conditions, the same water volume was used (except for the water volume stress conditions), with changes in salinity (NaCl: 25–200 mM), osmolarity (sorbitol, 50–300 mM) and temperature (10–40 °C), and with different forms of added mechanical stress (see Table S1).

For the stress combinations tested (see Table S1), initially 18 mL water was added (at t_0: immediately after seed soaking). The stress conditions were then added on sequentially in 6 mL water at t_1 (48 h after soaking) and t_2 (96 h after soaking) to provide the required conditions of salinity and osmolarity (final volume 30 mL). The mechanical stress was applied at 72 h (i.e., cutting off of one-third of the germinating sprouts). The stress-inducing NaCl and sorbitol solutions were added in manually with a pipette (without removal of the previous condition).

Following these treatments (i.e., after 144 h) the sprouts were collected and homogenised using a laboratory mill (A11 basic, IKA® Works, Staufen, Germany) to a fine powder and obtained particles were smaller than 0.25 mm in diameter. The water was removed by freeze-drying at −50 °C and 30 mTorr (to moisture level < 5%). The freeze-dried samples were stored at −20 °C for a maximum of three days.

2.3. Preparation of extractable and bound phenolics

Aliquots of the homogenised and freeze-dried germinated seeds were mixed with 99.9% methanol at a ratio of 1:9 (w/v). After 2 h of shaking (Tehtnica Železniki EV-403, Slovenia) at room temperature (22 ± 1 °C), the mixtures were centrifuged (Avanti JXN-26; Beckman Coulter, Krefeld, Germany) at 9793.9×*g* and 10 °C for 10 min. The methanol was evaporated off at 40 °C in vacuum evaporator (HT-4 series II GeneVac Technologies, Ipswich, UK), the residues were resuspended in water, and the samples were filtered (pore size, 0.45 μm). These filtered supernatants from the extractions contained the 'extractable phenolics'.

The solid residues following the methanol extraction were then treated by addition of 2 M sodium hydroxide (20 mL) and shaking for 4 h (Tehtnica Železniki EV-403, Slovenia) at room temperature. After this alkaline hydrolysis step, the hydrolysate samples that contained the released phenolics were acidified to pH 3 with concentrated formic acid. They were then filtered (Chromafil A-45/25 syringe filters; cellulose acetate, hydrophilic membrane; Macherey-Nagel, Düren, Germany) to obtain the samples with the phenolics that had been released by the alkaline hydrolysis (hereinafter referred to as the 'bound phenolics'). All of the extracts were stored at 2 °C until analysis for a maximum of three days.

In the present study, solid-phase extraction (SPE) cartridges (Strata-X RP; 100 mg; Phenomenex) were used to purify the crude extracts and to concentrate the phenolics. The SPE cartridges were initially preconditioned with 3 mL 99.9% methanol, followed by 3 mL water. The samples containing the extractable phenolics (5 mL) and bound phenolics (8 mL) were then added to the SPE cartridges, and allowed to enter the matrix. The cartridges were washed with 4 mL water and vacuum-dried for 2 min. The phenolics were then eluted from the cartridges with 2 mL 70% aqueous methanol. These methanolic eluates from the SPE-purified extracts then represented the corresponding extractable and bound fractions that contained the phenolics. Purified samples were stored at 2 °C until analysis, which were performed within one week.

2.4. Determination of total phenolics content

The TPCs were determined using the Folin–Ciocalteu method (Gutfinger, 1981). Appropriately diluted samples of the extractable and bound phenolics were mixed with water and Folin–Ciocalteu reagent. After 5 min of incubation, 20% sodium carbonate solution was added. The samples were incubated for 60 min in the dark at room temperature, and then the absorbance at 765 nm was measured using a UV–visible spectrophotometer (model 8453; Hewlett Packard, Waldbronn, Germany), with a 1-cm-light-path cuvette. A standard curve was prepared with Trolox, and the data are expressed as mg Trolox equivalents per g dry weight (mg TE/g DW).

2.5. HPLC-MS analysis

The samples containing the extractable and bound phenolics were analysed by HPLC (Thermo Dionex system; Thermo Scientific, San Jose, USA). The phenolics were detected at 280 nm, 310 nm and 350 nm. A C18 column was used (Gemini C18; 150 mm × 4.6 mm; 3 μm; Phenomenex, Torrance, USA), which was maintained at 25 °C. The elution solvents were 0.1% formic acid and 3% acetonitrile in double-distilled water (solvent A) and 0.1% formic acid and 3% water in acetonitrile (solvent B). The samples were eluted using the following discontinuous gradient: 0 → 15 min, 5% → 20% B; 15 → 20 min, 20% → 30% B; 20 → 25 min, 30% B; 25 → 30 min, 30% → 90% B; 30 → 45 min, 90% B; 45 → 50 min, 90% → 5% B (Wang et al., 2002). The injection volume was 20 μL, and the flow rate was 0.6 mL/min. All of the phenolics were identified using mass spectrometry (LTQ XL linear ion trap mass spectrometer; Thermo Fisher Scientific, San Jose, USA) with electrospray ionisation operating in negative-ion mode. All of the conditions for the mass spectrometry were the same as previously reported by Mikulic-Petkovsek, Koron, and Rusjan (2020). Identification of the individual phenolics was confirmed by comparisons of the retention times and spectra with standard databases, and by adding standard solutions to samples, followed by fragmentation (Supplementary Materials Fig. S1). The contents of *trans*-ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid and gallocatechin were calculated from the peak areas of the samples and the corresponding standards, and are expressed as μg per g dry weight (μg/g DW) of the germinated spelt seeds. For the compounds that were identified without standards, quantification was performed using similar compounds as standards. For compounds detected with known molecular weights but with unknown identities, the contents were also calculated from their peak areas and are expressed as *p*-coumaric acid equivalents (Supplementary Materials Table S2). *p*-Coumaric acid hexoside, epicatechin and apigenin hexoside pentoside were quantified according to *p*-coumaric acid, while 4-feruloylquinic acid was quantified according to ferulic acid.

2.6. In vitro tests of antioxidant activity

2.6.1. DPPH$^{\bullet}$-scavenging activity

The AAs of phenolics from germinated spelt seed extracts were determined using DPPH$^{\bullet}$ (Brand-Williams, Cuvelier, & Berset, 1995). The samples were added to 0.2 mM DPPH$^{\bullet}$ solution in 99.9% methanol. The mixtures were vortexed and incubated in the dark at room temperature for 60 min, and then the decreases in absorbance of the reaction mixtures were measured at 520 nm. Water was used as the blank solution. The AAs using DPPH$^{\bullet}$ (referred to as DPPH in Figures and Tables) are expressed as mg TE/g DW.

2.6.2. ABTS$^{\bullet+}$-scavenging activity

The scavenging activities of phenolics from the spelt extracts were also determined using ABTS$^{\bullet+}$, according to the method of Re et al. (1999), with some modifications. The working solution of ABTS$^{\bullet+}$ was mixed with phosphate buffer (pH 7.4), water and the samples, and incubated in the dark for 60 min. The AAs using ABTS$^{\bullet+}$ (referred to as ABTS in Figures and Tables) are expressed as mg TE/g DW.

2.6.3. $O_2^{\bullet-}$-scavenging activity

Measurement of the superoxide anion ($O_2^{\bullet-}$) scavenging activities (SASA) of the phenolics in the extracts of the spelt seeds were determined according to Robak and Gryglewski (1988). All of the reagents were prepared in 0.1 M phosphate buffer (pH 7.4). An aliquot of each sample (or 70% aqueous methanol as reference solution) was mixed with 150 μM nitroblue tetrazolium and 468 μM β-nicotinamide adenine dinucleotide. The reactions were started by addition of 60 μM phenazine methosulphate to the mixtures. After 5 min incubation at room temperature, the absorbance of the samples and the reference were measured at 560 nm against the blank (without phenazine methosulphate). The AAs using $O_2^{\bullet-}$ (referred to as SASA in Figures and Tables) are expressed as mg TE/g DW.

2.6.4. Coefficient of ROO$^{\bullet}$-scavenging activity

The AAs of the phenolics against the alkylperoxyl radical (ROO$^{\bullet}$) formed in an aqueous emulsion system of linoleic acid and β-carotene were determined according to Moure et al. (2000), with some modifications. A solution of β-carotene in chloroform was mixed with linoleic acid and Tween 20. After evaporation of the chloroform, water was added, and this was mixed well to form an emulsion. Aliquots of this emulsion that contained the germinated seed phenolics (or 70% aqueous methanol, for reference) were put into tubes, which were capped, and then they were thoroughly mixed. The tubes were incubated in a water bath at 50 °C. The final concentration of the samples in the emulsion was 15 mg TE/L. After a 100-min incubation, the absorbance was measured at 470 nm. A blank sample (emulsion without β-carotene) was prepared for background subtraction. The ROO$^{\bullet}$-scavenging activities are expressed as the coefficient of AA determined in the emulsion of β-carotene (C_{AA}). This was calculated as percentage inhibition in the test samples, relative to the reference.

2.7. In vivo tests

2.7.1. Determination of intracellular oxidation in the yeast Saccharomyces cerevisiae

The intracellular oxidation of the extractable and bound phenolics from the germinated spelt seed extracts was estimated using 2′,7′-dichlorofluorescein, which is widely used for the determination of hydrogen peroxide and hydroperoxides, and for studies of oxidative stress (Jakubowski & Bartosz, 1997).

The samples were added to the yeast (*S. cerevisiae*) suspensions and incubated for 2 h at 28 °C and 220 rpm. Samples of these yeast incubations were taken for determination of intracellular oxidation and cellular uptake. The yeast cells were sedimented by centrifugation (14000×*g*, 5 min) and washed with 50 mM potassium phosphate buffer (pH 7.8). The sediments were finally resuspended in 50 mM potassium phosphate buffer and incubated at 28 °C for 5 min. 2′,7′-Dichlorodihydrofluorescein diacetate was added to a final concentration of 10 μM (from a 1 mM stock solution in ethanol). After a 30-min incubation at 28 °C and 220 rpm, the fluorescence of the yeast cell suspensions was measured in a microplate reader (Safire II; Tecan, Switzerland). The excitation and emission wavelengths of the 2′,7′-dichlorofluorescein were measured at 488 nm and 520 nm, respectively. The analysis was carried out in triplicate. The fluorescence (F) was normalised to the optical densities (OD) of the yeast suspensions, with the data expressed as the samples relative to the control (untreated cells).

2.7.2. Determination of cellular uptake by the yeast Saccharomyces cerevisiae

The phenolics profiles were determined before and after the 2-h exposures of the yeast cells to the samples of the extractable and bound phenolics from the germinated spelt seed extracts. The yeast cell suspensions were centrifuged (14000×*g*, 5 min). The supernatants

obtained were filtered (pore size, 0.2 μm) and analysed by HPLC–mass spectrometry, to obtain the phenolics profiles. All of these experiments were performed in triplicate.

2.8. Statistical analysis

The seeds were germinated in two parallel sets, which were then combined. All of the analyses were performed in three parallel runs, on two separate extractions. The data are means ± standard deviation. One-way analysis of variance (ANOVA) was performed using the SPSS programme, as version 22 for Windows. The comparisons of the treatment means were based on Duncan's multiple range tests, with a significance level of 0.05. In addition, the Pearson's correlation coefficients (r) were determined, to express the strengths between two continuous variables. Multivariate statistical analysis (linear discriminant analysis; [LDA]) was performed to interpret the differences in all of the analysed samples.

3. Results and discussion

3.1. Characterisation of the crude extracts

The control treatment during the germination of the spelt seeds (from seed soaking) was defined as a water volume of 24 mL at 20 °C for 144 h. Following the preparation of the crude extracts (without SPE purification), the TPC of the extractable fraction was 8.3 mg TE/g DW, and of the bound fraction was 17.9 mg TE/g DW (Table 1).

A lower water volume of 12 mL significantly reduced the extractable and bound TPCs and their $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities. The same was obtained for increased salinity (NaCl concentration), except for the $ABTS^{\bullet+}$ scavenging activity of the bound fraction. Here, all of the additions of salt (25–200 mM) resulted in significantly greater $ABTS^{\bullet+}$ scavenging activities of the bound fraction compared to the control (Table 1). Increased osmolarity through increased sorbitol concentration and the lower and higher temperatures (10 °C, 30 °C) also generally significantly reduced the extractable and bound TPCs and $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities compared to the control, except for $DPPH^{\bullet}$ scavenging activity of the bound fraction (Table 1). Here, increased sorbitol and the changed temperatures resulted in significantly greater $DPPH^{\bullet}$ scavenging activities. The various types of mechanical stress significantly decreased TPC and $ABTS^{\bullet+}$ scavenging activity and increased $DPPH^{\bullet}$ scavenging activity of the extractable and bound fractions compared to the control (Table 1). Of interest, the combined stress conditions resulted in higher TPCs for the extractable and bound fractions, and they showed smaller increases for $ABTS^{\bullet+}$ scavenging activity compared to $DPPH^{\bullet}$ scavenging activity (Table 1).

As sugars, organic acids and other polar compounds in these crude extracts might affect the TPC and AA assays, SPE purification of the crude extracts was carried out before further analysis.

3.2. Total phenolic content of purified extracts

The selection for further analysis of these spelt seed extractable and bound fractions following SPE purification included the control germination (24 mL water at 20 °C for 144 h) and the four samples with the highest antioxidant potentials of the crude extracts for the individual stress combinations (Table 1: salinity; osmolarity; salinity + osmolarity; salinity + osmolarity + mechanical stress). The stress treatments for these samples are indicated in Tables 1, 2 and S1 as NN, SS, NS and NSM (respectively). These indicate the following treatments carried out at 25 °C, where t_0 was immediately after seed soaking, t_1 was 48 h after soaking, and t_2 was 96 h after soaking, with a final volume of 30 mL:

NN: t_0: water (18 mL); $t_1 + t_2$: 25 mM NaCl (added in 6 mL + 6 mL).

SS: t_0: water (18 mL); $t_1 + t_2$: 50 mM sorbitol (added in 6 mL + 6 mL).

NS: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); t_2: 50 mM sorbitol (added in 6 mL).

NSM: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); 72 h: mechanical stress applied; t_2: 50 mM sorbitol (added in 6 mL).

Here, the germination of these spelt seeds under the different combinations of stress (except for NSM) significantly increased the TPCs of these spelt seed extractable and bound fractions (Fig. 1). Of interest, before SPE purification (Table 1), the TPC of NSM was comparable to the other stress treatments (i.e., NN, SS, NS), and it was also greater than the control. As the phenolics are not the only contributors to the reductions in the phosphomolybdic/ phosphotungstic acid complexes in Folin–Ciocalteu reagent, this confirms that the Folin–Ciocalteu method is not specific for these compounds and will therefore often tend to overestimate their levels (Huang, Ou, & Prior, 2005). We can thus conclude here that some further compounds to those in the control and NN, SS and NS appear to have been generated as a result of the mechanical stress in these spelt seeds during their germination (i.e., NSM). These appear to have accumulated in particular in sample NSM during germination, and they might interfere with the TPC determination in an additive or enhancing manner. According to the properties of the SPE cartridges and solvents used, these further compounds might represent: (a) non-phenolic antioxidants (e.g., vitamins) removed by SPE purification; (b) less polar phenolics that were not bound to the columns during the loading; and/or (c) highly polar phenolics that were removed from the columns with the water wash.

Regardless of the germination treatments, notable differences between the extractable and bound phenolics were obtained, which indicated that approximately two-thirds of the phenolics from the germinated spelt seeds were not extracted with the methanol, and were only released after the alkaline hydrolysis. The TPCs of the extractable phenolics significantly increased under the stress treatments: NN, SS and NS. The bound phenolics increased under the stress treatments: NN and NS. On the other hand, the NSM combination resulted in 33% decrease in the TPC of the extractable fraction, and 18% decrease in the TPC of the bound fraction.

It should also be noted that rather than providing a comparison with the non-germinated seeds, the aim of the present study was to investigate the effects of these different stress conditions when applied during germination of the spelt seeds. The changes in the TPCs of these extractable and bound fractions indicated that transformations indeed occurred during germination.

3.3. HPLC-MS analysis of the individual phenolics

The stress conditions applied here had a major impact on the phenolics profiles of these SPE-purified extracts from germinated spelt seeds (Table 2). *p*-Coumaric acid, *trans*-ferulic acid, caffeic acid and *p*-hydroxybenzoic acid were detected in both the extractable and bound fractions. *Trans*-ferulic acid was the dominant phenolic acid detected in the present study. In particular, these phenolic acids were mainly extracted as their bound forms. This was particularly in case for *p*-coumaric and *trans*-ferulic acids, where their bound forms represented 99% of their totals.

For the extractable phenolic acids identified, all of these stress treatments (i.e., NN, SS, NS, NSM) resulted in decreased levels, with the most notable for NSM, compared to the control sample. Obviously, extractable phenolic acids were lost by cutting off the sprouts (i.e., the mechanical stress applied here; Table S1). The most notable was the decrease in *p*-coumaric acid (55%–75%).

On the other hand, when compared to the control, the contents of the bound phenolic acids increased under the treatments of NN, SS and NS. Falcinelli et al. (2017) and Gupta and De (2017) suggested that the effects of salinity on *p*-coumaric acid and ferulic acid are dependent on genotype, in terms of this salt sensitivity.

The NSM treatment, which included the mechanical stress, significantly increased the levels of bound *p*-coumaric acid, but had no significant effect on bound *trans*-ferulic acid and *p*-hydroxybenzoic acid. Conversely, here the caffeic acid levels were significantly lower, compared to the control (Fig. 2, Table 2). This makes sense, as the

Table 1
Total phenolics contents and antioxidant activities of the extractable and bound crude extracts of the germinated spelt seeds. Underlined: further purified with SPE and analysed (see Table S1). Bold: maximum values for an individual stress.

Stress	Germination treatment	Phenolics (mg TE/g DW)					
		Total Phenolics Content		DPPH assay		ABTS assay	
		Extractable	Bound	Extractable	Bound	Extractable	Bound
Water volume	24 mL H_2O, 20 °C (Control)	8.3 ± 0.3^{b}	17.9 ± 0.8^{B}	0.54 ± 0.05^{b}	1.85 ± 0.09^{B}	9.3 ± 0.5^{b}	24.5 ± 0.5^{B}
	12 mL H_2O, 20 °C	6.0 ± 0.2^{a}	14.5 ± 0.5^{A}	0.49 ± 0.01^{a}	1.57 ± 0.23^{A}	7.6 ± 0.2^{a}	21.1 ± 0.5^{A}
	18 mL H_2O, 20 °C	8.7 ± 0.2^{c}	18.8 ± 1.5^{Bc}	0.58 ± 0.02^{bc}	1.86 ± 0.15^{B}	**10.2 ± 0.5^{c}**	**27.4 ± 1.7^{C}**
	30 mL H_2O, 20 °C	**9.2 ± 0.2^{d}**	**19.6 ± 0.5^{C}**	**0.61 ± 0.00^{c}**	**2.14 ± 0.05^{C}**	**10.6 ± 0.2^{c}**	**28.1 ± 1.2^{C}**
Salinity	24 mL H_2O, 20 °C (Control)	**8.3 ± 0.3^{c}**	**17.9 ± 0.8^{C}**	0.54 ± 0.05^{b}	**1.85 ± 0.09^{C}**	9.3 ± 0.5^{c}	24.5 ± 0.5^{A}
	24 mL 25 mM NaCl, 20 °C	**8.1 ± 0.6^{c}**	**17.7 ± 0.9^{C}**	**0.62 ± 0.03^{c}**	1.73 ± 0.11^{BC}	**10.6 ± 0.5^{d}**	**38.9 ± 0.5^{D}**
	24 mL 50 mM NaCl, 20 °C	**7.9 ± 0.3^{c}**	15.9 ± 0.6^{B}	**0.64 ± 0.01^{c}**	1.70 ± 0.17^{BC}	9.2 ± 0.4^{c}	34.7 ± 0.8^{C}
	24 mL 100 mM NaCl, 20 °C	5.7 ± 0.6^{b}	15.1 ± 0.4^{B}	0.50 ± 0.03^{b}	1.59 ± 0.17^{AB}	6.9 ± 0.4^{b}	32.5 ± 1.9^{C}
	24 mL 200 mM NaCl, 20 °C	3.7 ± 0.2^{a}	11.5 ± 0.4^{A}	0.31 ± 0.01^{a}	1.49 ± 0.06^{A}	5.1 ± 0.1^{a}	26.8 ± 0.4^{B}
Osmolarity	24 mL H_2O, 20 °C (Control)	**8.3 ± 0.3^{d}**	**17.9 ± 0.8^{D}**	0.54 ± 0.05^{b}	1.85 ± 0.09^{AB}	9.3 ± 0.5^{c}	**24.5 ± 0.5^{D}**
	24 mL 50 mM sorbitol, 20 °C	**8.4 ± 0.2^{d}**	**17.9 ± 0.5^{D}**	**0.69 ± 0.01^{d}**	1.73 ± 0.07^{A}	**12.1 ± 0.1^{e}**	22.6 ± 1.4^{C}
	24 mL 125 mM sorbitol, 20 °C	7.0 ± 0.4^{c}	16.2 ± 0.6^{C}	0.64 ± 0.03^{c}	1.84 ± 0.08^{AB}	10.1 ± 0.4^{d}	20.5 ± 0.8^{B}
	24 mL 200 mM sorbitol, 20 °C	5.4 ± 0.2^{b}	14.7 ± 0.7^{B}	0.53 ± 0.03^{b}	1.83 ± 0.13^{AB}	8.7 ± 0.3^{b}	20.2 ± 0.7^{B}
	24 mL 300 mM sorbitol, 20 °C	4.0 ± 0.1^{a}	13.5 ± 0.3^{A}	0.40 ± 0.02^{a}	**1.91 ± 0.07^{B}**	7.3 ± 0.2^{a}	18.4 ± 0.3^{A}
Temperature	24 mL H_2O, 20 °C (Control)	**8.3 ± 0.3^{c}**	**17.9 ± 0.8^{C}**	**0.54 ± 0.05^{b}**	1.85 ± 0.09^{A}	**9.3 ± 0.5^{c}**	**24.5 ± 0.5^{B}**
	24 mL H_2O, 40 °C	–	–	–	–	–	–
	24 mL H_2O, 30 °C	5.0 ± 0.2^{b}	15.8 ± 0.4^{B}	**0.52 ± 0.03^{b}**	**2.17 ± 0.07^{B}**	5.2 ± 0.4^{a}	**22.7 ± 0.7^{B}**
	24 mL H_2O, 10 °C	3.7 ± 0.1^{a}	12.4 ± 0.6^{A}	0.34 ± 0.02^{a}	**2.21 ± 0.08^{B}**	8.3 ± 0.3^{b}	17.2 ± 0.2^{A}
Mechanical	24 mL H_2O, 20 °C (Control)	**8.3 ± 0.3^{c}**	**17.9 ± 0.8^{C}**	0.54 ± 0.05^{a}	1.85 ± 0.09^{A}	**9.3 ± 0.5^{c}**	**24.5 ± 0.5^{C}**
	24 mL H_2O + sandpaper, 20 °C	6.4 ± 0.3^{b}	14.7 ± 0.4^{A}	**0.60 ± 0.02^{b}**	2.17 ± 0.12^{B}	6.4 ± 0.3^{b}	17.0 ± 0.5^{AB}
	24 mL H_2O + drawing pins, 20 °C	5.6 ± 0.3^{a}	14.8 ± 0.7^{A}	0.56 ± 0.02^{ab}	2.31 ± 0.08^{C}	5.9 ± 0.1^{a}	16.3 ± 0.6^{A}
	24 mL H_2O + 72 h cutting 1/3 of sprouts, 20 °C	6.2 ± 0.1^{b}	16.3 ± 0.4^{B}	**0.60 ± 0.02^{b}**	**2.55 ± 0.01^{D}**	6.6 ± 0.1^{b}	17.4 ± 0.5^{AB}
	24 mL H_2O + shaking (3 h/day, 250 rpm), 20 °C	6.3 ± 0.1^{b}	16.3 ± 0.6^{B}	**0.60 ± 0.02^{b}**	2.25 ± 0.02^{BC}	6.4 ± 0.3^{b}	17.8 ± 0.3^{B}
Combined #1	24 mL H_2O, 20 °C (Control)	8.3 ± 0.3^{a}	17.9 ± 0.8^{A}	0.54 ± 0.05^{a}	1.85 ± 0.09^{A}	**9.3 ± 0.5^{b}**	**24.5 ± 0.5^{B}**
	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 25 mM NaCl, 20 °C	10.5 ± 0.3^{b}	20.7 ± 0.4^{B}	**1.54 ± 0.02^{b}**	2.84 ± 0.31^{B}	8.1 ± 0.3^{a}	22.3 ± 0.7^{A}
	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 50 mM NaCl, 20 °C	11.0 ± 0.3^{b}	20.8 ± 0.9^{B}	**1.56 ± 0.05^{b}**	3.04 ± 0.09^{BC}	7.9 ± 0.1^{a}	21.8 ± 0.7^{A}
(NN)	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 25 mM NaCl, 25 °C	**12.6 ± 0.4^{c}**	**25.5 ± 0.9^{D}**	**1.60 ± 0.04^{b}**	**3.57 ± 0.22^{D}**	**9.4 ± 0.3^{b}**	**25.9 ± 0.9^{B}**
	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 50 mM NaCl, 25 °C	**12.4 ± 0.3^{c}**	23.9 ± 0.7^{C}	**1.59 ± 0.04^{b}**	3.27 ± 0.13^{C}	**9.1 ± 0.3^{b}**	**25.4 ± 0.6^{B}**
Combined #2	24 mL H_2O, 20 °C (Control)	8.3 ± 0.3^{a}	17.9 ± 0.8^{A}	0.54 ± 0.05^{a}	1.85 ± 0.09^{A}	9.3 ± 0.5^{e}	**24.5 ± 0.5^{D}**
	t_0: 18 mL 25 mM NaCl, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 25 mM NaCl, 25 °C	9.0 ± 0.1^{b}	18.6 ± 0.4^{B}	0.73 ± 0.02^{b}	3.14 ± 0.25^{C}	7.7 ± 0.2^{b}	21.1 ± 0.8^{AB}
	t_0: 18 mL 25 mM NaCl, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 50 mM NaCl, 25 °C	8.8 ± 0.3^{b}	18.6 ± 0.3^{B}	0.77 ± 0.00^{c}	3.28 ± 0.15^{CD}	7.6 ± 0.2^{b}	20.0 ± 0.9^{A}
(SS)	t_0: 18 mL H_2O, t_1: 6 mL 50 mM sorbitol + t_2: 6 mL 50 mM sorbitol, 25 °C	10.3 ± 0.3^{c}	21.1 ± 0.4^{C}	0.84 ± 0.01^{d}	2.88 ± 0.08^{B}	8.5 ± 0.3^{c}	22.6 ± 0.3^{C}
	t_0: 18 mL 50 mM sorbitol, t_1: 6 mL 50 mM sorbitol + t_2: 6 mL 50 mM sorbitol, 25 °C	8.1 ± 0.4^{a}	17.6 ± 0.2^{A}	0.79 ± 0.02^{c}	3.41 ± 0.03^{DE}	7.0 ± 0.1^{a}	20.0 ± 0.1^{A}
(NS)	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 50 mM sorbitol, 25 °C	**13.9 ± 0.1^{e}**	**25.2 ± 0.5^{F}**	1.34 ± 0.02^{e}	4.74 ± 0.09^{F}	**9.9 ± 0.2^{f}**	**24.0 ± 0.4^{D}**
	t_0: 18 mL H_2O, t_1: 6 mL 50 mM sorbitol + t_2: 6 mL 25 mM NaCl, 25 °C	11.6 ± 0.3^{d}	24.1 ± 0.4^{E}	**1.38 ± 0.01^{f}**	**4.99 ± 0.11^{G}**	9.4 ± 0.1^{e}	21.8 ± 0.5^{BC}
(NSM)	t_0: 18 mL H_2O, t_1: 6 mL 25 mM NaCl + t_2: 6 mL 50 mM sorbitol + mechanical st., 25 °C	11.9 ± 0.1^{d}	22.6 ± 0.4^{D}	**1.37 ± 0.01^{ef}**	3.54 ± 0.65^{E}	9.0 ± 0.1^{d}	22.8 ± 0.2^{C}

Data are means ± standard deviation.
Germination at 40 °C led to the rotting of spelt seeds.
t_0, immediately after soaking; t_1, 48 h after soaking; t_2, 96 h after soaking
Different letters within the same treatment and fraction indicate significant differences ($P < 0.05$; Duncan's Multiple Range Test).

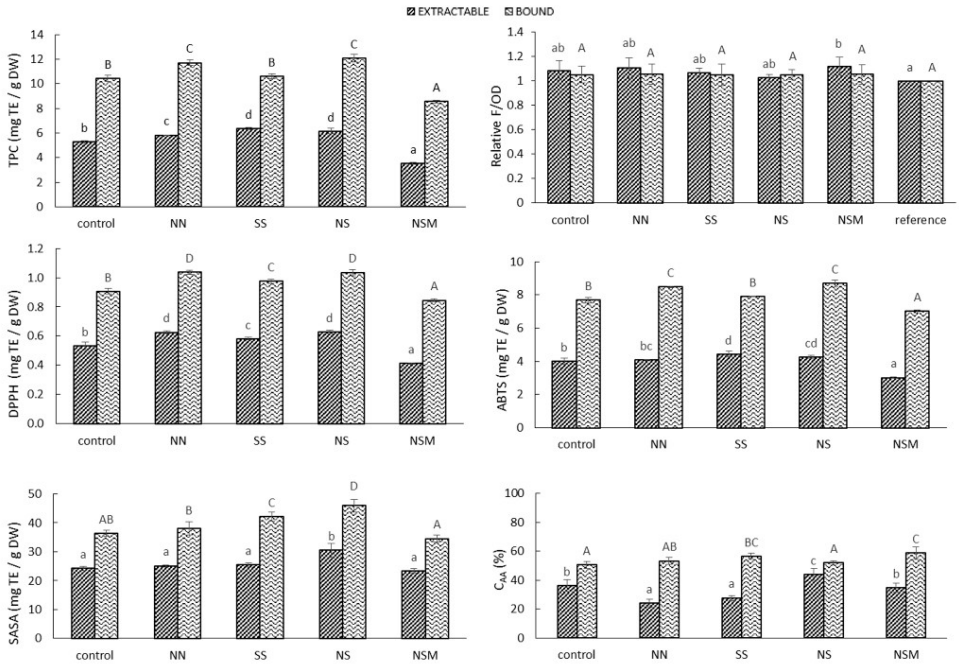

Fig. 1. Total phenolic content (TPC), antioxidant activity determined *in vivo* (intracellular oxidation in the yeast *Saccharomyces cerevisiae*; relative [F/OD]) and *in vitro* (scavenging activities against DPPH• (DPPH), ABTS•+ (ABTS), $O_2^{\bullet-}$ (SASA), ROO• (C_{AA}) radicals) for the extractable and bound fractions from the extractions of germinated spelt seed extracts following SPE purification. Control: t_0: 24 mL water, 20 °C 144 h; NN: t_0: water (18 mL); $t_1 + t_2$: 25 mM NaCl (added in 6 mL + 6 mL), at 25 °C; SS: t_0: water (18 mL); $t_1 + t_2$: 50 mM sorbitol (added in 6 mL + 6 mL), at 25 °C; NS: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); t_2: 50 mM sorbitol (added in 6 mL), at 25 °C; NSM: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); 72 h: mechanical stress applied; t_2: 50 mM sorbitol (added in 6 mL), at 25 °C. t_0, immediately after seed soaking; t_1, 48 h after soaking; t_2, 96 h after soaking. Data are means ± standard deviation, and different letters within the same fraction (extractable or bound) indicate significant differences ($P < 0.05$; Duncan's Multiple Range Test).

phenolics formed in the sprouts are initially present in their extractable forms, but over time they are transferred to other organelles and the cell walls, where they are bound in complex forms. By cutting off the sprouts, the bound phenolics were lost with the cut off part, while the newly formed phenolics would not yet be bound completely.

Overall, our observations here suggest that these treatments with NaCl and sorbitol had different effects on the transformations of the extractable and bound phenolic acids during seed germination. Thus, we have confirmed that different stress conditions during germination typically influence the ratio between these extractable and bound phenolics.

Ma et al. (2019) reported that the total ferulic acid levels in 144 h germinated barley treated with 60 mM NaCl was 1.21-fold higher than the control (untreated germinated barley).

The samples here that showed significantly improved *p*-coumaric acid and ferulic acid contents (i.e., particularly NS), also showed particularly efficient AAs and higher TPCs. This would indicate that these phenolic acids significantly contributed to the AAs of these spelt seeds germinated under the various stress conditions, and they might have the highest reactivities in the Folin–Ciocalteu assay. We also note that the extractable fractions had relatively strong AAs although they had lower levels of the phenolic acids, compared to the bound fractions. Other antioxidant compounds might indeed be seen as the unidentified peaks indicated in Table 2, and so these might also have contributed to the AAs here.

For these spelt seeds, compared to the bound fraction, the extractable fraction had not just the major phenolics indicated so far, but also a range of minor phenolics, including gallocatechin, *p*-coumaric acid hexoside, 4-feruloylquinic acid, epicatechin and apigenin hexoside pentoside, along with five unidentified compounds (Table 2). The treatment with NaCl (NN) significantly increased the levels of epicatechin (32%) and apigenin hexoside pentoside (45%). Furthermore, the impact of this increased salinity was even more pronounced for some of the unidentified compounds (Peak I, 145%); Peak III, 72%; Table 2). Also, the sequential use of NaCl and sorbitol (NS) promoted a large significant increase in *p*-coumaric acid hexoside (146%), with lesser, but still significant, increases for 4-feruloylquinic acid (27%) and some of the unidentified compounds (Peak II, 45%; Peak V, 29%; Table 2). According to these data, this large relative increase of *p*-coumaric acid hexoside indicates that it is an important complement to the stress

Table 2
Contents of the individual phenolics in the extractable and bound SPE-purified extracts following 144 h of treatments of the germinated spelt seeds with the different stress combinations.

Phenolics	Phenolics content under the given conditions (μg/g DW)									
	Control		NN		SS		NS		NSM	
	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound
Major										
p-Coumaric acid	12.0 ± 1.2^{d}	116.5 ± 8.8^{A}	3.9 ± 0.5^{b}	266.5 ± 5.8^{C}	3.9 ± 0.4^{b}	264.8 ± 3.8^{C}	5.5 ± 0.7^{c}	315.7 ± 18.8^{D}	2.9 ± 0.7^{a}	153.7 ± 9.7^{B}
trans-Ferulic acid	6.3 ± 1.6^{c}	964.4 ± 67.4^{A}	4.6 ± 0.5^{b}	1458.9 ± 42.0^{C}	4.7 ± 0.4^{b}	1287.7 ± 30.6^{B}	5.7 ± 0.6^{bc}	1517.5 ± 66.8^{C}	2.6 ± 0.9^{a}	957.3 ± 37.5^{A}
Caffeic acid	2.7 ± 0.5^{b}	14.1 ± 1.4^{B}	ND	16.4 ± 1.6^{CD}	ND	15.2 ± 0.7^{BC}	ND	16.6 ± 0.5^{D}	1.2 ± 0.4^{a}	10.3 ± 0.5^{A}
p-Hydroxybenzoic acid	26.7 ± 5.7^{c}	44.2 ± 3.3^{A}	19.1 ± 2.5^{b}	61.7 ± 9.3^{B}	14.9 ± 0.5^{a}	46.1 ± 5.4^{A}	14.1 ± 3.2^{a}	42.8 ± 8.9^{A}	14.5 ± 2.5^{a}	41.4 ± 2.0^{A}
Minor										
Gallocatechin	12.4 ± 8.4^{b}	ND	6.8 ± 0.9^{a}	ND	6.9 ± 0.7^{a}	ND	10.1 ± 1.5^{ab}	ND	5.8 ± 1.6^{a}	ND
p-Coumaric acid hexoside	1.5 ± 0.8^{a}	ND	2.8 ± 0.5^{b}	ND	2.3 ± 0.3^{b}	ND	3.6 ± 0.5^{c}	ND	1.3 ± 0.3^{a}	ND
4-feruloylquinic acid	7.5 ± 2.2^{a}	ND	9.2 ± 1.0^{b}	ND	9.3 ± 0.5^{b}	ND	9.6 ± 0.5^{b}	ND	6.4 ± 0.8^{a}	ND
Epicatechin	2.3 ± 1.0^{b}	ND	3.0 ± 0.4^{c}	ND	2.4 ± 0.2^{b}	ND	2.8 ± 0.2^{bc}	ND	1.2 ± 0.1^{a}	ND
Apigenin hexoside pentoside	13.0 ± 1.3^{b}	ND	18.9 ± 1.9^{d}	ND	13.6 ± 1.2^{b}	ND	16.3 ± 1.9^{c}	ND	9.4 ± 0.9^{a}	ND
Peak I	17.6 ± 3.4^{b}	ND	43.1 ± 3.9^{d}	ND	20.2 ± 1.4^{b}	ND	35.7 ± 4.9^{c}	ND	7.9 ± 0.9^{a}	ND
Peak II	7.3 ± 1.2^{b}	ND	9.9 ± 1.0^{c}	ND	6.7 ± 0.5^{b}	ND	10.5 ± 1.2^{c}	ND	3.4 ± 0.3^{a}	ND
Peak III	2.2 ± 0.9^{a}	ND	3.7 ± 0.3^{c}	ND	2.9 ± 0.2^{b}	ND	3.0 ± 0.3^{b}	ND	3.0 ± 0.5^{b}	ND
Peak IV	16.8 ± 2.1^{d}	ND	6.2 ± 0.8^{c}	ND	3.5 ± 0.2^{b}	ND	6.7 ± 0.5^{c}	ND	2.1 ± 0.5^{a}	ND
Peak V	16.0 ± 1.7^{c}	ND	12.1 ± 1.5^{b}	ND	14.2 ± 0.5^{c}	ND	20.6 ± 2.6^{d}	ND	8.9 ± 0.9^{a}	ND

Data are means ± standard deviation (n = 6).
Different small letters within a row (compound) indicate significant differences between the extractable contents ($P < 0.05$; Duncan's Multiple Range Test).
Different capital letters within a row indicated significant difference between the bound contents ($P < 0.05$; Duncan's Multiple Range Test).
Control: water (24 mL), 20 °C for 144 h.
NN: t_0: water (18 mL); $t_1 + t_2$: 25 mM NaCl (added in 6 mL + 6 mL), all at 25 °C
SS: t_0: water (18 mL); $t_1 + t_2$: 50 mM sorbitol (added in 6 mL + 6 mL), all at 25 °C.
NS: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); t_2: 50 mM sorbitol (added in 6 mL), all at 25 °C.
NSM: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); 72 h: mechanical stress applied; t_2: 50 mM sorbitol (added in 6 mL), all at 25 °C.
ND: not detectable.

defence in spelt seeds. Conversely, treatment with sorbitol alone did not have any notable effects on the contents of these minor compounds. When mechanical stress was added (NSM), there were generally decreases in the majority of these minor compounds (Table 2). This resulted in the maximum decrease among the identified minor phenolics for gallocatechin (53%), although an even greater decrease was found for the unidentified Peak IV (88%). However, at present the knowledge of the synthesis and regulation mechanism of these phenolic acids during germination is very limited.

Based on these data, we can conclude that the biosynthesis and transformation of these phenolics appears to depend on these stress conditions during germination, although not all of the phenolics are equally susceptible to such changes.

3.4. Antioxidant activities of the purified extracts

Considering the chemical diversity of the phenolics present in various foods and the interactions between these different molecules, the assessment of total AA appears to be a more useful marker than evaluation of the individual compounds. The antioxidant activities of these extractable and bound fractions from the germinated spelt seeds were determined using four different assays, referred to as $DPPH^{\bullet}$, $ABTS^{\bullet+}$, SASA (all expressed in TE) and C_{AA} (expressed in relative inhibition of oxidation). The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays are based on electron or hydrogen radical transfer mechanisms, and involve reduction of a coloured oxidant; as such, they are similar to the Folin–Ciocalteu assay (Dawidowicz & Olszowy, 2013). On the other hand, SASA is a competitive assay, where the antioxidant and detector (nitroblue tetrazolium) compete for $O_2^{\bullet-}$.

In general, in comparison to control, these free radical scavenging activities of the extractable and bound fractions were higher for the conditions of NN, SS and NS, while for NSM they were decreased (Fig. 1). The treatments NN and NS showed the greatest increase in $DPPH^{\bullet}$ scavenging activity of both the extractable and bound fractions. For the mechanical stress, as one-third of each sprout was cut off, these removed parts of the sprouts appear to have contained more antioxidants than the damaged sprouts (which continued to grow) could synthesise *de novo* in the remaining time.

Here, SS and NS showed the greatest increases in $ABTS^{\bullet+}$ scavenging activities of the extractable fraction, while NN and NS showed the greatest increases in $ABTS^{\bullet+}$ scavenging activities of the bound fraction. It appears that treatment with 50 mM sorbitol had a positive impact on the AAs of the extractable and bound TPCs. As a combination of 25 mM NaCl and 50 mM sorbitol, NS improved the scavenging activity against $O_2^{\bullet-}$ the most for both the extractable and bound fractions. Indeed, NS resulted in the same relative increase in SASA for both the extractable and bound fractions (26%), while SS resulted in an appreciably greater relative increase in SASA of the bound fraction compared to the extractable fraction. From this information, we can conclude that different compounds were synthesised under different stress conditions, where the selection between 50 mM sorbitol and 25 mM NaCl at t_1 (48 h) had the major impact.

The mechanism of bleaching of β-carotene in emulsified linoleic acid in water (as indicated by C_{AA}) is mediated by free radicals. The presence of various antioxidants can impede the extent of β -carotene bleaching by scavenging the free radical of linoleic acid and other free radicals that are formed in the system (Jayaprakasha, Singh, & Sakariah, 2001). The highest increase in C_{AA} observed among the extractable TPC was for NS,

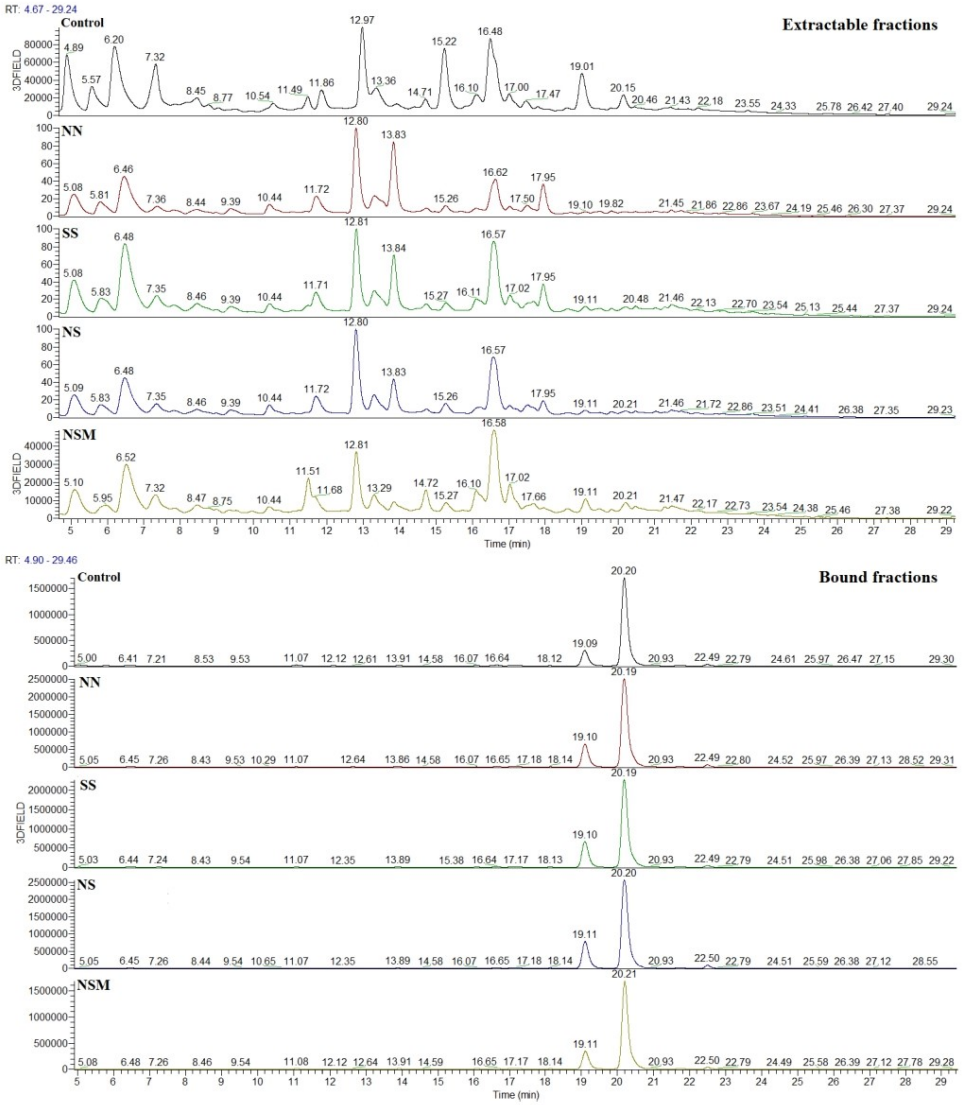

Fig. 2. Representative chromatograms of the extractable and bound fractions of the SPE-purified germinated spelt seed extracts (detected at 310 nm). Control: t_0: 24 mL water, 20 °C 144 h; NN: t_0: water (18 mL); $t_1 + t_2$: 25 mM NaCl (added in 6 mL + 6 mL), at 25 °C; SS: t_0: water (18 mL); $t_1 + t_2$: 50 mM sorbitol (added in 6 mL + 6 mL), at 25 °C; NS: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); t_2: 50 mM sorbitol (added in 6 mL), at 25 °C; NSM: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); 72 h: mechanical stress applied; t_2: 50 mM sorbitol (added in 6 mL), at 25 °C. t_0, immediately after seed soaking; t_1, 48 h after soaking; t_2, 96 h after soaking.

and the highest decreases were for NN and SS, as compared to the control. At 15 mg TE/L, the phenolics of the bound fraction was more effective as an ROO˙ scavenger than for the extractable fraction. The addition of the bound phenolics suppressed β -carotene bleaching by > 50%, which indicated the notable AAs in the emulsion regardless of the stress applied. It should be emphasised here that contrary to the DPPH˙, $ABTS^{\cdot+}$ and SASA assays, following treatment NSM, the bound fraction gave the highest C_{AA} enhancement compared to the control. The contribution of the bound phenolics is a lot greater than that of the extractable phenolics. However, a significantly higher C_{AA} was observed for the non-polar commercial antioxidant butylated hydroxytoluene (C_{AA}, 85%) compared to all of these tested samples. These results confirm polar paradox (Terpinc et al., 2011).

Based on these data here, we can assume that the combination of

NaCl and sorbitol is crucial for synthesis and/or transformation of less polar or non-polar extractable antioxidants. The addition of 25 mM NaCl after 48 h (t_1) and 96 h (t_2) of germination decreased the C_{AA} of the phenolics of the extractable fraction compared to the control sample. On the other hand, as already mentioned, the mechanical stress considerably increased the C_{AA} of the bound phenolics.

Our *in vitro* study suggests that germination under various abiotic stresses is a good way to improve the AA of spelt seeds. An appropriate germination process that includes controlled abiotic conditions such as temperature, water, salinity, light and mechanical damage is important to obtain germinated spelt seeds with higher nutritional value. Here, we can conclude that mechanical stress decreases TPC and AAs compared to the control (except for the C_{AA} assay). It might be that the mechanical stress resulted in synthesis and formation of less or non-polar bound antioxidants. Treatment with 25 mM NaCl at t_1 and 50 mM sorbitol at t_2 resulted in strong increases in the TPCs in the extractable and bound fractions, and for their AAs (except C_{AA} of the bound fraction). In addition, treatment with 50 mM sorbitol at t_1 and t_2 increased the extractable TPC and their AAs (according to $DPPH^{\bullet}$, $ABTS^{\bullet+}$), although on the other hand, sorbitol did not have the same effect on the bound fraction. The different individual phenolics in the various fractions will have led to their different AAs.

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are differently susceptible to phenolics of different chemical compositions. Nenadis and Tsimidou (2002) reported that the highest AAs determined by the $DPPH^{\bullet}$ scavenging activity are expressed by compounds with more than one hydroxyl group (e.g., caffeic acid), followed by monophenolics with methoxy (e.g., sinapinic, ferulic acids) or alkyl (e.g., butylated hydroxytoluene) substituents. In comparison with $DPPH^{\bullet}$, $ABTS^{\bullet+}$ reacts significantly better with monophenolics (e.g., ferulic, *p*-coumaric acids). Due to the high content of ferulic and *p*-coumaric acids in our germinated spelt seed extracts, the higher $ABTS^{\bullet+}$ than $DPPH^{\bullet}$ scavenging activity was expected. However, not only the phenolic composition and quantities, but also the reaction conditions (e.g., reaction mechanism, time, medium) and data interpretation can have a great influence on the results (Abramovič, Grobin, Ulrih, & Cigić, 2017).

3.5. Intracellular oxidation and cellular uptake

To our knowledge, no previous studies have determined the AAs of germinated spelt seeds *in vivo*, with the main objective of defining the relationships between the AAs *in vitro* and their phenolics. Based on *in vitro* studies, we cannot assume that the compounds obtained will show the same activities *in vivo*. Therefore, whether bioactive compounds will act as antioxidants or prooxidants in *in vitro* or *in vivo* systems depends on a number of factors, such as concentration, structure, the test system used, and the substrate to be protected by them (Maurya & Devasagayam, 2010).

As we could not predict cellular AAs according to the collected results determined *in vitro*, we measured intracellular oxidation using *S. cerevisiae* as a model organism. Initially, all of the extractable and bound fractions were tested for potential toxicity with the yeast at different concentrations according to cell viability, measured as colony forming units. These data indicated that the maximum concentration of the fractions of 60 μL/mL could be added to the yeast suspension without decrease in cell viability compared to the control. Therefore, the yeast cells were treated separately for 2 h with the extractable and bound fractions at 30 μL/mL and 60 μL/mL. These data did not show any significant differences in the intracellular oxidation between these concentrations (Fig. 1). Furthermore, the extractable and bound fractions of all of the analysed samples did not show any significant differences in intracellular oxidation compared to the reference (untreated cells).

To find an explanation why the analysed samples did not show significant differences in intracellular oxidation, further HPLC-MS analysis was performed for the cellular uptake of particular compounds from these fractions, in terms of before and after the treatments of the yeast cells. These data indicated that the compounds analysed showed little or no entry into the yeast cells (Fig. S2). Consequently, the extractable and bound fractions under the five conditions (control, NN, SS, NS, NSM) had no impact on intracellular oxidation of *S. cerevisiae*.

Cigut et al. (2011) tested the cellular uptake of three hydroxycinnamic acids and caffeic acid phenethyl ester (CAPE), and uptake into/on cell was found only for CAPE, while other tested compounds did not enter the cell. This was related to the polarities of these compounds, where CAPE is much less polar than the corresponding caffeic acid. Since CAPE did not decrease the intracellular oxidation, authors assumed that it probably remained in the cell membrane and therefore did not show any effects on intracellular oxidation. They pointed out that adsorption (accumulation at an interface) should be distinguished from absorption (penetration of a molecule throughout the membrane). However, cellular uptake (penetration through the cell) and decreased intracellular oxidation was seen only for a moderately polar fraction of the ethanolic extracts of propolis, mainly containing flavonoids. The latter coincides with our findings that identified compounds (which are all very polar) correlated very poorly with C_{AA} (Table S3). Considering the data obtained here, it can be concluded that the individual phenolics cannot enter the cell, and so probably they must undergo transformation beforehand. On the other hand, Masisi, Beta, and Moghadasian (2016) summarized in the literature review on *in vivo* antioxidant studies that phenolics from wheat bran are relatively well absorbed in humans and may enhance antioxidant status. The authors also highlighted the protective effect of wheat feruloyl oligosaccharides, which enhanced rat plasma antioxidant enzyme activity and lowered oxidized glutathione and malondialdehyde levels. Heleno et al. (2015) reported that ferulic and caffeic acids are metabolised after absorption, and that their metabolites (dihydroferulic acid, 4-vinyl guaiacol, 4-ethyl guaiacol and dihydrocaffeic acid, 4-vinyl catechol, 4-ethyl catechol etc.) circulate in conjugated forms in human plasma, and as such have strong AAs. Further studies of the phenolics in extractable and bound fractions of germinated spelt seeds are needed to understand the transition into cells and the specific mechanistic pathways of these molecules.

3.6. Pearson correlation analysis

3.6.1. Correlations between DPPH, ABTS, SASA, C_{AA} and TPC

As shown in Table S3, the activities revealed using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ strongly correlated with the extractable TPC and bound TPC, while the correlations between the SASA activities and the TPCs were moderate, although positive. In contrast, C_{AA} correlated negatively with the extractable and bound TPC. The strongest correlations observed between TPC and $ABTS^{\bullet+}$ scavenging activity are in agreement with Zhao et al. (2008) for barley. High correlations between $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities with TPC was also reported by Zhou, Laux, and Yu (2004) for wheat.

The lowest correlation coefficient was between C_{AA} and the other *in vitro* tests. The close relationships between TPC and AA (except C_{AA}) during germination further showed that the accumulation of phenolics is a very important factor for increased AA. All of these data showed that the polar phenolics in the germinated spelt seeds were the major $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ scavengers. However, the low correlations of extractable and bound C_{AA} with TPCs in the present study suggested that the phenolics in these germinated spelt seeds might be the weak suppressors of lipid peroxidation in the emulsion.

3.6.2. Correlations between TPC, DPPH, ABTS, SASA, C_{AA} and individual phenolic acids

From the data given in Table S3, we can suggest that *p*-coumaric, *trans*-ferulic and caffeic acids are the main contributors to the TPC and AAs of the bound fractions. Further, for the prediction of the TPC of the extractable fraction and their AAs, only the content of ferulic acid was reliable. Although the use of SPE eliminated most of the interference

from the samples, the sum of the antioxidant compounds identified was still significantly lower than the TPC of the purified extracts, which might indicate that some unknown compounds were present at lower concentrations and contributed significantly to TPC and AAs. Abramovič et al. (2017) showed that in food samples small variations in experimental protocols can lead to significant differences in reactivities of antioxidants. This confirms that AA is not an inherent property of a compound, but is strongly influenced by the nature of the reactive target species and by the environment in which the reaction takes place. As expected, according to the polar paradox, the bound phenolic acids analysed had smaller influence on C_{AA} than on the other *in vitro* AAs.

Among the phenolic acids analysed, bound *trans*-ferulic acid had the greatest influence on the DPPH$^{\bullet}$ scavenging activity from the bound fraction. Although ferulic acid has been reported to react poorly with the DPPH reagent, Foti, Daquino, and Geraci (2004) reported that the AA of phenolics evaluated with DPPH also depended on the phenol concentration used in the experiment. The latter coincides with spelt seed extracts containing a high content of *trans*-ferulic acid. Meanwhile, among the phenolic acids, the bound caffeic acid had the highest correlation with bound ABTS$^{\bullet+}$ scavenging activity. Indeed, it has been shown that caffeic acid is a more efficient antiradical compound than *p*-coumaric acid (Brand-Williams et al., 1995). On the other hand, bound *p*-hydroxybenzoic acid showed lower correlation with TPC and *in vitro* AAs. This is in agreement with Chen et al. (2020), who reported that hydroxycinnamic acids and their derivatives have good AAs. The presence of the ethylene —CH=CH— group in the hydroxycinnamic acids is considered to be key for the significantly higher AAs they show compared to hydroxybenzoic acids.

Bound *p*-coumaric, *trans*-ferulic and caffeic acids have stronger inhibitory effects on $O_2^{\bullet-}$ than other minor phenolics in these germinated spelt seed extracts, as the bound fraction showed lower correlation with SASA than the major individual phenolic acids. Indeed, ferulic acid has been reported to have excellent inhibitory effects on $O_2^{\bullet-}$, OH$^{\bullet}$ and NO$^{\bullet}$ (Ogiwara et al., 2002). Among the phenolic acids analysed, the extractable phenolic acids (i.e., *p*-coumaric, *trans*-ferulic, caffeic acids) demonstrated better activity against ROO$^{\bullet}$ in the emulsion, while the bound phenolic acids showed negative correlations with C_{AA}. Furthermore, the bound *p*-hydroxybenzoic acid had moderate correlation with TPC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ of the bound fraction. These data are consistent with various studies that have reported that bound phenolics show significantly higher antioxidant activities *in vitro* than extractable ones (Chen et al., 2017).

Which phenolics, either alone or in synergism with others, contribute the most to the biological effects of these germinated spelt seeds analysed remains an open question. It is worth noting that chemical interactions between different phenolics (e.g., synergism, antagonism) can take place, and might explain the data obtained in the present study.

3.7. Multivariate analysis

Linear discriminant analysis was performed to classify the extractable and bound fractions of these germinated spelt seed extracts (control, NN, SS, NS, NSM) based on their levels of phenolic acids (i.e., *p*-coumaric, *trans*-ferulic, caffeic, *p*-hydroxybenzoic) determined by HPLC-MS, their TPCs and their *in vitro* (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, SASA, C_{AA}) and *in vivo* (intracellular oxidation) AAs.

Altogether, 10 parameters were included in the analysis. Fig. 3 shows the discriminant score plots of the first two functions obtained by multivariate LDA for the extractable and bound fractions of these germinated spelt seed extracts. Overall, the accuracy of the placement of each sample into its corresponding group (extractable or bound fraction) was 100.0%. Furthermore, the first discriminant function (F1) explained 91% and the second discriminant function (F2) explained 5% of the total variance (Fig. 3). As can be seen, very good separation between the extractable and bound fractions was achieved for the samples. The discrimination between these different fractions is shown along the first discriminant function, with *p*-hydroxybenzoic acid, TPC, caffeic acid and *p*-coumaric acid, and for the second discriminant function, with *p*-coumaric acid, *trans*-ferulic acid, ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ as the most influential standardised canonical discriminant function coefficients. According to the Wilks' Lambda test, the low Wilks' Lambda value of 0.000 shows good separation between these fractions. The group of bound fractions is more heterogeneous, spread across the entire right side of the graph. In contrast, almost all of the samples from the extractable fractions are positioned in the upper left side of the graph (Fig. 3a). The initial variables in Fig. 3b show that the highest contents of all of the analysed parameters, except the intracellular oxidation, were measured in the bound fractions of these germinated spelt seeds. These initial parameters provided separation of the fractions in terms of the left and right sides of the graph. This shows that LDA is a suitable approach to discriminate between extractable and bound fractions according to the phenolics composition, TPC and *in vitro* and *in vivo* AAs. Indeed, the score plot provided from the LDA is very important once it projects all of the samples in a two-dimensional graph, where comparisons among

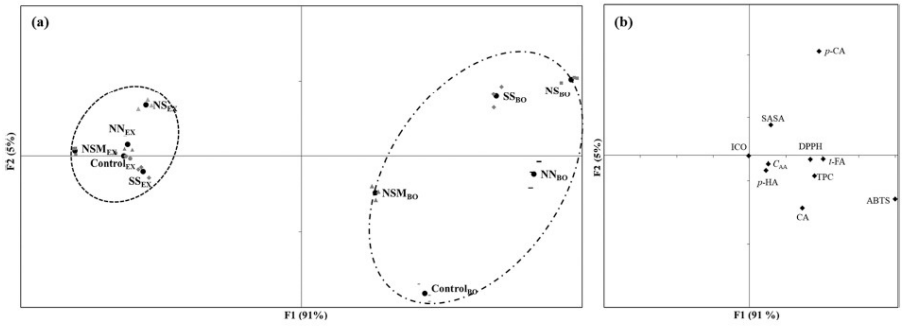

Fig. 3. (a) Discriminant score plot (F1 vs. F2) using linear discriminant analysis to classify extractable (EX) and bound (BO) fractions of the SPE-purified germinated spelt seed extracts based on the contents of *p*-coumaric acid (*p*-CA), *trans*-ferulic acid (*t*-FA), caffeic acid (CA) and *p*-hydroxybenzoic acid (*p*-HA), total phenolic content (TPC), scavenging activities against DPPH$^{\bullet}$ (DPPH), ABTS$^{\bullet+}$ (ABTS), $O_2^{\bullet-}$ (SASA), ROO$^{\bullet}$ (C_{AA}) radicals and intracellular oxidation (ICO). Control: t_0: 24 mL water, 20 °C 144 h; NN: t_0: water (18 mL); $t_1 + t_2$: 25 mM NaCl (added in 6 mL + 6 mL), at 25 °C; SS: t_0: water (18 mL); $t_1 + t_2$: 50 mM sorbitol (added in 6 mL + 6 mL), at 25 °C; NS: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); t_2: 50 mM sorbitol (added in 6 mL), at 25 °C; NSM: t_0: water (18 mL); t_1: 25 mM NaCl (added in 6 mL); 72 h: mechanical stress applied; t_2: 50 mM sorbitol (added in 6 mL), at 25 °C. t_0, immediately after seed soaking; t_1, 48 h after soaking; t_2, 96 h after soaking. (b) Plot of the correlations between the initial variables and the discriminant factors, here as F1 and F2.

samples can be performed based on the response parameters used in the study.

4. Conclusions

In this study, the germination of spelt seeds under different stress treatments was tested, with the aim being to increase the content of total and some individual antioxidants. Germination of spelt seeds with the addition of 25 mM NaCl and 50 mM sorbitol at 25 °C resulted in significant increases in the TPCs in the extractable and bound fractions and in their AAs (except C_{AA} of the bound fraction) compared to the control, where no stress was applied. Regardless of the stress condition applied, *trans*-ferulic, *p*-coumaric, caffeic and *p*-hydroxybenzoic acids were the major bound phenolics. Despite considerably lower content, extractable antioxidants were relatively more heterogeneous group. HPLC-MS analysis of samples before and after exposure of yeast cells indicated that the compounds analysed did not enter *S. cerevisiae* cells, or entered very poorly, and consequently had no impact on the intracellular oxidation of *S. cerevisiae*.

LDA satisfactorily classified the extractable and bound fractions of these germinated spelt seed extracts (control, NN, SS, NS, NSM). Indeed, the accuracy of the placement of each sample into its corresponding group as extractable or bound was 100%.

Our study suggests that the phenolics synthesised during seed germination under specific combined stress conditions (i.e., NN, SS, NS, NSM) could help to achieve increased levels of antioxidants.

CRediT authorship contribution statement

Marjeta Mencin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Helena Abramovič:** Writing - original draft, Writing - review & editing, Methodology, Supervision. **Polona Jamnik:** Writing - review & editing, Methodology, Supervision. **Maja Mikulič Petkovšek:** Data curation, Methodology, Software, Supervision, Writing - review & editing. **Robert Veberič:** Methodology, Writing - review & editing. **Petra Terpinc:** Conceptualization, Data curation, Methodology, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Assoc. Prof. Dr. Tomaž Požrl for kindly donating spelt seeds, and the Slovenian Research Agency for financial support through research programmes P4-0234 and P4-0013.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128704.

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Table S1. Different stress conditions applied during germination of the spelt seeds. All of the treatments were carried out over 144 h.

	Germination treatment			Germination conditions	
Stress	**t_0 = immediately after soaking**	**t_1 = 48 h after soaking**	**t_2 = 96 h after soaking**	**T (°C)**	**Mechanical stress**
Control	24 mL H_2O	/	/	20	
Water volume	12 mL H_2O	/	/	20	
	18 mL H_2O	/	/	20	
	30 mL H_2O	/	/	20	
Salinity	24 mL 25 mM NaCl	/	/	20	
	24 mL 50 mM NaCl	/	/	20	
	24 mL 100 mM NaCl	/	/	20	
	24 mL 200 mM NaCl	/	/	20	
Osmolarity	24 mL 50 mM sorbitol	/	/	20	
	24 mL 125 mM sorbitol	/	/	20	
	24 mL 200 mM sorbitol	/	/	20	
	24 mL 300 mM sorbitol	/	/	20	
Temperature	24 mL H_2O	/	/	40	
	24 mL H_2O	/	/	30	
	24 mL H_2O	/	/	10	
Mechanical stress	24 mL H_2O	/	/	20	Sprouts were in contact with sandpaper, which was on top of petri dish.
	24 mL H_2O	/	/	20	Sprouts were in contact with drawing pins, which were on top of petri dish.
	24 mL H_2O	/	/	20	After 72 h cutting 1/3 of sprouts.
	24 mL H_2O	/	/	20	Shaking of petri dish (3 h/day, 250 rpm).
Combined #1	18 mL H_2O	6 mL 25 mM NaCl	6 mL 25 mM NaCl	20	
	18 mL H_2O	6 mL 25 mM NaCl	6 mL 50 mM NaCl	20	
(NN)	18 mL H_2O	6 mL 25 mM NaCl	6 mL 25 mM NaCl	25	
	18 mL H_2O	6 mL 25 mM NaCl	6 mL 50 mM NaCl	25	
Combined #2	18 mL 25 mM NaCl	6 mL 25 mM NaCl	6 mL 25 mM NaCl	25	
	18 mL 25 mM NaCl	6 mL 25 mM NaCl	6 mL 50 mM NaCl	25	
(SS)	18 mL H_2O	6 mL 50 mM sorbitol	6 mL 50 mM sorbitol	25	
	18 mL 50 mM sorbitol	6 mL 50 mM sorbitol	6 mL 50 mM sorbitol	25	
(NS)	18 mL H_2O	6 mL 25 mM NaCl	6 mL 50 mM sorbitol	25	
	18 mL H_2O	6 mL 50 mM sorbitol	6 mL 25 mM NaCl	25	
(NSM)	18 mL H_2O	6 mL 25 mM NaCl	6 mL 50 mM sorbitol	25	After 72 h cutting 1/3 of sprouts.

2 **Table S2.** Identification of the phenolics in the SPE-purified extracts from the germinated spelt

3 seeds by negative-ion mode HPLC–mass spectrometry.

Phenolics	**[M-H]$^-$ (*m/z*)**	**MS^2 (*m/z*)**
p-Coumaric acid	163	119
trans-Ferulic acid	193	134, 149, 179
Caffeic acid	179	135
p-Hydroxybenzoic acid	137	93
Gallocatechin	305	261,219, 179, 165, 139, 137
p-Coumaric acid hexoside	325	163
4-feruloylquinic acid	367	193, 191, 173
Epicatechin	289	245
Apigenin hexoside pentoside	563	443
Peak I	371	193
Peak II	367	193
Peak III	595	519, 549, 323, 224, 192
Peak IV	307	261, 145
Peak V	563	164, 211

4

5

6 **Table S3.** Correlations among the total phenolic contents, *in-vitro* antioxidant activity tests, and

7 individual phenolic acids in the extractable and bound fractions.

	TPC	**DPPH**	**ABTS**	**SASA**	C_{AA}
Extractable fraction					
TPC	1.000				
DPPH	0.936	1.000			
ABTS	0.989	0.894	1.000		
SASA	0.606	0.672	0.548	1.000	
C_{AA}	-0.126	-0.105	-0.105	0.584	1.000
p-Coumaric acid	0.103	0.059	0.227	-0.043	0.343
trans-ferulic acid	0.684	0.666	0.750	0.488	0.307
Caffeic acid	-0.492	-0.543	-0.367	-0.510	0.256
p-Hydroxybenzoic acid	-0.001	0.013	0.110	-0.384	-0.087
Bound fraction					
TPC	1.000				
DPPH	0.956	1.000			
ABTS	0.990	0.978	1.000		
SASA	0.725	0.745	0.751	1.000	
C_{AA}	-0.704	-0.471	-0.618	-0.274	1.000
p-Coumaric acid	0.742	0.878	0.814	0.853	-0.067
trans-ferulic acid	0.871	0.966	0.926	0.785	-0.277
Caffeic acid	0.982	0.957	0.964	0.736	-0.651
p-Hydroxybenzoic acid	0.479	0.575	0.491	-0.098	-0.203

8 TPC, total phenolic content

9 Scavenging activities against: $DPPH^{\bullet}$ (DPPH), $ABTS^{\bullet+}$ (ABTS), $O_2^{\bullet-}$ (SASA) and $ROO^{\bullet}$ (C_{AA})

10 radicals were used for the Pearson's correlations.

11

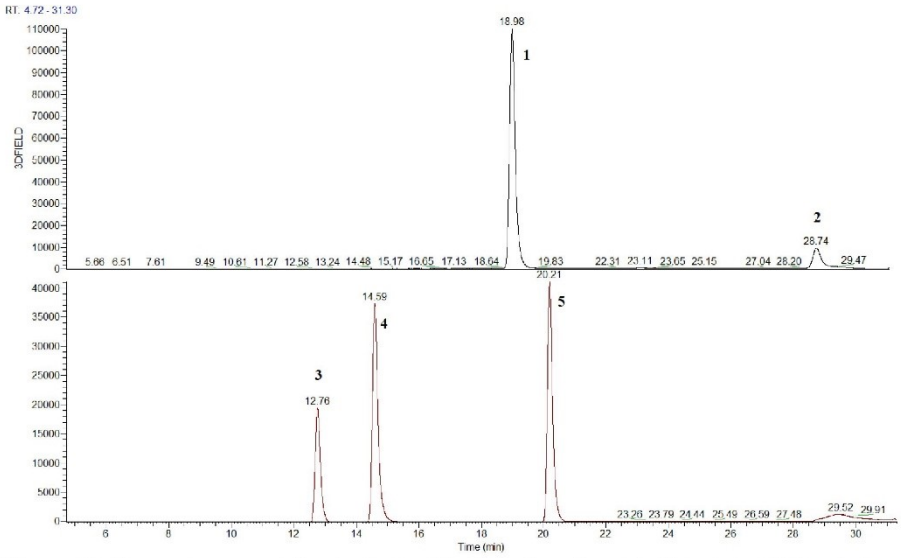

Figure S1. Chromatograms of the standards of the five phenolic acids (detected at 310 nm). 1, *p*-coumaric acid; 2, cinnamic acid; 3, *p*-hydroxybenzoic acid; 4, caffeic acid; 5, *trans*-ferulic acid.

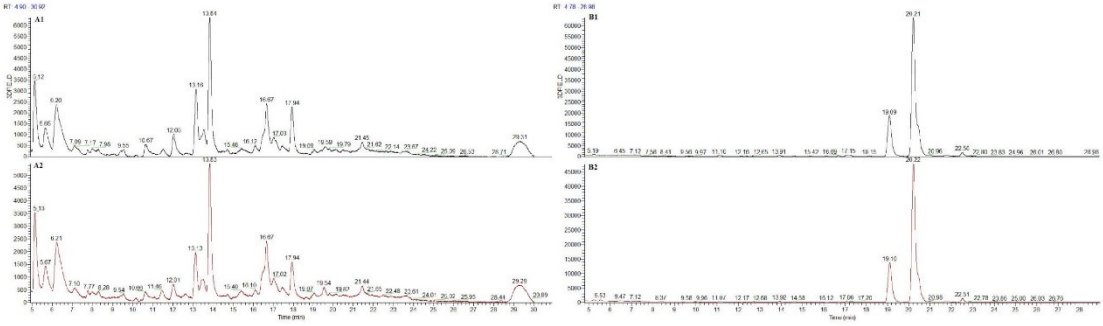

Figure S2. HPLC–mass spectrometry chromatograms (at 310 nm) of the phenolics in the supernatants after centrifugation of the suspensions of the yeast *S. cerevisiae* before (1) and after (2) 2 h exposure to the extractable (A) and bound (B) fractions from the spelt seeds.

2.2 IZBOLJŠANJE DOSTOPNOSTI IN BIOLOŠKE AKTIVNOSTI ANTIOKSIDANTOV NEOBDELANIH, KALJENIH IN ENCIMSKO OBDELANIH SEMEN PIRE (*Triticum spelta* L.) S FERMENTACIJO

Mencin M., Jamnik P., Mikulič Petkovšek M., Veberič R., Terpinc P. 2022a. Improving accessibility and bioactivity of raw, germinated and enzymatic-treated spelt (*Triticum spelta* L.) seed antioxidants by fermentation. Food Chemistry, 394: 133483, doi:10.1016/j.foodchem.2022.133483: 12 str.

Mlečnokislinska, alkoholna, kombinirana in spontana fermentacija neobdelanih, kaljenih in encimsko obdelanih pirinih semen je znatno izboljšala vsebnost ekstraktibilnih in vezanih fenolnih spojin in pomembno povečala razmerje med ekstraktibilnimi in vezanimi fenolnimi spojinami ter tako pozitivno vplivala na dostopnost pirinih antioksidantov. Največjo vsebnost ekstraktibilnih in vezanih posameznih fenolnih spojin in *in vitro* antioksidativno aktivnost smo določili v ekstraktih po fermentaciji kaljenih pirinih semen s *Saccharomyces cerevisiae*, medtem ko je bila za encimsko obdelana semena najučinkovitejša fermentacija z *Lactobacillus plantarum* (samostojno ali v kombinaciji s *S. cerevisiae*). Med ekstraktibilnimi fenolnimi spojinami se je najbolj povečala vsebnost *trans*-ferulne kisline in sicer v kaljenih semenih, fermentiranih s kvasovkami (2922 %); medtem ko smo pri vezanih fenolnih spojinah največji relativni prirast določili pri *cis*-ferulni kislini, prav tako pri fermentaciji s kvasovkami, vendar v neobdelanih pirinih semenih (466 %). Spontana fermentacija kaljenih in encimsko obdelanih vzorcev je najučinkoviteje znižala znotrajcelično oksidacijo, verjetno zaradi apigenin derivatov. Celični privzem vezanih hidroksicimetnih kislin je bil znatno večji kot privzem ekstraktibilnih hidroksicimetnih kislin; vendar so bile slednje učinkovitejši antioksidanti v *in vivo* pogojih.

Food Chemistry 394 (2022) 133483

Contents lists available at ScienceDirect

Food Chemistry

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Improving accessibility and bioactivity of raw, germinated and enzymatic-treated spelt (*Triticum spelta* L.) seed antioxidants by fermentation

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ARTICLE INFO

Keywords:
Spelt seeds
Fermentation
Lactobacillus plantarum
Saccharomyces cerevisiae
Extractable and bound phenolics
In vivo
Antioxidant activity
HPLC-MS

ABSTRACT

Lactic acid, alcoholic, combined and spontaneous fermentation of raw, germinated and enzymatic-treated spelt seeds significantly improved the content of extractable and bound phenolics and considerably increased the extractable:bound ratio, and therefore positively affected the accessibility of the spelt antioxidants. The highest extractable and bound individual phenolic contents and *in vitro* antioxidant activities of extracts were obtained following fermentation of germinated spelt seeds with *Saccharomyces cerevisiae*, while for enzymatic-treated seeds, *Lactobacillus plantarum* (alone or with *S. cerevisiae*) was the most effective. For extractable phenolics, *trans*-ferulic acid increased the most in yeast-fermented germinated seeds (2922%); for bound phenolics, *cis*-ferulic acid showed the greatest relative increase in yeast-fermented raw spelt seeds (466%). Spontaneous fermentation of germinated and enzymatic-treated samples decreased intracellular oxidation most effectively, probably due to apigenin derivatives. Cellular uptake of bound hydroxycinnamic acids was significantly higher than that of extractable hydroxycinnamic acids; however, the latter were more efficient *in vivo* antioxidants.

1. Introduction

Consumption of whole seeds can reduce the risk of type 2 diabetes, cardiovascular disease and obesity. *Triticum* seeds are considered a rich source of phenolic antioxidants (Călinoiu, Cătoi, & Vodnar, 2019; Mencin et al., 2021). Extractable fractions of raw spelt seeds were reported to contain a relatively low levels of syringic, gallic, caffeic (Gawlik-Dziki, Świeca, & Dziki, 2012), protocatechuic, sinapic and ferulic acids (Starzyńska-Janiszewska, Stodolak, Socha, Mickowska, & Wywrocka-Gurgul, 2019). However, the bound fraction contained much larger amounts of *p*-coumaric, *p*-hydroxybenzoic, ferulic, sinapic, and chlorogenic acids (Gawlik-Dziki et al., 2012). After germination, *p*-coumaric, *trans*-ferulic, caffeic and *p*-hydroxybenzoic acids were detected in both extractable and bound fractions. In extractable fraction, *p*-coumaric acid hexoside, 4-feruloylquinic acid, gallocatechin, epicatechin and apigenin hexoside pentoside were also detected (Mencin et al., 2021). However, Anson et al. (2009) determined low bioaccessibility of the most abundant ferulic acid from wheat fractions and bread (<1%), since it is esterified or covalently bound to arabinoxylan. Contrary, its bioaccessibility became higher when free ferulic acid was added to flour (~60%). In order to be metabolised and have their beneficial health-promoting effects, phenolic acids must be first released from the food matrix, and absorbed through the gastrointestinal tract.

New bioprocessing approach should be considered to increase extractable phenolics in the cereal matrix in order to improve its bioaccessibility. Fermentation, germination and enzymatic treatments represent new ways to improve the accessibility of bioactive compounds and offer the advantage of being more cost- and energy-efficient and environmentally friendly (Wang, He, & Chen, 2014).

The enzymes produced by microorganisms under fermentation conditions can modify plant constituents and subsequently enhance the release of phenolics from cereal matrices (Katina et al., 2007a). These enzymes can cut the chemical bonds of the bran phenolics from matrices such as rye bran (Katina et al., 2007b), buckwheat, wheat germ and barley (Đorđević, Šiler-Marinković, & Dimitrijević-Branković, 2010). Katina et al. (2007b) reported that fermentation of native and germinated rye with yeast increased the level of free phenolic acids 3 to10-fold, the highest increase being obtained with germinated rye. Dorđević et al. (2010) reported that TPC increased by 5% in buckwheat extract fermented with *Saccharomyces cerevisiae* and by 17% in extract

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https://doi.org/10.1016/j.foodchem.2022.133483
Received 11 February 2022; Received in revised form 12 May 2022; Accepted 12 June 2022
Available online 14 June 2022

fermented with *L. rhamnosus* compared to non-fermented sample. In wheat, TPC increased by 14% and 28% in extract fermented with *S. cerevisiae* and with *L. rhamnosus,* respectively, and in barley TPC increased by 13% and 23% in extract fermented with *S. cerevisiae* and with *L. rhamnosus,* respectively. Anson et al. (2009) reported that the most effective treatment was the combination of external enzymes and fermentation, which increased the bioaccessibility of ferulic acid from 1.1% to 5.5%. Furthermore, Koistinen et al. (2017) reported that ferulic acid bioaccessibility was significantly greater in the bread with the bioprocessed (yeast fermentation + enzymatic treatment) rye bran compared to bread made with native rye bran (88% vs. 51%, respectively).

S. cerevisiae has been shown to produce enzymes such as β-glucosidases, carboxylesterases and possibly feruloyl esterases (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004). *Lactobacillus plantarum* belongs to the lactic acid bacteria (LAB), which are most frequently encountered in the fermentation of plant samples in which phenolics are abundant.

However, the metabolism of phenolics is still largely unknown. Hydroxybenzoic and hydroxycinnamic acids can be decarboxylated to the corresponding phenol or vinyl derivatives by LAB or hydrogenated by phenolic acid reductases (Verni, Verardo, & Rizzello, 2019). Cinnamate carboxylyase activity, which decarboxylate coumaric and ferulic acids, has been demonstrated in *S. cerevisiae* strains as well (Verni et al., 2019). Further, feruloyl esterases, which can release ferulic acid and other cinnamic acids, have been described in LAB (Gänzle, 2014), and hypothesised in *S. cerevisiae* (Coghe et al., 2004). Several studies have investigated the impact on the release of phenolics of bioprocessing with baker's yeast and LAB, without and with addition of commercial enzymes, both in wheat bran (Coghe et al., 2004; Manini et al., 2014) and rye bran (Katina et al., 2007b; Koistinen, Katina, Nordlund, Poutanen, & Hanhineva, 2016). In all cases, significant increases have been seen, especially for ferulic acid.

Although the effect of fermentation on the release of bound phenolics and thus on improving their accessibility has been already explored, most studies have been conducted *in vitro*. In our research, we also determine antioxidant activity on the cellular organism (yeast), which gives us insight into *in vivo* environment. Our study showed for the first time the effects of individual and various combined bioprocessing techniques on (individual) phenolics content and their *in vitro* and *in vivo* antioxidant activity in *Triticum* seeds. The development of combinations of bioprocessing techniques seems to be a promising approach to improve the bioaccessibility of health-promoting compounds in cereals, which can be used as nutritional and quality (flavour, specific volume) improvers in breads and may contribute to better human nutrition, since bread is a daily consumed staple food. To the best of our knowledge, no studies have used lactic acid, alcoholic, combined (LAB + yeast) and spontaneous fermentation to investigate the effects of these fermentation types on different substrates (raw, germinated, enzymatic-treated spelt seeds). Therefore, the aim of this study was to investigate the effects of these different types of fermentation on the total and individual phenolics contents and *in vitro* and *in vivo* antioxidant activities in raw, germinated and enzymatic-treated spelt seeds. The aim was also to determine whether the combination of different bioprocessing techniques (i.e., fermentation, germination, enzymatic treatment) has synergistic effects on the total content and the individual profiles of the bioactive compounds.

2. Materials and methods

2.1. Materials

Spelt (*Triticum spelta* L. cv. Ostro) seeds were obtained from a local mill in the Dolenjska region of Slovenia. The germination of raw spelt seeds was performed according to Mencin et al. (2021), in the dark at 25 °C, for 144 h, with addition of 25 mM NaCl after 48 h of germination, and 50 mM sorbitol after 96 h. The enzymatic treatment of milled and freeze-dried raw spelt seeds was performed at 40 °C for 4 h, by the addition of cellulase (25 U/g dry weight [DW]), xylanase (5 U/g DW), feruloyl esterase (10 U/g DW), protease (50 U/g DW) and amylase (50 U/g DW). The fermentation was performed for milled and freeze-dried raw, germinated and enzymatic-treated spelt seeds. Before analysis, the raw, germinated, enzymatic-treated and fermented spelt seeds were freeze-dried at −50 °C and 30 mTorr, to a moisture level < 5%.

2.2. Chemicals

Methanol (99.9%), formic acid, glucose, De Man, Rogosa and Sharpe (MRS) broth, sodium hydroxide, sodium dihydrogen phosphate dihydrate and sodium carbonate were from Merck (Darmstadt, Germany). 2,2-Azino-bis-3-ethylbenzotiazolin-6-sulphonic acid (ABTS) reagent, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) reagent, Folin–Ciocalteu reagent, Trolox, sodium dihydrogen phosphate dihydrate, 2′,7′-dichlorodihydrofluorescein diacetate, *p*-hydroxybenzoic acid, *trans*-ferulic acid, *p*-coumaric acid, caffeic acid, gallocatechin, 4-vinylphenol, 4-vinylguaiacol and phenolphthalein were from Sigma-Aldrich (Steinheim, Germany). Agar, casein peptone and yeast extract were from Biolife (Milan, Italy). Phosphate-buffered saline was from Oxoid (Hampshire, England). All chemicals and reagents used for the present study were of analytical quality. Water (Milli-Q; Millipore) was used to prepare the working solutions.

2.3. Preparation of inoculum

The LAB *L. plantarum* (LMG 6907) used was originally isolated from plant material, and was obtained from the Institute of Dairy Science and Probiotics, Department of Animal Science, Biotechnical Faculty, University of Ljubljana (Slovenia). The yeast *S. cerevisiae* ZIM 2155 was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty, University of Ljubljana. Liquid MRS and liquid yeast extract peptone dextrose (YEPD) media were used to prepare the inoculum of *L. plantarum* and *S. cerevisiae*, respectively.

An aliquot of *L. plantarum* stock culture (50 μL) was inoculated into 20 mL sterilised liquid MRS medium. After overnight cultivation at 30 °C on a shaker at 150 rpm, 2 mL of the culture were centrifuged at 14000 × *g* for 5 min. The biomass obtained was washed once with saline followed by preparation of the *L. plantarum* suspension by adding 2 mL saline.

The *S. cerevisiae* culture was inoculated onto fresh YEPD agar and incubated at 28 °C for 72 h. A single yeast colony was transferred to 20 mL sterilised liquid YEPD medium and cultivated overnight at 28 °C on a shaker at 150 rpm. The procedure was then the same as for *L. plantarum*.

2.4. Fermentation

Optimisation of LAB, yeast and spontaneous fermentation conditions was performed on 10 g DW raw spelt seeds in 50 mL polyethylene centrifuge tubes. In a preliminary study, we optimised the substrate-to-saline ratio (1:1.5, 1:2), fermentation time (0 h, 24 h, 48 h, 72 h), temperature (25 °C, 30 °C) and inoculum volume added (1% [0.25 mL], 3% [0.75 mL]). We also found here that sterilisation of the spelt seeds resulted in enormous losses of the phenolics. Therefore, the spelt seed samples were not sterilised, as the primary goal was to increase the phenolics.

Four different types of fermentation were applied to raw, germinated and enzymatic-treated spelt seeds: (i) *L. plantarum* (lactic acid fermentation); (ii) *S. cerevisiae* (alcoholic fermentation); (iii) *L. plantarum* + *S. cerevisiae* (combined fermentation); and (iv) spontaneous fermentation (without adding inoculum). The optimised conditions were the substrate-to-saline ratio of 1:1.5 (10 g:15 mL), fermentation for 72 h at 30 °C under static conditions, and 3% (0.75 mL) inoculum. In the case of combined fermentation, 3% *L. plantarum* and 3% *S. cerevisiae* inoculum were added to the substrate. Spelt seeds treated by the same procedure

and inoculated with an equal volume of sterile saline (0.75 mL) were used as controls. All of the samples were freeze dried and then stored at −20 °C until further analysis. Fermentation of each sample was performed in parallel. The samples were collected before and after fermentation to measure pH, total titratable acidity (TTA) and CO_2 release and after fermentation to measure phenolics.

2.5. Preparation of extractable and bound phenolics

The extraction of extractable and bound phenolics was performed according to Mencin et al. (2021); briefly, aliquots of the homogenised and freeze-dried fermented seeds were mixed with 99.9% methanol at a ratio of 1:9 (w/v). After shaking for 2 h at room temperature, the samples were centrifuged (9793.9 × g, 10 min) and filtered (pore size, 0.45 μm). These filtered supernatants from the extractions contained the extractable phenolics. The solid residues after methanol extraction were treated by adding 2 M sodium hydroxide (20 mL) and shaking for 4 h at room temperature. After hydrolysis, the samples containing the released phenolics were acidified to pH 3 with concentrated formic acid. These filtered hydrolysates represented solution of bound phenolics. The extracts were further used for solid-phase extraction (SPE), to purify the crude extracts and to concentrate the phenolics. The SPE was carried out according to Mencin et al. (2021). The 70% methanolic eluates from the SPE-purified extracts represented the corresponding extractable and bound fractions that contained the phenolics. All of the fractions were stored at 2 °C until analysis.

2.6. Determination of pH, total titratable acidity and CO_2 release

The pH and TTA of the extracted samples was measured according to Yin et al. (2015) with some modifications by mixing 1 g sample with 9 mL distilled water, for 5 min. The pH was measured using a pH meter (50 + DHS; XS Instruments, Carpi, Italy). For determination of TTA, this suspension was titrated against 0.1 M NaOH, using 1% phenolphthalein as the indicator. The acidity was measured according to the point at which a pink colour was maintained for 30 s. TTA was expressed as the amount of NaOH used (mL). All of these samples were analysed in duplicate.

The amount of CO_2 released was determined by measuring the weight of polyethylene centrifuge tubes in which fermentation occurred every 24 h (Kanter et al., 2020). We calculating the weight loss after 72 h of fermentation compared to weight before fermentation. CO_2 release was expressed as g CO_2 released. These samples were analysed in duplicate.

2.7. LAB and yeast growth determination

Using 1 g of each fermented sample, this was added to and mixed with 9 mL sterile saline in a sterile test-tube. The fermented samples were diluted according to Koch, and spread onto MRS or YEPD agar plates. While MRS agar was used for LAB, YEPD agar was used for growth of yeasts. To measure the growth, the inoculated plates were incubated anaerobically in an anaerobic jar at 30 °C for 48 h (MRS), and aerobically at 28 °C for 48 h (YEPD). After this incubation, the numbers of LAB and yeast colonies formed were determined by counting the colony forming units (CFU). The results are expressed as $\log_{10}$ CFU/g fresh material.

2.8. Determination of total phenolics content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method (Mencin et al., 2021). Appropriately diluted samples of the extractable and bound phenolics were mixed with water and Folin–Ciocalteu reagent. After 5 min incubation, 20% sodium carbonate was added. The samples were incubated for 1 h at room temperature in the dark. Subsequently, absorbance was measured at 765 nm using a UV–visible spectrophotometer (model 8453; Hewlett Packard, Waldbronn, Germany). A standard curve was prepared with Trolox, and the data are expressed as mg Trolox equivalents per g DW (mg TE/g DW).

2.9. HPLC-MS analysis

The samples containing the extractable and bound phenolics were analysed by HPLC (Thermo Dionex system; Thermo Scientific, San Jose, USA). The phenolics were detected at 280 nm and 310 nm. A C18 column was used (Gemini; 150 mm × 4.6 mm; 3 μm; Phenomenex, Torrance, USA), which was maintained at 25 °C. The elution solvents were 0.1% formic acid and 3% acetonitrile in double-distilled water (solvent A), and 0.1% formic acid and 3% water in acetonitrile (solvent B). The samples were eluted using the following discontinuous gradient: 0 → 15 min, 5%→20% B; 15 → 20 min, 20%→30% B; 20 → 25 min, 30% B; 25 → 30 min, 30%→90% B; 30 → 45 min, 90% B; 45 → 50 min, 90%→5% B. The injection volume was 20 μL, and the flow rate was 0.6 mL/min. All of the phenolics were identified using mass spectrometry (LTQ XL linear ion trap mass spectrometer; Thermo Fisher Scientific, USA) with electrospray ionisation operating in negative-ion mode. All of the conditions for the mass spectrometry were the same as previously reported by Mikulic-Petkovsek, Koron, and Rusjan (2020). Identification of the individual phenolics was confirmed by comparisons of the retention times and spectra with standard databases, and by adding standard solutions to samples, followed by fragmentation (Table S1). The *trans*-ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid and gallocatechin levels were calculated from the peak areas of the samples and the corresponding standards, and are expressed as μg per g fermented spelt seeds DW (μg/g DW). HPLC-MS data showed that one peak had mass spectra nearly identical to those of *trans*-ferulic acid. This peak was tentatively identified as the *cis*-isomer of ferulic acid. It was quantified using the calibration curve of the *trans*-ferulic acid, as their mass spectra were similar. For the compounds that were identified without standards, quantification was performed using chemically similar compounds as standards. Apigenin hexoside pentoside (I, II, III) and unknown C-glycosyl derivative were quantified according to *p*-coumaric acid.

2.10. In vitro antioxidant activity.

2.10.1. DPPH$^{\bullet}$ scavenging activity

The antioxidant activities of the phenolics from the fermented spelt seed extracts were determined using DPPH$^{\bullet}$ (Mencin et al., 2021). The samples were added to 0.2 mM DPPH$^{\bullet}$ solution in 99.9% methanol. The mixtures were shaken and incubated for 1 h at room temperature in the dark. Then the decrease in absorbance of the reaction mixtures was measured at 520 nm. Water was used as the blank solution. The antioxidant activities using DPPH$^{\bullet}$ (referred to as DPPH in Figures and Tables) are expressed as mg TE/g DW.

2.10.2. ABTS$^{\bullet+}$ scavenging activity

The scavenging activities of phenolics in the fermented spelt extracts were determined using ABTS$^{\bullet+}$, according to the method of Mencin et al. (2021). The working solution of ABTS$^{\bullet+}$ was mixed with phosphate buffer (pH 7.4), water and the samples, and incubated in the dark for 1 h. The antioxidant activities using ABTS$^{\bullet+}$ (referred to as ABTS in Figures and Tables) are expressed as mg TE/g DW.

2.11. In vivo antioxidant activity

2.11.1. Determination of intracellular oxidation in the yeast Saccharomyces cerevisiae.

The antioxidant activity of the extractable and bound phenolics from the fermented spelt seed extracts was estimated using 2',7'-dichlorofluorescein, which is widely used for the determination of hydrogen peroxide and hydroperoxides, and for studies of oxidative stress

(Jakubowski & Bartosz, 1997).

The 70% methanolic extractable and bound fractions of the fermented and non-fermented spelt seeds were added to yeast (*S. cerevisiae*) suspensions and incubated for 2 h at 28 °C and 220 rpm. Then, samples of the yeast suspensions were taken for determination of intracellular oxidation. The yeast cells were sedimented by centrifugation (14000 × *g*, 5 min) and washed with 50 mM potassium phosphate buffer (pH 7.8). The sediments were finally resuspended in 50 mM potassium phosphate buffer and incubated at 28 °C for 5 min. 2′,7′-Dichlorodihydrofluorescein diacetate was added to a final concentration of 10 μM (from a 1 mM stock solution in ethanol). After a 30 min incubation at 28 °C and 220 rpm, the fluorescence of the yeast cell suspensions was measured in a Safire 2 microplate reader (Tecan, Männedorf, Switzerland), with the excitation and emission wavelengths of the 2′,7′-dichlorofluorescein measured at 488 nm and 520 nm, respectively. The analysis was carried out in triplicate. The fluorescence was normalised to the optical densities of the yeast suspensions (F/OD), with the data expressed as the samples relative to the reference (cells treated only with solvent; non-treated cells). The optical density was measured at 650 nm.

2.11.2. Determination of cellular uptake

The phenolic profiles were determined before and after the 2 h exposures of the yeast cells to the samples of the extractable and bound phenolics from the fermented spelt seed extracts. The yeast cell suspensions were centrifuged (14000 × *g*, 5 min). The supernatants obtained were filtered (pore size, 0.2 μm) and analysed by LC-MS, to obtain the phenolics profiles. All of these experiments were performed in duplicate.

2.12. Statistical analysis

The seeds were fermented in two parallel sets, which were then combined. All of the analyses were performed in three parallel runs, over two separate extractions. The data are means ± standard deviation. One-way analysis of variance (ANOVA) was performed using the SPSS programme (version 22 for Windows). The comparisons of the treatment means were based on Duncan's multiple range tests, with a significance level of 0.05. Multivariate statistical analysis (principal component analysis; [PCA]) was performed to interpret the differences in all of the analysed samples, using OriginPro 2015.

3. Results and discussion

3.1. Acidity and CO_2 release of fermented spelt seeds

The most acidic samples (pH < 4) were obtained when the spelt seeds were fermented with *L. plantarum* (Table 1). When *S. cerevisiae* was used as the starter culture, enhanced alcoholic fermentation and dominance of yeast over the competing indigenous flora occurred, as indicated by the high pH remaining after the fermentation (yeast produces lower concentrations of organic acids than LAB). The acidity was significantly higher in the lactic acid fermentations (i.e., lower pH; Table 1), which was reflected in the significantly higher TTA values. Katina et al. (2007b) also reported that pH of sourdough decreased to between 4 and 5 in yeast or spontaneous fermentation, with the most acidic sourdough obtained when native or germinated wholegrain rye was fermented with LAB. In our study, effective fermentation was also obtained without added microorganisms (i.e., spontaneous fermentation), with the acidity of raw, germinated and enzymatic-treated spelt seeds significantly higher following fermentation, compared to non-fermented samples. From Table 1, it can be seen that fermentation of germinated and enzymatic-treated spelt seeds resulted in significantly lower production of acids (i.e., higher pH) than fermentation of raw spelt seeds.

The fermentation activity of yeast expressed through released of CO_2: as shown in Table 1, CO_2 release was highest when raw, germinated and enzymatic-treated spelt seeds were fermented with *S. cerevisiae*. As yeast are part of the autochthonous microbiota of spelt seeds, CO_2 release was also seen during the spontaneous fermentation. Xu et al. (2019) also found that CO_2 production was the highest in bread dough prepared with sourdoughs fermented with *S. cerevisiae*. They also reported that fermentation with lactobacilli had no effect on CO_2 production in bread dough, suggesting that CO_2 production was mainly attributable to the yeast. Here, fermentation of the germinated spelt seeds resulted in higher CO_2 release than for raw and enzymatic-treated spelt seeds. Apparently, alcoholic fermentation was intensified due to the higher nutrient contents (e.g., nitrogen, sugars) in germinated seeds, and at the same time consumed more nutrients for their growth, so they began to use the organic acids produced as nutrients, and consequently the pH after 72 h of fermentation was significantly higher than in raw spelt seeds. It should also be noted that there were autochthonous microbiota present in our samples, part of which consists of both LABs and yeast. Therefore, the fermentation might behave differently than would be expected from fermentation of sterilised samples.

3.2. LAB and yeast growth determination

The LAB and yeast CFU increased when raw, germinated and enzymatic-treated spelt seeds were inoculated with *L. plantarum* and *S. cerevisiae* (Table 1). Interestingly, the LAB and yeast CFU were lower in the combined fermentation than in the individual *L. plantarum* and *S. cerevisiae* fermentations. Also, the LAB and yeast CFU were lower in the spontaneous fermentation than in the fermentation with the inoculated *L. plantarum* or *S. cerevisiae*. These data demonstrate that LAB and yeast showed higher adaptability to the germinated and enzymatic-treated spelt seeds. Moreover, the activation of α-amylases during spelt germination or addition of α-amylases during enzymatic treatment causes the hydrolysis of starch, yielding glucose, which makes spelt seeds an excellent substrate for LAB and yeast growth. Hole et al. (2012) found that fermentation of whole grain barley and oat flours by the selected LAB strains resulted in an increase of viable cell numbers from

Table 1
Analysis of the fermentations of the raw, germinated and enzymatic-treated spelt seeds.

Fermentation type	Raw spelt					Germinated spelt					Enzymatic treated spelt				
	pH	TTA (mL)	CO_2 (g)	LAB (log CFU/g)	Yeast (log CFU/g)	pH	TTA (mL)	CO_2 (g)	LAB (log CFU/g)	Yeast (log CFU/g)	pH	TTA (mL)	CO_2 (g)	LAB (log CFU/g)	Yeasts (log CFU/g)
L. plantarum	3.49^a	1.01^d	NA	8.15	NA	3.56^a	0.92^e	NA	8.38	NA	3.73^a	1.01^d	NA	8.26	NA
S. cerevisiae	4.26^c	0.55^b	0.58^b	NA	8.11	4.91^d	0.45^b	0.63^c	NA	8.40	4.39^c	0.52^b	0.40^c	NA	8.23
L. plantarum + *S. cerevisiae*	3.82^b	0.75^c	0.35^a	8.00	7.70	3.92^b	0.68^d	0.37^a	8.30	8.28	3.73^a	0.81^c	0.32^b	8.18	8.15
Spontaneous fermentation	4.11^c	0.59^b	0.37^a	6.78	6.48	4.30^c	0.52^c	0.49^b	6.95	6.78	4.20^b	0.57^b	0.24^a	6.78	6.70
Non-fermented	6.41^d	0.25^a	NA	NA	NA	6.01^e	0.29^a	NA	NA	NA	5.89^d	0.35^a	NA	NA	NA

LAB, lactic acid bacteria; TTA, total titratable acidity NA: not analyzed.
Different letters within the same column indicate significant differences ($P < 0.05$; Duncan's multiple range tests).

approximately 7 to 9 log CFU/mL after 18 h, regardless of strain and type of cereal flour. Călinoiu et al. (2019) reported that population of *S. cerevisiae* gradually increased after 48 h, *S. cerevisiae* reached about 7.5 log CFU/g for wheat bran. They also reported that wheat bran was considered good substrate for *S. cerevisiae* growth.

3.3. Effects of fermentation on total phenolics content

Fermented **raw** spelt seeds had significantly higher extractable and bound TPCs than their non-fermented counterpart (Table 2). Greater relative increases were seen for methanol-extractable phenolics, which can probably be attributed to enzymes available to increase the levels of the phenolics. Raw spelt seeds contain endogenous enzymes and indigenous microorganisms. During fermentation, the enzymes from the seeds and microorganisms contribute to modifications of the seed composition (Katina et al., 2007a). The extractable TPCs of the raw spelt seeds significantly increased during fermentation with *S. cerevisiae* + *L. plantarum* (100%) and *S. cerevisiae* (99%), compared to the non-fermented samples. The maximum increase in bound TPCs was obtained for the fermentation with *S. cerevisiae* (25%).

For the fermented **germinated** spelt seeds, 26% and 15% increases in extractable TPCs were seen for fermentation with *S. cerevisiae* and *S. cerevisiae* + *L. plantarum*, respectively. For bound TPCs, a significant increase was seen only for fermentation with *S. cerevisiae* (16%).

Interestingly, there were large increases in extractable TPCs in the fermented **enzymatic-treated** spelt seeds. The greatest increase in extractable TPCs was seen here for fermentation with *L. plantarum* (206%). The fermentation type also had an important role for the bound TPCs, although their relative increases were lower compared to the extractable TPCs. The greatest increase in bound TPCs was seen for fermentation with *S. cerevisiae* (31%). However, the absolute increases in bound TPCs were comparable to those of the extractable TPCs.

Regardless of the fermentation type, the bound TPCs were predominant over extractable phenolics. On the other hand, there were large differences in the ratios of extractable and bound TPCs between the different types of fermented spelt seeds. For the raw seeds, the extractable to bound TPCs (E/B) ratio was significantly lower than for the germinated or enzymatic-treated samples. The lowest E/B ratio was seen for the non-fermented raw spelt seeds (0.09), and here the E/B ratio showed an almost two-fold increase after fermentation. The highest E/B ratio was seen for the germinated spelt seeds fermented with *L. plantarum* + *S. cerevisiae* (0.56) and with *S. cerevisiae* (0.56). Among the enzymatic-treated spelt seeds, the highest E/B ratio was seen for fermentation with *L. plantarum* (0.54) and for spontaneous fermentation (0.54). Interestingly, the differences in E/B ratio between the fermented and non-fermented germinated seeds were much smaller than the differences between the fermented and non-fermented raw and enzymatic-treated samples.

This study showed that both the pre-processing of spelt seeds (germination, enzymatic treatment) before fermentation and the type of fermentation have effects on the changes in TPCs of the spelt seeds. The germinated spelt used in this study can be considered as enzyme-rich material for subsequent fermentation (Katina et al., 2007b). The combination of fermentation and germination leads to additive effects, as germination results in a higher amounts of fermentable sources (e.g., sugars, nitrogen) and these increase the concentrations of cell-wall-degrading enzymes, all of which can contribute to increased bioaccessibility of phenolic acids (Wang et al., 2014). Enzymatic treatment as a pre-processing technique also allows the phenolics to be freed from fibre esters, to improve the bioavailability of these compounds (Angelino et al., 2017). However, in the enzymatic treatment, only specific enzymes and concentrations and combinations were tested, whereas in nature during germination, the relationship between the specific substrate and the enzymes will already be optimised, and therefore higher efficiency can be expected. Our data can be explained on the basis that the levels of these bioactive compounds are modified during fermentation by the metabolic activity of the microorganisms. As many spelt phenolics are esterified and bound, the increase in extractable TPCs in fermented spelt seeds might be due to the actions of certain enzymes (depending on the microorganisms used), such as cellulases, esterases and β-glucosidases, which can potentially release bound phenolics during fermentation. Lee, Hung, and Chou (2008) found that the β-glucosidases produced by microorganisms during fermentation was responsible for the phenolic content increase in black bean koji.

Other studies have also reported increases in extractable TPCs after fermentation. In a study by Moore, Cheng, Su, and Yu (2006), three commercially available yeast preparations were shown to increase extractable TPCs by 50%, 100% and 69% in fermented wheat bran compared to their controls. Katina et al. (2007b) reported that the use of *S. cerevisiae* significantly contributed to increases in easily extractable TPCs, by 110%, while germination resulted in an 87% increase compared to non-germinated seeds. Dorđević et al. (2010) also reported that fermentation with *S. cerevisiae* increased extractable TPCs of buckwheat, wheat, barley and rye by 5%–23%. Furthermore, Călinoiu

Table 2
Total phenolic content (TPC) and *in-vitro* antioxidant activities (scavenging activities against DPPH• [DPPH] and ABTS•+ [ABTS] radicals) for the extractable and bound fractions from the fermentations of the raw, germinated and enzymatic-treated spelt seeds.

Spelt seeds	Fermentation type	TPC (mg TE/g DW)		Antioxidant activity (mg TE/g DW)			
				DPPH		ABTS	
		Extractable	Bound	Extractable	Bound	Extractable	Bound
Raw	*L. plantarum*	1.28 ± 0.01^{b}	8.64 ± 0.12^{B}	0.18 ± 0.00^{e}	1.05 ± 0.01^{D}	1.13 ± 0.02^{c}	8.02 ± 0.15^{C}
	S. cerevisiae	1.35 ± 0.02^{c}	9.74 ± 0.37^{C}	0.12 ± 0.01^{b}	1.16 ± 0.04^{E}	1.16 ± 0.01^{d}	8.54 ± 0.12^{D}
	L. plantarum + *S. cerevisiae*	1.36 ± 0.06^{c}	8.77 ± 0.12^{B}	0.14 ± 0.00^{c}	0.97 ± 0.02^{C}	1.14 ± 0.02^{cd}	8.05 ± 0.10^{C}
	Spontaneous fermentation	1.27 ± 0.01^{b}	8.92 ± 0.17^{B}	0.15 ± 0.00^{d}	0.96 ± 0.02^{B}	1.09 ± 0.01^{b}	7.57 ± 0.04^{B}
	Non fermented	0.68 ± 0.02^{a}	7.78 ± 0.08^{A}	0.06 ± 0.00^{a}	0.75 ± 0.03^{A}	0.44 ± 0.02^{a}	6.00 ± 0.03^{A}
Germinated	*L. plantarum*	6.30 ± 0.08^{a}	12.39 ± 0.35^{A}	0.73 ± 0.01^{b}	1.68 ± 0.02^{C}	4.51 ± 0.06^{b}	8.71 ± 0.07^{A}
	S. cerevisiae	7.77 ± 0.12^{c}	13.99 ± 0.37^{B}	1.05 ± 0.02^{d}	1.96 ± 0.02^{E}	5.56 ± 0.06^{e}	9.43 ± 0.37^{C}
	L. plantarum + *S. cerevisiae*	7.10 ± 0.10^{b}	12.58 ± 0.21^{A}	0.88 ± 0.04^{c}	1.72 ± 0.02^{D}	5.42 ± 0.09^{d}	9.04 ± 0.09^{B}
	Spontaneous fermentation	6.19 ± 0.04^{a}	12.09 ± 0.17^{A}	0.88 ± 0.00^{c}	1.48 ± 0.02^{B}	5.10 ± 0.05^{c}	8.44 ± 0.07^{A}
	Non fermented	6.15 ± 0.23^{a}	12.09 ± 0.32^{A}	0.63 ± 0.01^{a}	1.04 ± 0.02^{A}	4.25 ± 0.11^{a}	8.68 ± 0.20^{A}
Enzymatic treated	*L. plantarum*	3.92 ± 0.15^{d}	7.26 ± 0.21^{C}	0.49 ± 0.02^{e}	0.96 ± 0.03^{C}	2.93 ± 0.07^{b}	7.62 ± 0.23^{D}
	S. cerevisiae	3.49 ± 0.08^{b}	8.72 ± 0.18^{E}	0.39 ± 0.02^{c}	1.20 ± 0.04^{D}	3.11 ± 0.16^{cd}	7.13 ± 0.07^{B}
	L. plantarum + *S. cerevisiae*	3.55 ± 0.03^{b}	8.30 ± 0.17^{D}	0.41 ± 0.01^{d}	1.19 ± 0.02^{D}	3.04 ± 0.04^{bc}	7.31 ± 0.05^{BC}
	Spontaneous fermentation	3.76 ± 0.09^{c}	6.96 ± 0.27^{B}	0.37 ± 0.01^{b}	0.73 ± 0.02^{B}	3.22 ± 0.08^{d}	5.91 ± 0.18^{A}
	Non fermented	1.28 ± 0.05^{a}	6.66 ± 0.15^{A}	0.11 ± 0.00^{a}	0.63 ± 0.02^{A}	0.91 ± 0.01^{a}	7.53 ± 0.23^{CD}

Data are means ± standard deviation.
Different letters within the same fraction (extractable or bound) and the same type of spelt seeds (raw, germinated, enzymatic treated) indicate significant differences ($P < 0.05$; Duncan's multiple range tests).

et al. (2019) showed that in case of wheat bran, a 112% increase in TPC was observed after 72 h of fermentation, compared to the control. Hole et al. (2012) reported that fermentation with LAB also affected the changes in bound TPCs, by increasing their contents in barley and decreasing them in oats. They linked the increases in the bound fraction in barley to the increased soluble dietary fibre content and the consequent easier extractability of the bound fraction. Soluble dietary fibre appears to be a better substrate for feruloyl esterases compared to insoluble dietary fibre. The increase in soluble dietary fibre might be a consequence of the action of various endogenous polysaccharide-hydrolysing enzymes (Hole et al., 2012).

3.4. Effects of fermentation on the phenolics profile

The types of fermentation and substrate (i.e., raw, germinated, enzymatic treated) had major impacts on the phenolics profiles of the samples, as shown in Table 3. *p*-Coumaric, *trans*-ferulic, *cis*-ferulic, caffeic and *p*-hydroxybenzoic acids were detected in both the extractable and bound fractions.

In general, extractable *trans*-ferulic acid was predominant in the fermented germinated and enzymatic treated samples, while extractable *p*-hydroxybenzoic acid was predominant in the fermented raw samples. However, fermentation of **raw** spelt seeds significantly increased all of the extractable phenolic acids contents compared to the non-fermented sample (Fig. S1). Fermentation with *S. cerevisiae* both alone and in combination with *L. plantarum* resulted in the maximum increases in extractable *p*-coumaric (490%, 331%, respectively), *cis*-ferulic (180%, 273%), caffeic (469%, 331%) and *p*-hydroxybenzoic (1813%, 1332%) acids. At the same time, fermentation with *L. plantarum* alone resulted in the greatest increase in extractable *trans*-ferulic acid (1020%) of the raw spelt seeds. Moreover, fermentation with *S. cerevisiae* alone resulted in the greatest increases in bound *p*-coumaric (61%), *trans*-ferulic (9%), *cis*-ferulic (466%) and caffeic (33%) acids, while spontaneous fermentation and fermentation with *S. cerevisiae* + *L. plantarum* resulted in the greatest increases in bound *p*-hydroxybenzoic acid (14%, 11%, respectively) (Table 3, Fig. S1).

For **germinated** spelt seeds, fermentation with *S. cerevisiae* was the major factor for the increased levels of extractable *p*-coumaric (164%) and *trans*-ferulic (2922%) acids. It should be noted that the action of *S. cerevisiae* resulted in detection of *cis*-ferulic and caffeic acids, where their concentrations were relatively high compared to all of the other spelt seed samples. Instead, the greatest increase for extractable *p*-hydroxybenzoic acid (2020%) was seen for the germinated sample simultaneously fermented with *L. plantarum* and *S. cerevisiae* (Fig. S2). On the other hand, fermentation resulted in significant decreases in bound *p*-coumaric and *trans*-ferulic acids, except for fermentation with *S. cerevisiae*, which showed no significant difference compared to the non-fermented sample (Table 3, Fig. S2). Fermentation with *S. cerevisiae* resulted in the greatest increases in bound *cis*-ferulic and caffeic acids, while fermentation with *L. plantarum* resulted in the greatest increases in bound *p*-hydroxybenzoic acid.

Changes in pH during the different types of fermentation might have provided an optimal pH for the various cell wall degrading enzymes that originate from germinated spelt seeds. For example, the greatly

Table 3
Contents of the individual phenolic acids for the extractable and bound fractions from the fermentations of the raw, germinated and enzymatic-treated spelt seeds.

Spelt seeds	Fermentation type	Phenolic acids content (μg/g DW)									
		p-Coumaric acid		*trans*-Ferulic acid		*cis*-Ferulic acid		Caffeic acid		*p*-Hydroxybenzoic acid	
		Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound
Raw	*L. plantarum*	1.19 ± 0.08^{c}	37.98 ± 1.10^{B}	8.51 ± 0.23^{e}	727.46 ± 12.64^{B}	1.63 ± 0.05^{c}	15.32 ± 0.55^{B}	0.24 ± 0.03^{b}	8.40 ± 0.47^{A}	8.88 ± 0.21^{b}	14.13 ± 0.42^{A}
	S. cerevisiae	1.71 ± 0.11^{d}	53.86 ± 2.49^{C}	2.46 ± 0.14^{b}	807.92 ± 27.98^{C}	1.37 ± 0.07^{b}	43.44 ± 0.91^{D}	0.74 ± 0.03^{d}	11.41 ± 0.99^{B}	20.47 ± 0.30^{e}	14.01 ± 0.74^{A}
	L. plantarum + *S. cerevisiae*	1.25 ± 0.04^{c}	40.90 ± 1.30^{B}	3.95 ± 0.14^{c}	687.51 ± 9.17^{A}	1.83 ± 0.07^{d}	37.32 ± 1.48^{C}	0.56 ± 0.02^{c}	8.46 ± 0.43^{A}	15.32 ± 0.41^{c}	15.08 ± 0.58^{B}
	Spontaneous fermentation	1.09 ± 0.03^{b}	33.37 ± 1.17^{A}	4.94 ± 0.14^{d}	699.58 ± 11.52^{A}	1.65 ± 0.03^{c}	37.67 ± 2.46^{C}	0.73 ± 0.01^{d}	8.09 ± 0.28^{A}	18.78 ± 0.58^{d}	15.58 ± 0.37^{B}
	Non-fermented	0.29 ± 0.01^{a}	33.40 ± 1.80^{A}	0.76 ± 0.01^{a}	738.72 ± 24.25^{A}	0.49 ± 0.01^{a}	7.67 ± 0.18^{A}	0.13 ± 0.00^{a}	8.56 ± 0.18^{A}	1.07 ± 0.01^{a}	13.64 ± 0.59^{A}
Germinated	*L. plantarum*	8.78 ± 0.80^{d}	217.87 ± 5.78^{B}	69.23 ± 1.94^{d}	1219.56 ± 21.75^{B}	6.16 ± 0.28^{a}	22.36 ± 0.28^{A}	12.46 ± 0.54^{b}	19.63 ± 0.33^{B}	49.60 ± 1.90^{b}	53.86 ± 1.98^{E}
	S. cerevisiae	14.58 ± 0.15^{e}	310.94 ± 12.63^{C}	173.45 ± 4.78^{d}	1556.25 ± 39.49^{C}	46.25 ± 4.58^{d}	73.58 ± 4.72^{D}	18.71 ± 1.07^{d}	41.49 ± 2.82^{D}	80.55 ± 3.22^{d}	36.20 ± 1.64^{D}
	L. plantarum + *S. cerevisiae*	7.35 ± 0.10^{b}	208.02 ± 2.09^{B}	25.20 ± 1.57^{c}	1244.66 ± 13.10^{B}	9.65 ± 0.48^{b}	58.19 ± 2.47^{C}	9.11 ± 0.42^{a}	26.99 ± 0.77^{C}	113.41 ± 4.19^{e}	31.43 ± 2.66^{C}
	Spontaneous fermentation	8.14 ± 0.48^{c}	187.50 ± 3.18^{A}	16.83 ± 0.39^{b}	963.80 ± 27.34^{A}	14.11 ± 0.52^{c}	44.45 ± 2.07^{B}	16.07 ± 0.52^{c}	16.22 ± 0.15^{A}	63.69 ± 3.17^{c}	24.66 ± 0.91^{B}
	Non-fermented	5.52 ± 0.73^{a}	315.74 ± 18.78^{C}	5.74 ± 0.62^{a}	1517.52 ± 66.77^{C}	ND	ND	ND	16.63 ± 0.45^{A}	5.35 ± 1.23^{a}	16.21 ± 2.37^{A}
Enzymatic treated	*L. plantarum*	3.53 ± 0.31^{e}	31.66 ± 0.92^{B}	123.65 ± 5.07^{d}	434.63 ± 18.99^{B}	3.89 ± 0.14^{c}	18.97 ± 0.97^{A}	4.41 ± 0.27^{d}	5.69 ± 0.21^{B}	35.64 ± 1.51^{d}	54.61 ± 1.61^{E}
	S. cerevisiae	1.28 ± 0.08^{c}	283.49 ± 6.89^{E}	28.07 ± 2.51^{a}	698.32 ± 13.60^{C}	2.42 ± 0.22^{a}	58.03 ± 3.69^{B}	2.55 ± 0.25^{b}	6.75 ± 0.50^{C}	30.00 ± 0.83^{c}	27.80 ± 0.43^{B}
	L. plantarum + *S. cerevisiae*	0.85 ± 0.02^{b}	268.89 ± 4.41^{D}	48.34 ± 2.23^{b}	696.96 ± 6.15^{C}	3.53 ± 0.13^{b}	76.20 ± 3.11^{C}	3.64 ± 0.05^{c}	12.00 ± 0.50^{E}	56.89 ± 3.23^{e}	30.45 ± 0.99^{C}
	Spontaneous fermentation	1.68 ± 0.16^{d}	18.80 ± 0.30^{A}	55.45 ± 1.12^{c}	303.58 ± 11.07^{A}	4.25 ± 0.28^{d}	19.07 ± 0.42^{A}	2.63 ± 0.10^{b}	1.16 ± 0.05^{A}	12.51 ± 0.22^{b}	21.98 ± 0.77^{A}
	Non-fermented	0.36 ± 0.01^{a}	204.63 ± 4.95^{C}	25.52 ± 0.89^{a}	781.68 ± 8.10^{D}	ND	ND	0.47 ± 0.01^{a}	9.34 ± 0.20^{D}	3.37 ± 0.06^{a}	33.93 ± 0.85^{D}

Data are means ± standard deviation (n = 6).
Different small letters within a column (phenolic acid) and same type of spelt seeds (raw, germinated, enzymatic treated) indicate significant differences between the extractable contents ($P < 0.05$; Duncan's multiple range tests).
Different capital letters within a column and same type of spelt seeds indicated significant difference between the bound contents ($P < 0.05$; Duncan's multiple range tests).
ND: not detectable.

increased levels of extractable phenolic acids that occurred during alcoholic fermentation, as mainly *trans*-ferulic acid, were probably due to pH-mediated activation of feruloyl esterase, which has an optimum activity at pH 6.5 (Boskov Hansen et al., 2002). Anson et al. (2009) reported that treatment of wheat with *S. cerevisiae* can increase the bioaccessibility of ferulic acid. Furthermore, Moore et al. (2007) reported that strains of *S. cerevisiae* have been shown to produce enzymes, including β-glucosidases, carboxylesterases, and possibly feruloyl esterases. This suggests that feruloyl esterase activity can be attributed to the cereals or can originate from the yeast strain. However, more detailed studies of changes in the activities of the relevant enzymes during germination and fermentation of spelt seeds are required to determine the mechanisms responsible for the enhanced nutritional value of fermented spelt seeds.

In the **enzymatic-treated** spelt seeds, synergistic activity of *L. plantarum* and *S. cerevisiae* was optimal for the increase in the extractable *p*-hydroxybenzoic acid (1588%) (Fig. S3), while addition of *L. plantarum* alone resulted in the greatest increase in extractable *p*-coumaric (881%), *trans*-ferulic (385%) and caffeic (838%) acids. At the same time, fermentation with *L. plantarum* had a major negative impact on their bound forms (Table 3). Spaggiari et al. (2020) also reported that bound phenolic acids decreased during fermentation, especially *p*-coumaric, *trans*-ferulic and sinapic acids, probably due to the metabolic properties of microorganisms that can convert phenolic acids to other microbial metabolites. External enzymes had the potential to release the most insoluble bound phenolics from the spelt, which resulted in higher extractable phenolics contents and lower bound phenolics contents.

Interestingly, among the phenolic acids, a relatively high content of extractable caffeic acid was seen for the fermented raw, germinated and enzymatic-treated spelt seeds, suggesting some metabolic activity of the microorganisms occurred. Indeed, previous studies have indicated that *Lactobacillus* spp. can produce caffeic acid by hydrolysis from chlorogenic acid, which is present in wheat (Žilić et al., 2011).

The E/B ratio of phenolic acids increased up to 10-fold, 19-fold and 11-fold, after the fermentations of the raw, germinated and enzymatic-treated spelt seeds, respectively.

Interestingly, in the present study, we did not find fermentation metabolites of phenolic acids that can be formed by decarboxylation of *p*-coumaric, ferulic and caffeic acids. This is not in agreement with studies that have reported that *L. plantarum* has an inducible phenolic acid decarboxylase that decarboxylates *p*-coumaric, ferulic and caffeic acids to their corresponding vinyl derivatives (Filannino, Bai, Di Cagno, Gobbetti, & Gänzle, 2015). One of the possible reasons for this, as reported by Filannino, Di Cagno, and Gobbetti (2018), is that only extractable hydroxycinnamic acids can be decarboxylated to the corresponding vinyl derivatives, and in the present study, the non-fermented raw, germinated and enzymatic treated samples had very low contents of extractable phenolic acids. On the other hand, Coghe et al. (2004) reported that a higher content of ferulic acid does not necessarily lead to a higher content of 4-vinyl guaiacol.

In the fermented spelt seeds, compared to the bound fraction, the extractable fraction contained not only the main phenolics mentioned so far, but also a number of other phenolics, including apigenin hexoside pentoside I, apigenin hexoside pentoside II and apigenin hexoside pentoside III, which were present at relatively high contents, as well as an unknown C-gylcosyl derivative and gallocatechin (Table 4). All of the fermentation types increased the contents of gallocatechin in raw, germinated and enzymatic-treated samples, with the greatest increases in raw spelt seeds, by up to 641% (*L. plantarum* + *S. cerevisiae*). Interestingly, apigenin hexoside pentoside I and II decreased after fermentation of the raw spelt seeds, while apigenin hexoside pentoside III increased after fermentation with *S. cerevisiae* (24%), *L. plantarum* + *S. cerevisiae* (17%) and spontaneous fermentation (14%). In germinated and enzymatic-treated spelt seeds, fermentation with *L. plantarum* resulted in the greatest increases in apigenin hexoside pentoside I (329%, 319%, respectively), apigenin hexoside pentoside II (113%, 224%) and apigenin hexoside pentoside III (259%, 172%). Table 4 shows that *L. plantarum* metabolised apigenin derivatives better than *S. cerevisiae* in the germinated and enzymatic-treated spelt seeds, whereas *L. plantarum* and *S. cerevisiae* acted antagonistically when competing for the same substrates. Fermentation with *L. plantarum* resulted in the greatest increases in the unknown C-glycosyl derivative content of the raw (55%) and enzymatic-treated (239%) spelt seeds, while fermentation with *S. cerevisiae* resulted in the greatest increase in this unknown C-glycosyl derivative of germinated spelt seeds. Knowledge of the synthesis and regulation mechanism of these phenolics during fermentation is currently very limited.

The results of the present study are in agreement with those of Konopka, Tańska, Faron, and Czaplicki (2014), who reported that alcoholic fermentation increases free ferulic acid content of wheat and rye by 10-fold, and of sourdough by 11-fold. Antognoni, Mandrioli, Potente, Taneyo Saa, and Gianotti (2019) reported that three of eight strains of *L. plantarum* tested successfully enriched dough with free ferulic acid, but despite a relatively large increase, the free ferulic acid

Table 4
Contents of the individual phenolics for the extractable fraction from the fermentations of the raw, germinated and enzymatic-treated spelt seeds.

Spelt seeds	Fermentation type	Phenolics content (μg/g DW)				
		Apigenin hexoside pentoside			Unknown C-glycosyl derivative	Gallocatechin
		I	II	III		
Raw	*L. plantarum*	1.85 ± 0.13^{b}	0.63 ± 0.04^{a}	4.46 ± 0.23^{a}	0.62 ± 0.03^{e}	2.93 ± 0.20^{c}
	S. cerevisiae	2.27 ± 0.31^{c}	0.87 ± 0.05^{c}	5.76 ± 0.52^{b}	0.55 ± 0.01^{d}	3.22 ± 0.05^{d}
	L. plantarum + *S. cerevisiae*	1.98 ± 0.02^{b}	0.73 ± 0.03^{b}	5.47 ± 0.23^{b}	0.46 ± 0.01^{c}	3.26 ± 0.06^{d}
	Spontaneous fermentation	1.40 ± 0.05^{a}	0.97 ± 0.04^{d}	5.31 ± 0.09^{b}	0.27 ± 0.00^{a}	2.47 ± 0.15^{b}
	Non-fermented sample	2.47 ± 0.05^{d}	0.95 ± 0.02^{d}	4.66 ± 0.14^{a}	0.40 ± 0.01^{b}	0.44 ± 0.02^{a}
Germinated	*L. plantarum*	12.39 ± 1.50^{d}	18.60 ± 1.08^{d}	16.68 ± 0.18^{d}	3.46 ± 0.06^{a}	12.30 ± 0.43^{b}
	S. cerevisiae	10.10 ± 0.29^{c}	10.19 ± 0.55^{b}	6.73 ± 0.87^{b}	4.83 ± 0.13^{c}	20.03 ± 0.83^{d}
	L. plantarum + *S. cerevisiae*	7.31 ± 0.23^{b}	13.55 ± 0.52^{c}	12.81 ± 0.82^{c}	3.47 ± 0.03^{a}	12.37 ± 0.79^{b}
	Spontaneous fermentation	12.34 ± 0.45^{d}	17.36 ± 0.55^{d}	7.11 ± 0.67^{b}	4.07 ± 0.45^{b}	13.69 ± 0.34^{c}
	Non-fermented sample	2.89 ± 0.52^{a}	8.73 ± 0.97^{a}	4.65 ± 0.42^{a}	ND	10.08 ± 1.50^{a}
Enzymatic treated	*L. plantarum*	4.32 ± 0.13^{e}	1.46 ± 0.12^{e}	5.73 ± 0.18^{c}	1.56 ± 0.07^{e}	3.28 ± 0.37^{c}
	S. cerevisiae	2.10 ± 0.24^{b}	0.75 ± 0.03^{b}	4.74 ± 0.31^{b}	0.73 ± 0.02^{c}	3.08 ± 0.25^{c}
	L. plantarum + *S. cerevisiae*	2.53 ± 0.06^{d}	1.34 ± 0.02^{d}	5.51 ± 0.16^{c}	0.66 ± 0.01^{b}	2.50 ± 0.12^{b}
	Spontaneous fermentation	2.27 ± 0.07^{c}	0.94 ± 0.03^{c}	4.96 ± 0.40^{b}	1.12 ± 0.02^{d}	2.59 ± 0.13^{b}
	Non fermented sample	1.03 ± 0.02^{a}	0.45 ± 0.01^{a}	2.11 ± 0.02^{a}	0.46 ± 0.01^{a}	1.90 ± 0.13^{a}

Data are means ± standard deviation (n = 6).
Different letters within a column (phenolic) and same type of spelt seeds (raw, germinated, enzymatic treated) indicate significant differences between the extractable contents ($P < 0.05$; Duncan's multiple range tests).
ND: not detectable.

content was still much lower than that of the bound ferulic acids. Călinoiu et al. (2019) reported that ferulic acid increased significantly from day 1 of fermentation, and peaked at day 3; thereafter, it decreased. They explained this decrease by possible bioconversion of ferulic acid by yeast into vanillin. They also reported that apigenin-glucoside found in wheat bran had the highest increase (42%) after 72 h of yeast fermentation, compared to the control.

3.5. Effects of fermentation on in vitro antioxidant activity

Fermentation of the **raw** spelt seeds compared to germinated spelt seeds showed the greatest relative increases in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities for both the extractable and bound fractions (Table 2). However, for the DPPH$^{\bullet}$ scavenging activity of the extractable phenolics compared to the non-fermented samples, the greatest increase was seen for *L. plantarum* (200%) fermentation of raw spelt seeds. The DPPH$^{\bullet}$ scavenging activity of the bound phenolics of the raw spelt seeds was increased the most after fermentation by *S. cerevisiae* (55%). Further, fermentation with *S. cerevisiae* showed the greatest increase in ABTS$^{\bullet+}$ scavenging activity for the extractable (164%) and bound (42%) fractions.

Fermentation of the **germinated** spelt seeds with *S. cerevisiae* appeared to have a positive effect on the antioxidant activities of the extractable and bound phenolics. Indeed, fermentation with *S. cerevisiae* resulted in the greatest increases in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities for the extractable (67%, 31%, respectively) and bound (88%, 9%) phenolics. These increases in antioxidant activity following *S. cerevisiae* fermentation might be correlated to the increases in the extractable and bound TPCs also induced by *S. cerevisiae*. Instead, fermentation with *L. plantarum* and spontaneous fermentation had no significant effects on the ABTS$^{\bullet+}$ scavenging activities of the bound fractions from germinated seeds (Table 2).

Fermentation of the **enzymatic-treated** spelt seeds with *L. plantarum* showed the greatest increase in DPPH$^{\bullet}$ scavenging activity for the extractable fractions (345%), while fermentation with *S. cerevisiae* and *L. plantarum* + *S. cerevisiae* showed the greatest increases in DPPH$^{\bullet}$ scavenging activity of the bound fractions (90%, 89%, respectively). Spontaneous fermentation showed the lowest increases in DPPH$^{\bullet}$ scavenging activity for the extractable (236%) and bound (16%) fractions (Table 2). Interestingly, among the fermented enzymatic-treated spelt seeds, the greatest increase in ABTS$^{\bullet+}$ scavenging activity for the extractable fraction was with spontaneous fermentation (254%). At the same time, spontaneous fermentation decreased the ABTS$^{\bullet+}$ scavenging activity of the bound fraction by 22%. Overall for the enzymatic-treated spelt seeds, the extractable phenolics showed significantly greater relative increases in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities than the bound phenolics.

The fermented raw, germinated and enzymatic-treated spelt seeds with higher TPCs generally showed higher DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities. Đorđević et al. (2010) compared the antioxidant properties of raw seeds of wheat and rye with those fermented with *L. rhamnosus* or *S. cerevisiae*, and reported that the samples fermented with LAB showed greater radical scavenging activities. However, Moore et al. (2007) showed that fermentation of wheat bran for 48 h using a commercial yeast significantly increased the antioxidant properties. Xiao et al. (2015) also showed that 8-day fermentation significantly enhanced the TPCs and antioxidant activities of oats. These observations are consistent with the results of Zhang, Gao, Chen, and Wang (2014), who found that bioprocessing (6 days) by different strains increased the total antioxidant activity of wheat bran. Furthermore, Călinoiu et al. (2019) reported that there was a significant increase (34%) in DPPH radical inhibition capacity of wheat bran after 72 h of fermentation.

In the present study, the antioxidant properties depended not only on the type of fermentation, but also on the type of substrate. By fermenting raw samples or combining two bioprocessing techniques (e.g., enzymatic treatment + fermentation), we have obtained greatly improved antioxidant activities here. For fermented germinated samples, the increases in antioxidant activities were lower, although the germinated samples already showed higher extractable and bound TPCs and antioxidant activities.

The synergism between antioxidants in such extracts will result in antioxidant activities that are dependent not only on the concentration of the antioxidants, but also on the structures of and interactions between the antioxidants (Adebo & Medina-Meza, 2020). For this reason, samples with similar TPCs can vary greatly in terms of their antioxidant activities.

3.6. Effects of fermentation on in vivo antioxidant activity

To the best of our knowledge, no previous studies have determined the antioxidant activities of fermented spelt seeds *in vivo*. As the antioxidant activities of phenolics in the cell cannot be predicted on the basis of *in vitro* studies, we also determined the intracellular oxidation using *S. cerevisiae* as a model organism (Fig. 1). Here, the yeast cells were treated for 2 h with the extractable and bound fractions of the fermented and non-fermented samples at 60 μL/mL. This procedure was defined in our previous study (Mencin et al., 2021), in terms of the maximum concentration of methanol for maintained yeast cell viability compared to the control (non-treated cells).

For the extractable fractions of the **raw** spelt seeds, only those fermented with *L. plantarum* and *L. plantarum* + *S. cerevisiae* significantly decreased the intracellular oxidation, by 11% and 7%, respectively, compared to the reference (non-treated cells) (Fig. 1a). Instead, all of extractable fractions of fermented **germinated** spelt seeds showed decreased intracellular oxidation. Here, spontaneous fermentation induced the greatest decrease (-28%), followed by fermentation with *L. plantarum* + *S. cerevisiae* (-26%), *S. cerevisiae* (-19%) and *L. plantarum* (-11%) (Fig. 1b). Furthermore, for the extractable fractions of the **enzymatic-treated** samples, only those fermented with *S. cerevisiae* and spontaneously showed significantly decreased intracellular oxidation (-21%, –22%, respectively) (Fig. 1c).

In contrast, for the bound fractions of the fermented raw and germinated spelt seeds, there were no significant differences in intracellular oxidation compared to the reference (Fig. 1a, b). For the bound fractions of the fermented enzymatic-treated spelt seeds, only the spontaneously fermented sample showed decreased intracellular oxidation (-20%) (Fig. 1c).

Interestingly, among the extractable and bound fractions of the non-fermented samples, only the enzymatic-treated sample showed significant decreases in intracellular oxidation (-16%, −9%, respectively). Moreover, the bound fractions of the non-fermented raw and germinated samples showed significantly increased intracellular oxidation (7%, 5%, respectively). These bound fractions will have contained pro-oxidant substances that are reduced by fermentation; furthermore, Truusalu et al. (2004) demonstrated that specific lactobacilli can reduce pro-oxidant levels and oxidative stress indices, thus improving antioxidant activities.

These data for the antioxidant activities of the fermented raw, germinated and enzymatic-treated spelt seeds indicate differences between the *in vitro* and *in vivo* systems. To better understand which phenolics were responsible for the antioxidant activities in the yeast cells, their levels were determined using HPLC-MS before and after exposure of the cells to the extractable and bound fractions (i.e., as the phenolics uptake into the yeast cells). We measured decrease in phenolics concentration after exposure of the cells, but we do not know in what form phenolics enter the cells. In the group of hydroxycinnamic acids three compounds were identified, *p*-coumaric, *trans*-ferulic and caffeic acids, and in the group of flavonoids three apigenin derivatives were identified.

Our data indicated that in samples that showed no decrease in intracellular oxidation, the phenolics analysed, hardly, if at all, entered the yeast cell. Instead, the samples that showed significantly decreased

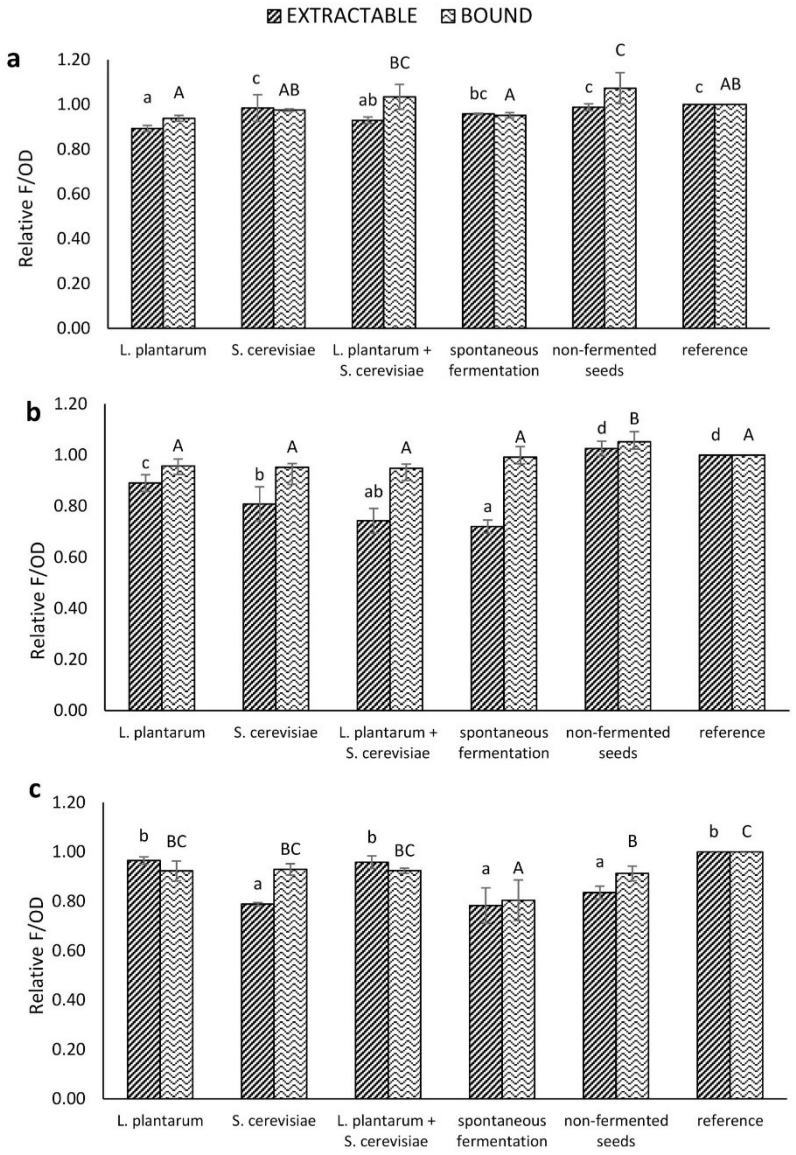

Fig. 1. Antioxidant activities determined *in vivo* (i.e., intracellular oxidation in the yeast *S. cerevisiae*) for the extractable and bound fractions for the fermented raw (**a**), germinated (**b**) and enzymatic-treated (**c**) spelt seeds, in comparison to the reference. Data are means ± standard deviation, expressed in terms of fluorescence of the yeast suspensions normalised to the optical densities (F/OD). Different letters within the same fraction (extractable, bound) indicate significant differences ($P < 0.05$; Duncan's multiple range tests).

intracellular oxidation indicted that some of the phenolics from the extractable and bound fractions had entered the cells, although with different uptake profiles (Table S2, Fig. S4, Fig. S5).

Interestingly, increased phenolics uptake did not necessarily result in higher *in vivo* antioxidant activity. For example, **raw** spelt seeds fermented with *L. plantarum* were characterised by similar *in vivo* antioxidant activities (Fig. 1), but by lower hydroxycinnamic acids uptake and higher apigenin derivatives uptake than samples fermented with

L. plantarum + *S. cerevisiae* (Table S2). Also, in the **germinated** spelt seeds fermented with *S. cerevisiae,* the total extractable phenolics uptake was significantly higher than in samples fermented with *L. plantarum* + *S. cerevisiae* and spontaneously fermented, in contrast to the smaller decrease in intracellular oxidation (Fig. 1b, Table S2). Furthermore, two-fold and three-fold more total extractable phenolics from the spontaneously fermented **enzymatic**-treated sample penetrated into the yeast cells than from the *S. cerevisiae* fermented and non-fermented samples, respectively (Table S2), but these showed no significant differences in the decreases in intracellular oxidation (Fig. 1).

The highest extractable hydroxycinnamic acids uptake for the fermented **raw** spelt seeds was seen for the samples simultaneously fermented with *L. plantarum* + *S. cerevisiae* (1.50 μg), with the highest apigenin derivatives uptake seen here for *L. plantarum* fermentation (3.08 μg) (Fig. S4). Considering that the sample fermented with *L. plantarum* had higher *in vivo* antioxidant activity, it can be concluded from this that the most represented flavonoids have greater effects on the reduction of the intracellular oxidation than the major hydroxycinnamic acids. These data are in agreement with Cigut, Polak, Gašperlin, Raspor, and Jamnik (2011), who reported that cellular uptake and decreases in intracellular oxidation were observed for propolis extracts that mainly contained flavonoids.

In the extractable fraction of the fermented **germinated** spelt seeds, the decrease in intracellular oxidation was mainly triggered by increased cellular uptake of apigenin hexoside pentosides (Table S2). The highest total apigenin hexoside pentosides uptake was 34.90 μg for spontaneous fermentation (Fig. S4), which also induced the greatest decrease in intracellular oxidation, while the highest extractable hydroxycinnamic acids uptake was 28.25 μg for the samples fermented with *S. cerevisae*. Here, *S. cerevisae* contributed to the high level of *trans*-ferulic acid uptake, while the co-presence of *L. plantarum* almost completely removed this effect. Interestingly, there was little or no uptake of extractable *p*-coumaric and ferulic acids for fermentation with *L. plantarum* and for spontaneous fermentation (Table S2). These data suggest that in addition to the TPCs that entered the cells, a key factor in the resulting antioxidant activity *in vivo* for the fermented raw and germinated spelt seeds was also the ratio between the different phenolics. When there were higher levels of flavonoids (i.e., apigenin derivatives) and lower levels of hydroxycinnamic acids, lower intracellular oxidation was seen.

For the bound phenolics fractions, only those from the spontaneously fermented and non-fermented enzymatic-treated samples showed any decrease in intracellular oxidation (Fig. S5, Table S2). The cellular uptake of bound hydroxycinnamic acids was significantly higher than that of the extractable hydroxycinnamic acids, as the bound forms were also present at significantly higher levels than the extractable forms. However, their effects on the decreases in intracellular oxidation were significantly smaller than those of the extractable hydroxycinnamic acids. We can conclude here that the extractable phenolics have significantly better *in vivo* antioxidant activities than the bound phenolics.

We have seen that spontaneous fermentation of germinated and enzymatic-treated samples showed the largest decreases in intracellular oxidation. Apparently, the diversity of the autochthonous microbiota of the spelt seeds contributed to greater diversity of metabolites formed, with new compounds that entered the cells more easily.

In our previous study (Mencin et al., 2021), we reported that the individual phenolics from spelt seeds germinated under different stress conditions did not enter the yeast cells, so it appears that they must undergo transformation beforehand. In the present study, we demonstrated that some of the fermented samples showed *in vivo* antioxidant activities. Furthermore, fermentation involves both the synthesis of new compounds as well as enzymatic transformation of various bioactive compounds already present (Heleno, Martins, Queiroz, & Ferreira, 2015).

In contrast, Cigut et al. (2011) reported that they tested the cellular uptake of *p*-coumaric, ferulic and caffeic acids, which did not enter the yeast cells, with uptake into the yeast cells only detected for caffeic acid phenethyl ester (CAPE). This was related to the polarities of these compounds, whereby CAPE is much less polar than the corresponding caffeic acid. Furthermore, Slatnar, Jakopic, Stampar, Veberic, and Jamnik (2012) showed that intracellular oxidation increased with higher consumption of hydroxycinnamic acids and lower consumption of anthocyanins in the cells. The differences in the antioxidant activities can therefore be ascribed to the individual molecular structures of the phenolics. Which of the phenolics (either alone or in synergism with others) contribute the most to the biological effects of these analysed fermented spelt seeds is still an open question.

3.7. Principal component analysis

Principal component analysis was performed to evaluate the extractable and bound fractions of the fermented and non-fermented raw, germinated and enzymatic-treated spelt seeds based on their individual phenolic acids contents and their TPCs, and on their *in vitro* ($DPPH^{\bullet}$, $ABTS^{\bullet+}$) and *in vivo* (intracellular oxidation) antioxidant activities.

Principal component 1 (PC1) explained up to 63.9% of the total variance, and PC2 explained 18.7%. Thus, as shown in the two-dimensional representation in Figure 2, 82.6% of the variability in the experimental data can be explained. The samples were separated along PC1 by differences in TPCs, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and *p*-coumaric, *trans*-ferulic, *cis*-ferulic and caffeic acids. Then PC2 separated the samples based on intracellular oxidation and *p*-hydroxybenzoic acid.

Fig. 2a shows that there are six groups that represent the extractable and bound fractions of the fermented raw, germinated and enzymatic-treated spelt seeds. These groups do not overlap, so clear separation between the different types of fermented samples is obtained. Almost all of the extractable fractions are positioned in the left side (Fig. 2a), with the exception of the extractable fraction of the germinated spelt seeds fermented with *S. cerevisiae*, which has significantly higher contents of phenolic acids and TPCs, and greater *in vitro* antioxidant activities. In contrast, the groups of the bound fractions are spread across the right side, with the exception of the bound fraction of spontaneously fermented enzymatic-treated spelt seeds, which showed significantly greater decreases in intracellular oxidation in the yeast compared to the other bound fractions.

It can also be seen that the extractable fractions of the fermented raw spelt seeds are positioned close to each other in the lower left quadrant, which suggests certain similarities in the characteristics of their phenolics; moreover, the corresponding bound fractions are also positioned close to each other. On the other hand, the groups of extractable and bound fractions of the fermented germinated and enzymatic-treated spelt seeds are more heterogeneous, which suggests that there were greater differences among these fractions. It is interesting to note that the extractable and bound fractions of the non-fermented raw, germinated and enzymatic-treated samples are positioned differently in Fig. 2a than the corresponding fermented samples, which suggests that there were much greater differences between these samples, with the exception of the bound fraction of the enzymatic-treated spelt seeds.

The initial parameters in Fig. 2b show that except for *p*-hydroxybenzoic acid and intracellular oxidation, the highest levels of all of the analysed parameters were seen for the bound fractions of these fermented and non-fermented spelt seeds.

4. Conclusions

The combination of bioprocessing techniques was the most efficient way to significantly increase the contents of extractable and bound TPCs, individual phenolic acids, and their *in vitro* (and in some cases *in vivo*) antioxidant activities. Fermentation of raw, germinated and enzymatic-treated spelt seeds significantly increased the E/B ratio and thus had a positive effect on the accessibility of the spelt antioxidants.

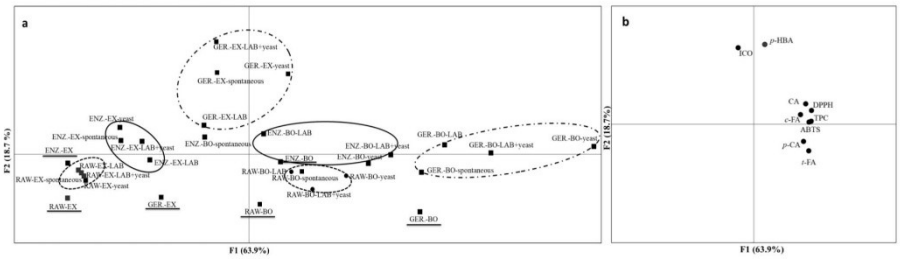

Fig. 2. (**a**) Score plot (F1 vs. F2) using principal component analysis to classify the extractable (EX) and bound (BO) fractions of the fermented and non-fermented raw, germinated (GER) and enzymatic-treated (ENZ) spelt seeds. The six groups that represent the extractable and bound fractions of the fermented raw, germinated and enzymatic-treated spelt seeds are also shown. LAB, fermentation with *L. plantarum*; yeast, fermentation with *S. cerevisiae*; spontaneous, spontaneous fermentation. (**b**) Loading plot of the correlations between the initial variables and the discriminant factors, here as F1 and F2. TPC, total phenolics content; DPPH, ABTS, scavenging activities against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$; ICO, intracellular oxidation; *p*-CA *p*-coumaric acid content; *t*-FA, *trans*-ferulic acid content; *c*-FA, *cis*-ferulic acid content; CA, caffeic acid content; *p*-HBA, *p*-hydroxybenzoic acid content.

These data show that the highest extractable and bound individual phenolics contents and *in vitro* antioxidant activities of these extracts were obtained for fermentation of the germinated spelt seeds with *S. cerevisiae*, while for the enzymatic-treated seeds, *L. plantarum* (alone or with *S. cerevisiae*) was the most effective. For extractable phenolics, *trans*-ferulic acid increased the most in yeast-fermented germinated seeds (2922%); for bound phenolics, *cis*-ferulic acid showed the greatest relative increase in yeast-fermented raw spelt seeds (466%).

The samples that showed *in vivo* antioxidant activities were mainly the extractable fractions of the fermented raw, germinated and enzymatic-treated spelt seeds, where spontaneous fermentation of the germinated and enzymatic-treated samples decreased the intracellular oxidation most effectively. Among the bound fractions for the enzymatic-treated spelt seeds, only the spontaneously fermented and non-fermented samples showed *in vivo* antioxidant activities.

The present study suggests that fermentation of spelt seeds can be used to enrich the extractable phenolics contents and antioxidant activities, which could improve their bioaccessibility. However, more detailed studies of the changes in microbial populations and activities of the relevant enzymes during the fermentation of spelt seeds are required to determine the precise mechanisms that provide fermented spelt seeds with improved nutritional value.

CRediT authorship contribution statement

Marjeta Mencin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Polona Jamnik:** Writing – review & editing, Methodology, Supervision. **Maja Mikulič Petkovšek:** Data curation, Methodology, Supervision, Writing – review & editing. **Robert Veberič:** Writing – review & editing. **Petra Terpinc:** Conceptualization, Data curation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the Slovenian Research Agency for financial support through research programmes P4-0234 and P4-0013.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133483.

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Table S1. HPLC–mass spectrometry identification of the phenolics in the extracts from the fermentations of the raw, germinated and enzymatic-treated spelt seeds.

Phenolic	**$[M-H]^-$ (*m/z*)**	**Fragment ions (*m/z*)**
p-Coumaric acid	163	119
trans-Ferulic acid	193	134, 149, 179
cis-Ferulic acid	193	134, 149, 179
Caffeic acid	179	135
p-Hydroxybenzoic acid	137	93
Gallocatechin	305	261, 219, 179, 165, 139, 137, 221, 125
Apigenin hexoside pentoside	563	443, 353, 383
Unknown C-glycosyl derivative	723	677, 451, 225

Table S2. Cellular uptake for the extractable and bound phenolics from the fermentations of the raw, germinated and enzymatic-treated spelt seeds that showed decreased intracellular oxidation in yeast cells.

Spelt seeds	Fermentation type	Cellular uptake of phenolic (μg)								
		p-Coumaric acid		*trans*-Ferulic acid		Caffeic acid		Extractable apigenin hexoside pentoside		
		Extractable	Bound	Extractable	Bound	Extractable	Bound	I	II	III
Raw	*L. plantarum*	0.12	/	0.22	/	0.63	/	0.83	0.37	1.88
	L. plantarum + *S. cerevisiae*	0.38	/	1.12	/	ND	/	0.67	0.13	1.35
Germinated	*L. plantarum*	0.02	/	0.00	/	1.74	/	3.09	2.85	5.25
	S. cerevisiae	2.02	/	24.73	/	1.50	/	3.53	14.06	4.18
	L. plantarum + *S. cerevisiae*	0.56	/	1.74	/	1.32	/	3.08	11.98	5.65
	Spontaneous fermentation	0.00	/	0.00	/	0.55	/	3.08	21.61	10.21
Enzymatic treated	*S. cerevisiae*	0.80	/	10.90	/	0.38	/	0.97	2.41	1.58
	Spontaneous fermentation	0.00	26.91	29.87	287.31	0.70	0.86	1.03	2.32	3.28
	Non-fermented	0.18	45.42	8.91	182.49	0.52	ND	0.29	0.16	0.71

ND: not detectable.

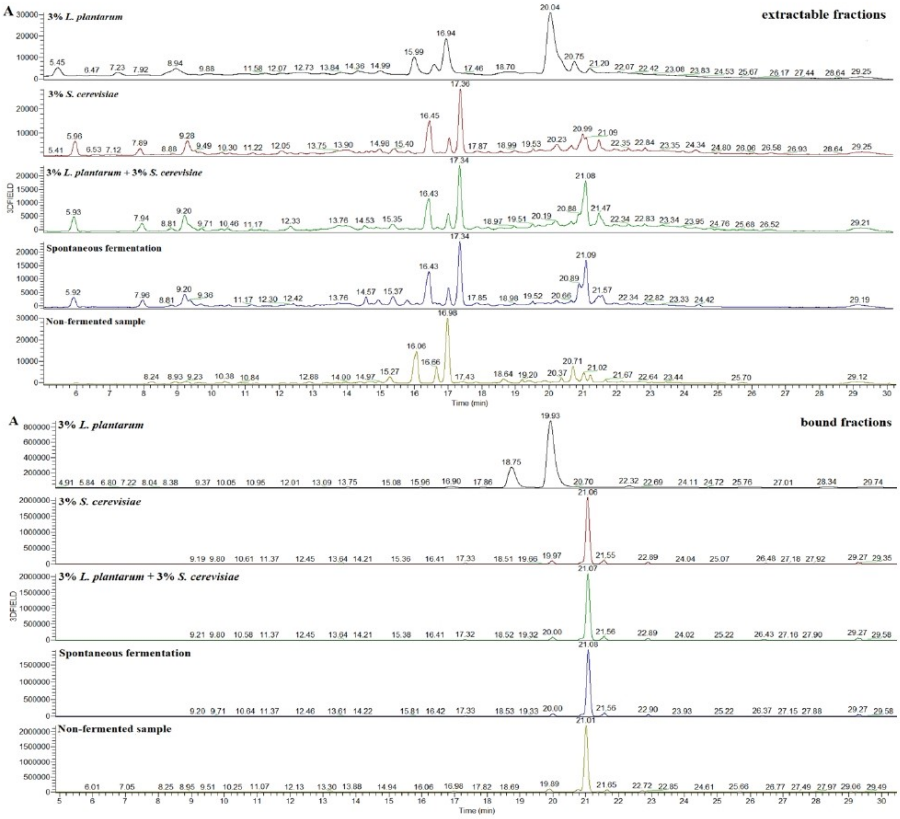

Figure S1. Representative chromatograms of the extractable (top) and bound (bottom) fractions of the raw spelt seeds (detected at 310 nm) for the different fermentation types (as indicated left).

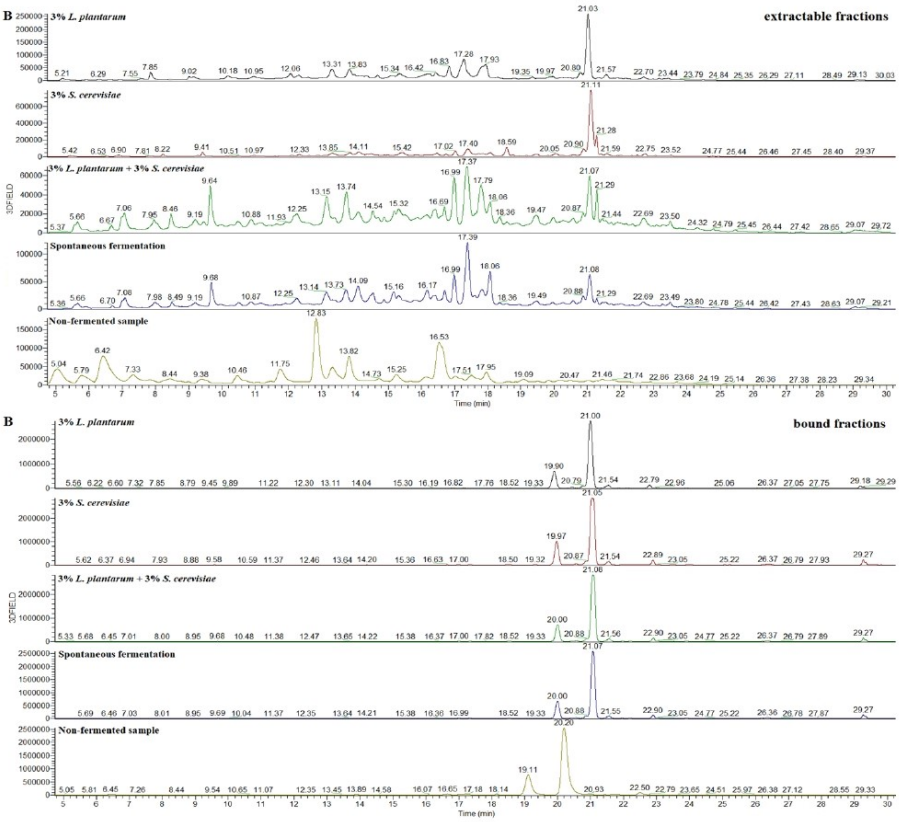

Figure S2. Representative chromatograms of the extractable (top) and bound (bottom) fractions of the germinated spelt seeds (detected at 310 nm) for the different fermentation types (as indicated left).

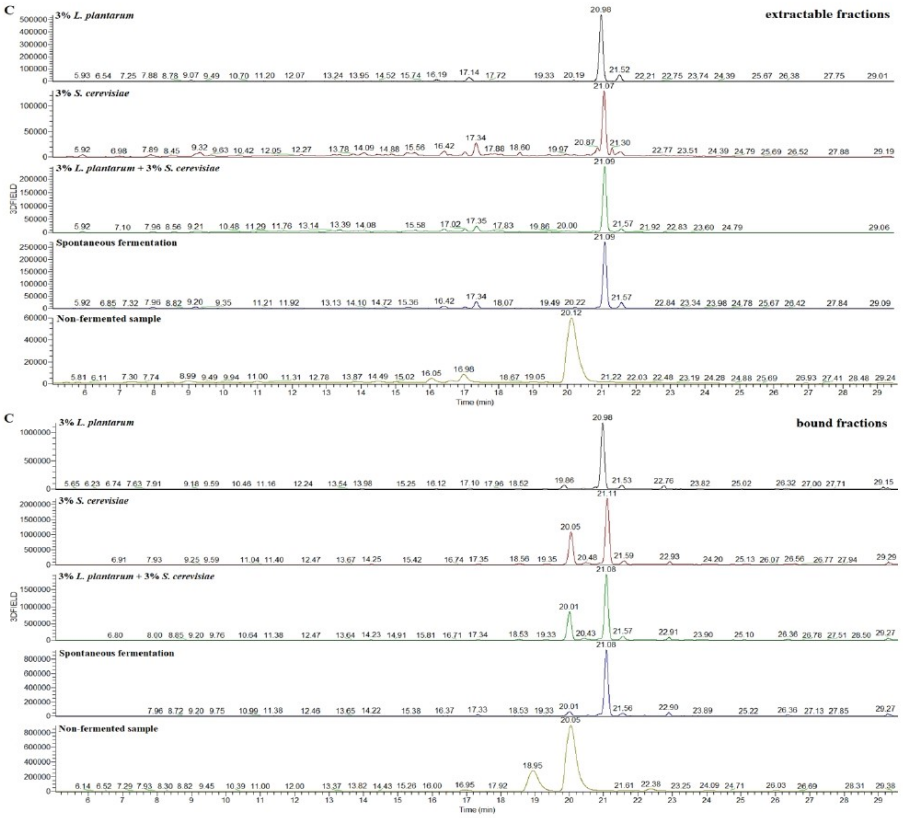

Figure S3. Representative chromatograms of the extractable (top) and bound (bottom) fractions of the enzymatic-treated spelt seeds (detected at 310 nm) for the different fermentation types (as indicated left).

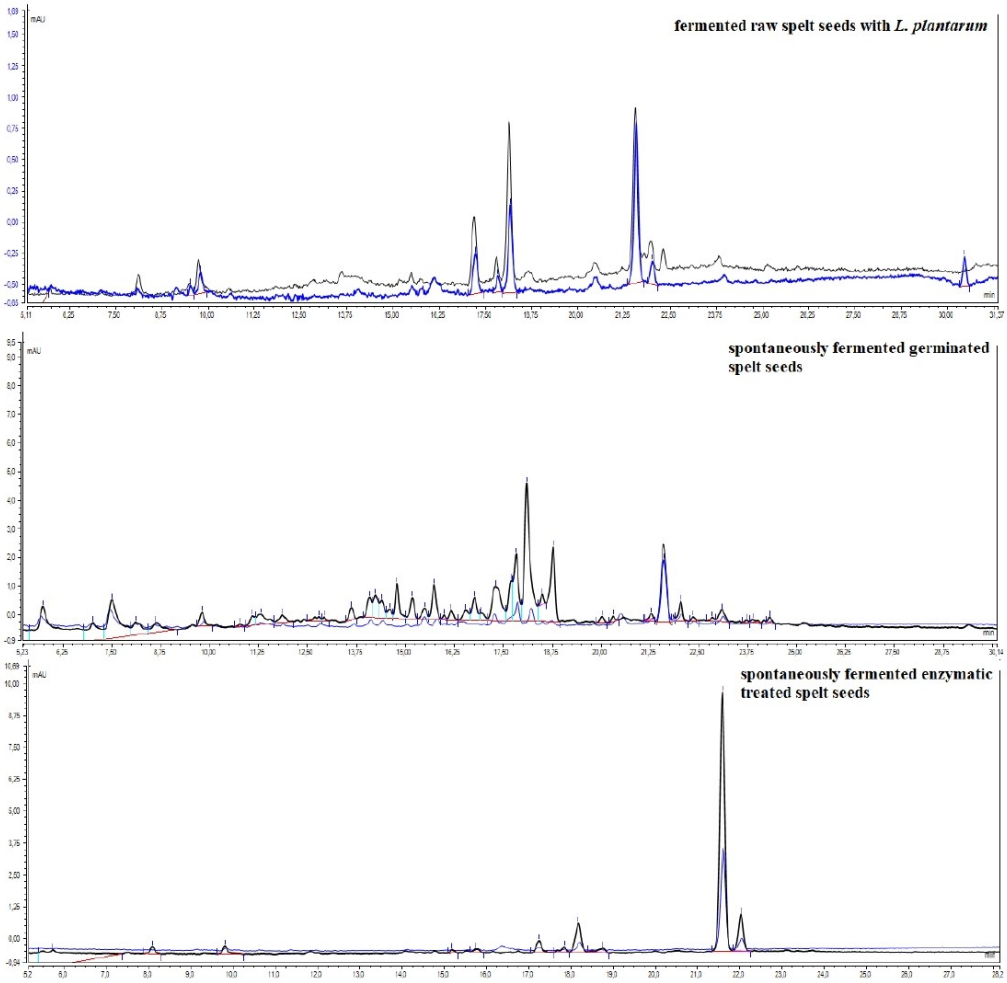

Figure S4. Representative HPLC chromatograms (detected at 310 nm) for the phenolics in the supernatants after centrifugation of the suspensions of the yeast *S. cerevisiae* before (black) and after (blue) 2 h exposure to the extractable fractions from the fermented raw, germinated and enzymatic-treated spelt seeds.

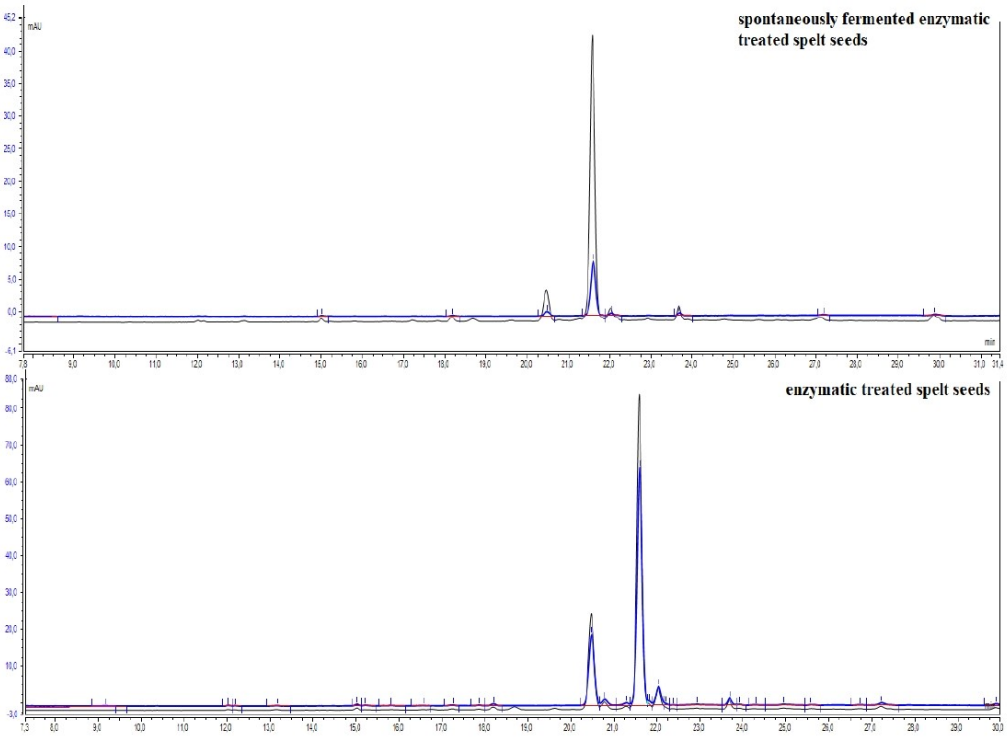

Figure S5. Representative HPLC chromatograms (detected at 310 nm) for the phenolics in the supernatants after centrifugation of the suspensions of the yeast *S. cerevisiae* before (black) and after (blue) 2 h exposure to the bound fractions from the fermented and non-fermented enzymatic-treated spelt seeds.

2.3 ENCIMSKA OBDELAVA NEOBDELANIH, KALJENIH IN FERMENTIRANIH SEMEN PIRE (*Triticum spelta* L.) IZBOLJŠA DOSTOPNOST IN ANTIOKSIDATIVNO AKTIVNOST NJIHOVIH FENOLNIH SPOJIN

Mencin M., Jamnik P., Mikulič Petkovšek M., Veberič R., Terpinc P. 2022b. Enzymatic treatments of raw, germinated and fermented spelt (*Triticum spelta* L.) seeds improve accessibility and antioxidant activity of their phenolics. LWT, 169: 114046, doi:10.1016/j.lwt.2022.114046: 13 str.

Namen naše študije je bil raziskati učinek celulaze, ksilanaze, feruloil esteraze, α-amilaze in proteaze na sproščanje fenolnih spojin iz semen pire ter ugotoviti vpliv kombiniranja encimske obdelave, kaljenja ali fermentacije na vsebnost fenolnih spojin in njihovo *in vitro* in celično antioksidativno aktivnost. Obdelava pirinih semen z različnimi kombinacijami celulaze, ksilanaze, feruloil esteraze, α-amilaze in proteaze je povečala sproščanje vezanih fenolnih spojin iz matrice pirinih semen. Vzorci, obdelani z vsemi petimi encimi skupaj, so pokazali znatno povečanje vsebnosti ekstraktibilne *p*-kumarne, ferulne in *p*-hidroksibenzojske kisline, učinkovitejšo antioksidativno aktivnost, še posebno pri ABTS testu, in največjo vsebnost ekstraktibilnih TPC. S kombiniranjem encimske obdelave s kaljenjem oz. s fermentacijo so se ekstraktibilne TPC povečale za 28 % oz. 458 %. Kombinacija encimske obdelave s kaljenjem oz. fermentacijo je nadalje vodila do velikega povečanja vsebnosti ekstraktibilne *trans*-ferulne kisline (5899 % oz. 8263 %). Vezana frakcija encimsko obdelanih neobdelanih semen je imela nižjo antioksidativno aktivnost v celici kot pripadajoča ekstraktibilna frakcija, vendar pa kar 24-krat večji celični privzem hidroksicimetnih kislin. Analiza glavnih komponent je pokazala nekatere podobnosti med ekstraktibilnimi in vezanimi frakcijami encimsko obdelanih neobdelanih, kaljenih in fermentiranih vzorcev, predvsem na račun povečanja vsebnosti ekstraktibilnih kislin, še posebej *cis*-ferulne, kavne in *p*-hidroksibenzojske kisline.

LWT - Food Science and Technology 169 (2022) 114046

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Enzymatic treatments of raw, germinated and fermented spelt (*Triticum spelta* L.) seeds improve the accessibility and antioxidant activity of their phenolics

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ARTICLE INFO

Keywords:
Spelt seeds
Hydrolytic enzymes
Extractable and bound phenolics
Cellular antioxidant activity
HPLC-MS

ABSTRACT

Our study aimed to investigate the effect of cellulase, xylanase, esterase, α-amylase and protease on the enhanced release of phenolics from spelt seeds, and to demonstrate the co-effect of enzymatic treatment, germination or fermentation on the spelt phenolics content and their *in vitro* and cellular antioxidant activities. Bound phenolics of spelt seeds were successfully released by different combinations of cellulase, xylanase, feruloyl esterase, α-amylase and protease. Samples treated with all five enzymes together showed significantly improved extractable *p*-coumaric, ferulic and *p*-hydroxybenzoic acid contents, particularly efficient antioxidant activity in $ABTS^{\bullet+}$ scavenging activity and the highest extractable TPCs. By combining enzymatic treatment with germination and fermentation, extractable TPCs were increased by 28% and 458%, respectively. A combination of enzymatic treatment with germination and fermentation led to enormously increased extractable *trans*-ferulic acid content (5899% and 8263%, respectively). The bound fraction of enzymatic treated raw seeds showed lower decrease in intracellular oxidation than the corresponding extractable fraction, but a 24-fold higher uptake of hydroxycinnamic acids. Principal component analysis showed some similarities between extractable and bound fractions of enzymatic treated raw, germinated and fermented samples, mainly due to the increase in extractable acids content (biochemical parameters), especially *cis*-ferulic, caffeic and *p*-hydroxybenzoic acids.

1. Introduction

Since there is increasing evidence of the role of bioactive compounds in disease prevention, research has been encouraged to improve the bioaccessibility and bioavailability of bioactive compounds in humans. Spelt (*Triticum spelta* L.) is an ancient subspecies of common wheat (*Triticum aestivum* L.). *Triticum* bran is a good source of dietary fibre (36–52 g/100 g of dry weight), consisting mainly of arabinoxylans (70%), cellulose (24%), and β-glucan (6%) (Bautista-Expósito et al., 2020). Bound phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids, can be bound to lignin via ether linkages with hydroxyl groups from the aromatic ring or to structural carbohydrates and proteins via ester linkages with carboxyl groups (Domínguez-Rodríguez et al., 2017). Extractable fractions of raw spelt seeds were reported to contain a relatively low levels of syringic, gallic, caffeic (Gawlik-Dziki et al., 2012), protocatechuic, sinapic and ferulic acids (Starzyńska-Janiszewska et al., 2019), while the bound fraction contained much larger amounts of *p*-coumaric, *p*-hydroxybenzoic, ferulic, sinapic, and chlorogenic acids (Gawlik-Dziki et al., 2012). However, not all of these compounds were confirmed in our recent study (Mencin et al., 2022).

Kern et al. (2003) reported that only extractable phenolic acids are available for absorption in the human small or large intestines (i.e., bioaccessible), whereas absorption of the predominantly bound phenolic acids in wheat bran has been shown to be minimal. In light of this, releasing the bound phenolic acids in spelt seeds prior to consumption by enzymatic treatment could be a strategy to improve their bioaccessibility in humans.

Enzymatic treatment is a specific and effective method for releasing bound phenolic acids from cell wall materials (Chen et al., 2015). Cell wall hydrolysing enzymes (cellulases, xylanases, etc.) have been effectively used to release bound phenolics by breaking up cell wall material from wheat bran (Acosta-Estrada et al., 2014; Moore et al., 2006; Sørensen et al., 2003). Bei et al. (2018) reported that in fermented and enzymatic treated oat the contents of gallic, chlorogenic and ferulic acid

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https://doi.org/10.1016/j.lwt.2022.114046
Received 30 May 2022; Received in revised form 26 September 2022; Accepted 29 September 2022
Available online 6 October 2022

in the soluble fraction increased by 42%, 86% and 46%, respectively. Anson et al. (2009) reported that the most effective treatment was the combination of external enzymes and fermentation, which increased the bioaccessibility of ferulic acid from 1.1% to 5.5%. Furthermore, Koistinen et al. (2017) reported that ferulic acid bioaccessibility was significantly greater in the bread with the bioprocessed (yeast fermentation + enzymatic treatment) rye bran compared to bread made with native rye bran (88% vs. 51%, respectively). Feruloyl esterases (hereinafter referred to as esterases) are a subclass of esterases that hydrolyse the ester bond between hydroxycinnamic acids (e.g., ferulic acid) and the hemicellulose present in plant cell walls. They are also capable of cleaving diferulic bridges between xylan chains, opening the structures and releasing lignin. According to previous publications (Moore et al., 2006; Sancho et al., 2001; Sørensen et al., 2003), synergistic interaction occurs during xylanase catalysing degradation of polysaccharides into lower molecular weight fragments (suitable as esterase substrates), and the esterase releasing phenolic groups (that facilitate the accessibility of xylanase to the cell wall backbone). Several enzymes (α-amylase, protease, etc.) have also been used in connection with cell wall degradation. In addition, proteins and starch are also present in the spelt matrix. Proteins constitute up to 15–17% of the bran layer; protease has therefore been explored for bran degradation (Singh et al., 2016). Phenolic acids, with their carboxyl and hydroxyl groups, are able to bond with starch and other polysaccharides via hydrogen bonds, chelation or covalent bonds (Renard et al., 2017; Yu et al., 2001; Zhu, 2018). Hydrolysis of starch by α-amylase can therefore release phenolic acids. The outer layers of cereal grains (bran) contain the highest content of total phenolics, while their content in the starch-rich endosperm is much lower (Yu et al., 2001).

Enzymatic treatment is considered to be environmentally friendly, energy efficient, specific, and releases only certain compounds without damaging other valuable chemicals (Ferri et al., 2020).

Although the effect of enzymatic treatment on the release of bound phenolics and subsequent accessibility has been already explored, most studies have been conducted *in vitro*. In present research we determine antioxidant activity on the cellular organism (*S. cerevisiae*), which gives us insight into *in vivo* environment. After determination of optimal concentration of individual enzymes, we tested their different combinations. Our aim was to investigate the effect of cellulase, xylanase, esterase, α-amylase and protease (alone or in combination), on the enhanced release of phenolics from spelt seeds, as well as to present the effect of these enzymes on the distribution of total and individual phenolics between extractable and bound fractions. In addition, the co-effect of optimal enzymatic treatment, germination or fermentation on the content of spelt phenolics and their *in vitro* and cellular antioxidant activities (AAs) was investigated.

2. Materials and methods

2.1. Materials

Spelt (*Triticum spelta* L. cv. Ostro) seeds were obtained from the Dolenjska region of Slovenia. Germination of spelt seeds was carried out according to Mencin et al. (2021), under specific abiotic stress (darkness, at 25 °C for 144 h, with the addition of 25 mmol/L NaCl after 48 h and 50 mmol/L sorbitol after 96 h of germination). Fermentation of milled and freeze-dried raw spelt seeds was carried out for 72 h at 30 °C under static conditions, the sample-to-saline ratio 1:1.5 (10 g:15 mL) and the addition of 0.75 mL of *Saccharomyces cerevisiae* inoculum. Enzymatic treatment of milled and freeze-dried raw spelt seeds was performed at 40 °C for 4 h, with the addition of cellulase (25 U/g DW), xylanase (5 U/g DW), feruloyl esterase (10 U/g DW), protease (50 U/g DW) and α-amylase (50 U/g DW) and their combinations. Furthermore, enzymatic treatment with the addition of all five enzymes together was performed in milled and freeze-dried germinated and fermented spelt seeds. Before analysis, the raw, germinated, fermented and enzymatic treated spelt seeds were freeze-dried at −50 °C and 30 mTorr (to a moisture level <5%).

2.2. Enzymes and chemicals

Methanol (99.9%), formic acid, sodium hydroxide, sodium dihydrogen phosphate dihydrate and sodium carbonate were from Merck (Darmstadt, Germany). 2,2-Azino-bis-3-etilbenzotiazolin-6-sulfonic acid diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, Trolox, 2′,7′-dichlorodihydrofluorescein diacetate, acetic acid, *p*-hydroxybenzoic acid, *trans*-ferulic acid, *p*-coumaric acid, caffeic acid, gallocatechin, α-amylase (EC 232-560-9) from *Bacillus amyloliquefaciens* (enzyme activity 250 U/g) and protease (EC 232-752-2) from *Aspergillus oryzae* (enzyme activity 500 U/g) were from Sigma-Aldrich (Steinheim, Germany). Cellulase (EC 3.2.1.4) from *Talaromyces emersonii* (enzyme activity 70 U/mg), xylanase (EC 3.2.1.8) from *Aspergillus niger* (enzyme activity 79 U/mg) and feruloyl esterase (EC 3.1.1.73) from rumen microorganism (enzyme activity 30 U/mg) were from Megazyme (Wicklow, Ireland). Phosphate-buffered saline (PBS) was from Oxoid (Hampshire, England). All of the chemicals and reagents used for the present study were of analytical quality. Water (Milli-Q; Millipore) was used to prepare the working solutions.

2.3. Optimisation of enzymatic treatment

In this study, the effect of time (2, 4 and 6 h), the sample-to-buffer ratio (1:2, 1:4 and 1:6 (w/v)) and concentration of enzymes (cellulase (10, 25, 50 U/g spelt seed (on dry weight)), xylanase (5, 10, 20 U/g), esterase (5, 10, 20 U/g), α-amylase (12.5, 25, 50 U/g) and protease (12.5, 25, 50 U/g)) were optimised. The enzymes were selected based on the structural composition of spelt seeds. The most suitable conditions (temperature and pH) for each enzyme were taken from the manufacturers' recommendations, namely, in 100 mmol/L sodium acetate buffer (pH 4.5) at 40 °C for cellulase and xylanase; in 100 mmol/L sodium phosphate buffer (pH 6) at 40 °C for esterase, α-amylase and protease. The same conditions were also used for the samples without the presence of enzymes in order to prepare a control sample.

We selected the optimal conditions for enzymatic treatment of spelt seeds by analysing extractable and bound total and individual phenolics and their antioxidant activity (AA) (data not shown). The optimised conditions for enzymatic treatment were a sample-to-buffer ratio of 1:4 (5 g: 20 mL) and time 4 h. The optimal enzyme concentrations were for cellulase 25 U/g DW, xylanase 5 U/g DW, esterase 10 U/g DW, α-amylase 50 U/g DW and protease 50 U/g DW.

2.4. Enzymatic treatment of spelt seeds

After optimising the enzymatic treatment conditions for each enzyme separately, the enzymatic treatment was performed on raw spelt seeds by different enzymes separately and in combination: cellulase (C); xylanase (X); esterase (E); cellulase + xylanase (C + X); xylanase + esterase (X + E); cellulase + xylanase + esterase (C + X + E); α-amylase (A); protease (P); esterase + α-amylase (E + A); esterase + protease (E + P); cellulase + xylanase + esterase + α-amylase (C + X + E + A); cellulase + xylanase + esterase + protease (C + X + E + P); cellulase + xylanase + esterase + α-amylase + protease (C + X + E + A + P). We selected these combinations based on other studies on the enzymatic treatment of cereal seeds. At the same time, we conducted preliminary experiments to determine which combinations gave the best results (results not shown).

Enzymatic treatments were performed on 5 g of spelt seeds mixed with 100 mmol/L sodium acetate (pH 4.5) or sodium phosphate (pH 6) buffer in 50 mL polyethylene centrifuge tubes. The tubes were rotated at 70 rpm in a Roto-Therm incubated rotator (Benchmark, Edison, New Jersey; USA). The enzymes were added after pre-equilibration for 5 min at 40 °C. Upon completion, the sample tubes were placed on ice for 5

min and then cooled with liquid nitrogen to inactivate the enzymes. The samples were freeze-dried and then stored at −20 °C until further analysis.

We extended our research with enzymatic treatment of germinated and fermented spelt seeds. Germinated and fermented spelt seeds mixed with phosphate buffer were treated with C + X + E + A + P, due to the highest results of extractable total phenolic content (TPC) and extractable *trans*-ferulic acid in the raw spelt seeds (Table 1). The following steps were performed according to the procedure described above.

2.5. Extraction of extractable and bound phenolics

Extraction of extractable and bound phenolics was performed according to Mencin et al. (2021). Filtered supernatants from the extractions with methanol contained the extractable phenolics. After hydrolysis of solid residues with 2 mol/L sodium hydroxide, the samples containing the released phenolics were acidified to pH 3 with concentrated formic acid and centrifuged. These filtered hydrolysates represented a solution of bound phenolics.

The extracts were further used for solid-phase extraction (SPE) to purify the extracts and to concentrate the phenolics. SPE was performed according to Mencin et al. (2021). The 70% methanolic eluates from the SPE-purified extracts then represented the corresponding extractable and bound fractions of phenolics. All of the samples were stored at 2 °C until analysis.

2.6. Determination of total phenolics content

The total phenolics contents (TPCs) were determined using the Folin–Ciocalteu method as described in our previous publication (Mencin et al., 2021). Appropriately diluted samples were mixed with water and Folin–Ciocalteu reagent. After 5 min of incubation, 20 g/100 mL sodium carbonate solution was added. Absorbance was measured at 765 nm using a UV–visible spectrophotometer (model 8453; Hewlett Packard, Waldbronn, Germany) after 1 h of incubation at room temperature in the dark. A standard curve was prepared with Trolox solution (6.75–27 μg/mL), and the final results are expressed as mg Trolox equivalents per g dry weight (mg TE/g DW).

2.7. Phenolics profile

The samples were analysed by HPLC (Thermo Dionex system; Thermo Scientific, San Jose, USA). The phenolics were detected at 280 nm, 310 nm and 350 nm. A C18 column was used (Gemini C18; 150 mm × 4.6 mm; 3 μm; Phenomenex, Torrance, USA), which was maintained at 25 °C. The elution solvents were 0.1% formic acid and 3% acetonitrile in double-distilled water (solvent A) and 0.1% formic acid and 3% water in acetonitrile (solvent B). The samples were eluted using the following discontinuous gradient: 0 → 15 min, 5% → 20% B; 15 → 20 min, 20% → 30% B; 20 → 25 min, 30% B; 25 → 30 min, 30% → 90% B; 30 → 45 min, 90% B; 45 → 50 min, 90% → 5% B. The injection volume was 20 μL, and the flow rate was 0.6 mL/min. All of the phenolics were identified using mass spectrometry (LTQ XL linear ion trap mass spectrometer; Thermo Fisher Scientific, San Jose, USA) with electrospray ionisation operating in negative-ion mode. The analyses were carried out using full scan data-dependent MS^n scanning from m/z 115 to 1900. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 60 and 15 units, the source voltage was 3 kV, and normalized collision energy was between 20% and 35%. Spectral data were elaborated using the Excalibur software (Thermo Scientific). Identification and quantification of phenolics were performed on the basis of their UV-VIS spectra and MS spectra and by chromatographic comparison with standards as fully described in our previous investigation (Mencin et al., 2021). The content of individual phenolics was calculated using standard curves of different phenolics. To obtain standard curves, five different concentrations for each phenolic were injected three times. HPLC-MS data

Table 1
Total phenolic content (TPC), *in vitro* antioxidant activities (scavenging activities against $DPPH^{\bullet}$ (DPPH) and $ABTS^{\bullet+}$ (ABTS) radicals) and individual phenolic acids content for the extractable and bound fractions from the raw spelt seeds treated with different combinations of enzymes.

Enzymatic treatment	TPC		DPPH		ABTS		*p* Coumaric acid		*trans* Ferulic acid		Caffeic acid		*p* Hydroxybenzoic acid	
	mg TE/g DW		mg TE/g DW		mg TE/g DW		μg/g DW		μg/g DW		μg/g DW		μg/g DW	
	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound
C	1.84^{e}	4.97^{C}	0.39^{h}	0.81^{H}	1.39^{e}	5.72^{D}	1.13^{c}	54.90^{B}	4.94^{c}	653.85^{D}	0.79^{d}	10.72^{J}	25.56^{f}	**38.79**^{J}
X	1.29^{b}	5.04^{C}	0.30^{f}	**0.86**^{I}	0.94^{b}	5.69^{D}	0.56^{b}	63.39^{C}	2.81^{b}	696.62^{E}	0.34^{a}	**11.42**^{J}	22.53^{e}	30.33^{G}
E	1.47^{c}	4.28^{A}	0.46^{i}	0.48^{D}	1.09^{c}	4.78^{A}	1.91^{f}	50.27^{B}	7.38^{de}	515.65^{A}	1.45^{g}	6.83^{DE}	24.82^{f}	37.50^{I}
C + X	2.15^{f}	4.64^{B}	0.49^{j}	0.44^{C}	1.47^{f}	5.53^{CD}	3.01^{h}	48.95^{B}	6.22^{cd}	543.95^{C}	**4.45**^{m}	5.53^{A}	28.44^{h}	28.77^{F}
X + E	1.83^{e}	4.81^{BC}	0.52^{k}	0.49^{D}	1.29^{d}	5.25^{BC}	2.33^{g}	54.08^{B}	79.13^{j}	685.01^{E}	1.82^{h}	6.22^{BC}	24.51^{f}	33.14^{H}
C + X + E	1.91^{e}	4.18^{A}	**0.54**^{l}	0.36^{A}	2.27^{i}	5.05^{AB}	1.79^{f}	48.15^{B}	77.15^{i}	485.04^{A}	1.89^{j}	6.42^{BCD}	26.81^{g}	23.42^{E}
A	2.19^{g}	5.01^{C}	0.22^{c}	0.57^{E}	1.84^{g}	**8.88**^{J}	1.38^{d}	**354.15**^{I}	5.83^{e}	**1128.23**^{J}	0.43^{b}	6.08^{B}	18.11^{d}	14.74^{B}
P	2.58^{h}	5.69^{D}	0.26^{d}	0.72^{G}	2.45^{j}	7.12^{G}	3.16^{i}	245.23^{H}	8.09^{e}	840.06^{H}	2.82^{j}	6.34^{BC}	26.68^{g}	8.51^{A}
E + A	1.56^{d}	**7.31**^{G}	0.16^{b}	0.58^{E}	1.44^{f}	8.48^{I}	1.57^{ef}	211.61^{EF}	8.48^{e}	932.58^{I}	0.57^{c}	6.61^{CD}	14.82^{c}	18.63^{C}
E + P	2.87^{j}	6.36^{E}	0.27^{e}	0.63^{F}	2.60^{k}	7.24^{G}	3.80^{j}	220.93^{G}	14.75^{f}	763.85^{G}	0.99^{e}	7.12^{E}	33.63^{j}	20.96^{D}
C + X + E + A	2.24^{g}	**7.27**^{G}	0.24^{d}	0.49^{D}	1.90^{h}	8.52^{I}	1.83^{f}	217.20^{FG}	40.08^{g}	729.86^{F}	1.09^{f}	7.63^{F}	10.90^{b}	18.56^{C}
C + X + E + P	3.22^{l}	6.33^{E}	0.34^{g}	0.63^{F}	**2.69**^{j}	6.76^{F}	**4.78**^{k}	185.29^{D}	46.39^{h}	707.16^{E}	3.83^{l}	8.07^{G}	**37.69**^{J}	9.37^{A}
C + X + E + A + P	**3.46**^{k}	6.66^{F}	0.36^{g}	0.63^{F}	**2.70**^{j}	7.53^{H}	3.28^{i}	204.63^{E}	**86.18**^{k}	781.68^{G}	3.08^{k}	9.34^{H}	29.36^{h}	33.93^{H}
Non treated	0.68^{a}	7.78^{H}	0.06^{a}	0.75^{G}	0.44^{a}	6.00^{E}	0.29^{a}	33.40^{A}	0.76^{a}	738.72^{F}	0.49^{b}	7.67^{F}	0.13^{a}	8.56^{A}

Data are means (n = 6).
Different letters within the same fraction (extractable or bound) indicate significant differences ($P < 0.05$; Duncan's Multiple Range Test).
C: cellulase; X: xylanase; E: feruloyl esterase; A: α-amylase; P: protease.

showed that one peak had mass spectra nearly identical to those of *trans*-ferulic acid. This peak was tentatively identified as the *cis*-isomer of ferulic acid. It was quantified using the calibration curve of *trans*-ferulic acid, since their mass spectra were similar (Table S1). For compounds that were identified without standards, quantification was performed using similar compounds as standards. Apigenin hexoside pentoside (I, II, III) and an unknown C-glycosyl derivative were quantified according to *p*-coumaric acid.

2.8. In vitro tests of antioxidant activity

2.8.1. DPPH$^{\bullet}$ scavenging activity

The AAs of phenolics from enzymatic treated seeds were determined using DPPH$^{\bullet}$, according to the method described in our previous publication (Mencin et al., 2021). Appropriate dilutions of the spelt seed extracts were mixed with 0.2 mmol/L DPPH$^{\bullet}$ solution in 99.9% methanol. The decrease in absorbance was measured at 520 nm after 1 h of incubation at room temperature in the dark. The AAs using DPPH$^{\bullet}$ (referred to as DPPH in Figures and Tables) was obtained from the calibration curve, which was made by preparing various concentrations of a Trolox solution (2.5–10.01 μg/mL) in methanol. The final results are expressed as mg TE/g DW.

2.8.2. ABTS$^{\bullet+}$ scavenging activity

The scavenging activities of phenolics from the spelt extracts were also determined using ABTS$^{\bullet+}$, according to the method described by Mencin et al. (2021). The working solution of ABTS$^{\bullet+}$ was mixed with phosphate buffer (0.3 mol/L, pH 7.4), water, and the appropriately diluted samples and incubated in the dark for 1 h. Then the decrease in absorbance at 734 nm was measured. AAs using ABTS$^{\bullet+}$ (referred to as ABTS in Figures and Tables) was obtained from the calibration curve, which was made by preparing various concentrations of a Trolox solution (2.5–10.01 μg/mL) in water. The final results are expressed as mg TE/g DW.

2.9. Cellular antioxidant activity

2.9.1. Determination of intracellular oxidation in the yeast Saccharomyces cerevisiae

The intracellular oxidation of the extractable and bound phenolics from the enzymatic treated spelt seed extracts was estimated using 2′,7′-dichlorofluorescein, which reacts with oxidants, thus revealing the presence of reactive oxygen species (ROS) (Petelinc et al., 2013).

The 70% methanolic extractable and bound fractions were added to the yeast (*S. cerevisiae*) suspensions and incubated for 2 h at 28 °C and 220 rpm. Samples of yeast incubations were then taken for determination of intracellular oxidation and cellular uptake. After 2 h of treatment, the yeast from cell suspensions were sedimented by centrifugation (14000×*g*, 5 min) and washed with 50 mmol/L potassium phosphate buffer (pH 7.8). The sediments were finally resuspended in 50 mmol/L potassium phosphate buffer and incubated at 28 °C for 5 min. The ROS-sensing dye, 2′,7′-dichlorodihydrofluorescein diacetate, was added to a final concentration of 10 μmol/L (from a 1 mmol/L stock solution in 96% ethanol). After a 30 min incubation at 28 °C and 220 rpm, the fluorescence of the yeast cell suspensions was measured in a microplate reader (Safire II; Tecan). The excitation and emission wavelengths of the 2′,7′-dichlorofluorescein were measured at 488 nm and 520 nm, respectively. The fluorescence (F) was normalized to the optical densities (OD) of the yeast suspensions, with the data expressed as the samples relative to the control (non-treated cells). The analysis was carried out in triplicate.

2.9.2. Determination of cellular uptake by the yeast Saccharomyces cerevisiae

To study the cellular uptake of the phenolics, the phenolic profile was determined in the PBS buffer immediately after the addition of 70% methanolic extractable or bound fractions (i.e., before yeast exposure) and after 2 h of exposure of yeast cells to extractable or bound fractions. The yeast cell suspensions were centrifuged (14000×*g*, 5 min). The supernatants obtained were filtered (pore size 0.2 μm) and analysed by HPLC–MS, to obtain the phenolics profiles. All of these experiments were performed in duplicate.

2.10. Statistical analysis

The seeds were enzymatic treated in two parallel sets, which were then combined. All of the analyses were performed in three parallel runs, on two separate extractions. The difference among various enzymatic treatments of raw spelt seeds was analysed with one-way analysis of variance (ANOVA) and Duncan's post-hoc test. The difference among enzymatic treated raw, germinated and fermented spelt seeds and their non-treated counterparts was analysed with the paired-samples *t*-test. The level of statistical significance in all of the tests was set at $p < 0.05$. The statistical analysis was performed using the SPSS programme, as version 22 for Windows. Multivariate statistical analysis (principal component analysis; [PCA]) was performed to interpret the differences in all of the analysed samples by OriginPro 2015.

3. Results and discussion

3.1. Effect of enzymatic treatment on total phenolic content

3.1.1. Effect of different enzymatic treatments on raw spelt seeds

The effects of five plant matrix degrading enzymes (alone or in combination), namely cellulase (C), xylanase (X), esterase (E), α-amylase (A) and protease (P) on the extractable and bound TPCs of spelt seeds were investigated in this study (Table 1). The enzymatic treatments improved the yield of extractable TPCs from spelt seeds up to 5-fold compared to the non-treated sample, whereas the bound TPCs decreased after enzymatic treatments.

Among different enzymatic treatments, the C + X + E + A + P treatment increased the extractable TPC the most. Interestingly, treatments with protease, alone or in combination with other enzymes, have a greater effect on the release of extractable TPCs than other treatments (Table 1). In comparison to the non-treated raw spelt seeds, which yielded 0.68 mg TE/g DW, P, E + P, C + X + E + P, and C + X + E + A + P treatments significantly increased the yields of extractable TPCs by 279%, 322%, 374% and 409%, respectively. According to our results, esterase alone did not significantly increase extractable TPCs, suggesting that this esterase may not be able specifically to hydrolyse hydroxycinnamate ester linkages in spelt seeds, due to the possible steric hindrance caused by the polysaccharide structure and the limited migration of the enzyme under the enzymatic treatment conditions.

Among the enzymatic treatments, the highest bound TPC was induced by treatments with amylase in combination with esterase (E + A and C + X + E + A) (Table 1).

Treatment with C + X + E + A + P resulted in an increase in the proportion of the extractable TPCs over total phenolics (extractable + bound) from 8% to 34%. All three experiments that included a combination of esterase and protease showed a great ability to release bound TPCs into extractable TPCs (Table 1). This result suggests that esterase in synergism can hydrolyse the ester- or ether-bond between phenolics and the cell wall structure of spelt seeds and transform bound phenolics into extractable form (Acosta-Estrada et al., 2014). Interestingly, protease was able significantly to increase the releasability of extractable TPCs from spelt seeds, suggesting that this enzyme preparation may be involved in the hydrolysation of ester and/or glycosidic bonds. Furthermore, Everette et al. (2010) showed that many non-phenolic compounds exhibit considerable reactivity toward the Folin–Ciocalteu reagent, including proteins that can vary considerably in their reactivity toward the Folin–Ciocalteu reagent. Contrary to our expectations, we found that treatments with xylanase, esterase and cellulase, alone or in

combination with each other, were less efficient. However, enzymatic treatment could not completely convert bound phenolics to the extractable form because the cell wall matrix of spelt seeds might contain arabinoxylans, which cannot be hydrolysed by enzymes (Moore et al., 2006).

Similar data were reported by Sungsopha et al. (2009), implying that after treatment of rice bran with α-amylase and protease, extractable TPCs were increased by 476% compared to non-treated samples. Moore et al. (2006) reported that treatment with commercial proteases at a level of 221 U/g resulted in the greatest increases in extractable TPC, by 5.6- and 5.1-fold for Akron and Jagalene wheat bran, respectively. Meanwhile, esterases were not able to improve TPC release from the same samples. Radenkovs et al. (2014) suggested that enzymatic treated rye bran with α-amylase and Viscozyme L (containing a wide range of carbohydrases) had the highest TPC increase (136%) compared to the non-treated sample. Alrahmany and Tsopmo (2013) also reported that four different carbohydrases increased the extractable TPC in oat bran.

3.1.2. Effect of enzymatic treatment on germinated and fermented spelt seeds

Table 2 shows the contents of the extractable and bound TPCs in germinated and fermented spelt seeds following enzymatic treatment (C + X + E + A + P). After **germination** the extractable and bound TPCs were 6.15 and 12.09 mg TE/g DW, respectively. By combining germination and enzymatic treatment, the extractable TPCs increased by 28% and 128% compared to the non-treated germinated spelt seeds and C + X + E + A + P treated raw seeds, respectively. Furthermore, the bound TPC of enzymatic treated germinated spelt seeds was 18% lower than that of germinated seeds and 48% higher than that of C + X + E + A + P treated raw seeds.

After **fermentation** with *S. cerevisiae*, the extractable and bound TPCs were 1.35 and 9.74 mg TE/g DW, respectively. The extractable TPC of enzymatic treated fermented spelt seeds was 458% higher than that of only fermented spelt seeds and 119% higher than that of raw seeds treated with C + X + E + A + P. Moreover, the bound TPC of enzymatic treated fermented spelt seeds was 22% lower than that of only fermented spelt seeds and 15% higher than only C + X + E + A + P treated raw seeds.

Our previous study found that germination under abiotic stress (Mencin et al., 2021) or fermentation with *S. cerevisiae* by itself significantly enhances TPC and AA compared to raw spelt seeds. The results of the current study revealed that additional enzymatic treatment distinctly increased the extractable TPCs when compared to solely germination or fermentation. It is worth mentioning that enzymatic treatment after germination and fermentation resulted in an increase in the proportion of extractable TPCs over total phenolics (extractable + bound) from 34% to 44% and from 12% to 50%, respectively. This could be due to the complexity of plant cell wall structures and the limitation of specific enzymes produced by *S. cerevisiae* during fermentation or activated during germination. The results of the present study further imply that the sequential application of germination/fermentation and enzymes (C + X + E + A + P) may not only favour the conversion of insoluble phenolics to extractable ones, but also weakens the ether bonds between insoluble phenolics and cell wall components, making the phenolics that are still in insoluble form easier to extract (Bei et al., 2018).

Furthermore, Liu et al. (2017) reported that fermentation with two lactic acid bacteria improved the extractable TPC from rice bran pre-treated with α-amylase. They also indicated that fermentation and treatment with amylase, protease and cellulase of rice bran significantly increased extractable TPCs, by 59% compared to the non-treated sample. Wang et al. (2018) reported that fermentation combined with enzymatic treatment increased the extractable TPC of guava leaves tea by 22% compared to the fermentation process. Sungsopha et al. (2009) observed that both germination and α-amylase and protease treatment of rice improved extractable TPCs by 5-times compared to non-treated rice bran.

3.2. Phenolics profile

3.2.1. Effect of different enzymatic treatments on raw spelt seeds

Trans-ferulic acid was the dominant bound phenolic acid detected in the present study. In the case of *p*-coumaric and *trans*-ferulic acids, their bound forms represented more than 90% of their totals (Table 1). All enzymatic treatments increased the content of extractable *p*-coumaric, *trans*-ferulic, caffeic and *p*-hydroxybenzoic acids, with the highest increase of 16-fold (C + X + E + P), 113-fold (C + X + E + A + P), 9-fold (C + X) and 290-fold (C + X + E + P), respectively, compared to the non-treated sample. The enzymes that contributed the most were protease (P; E + P; C + X + E + P; C + X + E + A + P) for extractable *p*-coumaric and *p*-hydroxybenzoic acids, a mixture of xylanase and esterase (X + E; C + X + E + A + P; C + X + E) for extractable *trans*-ferulic acid and a mixture of cellulase and xylanase (C + X; C + X + E + P; C + X + E + A + P) for caffeic acid. In contrast, treatments with xylanase had a negative impact on extractable *p*-coumaric, *trans*-ferulic and caffeic acids.

The samples that showed significantly improved extractable *p*-coumaric, ferulic and *p*-hydroxybenzoic acids contents (i.e., particularly C + X + E + A + P; C + X + E + P), also showed particularly efficient AAs in $ABTS^{\bullet+}$ scavenging activity and higher extractable TPCs. This would indicate that these phenolic acids significantly contribute to the AA of these spelt seeds and they may have the highest reactivities in the Folin–Ciocalteu assay. We also found that the extractable fractions exhibited relatively strong AAs although they had lower levels of phenolic acids, compared to the bound fractions.

Table 2
Total phenolic content (TPC) and *in vitro* antioxidant activities (scavenging activities against $DPPH^{\bullet}$ (DPPH) and $ABTS^{\bullet+}$ (ABTS) radicals) for the extractable and bound fractions from raw, germinated and fermented spelt seeds treated with all five enzymes (C + X + E + A + P).

Spelt seeds	Enzymatic treatment	TPC		DPPH		ABTS	
		mg TE/g DW		mg TE/g DW		mg TE/g DW	
		Extractable	Bound	Extractable	Bound	Extractable	Bound
Germinated	C + X + E + A + P	7.85^{b}	9.86^{A}	0.85^{b}	1.10^{B}	5.43^{b}	6.65^{A}
	non-treated	6.15^{a}	12.09^{B}	0.63^{a}	1.04^{A}	4.25^{a}	8.68^{B}
Fermented	C + X + E + A + P	7.53^{b}	7.63^{A}	0.78^{b}	0.92^{A}	5.83^{b}	5.7^{A}
	non treated	1.35^{a}	9.74^{B}	0.12^{a}	1.16^{B}	1.16^{a}	8.54^{B}
Raw	C + X + E + A + P	3.46^{b}	6.66^{A}	0.36^{b}	0.63^{A}	2.70^{b}	7.53^{B}
	non treated	0.68^{a}	7.78^{B}	0.06^{a}	0.75^{B}	0.44^{a}	6.00^{A}

Data are means.
Different letters within the same fraction (extractable or bound) and the same type of spelt seeds (raw, germinated, fermented) indicate significant differences ($P < 0.05$; Paired-Samples T Test).
C + X + E + A + P: sample was treated with cellulase (C); xylanase (X); feruloyl esterase (E); α-amylase (A); protease (P).

As can be observed from Table 1, treatments with amylase or protease alone showed the highest increase in bound *p*-coumaric and *trans*-ferulic acids contents, while the combination of cellulase, xylanase or esterase (C + X + E) caused a 34% reduction of bound ferulic acid. The highest increase in bound caffeic acid content was shown by treatment with xylanase alone, while treatment with cellulase alone was the best option for the bound *p*-hydroxybenzoic acid content.

Following cross-linking through the formation of dimers, trimers and tetramers, ferulic acid is thought to influence the physical and textural attributes of plant-based foods. The combination of xylanase and esterase (X + E) alone or in combination with other enzymes caused the highest increase in extractable *trans*-ferulic acid content, since xylanases randomly cleave the β-1,4 xylan backbone, whereas esterases are capable of releasing ferulic acid (Sancho et al., 2001). The addition of appropriate enzymes therefore has the potential to act synergistically and partly release bound phenolic acids from spelt seeds, thereby improving their bioaccessibility. We suggest that very few bound phenolics were synthesised by endogenous enzymes during this process.

Spelt bran is a rich source of dietary fibres, but it also contains 41% of starch. When considering destarched bran, the arabinose plus xylose content reached 31% for spelt (Escarnot et al., 2012). Treatment with α-amylase (A) released one of the lowest amounts of extractable phenolic acids; consequently, the content of bound fractions (especially *p*-coumaric and ferulic acids) remained the highest. We suggest that starch granules location in the plant cell might be a possible reason, since phenolic acids are usually bound to non-starch polysaccharides. Interestingly, the extractable TPC was quite high when treated with amylase. It should be noted that the Folin–Ciocalteu reagent is nonspecific, since it also responds to reducing sugars, which are a product of α-amylase activity. Alrahmany and Tsopmo (2012) reported that concentration of reducing sugar increased in α-amylase treated oat bran when compared to control (no enzyme). They also reported that the increase of reducing sugars in samples was associated with an increase in amount of other extractable phenolics. Zeng et al. (2018) reported that reducing sugars exhibited significant positive relationships with the free phenolic content in brown rice. During α-amylase treatment, the starch was remarkably degraded, which facilitated the release of phenolics from the cereal matrix, therefore, these phenolics became extractable (Zeng et al., 2018). In addition, some phenolics and starch could intensely interact to form non-covalent complex through hydrophobic

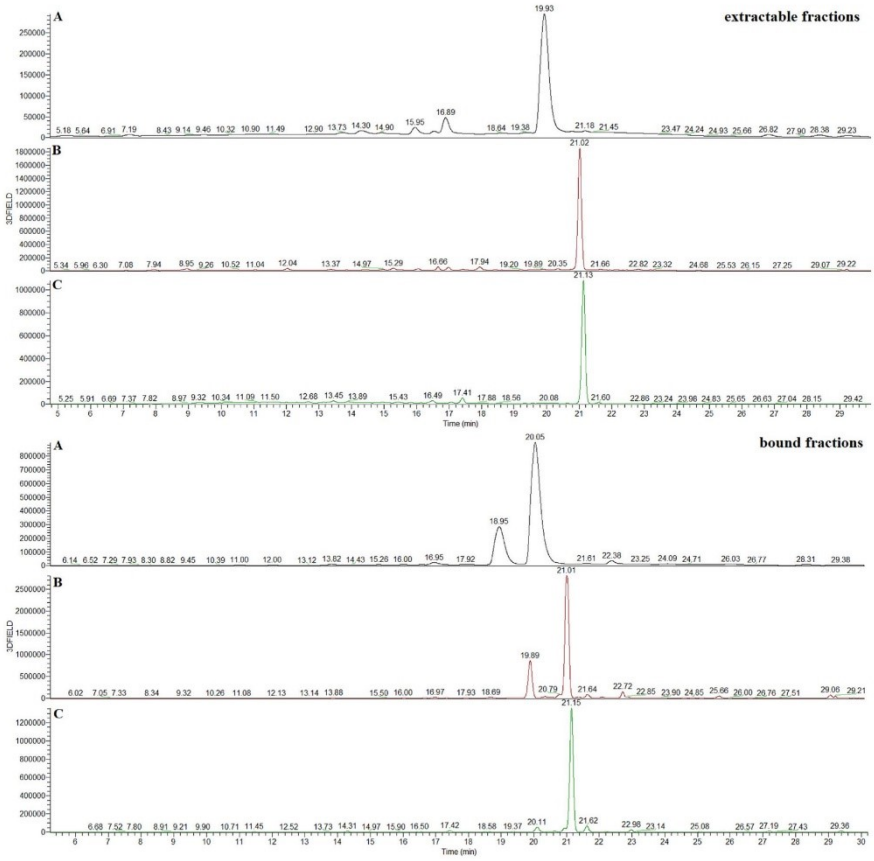

Fig. 1. Representative chromatograms of the extractable and bound fractions of raw (A), germinated (B) and fermented (C) spelt seeds (detected at 310 nm) treated with a combination of five enzymes (cellulase, xylanase, feruloyl esterase, α-amylase, protease).

effect and hydrogen bonds. These phenolics cannot be extracted by the organic solvents (Zhu, 2018).

Simultaneous application of all five enzymes (C + X + E + A + P) resulted in the degradation of cellulose, arabinoxylans, polysaccharides and protein and, subsequently, a decrease in the molecular mass and size of cell wall components, a break in the cross-linking mechanisms within the cell wall structure, and thus enhance the release of phenolics from spelt seeds (Fig. 1). Our findings are in agreement with previous publications. Rakariyatham et al. (2020) reported that cellulase increased the release of extractable o-coumaric acid, Peixoto Araujo et al. (2019) reported that treatment with protease and cellulase enhanced the recovery of extractable phenolic acids, such as *p*-hydroxybenzoic and ferulic acids. Furthermore, Moore et al. (2006) found that commercial β-glucanase enzymes were the most efficient enzyme that could convert 50% of the bound ferulic acid in wheat bran to the extractable form.

3.2.2. *Effect of enzymatic treatment on germinated and fermented spelt seeds*

In **germinated** spelt seeds, enzymatic treatment induced a significant increase of extractable *p*-coumaric (134%), *trans*-ferulic (5899%) and *p*-hydroxybenzoic (659%) acids compared to non-treated germinated seeds. Compared to enzymatic treated raw seeds, the C + X + E + A + P treatment of germinated seeds increased extractable *p*-coumaric (295%), *trans*-ferulic (300%) and *p*-hydroxybenzoic (38%) acids (Table 3; Fig. 1).

In **fermented** spelt seeds, enzymatic treatment induced a significant increase in extractable *p*-coumaric (436%), *trans*-ferulic (8263%), *cis*-ferulic (538%), caffeic (1534%) and *p*-hydroxybenzoic (54%) acids compared to non-treated fermented seeds. Compared to enzymatic treated raw seeds, the C + X + E + A + P treatment of fermented seeds increased extractable *p*-coumaric (179%), *trans*-ferulic (139%), caffeic (293%) and *p*-hydroxybenzoic (7%) acids (Table 3, Fig. 1).

Enzymatic treatment of **germinated** and **fermented** seeds negatively affected bound *p*-coumaric acid (21% and 53%), *trans*-ferulic acid (19% and 43%), and caffeic acid (21% and 36%) (Table 3). In contrast, in bound *p*-hydroxybenzoic acid, the enzymatic treatment of germinated and fermented seeds showed an increase by 218% and 144%, respectively, compared to their non-treated counterparts (Table 3). Because of the cooperation between enzymatic treatment and germination or fermentation, we detected bound *cis*-ferulic acid, which was below the detection limit in enzymatic treated raw seeds.

After **germination**, enzymatic treatment resulted in an increase in the proportion of extractable *p*-coumaric, *trans*-ferulic, caffeic and *p*-hydroxybenzoic acids over the total phenolic acid content (extractable + bound form) from 2% to 5%, 0.4%–22%, 0%–47% and from 25% to 44%, respectively. Furthermore, enzymatic treatment after **fermentation** also resulted in an increase in the proportion of extractable *p*-coumaric, *trans*-ferulic, *cis*-ferulic and caffeic acids over the total phenolic acid from 3% to 26%, 0.3%–31%, 3%–22%, and from 6% to 62%, respectively. Contrary, the proportion of extractable *p*-hydroxybenzoic acid decreased, despite its higher accessibility (Table 3).

Bei et al. (2018) reported that treatment with cellulase and fermentation of oats significantly increased the contents of extractable *p*-hydroxybenzoic and ferulic acid, by 34% and 46%, respectively, compared to the raw oat sample.

Enzymatic treated germinated seeds had the highest absolute content of extractable phenolic acids. A variety of enzymes could be directly or indirectly involved in the formation of extractable phenolics during spelt seed germination. Since in our study the enzymatic treatment conditions were mild (pH 6, 40 °C), these were also ideal conditions for better activity of endogenous enzymes (esterase, protease, amylase etc.) (Rakariyatham et al., 2020). Due to the metabolic activity of the microbes, fermentation also leads to structural degradation of the cell wall, resulting in the release of various bioactive compounds (Adebo & Medina-Meza, 2020). The composition of the microbiota and its development are important factors affecting fermentation and the quality of the final product. Metabolism of substrates by microorganisms depends on environmental conditions during fermentation such as temperature, water activity, oxygen availability, and pH. The biotransformation of hydroxycinnamic acids, such as *p*-coumaric, ferulic or caffeic acids to their corresponding vinyl derivatives, by lactic acid bacteria and yeast's decarboxylases or reductases has been documented in previous studies (Leonard et al., 2021).

In our enzymatic treated spelt seeds, compared to the bound fraction, the extractable fraction contained a number of minor phenolics, including apigenin hexoside pentoside I, II and III, an unknown C-

Table 3
Contents of individual phenolics for the extractable and bound fractions from raw, germinated and fermented spelt seeds treated with five enzymes (C + X + E + A + P).

Phenolics content (μg/g DW)		Spelt seeds					
		Germinated		Fermented		Raw	
		C + X + E + A + P	non-treated	C + X + E + A + P	non-treated	C + X + E + A + P	non-treated
Major							
***p*-Coumaric acid**	**Extractable**	12.94^{b}	5.52a	9.16b	1.71^{a}	3.28^{b}	0.29^{a}
	Bound	250.03^{A}	315.74B	25.46A	53.86^{B}	204.63^{B}	33.40^{A}
***trans*-Ferulic acid**	**Extractable**	344.36^{b}	5.74a	205.73b	2.46^{a}	86.18^{b}	0.76^{a}
	Bound	1236.32^{A}	1517.52B	462.62A	807.92^{B}	781.68^{B}	738.72^{A}
***cis*-Ferulic acid**	**Extractable**	13.69	ND	8.74b	1.37^{a}	ND	0.49
	Bound	22.69	ND	30.88A	43.44^{B}	ND	7.67
Caffeic acid	**Extractable**	11.80	ND	12.09b	0.74^{a}	3.08^{b}	0.13^{a}
	Bound	13.12^{A}	16.63B	7.32A	11.41^{B}	9.34^{B}	8.56^{A}
***p*-Hydroxybenzoic acid**	**Extractable**	40.60^{b}	5.35a	31.49b	20.47^{a}	29.36^{b}	1.07^{a}
	Bound	51.59^{B}	16.21A	34.15B	14.01^{A}	33.93^{B}	13.64^{A}
Minor							
Apigenin hexoside pentoside I	**Extractable**	6.91^{b}	2.89a	8.87b	2.27^{a}	4.30^{b}	2.47^{a}
Apigenin hexoside pentoside II	**Extractable**	18.29^{b}	8.73a	3.10b	0.87^{a}	1.86^{b}	0.95^{a}
Apigenin hexoside pentoside III	**Extractable**	10.50^{b}	4.65a	12.99b	5.76^{a}	8.20^{b}	4.66^{a}
Unknown C-glycosyl derivative	**Extractable**	3.41	ND	2.97^{b}	0.55^{a}	2.99^{b}	0.40^{a}
Gallocatechin	**Extractable**	40.65^{b}	10.08^{a}	22.71^{b}	3.22^{a}	4.30^{b}	0.44^{a}

Data are means (n = 6).
Different small letters within a row (compound) and same type of spelt seeds (raw, germinated, fermented) indicate significant differences between the extractable contents ($P < 0.05$; Paired-Samples T Test).
Different capital letters within a row and the same type of spelt seeds indicate significant difference between the bound contents ($P < 0.05$; Paired-Samples T Test).
C + X + E + A + P: sample was treated with cellulase (C); xylanase (X); feruloyl esterase (E); α-amylase (A); protease (P).
ND: not detectable.

gylcosyl derivative and gallocatechin, which were present in quite high contents (Table 3). Enzymatic treatment (C + X + E + A + P) increased gallocatechin content in raw, germinated and fermented samples the most, by 877%, 303% and 605%, respectively, compared to the corresponding non-treated samples. Apigenin hexoside pentosides I, II and III increased after enzymatic treatment of raw, germinated and fermented spelt seeds, with the highest increases in enzymatic treated fermented spelt seeds (291%, 256% and 126%, respectively). Enzymatic treatment resulted in a large increase in the unknown C-glycosyl derivative of raw (648%) and fermented (440%) spelt seeds. We did not observe any C-glycosyl derivative in non-treated germinated spelt seeds, whereas we found 3.41 μg/g DW of C-glycosyl derivative in enzymatic treated seeds (Table 3).

Based on the data presented in recent review (Bento-Silva et al., 2020), it can be concluded that the biosynthesis and transformation of phenolics seems to depend on the food matrix and its processing, although not all phenolics are equally susceptible to such changes. Germination of seeds significantly increase the content of phenolics, possible reason is that the activity of phenylalanine ammonia lyase, which catalyzes the pathways responsible for biosynthesis of phenolics, is enhanced during germination (Nkhata et al., 2018). Another possible explanation is that there could be hydrolysis of bound phenolic compounds as well as *de novo* biosynthesis of phenolics in the embryonic axis of the sprouts (Nkhata et al., 2018). During fermentation, microorganisms break down spelt seed matrix, resulting in the release of bound phenolics. It has been previously reported that lactic acid bacteria and yeast possess β-glucosidase, which can cleave glucoside bonds between phenolics and sugars, thereby releasing phenolics (Nkhata et al., 2018). Available enzymes during fermentation and/or produced by fermenting microorganisms could also break down ester bonds, hydrolyse β-glucosidic bonds, and distort the hydroxyl groups in phenolic structures, releasing free phenolics and other antioxidants (Adebo & Medina-Meza, 2020). The increased content of phenolics after fermentation is also influenced by a more favourable ratio between soluble and insoluble dietary fibres in cereal products. Such redistribution of dietary fiber is desirable from a nutritional perspective, and soluble fiber is an excellent substrate for feruloyl esterases (Hole et al., 2012). According to our results, (Tables 2 and 3) fermented and germinated seeds thus represent a substrate more accessible to the exogenous enzymes than non-bioprocessed seeds.

Regarding the most abundant antioxidant in spelt, microbial degradation of ferulic acid includes oxidation or reduction of the side chain, forming vanillin and eventually vanillic acid via the oxidation of the aldehyde group; and finally, the formation of guaiacol and protocatechuic acid as common intermediates (Bento-Silva et al., 2020). Three main ferulic acid metabolites were found in wheat sourdough, whereas their concentrations were strains dependent: vinylguaiacol, ethylguaiacol, dehydroferulic acid (Ripari et al., 2019); however, none of them was detected in our bioprocessed spelt seeds. One of the possible reasons for this, as we reported in our previous study (Mencin et al., 2022), is that only extractable hydroxycinnamic acids can be decarboxylated to the corresponding vinyl derivatives, and in the present study, the non-enzymatic treated raw, germinated and fermented samples had very low contents of extractable phenolic acids. On the other hand, a higher content of ferulic acid does not necessarily lead to a higher content of 4-vinyl guaiacol.

3.3. Effect of enzymatic treatment on in vitro antioxidant activity

3.3.1. Effect of different enzymatic treatments on raw spelt seeds

All enzymatic treatments increased the DPPH$^{\bullet}$ scavenging activity of extractable TPCs, while the DPPH$^{\bullet}$ scavenging activity of bound TPC decreased in almost all treatments, except for the treatments with cellulase (C) and xylanase (X).

The seeds enzymatically treated with a combination of cellulase, xylanase and esterase (C + X + E) showed the highest AA increase (800%) in DPPH$^{\bullet}$ scavenging activity of extractable phenolics (Table 1). Interestingly, the treatments with α-amylase (A) or protease (P) and their combinations (E + A; C + X + E + A) showed the lowest DPPH$^{\bullet}$ scavenging activity of extractable phenolics. The highest AA in the DPPH$^{\bullet}$ scavenging activity of bound phenolics was shown by xylanase (X) or cellulase (C).

Compared to the non-treated raw spelt seeds, C + X + E + P and C + X + E + A + P treatments increased the ABTS$^{\bullet+}$ scavenging activity for extractable TPCs the most, by 511% and 514%, respectively, and the treatment with α-amylase increased the activity for bound TPCs by 48% (Table 1). Treatments with xylanase (X) showed the lowest ABTS$^{\bullet+}$ scavenging activity of the extractable fraction, while treatments with esterase (E) showed the lowest AA for the bound fraction.

The activities revealed using ABTS$^{\bullet+}$ strongly positively correlated with the extractable TPC (R = 0.905) and bound TPC (R = 0.778), while the correlations between the DPPH$^{\bullet}$ activities and the extractable TPC (R = −0.155) and bound TPC (R = 0.097) were negligible. The strongest correlations observed between TPC and ABTS$^{\bullet+}$scavenging activity are in agreement with Zhao et al. (2008) for barley. All of these data showed that the polar phenolics in the enzymatic treated spelt seeds were the major ABTS$^{\bullet+}$ scavengers. The enzymatic treatment increased the AA of the seeds, probably by releasing polar antioxidant compounds bound to the cell wall and/or by hydrolysing biopolymers, such as polypeptides and polysaccharides (Azmir et al., 2013). However, the low correlations of extractable and bound DPPH$^{\bullet}$ activities with TPCs in the present study suggested that phenolics in these treated spelt seeds might react poorly with the DPPH reagent. In that respect, Li et al. (2009) also found that there was no direct correlation between DPPH inhibitory activity and TPCs of Radix *Angelica sinensis*.

The increase in the level of phenolic acids (Table 1) after enzymatic treatments indicates the possibility of enhancing AA, since phenolic acids are supposed to have greater antioxidant power (Netzel et al., 2000). Indeed, this study showed that extractable ferulic acid was the major ABTS$^{\bullet+}$ scavengers among obtained phenolic acids. Their AAs follow the sequence: caffeic acid > ferulic acid > *p*-coumaric acid (Ota et al., 2011).

Although the bound TPCs from samples treated with α-amylase or protease and their combinations were lower than in the non-treated sample, the AA in the ABTS$^{\bullet+}$ scavenging activity was higher for the enzymatic treated samples (Table 1). Several studies have demonstrated that the AA of phenolics is influenced by synergistic or antagonistic interaction with other compounds or with themselves (Adebo & Medina-Meza, 2020).

3.3.2. Effect of enzymatic treatment on germinated and fermented spelt seeds

Compared to the non-treated raw spelt seeds, **germination** significantly increased the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of extractable and bound fractions (Table 2). Enzymatic treatment further increased the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of the extractable fraction by 35% and 28%, respectively, when compared to only germinated spelt seeds, and when compared to enzymatic treated raw seeds, the values increased by 136% and 101%, respectively. Furthermore, when compared to germinated spelt seeds and enzymatic treated raw seeds, enzymatic treatment increased the DPPH$^{\bullet}$ scavenging activity of bound fraction by 6% and 75%, respectively, and the ABTS$^{\bullet+}$ scavenging activity decreased by 23% and 12%, respectively (Table 2).

Compared to the non-treated raw spelt seeds, yeast **fermentation** significantly increased the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of extractable and bound fractions (Table 2). The enzymatic treatment further increased the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of the extractable fraction by 117% and 116%, respectively, when compared to the enzymatic treated raw seeds, and when compared to only fermented seeds, the values increased by 550% and 403%, respectively. Furthermore, enzymatic treatment increased the DPPH$^{\bullet}$ scavenging activity of the bound fraction by 46% compared to enzymatic treated raw spelt

seeds, but decreased the $ABTS^{\bullet+}$ scavenging activity by 24%. Compared to only fermented spelt seeds $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities decreased by 21% and 33%, respectively (Table 2).

In the current study, the AA determined by an $ABTS^{\bullet+}$ was significantly and consistently higher than that of $DPPH^{\bullet}$. Due to the significantly high content of ferulic and *p*-coumaric acids in our enzymatic treated spelt seeds (Table 3), the obtained results were expected, since $ABTS^{\bullet+}$ reacts significantly better with mono-phenolics (e.g., ferulic acid, *p*-coumaric acid) than $DPPH^{\bullet}$ (Nenadis & Tsimidou, 2002). There are many variations in these $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, particularly regarding the choice of solvent for the assay. It has already been shown that the composition of the solvent and the pH have a great influence on the reactivity of the antioxidants in these $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays (Abramovič et al., 2017). However, it was reported that the type of assay and the solvent composition have similar influences on the reactivity of a particular antioxidant, either pure or as part of a complex matrix (Abramovič et al., 2018). Therefore, we determined the antioxidant activity of bioprocessed spelt seed with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ in different media. Since the chemical environment has different influences on the reactivity of antioxidants, changes in the relative composition of antioxidants might be reflected in different reactivities (Terpinc et al., 2016). In systems such as the $DPPH^{\bullet}$ scavenging assay, possible interactions of phenolics with the solvent may be important. Foti et al. (2004) reported that only the fraction of molecules that are not hydrogen bonded to the solvent are capable of transferring a hydrogen atom to a free radical. Therefore, the rate of abstraction of hydrogen atom from phenolic is significantly affected by strong hydrogen-bond-accepting solvents like methanol.

Terpinc et al. (2011) reported that phenolic acids can form an intramolecular hydrogen bond between the carbonyl oxygen in the –COOH group and the H atom of the –OH group. These intramolecular hydrogen bondings are probably more common in water-containing systems; it is likely that hydrogen bonds are formed competitively between water or ethanol and the carbonyl group. It has been shown that these interactions can be important because they decrease reactivity. Therefore, media of different polarity, pH of solvents, hydrogen atom abstraction and/or electron transfer, intra- and intermolecular hydrogen bonds, molecular structure of phenolics, etc. have all been factors affecting radical scavenging activity.

Our results confirmed that combining germination or fermentation with enzymatic treatment is a good strategy for improving the AA of extractable TPCs. Releasing the bound spelt antioxidants led to the increased bioaccessibility of free phenolics. According to Wang et al. (2018), there are two possible explanations for improving AA: one was to increase the solubility of the TPCs from spelt seeds and the other was to obtain phenolics with higher bioactivities using enzymatic treatment.

Min et al. (2012) also reported that the enhancement of AA was closely related to the increase of phenolics in rice bran. Alrahmany and Tsopmo (2013) reported that four different carbohydrases increased the total AA in oat bran. Sungsopha et al. (2009) reported that AA was significantly increased after germination and enzymatic treatment of rice bran. Radenkovs et al. (2014) reported that the highest scavenging effect of rye bran extracts on $DPPH^{\bullet}$ was observed in enzymatic treated bran.

3.4. *Effect of enzymatic treatment on cellular antioxidant activity*

To our knowledge, no previous study has determined the AAs of raw, germinated and fermented spelt seeds treated with C + X + E + A + P in the cells. Since AA of phenolics in the cell cannot merely be predicted based on their *in vitro* studies, we measured intracellular oxidation using *S. cerevisiae* as a model organism. The yeast cells were treated for 2 h with extractable and bound fractions of enzymatic treated and non-treated samples at 60 μL/mL. This had been determined in our previous study (Mencin et al., 2021) as a maximum concentration of fractions without a decrease in cell viability compared to the control (non-treated cells).

The extractable fraction of enzymatic treated **raw** spelt seeds decreased the intracellular oxidation by 16%, in contrast to non-treated seeds, where no changes in intracellular oxidation were observed compared to the reference (non-treated cells) (Fig. 2a). The extractable fraction of enzymatic treated **germinated** spelt seeds decreased the intracellular oxidation by 6%, but non-treated germinated seeds showed no decrease in intracellular oxidation (Fig. 2b). Furthermore, among extractable fractions of **fermented** seeds, only the enzymatic treated sample showed a significant decrease in intracellular oxidation, by 18% (Fig. 2c).

In contrast, the bound fractions of enzymatic treated germinated and fermented spelt seeds did not show significant differences in intracellular oxidation compared to the reference (Fig. 2b and c). Among the bound fractions, only the enzymatic treated raw seeds showed a 9% decrease in intracellular oxidation (Fig. 2a). Moreover, the bound fractions of the non-treated raw and germinated samples showed an increase in intracellular oxidation by 7% and 5%, respectively. These bound fractions obviously contain prooxidant substances that increase intracellular oxidation.

The results concerning AA of enzymatic treated raw, germinated and

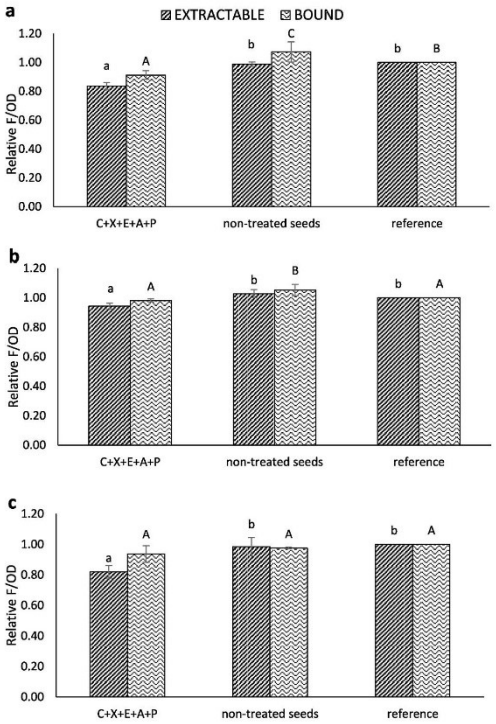

Fig. 2. Antioxidant activity determined *in vivo* (intracellular oxidation in the yeast *Saccharomyces cerevisiae*; relative [F/OD]) for the extractable and bound fractions of: (a) raw, (b) germinated and (c) fermented spelt seeds treated with a combination of five enzymes (C + X + E + A + P: cellulase, xylanase, feruloyl esterase, α-amylase, protease), in comparison to the reference. Data are means ± standard deviation, and different letters within the same fraction (extractable or bound) indicate significant differences ($P < 0.05$; Duncan's Multiple Range Test).

fermented spelt seeds indicate significant differences between *in vitro* and cellular AAs (Table 2, Fig. 2). The activities revealed using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ strongly correlated (r = 0.997; r = 0.983, respectively) with the extractable TPC. In contrast, cellular AA correlated negatively with the extractable TPC (r = −0.455) and DPPH$^{\bullet}$ (r = −0.518) and ABTS$^{\bullet+}$ (r = −0.284) scavenging activities. Furthermore, cellular AA correlated negatively with the bound TPC (r = −0.998) and DPPH$^{\bullet}$ scavenging activity (r = −0.956), whereas it showed a low but positive correlation with ABTS$^{\bullet+}$ scavenging activity (r = 0.326).

Better to understand which phenolics are responsible for AA in yeast cells, their levels were determined using HPLC-MS, before and after exposure of the cells. We measured a decrease in phenolics contents after exposure of the cells, but we do not know in what form the phenolics enter the cells. Three compounds were identified in the group of hydroxycinnamic acids, *p*-coumaric, *trans*-ferulic and caffeic acids, and three apigenin derivatives were identified in the group of flavonoids.

Our data indicated that in samples that showed no decrease in intracellular oxidation, the phenolics analysed, hardly, if at all, entered the yeast cell. Meanwhile, samples that significantly decreased intracellular oxidation showed that the tested compounds entered the cell, but their uptake was varied (Table S2, Fig. S1).

Interestingly, an increased phenolic uptake did not necessarily result in higher cellular AA. For example, the bound fraction of enzymatic treated raw spelt seeds was characterized by a lower cellular AA, but a 24-fold, 12-fold and 8-fold higher hydroxycinnamic acids uptake than the extractable fractions of enzymatic treated raw, germinated and fermented samples, respectively (Table S2). Moreover, the extractable phenolics of enzymatic treated germinated seeds showed lower cellular AA, but uptake of the tested extractable phenolics was 3.2-fold and 1.1-fold higher than the extractable phenolics of enzymatic treated raw and fermented samples, respectively.

Among extractable fractions of enzymatic treated seeds, the highest hydroxycinnamic acids uptake (28.61 μg) and apigenin derivatives uptake (14.98 μg) was measured in fermented and germinated seeds, respectively. The extractable fraction of enzymatic treated fermented seeds has comparable cellular AA to enzymatic treated raw seeds. Despite this, in enzymatic treated fermented seeds, a 3-fold higher hydroxycinnamic acid uptake, and a 1.7-fold higher apigenin derivatives uptake was measured.

In our previous study (Mencin et al., 2021), we reported that germinated spelt seeds under different abiotic stresses showed no effects on intracellular oxidation in *S. cerevisiae* and individual phenolics could not enter the cell, so they must probably undergo transformation beforehand. In contrast, in our recent study, we showed that extractable fractions of fermented raw, germinated and enzymatic treated spelt seeds showed a decrease in intracellular oxidation, probably due to apigenin derivatives. However, in the present study, the extractable fractions of enzymatic treated germinated seeds with the highest apigenin derivatives uptake showed the lowest decrease in intracellular oxidation, suggesting that other phenolics contributed more to the decrease in intracellular oxidation.

The cellular uptake of bound hydroxycinnamic acids is much higher than that of extractable acids, since the bound form is also present in a significantly higher concentration than the extractable ones. Obviously, the amount of phenolics that can enter the cell also depends on the content of the phenolics in the fraction itself. However, their effect on decreasing intracellular oxidation was significantly lower than that of extractable acids. The results suggest that not only the amount of phenolics entering the cell is important, but also the form in which they enter the cell. Therefore, the extractable form of phenolics is crucial for entering the cell.

Enzymatic treatment of fermented samples decrease intracellular oxidation the most. Apparently, the fermentation of spelt seeds with *S. cerevisiae* can convert phenolic acids to other microbial metabolites and new compounds that enter the cell more easily. Furthermore, the addition of external enzymes after fermentation had the potential to release even more insoluble bound phenolics from spelt. The differences in cellular AA could be ascribed to the individual molecular structures of phenolics. We have only studied the phenolics that are present in the highest amounts in spelt seeds. Which other phenolics, either alone or in synergism with others, contribute to cellular AA is still an open question.

3.5. Principal component analysis

Principal component analysis (PCA) is one of the most classical and widely used techniques to reduce dimensionality and increase interpretability of large datasets. Similarities and differences between groups of samples can be highlighted. The position of each variable in the PCA biplot (Fig. 3) describes its relationship to the other variables. Variables which are close have high correlations. Variables on the same side of the origin (0,0) are positively correlated and those on the opposite side of origin are negatively correlated.

The first PCA was performed to evaluate the extractable fractions of raw spelt seeds treated with different combinations of enzymes based on different biochemical parameters; their phenolic acids contents (i.e., *p*-coumaric, *trans*-ferulic, caffeic, *p*-hydroxybenzoic acids), their TPCs, and their *in vitro* (DPPH$^{\bullet}$, ABTS$^{\bullet+}$) AAs (Fig. 3a). For the first PCA, only the extractable fractions of the enzymatic treated samples were used because the differences in biochemical parameters between the bound and extractable fractions were too large to show good separation between the extractable fractions in the graph. Principal component 1 (PC1) explained up to 55.5% of total variance and PC2 explained 22.0%. Thus, the presented two-dimensional graph was able to explain 77.5% of the variability in the experimental data (Fig. 3a). Samples were separated along the first PC by differences observed in TPC, ABTS$^{\bullet+}$, *p*-coumaric acid, caffeic acid and *p*-hydroxybenzoic acid. The second PC separated the samples based on DPPH$^{\bullet}$ and *trans*-ferulic acid. As can be seen in Fig. 3a, there are four groups representing the extractable fractions of raw spelt seeds treated with different enzymes and their combinations. It can be observed that the groups do not overlap, so a clear separation can be observed between samples treated with different enzymes and their combinations. Extractable fractions of samples treated with α-amylase alone or in combination (E + A and C + X + E + A) are positioned in the left lower side of the graph (Fig. 3a). These samples showed lower DPPH$^{\bullet}$ scavenging activity and content of extractable *trans*-ferulic, caffeic and *p*-hydroxybenzoic acids. The extractable fractions of samples treated with protease alone or in combination (P; E + P; C + X + E + P; C + X + E + A + P) are positioned in the right lower side of the graph (Fig. 3a). These samples had the highest extractable TPCs, ABTS$^{\bullet+}$ scavenging activities and contents of *p*-coumaric acid. In contrast, the extractable fractions of samples treated with cellulase (C), xylanase (X) and esterase (E) individually are positioned in the left upper side of the graph. These samples had the lowest extractable TPCs, ABTS$^{\bullet+}$ scavenging activities and content of *p*-coumaric acid. The last group represents the extractable fraction of samples treated with a combination of cellulase, xylanase and esterase (C + X; X + E; C + X + E). These samples showed the highest DPPH$^{\bullet}$ scavenging activities, and higher contents of extractable *trans*-ferulic acid (Fig. 3a). The biochemical parameters allowed separation of the fractions in terms of the left and right sides of the graph.

The second PCA was performed to evaluate the extractable and bound fractions of enzymatic treated (C + X + E + A + P) and non-treated raw, germinated and fermented spelt seeds based on their biochemical parameters (phenolic acids contents (i.e., *p*-coumaric, *trans*-ferulic, *cis*-ferulic, caffeic, *p*-hydroxybenzoic acids), their TPCs and their *in vitro* (DPPH$^{\bullet}$, ABTS$^{\bullet+}$) and cellular AAs (ICO)) (Fig. 3b). PC1 explained up to 60.3% of total variance and PC2 explained 17.9%. Thus, the presented two-dimensional graph was able to explain 78.2% of the variability in the experimental data (Fig. 3b). Samples were separated along the first PC by differences observed in TPC, DPPH$^{\bullet}$, ABTS$^{\bullet+}$, *p*-coumaric acid, *trans*-ferulic acid and caffeic acid. The second PC separated the samples based on *p*-hydroxybenzoic acid, *cis*-ferulic acid and

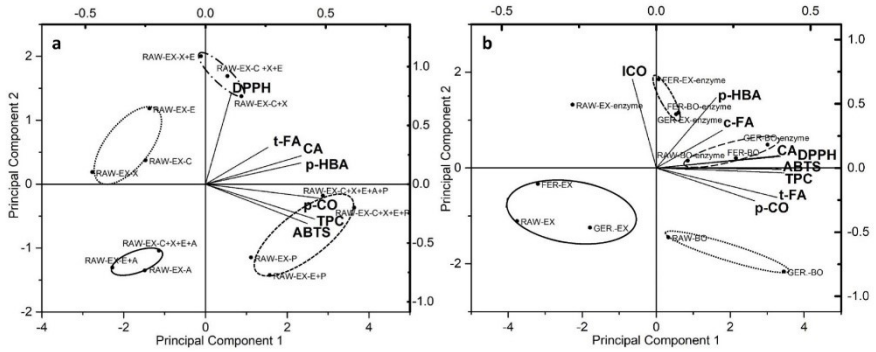

Fig. 3. **(a)** PCA biplot of the first two principal components from analysis of extractable (EX) fractions of raw spelt seeds treated with different enzymes. **(b)** PCA biplot of the first two principal components from analysis of extractable and bound (BO) fractions of enzymatic treated (enzyme: C + X + E + A + P) and non-treated raw, germinated (GER) and fermented (FER) spelt seeds. *p*-CO: *p*-coumaric acid; *t*-FA: *trans*-ferulic acid; *c*-FA: *cis*-ferulic acid; CA: caffeic acid; *p*-HBA: *p*-hydroxybenzoic acid; TPC: total phenolic content; DPPH: DPPH$^{\bullet}$ scavenging activity; ABTS: ABTS$^{\bullet+}$ scavenging activity; ICO: intracellular oxidation; C: cellulase; X: xylanase; E: feruloyl esterase; A: α-amylase; P: protease.

intracellular oxidation. From Fig. 3b it can be seen that there are four groups of samples and a sample that cannot be assigned to any group. A clear separation can be observed between extractable and bound fractions of enzymatic treated and non-treated samples. It can be seen that the extractable fractions of non-treated raw, germinated and fermented spelt seeds are positioned close to each other in the lower left quadrant, indicating some similarities in phenolic characteristics. On the other hand, the associated bound fractions are positioned in the lower right quadrant; the exception is the fermented sample. This group is more heterogeneous, suggesting that there are greater differences between these fractions. The position of the bound fraction of germinated spelt seeds indicated that it contained the highest levels of *p*-coumaric and *trans*-ferulic acids. In addition, the bound fractions of raw and germinated seeds showed prooxidant activity in the yeast cell, so the ICO parameter is positioned on the opposite side of the graph. Furthermore, the bound fraction of fermented spelt seeds showed more similarities with bound fractions of enzymatic treated raw and germinated spelt seeds, showing higher DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities and a higher content of caffeic acid. Bound fraction of the enzymatic treated fermented seeds shows greater similarity to the extractable fractions of enzymatic treated germinated and fermented seeds, as indicated by significantly lower contents of *p*-coumaric and *trans*-ferulic acids than in the bound fractions of enzymatic treated raw and germinated seeds. The extractable fractions of enzymatic treated raw and fermented spelt seeds are positioned close to the ICO parameter (Fig. 3b), suggesting that these two fractions showed the highest cellular AAs. It is interesting to note that extractable fractions of the non-treated raw, germinated and fermented samples have a significantly different position in the graph than the corresponding bound fractions, suggesting that there are much greater differences between these samples (Fig. 3b). On the other hand, the extractable fractions of enzymatic treated raw, germinated and fermented samples positioned closer to the corresponding bound fractions, suggesting that there are some similarities between these samples, mainly due to the increase in extractable phenolic acids due to the release of bound acids.

4. Conclusions

This study demonstrates an approach whereby treatments with specific enzymes alone or in combinations significantly improves the extractable TPCs and *in vitro* AAs of raw spelt seeds. The sample (i.e., C + X + E + A + P) that exhibited significantly improved extractable *p*-coumaric, *trans*-ferulic and *p*-hydroxybenzoic acids contents, also showed particularly efficient AA in ABTS$^{\bullet+}$ scavenging activity and higher extractable TPCs.

Additionally, combining biotechnological processes was clearly the most efficient way of releasing a large amount of bound phenolics in the extractable form and improve their bioaccessibility. In germinated and fermented spelt seeds, enzymatic treatment resulted in a significant increase in extractable TPCs and their *in vitro* AAs, as well as in extractable *p*-coumaric, ferulic, caffeic and *p*-hydroxybenzoic acids. Due to the combination of germination or fermentation with enzymatic treatment, especially *trans*-ferulic acid contents enormously increased (5899% and 8263%, respectively) in the extractable fraction. At the same time, enzymatic treatment of germinated and fermented seeds decreased content of bound phenolics and their AAs, although they still represented a predominant proportion of TPCs.

Among extractable fractions, the enzymatic treated raw, germinated and fermented seeds expressed cellular AA, while among the bound fractions, only the enzymatic treated raw seeds showed cellular AA. Our results suggest that increased phenolic uptake did not necessarily result in higher cellular AA, since the bound fraction of enzymatic treated raw spelt seeds showed lower AA cellular, but much higher hydroxycinnamic acids uptake than the extractable fractions, which showed cellular AA.

Ferulic acid, as the predominant phenolic in bioprocessed spelt seeds, is known to inhibit diseases caused by oxidative stress - preventing various cancers, cardiovascular and neurodegenerative diseases. Consumption of bound phenolics is associated with enhanced antioxidant and anti-inflammatory activity, and many evidences point to their preventive role in the development of intestinal diseases (Fardet, 2010).

The development of combinations of bioprocessing techniques seems to be a promising approach to improve the bioaccessibility of health-promoting compounds in cereals. Further studies are needed to explore possible transformation of phenolics to their more potent antioxidant compounds. This will help to convert the complex phenolics to their simple phenolic acids, which could increase the bioaccessibility of these phenolics. For this reason, the enzymatic release of phenolics could be useful in the food and nutraceutical industries.

CRediT authorship contribution statement

Marjeta Mencin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Polona Jamnik:** Writing – review & editing, Methodology,

Supervision. **Maja Mikulič Petkovšek:** Data curation, Methodology, Supervision, Writing – review & editing. **Robert Veberič:** Writing – review & editing. **Petra Terpinc:** Conceptualization, Data curation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank the Slovenian Research Agency for financial support through research programmes P4-0234 and P4-0013.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114046.

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Table S1. Identification of phenolics in extracts from enzymatic treated and non-treated raw, germinated and fermented spelt seeds by HPLC–MS^n in negative-ion mode.

Phenolics	**[M-H]$^-$ (*m/z*)**	**Fragment ions (*m/z*)**
p-Coumaric acid	163	119
trans-Ferulic acid	193	134, 149, 179
cis-Ferulic acid	193	134, 149, 179
Caffeic acid	179	135
p-Hydroxybenzoic acid	137	93
Gallocatechin	305	261, 219, 179, 165, 139, 137, 221, 125
Apigenin hexoside pentoside	563	443, 353, 383
Unknown C-glycosyl derivative	723	677, 451, 225

Table S2. Cellular uptake of extractable or bound phenolics from samples that showed a decrease in intracellular oxidation in yeast cells.

	Spelt seeds			
	Raw		**Germinated**	**Fermented**
	Extractable	**Bound**	**Extractable**	**Extractable**
Cellular uptake of phenolics (µg)	**C+X+E+A+P**	**C+X+E+A+P**	**C+X+E+A+P**	**C+X+E+A+P**
***p*-Coumaric acid**	0.18	45.42	0.48	0.89
***trans*-Ferulic acid**	8.91	182.49	18.85	26.38
Caffeic acid	0.52	0.00	0.29	1.34
Apigenin hexoside pentoside I	0.29	ND	0.06	0.56
Apigenin hexoside pentoside II	0.16	ND	1.53	0.15
Apigenin hexoside pentoside III	0.71	ND	13.39	1.31

C+X+E+A+P: sample was treated with cellulase (C); xylanase (X); feruloyl esterase (E); α-amylase (A); protease (P).

ND: not detectable.

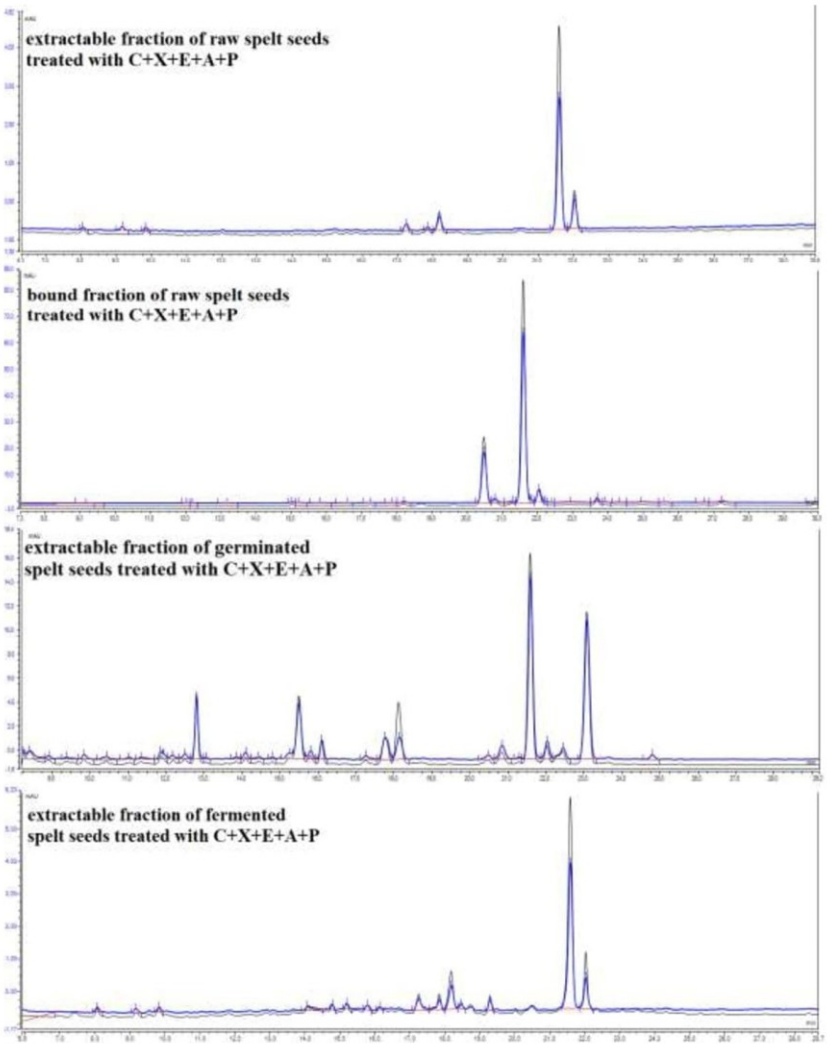

Fig. S1. HPLC chromatograms (at 310 nm) of the phenolics in the supernatants after centrifugation of the suspensions of the yeast *S. cerevisiae* before (black line) and after (blue line) 2 h exposure to the extractable or bound fractions from the enzymatic treated raw, germinated and fermented spelt seeds.

2.4 SIMULACIJA GASTROINTESTINALNE PREBAVE BIOLOŠKO OBDELANIH PIRINIH SEMEN: BIOLOŠKA DOSTOPNOST IN AKTIVNOST FENOLNIH SPOJIN

Mencin M., Mikulič Petkovšek M., Veberič R., Terpinc P. 2022c. Simulated gastrointestinal digestion of bioprocessed spelt seeds: bioaccessibility and bioactivity of phenolics. Antioxidants, 11, 9: 1703, doi:10.3390/antiox11091703: 20 str.

Cilj te raziskave je bil ovrednotiti vpliv različnih biotehnoloških procesov na izboljšanje biološke dostopnosti fenolnih spojin iz pirinih semen. Kljub negativnemu vplivu gastrointestinalne prebave je fermentacija kaljenih semen znatno povečala biološko dostopnost skupnih fenolnih spojin in njihovo antioksidativno aktivnost v primerjavi s prebavljenimi neobdelanimi semeni. Encimsko obdelana fermentirana semena so pokazala največjo relativno biološko dostopnost *p*-kumarne in *trans*-ferulne kisline, medtem ko je bila njihova absolutna vsebnost znatno večja pri »kaljenih + fermentiranih« semenih. Naša raziskava nakazuje, da predobdelava pirinih semen s hidrolitičnimi encimi omogoča lažji dostop mikroorganizmov do strukturnih elementov, zaradi česar smo uspeli določiti večjo vsebnost ekstraktibilne in vezane *trans*-ferulne kisline. Znatno višjo biološko stabilnost fenolnih spojin smo določili pri neobdelanih semenih. Ker so antioksidanti istega ekstrakta pokazali drugačno relativno zmanjšanje sposobnosti lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov v primerjavi z neobdelanimi semeni ali njihovimi pripadajočimi neprebavljenimi ekstrakti, predvidevamo, da med prebavo pride do kvalitativnih sprememb v sestavi ekstraktov. Pomembno je, da z biološko obdelavo povečamo vsebnost ekstraktibilnih antioksidantov v semenih, saj se njihova vsebnost med prebavo močno zmanjša.

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Simulated Gastrointestinal Digestion of Bioprocessed Spelt Seeds: Bioaccessibility and Bioactivity of Phenolics

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Abstract: The goal of this research was to evaluate the impact of different bioprocessing techniques on improved bioaccessibility of phenolics from spelt seeds. Despite the negative influence of gastrointestinal digestion, fermentation of germinated seeds significantly increased the bioaccessibility of total phenolics and their antioxidant activity compared to digested raw seeds. Enzymatic treated fermented seeds showed the highest relative bioaccessibility of *p*-coumaric and *trans*-ferulic acids, while their absolute contents were significantly higher in "germinated + fermented" seeds. Our research suggests that pretreatment of spelt seeds with hydrolytic enzymes improves access of fermenting microorganisms to structural elements, resulting in an increased content of extractable and bound *trans*-ferulic acid. Significantly higher biostability of phenolics was observed in raw seeds. Some major quality changes in the composition of extracts were observed under simulated in vitro digestion, since antioxidants of the same extract showed a different relative decrease in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities compared to the raw seeds or their corresponding undigested counterparts. It is therefore important to increase the content of extractable antioxidants in seeds by bioprocessing, since they are strongly diminished during digestion.

Keywords: spelt seeds; bioaccessibility; biostability; bioprocessing; in vitro digestion; extractable and bound phenolics

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Citation: Mencin, M.; Mikulič Petkovšek, M.; Veberič, R.; Terpinc, P. Simulated Gastrointestinal Digestion of Bioprocessed Spelt Seeds: Bioaccessibility and Bioactivity of Phenolics. *Antioxidants* **2022**, *11*, 1703. https://doi.org/10.3390/antiox11091703

Academic Editor: Stanley Omaye

Received: 8 August 2022
Accepted: 27 August 2022
Published: 30 August 2022

1. Introduction

Foods rich in antioxidants have attracted attention, since their consumption plays an essential role in the prevention of various diseases. In order to exert a health-promoting effect, phytochemicals must first survive food processing, be successfully released from the food matrix, be able to pass through the intestinal epithelia, and express (as the parent compound or its metabolite) bioactive properties in the target tissue [1]. Phenolics in cereals can prevent oxidation and reactive oxygen species (ROS) formation, but their antioxidant properties depend not only on their structure and concentrations in cereals but also on their bioaccessibility [2]. Bioaccessibility refers to the amount of compound released from the solid food matrix into the intestine during gastrointestinal (GI) digestion [3]. Approximately 99% of spelt major phenolics, *trans*-ferulic and *p*-coumaric acids, are present in an insoluble form and are bound to indigestible cell wall components [4]; their structural position within these building blocks may therefore hinder the access of feruloyl esterase, cellulase, and xylanase, enzymes that can release bound phenolics [5]. Soluble phenolics, which are located in vacuole, are generally more likely to be absorbed into systemic circulation, while unabsorbed phenolics are transported to the colon, where they can be biotransformed by microbial fermentation [6].

Bioprocessing techniques (germination/fermentation/enzymatic treatment) have been shown to enhance the bioaccessibility of phenolics from cereal bran incorporated into

food matrices prior to consumption. Koistinen et al. [7] investigated the impacts of yeast fermentation and enzymatic treatment on the bioaccessibility of phenolics from wheat bread enriched with bioprocessed rye bran using an in vitro colon model. They showed that phenolic acids were released more extensively in bioprocessed bread, which was confirmed by a significant decrease in bound phenolic acids in enriched bread compared to non-enriched bread, following enzymatic digestion and dialysis. Furthermore, Anson et al. [2] showed that a combination of external enzymes and fermentation was the most effective treatment that increased the bioaccessibility of ferulic acid from 1.1% to 5.5%.

The in vitro GI digestion model is an effective tool for evaluating the bioaccessibility of bioactive compounds, because it simulates the oral, gastric and small intestinal phases and, occasionally, fermentation in the colon [8]. Indeed, it is relatively inexpensive, rapid, not subject to ethical constraints, conditions can be controlled, sampling is simple and results are reproducible. In vitro digestion models have already been used to mimic the physiological release of phenolics in some cereals and cereal-based products [9,10]. However, the release of phenolics from bioprocessed *Triticum* matrix under digestion has not yet been reported. The objectives of the present study were therefore (a) to determine the content of total and individual phenolics and their antioxidant activity after homogenised spelt seeds that had been subjected to in vitro GI digestion (bioaccessible phenolics), and (b) to evaluate the percentage of bioaccessibility of these same phenolics after the seeds had been treated with different bioprocessing techniques and their combinations (germination/fermentation/enzymatic treatment).

2. Materials and Methods

2.1. Chemicals

Methanol (99.9%), formic acid, sodium hydroxide, sodium dihydrogen phosphate dehydrate, sodium carbonate and calcium chloride dehydrate ($CaCl_2(H_2O)_2$) were purchased from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) reagent, 2,2-Azino-bis-3-etilbenzotiazolin-6-sulfonic acid (ABTS) reagent, Trolox, hydrochloric acid, *p*-coumaric acid, *trans*-ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, gallocatechin, 4-vinylphenol, 4-vinylguaiacol, α-amylase (EC 232-565-6) from porcine pancreas (enzyme activity 5 U/mg solid), pepsin (EC 232-629-3) from porcine gastric mucosa (enzyme activity ≥ 2500 U/mg protein) and pancreatin (EC 232-468-9) from porcine pancreas (4 × USP specifications) were purchased from Sigma-Aldrich (Steinheim, Germany). Bile bovine (EC 232-369-0) was from Millipore (ZDA). Potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), and magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium carbonate ($(NH_4)_2CO_3$) was from Honeywell/Riedel-de Haen (Seelze, Germany). All chemicals and reagents used were of analytical quality. For preparation of working solutions ultrapure water (Milli-Q; Millipore, Bedford, MA, USA) was used.

2.2. Sample Preparation

Spelt (*Triticum spelta* L. cv. Ostro) seeds were obtained from Slovenia. Seeds were germinated according to Mencin et al. [4] under specific abiotic stress (darkness, at 25 °C for 144 h, with the addition of 25 mM NaCl after 48 h and 50 mM sorbitol after 96 h of germination) and then immediately milled (particles size < 0.25 mm), freeze-dried, and stored at –20 °C. The milled and freeze-dried raw spelt seeds were fermented according to [11], carried out for 72 h at 30 °C under static conditions, a sample-to-saline ratio 1:1.5 (10 g:15 mL), and with the addition of 0.75 mL of *Saccharomyces cerevisiae* inoculum. Enzymatic treatment of milled and freeze-dried raw spelt seeds was carried out at 40 °C for 4 h, with the addition of cellulase (C) (25 U/g DW), xylanase (X) (5 U/g DW), feruloyl esterase (E) (10 U/g DW), protease (P) (50 U/g DW), and α-amylase (A) (50 U/g DW). Other samples we used for in vitro GI digestion were: germinated spelt seeds treated with a combination of all five enzymes (C+X+E+A+P) (hereinafter referred to as "germinated + en-

zymatic treated"); fermented seeds with *S. cerevisiae* treated with C+X+E+A+P (hereinafter referred to as "fermented + enzymatic treated"), germinated spelt seeds fermented with *S. cerevisiae* (hereinafter referred to as "germinated + fermented"), and enzymatic treated seeds (C+X+E+A+P) fermented with *L. plantarum* (hereinafter referred to as "enzymatic treated + fermented"). Prior to analysis, raw and bioprocessed spelt seeds were freeze-dried (–50 °C and 30 mTorr) to a moisture content < 5%. We selected optimal treatments of spelt seeds based on our previous studies on the germination, fermentation, and enzymatic treatment of spelt seeds.

2.3. Simulated In Vitro Gastrointestinal Digestion of Spelt Seeds

In vitro GI digestion consisted of three-steps (oral, gastric and intestinal digestion) and was adapted from Brodkorb et al. [12] and Minekus et al. [13], with slight modifications. The solutions used to simulate GI digestive fluids (SSF: simulated salivary fluid, SGF: simulated gastric fluid, and SIF: simulated intestinal fluid) were prepared according to Table 1. Briefly, in oral digestion, 1.67 g of sample (raw or bioprocessed spelt seeds) was placed into a 50 mL polyethylene centrifuge tube and mixed with 2.67 mL of SSF to create a thin paste-like consistency. We then added 31.3 mg of α-amylase (to achieve 75 U/mL in the final mixture), followed by 12.5 µL of 0.3 M calcium chloride dehydrate and 666 µL of water and thoroughly mixed. The solution was incubated in a Roto-Therm incubated rotator (Benchmark, Edison, NJ, USA) at 37 °C for 2 min at constant rotation combined with oscillations. Afterward, gastric digestion was continued by the immediate addition of 4 mL SGF, 8 mg of pepsin (to achieve 2000 U/mL in the final mixture) and 2.5 µL of 0.3 M calcium chloride dehydrate. The mixture was intensively mixed, the pH of the solution was adjusted to 3 with 1 M HCl, and the volume was brought to 10 mL with water. The mixture was incubated in a Roto-Therm incubated rotator at 37 °C for 2 h. In order to simulate intestinal digestion, 8 mL of SIF, 20 mg of pancreatin (to achieve 100 U/mL of trypsin activity in the final mixture), 80 mg of bile bovine (to achieve 10 mM in the final mixture), and 20 µL of 0.3 M calcium chloride dehydrate were then added. The mixture was afterwards intensively mixed, and the pH of the solution was adjusted to 7 with 1 M NaOH and the volume was brought to 20 mL with water. The mixture was incubated in a Roto-Therm incubated rotator at 37 °C for 2 h.

Table 1. Composition of stock solutions of simulated digestion fluids. SSF: simulated salivary fluid, SGF: simulated gastric fluid, and SIF: simulated intestinal fluid.

		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt Solution	**Stock Concentration (mol/L)**	**mL of Stock Added to Prepare 0.4 L (mL)**	**Final Salt Concentration in SSF (mmol/L)**	**mL of Stock Added to Prepare 0.5 L (mL)**	**Final Salt Concentration in SGF (mmol/L)**	**mL of Stock Added to Prepare 1 L (mL)**	**Final Salt Concentration in SIF (mmol/L)**
KCl	0.5	15.1	15.1	8.6	6.9	17	6.8
KH_2PO_4	0.5	3.7	3.7	1.1	0.9	2	0.8
$NaHCO_3$	1	6.8	13.6	15.6	25	106.3	85
NaCl	2	/	/	14.8	47.2	24	38.4
$MgCl_2(H_2O)_6$	0.15	0.5	0.15	0.5	0.12	2.8	0.33
$(NH_4)_2CO_3$	0.5	0.06	0.06	0.6	0.5	/	/
$CaCl_2(H_2O)_2$	0.3	/	1.50	/	0.15	/	0.6

At the end of GI digestion, samples were immediately placed in an ice bath for 10 min to deactivate enzymes and then centrifuged for 10 min at 9793.9× *g* and 10 °C. The aliquots of supernatants (5 mL) were collected, filtered (pore size, 0.45 µm) and stored at 4 °C. The remaining residue was freeze-dried and stored at –20 °C until further analysis.

A reference blank (without the added sample) was incubated under the same conditions and used to determine phenolics content and antioxidant activity for the correction of interference from the digestive enzymes and buffers. Under the same enzymatic and

incubation conditions, proteins (enzymes) can interfere with the analysis of phenolics (especially with the Folin-Ciocalteu reagent). Our controls (undigested samples) were spelt seeds exposed to simulated digestive fluids without enzymes or bile. For each sample employed for the in vitro GI digestion, three replicates were used.

2.4. Extraction of Extractable and Bound Phenolics

Extractable and bound phenolics were extracted according to the extraction procedure described in Mencin et al. [4]. Briefly, 1 g of the homogenised and freeze-dried undigested and digested spelt seeds were mixed with 9 mL of absolute methanol. After shaking for 2 h at room temperature, the samples were centrifuged (9793.9× *g*, 10 min) and filtered (pore size, 0.45 µm). These filtered supernatants from the methanolic extraction contained the extractable phenolics. The solid residues after methanol extraction were treated by adding 20 mL of 2 M sodium hydroxide and shaking for 4 h at room temperature. After hydrolysis, the samples were acidified to pH 3 with concentrated formic acid. These filtered hydrolysates represented a solution of bound phenolics.

The supernatants and hydrolysates were further used for solid-phase extraction (SPE) for isolation and concentration of the phenolics from the extracts. SPE was performed according to [14]. Briefly, 100 mg Strata-X RP cartridges (Phenomenex, Torrance, CA, USA) were first preconditioned with 3 mL of 99.9% methanol, and then with 3 mL of water. The samples containing the extractable (5 mL) or bound (8 mL) phenolics were then added to the SPE cartridges and allowed to penetrate the matrix. The cartridges were washed with 4 mL of water and vacuum dried for 2 min. The phenolics were then eluted with 2 mL of 70% aqueous methanol. These methanolic eluates represented the corresponding extractable and bound fractions of phenolics. Until analysis, all samples were stored at 2 °C.

2.5. Determination of Total Phenolics Content

Total phenolics contents (TPCs) were determined using the Folin–Ciocalteu method according to the method described in our previous publication [4]. Appropriately diluted samples were mixed with water, Folin-Ciocalteu reagent, and after 5 min with 20% sodium carbonate. The mixtures were allowed to incubate for 1 h at room temperature in the dark. Absorbance was read at 765 nm using a UV-visible spectrophotometer (model 8453; Hewlett Packard, Waldbronn, Germany). A standard curve was prepared with Trolox, and the final results are expressed as mg Trolox equivalents per g dry weight of freeze-dried spelt seeds (mg TE/g DW).

2.6. Phenolics Profile

Analysis of individual phenolics was performed on HPLC (Thermo Dionex system; Thermo Scientific, San Jose, CA, USA), using a C18 column (Gemini C18; 150 mm × 4.6 mm; 3 µm; Phenomenex, Torrance, CA, USA) and an UV detector set at 280 nm and 310 nm. All phenolics were identified by mass spectrometry (LTQ XL linear ion trap mass spectrometer; Thermo Fisher Scientific, San Jose, CA, USA) with electrospray ionisation operating in negative- ion mode. All mass spectrometry conditions were described in our previous publication [4]. Identification and quantification of phenolics were confirmed by comparisons of their UV-VIS spectra and MS spectra and retention times with external standards and by internal standards, followed by fragmentation, as fully described in our previous investigation [4]. Concentrations of *p*-coumaric, *trans*-ferulic, caffeic and *p*-hydroxybenzoic acids and gallocatechin were calculated from the peak areas of the samples and the corresponding standards. Concentrations were expressed as µg per g spelt seeds DW (µg/g DW). One peak was tentatively identified as the *cis*-isomer of ferulic acid and was quantified using the calibration curve of *trans*-ferulic acid. For the compounds lacking standards, quantification was carried out using similar compounds as standards. Apigenin hexoside pentoside (I, II, III) and unknown C-glycosyl derivative were quantified according to *p*-coumaric acid.

2.7. Determination of Antioxidant Activity

2.7.1. DPPH• Scavenging Activity

The antioxidant activity of phenolics from undigested and digested spelt seeds was determined using the DPPH assay as described in our previous paper [4]. Samples were added to 0.2 mM DPPH• solution in 99.9% methanol. After the incubation period (1 h, at room temperature in the dark), the decrease in absorbance of the mixtures was measured at 520 nm. The final results are expressed as mg TE/g DW.

2.7.2. ABTS•+ Scavenging Activity

The scavenging activities of spelt seeds phenolics were also determined using ABTS assay, according to the method described by Mencin et al. [4]. The working solution of ABTS•+ was mixed with phosphate buffer (pH 7.4), water and the samples. The mixture was then incubated for 1 h in the dark. The decrease in absorbance of the reaction mixtures was measured at 734 nm. The final results are expressed as mg TE/g DW.

2.8. Bioaccessibility and Biostability of Phenolics after Digestion

After GI digestion, we obtained two fractions: a soluble-extractable fraction (bioaccessible) (supernatant of digestion) and an insoluble-bound fraction (biostable) (residue of digestion). The relative bioaccessibility represents the percentage of phenolics released after simulated GI digestion that could become available for absorption into systemic circulation. The relative biostability indicates the percentage of phenolics that remain in the digested residue and are not released into the digestive tract. For total and individual phenolics, the proportion released from the spelt seed matrix into the digestive fluids and the proportion insoluble during digestion were calculated as follows:

$$\text{Relative bioaccessibility (\%)} = (\text{Supernatant/Undigested}) * 100 \quad (1)$$

$$\text{Relative biostability (\%)} = (\text{Residue /Undigested}) * 100 \quad (2)$$

where Supernatant (extractable phenolics) is the content of total (TPC) or individual phenolics in the supernatant after GI digestion, Residue (bound phenolics) is the TPC or individual phenolics content in the residue after digestion, and Undigested is the total phenolic content (extractable + bound) in samples exposed to simulated digestive fluids without enzymes or bile. All values were corrected (due to interference from the digestive enzymes and buffers) with reference blank samples.

2.9. Statistical Analysis

All analyses were performed in three parallel runs with two separate extractions. One-way analysis of variance (ANOVA) was performed for each parameter, followed by a Duncan's post-hoc test to determine significantly different means ($p < 0.05$) using the SPSS programme, version 22 for Windows (IBM, Armonk, NY, USA). In addition, the Pearson's correlation coefficients (r) were determined to express the strength between two continuous variables. Multivariate statistical analysis (principal component analysis; PCA) was performed using OriginPro 2015 (OriginLab, Northampton, MA, USA) to interpret the differences in the analysed samples.

3. Results and Discussion

3.1. Total Phenolic Content

Considering the limitations of the Folin-Ciocalteu assay, the data obtained should always be interpreted with great caution, especially in situations in which, in addition to the studied extract or compound, the system contains complex food matrices. Because the Folin-Ciocalteu reagent can be reduced non-specifically by ascorbates, reducing sugars, aromatic amines, organic acids, fatty acids, proteins, and small peptides [15], we used a reference blank (without the added sample) to correct interference from the digestive

enzymes and buffers. However, the contribution of digestive fluids, enzymes and bile to TPC is usually overlooked in these types of studies.

The content of TPCs for samples exposed to simulated digestive fluids without enzymes and bile (undigested samples) and samples after in vitro GI digestion (digested samples) are presented in Table 2. The TPCs of undigested seeds showed a predominance of bound phenolics in raw and with a single bioprocessing technique treated seeds, whereas the concentrations of extractable and bound phenolics were quite similar when different bioprocessing techniques were combined. Bioprocessing techniques had already resulted in an increased proportion of extractable TPCs over the total spelt phenolics in our previous experiments [4,11], but the seeds were analysed in the absence of digestive fluids.

Table 2. Total phenolic content (TPC) and scavenging activities against DPPH$^{\bullet}$ [DPPH] and ABTS$^{\bullet+}$ [ABTS] radicals of undigested and digested spelt seeds in extractable and bound fractions.

	TPC		DPPH		ABTS	
	mg TE/g DW		mg TE/g DW		mg TE/g DW	
Sample	**Extractable**	**Bound**	**Extractable**	**Bound**	**Extractable**	**Bound**
Undigested						
raw	1.28 ^{a}	6.73 ^{D}	0.04 ^{a}	0.85 ^{EF}	1.30 ^{a}	6.98 ^{F}
germinated	4.85 ^{e}	10.76 ^{G}	0.43 ^{d}	1.50 ^{H}	4.21 ^{d}	8.90 ^{HI}
fermented	3.89 ^{c}	7.77 ^{E}	0.26 ^{b}	0.87 ^{F}	4.96 ^{e}	7.13 ^{F}
enzymatic treated	4.52 ^{de}	6.13 ^{C}	0.89 ^{g}	0.80 ^{E}	6.31 ^{f}	6.01 ^{E}
germinated + enzymatic treated	7.29 ^{h}	9.90 ^{F}	0.91 ^{g}	1.69 ^{J}	6.97 ^{g}	8.14 ^{G}
fermented + enzymatic treated	8.57 ^{i}	5.72 ^{BC}	1.21 ^{i}	0.79 ^{E}	7.45 ^{h}	5.61 ^{CD}
germinated + fermented	14.19 ^{k}	13.08 ^{H}	1.57 ^{k}	1.84 ^{K}	9.61 ^{i}	9.05 ^{I}
enzymatic treated +fermented	8.02 ^{i}	6.10 ^{C}	1.15 ^{h}	0.85 ^{EF}	7.16 ^{g}	5.83 ^{DE}
Digested						
raw	1.48 ^{a}	5.98 ^{BC}	0.06 ^{a}	0.70 ^{D}	1.35 ^{a}	6.10 ^{E}
germinated	5.62 ^{f}	7.39 ^{E}	0.70 ^{e}	0.40 ^{B}	4.28 ^{d}	7.21 ^{F}
fermented	4.22 ^{cd}	5.39 ^{B}	0.23 ^{b}	0.35 ^{AB}	2.33 ^{c}	6.09 ^{E}
enzymatic treated	2.92 ^{b}	4.13 ^{A}	0.24 ^{b}	0.56 ^{C}	1.94 ^{b}	5.36 ^{BC}
germinated + enzymatic treated	6.19 ^{g}	9.71 ^{F}	0.75 ^{f}	1.32 ^{G}	4.28 ^{d}	7.26 ^{F}
fermented + enzymatic treated	3.85 ^{c}	4.53 ^{A}	0.36 ^{c}	0.36 ^{AB}	2.28 ^{c}	5.15 ^{B}
germinated + fermented	10.19 ^{j}	12.48 ^{H}	1.28 ^{j}	1.60 ^{I}	6.24 ^{f}	8.57 ^{H}
enzymatic treated + fermented	4.14 ^{c}	4.34 ^{A}	0.44 ^{d}	0.31 ^{A}	2.37 ^{c}	4.51 ^{A}

Results were expressed as the mean of three replicates. Means with different small letters within a column indicate significant difference between the extractable phenolic contents ($p < 0.05$; Duncan's multiple range test). Means with different capital letters within a column indicate significant difference between the bound phenolic contents ($p < 0.05$; Duncan's multiple range test).

As shown in Table 2, in vitro digestion had a different effect on the content of phenolics in the extractable (bioaccessible) and bound (non-bioaccessible) fractions. Among the digested samples, the lowest extractable TPC was determined in raw seeds and the lowest bound TPC in enzymatic treated seeds (alone or in combination), while the highest extractable and bound TPCs were determined for "germinated + fermented" seeds. The considerable increase in extractable TPCs in the bioprocessed spelt seeds (a max 7-fold increase was observed in "germinated + fermented" seeds) compared to raw seeds after GI digestion may be due to the hydrolysis of various spelt fibre polymers resulting from the activity of the cereal and microbial enzymes, which may lead to a structural degradation of bran cell walls. Among the digested samples, germinated samples alone or in combination with fermentation or enzymatic treatment had higher extractable and bound TPCs than other bioprocessed samples. Only germinated seeds showed a statistically significant increase in extractable TPCs (by 16%) after digestion compared to the corresponding undigested seeds. The increase in the amount of extractable phenolics in digested germinated seeds may be the result of digestive enzymes and bile salts acting on the modified spelt seed matrix and facilitating the release of bound phenolics into the digestive juice [16]. Furthermore, the transition from an acidic to an alkaline environment

leads to deprotonation of hydroxyl moieties of aromatic rings, which may have contributed to the increased extractable TPC of digested germinated seeds [17]. A possible reason may also be the structure of germinated seeds, since starch in germinated seeds is generally more digestible due to the enzymatically modified structure of starch granules, thin cell walls and readily available mono- and disaccharides. Polysaccharides in the cell walls are hydrolyzed during germination by de novo synthesized enzymes, resulting in changes in the composition of insoluble and soluble dietary fibre of cereal seeds [18]. Analogously, bound TPCs significantly decreased in almost all digested samples compared to the undigested ones, whereby the highest decrease (33%) was observed in enzymatic treated seeds. The proportion of bound TPCs to total TPCs was highest (80%) for digested raw seeds, and lowest (51%) in digested "enzymatic treated + fermented" seeds. This decrease in bound TPCs in digested samples indicates their partial conversion to extractable forms during digestion, although the quantitative changes are generally not reflected in an increase in extractable fractions.

The digestive enzymes hydrolyze starch and proteins, which may favour the release of bound phenolics. On the other hand, decreases in the content of extractable TPCs in samples after digestion are in agreement with the studies of Ortega et al. [19] and Ydjedd et al. [20], who reported a significant decrease in free phenolics after GI digestion of carob flour. Furthermore, Chait et al. [21] reported that, after the intestinal phase of digestion, the TPC values in soluble free, soluble conjugated and bound fractions of carob decreased drastically by 28%, 66%, and 68%, respectively, compared to the undigested carob sample.

It can be concluded from these results that it is important to increase the content of extractable phenolics in seeds by bioprocessing techniques precisely because of losses during digestion. As can be seen from our research, the content of bioaccessible phenolics in bioprocessed seeds is significantly higher (up to 589% in "germinated + fermented") than in raw seeds.

3.2. Phenolic Profiles

The individual extractable and bound phenolic acids obtained from raw and bioprocessed spelt seeds before and after in vitro GI digestion are shown in Table 3. Bound *trans*-ferulic acid was the most abundant phenolic compound in all digested samples, representing as much as 91% in raw seeds and only 66% in "enzymatic treated + fermented" seeds of the total concentration of all detected phenolic acids. As mentioned earlier, *trans*-ferulic acid is linked to arabinoxylans and lignin in plant cell walls in the form of covalent ester and ether linkages, and these cross-links protect cell-wall carbohydrates from microbial attack and enzymatic hydrolysis [5]. The combined effect of yeast fermentation and applied hydrolytic enzymes on spelt bran improves the extractable ferulic acid at a rate higher than spelt seeds subjected only to the fermentation process. The mixture of enzymes used in our study for the treatment of spelt seeds enables the hydrolysis of various spelt polymers, thus improving the solubility and break down of the complex cell wall structures in the bran. One of the enzymes used in our study was a feruloyl esterase, which is able to cleave the ester-bound ferulic acid of the cell-wall polymers in spelt, resulting in the release of extractable ferulic acid (unpublished results). This is in agreement with Wang et al. [5], who reported that rumen microbes are capable of breaking down the ester linkages within plant cell walls by secreting feruloyl esterase, the latter having synergistic effects with xylanase and cellulase. The breakdown of these bonds makes the cell wall more susceptible to enzymatic attack during rumen fermentation and increases cell wall degradability. On the other hand, the authors also reported that the ether bonds between ferulic acid and lignin cannot be cleaved in the rumen. Based on our results, we suggest that pretreatment of spelt seeds with external hydrolytic enzymes increases the accessibility of bound *trans*-ferulic acid for attack by degradative enzymes present in secretions of fermenting microbes.

Table 3. Contents of individual phenolic acids of undigested and digested spelt seeds in extractable and bound fractions.

Sample	Phenolic Acids (μg/g DW)									
	p-Coumaric Acid		*trans*-Ferulic Acid		*cis*-Ferulic Acid		Caffeic Acid		*p*-Hydroxybenzoic Acid	
	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound
Undigested										
raw	0.27 ^{a}	28.86 ^{A}	0.59 ^{a}	834.99 ^{E}	0.30 ^{a}	37.14 ^{E}	0.29 ^{a}	7.87 ^{E}	1.20 ^{a}	12.57 ^{B}
germinated	3.54 ^{d}	221.60 ^{E}	9.19 ^{bc}	1466.45 ^{J}	4.75 ^{c}	80.77 ^{K}	2.92 ^{e}	13.98 ^{I}	14.24 ^{f}	18.13 ^{D}
fermented	1.09 ^{b}	32.11 ^{BC}	3.58 ^{a}	965.96 ^{G}	1.56 ^{b}	54.02 ^{G}	0.90 ^{b}	5.17 ^{BC}	9.21 ^{c}	15.30 ^{C}
enzymatic treated	6.37 ^{h}	36.07 ^{C}	140.68 ^{j}	578.91 ^{E}	8.14 ^{f}	37.86 ^{E}	5.06 ^{h}	8.46 ^{E}	24.81 ^{i}	30.12 ^{EF}
germinated + enzymatic treated	7.90 ^{j}	265.04 ^{G}	111.56 ^{i}	1351.42 ^{I}	7.62 ^{ef}	61.57 ^{H}	3.93 ^{g}	14.48 ^{I}	12.19 ^{de}	49.63 ^{G}
fermented + enzymatic treated	7.95 ^{j}	38.21 ^{C}	345.74 ^{l}	532.01 ^{CD}	22.87 ^{h}	26.87 ^{B}	7.63 ^{j}	6.39 ^{D}	36.29 ^{j}	29.62 ^{E}
germinated + fermented	15.31 ^{l}	298.17 ^{H}	68.32 ^{g}	1699.14 ^{K}	30.84 ^{i}	77.15 ^{J}	22.56 ^{l}	14.40 ^{I}	97.27 ^{m}	31.50 ^{EF}
enzymatic treated + fermented	6.84 ^{i}	35.67 ^{C}	320.92 ^{k}	549.15 ^{DE}	19.17 ^{g}	30.38 ^{C}	6.42 ^{i}	6.30 ^{D}	70.08 ^{l}	29.34 ^{E}
Digested										
raw	0.44 ^{a}	28.07 ^{A}	0.61 ^{a}	820.05 ^{F}	0.49 ^{a}	33.27 ^{D}	0.53 ^{a}	7.59 ^{E}	2.27 ^{b}	11.18 ^{B}
germinated	4.06 ^{e}	185.19 ^{D}	14.39 ^{c}	1210.44 ^{H}	6.36 ^{d}	44.52 ^{F}	3.27 ^{f}	9.19 ^{F}	18.39 ^{g}	17.60 ^{D}
fermented	2.25 ^{c}	27.80 ^{A}	4.26 ^{ab}	805.45 ^{F}	2.05 ^{b}	33.76 ^{D}	1.35 ^{c}	2.61 ^{A}	11.57 ^{d}	10.48 ^{B}
enzymatic treated	3.69 ^{d}	33.56 ^{BC}	12.48 ^{c}	493.10 ^{B}	2.10 ^{b}	28.01 ^{BC}	2.40 ^{d}	8.65 ^{EF}	11.26 ^{d}	16.62 ^{CD}
germinated + enzymatic treated	5.70 ^{g}	238.38 ^{F}	34.95 ^{d}	1209.11 ^{H}	7.26 ^{e}	55.35 ^{G}	5.24 ^{h}	11.05 ^{G}	21.03 ^{h}	29.64 ^{E}
fermented + enzymatic treated	3.90 ^{de}	39.17 ^{C}	41.55 ^{e}	508.46 ^{BC}	2.04 ^{b}	21.90 ^{A}	3.73 ^{g}	5.73 ^{C}	26.82 ^{i}	9.73 ^{AB}
germinated + fermented	11.05 ^{k}	293.85 ^{H}	85.61 ^{h}	1668.17 ^{K}	31.45 ^{i}	65.84 ^{I}	15.93 ^{k}	12.84 ^{H}	52.57 ^{k}	31.75 ^{F}
enzymatic treated + fermented	5.32 ^{f}	36.29 ^{C}	55.21 ^{f}	382.21 ^{A}	6.31 ^{d}	22.23 ^{A}	5.06 ^{h}	4.95 ^{B}	53.46 ^{k}	8.30 ^{A}

Data are means ($n = 6$). Means with different small letters within a column (phenolic acid) indicate significant difference between the extractable phenolic contents ($p < 0.05$; Duncan's multiple range test). Means with different capital letters within a column indicated significant difference between the bound phenolic contents ($p < 0.05$; Duncan's multiple range test).

Among digested spelt seeds, the "germinated + fermented" seeds showed the highest content of all extractable and bound phenolic acids. A combination of germination and fermentation increased the extractable and bound *p*-coumaric, *trans*-ferulic, cis-ferulic, caffeic, and *p*-hydroxybenzoic acids by 25-fold and 10-fold, 140-fold and two -fold, 64-fold and two-fold, 30-fold and two -fold, 23-fold and two -fold, respectively, compared to digested raw seeds. It seems as if during germination an optimal quantity of spelt native enzyme was produced (or activated) when compared to external enzymatic treatment of seeds before fermentation.

After GI digestion, the contents of bound phenolic acids were decreased compared to the undigested samples. Most of the extractable phenolic acids in digested samples were also decreased, except for raw, germinated and fermented seeds. In contrast, extractable *trans*-ferulic acid was increased after digestion in "germinated + fermented" seeds by 25%, and extractable caffeic and *p*-hydroxybenzoic acids were increased in "germinated + enzymatic treated" seeds by 33% and 73%, respectively. After GI digestion, the extractable *trans*-ferulic and cis-ferulic acids content decreased strongly in some bioprocessed seeds, showing a loss in *trans*-ferulic acid, from 69% ("germinated + enzymatic treated" seeds) to 91% (enzymatic treated seeds) compared to corresponding undigested samples. Although the percentage of phenolic acids decreased after digestion is high, the content of phenolic acids in bioprocessed seeds was significantly higher than in raw seeds. It is important that our spelt seeds were bioprocessed, since large losses occur during GI digestion. Furthermore, if our bioprocessed samples had a much higher initial phenolic acids content than the raw sample, consequently, more phenolics would survive the digestion process. Among digested samples, the combinations of bioprocessed techniques had higher levels of extractable phenolic acids than raw seeds or those treated with an individual bioprocessed technique.

It is interesting that, although the extractable *trans*-ferulic acid content in the undigested seeds was 2.2–46.9-fold higher than that of extractable *p*-coumaric acid (Table 3), the content of extractable *trans*-ferulic acid in digested seeds was only 1.4–10.7-fold higher than that of extractable *p*-coumaric acid, suggesting a much higher bioaccessibility of *p*-coumaric acid than *trans*-ferulic acid. Furthermore, the same trend was also observed when we compared the contents of extractable *trans*-ferulic acid with caffeic and *p*-hydroxybenzoic acids, suggesting lower bioaccessibility of *trans*-ferulic acid than the other three phenolic acids.

In the gastric phase, mainly the proteins present in seeds are digested and some of the phenolics bound with proteins may be released at this point. It is noteworthy that phenolics released in the gastric phase can also be labile due to the low pH (pH 3). However, some authors have previously shown that phenolic acids, such as coumaric, ferulic and caffeic acids, are absorbed from the stomach [22,23]. In the simulated digestion model performed by [24], it was shown that bound phenolics were released from wheat insoluble dietary fibre to a greater extent during the intestinal digestion stage than during the gastric digestion stage. In addition, a decrease of some phenolics in barley, chia seeds, pomegranate peel and carob flour has been reported after GI digestion [19,25]. It should be noted that, despite the decrease in the concentration of bound phenolic acids during GI digestion, their degradation remains partial. The drastic losses of phenolic acids after GI digestion are probably due to various factors: interactions with other dietary compounds, such as fibre, protein, carbohydrate; chemical reactions, mainly oxidation and polymerization, leading to the formation of other phenolic derivatives; or changes in molecular structure due to enzymatic action and, consequently, in its solubility [19].

In our undigested and digested raw and bioprocessed spelt seeds, compared to the bound fraction, the extractable fraction contained a number of phenolics, including gallocatechin, apigenin hexoside pentoside I, II, and III, and an unknown C-gylcosyl derivative, which were present in quite high contents (Table 4). Bioprocessing techniques significantly increased gallocatechin and C-gylcosyl derivative content in digested seeds compared to the raw seeds. However, apigenin derivatives showed a different trend in some bioprocessed seeds.

Table 4. Contents of individual flavonoids of undigested and digested spelt seeds in extractable fraction.

	Flavonoids (μg/g DW)				
	Gallocatechin	Apigenin			Unknown C-Glycosyl Derivative
		I	II	III	
Sample	Extractable	Extractable	Extractable	Extractable	Extractable
Undigested					
raw	1.01 ± 0.13 [a]	4.28 ± 0.06 [a]	1.36 ± 0.04 [a]	8.95 ± 0.29 [d]	0.45 ± 0.03 [a]
germinated	7.16 ± 0.28 [e]	6.17 ± 0.13 [d]	7.72 ± 0.30 [i]	7.21 ± 0.29 [b]	2.86 ± 0.16 [e]
fermented	3.42 ± 0.13 [b]	5.88 ± 0.15 [d]	2.36 ± 0.14 [c]	9.70 ± 0.29 [e]	1.32 ± 0.03 [b]
enzymatic treated	6.24 ± 0.26 [d]	8.47 ± 0.23 [g]	6.02 ± 0.19 [g]	13.02 ± 0.40 [g]	4.82 ± 0.11 [j]
germinated + enzymatic treated	31.21 ± 1.48 [l]	7.56 ± 0.22 [f]	7.33 ± 0.25 [h]	10.69 ± 0.27 [f]	4.70 ± 0.12 [j]
fermented + enzymatic treated	22.95 ± 0.59 [i]	9.85 ± 0.28 [i]	5.03 ± 0.22 [f]	15.28 ± 0.31 [h]	4.04 ± 0.10 [i]
germinated + fermented	39.57 ± 1.00 [m]	23.21 ± 0.57 [k]	18.95 ± 0.68 [j]	35.19 ± 0.95 [j]	7.13 ± 0.37 [k]
enzymatic treated + fermented	24.37 ± 0.69 [j]	9.23 ± 0.38 [h]	5.17 ± 0.15 [f]	15.67 ± 0.25 [h]	2.11 ± 0.13 [d]
Digested					
raw	1.00 ± 0.17 [a]	4.33 ± 0.06 [a]	3.74 ± 0.20 [e]	10.66 ± 0.15 [f]	1.82 ± 0.04 [c]
germinated	11.34 ± 0.22 [f]	4.78 ± 0.16 [b]	5.92 ± 0.52 [g]	5.98 ± 0.30 [a]	2.83 ± 0.14 [e]
fermented	3.60 ± 0.07 [bc]	4.26 ± 0.31 [a]	1.90 ± 0.05 [b]	7.91 ± 0.51 [c]	3.37 ± 0.09 [g]
enzymatic treated	3.93 ± 0.27 [c]	4.27 ± 0.18 [a]	2.11 ± 0.09 [bc]	8.03 ± 0.41 [c]	3.44 ± 0.11 [g]
germinated + enzymatic treated	32.13 ± 0.84 [k]	7.49 ± 0.71 [f]	7.58 ± 0.34 [hi]	7.75 ± 0.50 [c]	8.20 ± 0.21 [l]
fermented + enzymatic treated	20.93 ± 0.92 [h]	6.93 ± 0.61 [e]	2.82 ± 0.18 [d]	7.78 ± 0.44 [c]	3.72 ± 0.22 [h]
germinated + fermented	25.89 ± 0.43 [k]	18.19 ± 0.75 [j]	19.27 ± 0.57 [k]	16.83 ± 0.82 [i]	10.62 ± 0.38 [m]
enzymatic treated + fermented	17.51 ± 0.81 [g]	5.44 ± 0.54 [c]	2.84 ± 0.16 [d]	9.79 ± 0.35 [e]	3.14 ± 0.16 [f]

Data are means ± standard deviation. Means with different letters within the same column indicated significant difference between the extractable flavonoid contents ($p < 0.05$; Duncan's multiple range test).

Among digested samples, the "germinated + fermented" seeds had the highest content of all flavonoids detected, except for gallocatechin, which was the highest in "germinated + enzymatic treated" seeds. After GI digestion, compared with undigested samples, the contents of most flavonoids decreased. However, especially the unknown C-glycosyl derivative showed an increase after GI digestion in all samples, except in germinated, enzymatic treated and "fermented + enzymatic treated" seeds. Interestingly, after digestion, the raw seeds showed increased contents of apigenin I, II and C-glycosyl derivative, by 175%, 19% and 304%, respectively, compared to corresponding undigested samples. Apigenin II content also increased in digested "germinated + fermented" seeds by 2% compared to undigested sample. Gallocatechin increased by 58% after digestion only in germinated seeds. This increase in flavonoids contents may be related to hydrolysis of complex compounds from their glycoside to aglycone forms [19]. According to the research of [24], comparable amounts of wheat flavones were released in an acidic gastric environment and alkaline intestinal environment. They speculated that the increase in the amounts of flavonoids after digestion was due to the cleavage of the C-ring and reduction of double bonds.

3.3. Antioxidant Activity

The antioxidant activity of cereal extracts is mainly associated with their phenolics. However, the antioxidant properties of phenolics may change due to chemical transformations produced by various mechanisms during GI digestion. To evaluate the influence of GI digestion on the antioxidant activity of raw and bioprocessed spelt seeds, two assays were performed ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$).

Compared to the raw seeds, bioprocessing techniques significantly increased the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities of extractable fractions in digested samples (Table 2). After GI digestion, the extractable DPPH values were 21-fold higher, while bound DPPH values were 2.3-fold higher in "germinated + fermented" seeds than in digested raw seeds. The same combination of bioprocessing techniques was also optimal according to

the ABTS assay, but it should be stressed that the relative increase was not comparable with that of the DPPH method. Extractable and bound fraction of "germinated + fermented" seeds expressed 4.6-fold and 1.4-fold higher reactivity against $ABTS^{\bullet+}$, respectively. The obtained results indicate some serious quality changes in the composition of extracts under simulated in vitro digestion, since the antioxidants of the same extract showed a different response against different free radicals compared to the raw seeds. Similar variations were also observed when digested samples were compared with their undigested counterparts. For example, DPPH values of extractable phenolics of germinated seeds showed a great increase (63%) after GI digestion with reference to the undigested sample, while for ABTS, the increase was minimal (2%) for the same treatment. According to the lower reactivity of ferulic acid in the DPPH assay in comparison to the ABTS assay [4], and results presented in Tables 2 and 3, we assume that changes in the composition of antioxidants could be related to minor unknown compounds with important antioxidant activity, which were released or formed during GI digestion. The higher antioxidant activity observed after digestion could be attributed to pH changes and deprotonation of the hydroxyl groups present on the aromatic rings of the phenolics [21]. This could also be related to the structural changes of the phenolic molecules or liberation of new compounds having higher antioxidant activity [21]. Other bioprocessed samples showed a decrease or no significant difference in extractable $DPPH^{\bullet}$ scavenging activity, with the highest decrease of 70% in "fermented + enzymatic treated" seeds compared to corresponding undigested seeds. This lower activity could be due to the lower TPC and/or the fact that some phenolics can be converted into different structural forms with different chemical properties, due to their sensitivity to neutral pH [20]. According to Rice-Evans et al. [26], the chemical structure of phenolics also plays a role in free radical-scavenging activity, which mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic rings of phenolic molecules. The DPPH assay may involve electron transfer reactions and acids and alkalis present in extracts may affect the ionization equilibrium of phenolics, leading to a reduction or enhancement of the reaction rate [27]. In addition to possible intermolecular interactions (hydrogen bonds) of phenolic acids with the solvent in the reaction mixture, which can decrease their reactivity in the antioxidant assay, phenolic acids can also form intramolecular hydrogen interactions, which may also affect the transfer of a hydrogen atom to a free radical [28].

All antioxidant activities of bound fractions tested decreased strongly after digestion in comparison to the undigested seeds. This reduction in activity could be due to the decrease in bound TPCs after digestion. The antioxidant activity is tightly related to phenolics content and composition. Similarly, Correa-Betanzo et al. [29] showed that $DPPH^{\bullet}$ scavenging activity of blueberry extracts decreased over 50% after intestinal digestion. Chen et al. [30] also reported that the DPPH values of different sesame varieties were significantly lower after digestion than before digestion. On the other hand, Chait et al. [21] reported that DPPH and ABTS levels of carob phenolics in soluble free form showed a significant increase to 107 mg Gallic equivalents (GAE)/g and 399 mg TE/g, respectively, under GI digestion compared to undigested extract.

Coefficients of correlation (r) were calculated to explain the relationship between TPCs of extractable and bound fractions and their antioxidant activities ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$). Before and after GI digestion, the extractable fraction showed a positive and strong correlation between TPC and the antioxidant activity measured with DPPH (r = 0.923 and r = 0.978, respectively) and ABTS (r = 0.918 and r = 0.973, respectively) assays. Furthermore, before and after GI digestion, the DPPH (r = 0.951 and r = 0.911, respectively) and ABTS (r = 0.959 and r = 0.945, respectively) values found in bound fractions were also strongly positively correlated with TPC. These results agree with several previous studies [21,31], which reported a high correlation between TPC and antioxidant activities. Thus, these results indicate that phenolics widely contributed to the antioxidant activity of spelt seeds. Moreover, the antioxidant activity of phenolics depends essentially on their molecular structure. It has been shown that the CH=CH-COOH grouping of hydroxycinnamic acids

ensures a greater capacity to transfer a proton and subsequently to stabilize radicals than the carboxyl (COOH) grouping of hydroxybenzoic acids. In addition, flavonoids can act as proton or electron donors, which also lead to a good correlation with them. Cereal extracts are very complex mixtures of many different compounds with different activities. The different activity may be due to the synergistic or antagonistic action of these compounds [26].

The reduction in antioxidant activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) observed in our study and in the work of other authors during the intestinal part of in vitro digestion can be explained by the structural reorganisation of some bioactive compounds when the pH is changed to slightly alkaline. Moreover, these compounds gain the ability to react and bind with other components of the seed matrix, leading to a decrease in their antioxidant activity [32].

3.4. Bioaccessibility and Biostability of Digested Phenolics

3.4.1. Bioaccessibility and Biostability of Total Phenolics Content in Analysed Spelt Seeds

After in vitro digestion, part of the phenolics from spelt seeds were released into the digestion supernatant, indicating that they became bioaccessible and could be absorbed by our body. The bioaccessibility of TPCs of the analysed spelt seeds was evaluated after GI digestion by Folin-Ciocalteu assay compared to corresponding undigested samples. Figure 1A shows the percentage of TPCs bioaccessibility for all samples, estimated by GI digestion. The seeds that showed the highest bioaccessibility were "germinated + fermented" (37.4%), followed by fermented, germinated and "germinated + enzymatic treated". These samples also showed higher extractable TPCs than other digested samples; furthermore, "germinated + fermented" seeds also showed the highest content of extractable phenolic acids and contained most flavonoids after digestion. The raw seeds showed the lowest bioaccessibility (18.4%). It was shown that bioprocessing of spelt seeds increases the bioaccessibility of phenolics by 47% ("fermented + enzymatic treated") to 103% ("germinated + fermented") compared to raw seeds. The great increase in bioaccessibility in bioprocessed seeds may be due to the hydrolysis of different spelt fibre polymers by hydrolytic enzymes, which may lead to a structural degradation of bran cell walls [33]. The lower bioaccessibility of phenolics in raw seeds may be due to the fact that most phenolics in plants are conjugated to other molecules, such as carbohydrates, cellulose, and lignin, which are resistant to digestion.

Part of the phenolics remained in the digested spelt seeds residue, indicating that they are not bioaccessible (bound phenolics). We further examined the biostability of these phenolics (Figure 1B). In raw seeds, a significantly higher biostability of phenolics was observed compared to bioprocessed seeds. The biostability of TPC in raw seeds was from 31% to 141% higher than in other bioprocessed seeds. The lowest biostability of TPC was seen for "enzymatic treated + fermented" and "fermented + enzymatic treated" seeds. Among bioprocessed seeds, the highest biostability of TPC was observed for "germinated + enzymatic treated" seeds. Since the percentage of biostability is calculated based on the total (extractable + bound) phenolics of the undigested samples, the biostability of raw seeds was consequently higher, since the content of extractable phenolics is significantly higher in bioprocessed seeds. Although raw seeds had the highest percentage of phenolics biostability, the content of biostable phenolics in the "germinated + fermented" seeds was significantly higher (2-fold) than in raw seeds. The more stable the phenolics are during GI digestion, the more they continue to enter the colon, where the microbiota metabolizes phenolics into products that can be absorbed by our body. However, Ren et al. [34] reported that most phenolics with multiple hydroxyl groups are highly unstable. One of the ways of improving the bioaccessibility or biostability of phenolics is encapsulation of active ingredients.

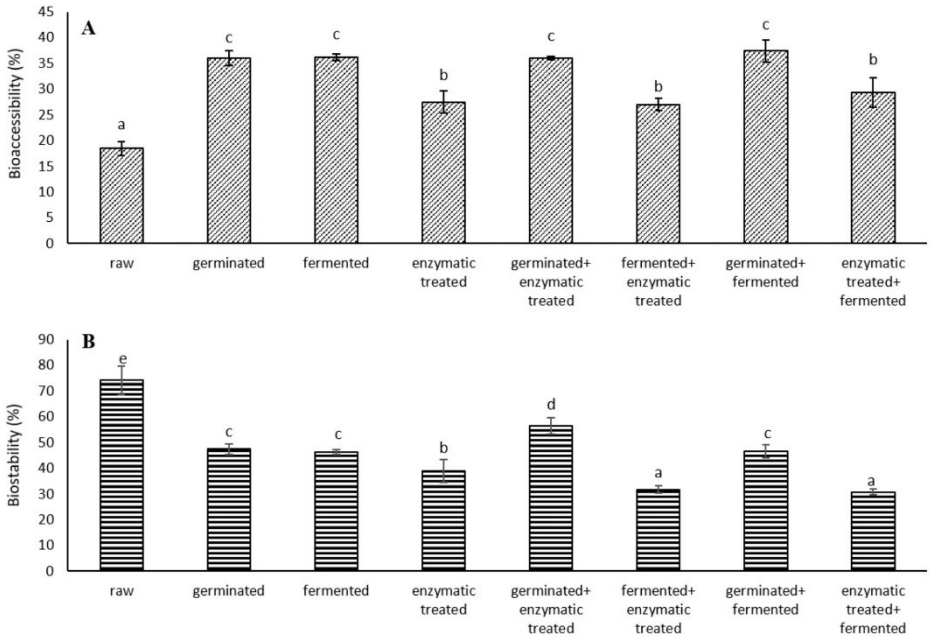

Figure 1. (**A**) Bioaccessibility and (**B**) biostability of total phenolic content (TPC) from raw and bioprocessed spelt seeds after in vitro digestion. The percentages were calculated with respect to corresponding undigested spelt seed TPCs. Results were expressed as the mean of three replicates and the error bars indicate standard deviation. Different letters indicate significant differences ($p < 0.05$; Duncan's Multiple Range Test).

3.4.2. Bioaccesebility and Biostability of Individual Phenolics in Analysed Spelt Seeds

The bioaccessibility of individual phenolic acids and flavonoids is shown in Table 5. Bioprocessing of spelt seeds increased the bioaccessibility of phenolic acids compared to raw seeds. The bioaccessibility of phenolic acids varied greatly among differently treated spelt seeds.

Bioprocessing of spelt seeds by fermentation and enzymatic treatment, alone or in combination, showed the highest bioaccessibility of *p*-coumaric acid. However, the amount of released (extractable) *p*-coumaric acid after digestion was the highest in "germinated + fermented" seeds, although the proportion of extractable form against the bound form was completely different in these spelt seeds. The most effective bioprocessing techniques were their combinations, which increased *trans*-ferulic acid bioaccessibility by 24–63-fold compared to raw seeds. Similar results were also observed by Anson et al. [2], who reported that the most effective bioprocessing technique was a combination of fermentation and enzymatic treatment of wheat bran, which increased bioaccessibility of ferulic acid by 5-fold compared to native bran. Bioprocessing techniques resulted in an increase in cis-ferulic acid bioaccessibility from 2.8-fold in fermented seeds to 22-fold in "germinated + fermented" seeds compared to raw seeds. The bioaccessibility of caffeic acid increased from 2.7-fold in enzymatic treated seeds to 6.6-fold in "germinated + fermented" seeds and the bioaccessibility of *p*-hydroxybenzoic acid increased from 1.2-fold in enzymatic treated seeds to 3.4-fold in germinated seeds compared to raw seeds. Among phenolic acids, *p*-hydroxybenzoic acid showed the highest bioaccessibility, from 16.5% (raw seeds) to 56.8% (germinated seeds). The proportion of extractable *p*-hydroxybenzoic acid over total (extractable + bound)

p-hydroxybenzoic acid was drastically higher than in case of other phenolic acids, ranging from 17% to 87%. It is interesting that the digested "germinated + fermented" seeds, which showed the highest content of bioaccessible *p*-coumaric, *trans*-ferulic and *p*-hydroxybenzoic acids, did not show the highest percentage of bioaccessibility. The reason was the content of extractable and bound phenolic acids in undigested seeds. In undigested "germinated + fermented" seeds, the content of bound *p*-coumaric and *trans*-ferulic acids, as well as the content of extractable *p*-hydroxybenzoic acid, was significantly higher than in other undigested samples, which we considered when calculating bioaccessibility.

Table 5. Percentages of bioaccessibility and biostability for individual phenolics from raw and bioprocessed spelt seeds after in vitro digestion.

Digested Spelt Seeds	*p*-Coumaric Acid	*trans*-Ferulic Acid	*cis*-Ferulic Acid	Caffeic Acid	*p*-Hydroxybenzoic Acid	Gallocatechin	Apigenin			Unknown C-glycosyl Derivative
							I	II	III	
Bioaccessibility (%)										
raw	1.5 ^{a}	0.1 ^{a}	1.3 ^{a}	6.5 ^{a}	16.5 ^{a}	43.1 ^{a}	45.6 ^{e}	83.4 ^{g}	61.0 ^{e}	10.9 ^{b}
germinated	1.8 ^{b}	1.0 ^{c}	7.4 ^{e}	19.3 ^{c}	56.8 ^{g}	85.9 ^{f}	32.5 ^{b}	32.7 ^{b}	33.5 ^{a}	8.1 ^{a}
fermented	6.8 ^{e}	0.4 ^{b}	3.7 ^{b}	22.2 ^{d}	47.2 ^{e}	66.9 ^{d}	39.1 ^{d}	36.2 ^{d}	44.2 ^{d}	22.8 ^{f}
enzymatic treated	8.7 ^{f}	1.7 ^{d}	4.6 ^{d}	17.8 ^{b}	20.5 ^{b}	41.2 ^{a}	30.4 ^{a}	21.4 ^{a}	37.6 ^{b}	16.8 ^{c}
germinated + enzymatic treated	2.1 ^{c}	2.4 ^{e}	10.5 ^{f}	28.5 ^{f}	34.0 ^{c}	85.3 ^{f}	47.2 ^{f}	45.5 ^{e}	35.5 ^{b}	20.1 ^{e}
fermented + enzymatic treated	8.5 ^{f}	4.7 ^{f}	4.1 ^{c}	26.6 ^{e}	40.7 ^{d}	78.6 ^{e}	47.8 ^{f}	34.9 ^{c}	34.9 ^{a}	21.7 ^{f}
germinated + fermented	3.5 ^{d}	4.8 ^{f}	29.1 ^{h}	43.1 ^{h}	40.8 ^{d}	58.1 ^{b}	61.5 ^{g}	69.8 ^{f}	36.6 ^{b}	26.0 ^{g}
enzymatic treated + fermented	12.5 ^{g}	6.3 ^{g}	12.7 ^{g}	39.8 ^{g}	53.8 ^{f}	61.5 ^{c}	36.4 ^{c}	30.6 ^{b}	41.2 ^{c}	17.4 ^{d}
Biostability (%)										
raw	96.4 ^{f}	98.1 ^{f}	88.9 ^{e}	93.1 ^{h}	81.2 ^{h}	48.4 ^{f}	48.3 ^{e}	53.0 ^{h}	42.7 ^{e}	91.7 ^{e}
germinated	82.3 ^{b}	82.0 ^{d}	52.1 ^{b}	54.4 ^{e}	54.4 ^{g}	37.0 ^{e}	48.5 ^{e}	40.9 ^{e}	59.3 ^{g}	89.0 ^{d}
fermented	83.7 ^{b}	83.1 ^{d}	60.8 ^{c}	43.0 ^{d}	42.8 ^{e}	29.4 ^{c}	13.8 ^{a}	43.7 ^{f}	29.9 ^{b}	80.9 ^{c}
enzymatic treated	79.1 ^{a}	68.5 ^{c}	60.9 ^{c}	64.0 ^{f}	30.2 ^{d}	35.5 ^{d}	39.2 ^{d}	37.1 ^{d}	38.4 ^{d}	74.6 ^{b}
germinated + enzymatic treated	87.3 ^{d}	82.6 ^{d}	80.0 ^{d}	60.0 ^{g}	47.9 ^{f}	7.6 ^{a}	53.7 ^{f}	51.9 ^{g}	47.9 ^{f}	88.3 ^{d}
fermented + enzymatic treated	84.8 ^{c}	57.9 ^{b}	44.0 ^{a}	40.9 ^{c}	14.8 ^{b}	13.2 ^{b}	20.0 ^{c}	33.3 ^{b}	32.4 ^{c}	75.9 ^{b}
germinated + fermented	93.7 ^{e}	94.4 ^{e}	61.0 ^{c}	34.7 ^{a}	24.7 ^{c}	8.0 ^{a}	20.4 ^{c}	30.1 ^{a}	22.8 ^{a}	81.6 ^{c}
enzymatic treated + fermented	85.4 ^{c}	43.9 ^{a}	44.9 ^{a}	38.9 ^{b}	8.3 ^{a}	13.8 ^{b}	18.6 ^{b}	35.7 ^{c}	33.7 ^{c}	65.4 ^{a}

Results were expressed as the mean of three replicates. Different letters within the same column (phenolic acids) indicate significant differences ($p < 0.05$; Duncan's multiple range test).

Phenolics that stay in the digested residue (bound phenolics) and are not released into the digestive system are biostable. Several authors pointed out that the stability of phenolics during the GI digestion process is strongly influenced by their chemical structure, as phenolics have different sensitivities to pH variations and digestive enzymes activity [35,36]. The biostability or, as some authors call it, the recovery of phenolic acids, is presented in Table 5. In the digestion residue, the highest percentage of phenolic acids remained biostable in raw seeds, which is consistent with TPC biostability. This is quite logical, since the raw seeds are not pre-treated with bioprocessing techniques that make the material more accessible to digestive enzymes. Consequently, raw seeds have the highest proportion of phenolics bound to various components of the cell wall. Sęczyk et al. [37] suggested that food matrices with a high content of insoluble dietary fibre and proteins

have stronger interactions with phenolics and lower digestibility. Xu et al. [38] reported that non-starch polysaccharides protect the phenolics bound to them from enzymes in the mouth, stomach and small intestine. When the non-starch polysaccharides enter the colon, they can be digested by the colon microbiota, releasing phenolics that protect us against colon cancer. In general, *p*-coumaric and *trans*-ferulic acids showed the highest biostability, while *p*-hydroxybenzoic acid showed the lowest biostability. Tomé-Sánchez et al. [17] also reported that ferulic acid derivatives of barley and wheat sprouts have high stability to digestion conditions. Interestingly, the lowest biostability of *p*-hydroxybenzoic acid was found in "enzymatic treated + fermented" seeds; it was almost 10-fold lower than in raw seeds. However, the bioaccessibility of *p*-hydroxybenzoic acid was significantly higher in "enzymatic treated + fermented" seeds than in raw seeds, so it could be suggested that some of the bound *p*-hydroxybenzoic acid was released from food matrix into the digestive fluid during digestion and may also change its structure to form new derivatives. A similar trend was also observed for other phenolic acids.

Despite the substantial increase in the bioaccessibility of phenolic acids achieved by bioprocessing, the major part of the phenolic acids remained in the non-bioaccessible (biostable) fraction that will enter the colon. Fermentation of cell-wall structures in the colon by bacterial enzymes is expected to facilitate the release of phenolic acids that were not accessible in the small intestine. It is therefore important to understand how the digestion process affects the phenolic structure and stability, since this, in turn, influences bioaccessibility of phenolics and their potential beneficial effects in cells of the gut epithelium [29].

As reported by Koistinen et al. [7], the bioaccessibility of ferulic acid was significantly higher in bioprocessed rye bread (yeast fermentation + enzymatic treatment) than in non-bioprocessed rye bread (88% vs. 51%, respectively). Gullon et al. [39] also reported that flavonoid bioaccessibility (64%) was higher than that of phenolics (36%). Kroon et al. [40] also indicated that only 2.6% of ferulic acid was released from wheat under GI digestion. Hemery et al. [41] reported that in whole-grain and bran-rich breads, the bioaccessibility ranged from 6% to 15% for *p*-coumaric acid, and from 2.5% to 5% for ferulic acid. The above data are difficult to compare directly, because to the best of our knowledge, studies about the effects of digestion on bioaccessibility often use different calculations for bioaccessibility or biostability.

The increase in extractable flavonoids in undigested spelt seeds caused by bioprocessing was not necessarily reflected in an increase in bioaccessible flavonoids during digestion of samples (Table 5). The highest content of bioaccessible gallocatechin had "germinated + enzymatic treated" seeds and during digestion there was no decrease. Furthermore, germinated seeds showed the highest increase in gallocatechin after digestion and, consequently, the bioaccessibility was also the highest. Enzymatic treated seeds showed the lowest bioaccessibility of gallocatechin, which is probably due to the fact that gallocatechin content decreased significantly after digestion (by 37%). Interestingly, apigenin II and III showed the highest bioaccessibility in raw seeds, probably due to the greater proportion of the extractable form, which increased significantly after digestion (by 175% and 19%, respectively). The bioprocessed seeds showed significantly lower bioaccessibility of apigenin II and III, although germination, alone or in combination, showed significantly higher bioaccessible apigenin II content and "germination + fermentation" showed significantly higher apigenin III content. The highest bioaccessibility of apigenin I and an unknown C-glycosyl derivative was found in "germinated + fermented" seeds. The lowest bioaccessibility of apigenin I was found in enzymatic treated seeds, which also showed the highest decrease in apigenin I content after digestion (50%). The lowest bioaccessibility of C-glycosyl derivative was found in germinated seeds.

The highest biostability of gallocatechin, apigenin II and C-glycosyl derivative was found in raw seeds. The highest biostability of apigenin I and III was found in "germinated + enzymatic treated" seeds and germinated seeds, respectively. It is interesting to note that, among tested flavonoids, the C-glycosyl derivative showed the highest biostability; it was

more than 65%. Furthermore, the C-glycosyl derivative was only up to 26% bioaccessible. On the other hand, gallocatechin generally showed the lowest biostability.

The above results suggest that during GI digestion of spelt seeds, various changes may have occurred in phenolic acids and flavonoids, such as (i) a change in chemical structure, (ii) increased or decreased solubility, or (iii) interaction with other compounds, affecting their bioaccessibility [39]. Adom and Liu [42] reported that insoluble bound phenolics could resist GI digestion to reach the colon, because cell wall components are difficult to digest. The higher fibre and protein content could partially prevent digestive enzymes from releasing the bound phenolics, thus limiting their bioaccessibility. Furthermore, β-glucan is a soluble, viscous dietary fibre that may contribute to the low phenolics bioaccessibility, since ß-glucans can form viscous gels that can trap phytochemicals, including phenolics. This may also explain part of the particularly lower bioaccessibility in the spelt seeds found in this study, since Triticum bran is a good source of dietary fibre, consisting mainly of arabinoxylans, cellulose, and β-glucan [33].

Zeng et al. [43] reported that although wheat possessed the highest TPC and antioxidant activity, the bioaccessible phenolics were lower than those of brown rice and oat. This suggests that cereals with the most abundant phenolics are not necessarily those with the highest bioaccessibility. The cereal matrix appears to be a crucial factor in their digestibility and stability during digestion, affecting bioaccessibility. Although ferulic acid is the most abundant phenolic compound in spelt, the bioaccessibility of ferulic acid (up to 6.3%) was significantly lower than that of TPC (up to 37.4%). A possible reason for this large discrepancy was that many of the compounds present in the supernatant of the digested samples were unknown.

3.5. Pearson Correlation Analysis

3.5.1. Correlations between the Proportion of Extractable Phenolics and Bioaccessibility

There were large differences in the bioaccessibility of phenolic acids in different spelt seeds. The correlation between the proportion of extractable *p*-coumaric (r = 0.997), *trans*-ferulic (r = 0.942), cis-ferulic (r = 0.960) and caffeic (r = 0.976) acids over the total (extractable + bound) phenolic acid in the digested samples and the bioaccessibility (refers to undigested samples) was very strong, while the correlation between the proportion of extractable *p*-hydroxybenzoic acid and its bioaccessibility was less strong (r = 0.737). Interestingly, the proportion of extractable TPC over the total TPC in digested samples correlated only moderately (r = 0.653) with TPC bioaccessibility. The lower correlation between extractable TPC and bioaccessibility was probably also influenced by some flavonoids that showed moderate correlation with bioaccessibility. The proportion of gallocatechin (r = 0.687), apigenin I (r = 0.538), III (r = 0.536) over the total of digested samples correlated moderately with their bioaccessibility, while apigenin II (r = 0.883) and C-glycosyl derivative (r = 0.956) correlated strongly with their bioaccessibility. It can therefore be concluded that the increase in bioaccessibility of hydroxycinnamic acids might be related to the increase in the proportion of extractable phenolic acids in spelt seeds. Mateo Anson et al. [44] also demonstrated a strong correlation between the proportion of free ferulic acid and bioaccessibility among five breads.

3.5.2. Correlations between Extractable Phenolic Acids of Digested and Undigested Spelt Seeds

The correlations between the amounts of extractable phenolic acids in digested spelt seeds and the amounts of extractable phenolic acids in the corresponding undigested seeds were calculated. The r-values were 0.966, 0.946, 0.938, 0.706 and 0.445 for extractable caffeic, *p*-coumaric, *p*-hydroxybenzoic, cis-ferulic and *trans*-ferulic acids, respectively. The strong positive correlation suggests that the higher level of caffeic, *p*-coumaric and *p*-hydroxybenzoic acids in undigested spelt seeds results in a higher level of bioaccessible caffeic, *p*-coumaric and *p*-hydroxybenzoic acids. Meanwhile, the correlation between extractable *trans*-ferulic acid in undigested and digested samples was moderately positive.

This suggests that, although spelt seeds could be considered as a ferulic acid-rich source, a higher extractable ferulic acid content before digestion is not necessarily associated with a higher content after digestion.

3.6. Principal Component Analysis

PCA (Figure 2) was used to explore similarities among the quantities of total and individual phenolics and their antioxidant activities in the bioaccessible fraction of spelt seeds and their percentage of bioaccessibility. Principal component 1 (PC1) corresponded to 69.4% of the data variation and differentiated spelt seeds according to the amounts of *p*-coumaric, *trans*-ferulic, *cis*-ferulic, and caffeic acids, bioaccessibility of *cis*-ferulic and caffeic acids, TPC and its bioaccessibility, and DPPH assay. PC2 explained 17.4% of the data variance due the amounts of *p*-hydroxybenzoic acid and its bioaccessibility, bioaccessibility of *p*-coumaric and *trans*-ferulic acids, and ABTS assay. Notably, germination, alone or in combination, showed the highest bioaccessible TPC and its percentage of bioaccessibility (bioaccessible TPC/totals in undigested sample), as well as the highest antioxidant activity against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ and content of bioaccessible cis-ferulic and caffeic acids. More specifically, "germinated + fermented" seeds showed the highest content of bioaccessible TPC, individual phenolic acids and their antioxidant activities. Interestingly, "enzymatic treated + fermented" seeds are positioned in the right upper side of the graph and showed the highest percentage of bioaccessibility of *p*-coumaric and *trans*-ferulic acids, while the content of these bioaccessible phenolic acids was significantly higher in "germinated + fermented" seeds (108% and 55%, respectively). "Fermented + enzymatic treated" seeds were distinguished by a high bioaccessibility of *p*-coumaric and *trans*-ferulic acids. Enzymatic treated seeds were characterised by higher bioaccessibility of *p*-coumaric acid. Raw seeds are positioned in the lower left side of the graph and showed the lowest content of bioaccessible TPC, individual phenolic acids and their antioxidant activities, as well as showing the lowest percentage of bioaccessibility of TPC and individual phenolic acids.

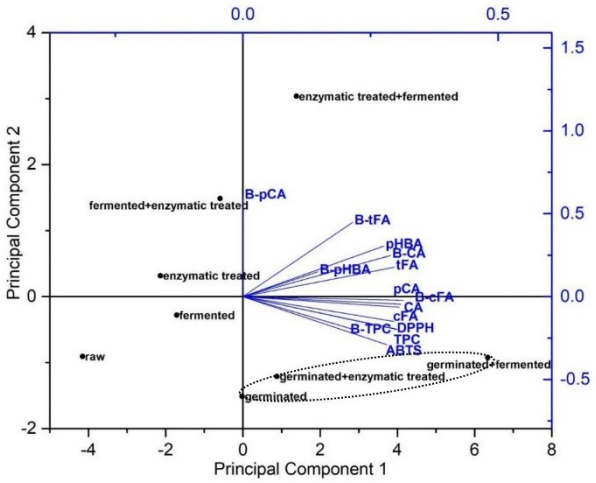

Figure 2. Principal component analysis (PCA) biplot of content of bioaccessible *p*-coumaric (pCA), *trans*-ferulic (tFA), *cis*-ferulic (cFA), caffeic (CA) and *p*-hydroxybenzoic (pHBA) acids, total phenolic content (TPC), scavenging activities against DPPH$^{\bullet}$ (DPPH) and ABTS$^{\bullet+}$ (ABTS) radicals and bioaccessibility of *p*-coumaric acid (B-pCA), *trans*-ferulic acid (B-tFA), *cis*-ferulic acid (B-cFA), caffeic acid (B-CA), *p*-hydroxybenzoic acid (B-pHBA) and total phenolic content (B-TPC) of raw and bioprocessed spelt seeds.

Overall, the results presented here showed that the bioprocessing of spelt seeds significantly improved the bioaccessibility of TPC and individual phenolic acids compared to raw seeds. In general, the combination of germination and fermentation was the most effective bioprocessing technique, showing the highest content of total and individual bioaccessible phenolics and higher bioaccessibility than other digested samples.

4. Conclusions

Bioprocessing techniques aimed at improving the bioaccessibility of phenolics from cereal products may represent the most promising approach to improving health benefits at the systemic level. The study showed a statistically significant effect of bioprocessing techniques on the content of bioaccessible TPC, individual phenolics and their antioxidant activities. Raw seeds showed the lowest content of bioaccessible TPC and individual phenolics, as well as their antioxidant activities. A combination of bioprocessing techniques, especially "germination + fermentation", was the most effective method to drastically improve the content of total and individual bioaccessible phenolics and their antioxidant activities. However, bioaccessibility of *p*-coumaric and *trans*-ferulic acids was the highest in "enzymatic treated + fermented" seeds. The application of the in vitro digestion model allowed a more detailed characterisation of the content of individual phenolics in spelt seeds that are actually accessible to the human body. However, in cereals, most phenolics are in bound form and may not be released during GI digestion but only in the colon. We should therefore highlight a problem that exists when using only GI digestion, namely the lack of a fermentation stage in the large intestine, where phenolics are highly metabolized. We do not therefore have a complete picture of the bioaccessibility of phenolics in the human body.

Author Contributions: Conceptualization, M.M. and P.T.; methodology, M.M., M.M.P., R.V. and P.T.; software, M.M.P.; validation, M.M., M.M.P. and P.T.; formal analysis, M.M. and M.M.P.; investigation, M.M. and P.T.; resources, P.T.; data curation, M.M. and P.T.; writing—original draft preparation, M.M. and P.T.; writing—review and editing, M.M., M.M.P., R.V. and P.T.; visualization, M.M. and P.T.; supervision, P.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Slovenian Research Agency, through research programmes P4-0234 (Integrated food technology and nutrition) and P4-0013 (Horticulture).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained in this article.

Acknowledgments: The authors would like to thank the Slovenian Research Agency for financial support through research programmes P4-0234 and P4-0013.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Osnovni namen naše raziskave je bil z različnimi biotehnološkimi procesi obdelati semena pire (*Triticum spelta* L.), s čimer bi izboljšali biološko dostopnost fenolnih spojin, njihovo vsebnost in antioksidativno aktivnost. Zanimala nas je tako vsebnost ekstraktibilnih in vezanih TPC, določena s Folin-Ciocalteu metodo, njuno razmerje, hkrati pa tudi identifikacija in kvantifikacija posameznih fenolnih spojin, določena s HPLC-MS/MS metodo. Ekstraktibilne fenolne spojine smo ekstrahirali z absolutnim metanolom. Zaradi več hidroksilnih skupin, ki so polarne, in aromatskega obroča, ki je nepolaren, je večina fenolnih spojin dobro topna v polarnih in srednje nepolarnih topilih. Po metanolni ekstrakciji pa netopne vezane fenolne spojine praviloma še vedno ostajajo vezane na celične stene (Domínguez-Rodríguez in sod., 2017), zato smo jih s pomočje alkalne hidrolize sprostili iz vezane oblike. Za njihovo izolacijo smo razvili nov pristop in ga tudi optimizirali (Priloga C) (Mencin in sod., 2021b).

3.1.1 Vpliv kaljenja na vsebnost fenolnih spojin in njihovo antioksidativno aktivnost

Prvi sklop raziskav je obsegal iskanje optimalne kombinacije pogojev abiotskega stresa z namenom izboljšanja fenolnega profila in aktivnosti ekstraktibilnih in vezanih antioksidantov iz semen kaljene pire. Vsebnost fenolnih spojin, ki jih neka rastlina sintetizira in kopiči med kaljenjem, je predvsem odvisna od odziva rastline na stresne dejavnike (Hübner in Arendt, 2013). Večina raziskav je poročala, da se med kaljenjem poveča vsebnost topnih fenolnih spojin, kar pripisujejo sintezi *de novo* in različnim biološkim transformacijam (Gan in sod., 2017; Kim in sod., 2018). Kar se tiče vezanih fenolnih spojin, raziskave poročajo, da na začetku kaljenja njihova vsebnost pada, kasneje pa začne ponovno naraščati (Hung in sod., 2011). Domneva se, da je vsebnost vezanih fenolnih spojin odvisna od njihove stopnje sprostitve in konjugacije. V zgodnji fazi kaljenja se ogljikovi hidrati in proteini razgradijo, hkrati pa se opazi povečanje vsebnosti enostavnih sladkorjev in aminokislin (Wang in sod., 2014), zato se tudi vezane fenolne spojine, konjugirane s komponentami celične stene, začnejo sproščati. Z daljšim časom kaljenja se tvorijo rastlinske celice z novimi celičnimi stenami in sintetizirane topne fenolne spojine se lahko vežejo z novo nastalimi komponentami celične stene in tako tvorijo nove vezane fenolne spojine. Tako so vezane fenolne spojine vključene v dinamičen proces, njihovo sproščanje in stopnja konjugacije pa se lahko razlikuje med različnimi vrstami kaljenih semen (Gan in sod., 2017). Antioksidativna aktivnost je najbolj pogosto raziskovana biološka aktivnost kaljenih semen in večina raziskav kaže, da kaljenje znatno izboljša antioksidativno aktivnost topnih ekstraktibilnih fenolnih spojin, v primerjavi z nekaljenimi semeni. Povečano antioksidativno aktivnost lahko pripišemo povečanju vsebnosti antioksidativnih komponent v kaljenih semenih. Rezultati raziskav kažejo, da se med kaljenjem žit poveča tudi antioksidativna aktivnost vezanih fenolnih spojin (Ti in sod., 2014; Žilić in sod., 2015).

V naši raziskavi smo ugotavljali vpliv različnih stresnih pogojev na vsebnost ekstraktibilnih in vezanih fenolnih spojin ter njihovo *in vitro* in *in vivo* antioksidativno aktivnost v 144 h kaljenih pirinih semenih. Pred kaljenjem smo mehansko oluščena semena pire pregledali ter poškodovana semena odstranili. Različne stresne dejavnike smo preverjali na neprečiščenih ekstraktih. Kontrolo so predstavljala pirina semena, ki smo jim dodali 24 mL vode in so 144 h kalila pri 20 °C. Opozoriti je potrebno, da je bil cilj študije raziskati vplive različnih stresnih pogojev, apliciranih med kaljenjem pirinih semen, in ne učinek samega kaljenja na fenolne spojine in njihov antioksidativni potencial, zato smo za kontrolo izbrali semena, kaljena pri optimalnih pogojih, in ne nekaljenih semen. Po končanem kaljenju smo semena tudi vizualno pregledali glede morebitne prisotnosti plesni. Pri semenih, ki so kalila pri 40 °C smo našli prisotno plesen, zato smo ta semena izločili iz nadaljnih analiz. Manjši dodatek vode semenom (12 mL) je znatno zmanjšal vsebnost ekstraktibilnih in vezanih TPC ter njihovo sposobnost lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov. Vse dodane koncentracije NaCl (25–200 mM) so rezultirale v znatnem povečanju sposobnosti lovljenja $ABTS^{\bullet+}$ radikalov vezane freakcije, v primerjavi s kontrolo. Povečana osmolarnost ob dodatku raztopine sorbitola in kaljenje pri 10 °C oz. 30 °C so znatno znižali vsebnost ekstraktibilnih in vezanih TPC in njihovo sposobnost lovljenja $ABTS^{\bullet+}$ radikalov. Po drugi strani pa sta dodatek raztopine sorbitola in sprememba temperature kaljenja znatno izboljšala sposobnost lovljenja $DPPH^{\bullet}$ radikalov. Mehanske poškodbe kalčkov so vodile k zmanjšanju vsebnosti ekstraktibilnih in vezanih TPC, medtem ko je bil učinek na antioksidativno aktivnost ekstraktibilne in vezane frakcije odvisen od vrste prostih radikalov; negativen v primeru $ABTS^{\bullet+}$ in pozitiven v primeru $DPPH^{\bullet}$ radikalov. Nadalje smo v našem poskusu ugotavljali vpliv kombiniranja različnih stresorjev, kar je rezultiralo v višjih vsebnostih ekstraktibilnih in vezanih TPC. Znatno se je povečala tudi sposobnost lovljenja $DPPH^{\bullet}$ radikalov, medtem ko se je sposobnost lovljenja $ABTS^{\bullet+}$ radikalov minimalno povečala v primerjavi s kontrolo. Ker lahko prisotni sladkorji, organske kisline in druge polarne komponente v neprečiščenih pirinih ekstraktih vplivajo na TPC in antioksidativno aktivnost, smo pred nadaljnjimi analizami vzorce prečistili z ekstrakcijo na trdno fazo (SPE). Štiri vzorce, ki so pokazali najvišjo antioksidativno aktivnost pri neprečiščenih ekstraktih, smo prečistili in jim nadalje določili vsebnost skupnih in individualnih fenolnih spojin ter njihovo *in vitro* in *in vivo* antioksidativno aktivnost. Ti vzorci so bili: dodatek raztopine NaCl (25 mM) po 48 h in 96 h kaljenja (vzorec NN); dodatek raztopine sorbitola (50 mM) po 48 h in 96 h kaljenja (vzorec SS); dodatek raztopine NaCl (25 mM) po 48 h in dodatek raztopine sorbitola (50 mM) po 96 h kaljenja (vzorec NS); dodatek raztopine NaCl po 48 h in dodatek raztopine sorbitola po 96 h kaljenja z apliciranim mehanskim stresom pri 72 h kaljenja (vzorec NSM). Vzorci so bili kaljeni pri 25 °C, 144 h. Ne glede na abiotski stres, so bile opazne razlike v kvantiteti med ekstraktibilnimi in vezanimi fenolnimi spojinami, saj se približno dve tretjini fenolnih spojin iz kaljenih semen ni ekstrahiralo z metanolom, ampak so se sprostile šele po alkalni hidrolizi. Ugotovili smo, da kaljenje pirinih semen ob dodatku 25 mM NaCl in 50 mM sorbitola brez aplicirane mehanske poškodbe med preizkušenimi kombinacijami abiotskega stresa v splošnem najbolj pripomore k povečanju vsebnosti fenolnih spojin in

učinkovitejšemu lovljenje prostih radikalov ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $ROO^{\bullet}$) v primerjavi s kontrolo. Ferulna, *p*-kumarna, kavna in *p*-hidroksibenzojska kislina so bile glavne predstavnice identificiranih vezanih antioksidantov v kaljeni piri; pri *p*-kumarni in *trans*-ferulni kislini je vezana oblika predstavljala kar 99 % od skupne vsebnosti posamezne kisline. Vsi štirje stresni pogoji (NN, SS, NS, NSM) so zmanjšali vsebnost posameznih ekstraktibilnih fenolnih kislin, največje zmanjšanje pa smo zaznali pri vzorcu NSM v primerjavi s kontrolo. Očitno smo ekstraktibilne fenolne kisline zgubili z apliciranjem mehanskega stresa, saj smo eno tretjino kalčkov odrezali. Po drugi strani pa se je vsebnost vezanih fenolnih kislin zvišala v vzorcih, ki so bili izpostavljeni povišani slanosti in osmolarnosti brez apliciranega mehanskega stresa. NSM obdelava je znatno zvišala le vsebnost vezane *p*-kumarne kisline. Ne glede na vse, naši rezultati nakazujejo, da imajo obdelave z raztopinama NaCl in sorbitola različne vplive na transformacije ekstraktibilnih in vezanih fenolnih kislin med kaljenjem pirinih semen. Tako lahko potrdimo, da različni stresni pogoji značilno vplivajo na razmerje med ekstraktibilnimi in vezanimi fenolnimi spojinami. Zanimivo je, da so vzorci, ki so pokazali povečanje vsebnosti vezane *p*-kumarne in *trans*-ferulne kisline, pokazali tudi višje TPC in boljšo antioksidativno aktivnost, glede na kontrolo. To nakazuje na pomembno vlogo teh dveh fenolnih kislin pri izražanju celokupne antioksidativne aktivnosti kaljenih pirinih semen in reaktivnosti na Folin-Ciocalteu reagent. Prav tako smo opazili, da so ekstraktibilne frakcije izkazovale relativno visoko antioksidativno aktivnost, čeprav so vsebovale manj fenolnih kislin v primerjavi z vezanimi frakcijami. Sestava ekstraktibilne frakcije je bila precej bolj heterogena, a koncentracijsko manj pomembna. Katera fenolna kislina je najbolj prispevala k aktivnosti kaljenih semen, ostaja še vedno odprto vprašanje. Omeniti velja, da so ekstraktibilne frakcije kaljenih semen vsebovale kar nekaj neznanih spojin, ki jih nismo identificirali in bi lahko pomembno prispevale k vsebnosti TPC in antioksidativni aktivnosti.

Kaljena semena, ki so bila izpostavljena dodatku raztopin 25 mM NaCl in 50 mM sorbitola, so na splošno pokazala največjo vsebnost ekstraktibilnih in vezanih TPC in izražala najboljšo antioksidativno aktivnost tako v homogenem (vodna ali organska raztopina) kot tudi v heterogenem (emulzija linolne kisline v vodi) mediju, ter največjo vsebnost vezane *p*-kumarne, *trans*-ferulne in kavne kisline. Chu in sod. (2020) so v kaljenem kitajskem divjem rižu določili precejšnjo pozitivno linearno korelacijo (r = 0,834–0,862) med aktivnostjo PAL, ki transformira fenilalanin v cimetno kislino, in vsebnostjo *p*-kumarne, ferulne in *p*-hidroksibenzojske kisline. Na splošno obstajajo tri možne poti za nastanek ekstraktibilnih fenolnih spojin med kaljenjem. Prvič, njihovo prirast lahko pripišemo sproščanju vezanih spojin. Hidrolitični encimi povzročijo hidrolizo strukturnih komponent, del katerih so tudi fenolne spojine. Drugič, med kaljenjem semen se lahko proteini, škrob in lipidi razgradijo v aminokisline, glukozo in acetil CoA, ki predstavljajo neposredne ali posredne substrate za *de novo* sintezo fenolnih spojin. Posledično je pomembno tudi povečanje aktivnosti encimov, ki so odgovorni za pretvorbo teh substratov v fenolne spojine (Carciochi in sod., 2016). Tretjič, tudi regulacija prekomerne koncentracije ROS je ena izmed možnih poti za

povečanje ekstraktibilnih fenolnih spojin. Med kaljenjem semen se namreč tvorijo številne ROS, več prostih radikalov bi lahko spodbudilo gen, ki sproži produkcijo encimov za zaščito organelov pred napadom prostih radikalov. Po drugi strani se lahko vsebnost ekstraktibilnih fenolnih spojin med kaljenjem tudi zmanjša. Eden od mehanizmov je, da so slednje vključene v nevtralizacijo ROS. Ekstraktibilne fenolne spojine lahko donirajo vodik ali elektron za lovljenje prostih radikalov, ki tako postanejo relativno stabilni radikali fenolnih spojin. Nadalje, med kaljenjem se v semenih poveča količina encimov fenol-oksidaz in peroksidaz. Ti encimi lahko oksidirajo široko paleto donorjev vodika, vključno s fenolnimi kislinami, flavonoidi, polifenoli, itd. (Richard-Forget in Gauillard, 1997) in posledično pripomorejo k zmanjšanju vsebnosti ekstraktibilnih fenolnih spojin. Drugi mehanizem, ki je lahko odgovoren za zmanjšanje vsebnosti ekstraktibilnih fenolnih spojin je, da se lahko ekstraktibilne spojine prenesejo na celično steno z vezikli, kjer se transformirajo v vezano obliko (Xu in sod., 2020).

Da bi podali čim bolj celovito oceno antioksidativne aktivnosti prisotnih fenolnih spojin v naši rastlinski matrici, smo uporabili različne analizne metode. Metodi določanja sposobnosti lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov temeljita na mehanizmu prenosa elektrona ali vodikovega radikala in vključujeta redukcijo obarvanega oksidanta, kot taki sta podobni Folin-Ciocalteu testu (Dawidowicz in Olszowy, 2013). Pomembno je poudariti, da sta $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikala različno dovzetna za reakcije s fenolnimi spojinami različnih kemijskih struktur. Nenadis in Tsimidou (2002) sta poročala, da so najvišjo antioksidativno aktivnost, določeno z metodo lovljenja $DPPH^{\bullet}$ radikala, pokazale spojine z več hidroksilnimi skupinami (npr. kavna kislina), sledile so jim monofenolne spojine z metoksi skupino (npr. sinapinska, ferulna kislina). V primerjavi z $DPPH^{\bullet}$, $ABTS^{\bullet+}$ radikal bistveno bolje reagira z monofenolnimi spojinami (ferulno, *p*-kumarno kislino). Ker kaljena pirina semena vsebujejo veliko *p*-kumarne in ferulne kisline, je bila večja sposobnost lovljenja $ABTS^{\bullet+}$ radikalov kot $DPPH^{\bullet}$ pričakovana. Prav tako so Abramovič in sod. (2017) poročali, da sestava topila in pH močno vplivata na reaktivnost antioksidantov pri DPPH in ABTS testu. Določitev sposobnosti lovljenja superoksidnega anionskega radikala ($O_2^{\bullet-}$) je metoda, kjer antioksidant in tarčna molekula (nitrotetrazol modro) tekmujeta za $O_2^{\bullet-}$. Naša analiza kakovosti pirinih antioksidantov je pokazala, da njihovo sposobnost lovljenja $DPPH^{\bullet}$, $ABTS^{\bullet+}$ in $O_2^{\bullet-}$ radikalov najbolj izboljšamo, če semena izpostavimo povišani slanosti in osmolarnosti (vzorec NS). Glede na dobljene rezultate lahko predvidevamo, da se različne spojine sintetizirajo pri različnih stresnih pogojih, pri čemer je bila odločilna izbira med dodatkom 50 mM sorbitola ali 25 mM NaCl po 48 h kaljenja. Metoda beljenja β-karotena se od ostalih treh metod razlikuje po tem, da reakcija med antioksidantom in radikalom poteka v sistemu emulzije linolne kisline v vodi. Ker so živila po svoji sestavi večfazni in heterogeni sistemi, predstavlja ta metoda enega bolj realnih medijev, saj antioksidativna aktivnost spojin ni odvisna le od sposobnosti lovljenja radikalov, ampak tudi od porazdelitve med vodno in lipidno fazo. Antioksidanti, prisotni v analiziranem vzorcu, tekmujejo z β-karotenom v reakciji s peroksilnimi ($ROO^{\bullet}$) radikali. V naši raziskavi se je kot najučinkovitejši med

ekstraktibilnimi TPC izkazal vzorec NS. Ugotovili smo, da so pri koncentraciji 15 mg ekvivalent Troloksa (TE)/L bolj učinkoviti lovilci $ROO^{\bullet}$ radikalov vezane fenolne spojine kot ekstraktibilne. Dodatek vezanih fenolnih spojin je pri vseh obdelavah upočasnil beljenje β-karotena vsaj za polovico, kar nakazuje na opazno antioksidativno aktivnost v emulziji ne glede na uporabljene stresne dejavnike. Vezani antioksidanti iz mehansko poškodovanih kalčkov so pokazali slabšo učinkovitost pri lovljenju $DPPH^{\bullet}$, $ABTS^{\bullet+}$ in $O_2^{\bullet-}$ radikalov, po drugi strani pa je mehanski stres izboljšal njihovo antioksidativno učinkovitost v emulziji. Glede na rezultate predvidevamo, da mehanski stres rezultira v sintezi in tvorbi manj polarnih oz. nepolarnih fenolnih spojin. Naša *in vitro* raziskava kaže, da je kaljenje pod abiotskim stresom dober način za izboljšanje antioksidativne aktivnosti pirinih semen. Proces kaljenja, ki vključuje kontrolirane stresne pogoje, kot so temperatura, svetloba, voda, slanost, povečana osmolarnost in mehanske poškodbe, nam omogoča pridobitev pirinih semen s povečano hranilno vrednostjo.

Ali bodo bioaktivne spojine delovale kot antioksidanti v *in vivo* sistemih, je odvisno od številnih faktorjev, kot so koncentracija, struktura in substrat, ki ga morajo zaščititi (Maurya in Devasagayam, 2010). Na podlagi rezultatov, določenih z *in vitro* metodami, ne moremo predvideti antioksidativne aktivnosti kaljenih semen v *in vivo* sistemu, zato smo določali znotrajcelično oksidacijo (ICO) v modelnem organizmu *S. cerevisiae.* Ugotovili smo, da ekstraktibilne in vezane frakcije kaljenih pirinih semen niso pokazale nobene statistično značilne razlike v znotrajcelični oksidaciji v primerjavi z netretiranimi celicami modelnega organizma. S pomočjo HPLC-MS/MS metode smo nadalje ugotavljali celični privzem posameznih fenolnih spojin ekstraktibilnih in vezanih frakcij, pred in po tretiranju kvasnih celic. Sklepamo, da glavni antioksidanti iz kaljenih pirinih semen ne morejo direktno vstopiti v celico in jo zaščititi pred oksidacijo, ampak se morajo pred tem najverjetneje preoblikovati. Cigut in sod. (2011) so ugotavljali celični privzem treh hidroksicimetnih kislin in fenetilnih estrov kavne kisline (CAPE), prehod v celico je bil ugotovljen samo pri CAPE, medtem ko ostale analizirane spojine niso vstopile v celico. Kot poročajo avtorji, je prehod spojin v celico povezan s polarnostjo spojine; CAPE so veliko manj polarni kot pripadajoča kavna kislina. Kljub temu, da so CAPE vstopili v celico, pa tam niso znižali znotrajcelične oksidacije, zato avtorji predvidevajo, da so verjetno ostali na celični membrani. Celični privzem in hkrati tudi znižanje znorajcelične oksidacije so opazili le pri srednje polarni frakciji etanolnih ekstraktov propolisa, ki je večinoma vsebovala flavonoide. Slednje sovpada z našo ugotovitvijo, da identificirane spojine, ki so bolj ali manj polarne, zelo slabo korelirajo s koeficientom antioksidativne aktivnosti določene v emulziji (C_{AA}). Zanimivo je, da so Masisi in sod. (2016) v pregledu literature o *in vivo* raziskavah antioksidantov izpostavili, da se fenolne spojine iz pšeničnih otrobov relativno dobro absorbirajo v GI traktu in lahko delujejo antioksidativno.

Naša raziskava kaže, da je biosinteza in transformacija fenolnih spojin odvisna od specifičnih stresnih razmer med kaljenjem. Kaljenje pod abiotskim stresom velja za varen,

dostopen in enostaven proces, zato pričakujemo, da bi bili potencialni izdelki z izboljšano prehransko vrednostjo dobro sprejeti med potrošniki.

3.1.2 Vpliv fermentacije na vsebnost fenolnih spojin in njihovo antioksidativno aktivnost

Nadalje smo z uporabo fermentacije pirinih semen želeli izboljšati dostopnost fenolnih spojin in njihovo antioksidativno aktivnost. Različne tipe fermentacij smo kombinirali z ostalima biotehnološkima procesoma – kaljenjem in encimsko obdelavo. Naši vzorci niso bili sterilizirani, saj smo glede na preliminarne poskuse ugotovili, da s sterilizacijo izgubljamo enormne količine fenolnih spojin, naš primarni cilj pa je bilo povečanje njihove vsebnosti. Vpliv fermentacije na fenolne spojine je v glavnem odvisen od vrste uporabljenih mikroorganizmov in pogojev fermentacije, kot sta temperatura in čas. Mlečnokislinska, alkoholna, kombinirana (mlečnokislinska + alkoholna) in spontana fermentacija neobdelane, kaljene in encimsko obdelane pire so znatno izboljšale vsebnost ekstraktibilnih in vezanih fenolnih spojin ter pomembno zmanjšale razmerje med vezanimi in ekstraktibilnimi fenolnimi spojinami in tako pozitivno vplivale na dostopnost pirinih antioksidantov. Zanimivo je, da smo zaznali kar velike razlike v razmerju med ekstraktibilnimi in vezanimi TPC pri različnih vrstah fermentiranih semen (neobdelana, kaljena, encimsko obdelana). Pri neobdelanih semenih je bilo razmerje med ekstraktibilnimi in vezanimi TPC znatno manjše kot pri kaljenih ali encimsko obdelanih semenih. Najnižje razmerje med ekstraktibilnimi in vezanimi TPC je bilo ugotovljeno pri neobdelani nefermentirani piri, po fermentaciji pa se je razmerje povečalo za kar 2-krat. Največje razmerje smo našli pri kaljenih semenih, hkrati fermentiranih z *L. plantarum* in *S. cerevisiae* in samo s *S. cerevisiae*. Ne glede na vrsto fermentacije so vezane fenolne spojine prevladovale. Naša raziskava kaže, da tako biološka obdelava pirinih semen s kaljenjem oz. encimsko obdelavo pred fermentacijo, kot tudi vrsta fermentacije vplivata na spremembe vsebnosti skupnih fenolnih spojin. Kaljena semena, uporabljena v tej raziskavi, se lahko obravnava kot material, bogat z encimi za sledečo fermentacijo (Katina in sod., 2007a). Kombinacija kaljenja in fermentacije vodi do sinergističnih učinkov, saj kaljenje prispeva k večji količini fermentabilnih virov (sladkor/dušik), hkrati pa tako kaljenje kot fermentacija pripomoreta k večji koncentraciji in aktivnosti encimov, ki razgrajujejo celične stene, kar lahko prispeva k večji biološki dostopnosti fenolnih kislin (Wang in sod., 2014). Encimska obdelava, kot tehnika za biološko obdelavo semen, omogoča sprostitev fenolnih spojin iz komponent celične stene in tako izboljša biološko dostopnost teh spojin (Angelino in sod., 2017). Vsebnosti bioaktivnih spojin se med fermentacijo spreminjajo zaradi metabolne aktivnosti mikroorganizmov. Med fermentacijo pirinih semen lahko prisotni encimi v semenih in/ali encimi, ki jih proizvajajo mikroorganizmi, razgradijo estrske vezi in hidrolizirajo β-glukozidne vezi, pri čemer se sproščajo fenolne spojine (Adebo in Medina-Meza, 2020). Poudariti pa moramo, da na vsebnost fenolnih spojin med fermentacijo lahko vpliva tudi endogena sinteza fenolnih spojin v mikroorganizmih (Chrzanowski, 2020; Marienhagen in Bott, 2013).

Katina in sod. (2007a) so poročali, da je fermentacija kaljenih rženih semen z uporabo kvasovke *S. cerevisiae* znatno povečala vsebnost skupnih fenolnih kislin in sicer za kar 110 % (zgolj s kaljenjem je bilo povečanje 87 %) v primerjavi z nekaljenimi semeni. Fermentacija je bistveno pripomogla k dvigu vsebnosti prostih fenolnih kislin, zabeležili pa so tudi dvig vsebnosti zaestrenih, glikoziliranih in vezanih fenolnih kislin. Liu in sod. (2017) so poročali, da je fermentacija z dvema MKB izboljšala vsebnost ekstraktibilnih TPC v riževih otrobih, ki so bili predhodno obdelani z α-amilazami. Ugotovili so tudi, da je fermentacija in obdelava z α-amilazami, proteazami in celulazami riževih otrobov povečalo ekstraktibilne TPC za 59 % glede na neobdelan vzorec. Hole in sod. (2012) so pokazali, da fermentacija z MKB poveča vsebnost vezanih TPC v ječmenu, po drugi strani pa zmanjšuje njihovo vsebnost v ovsu. Povečanje vsebnosti vezanih fenolnih spojin v ječmenu so povezali s povečano vsebnostjo topnih prehranskih vlaknin in posledično lažjo ekstrakcijo vezane frakcije. Zdi se, da so topne prehranske vlaknine boljši substrat za feruloil esteraze v primerjavi z netopnimi prehranskimi vlakninami. Povečanje topnih prehranskih vlaknin je lahko posledica delovanja različnih endogenih encimov, ki hidrolizirajo polisaharide (Hole in sod., 2012).

Največjo vsebnost posameznih ekstraktibilnih in vezanih fenolnih spojin in njihovo *in vitro* antioksidativno aktivnost smo določili pri kaljenih semenih fermentiranih s kvasovko *S. cerevisiae*, medtem ko je bila pri encimsko obdelani piri najučinkovitejša fermentacija z MKB *L. plantarum*. Med ekstraktibilnimi fenolnimi spojinami se je vsebnost *trans*-ferulne kisline najbolj povečala in to za kar 2922 % pri s kvasovko *S. cerevisiae* fermentirani kaljeni piri. Med vezanimi fenolimi spojinami je *cis*-ferulna kislina pokazala največje relativno povečanje vsebnosti pri neobdelani piri, fermentirani s kvasovko, in sicer za 466 %. Spremembe pH med različnimi vrstami fermentacije bi lahko zagotovile pH, optimalen za delovanje različnih encimov, ki razgrajujejo celične stene. Na primer, močno povišana vsebnost ekstraktibilnih fenolnih kislin, predvsem *trans*-ferulne kisline, ki se je pojavila med alkoholno fermentacijo, je bila lahko posledica aktivacije feruloil esteraze zaradi spremembe pH (Boskov Hansen in sod., 2002). Anson in sod. (2009) so poročali, da lahko fermentacija pšenice s *S. cerevisiae* poveča biološko dostopnost ferulne kisline. Nadalje so Moore in sod. (2006) dokazali, da sevi *S. cerevisiae* proizvajajo encime, vključno z β-glukozidazami, karboksilesterazami in morda tudi feruloil esterazami. To nakazuje, da je izvor feruloil esteraz mogoče pripisati semenom ali pa kvasovkam. Rezultati naše raziskave se ujemajo tudi z rezultati avtorjev Konopka in sod. (2014), ki so poročali, da alkoholna fermentacija poveča vsebnost proste ferulne kisline v pšenici in rži za kar 10-krat. Prav tako so Antognoni in sod. (2019) ugotovili, da sevi bakterije *L. plantarum* uspešno obogatijo testo s prosto ferulno kislino, vendar pa je bila vsebnost proste ferulne kisline, kljub razmeroma velikemu povečanju, še vedno precej nižja od vsebnosti vezane oblike.

Encimska obdelava in fermentacija sta učinkovali sinergistično na vsebnost posameznih fenolnih kislin, še posebej fermentacija z MKB, ki je rezultirala v velikem povečanju

vsebnosti ekstraktibilne *p*-kumarne (881 %), *trans*-ferulne (385 %) in kavne (838 %) kisline. Hkrati pa je ista fermentacija imela negativen vpliv na njihove vezane oblike. O zmanjšanju vsebnosti vezanih fenolnih kislin, še posebno *p*-kumarne in *trans*-ferulne kisline med fermentacijo, so poročali tudi Spaggiari in sod. (2020). Predvidevali so, da razlog tiči v metabolnih lastnostih mikroorganizmov, ki lahko transformirajo fenolne kisline v različne metabolite. V naši raziskavi se je pokazalo, da imajo eksterni encimi potencial za sproščanje najbolj netopnih vezanih fenolnih spojin iz pirinih semen, saj je prišlo do povečanja vsebnosti ekstraktibilne in zmanjšanja deleža vezane frakcije. Razmerje med količinami identificiranih ekstraktibilnih in vezanih fenolnih kislin se je po fermentaciji povečalo za kar 10-krat pri neobdelanih semenih, za 19-krat pri kaljenih semenih in za 11-krat pri encimsko obdelanih semenih.

Metabolizem fenolnih kislin v MKB poteka s pomočjo reduktaz in dekarboksilaz. Fenolne kisline se dekarboksilirajo v ustrezne fenolne in vinilne derivate. Fermentacija z različnimi vrstami mikroorganizmov vključuje različne encimske reakcije, posledično se sproščajo različne fenolne spojine. Kar zadeva količinsko najbolj zastopano fenolno spojino v pirinih semenih, ferulno kislino, mikrobna razgradnja ferulne kisline vključuje oksidacijo ali redukcijo stranske verige, pri čemer nastane vanilin, sledi nastanek vanilinske kisline z oksidacijo aldehidne skupine in na koncu še tvorba gvajakola in protokatehujske kisline (Bento-Silva in sod., 2020). Metabolizem ferulne kisline s pomočjo *L. plantarum* in *S. cerevisiae* med fermentacijo poteka predvsem po dveh presnovnih poteh. Prva vključuje aktivnost dekarboksilaz, ki so odgovorne za pretvorbo ferulne kisline v 4-vinil gvajakol. Aktivnost dekarboksilaz so identificirali tako v MKB kot tudi v kvasovki. Druga pot pa je redukcija ferulne kisline v dihidroferulno kislino, ki jo povzročajo reduktaze fenolnih kislin (Boudaoud in sod., 2021). Reduktazna aktivnost je bila identificirana v MKB, medtem ko je pri kvasovki *S. cerevisiae* niso odkrili. Zanimivo je bilo, da v naši raziskavi nismo našli in identificirali pričakovanih metabolitov *p*-kumarne, ferulne in kavne kisline. Rezultati so bili tako v nasprotju z raziskavami, ki so poročale, da bakterija *L. plantarum* proizvaja dekarboksilaze, ki so sposobne dekarboksilirati *p*-kumarno, ferulno in kavno kislino do pripadajočih vinil derivatov (Filannino in sod., 2015). Eden od možnih razlogov, zakaj nismo našli metabolitov fenolnih kislin je, da je mogoče dekarboksilirati samo ekstraktibilne fenolne kisline v ustrezne vinil derivate (Filannino in sod., 2018). V naši raziskavi pa so neobdelana, kaljena in encimsko obdelana semena pred fermentacijo vsebovala nizko vsebnost ekstraktibilnih fenolnih kislin. Po drugi strani pa so Coghe in sod. (2004) poročali, da višja vsebnost ferulne kisline ne vodi nujno do višje vsebnosti 4-vinil gvajakola. V ekstraktibilni frakciji fermentiranih pirinih semen smo identificirali in kvantificirali tudi pet flavonoidov (apigenin derivati, *C*-glikozil derivat in galokatehin), ki so bili prisotni v dokaj visokih koncentracijah. Poznavanje mehanizmov sinteze in regulacije fenolnih spojin med fermentacijo je trenutno zelo omejeno.

Na splošno so fermentirana neobdelana, kaljena in encimsko obdelana pirina semena, ki so vsebovala večje vsebnosti TPC izkazala tudi večjo sposobnost lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov. V naši raziskavi antioksidativne lastnosti niso bile odvisne samo od vrste fermentacije, temveč tudi od vrste substrata. S fermentacijo neobdelanih semen ali s kombinacijo tehnik za biološko obdelavo (npr. encimsko obdelavo in fermentacijo) smo dosegli močno izboljšanje antioksidativne aktivnosti. Pri fermentiranih kaljenih semenih je bilo relativno povečanje antioksidativne aktivnosti manjše, vendar pa je bila absolutna antioksidativna aktivnost večja kot pri ostalih biološko obdelanih vzorcih. Sinergizem med antioksidanti v biološko obdelanih vzorcih rezultira v antioksidativnih aktivnostih, ki niso odvisne le od koncentracije antioksidantov, temveč tudi od njihove strukture in interakcij med antioksidanti (Adebo in Medina-Meza, 2020). Zaradi tega se lahko vzorci s podobno vsebnostjo skupnih fenolnih spojin močno razlikujejo glede na svojo antioksidativno aktivnost.

Pri določanju antioksidativne aktivnosti fermentiranih pirinih semen v celici modelnega organizma *S. cerevisiae* smo ugotovili, da so vzorci, ki so pokazali antioksidativno aktivnost *in vivo,* bile predvsem ekstraktibilne frakcije fermentiranih neobdelanih, kaljenih in encimsko obdelanih semen, kjer je spontana fermentacija kaljenih in encimsko obdelanih semen najučinkoviteje znižala znotrajcelično oksidacijo v modelnem organizmu. Očitno je pestrost avtohtone mikrobiote pirinih semen pripomogla k večji raznolikosti nastalih metabolitov, ki so lažje vstopili v celico. Pokazali smo, da nekatere ekstraktibilne in vezane frakcije fermentiranih pirinih semen izkazujejo antioksidativno aktivnost v *in vivo* sistemu. Gre za pomembno odkritje, saj fenolne spojine samo kaljenih pirinih semen pri izpostavitvi abiotskemu stresu niso vstopile v modelni organizem in niso izkazale antioksidativne aktivnosti *in vivo.* Fermentacija vključuje sintezo novih spojin, kot tudi encimsko transformacijo že prisotnih različnih bioaktivnih spojin. Naši rezultati so pokazali, da je ključni dejavnik za izkazano antioksidativno aktivnost *in vivo* za fermentirana neobdelana in kaljena semena razmerje med različnimi fenolnimi spojinami, ki vstopajo v celico. Pri večji količini flavonoidov in manjši količini hidroksicimetnih kislin, ki so prehajale v celico, smo zabeležili nižjo znotrajcelično oksidacijo. Med vezanimi frakcijami sta samo tisti iz spontano fermentiranih in nefermentiranih encimsko obdelanih semen pokazali znižanje znotrajcelične oksidacije. Celični privzem vezanih hidroksicimetnih kislin je bil znatno večji kot pri ekstraktibilnih kislinah, vendar so bile slednje učinkovitejše pri znižanju znotrajcelične oksidacije. Tako lahko sklepamo, da imajo ekstraktibilne fenolne spojine boljšo antioksidativno aktivnost *in vivo* kot vezane fenolne spojine.

Pri drugem sklopu raziskav smo ugotovili, da je kombiniranje biotehnoloških procesov najučinkovitejši način za znatno povečanje vsebnosti ekstraktibilnih in vezanih skupnih in posameznih fenolnih spojin, njihove antioksidativne aktivnosti *in vitro* ter v nekaterih primerih *in vivo.* Naši rezultati kažejo, da se lahko fermentacija pirinih semen uporablja za

izboljšanje vsebnosti fenolnih spojin in antioksidativne aktivnosti, kar bi lahko dodatno izboljšalo njihovo biološko dostopnost.

3.1.3 Vpliv encimske obdelave na vsebnost fenolnih spojin in njihovo antioksidativno aktivnost

Tretji biotehnološki proces, s katerim smo biološko obdelali pirina semena, je encimska obdelava. V raziskavi smo z različnimi kombinacijami uporabe celulaz (C), ksilanaz (X), feruloil esteraz (E), α-amilaz (A) in proteaz (P) uspešno sprostili vezane fenolne spojine pirinih semen. Encimske obdelave so izboljšale vsebnost ekstraktibilnih TPC pirinih semen do 5-krat v primerjavi z neobdelanimi semeni, medtem ko se je vsebnost vezanih fenolnih spojin zmanjšala po encimski obdelavi. Med vsemi različnimi encimskimi obdelavami je obdelava pirinih semen z vsemi petimi encimi (C+X+E+A+P) najbolj povečala vsebnost ekstraktibilnih TPC. Naši rezultati so pokazali, da uporaba samo feruloil esteraz pri obdelavi semen ne poveča bistveno ekstraktibilnih TPC, kar nakazuje na to, da uporabljene esteraze niso sposobne samostojno hidrolizirati estrskih povezav med hidroksicimetnimi kislinami in hemicelulozo v pirinih semenih zaradi možne sterične ovire, ki jo povzroča struktura polisaharidov, s čimer je omejena tudi migracija encimov. So pa zato vse tri obdelave, ki so vključevale kombinacijo feruloil esteraz in proteaz, pokazale veliko sposobnost sproščanja vezanih TPC v ekstraktibilne. Ti rezultati potrjujejo, da feruloil esteraze in proteaze sodelujejo pri razgradnji celične stene, tako da feruloil esteraze hidrolizirajo estrsko in etrsko vez med fenolnimi spojinami in strukturnimi komponentami celične stene pirinih semen ter tako sprostijo vezane fenolne spojine (Acosta-Estrada in sod., 2014). Proteaze pa so vključene v hidrolizo strukturnih proteinov, na katere so vezane fenolne spojine, posledično pa se poveča sposobnost sproščanja vezanih TPC iz pirinih semen. Poleg tega so Everette in sod. (2010) pokazali, da številne nefenolne spojine kažejo precejšnjo reaktivnost s Folin-Ciocalteu reagentom, vključno s proteini, ki pa se lahko po svoji reaktivnosti do Folin-Ciocalteu reagenta precej razlikujejo. V nasprotju z našimi pričakovanji smo ugotovili, da so bile obdelave s ksilanazami, esterazami in celulazami, posamezno ali v kombinaciji, manj učinkovite pri sproščanju vezanih TPC. Encimska obdelava ne more v celoti pretvoriti vezanih fenolnih spojin v ekstraktibilno obliko, saj lahko matrica celične stene pirinih semen vsebuje strukturne elemente, ki jih encimi ne morejo hidrolizirati (Moore in sod., 2006).

Kaljenje pirinih semen pod abiotskim stresom in fermentacija semen s kvasovko *S. cerevisiae* sta znatno povečala vsebnosti TPC in njihovo antioksidativno aktivnost glede na neobdelana pirina semena. Rezultati raziskave, kjer smo ugotavljali vpliv encimske obdelave pirinih semen, pa so pokazali, da je encimska obdelava kaljenih in fermentiranih semen dodatno povečala vsebnost ekstraktibilnih TPC v primerjavi s samo kaljenimi oz. fermentiranimi semeni. Omeniti velja še, da je encimska obdelava kaljenih in fermentiranih semen rezultirala v povečanju deleža ekstraktibilnih TPC glede na skupne TPC (ekstraktibilne + vezane) s 34 % na 44 % in z 12 % na 50 %. Rezultati nadalje kažejo, da zaporedna uporaba kaljenja/fermentacije in encimske obdelave (C+X+E+A+P) poleg

transformacije netopnih fenolnih spojin v topne ekstraktibilne, omogoča oslabitev estrske in etrske vezi med netopnimi fenolnimi spojinami in komponentami celične stene, zaradi česar fenolne spojine, ki so še v netopni obliki, lažje ekstrahiramo in tako določimo njihovo večjo vsebnost. Slednje sovpada z ugotovitvami Bei in sod. (2018).

Vse encimske obdelave so povečale vsebnost ekstraktibilne *p*-kumarne, *trans*-ferulne, kavne in *p*-hidroksibenzojske kisline, v primerjavi z neobdelanim vzorcem. Vzorci, ki so pokazali znatno povečanje vsebnosti ekstraktibilne *p*-kumarne, ferulne in *p*-hidroksibenzojske kisline (zlasti C+X+E+A+P in C+X+E+P), so bili posebno učinoviti pri lovljenju $ABTS^{\bullet+}$ radikalov in so vsebovali večjo vsebnost ekstraktibilnih TPC. Smatramo, da so te fenolne kisline znatno prispevale k antioksidativni aktivnosti pirinih semen in da izkazujejo večjo reaktivnost v Folin-Ciocalteu testu. Kombinacija ksilanaz in esteraz je povzročila največje povečanje vsebnosti ekstraktibilne *trans*-ferulne kisline, saj ksilanaze naključno cepijo β-1,4 ksilansko strukturo, medtem ko so esteraze sposobne sproščati ferulno kislino (Sancho in sod., 2001). Pirini otrobi so bogat vir prehranskih vlaknin, vsebujejo pa tudi okoli 41 % škroba (Escarnot in sod., 2012). Obdelava z α-amilazami je sprostila zelo malo vezanih fenolnih kislin, posledično je vsebnost vezanih fenolnih spojin, še posebej *p*-kumarne in ferulne kisline, ostala visoka. Predvidevamo, da je možen razlog lokacija škrobnih zrnc v rastlinski celici, saj se nahajajo predvsem v endospermu, večina fenolnih kislin v piri pa je vezanih na neškrobne polisaharide v otrobih. Zanimivo pa je, da je bila vsebnost ekstraktibilnih TPC pri obdelavi z α-amilazami precej visoka, kar je lahko posledica dejstva, da je Folin-Ciocalteu reagent nespecifičen, saj se odziva tudi na reducirajoče sladkorje, ki so produkt aktivnosti α-amilaz. Sočasna uporaba vseh petih encimov je povzročila razgradnjo celuloze, arabinoksilanov, ostalih polisaharidov in proteinov ter posledično zmanjšala molekulsko maso in velikost komponent celične stene, prekinila vezi med komponentami celične stene in fenolnih spojin in tako povečala sproščanje fenolnih spojin iz pirinih semen. Naše ugotovitve se ujemajo z rezultati drugih raziskav, tako so Rakariyatham in sod. (2020) poročali, da je obdelava s celulazami povečala sproščanje *o*-kumarne kisline. Peixoto Araujo in sod. (2019) so ugotovili, da je obdelava s proteazami in celulazami povečala vsebnost ekstraktibilnih fenolnih kislin, kot sta *p*-hidroksibenzojska in ferulna kislina. V naši raziskavi je kombinacija kaljenja in encimske obdelave ter fermentacije in encimske obdelave pirinih semen privedla do izjemnega povečanja vsebnosti ekstraktibilne *trans*-ferulne kisline, za kar 5899 % in 8263 %. Encimsko obdelana kaljena semena so imela največjo absolutno vsebnost ekstraktibilnih fenolnih kislin. Različni encimi so lahko med kaljenjem pirinih semen neposredno ali posredno vključeni v tvorbo ekstraktibilnih fenolnih spojin. Blagi pogoji encimske obdelave (pH 6, 40 °C) v naši raziskavi so bili hkrati idealni za boljšo aktivnost endogenih encimov (esteraze, proteaze, amilaze, itd.) (Rakariyatham in sod., 2020). Kaljena in fermentirana semena so se, v primerjavi z neobdelanimi pirinimi semeni, izkazala za znatno boljši substrat za obdelavo z eksternimi encimi. Predvidevamo lahko, da so predhodno obdelana pirina semena omogočila dodanim encimom lažji dostop do njihovih substratov kot neobdelana.

Antioksidativne aktivnosti encimsko obdelanih pirinih semen, ki so bile ugotovljene z uporabo $ABTS^{\bullet+}$ radikalov, so močno pozitivno korelirale z vsebnostjo ekstraktibilnih (r = 0,905) in vezanih (r = 0,778) TPC, medtem ko so bile korelacije med DPPH testom in vsebnostjo ekstraktibilnih (r = -0,155) in vezanih (r = 0,097) TPC zanemarljive. Ti podatki kažejo, da so verjetno polarne fenolne spojine v encimsko obdelanih pirinih semenih glavni lovilci $ABTS^{\bullet+}$ radikalov. Encimska obdelava je povečala antioksidativno aktivnost semen verjetno s sproščanjem polarnih antioksidantov, vezanih na celično steno, in/ali s hidrolizo biopolimerov, kot so polipeptidi in polisaharidi (Azmir in sod., 2013). Hkrati pa na reaktivnost fenolnih spojin vpliva tudi izbor metode, tako Folin-Ciocalteu test najbolje korelira z ABTS testom, ker sta oba izvedena v rahlo alkalnem vodnem mediju (pH 7,4), medtem ko je DPPH test izveden v metanolu (Abramovič in sod., 2017). Po drugi strani pa nizka korelacija med antioksidativno aktivnostjo, določeno z DPPH testom, in vsebnostjo ekstraktibilnih in vezanih TPC kaže, da fenolne spojine v encimsko obdelanih semenih slabše reagirajo z DPPH reagentom. V naših raziskavah so bile vrednosti antioksidativne aktivnosti, določene z ABTS testom, znatno višje od tistih določenih z DPPH testom. Zaradi visoke vsebnosti *p*-kumarne in ferulne kisline v biološko obdelanih pirinih semenih so bili dobljeni rezultati pričakovani, saj $ABTS^{\bullet+}$ radikal bistveno bolje reagira z monofenolnimi spojinami kot $DPPH^{\bullet}$ radikal. Naši razultati so potrdili, da je kombinacija kaljenja oziroma fermentacije z encimsko obdelavo dobra strategija za izboljšanje antioksidativne aktivnosti ekstraktibilnih fenolnih spojin. Glede na raziskavo Wang in sod. (2018) obstajata dve možni razlagi za to: prva je, da smo z encimsko obdelavo (biološko obdelavo) povečali topnost fenolnih spojin iz pirinih semen, druga pa je, da smo pridobili fenolne spojine z večjo antioksidativno aktivnostjo.

Pri določanju antioksidativne aktivnosti v celici modelnega organizma *S. cerevisiae* smo ugotovili, da so samo ekstraktibilne frakcije semen, ki so bila podvržena kombinacijam biotehnoloških procesov, znižale znotrajcelično oksidacijo, medtem ko vezane frakcije encimsko obdelanih kaljenih in fermentiranih semen niso pokazale nobenih statistično značilnih razlik v znotrajcelični oksidaciji v primerjavi s kontrolo. Zanimivo je, da je med vezanimi frakcijami samo encimska obdelava neobdelanih semen pokazala znižanje znotrajcelične oksidacije v kvasovki. Nasprotno, vezane frakcije neobdelanih semen in samo kaljenih semen so celo zvišale znotrajcelično oksidacijo, zato sklepamo, da omenjeni frakciji vsebujeta prooksidativne snovi, ki povečujejo znotrajcelično oksidacijo. Znano je, da z *in vitro* testi določanja antioksidativne aktivnosti ne moremo predvideti, kakšna bo antioksidativna aktivnost *in vivo*, to ponazarjajo tudi negativne korelacije med antioksidativno aktivnostjo, določeno z DPPH testom oz. ABTS testom, ter antioksidativno aktivnostjo, določeno v celici kvasovke. Zanimivo je, da večji celični privzem fenolnih spojin ne pomeni nujno tudi večje antioksidativne aktivnosti v celici. Vezana frakcija encimsko obdelanih neobdelanih semen je pokazala nižjo antioksidativno aktivnost v celici modelnega organizma kot pripadajoča ekstraktibilna frakcija, medtem ko je bil celični privzem vezanih hidroksicimetnih kislin, določen s HPLC-MS/MS metodo kot razlika v

koncentraciji fenolnih kislin pred in po tretiranju kvasnih celic, kar 24-krat višji od ekstraktibilnih. Rezultati kažejo, da ni pomembna samo količina fenolnih spojin, ki vstopajo v celico, ampak tudi oblika (ekstraktibilna/vezana), v kateri vstopijo v celico. Zato je ekstraktibilna oblika fenolnih spojin ključna za vstop v celico. Ugotovili smo, da encimska obdelava fermentiranih semen najbolj zniža znotrajcelično oksidacijo. Očitno fermentacija pirinih semen s kvasovko *S. cerevisiae* lahko pretvori fenolne kisline v metabolite in nove spojine, ki lažje vstopajo v celico. Poleg tega je dodajanje eksternih encimov po fermentaciji omogočilo še dodatno sproščanje netopnih vezanih fenolnih spojin iz pirinih semen.

Naši rezultati so prvič pokazali, da je mogoče encimsko obdelavo uporabiti v kombinaciji z drugimi biotehnološkimi procesi (kaljenje, fermentacija) za povečanje vsebnosti ekstraktibilih fenolnih spojin in njihove antioksidativne aktivnosti in tako izboljšati potencialno biološko dostopnost antioksidantov pirinih semen. Vzorci, ki so bili obdelani z vsemi petimi hidrolitičnimi encimi skupaj, so pokazali znatno izboljšanje vsebnosti ekstraktibilne *p*-kumarne, ferulne in *p*-hidroksibenzojske kisline, skupnih fenolnih spojin in učinkovitejšo *in vitro* antioksidativno aktivnost. Potrebne pa so nadaljnje študije za raziskovanje možne transformacije fenolnih spojin v njihove metabolite, ki imajo večjo antioksidativno aktivnost in lažje vstopajo v celico. Iz tega razloga bi lahko bilo encimsko sproščanje fenolnih spojin uporabno tako v prehrambeni kot tudi nutracevtski industriji.

3.1.4 Vpliv biološke obdelave pirinih semen na biološko dostopnost fenolnih spojin

Da bi fenolne spojine pirinih semen imele zdravju koristne učinke, se morajo uspešno sprostiti iz matrice zaužite hrane, hkrati morajo prestati pogoje GI trakta in v tarčnem tkivu nato izkazati bioaktivne lastnosti (Shahidi in Pan, 2021). Biotehnološki procesi (kaljenje, fermentacija, encimska obdelava) dokazano izboljšajo biološko dostopnost fenolov iz žitnih semen. Tako so Koistinen in sod. (2017) ugotovili, da se biološka dostopnost ferulne kisline znatno poveča v kruhu iz biološko obdelanih (fermentacija + encimska obdelava) rženih otrobov v primerjavi s kruhom iz navadnih rženih otrobov (88 % proti 51 %). Prav tako so Anson in sod. (2009) poročali, da je bila kombinacija uporabe eksternih encimov in fermentacije učinkovit pristop, saj je povečala biološko dostopnost ferulne kisline z 1 % na skoraj 6 %. Namen naše raziskave je bil oceniti vpliv različnih tehnik biološke obdelave na izboljšanje biološke dostopnosti fenolnih spojin iz pirinih semen. Uporabili smo *in vitro* prebavni model, s katerim smo posnemali prebavo v ustih, želodcu in tankem črevesu. Med prebavljenimi vzorci smo največjo vsebnost ekstraktibilnih in vezanih TPC našli pri kaljenih semenih, fermentiranih s *S. cerevisiae*. Glede na neobdelana prebavljena semena je bila vsebnost ekstraktibilnih TPC za kar 7-krat večja, najverjetneje kot posledica delovanja žitnih in mikrobnih encimov, ki so povzročili strukturno degradacijo celične stene otrobov. Zanimivo, da so samo kaljena semena po prebavi pokazala statistično značilno povečanje vsebnosti ekstraktibilnih TPC (16 %) glede na pripadajoči neprebavljen vzorec. Povečanje vsebnosti ekstraktibilnih fenolnih spojin po prebavi je lahko rezultat prebavnih encimov in

žolčnih soli, ki delujejo na matrico pirinih semen in tako olajšajo sproščanje vezanih fenolnih spojin v prebavni sok (Zhang in sod., 2017). Poleg tega, prehod iz kislega v alkalno okolje vodi do deprotonacije hidroksilnih skupin aromatskih obročev, kar bi lahko prispevalo k povečanju vsebnosti ekstraktibilnih TPC prebavljenih kaljenih semen (Tomé-Sánchez in sod., 2021). Možen razlog je lahko tudi struktura kaljenih semen, saj je škrob v kaljenih semenih praviloma bolj prebavljiv zaradi encimsko modificirane strukture škrobnih zrn, tankih celičnih sten ter lažje dostopnih mono- in disaharidov. Polisaharidi v celičnih stenah so med kaljenjem hidrolizirani z *de novo* sintetiziranimi encimi, kar povzroči spremembe v sestavi netopnih in topnih prehranskih vlaknin žitnih semen (Xu in sod., 2021). Vsebnost vezanih TPC se je znatno zmanjšala v skoraj vseh prebavljenih vzorcih v primerjavi z neprebavljenimi. Delež vezanih TPC glede na skupne TPC (ekstraktibilni + vezani) je bil najvišji (80 %) pri prebavljenih neobdelanih semenih in najnižji (51 %) pri prebavljenih encimsko obdelanih semenih, fermentiranih z *L. plantarum*. Zmanjšanje vsebnosti vezanih TPC v prebavljenih vzorcih kaže na njihovo delno pretvorbo v ekstraktibilno obliko med prebavo, čeprav se kvantitativne spremembe na splošno ne odražajo v povečanju ekstraktibilne frakcije. Do podobnih rezultatov so prišli tudi Ortega in sod. (2011) in Ydjedd in sod. (2017), saj so poročali o znatnem upadu količine prostih fenolnih spojin rožičeve moke po GI prebavi. Poleg tega so Chait in sod. (2020) poročali, da so se po fazi prebave v tankem črevesu vrednosti TPC v topni prosti (-28 %), topni konjugirani (-66 %) in vezani (-68 %) frakciji rožičeve moke drastično zmanjšale v primerjavi z neprebavljenim vzorcem. Rezultati naše raziskave kažejo, da je vsebnost biološko dostopnih fenolnih spojin v biološko obdelanih semenih znatno večja (do 589 % v "kaljenih + fermentiranih" semenih) kot v neobdelanih semenih.

Kombinacija encimske obdelave in fermentacije ima večji vpliv na povečanje vsebnosti ekstraktibilne ferulne kisline v pirinih semenih kot sam proces fermentacije. Na podlagi naših rezultatov predvidevamo, da obdelava pirinih semen z eksternimi hidrolitičnimi encimi poveča dostopnost vezane *trans*-ferulne kisline za delovanje hidrolitičnih encimov fermentacijskih mikroorganizmov. Med prebavljenimi vzorci so "kaljena + fermentirana" semena pokazala največjo vsebnost vseh identificiranih ekstraktibilnih in vezanih fenolnih kislin. Zdi se, kot da med kaljenjem pire nastaja oz. se aktivira optimalna količina encimov za *de novo* sintezo fenolnih spojin in za razgradnjo komponent celične stene, kar poveča tudi vsebnost fermentabilnih virov za mikroorganizme, posledično kaljena semena predstavljalo dober substrat za delovanje mikroorganizmov. Po GI prebavi se je vsebnost vezanih fenolnih kislin zmanjšala v primerjavi z neprebavljenimi vzorci. Prav tako se je vsebnost večine ekstraktibilnih fenolnih kislin zmanjšala v prebavljenih vzorcih. Po drugi strani pa se je vsebnost ekstraktibilne *trans*-ferulne kisline povečala po prebavi v "kaljenih + fermentiranih" semenih za 25 %, prav tako sta se po prebavi povečali vsebnosti ekstraktibilne kavne (33 %) in *p*-hidroksibenzojske (73 %) kisline v "kaljenih + encimsko obdelanih" semenih. Po GI prebavi se je vsebnost *trans*- in *cis*-ferulne kisline drastično zmanjšala v nekaterih biološko obdelanih semenih. Izguba *trans*-ferulne kisline po prebavi

je bila od 69 % ("kaljena + encimsko obdelana" semena) do 91 % (encimsko obdelana semena) v primerjavi s pripadajočimi neprebavljenimi vzorci. Drastične izgube fenolnih kislin po GI prebavi so verjetno posledica spremembe v molekulski strukturi fenolnih kislin zaradi različnih kemijskih reakcij, predvsem oksidacije in polimerizacije, in zaradi encimskega delovanja, posledično lahko pride do spremembe v njihovi topnosti (Ortega in sod., 2011). Pomembno je, da so semena biološko obdelana, saj pri prebavi prihaja do velikih izgub. Če imajo naši biološko obdelani vzorci znatno večjo izhodiščno vsebnost fenolnih kislin kot neobdelani vzorec, bo posledično več fenolnih spojin prestalo proces prebave. Tako so imela po prebavi biološko obdelana semena znatno večjo vsebnost ekstraktibilne *trans*-ferulne kisline in sicer od 7-krat (fermentirana semena) do 140-krat ("kaljena + fermentirana" semena) v primerjavi z neobdelanimi semeni. Med prebavljenimi vzorci so dale kombinacije tehnik več ekstraktibilnih fenolnih kislin kot neobdelana semena ali tista, obdelana zgolj s posamezno tehniko biološke obdelave.

Antioksidativne lastnosti fenolnih spojin se lahko spremenijo zaradi kemijskih transformacij, ki so posledica različnih mehanizmov med GI prebavo. V primerjavi z neobdelanimi semeni so biotehnološki procesi znatno povečali sposobnost lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov ekstraktibilnih frakcij prebavljenih vzorcev. Po prebavi so bile DPPH vrednosti ekstraktibilnih fenolnih spojin v "kaljenih + fermentiranih" semenih 21-krat večje, medtem ko so bile DPPH vrednosti vezanih fenolnih spojin 2-krat večje kot v prebavljenih neobdelanih semenih. Ista kombinacija tehnik biološke obdelave semen je bila optimalna tudi pri ABTS testu, vendar pa je potrebno poudariti, da relativno povečanje ni bilo primerljivo z DPPH metodo. Ekstraktibilna frakcija "kaljenih + fermentiranih" semen je pokazala 4,6-krat večjo reaktivnosti proti $ABTS^{\bullet+}$ radikalu, medtem ko je vezana frakcija pokazala 1,4-krat večjo reaktivnost kot prebavljena neobdelana semena. Dobljeni rezultati kažejo na spremembe v sestavi ekstraktov med simulirano *in vitro* prebavo, saj so antioksidanti istega ekstrakta pokazali drugačen odziv proti različnim prostim radikalom v primerjavi z neobdelanimi semeni. Podobne razlike smo opazili tudi, ko smo prebavljene vzorce primerjali z neprebavljenimi. Na primer, DPPH vrednosti ekstraktibilnih fenolnih spojin kaljenih semen so pokazale veliko povečanje (63 %) po GI prebavi glede na neprebavljen vzorec, medtem ko je bilo povečanje ABTS vrednosti po prebavi zanemarljivo (2 %). Večjo antioksidativno aktivnost po prebavi bi lahko pripisali spremembam pH in deprotonaciji hidroksilnih skupin, prisotnih na aromatskih obročih fenolnih spojin (Chait in sod., 2020). To bi lahko bilo povezano tudi s strukturnimi spremembami molekul fenolnih spojin ali s sproščanjem novih spojin z večjo antioksidativno aktivnostjo (Chait in sod., 2020). Drugi biološko obdelani vzorci so pokazali zmanjšanje sposobnosti lovljenja $DPPH^{\bullet}$ in $ABTS^{\bullet+}$ radikalov ekstraktibilnih fenolnih spojin, z največjim zmanjšanjem 70 % pri "fermentiranih + encimsko obdelanih" semenih v primerjavi z neprebavljenimi vzorci. Upad antioksidativne aktivnosti med prebavo je lahko posledica nižje vsebnosti skupnih fenolnih spojin oz. dejstva, da je mogoče nekatere fenolne spojine pretvoriti v različne derivate z različnimi kemijskimi lastnostmi, zaradi njihove občutljivosti na nevtralni pH v fazi prebave

v tankem črevesu (Ydjedd in sod., 2017). Poleg možnih medmolekulskih interakcij (vodikove vezi) fenolnih kislin s topilom v reakcijski mešanici, ki lahko zmanjšajo njihovo reaktivnost v antioksidativnih testih, lahko fenolne kisline tvorijo tudi intramolekularne vodikove interakcije, kar lahko vpliva na prenos vodikovega atoma do prostega radikala (Terpinc in Abramovič, 2010). Antioksidativna aktivnost fenolnih spojin je odvisna od njihove molekulske strukture. Dokazano je, da CH=CH-COOH skupina hidroksicimetnih kislin zagotavlja večjo sposobnost prenosa vodikovega atoma in posledično stabilizacijo radikalov kot karboksilna (COOH) skupina hidroksibenzojskih kislin (Rice-Evans in sod., 1996). Ekstrakti žitaric so zelo kompleksne mešanice številnih različnih spojin z različnimi aktivnostmi.

Po *in vitro* prebavi se je del fenolnih spojin iz pirinih semen sprostil v supernatant in te molekule so predstavljale biološko dostopne fenolne spojine, ki jih naše telo lahko absorbira. "Kaljena + fermentirana" semena so pokazala najvišjo biološko dostopnost TPC (37,4 %), poleg tega so ta semena pokazala tudi najvišjo vsebnost biološko dostopnih (ekstraktibilnih) fenolnih kislin in flavonoidov, kvantificiranih s HPLC-MS/MS. Najnižjo biološko dostopnost TPC so pokazala neobdelana semena (18,4 %). Manjšo biološko dostopnost fenolnih spojin v neobdelanih semenih pripisujemo dejstvu, da je večina fenolih spojin v rastlinah konjugiranih z drugimi molekulami, kot so ogljikovi hidrati, celuloza in lignin, ki so odporni na prebavo. Pokazali smo, da biološka obdelava pirinih semen poveča biološko dostopnost fenolnih spojin za najmanj 47 % ("fermentirana + encimsko obdelana" semena) pa vse do 103 % ("kaljena + fermentirana" semena) v primerjavi z neobdelanimi semeni. Del fenolnih spojin je po prebavi ostal v prebavljenem ostanku pirinih semen, kar kaže, da niso biološko dostopni, vendar pa so biološko stabilni (vezani fenoli), saj preživijo proces prebave. Pri neobdelanih semenih smo opazili znatno višjo biološko stabilnost fenolnih spojin v primerjavi z biološko obdelanimi semeni. Biološka stabilnost TPC v neobdelanih semenih je bila od 31 % do 141 % večja kot v biološko obdelanih semenih. Čeprav so imela neobdelana semena najvišji odstotek biološko stabilnih fenolnih spojin, je bila vsebnost biološko stabilnih fenolnih spojin v "kaljenih + fermentiranih" semenih znatno večja (2-krat) kot v neobdelanih semenih. Bolj kot so fenolne spojine stabilne med GI prebavo, več jih vstopa naprej v debelo črevo, kjer jih prisotna mikrobiota presnavlja v produkte, ki jih naše telo lažje absorbira. Vendar pa so Ren in sod. (2022) poročali, da je večina fenolnih spojin z več hidroksilnimi skupinami nestabilnih. Eden od načinov za izboljšanje biološke dostopnosti oz. stabilnosti fenolnih spojin je s pomočjo njihove inkapsulacije (Peanparkdee in Iwamoto, 2022). Biološka dostopnost posameznih fenolnih kislin se med različno biološko obdelanimi pirinimi semeni zelo razlikuje. Biološka obdelava semen s fermentacijo in encimsko obdelavo, posamezno ali v kombinaciji, je pokazala največjo biološko dostopnost *p*-kumarne kisline, vendar pa je bila količina biološko dostopne *p*-kumarne kisline po prebavi največja pri "kaljenih + fermentiranih" semenih. V primeru *trans*-ferulne kisline so se še posebej izkazale kombinacije dveh različnih tehnik biološke obdelave, ki so njeno biološko dostopnost povečale za od 24-krat ("kaljena + encimsko obdelana" semena)

do 63-krat ("encimsko obdelana + fermentirana" semena) v primerjavi z neobdelanimi semeni. Podobne rezultate so opazili tudi Anson in sod. (2009), ki so poročali, da je bila najučinkovitejša tehnika biološke obdelave kombinacija fermentacije in encimske obdelave pšeničnih otrobov, saj je povečala biološko dostopnost ferulne kisline za 5-krat v primerjavi z nativnimi otrobi. Med fenolnimi kislinami je *p*-hidroksibenzojska kislina pokazala največjo biološko dostopnost, do 57 %. Več avtorjev je izpostavilo, da na stabilnost fenolnih spojin med procesom GI prebave močno vpliva njihova kemijska struktura, saj imajo fenolne spojine različno občutljivost na variacije pH in aktivnost prebavnih encimov (Goulas in Hadjisolomou, 2019; Lima in sod., 2019). Po prebavi je največji odstotek fenolnih kislin ostal biološko stabilen v neobdelanih semenih, kar je skladno s stabilnostjo TPC. Ker neobdelana semena niso predhodno obdelana z biotehnološkimi procesi, ostaja substrat težje dostopen za prebavne encime. Posledično imajo neobdelana semena največji delež fenolnih spojin, vezanih na različne komponente celične stene. Sęczyk in sod. (2021) so poročali, da so matrice z visoko vsebnostjo netopnih prehranskih vlaknin in proteinov pokazale močnejše interakcije s fenolnimi spojinami in manjšo prebavljivost. Nadalje so Xu in sod. (2020) poročali, da neškrobni polisaharidi ščitijo nanje vezane fenolne spojine pred encimi v ustih, želodcu in tankem črevesu. Na splošno sta *p*-kumarna in *trans*-ferulna kislina pokazali največjo biološko stabilnost, medtem ko je *p*-hidroksibenzojska kislina pokazala najmanjšo stabilnost. Tomé-Sánchez in sod. (2021) so poročali, da imajo derivati ferulne kisline ječmenovih in pšeničnih kalčkov visoko stabilnost na prebavne pogoje. Kljub bistvenemu povečanju biološke dostopnosti fenolnih kislin, doseženim z uporabo različnih biotehnoloških procesov, je večji del fenolnih kislin ostal v biološko nedostopni (stabilni) obliki, ki nadalje vstopa v debelo črevo. V debelem črevesu naj bi fermentacija komponent celične stene z delovanjem bakterijskih encimov olajšala sproščanje fenolnih kislin, ki v tankem črevesu niso bile dostopne. Zato je pomembno razumeti, kako proces prebave vpliva na strukturo in stabilnost fenolnih spojin, saj to posledično vpliva na njihovo biološko dostopnost in možne koristne učinke v celicah črevesnega epitelija (Correa-Betanzo in sod., 2014). Zeng in sod. (2016) so poročali, da je bila vsebnost biološko dostopnih fenolnih spojin v pšenici manjša kot pri rjavem rižu in ovsu kljub večji vsebnosti TPC in močneje izraženi antioksidativni aktivnosti. To nakazuje, da žita z največjo vsebnostjo fenolnih spojin niso nujno tista z najvišjo biološko dostopnostjo. Zdi se, da je žitna matrica ključni dejavnik pri prebavljivosti in stabilnosti fenolnih spojin med prebavo. Čeprav je ferulna kislina najpogostejša fenolna spojina v piri, je bila v naši raziskavi njena biološka dostopnost (maks. 6,3 %) znatno nižja od biološke dostopnosti TPC (maks. 37,4 %). Možen razlog za tako veliko razliko v dostopnosti je, da je bila identiteta številnih spojin, prisotnih v supernatantu prebavljenih vzorcev, neznanih.

Biotehnološki procesi (kaljenje/fermentacija/encimska obdelava), katerih cilj je izboljšati biološko dostopnost fenolnih spojin iz žitnih izdelkov, so lahko obetaven pristop k izboljšanju zdravja ljudi na sistemski ravni. Naša raziskava je pokazala statistično značilen učinek biotehnoloških procesov na povečanje vsebnosti biološko dostopnih skupnih,

posameznih fenolnih spojin in njihove antioksidativne aktivnosti v primerjavi z neobdelanimi semeni. Kombinacija biotehnoloških procesov, zlasti kaljenja in fermentacije, je bila najučinkovitejša metoda za drastično povečanje vsebnosti skupnih in posameznih biološko dostopnih fenolnih spojin in njihove antioksidativne aktivnosti. Biološka dostopnost *p*-kumarne in *trans*-ferulne kisline pa je bila največja pri »encimsko obdelanih + fermentiranih« semenih. Uporaba modela *in vitro* prebave je omogočila podrobnejši vpogled v posamezne fenolne spojine pirinih semen, ki so človeku dejansko dostopne. Vendar pa se je v naši študiji potrdilo, da se v žitih večina fenolnih spojin nahaja v vezani obliki in se ne sprostijo med GI prebavo, temveč šele v debelem črevesu. Poudariti je potrebno, da naša simulacija prebave ni zajela pomembne stopnje - fermentacije v debelem črevesu, kjer so fenolne spojine podvržene metabolizmu s strani naravno prisotne črevesne mikrobiote.

3.2 PRISPEVEK K RAZVOJU ZNANOSTI

Pridobljene informacije se lahko uporabljajo za izboljšanje prehranske vrednosti kruha in drugih izdelkov iz žit. Kolikor nam je znano, nobena raziskava o vplivu biotehnoloških procesov na izboljšanje dostopnosti fenolnih spojin iz rastlinskega materiala ni zajemala tako širokega spektra biotehnoloških procesov, optimizacije posameznih tehnik in njihovih kombinacij. Prav tako smo pridobljenim ekstraktom poleg *in vitro* antioksidativne aktivnosti določali še antioksidativno aktivnost v celičnem organizmu *S. cerevisiae,* ki nam daje vpogled v živo celico. Z namenom omejevanja številnih kroničnih bolezni različne zdravstvene organizacije doma (Nacionalni program o prehrani in telesni dejavnosti za zdravje 2015-2025) in po svetu (poročilo Svetovne zdravstvene organizacije za obdobje 2012-2016) spodbujajo večje uživanje polnozrnatih izdelkov. Ravno v vključevanju biotehnološko obdelanih semen v živila, ki jih uživamo na dnevni ravni, vidimo velik potencial za vnos bioaktivnih komponent, ki so tekom procesiranja žit sicer praviloma odstranjena. Menimo, da bodo rezultati doktorske disertacije pomembno dopolnili področje izboljšanja biološke dostopnosti fenolnih spojin iz semen žit. Izsledki raziskave odpirajo možnosti za razvoj funkcionalnih živil, saj ravno s hranili osiromašena hrana znatno pripomore k večji pojavnosti kroničnih bolezni. S tem bi prispevali k večjemu vnosu antioksidantov v telo, posledično pa bi pozitivno vplivali na svoje zdravje. Kaljenje, fermentacija in encimska obdelava pirinih semen znatno povečajo dostopnost fenolnih spojin na varen, poceni in tehnološko enostaven način, zaradi naravnega pristopa pa nenazadnje verjamemo tudi, da bi bili potencialni izdelki z izboljšano prehransko vrednostjo dobro sprejeti med potrošniki.

3.3 SKLEPI

Hipotezo, da kaljenje pirinih semen pod abiotskim stresom pripomore k izboljšanju antioksidativne aktivnosti, smo delno potrdili. Potrebno je poudariti, da vsi pogoji abiotskega stresa niso rezultirali v izboljšanju antioksidativne aktivnosti v primerjavi s kontrolo in da so bili praviloma učinkovitejši, če smo se odločili za souporabo različnih vrst abiotskega

stresa. Kaljenje pirinih semen ob dodatku 25 mM NaCl po 48 h in 50 mM sorbitola po 96 h, brez aplicirane mehanske poškodbe je znatno pripomoglo tako k povečanju vsebnosti ekstraktibilnih in vezanih fenolnih spojin, kot tudi k učinkovitejšemu lovljenju prostih radikalov $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$ v primerjavi s kontrolo. Medtem, ko je kaljenje pri povišani slanosti in osmolarnosti z apliciranim mehanskim stresom rezultiralo v najboljši antioksidativni učinkovitosti vezane frakcije pri lovljenju $ROO^{\bullet}$ radikalov v emulziji.

Hipotezo, da je učinkovitost fermentacije z namenom povečanja vsebnosti fenolnih antioksidantov večja z uporabo izbranih mikroorganizmov (kvasovk, MKB) kot v primeru spontane fermentacije, nismo potrdili. Učinkovito fermentacijo smo dosegli tudi brez inokuliranih mikroorganizmov, saj smo pri vseh vrstah fermentacij opazili sproščanje CO_2 in vzorcem po zaključku poskusa določili znatno višjo kislost v primerjavi z nefermentiranimi semeni. Čeprav je bila koncentracija MKB in kvasovk pri spontani fermentaciji manjša kot pri fermentaciji z inokuliranimi MKB *L. plantarum* in kvasovkami *S. cerevisiae*, se to neposredno ni izrazilo v vsebnosti fenolnih spojin. Pri kaljenih semenih je spontana fermentacija pokazala najmanjšo vsebnost ekstraktibilnih in vezanih TPC, v encimsko obdelanih semenih je pokazala najmanjšo vsebnost vezanih posameznih fenolnih kislin, a največjo vsebnost ekstraktibilnih TPC, hkrati pa je rezultirala v največjem povečanju vsebnosti vezane *p*-hidroksibenzojske kisline v neobdelanih semenih. Pestrost avtohtone mikrobiote pirinih semen povzroča nastanek raznolikih metabolitov, ki lažje vstopajo v celico, saj je spontana fermentacija kaljenih in encimsko obdelanih semen najučinkoviteje znižala znotrajcelično oksidacijo.

Hipotezo, da kaljenje in fermentacija učinkujeta sinergistično na povečanje dostopnosti fenolnih spojin, smo potrdili. Kombinacija kaljenja in fermentacije je imela vzajemen učinek, saj kaljenje prispeva k večji količini fermentabilnih snovi (sladkor, dušik), hkrati pa oba procesa pripomoreta k večji koncentraciji in aktivnosti hidrolitičnih encimov. Največjo vsebnost posameznih ekstraktibilnih in vezanih fenolnih spojin ter biološko dostopnih TPC smo med vsemi poskusi določili pri kaljenih semenih, fermentiranih s kvasovko *S. cerevisiae*.

Hipotezo, da so vsebnosti posameznih fenolnih spojin odvisne od pogojev fermentacije in kaljenja, smo potrdili. Različni stresni pogoji med kaljenjem značilno vplivajo na vsebnost posameznih ekstraktibilnih in vezanih fenolnih spojin ter na njihove medsebojne transformacije. Nadalje smo ugotovili, da na vsebnost posameznih fenolnih spojin vpliva tako tip fermentacije, kot tudi vrsta substrata (neobdelano, kaljeno, encimsko obdelano seme). Pri kaljenih semenih je uporaba *S. cerevisiae* najučinkoviteje povečala vsebnost ekstraktibilne *p*-kumarne in *trans*-ferulne kisline. Največje povečanje vsebnosti ekstraktibilne *p*-hidroksibenzojske kisline smo našli v kaljenih semenih, sočasno fermentiranih z *L. plantarum* in *S. cerevisiae.* Fermentacija kaljenih semen je praviloma rezultirala v zmanjšanju vsebnosti vezane *p*-kumarne in *trans*-ferulne kisline, največje povečanje količine vezane *cis*-ferulne in kavne kisline smo našli pri kaljenih semenih, fermentiranih s *S. cerevisiae*, medtem ko je fermentacija z *L. plantarum* rezultirala v

največjem povečanju vsebnosti *p*-hidroksibenzojske kisline. Uporaba različnih mikroorganizmov specifično vpliva tudi na pH, ki lahko posledično predstavlja optimalno okolje za delovanje različnih encimov, ki razgrajujejo celično steno in izvirajo iz kaljenih pirinih semen.

Hipotezo, da antioksidativna aktivnost, določena v celicah kvasovke *S. cerevisiae,* ni v korelaciji z antioksidativno aktivnostjo, določeno z različnimi *in vitro* testi, smo potrdili. Korelacije med i*n vitro* antioksidativno aktivnostjo pirinih semen, kaljenih pod različnimi stresnimi pogoji, in zmanjšanjem znotrajcelične oksidacije (ICO) v kvasovki nismo ugotovili. Tudi pri fermentiranih vzorcih korelacije med *in vitro* in *in vivo* antioksidativno aktivnostjo nismo našli. Močne pozitivne korelacije smo določili zgolj med vrednostmi DPPH in zmanjšanjem ICO ekstraktibilne frakcije neobdelanih semen; med vrednostmi ABTS in zmanjšanjem ICO ekstraktibilne frakcije encimsko obdelanih semen in med vrednostmi DPPH oz. ABTS ter zmanjšanjem ICO vezane frakcije kaljenih semen. Prav tako pri encimsko obdelanih vzorcih nismo ugotovili korelacije med *in vitro* in *in vivo* antioksidativno aktivnostjo.

Glavni namen doktorske disertacije je bil z različnimi biotehnološkimi procesi izboljšati dostopnost vezanih fenolnih spojin, ki prevladujejo v pirinih semenih. Z uspešno uporabo biotehnoloških procesov smo v sklopu štirih zastavljenih poskusov ugotovili:

- Biosinteza in transformacija fenolnih spojin je odvisna od specifičnih stresnih razmer med kaljenjem. V kombinaciji z ustreznimi pogoji abiotskega stresa je kaljenje pirinih semen pri 25 °C ob dodatku 25 mM NaCl in 50 mM sorbitola brez aplicirane mehanske poškodbe znatno pripomoglo k povečanju vsebnosti ekstraktibilnih in vezanih fenolnih spojin in k učinkovitejšem lovljenju prostih radikalov ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $ROO^{\bullet}$), kot če so semena kalila brez stresa.
- Glavne identificirane fenolne spojine kaljenih pirinih semen ne morejo direktno vstopiti v celico modelnega organizma *S. cerevisiae* in jo zaščititi pred oksidacijo.
- Kombiniranje fermentacije s kaljenjem ali encimsko obdelavo je sinergistično povečalo vsebnost ekstraktibilnih in vezanih fenolnih spojin ter njihove antioksidativne aktivnosti.
- Največjo absolutno vsebnost ekstraktibilnih in vezanih posameznih fenolnih kislin smo določili pri kaljenih pirinih semenih, fermentiranih s *S. cerevisiae.* Poleg tega je uporaba kvasovke omogočila tudi največji relativni prirast identificiranih spojin in sicer ekstraktibilne *trans*-ferulne kisline (2922 %) v primeru kaljenih pirinih semen in vezane *cis*-ferulne kisline (466 %) v primeru neobdelanih semen.
- Antioksidativna aktivnost fermentiranih semen, določena v celicah kvasovke *S. cerevisiae,* praviloma ni bila v korelaciji z *in vitro a*ntioksidativno aktivnostjo. Vzorci, ki so pokazali antioksidativno aktivnost *in vivo*, so bile predvsem ekstraktibilne frakcije fermentiranih neobdelanih, kaljenih in encimsko obdelanih semen.

- Obdelava pirinih semen z vsemi petimi hidrolitičnimi encimi hkrati je najbolj povečala vsebnost ekstraktibilnih TPC. Encimska obdelava kaljenih in fermentiranih pirinih semen je izrazito povečala ekstraktibilne TPC v primerjavi s samo kaljenimi oz. fermentiranimi semeni. Dodatek encimov k delno modificiranim vzorcem je rezultiral v povečanem deležu ekstraktibilnih TPC glede na skupne (ekstraktibilne + vezane) TPC in sicer s 34 % na 44% pri kaljenih in z 12 % na 50 % pri fermentiranih semenih.
- Kombinacija kaljenja in encimske obdelave ter fermentacije in encimske obdelave semen je privedla do velikega povečanja vsebnosti ekstraktibilne *trans*-ferulne kisline, za kar 5899 % in 8263 %. Samo ekstraktibilne frakcije encimsko obdelanih neobdelanih, kaljenih in fermentiranih semen ter vezana frakcija encimsko obdelanih neobdelanih semen so znižale znotrajcelično oksidacijo.
- Vsebnost biološko dostopnih TPC v kaljenih semenih, fermentiranih s *S. cerevisiae,* je bila kar 7-krat višja kot pri prebavljenih neobdelanih semenih. Prav tako je isti vzorec pokazal največjo vsebnost vseh identificiranih ekstraktibilnih in vezanih fenolnih kislin med prebavljenimi vzorci. Pri neobdelanih semenih smo resda opazili znatno večjo biološko stabilnost fenolnih spojin, a hkrati je bilo slednjih koncentracijsko 2-krat manj kot v »kaljenih + fermentiranih« semenih. Med GI prebavo prihaja do velikih izgub tako ekstraktibilnih kot tudi vezanih fenolnih spojin, zato je pomembno, da so semena biološko obdelana. Znatno večja izhodiščna vsebnost fenolnih spojin v tako pripravljenih vzorcih sovpada z večjo količino fenolnih spojin, ki bo uspešno prešla proces prebave.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Pira predstavlja pomemben vir vlaknin in nanje vezanih antioksidantov. Glavni predstavniki fenolnih spojin v pirinih semenih so ferulna, *p*-kumarna, kavna in *p*-hidroksibenzojska kislina. Te fenolne kisline imajo nizko biološko dostopnost, saj so v večini vezane na strukturne elemente celične stene. Da bi izkazale svoje blagodejne učinke na zdravje, se morajo fenolne spojine sprostiti iz matrice hrane in biti dostopne v prebavilih. Biološka obdelava živil lahko učinkovito izboljša dostopnost zaužitih bioaktivnih spojin. Čeprav je bil vpliv nekaterih biotehnoloških procesov na sproščanje vezanih fenolnih spojin in na njihovo dostopnost predhodno že raziskan, smo v naši raziskavi uporabo biotehnoloških procesov in njihovih kombinacij močno razširili, prav tako smo poleg določanja antioksidativne aktivnosti *in vitro*, ugotavljali antioksidativno aktivnost v celicah kvasovke *S. cerevisiae,* s čimer smo dobili vpogled v živo celico. Uporaba biotehnoloških procesov (kaljenja, fermentacije, encimske obdelave) predstavlja nove načine za izboljšanje dostopnosti bioaktivnih spojin ter ponuja določene prednosti, saj so stroškovno in energetsko učinkoviti ter okolju prijazni.

Kaljenje je proces, ki zmehča strukturo zrna, zmanjša vsebnost antinutrientov in izboljša biološko dostopnost prehransko pomembnih komponent. Zato je bil cilj prvega sklopa raziskav s kaljenjem pirinih semen pod različnimi stresnimi pogoji povečati vsebnost ekstraktibilnih in vezanih fenolnih spojin. Ugotovili smo, da kaljenje semen pri 25 °C ob dodatku 25 mM NaCl in 50 mM sorbitola brez aplicirane mehanske poškodbe znatno pripomore k povečanju vsebnosti ekstraktibilnih in vezanih fenolnih spojin in njihove antioksidativne aktivnosti *in vitro.* Fenolne spojine kaljenih semen niso pokazale antioksidativne aktivnosti v celici modelnega organizma *S. cerevisiae*, ker identificirane spojine v intaktni obliki niso mogle vstopiti v celico in jo zaščititi pred oksidacijo. Ugotovili smo, da različni stresni pogoji med kaljenjem značilno vplivajo na vsebnost posameznih ekstraktibilnih in vezanih fenolnih spojin.

V drugem sklopu raziskav smo ugotavljali vpliv različnih tipov fermentacije (mlečnokislinske, alkoholne, kombinirane, spontane) na vsebnost ekstraktibilnih in vezanih fenolnih spojin in na njihovo *in vitro* in *in vivo* antioksidativno aktivnost. Nadalje smo želeli ugotoviti, ali lahko kombiniranje biotehnoloških procesov še dodatno poveča izplen fenolnih spojin in učinkovitost pridobljenih antioksidantov. Vsebnost ekstraktibilnih fenolnih spojin se je v primerjavi z nefermentiranimi vzorci bistveno povečala neodvisno od izbranih pogojev fermentacije. Kombiniranje biotehnoloških procesov je sinergistično povečalo vsebnost ekstraktibilnih in vezanih antioksidantov. Največjo absolutno vsebnost posameznih ekstraktibilnih in vezanih fenolnih spojin in *in vitro* antioksidativno aktivnost smo v primeru kaljenih semen določili z uporabo *S. cerevisiae,* medtem ko je bila za encimsko obdelana semena najučinkovitejša fermentacija z *L. plantarum* (samostojno ali v kombinaciji s *S.*

cerevisiae). Med ekstraktibilnimi spojinami se je relativno najbolj povečala vsebnost *trans*-ferulne kisline, in to za kar 2922 % v kaljenih semenih, fermentiranih s *S. cerevisiae.* Pri vezanih fenolnih spojinah pa se je najbolj povečala količina *cis*-ferulne kisline, za 466 % v neobdelanih semenih, prav tako fermentiranih zgolj s *S. cerevisiae.* Antioksidativna aktivnost *in vitro* praviloma ni bila v korelaciji z antioksidativno aktivnostjo, določeno v celicah kvasovke. Vzorci, ki so pokazali antioksidativno aktivnost *in vivo*, so bile predvsem ekstraktibilne frakcije fermentiranih neobdelanih, kaljenih in encimsko obdelanih semen. Spontana fermentacija kaljenih in encimsko obdelanih vzorcev je najbolj učinkovito znižala znotrajcelično oksidacijo. Celični privzem vezanih hidroksicimetnih kislin je bil znatno večji kot privzem ekstraktibilnih kislin; vendar so bile slednje učinkovitejši antioksidanti v *in vivo* sistemu. Glede na našo študijo predvidevamo, da bi se fermentacija pirinih semen lahko uporabila za izboljšanje vsebnosti, antioksidativne aktivnosti in celičnega privzema fenolnih spojin.

Tretji sklop raziskav prikazuje pristop, pri katerem obdelava s specifičnimi encimi (celulaze, ksilanaze, feruloil esteraze, proteaze, α-amilaze) posamezno ali v kombinaciji znatno izboljša vsebnost ekstraktibilnih TPC in njihovo *in vitro* antioksidativno aktivnost. Semena, obdelana z vsemi petimi encimi naenkrat, so imela znantno povečano vsebnost ekstraktibilne *p*-kumarne, *trans*-ferulne in *p*-hidroksibenzojske kisline, ekstraktibilnih TPC, prav tako so bila še posebno učinkovita pri lovljenju $ABTS^{\bullet+}$ radikalov. Najučinkovitejši način za sproščanje večjih količin vezanih fenolnih spojin v ekstraktibilno obliko je bilo kombiniranje biotehnoloških procesov. Pri kaljenih in fermentiranih semenih je encimska obdelava povzročila znatno povečanje vsebnosti ekstraktibilnih TPC in njihove *in vitro* antioksidativne aktivnosti, prav tako se je povečala vsebnost posameznih fenolnih kislin. Zaradi kombiniranja kaljenja in encimske obdelave se je vsebnost ekstraktibilne *trans*-ferulne kisline povečala za kar 5899 % v primerjavi s kaljenimi semeni, pri kombiniranju fermentacije in encimske obdelave pa za kar 8263 % v primerjavi s fermentiranimi semeni. Hkati pa je encimska obdelava kaljenih in fermentiranih pirinih semen negativno vplivala na vsebnost vezanih fenolnih spojin in njihovo antioksidativno aktivnost, kljub temu je ta oblika še vedno predstavljala prevladujoč delež skupnih fenolnih spojin. Med ekstraktibilnimi frakcijami so encimsko obdelana neobdelana, kaljena in fermentirana semena izkazala antioksidativno aktivnost v celici, medtem ko so med vezanimi frakcijami to lastnost izkazala le encimsko obdelana neobdelana semena.

Vpliv posameznih biotehnoloških procesov in njihovih kombinacij na izboljšanje biološke dostopnosti fenolnih spojin iz pirinih semen smo ugotavljali z uporabo statičnega *in vitro* prebavnega modela, s katerim smo posnemali prebavo v ustih, želodcu in tankem črevesu. Raziskava je pokazala statistično značilen vpliv biotehnoloških procesov na vsebnost biološko dostopnih TPC, posameznih fenolnih spojin in njihovo antioksidativno aktivnost, kljub temu, da je med procesom prebave prišlo do velikih izgub fenolnih spojin. Neobdelana semena so imela najmanjšo vsebnost biološko dostopnih TPC in posameznih fenolnih spojin.

Kombinacija biotehnoloških procesov, zlasti kaljenja in alkoholne fermentacije, se je izkazala za najučinkovitejši pristop. Biološka dostopnost *p*-kumarne in *trans*-ferulne kisline je bila največja pri encimsko obdelanih semenih, fermentiranih z *L. plantarum*, medtem ko je bila njuna absolutna vsebnost največja v kaljenih semenih, fermentiranih s *S. cerevisiae*. Največjo biološko stabilnost fenolnih spojin smo po pričakovanju določili v neobdelanih semenih, kjer so bili substrati slabše dostopni za prebavne encime. Izkazalo se je, da imajo biološko obdelana semena bistveno večjo začetno vsebnost fenolnih kislin kot neobdelana, posledično jih tudi več vstopa v debelo črevo.

4.2 SUMMARY

Spelt is an important source of dietary fiber and antioxidants bound to it. The main representatives of phenolic compounds in spelt seeds are ferulic, *p*-coumaric, caffeic and *p*-hydroxybenzoic acids. These phenolic acids have low bioaccessibility, since they are mostly bound to the structural elements of the cell wall. To exert their health-promoting effects, phenolic compounds must be released from the food matrix and be accessible in the digestive system. Bioprocessing of foods can effectively improve the accessibility of ingested bioactive compounds. Although the influence of some biotechnological processes on the release of bound phenolic compounds and their accessibility has been studied previously, our research has greatly expanded the use of biotechnological processes and their combinations, and in addition to determining antioxidant activity *in vitro*, we have determined cellular antioxidant activity in yeast *S. cerevisiae*, which provides insight into the living cell. The use of biotechnological processes (germination, fermentation, enzymatic treatment) represents a new way to improve the accessibility of bioactive compounds and offers certain advantages, as they are cost and energy efficient and environmentally friendly.

Germination is a process that softens seed structure, reduces the content of antinutrients, and improves bioaccessibility of nutritionally important components. Therefore, the aim of the first series of research was to increase the content of extractable and bound phenolic compounds by germination of spelt seeds under different stress conditions. We found that germination of seeds at 25 °C with the addition of 25 mM NaCl and 50 mM sorbitol solutions without applied mechanical damage significantly increased the content of extractable and bound phenolic compounds and their antioxidant activity *in vitro*. The phenolic compounds of the germinated seeds did not show antioxidant activity in the cell of the model organism *S. cerevisiae*, because the identified compounds in their intact form could not enter the cell and protect it from oxidation. We found that different stress conditions during germination typically affect the content of individual extractable and bound phenolic compounds.

In the second set of research experiments, we determined the influence of different types of fermentation (lactic acid, alcoholic, combined, spontaneous) on the content of extractable and bound phenolic compounds and on their *in vitro* and *in vivo* antioxidant activity.

Furthermore, we also determined whether the combination of biotechnological processes can further increase the yield of phenolic compounds and their antioxidant activity. The content of extractable phenolic compounds increased significantly compared to non-fermented samples, regardless of the selected fermentation conditions. The combination of biotechnological processes had synergistic effects on increasing the content of extractable and bound antioxidants. The highest absolute content of individual extractable and bound phenolic compounds and *in vitro* antioxidant activity were obtained after fermentation of germinated seeds with *S. cerevisiae*, while for enzymatic treated seeds, fermentation with *L. plantarum* (alone or in with *S. cerevisiae*) was the most effective. Among extractable phenolic compounds, the content of *trans*-ferulic acid increased relatively the most in the germinated seeds fermented with *S. cerevisiae*, by 2922%. Among bound phenolic compounds, content of *cis*-ferulic acid increased the most in the raw seeds fermented with *S. cerevisiae*, by 466%. Antioxidant activity *in vitro* generally did not correlate with antioxidant activity determined in yeast cells. The samples that showed antioxidant activity *in vivo* were mainly the extractable fractions of fermented raw, germinated and enzymatic treated seeds. Spontaneous fermentation of germinated and enzymatic treated samples most effectively decreased intracellular oxidation. Cellular uptake of bound hydroxycinnamic acids was significantly higher than that of extractable acids; however, the latter were more efficient antioxidants in the *in vivo* system. According to our study, we suggest that fermentation of spelt seeds can be used to improve the content, antioxidant activity and cellular uptake of phenolic compounds.

The third set of research experiments demonstrates an approach in which treatment with specific enzymes (cellulases, xylanases, feruloyl esterases, proteases, α-amylases) individually or in combination significantly improves the content of extractable TPCs and their *in vitro* antioxidant activity. Seeds treated with all five enzymes simultaneously had significantly higher content of extractable *p*-coumaric, *trans*-ferulic and *p*-hydroxybenzoic acids, as well as extractable TPCs, and were also particularly effective in scavenging activity against $ABTS^{\bullet+}$ radicals. The most effective way to release larger amounts of bound phenolic compounds into an extractable form was to combine biotechnological processes. In germinated and fermented seeds, enzymatic treatment resulted in a significant increase in the content of extractable TPCs and their *in vitro* antioxidant activity, as well as in the content of individual phenolic acids. The combination of germination and enzymatic treatment enormously increased the content of extractable *trans*-ferulic acid by 5899% compared to germinated seeds, and the combination of fermentation and enzymatic treatement increased its content by 8263% compared to fermented seeds. However, enzymatic treatment of germinated and fermented spelt seeds had a negative effect on the content of bound phenolic compounds and their antioxidant activity, although they still represented a predominant proportion of total phenolic compounds. Among the extractable fractions, the enzymatic treated raw, germinated, and fermented seeds showed cellular

antioxidant activity, while among the bound fractions, only the enzymatic treated raw seeds showed cellular antioxidant activity.

The influence of individual biotechnological processes and their combinations on improving the bioaccessibility of phenolic compounds from spelt seeds was determined using a static *in vitro* digestion model simulating oral, gastro and intestinal digestion. The study showed a statistically significant effect of the biotechnological processes on the content of bioaccessible TPCs, individual phenolic compounds and their antioxidant activity, despite that large losses of phenolic compounds occured during the digestion process. Raw seeds showed the lowest content of bioaccessible TPCs and individual phenolic compounds. The combination of biotechnological processes, especially germination and alcoholic fermentation, has proven to be the most effective approach. The bioaccessibility of *p*-coumaric and *trans*-ferulic acids was the highest in enzymatic treated seeds fermented with *L. plantarum*, while their absolute content was the highest in germinated seeds fermented with *S. cerevisiae*. As expected, the greatest biostability of phenolic compounds was determined in raw seeds, where the substrates were less accessible to digestive enzymes. It turned out that bioprocessed seeds have a significantly higher initial content of phenolic acids than raw seeds, and as a result, more phenolic compounds enter the colon.

5 VIRI

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ZAHVALA

Tekom doktorskega študija sem imela ta privilegij, da sem se lahko večkrat zahvaljevala, saj je zahvala vedno odziv na neko dobro, plemenito dejanje, ki si ga kot posameznik deležen.

Zahvale začenjam z največjo zahvalo in ta gre moji mentorici doc. dr. Petri Terpinc. V teh štirih letih si mi bila, draga Petra, več ko le mentorica, bila si tudi zaupnica, ki sem ji lahko povedala svoje skrbi, dvome in čisto življenjske probleme. Si mentorica, ki mi je dajala svobodo za razvoj idej, podpirala si me pri delu in me s svojimi nasveti usmerjala v pravo smer. V posebno zadovoljstvo so mi bili najini pogovori. Zaradi tebe so bila ta štiri leta res lepa. Hvala tudi za vse vikende in prosti čas, ki si ga namenila za popravljanje najinih člankov.

Hvala vsem članom komisije, prof. dr. Blažu Cigiću, prof. dr. Heleni Prosen, doc. dr. Borisu Kovaču, za vaš trud, za vse vaše predloge in popravke, s katerimi smo izboljšali našo doktorsko nalogo.

Velika zahvala velja prof. dr. Rajku Vidrihu, ki mi je pred štirimi leti predlagal naj se prijavim za mlado raziskovalko. Hvala vam, da vas je vedno zanimalo kako sem, kako mi gre delo, koliko člankov sem že napisala in hvala, da se v zadnjih mesecih tako trudite glede moje nadaljnje zaposlitve.

Posebna zahvala gre trem profesoricam, s katerimi sem imela čast sodelovati, prof. dr. Heleni Abramovič, prof. dr. Poloni Jamnik in prof. dr. Maji Mikulič Petkovšek. Hvala vsaki posebej za možnost sodelovanja, za to, da ste bile vedno pripravljene pomagati ter za vedno lepe in spodbujajoče besede. Hvala vam.

Zahvala velja vsem zaposlenim na Katedri za tehnologije rastlinskih živil in vino, za to da sem se dobro počutila ob vas, za dobro voljo in seveda hrano.

Hvala Doris, Saši in Niku za vse trenutke v laboratoriju, za vse fore, smeh, pogovore, za vso pomoč in nasvete. Hvala tudi Hani, ki me je ob popoldnevih vedno nasmejala.

Ena največjih zahval gre sigurno mojemu službenemu cimru s katerim sem preživela največ časa. Andrej, hvala ti za tvojo “laganost” s katero si me znal pomiriti, ko sem paničarila, za vse fore, prigode in različna mnenja, zaradi tebe je bil marsikateri dan lažji in lepši. Hvala za tvoje nasvete pri “optimizaciji” mojega dela v laboratoriju, za vse epruvete, ki si jih pomil namesto mene. Hvala, ker nisi bil samo sodelavec, ampak si bil prijatelj. Hvala lepa ☺.

Velika hvala gre mojim staršem, ki sta me celo življenje spodbujala pri izobraževanju in sta mi velik zgled delavnosti, pridnosti in poštenosti. Hvala, da me še vedno marsikdaj razvajata z vnaprej pripravljenim kosilom …

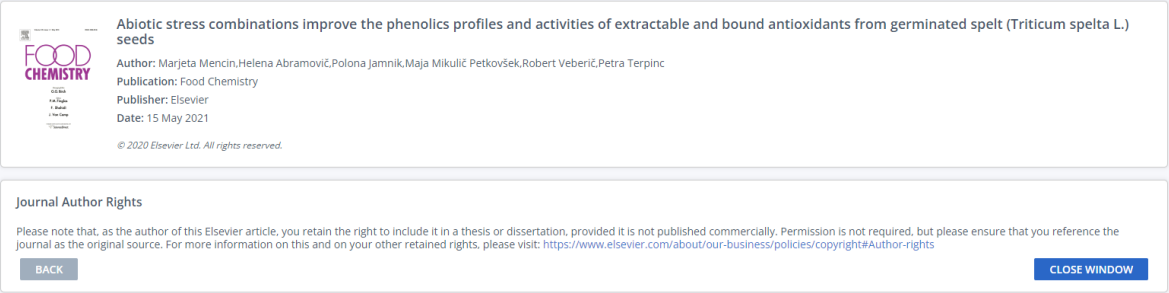
Posebna zahvala gre moji sestri Katji in mojim prijateljem, še posebej Janu in Kaji, za vse pogovore, za vse hece, za vse spodbude. Kakšen privilegij je, da vas imam.

Začela sem s prvo zahvalo, ki je bila na službenem področju najpomembnejša, zaključujem pa z zahvalo osebi, ki je v mojem življenju najpomembnejša, mojemu možu. Timi hvala ti, da me imaš rad tako kot sem, hvala ker ne kompliciraš, hvala za vse spodbudne in lepe besede, ki mi jih nameniš, hvala ker si me v zadnjem mesecu dobro prenašal in ker si me znal pomiriti. Hvala, ker se tako trudiš za najin odnos. Rada sem tvoja žena. ☺

Na koncu še hvala Njemu, ki je na mojo pot pripeljal vse te ljudi. Srečnica sem! ☺

PRILOGE

PRILOGA A: Dovoljenje za uporabo članka Mencin in sod. (2021a) iz revije Food Chemistry.



Abiotic stress combinations improve the phenolics profiles and activities of extractable and bound antioxidants from germinated spelt (Triticum spelta L.) seeds

Author: Marjeta Mencin,Helena Abramovič,Polona Jamnik,Maja Mikulič Petkovšek,Robert Veberič,Petra Terpinc
Publication: Food Chemistry
Publisher: Elsevier
Date: 15 May 2021

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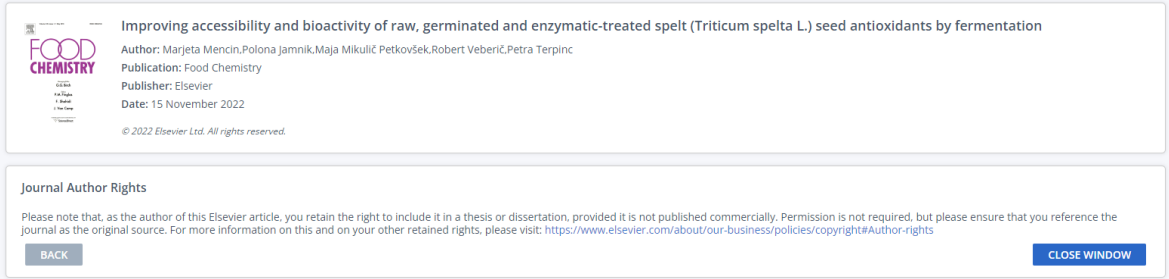

Improving accessibility and bioactivity of raw, germinated and enzymatic-treated spelt (Triticum spelta L.) seed antioxidants by fermentation

Author: Marjeta Mencin,Polona Jamnik,Maja Mikulič Petkovšek,Robert Veberič,Petra Terpinc
Publication: Food Chemistry
Publisher: Elsevier
Date: 15 November 2022

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antioxidants

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Article

Development and Optimisation of Solid-Phase Extraction of Extractable and Bound Phenolic Acids in Spelt (*Triticum spelta* L.) Seeds

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Abstract: A solid-phase extraction (SPE) technique was developed and optimised for isolation and concentration of extractable and bound phenolic acids from germinated spelt seeds, for analysis by liquid chromatography–mass spectrometry. Samples initially underwent solvent extraction under different conditions to maximise the yield of phenolic antioxidants. Optimal extraction conditions for extractable phenolics were absolute methanol as solvent, sample-to-methanol ratio 1:9, and reconstitution in non-acidified water. The bound phenolics were extracted from sample pellets using hydrolysis with 2 M NaOH, acidification of the hydrolysate with formic acid, and simultaneous isolation and purification using Strata X polymeric RP tubes. Compared to liquid-liquid extraction, this direct SPE protocol has significant advantages in terms of higher extraction efficiencies of total and individual phenolics and their antioxidant activities. These data suggest that direct SPE represents a rapid and reliable method for quantitative analysis of both the extractable and the commonly overlooked bound phenolics in *Triticum spelta* seeds.

Keywords: spelt phenolics; extraction; hydrolysis; solid-phase extraction; liquid-liquid extraction; antioxidant activity; LC-MS

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Citation: Mencin, M.; Mikulic-Petkovsek, M.; Veberič, R.; Terpinc, P. Development and Optimisation of Solid-Phase Extraction of Extractable and Bound Phenolic Acids in Spelt (*Triticum spelta* L.) Seeds. *Antioxidants* **2021**, *10*, 1085. https://doi.org/10.3390/antiox10071085

Academic Editors: Irina Ioannou and Cédric Peyrot

Received: 2 June 2021
Accepted: 1 July 2021
Published: 5 July 2021

1. Introduction

Spelt (*Triticum spelta* L.) is an ancient form of wheat, and it is cultivated in several central European countries. Over the past few decades, it has attracted renewed interest as a healthier, more natural, less 'over-bred' cereal compared to modern common wheat (*Triticum aestivum* L.). More recently, this has been combined with increased attention on the phenolics in the whole grain of *Triticum* species [1–3]. Our previous study suggested that germination of spelt seeds under specific combined stress conditions can significantly improve their total phenolics content (TPC), along with the levels of the individual phenolics and their scavenging activities against different free radicals (e.g., $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $ROO^{\bullet}$) [4].

Phenolics can occur as soluble free phenolics in the vacuole of plant cells, while their soluble conjugates are covalently bound or esterified to sugars and other low molecular mass components. These soluble phenolics are often referred to as 'extractable', and they are generally extracted from food matrices using different combinations of aqueous and organic solvents. However, phenolics can also be covalently bound to cell-wall materials through ester, ether and carbon–carbon bonds, or entrapped in the macromolecules of food matrices through hydrophobic interactions and hydrogen bonding [5]. These represent the insoluble 'bound' phenolics.

Similar to other cereals, these bound phenolics of the grain of modern and ancient varieties of wheat mostly remain in the solid residues after conventional solvent extraction.

Their quantification can be improved using acidic [6], alkaline [7] and enzymatic [8] hydrolysis. Zhang et al. [5] recently highlighted the roles for *Triticum* phenolics in human health, and therefore a suitable extraction method for obtaining these bound components is much in demand, especially as they have often been ignored.

A wide range and combination of techniques have been used to isolate and purify these extractable and bound phenolics from the wheat matrix [9]. For example, solid-phase extraction (SPE) is a popular method that uses a solid phase and a liquid phase to isolate analytes from solutions. SPE has usually been used as a 'clean-up' procedure prior to chromatographic or other analytical methods that can then be used to quantify various analytes. SPE based on reversed-phase polymeric sorbents allows the extraction of phenolics and the removal of sugars and other highly polar compounds (e.g., organic acids, amino acids, proteins) [10]. Many studies have focused on the extraction and SPE purification of the extractable phenolics from cereals [11–15] and reported that SPE clean-up step allows high recoveries and good precision for extractable phenolic acids in a different cereals. To the best of our knowledge, the application and optimisation of SPE for the extraction and purification of bound phenolics extracts has not yet been reported. The optimisation of extraction and SPE purification for particularly bound phenolics would be helpful for their more routine analysis.

liquid-liquid extraction (LLE) can also be used to enrich high-molecular-weight phenolics and to clean-up extracts using suitable solvents [16]. LLE is one of the most widely used technique applied to extraction of phenolics from different cereals [17–20]. Although LLE is inexpensive, it involves the use of organic solvents (e.g., often highly toxic diethyl ether, either alone or in combination with ethyl acetate) and requires longer extraction times. This can also result in extract degradation.

Although the equipment required for SPE is more expensive than for LLE, the use of SPE can avoid many of the problems associated with LLE, such as incomplete phase separations, unsatisfying recoveries, use and disposal of large quantities of organic solvents, among others [21].

The main objective of the present study was to develop an extraction and purification protocol for analysis of spelt seed phenolics, to facilitate their isolation for subsequent characterisation and quantification, and to also overcome the drawbacks of the previously established LLE method. We strongly believe that the direct SPE method presented here will also be useful for other *Triticum* species, as they are genetically and anatomically very similar to spelt.

2. Materials and Methods

2.1. Materials

Spelt (*Triticum spelta* L. cv. Ostro) seeds were obtained from a local mill in the Dolenjska region of Slovenia. The seeds were stored in the dark at 1 °C until analysis. The preparation of the germinated spelt seeds was reported in detail by Mencin et al. [4].

Methanol (99.9%), formic acid, sodium hydroxide and sodium carbonate were from Merck (Darmstadt, Germany). 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) reagent, the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) reagent, Folin–Ciocalteu reagent, Trolox, sodium dihydrogen phosphate dehydrate, *p*-hydroxybenzoic acid, *trans*-ferulic acid, *p*-coumaric acid and caffeic acid were from Sigma-Aldrich (Steinheim, Germany). All of the chemicals and reagents used for the present study were of analytical quality. Water (Milli-Q; Millipore, Billerica, MA, USA) was used to prepare the working solutions.

2.2. Preparation of Crude Extractable and Bound Fractions

In recent years, a lot of research has been carried out on germinated grain, with aim to improve the nutritional value. Therefore, we optimised the following methods for use with germinated seeds.

For the preparation of crude extractable and bound fractions, 1.0 g germinated seeds were milled (A11 basic; IKA-Werke GmbH, Staufen im Breisgau, Germany) and freeze-

dried (VirTis AdVantage Pro Freeze Dryer, SP Scientific, Gardiner, NY, USA) in different volumes of water or methanol, and left shaking (EV-403; Tehnica, Železniki, Slovenia) for 2 h. The initial investigations indicated that pure methanol provided greater extraction for the more prevalent phenolic acids in the spelt seeds, and therefore the further optimisation was carried out with only methanol as the extraction solvent.

The aliquots of the spelt seeds were mixed with 99.9% methanol at a ratio of freeze-dried seeds to methanol of 1:9 or 1:6 (*w/v*). After 2 h of extraction at room temperature, the mixtures were centrifuged at 9793.9× *g* for 10 min. The supernatant was then removed as the 'crude extractable fraction', and the residue was processed further for the bound fraction.

2.3. Preparations for Direct Solid-Phase Extraction of Phenolics

2.3.1. Preparation of Extractable Phenolics for Solid-Phase Extraction

Following filtration of the crude extractable phenolics fraction (pore size, 0.45 µm, Macherey-Nagel, Duren, Germany), this was further processed for direct SPE according to one of two different procedures. For the first, an aliquot of the supernatant was diluted to 10% methanol with water, and then applied to an SPE column. For the second, the solvent was evaporated off an aliquot of the supernatant (5 mL) in a vacuum evaporator (HT-4 series II; GeneVac Technologies, Ipswich, UK), and the residue was re-suspended in 5 mL water (pH 7, if not specified otherwise) (Figure 1). The samples were then filtered and applied to an SPE column.

2.3.2. Preparation of Bound Phenolics for Solid-Phase Extraction

After the removal of the supernatant from the methanol extraction of the spelt seeds (i.e., the 'crude extractable phenolics'), the solid residue was treated with 2 M sodium hydroxide (NaOH; 10, 15, 20 mL) with shaking for 4 h (EV-403; Tehnica, Železniki, Slovenia) at room temperature. After this alkaline hydrolysis step, the hydrolysed sample that contained the previously bound, but now released, phenolics (i.e., the 'crude bound phenolics') was acidified to pH 2 with 6 M HCl, or to pH 3 with concentrated formic acid (Figure 2). These samples were then filtered (pore size, 0.45 µm; Macherey-Nagel, Duren, Germany) and loaded onto an SPE column.

2.4. liquid-liquid Extraction of Phenolics

Preparation of Free Phenolic Extract

The solvent from 5 mL of the supernatant for the crude extractable phenolics was evaporated off, and the dry methanolic extract was redissolved in 5 mL water acidified with formic acid (pH 3). This was then extracted three times with ethyl acetate (5 mL) for 5 min. The combined ethyl acetate fractions were evaporated to dryness at 30 °C, and the residue was reconstituted in methanol and filtered through a 0.45 µm membrane filter (Chromafil A-45/25 syringe filters; cellulose acetate, hydrophilic membrane; Macherey-Nagel, Duren, Germany). This provided the LLE 'free phenolics fraction' (Figure 1). For additional SPE purification of the ethyl acetate extracts (i.e., LLE + SPE), the residue following evaporation was reconstituted in water instead (Figure 1).

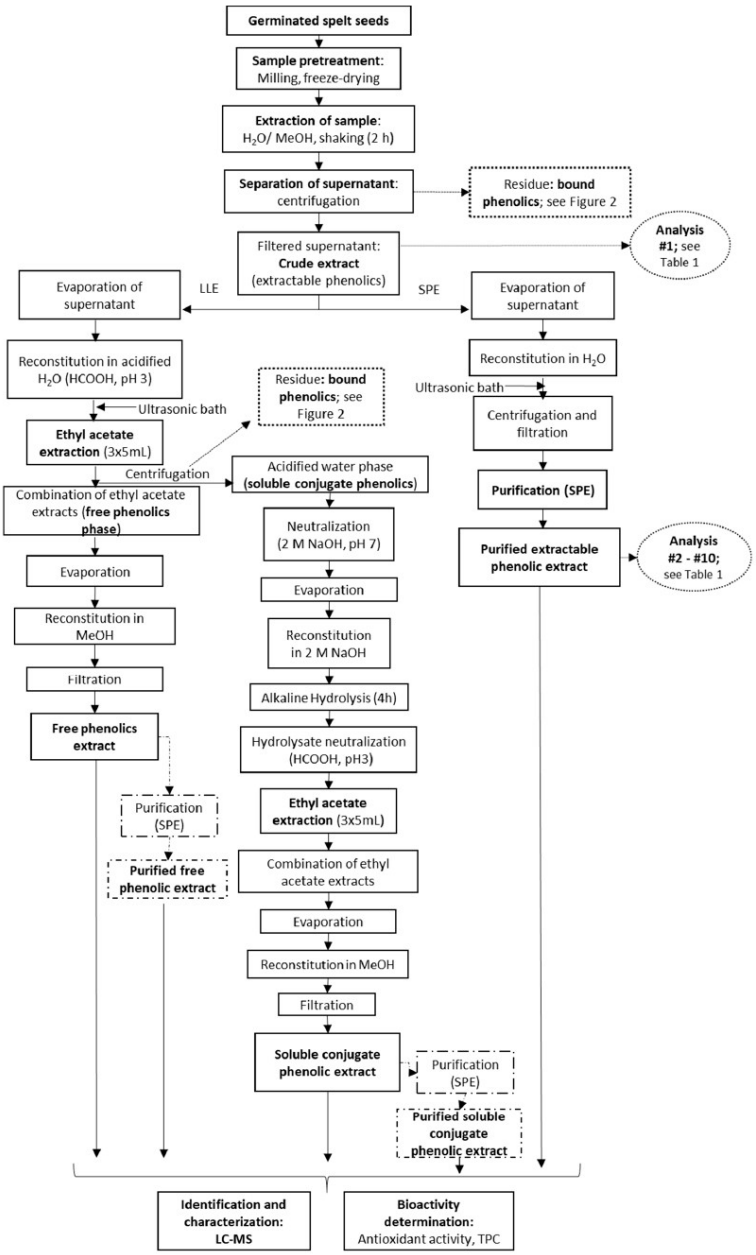

Figure 1. Processing of the germinated spelt (*Triticum spelta*) seeds to obtain the extractable phenolics through direct purification by solid-phase extraction (SPE) and the free and soluble conjugate phenolics through liquid-liquid extraction (LLE) without and with SPE purification. For analyses indicated, see Table 1.

Table 1. Effects on total phenolics content (TPC), DPPH• scavenging activity (DPPH) and individual phenolic acid content for the different protocols for the extractable and bound phenolics of the germinated spelt seeds.

Fraction	No.	Extraction	Extractable Fraction		Bound Fraction		In Vitro Assays (mg TE/g DW)			Phenolic Acid Content (µg/g DW)			
		Sample–to–Solvent Ratio (*w/v*)	Reconstitution	SPE (mL)	Hydrolysis: 2 M NaOH (mL)	Neutralisation	SPE (mL)	TPC	DPPH	*p*-Coumaric Acid	*trans*-Ferulic Acid	Caffeic Acid Derivatives	*p*-Hydroxy-Benzoic Acid
Crude	#1	MeOH 1:9	-	-	-	-	-	9.19	0.94	5.79	9.12	9.63	55.12
Extractable	#2	MeOH 1:9	5 mL H_2O (pH 7)	3	na	na	na	4.10 ^{b}	0.52 ^{b}	3.37 ^{b}	6.19 ^{b}	4.68 ^{a}	18.59 ^{b}
	#3	H_2O 1:9	5 mL H_2O (pH 7)	3	na	na	na	3.95 ^{a}	0.45 ^{a}	3.06 ^{a}	5.43 ^{a}	5.72 ^{b}	14.12 ^{a}
	#4	MeOH 1:6	5 mL H_2O (pH 7)	3	na	na	na	3.01 ^{a}	0.46 ^{a}	3.29 ^{a}	6.06 ^{a}	4.70 ^{a}	16.78 ^{a}
	#5	MeOH 1:9	30 mL 10% MeOH	30	na	na	na	3.51 ^{a}	0.56 ^{c}	1.97 ^{a}	4.48 ^{a}	4.01 ^{a}	12.51 ^{a}
	#6		5 mL H_2O (pH 7)	3	na	na	na	4.10 ^{b}	0.52 ^{a}	3.37 ^{b}	6.19 ^{b}	4.68 ^{b}	18.59 ^{b}
	#7		5 mL acidified H_2O (pH 3, HCOOH)	3	na	na	na	4.39 ^{c}	0.53 ^{b}	3.42 ^{b}	5.92 ^{b}	4.72 ^{b}	23.62 ^{c}
	#8		5 mL acidified H_2O (pH 2, HCl)	3	na	na	na	4.47 ^{c}	0.55 ^{c}	3.52 ^{b}	6.04 ^{b}	4.79 ^{b}	23.93 ^{c}
	#9		5 mL H_2O (pH 7)	3	na	na	na	4.10 ^{a}	0.52 ^{a}	3.37 ^{a}	6.19 ^{a}	4.68 ^{a}	18.59 ^{a}
	#10		5 mL H_2O (pH 7)	5	na	na	na	4.28 ^{b}	0.54 ^{b}	3.64 ^{b}	6.97 ^{b}	5.10 ^{b}	20.59 ^{b}

Table 1. *Cont.*

Fraction	No.	Extraction	Extractable Fraction		Bound Fraction		In Vitro Assays (mg TE/g DW)			Phenolic Acid Content (µg/g DW)			
		Sample–to–Solvent Ratio (*w/v*)	Reconstitution	SPE (mL)	Hydrolysis: 2 M NaOH (mL)	Neutralisation	SPE (mL)	TPC	DPPH	*p*-Coumaric Acid	*trans*-Ferulic Acid	Caffeic Acid Derivatives	*p*-Hydroxy-Benzoic Acid
Bound	#11	MeOH 1:9	na	na	10	HCOOH (pH 3)	3	6.18 ^{a}	0.64 ^{a}	55.19 ^{a}	314.99 ^{a}	7.06 ^{a}	27.40 ^{a}
	#12		na	na	15	HCOOH (pH 3)	3	7.57 ^{b}	0.81 ^{c}	64.94 ^{b}	396.05 ^{b}	13.04 ^{b}	36.41 ^{b}
	#13		na	na	20	HCOOH (pH 3)	3	7.58 ^{b}	0.74 ^{b}	77.33 ^{c}	534.37 ^{c}	21.82 ^{c}	44.16 ^{c}
	#14		na	na	20	HCl (pH 2)	3	7.88 ^{a}	0.73 ^{a}	61.96 ^{a}	363.36 ^{a}	16.86 ^{a}	27.68 ^{a}
	#15		na	na	20	HCOOH (pH 3)	3	10.20 ^{b}	1.21 ^{b}	77.33 ^{b}	534.37 ^{b}	21.82 ^{b}	44.16 ^{b}
	#16		na	na	20	HCOOH (pH 3)	3	7.58 ^{a}	0.74 ^{a}	77.33 ^{a}	534.37 ^{a}	21.82 ^{b}	44.16 ^{a}
	#17		na	na	20	HCOOH (pH 3)	5	7.49 ^{a}	0.80 ^{b}	86.38 ^{b}	552.13 ^{b}	18.00 ^{a}	42.51 ^{a}
	#18		na	na	20	HCOOH (pH 3)	8	13.38 ^{b}	1.17 ^{c}	92.92 ^{c}	563.85 ^{c}	18.86 ^{a}	48.96 ^{b}

Data are means ± SD ($n = 3$). Grouping of datasets indicate data included in the statistical analysis across each dataset. No., Numbered methodology for cross-reference with Figure 1; Figure 2; na, not applicable. SPE, loading sample on SPE tube. Different small letters within a column (TPC, DPPH, phenolic acid) indicate significant differences within the individual datasets for comparisons within the extractable and within the bound contents ($p < 0.05$; Duncan's multiple range tests). Note: For clarity for the statistical analysis, the following samples are repeated: #2, #6, #9; #13, #15, #16.

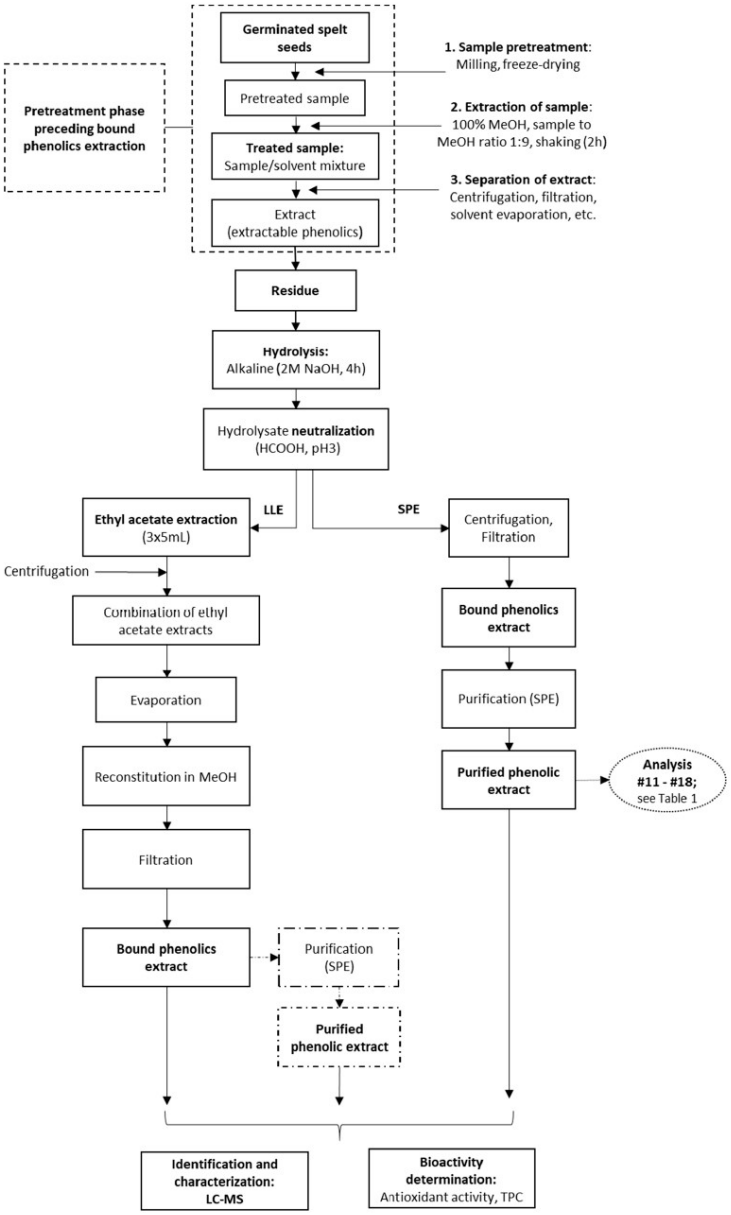

Figure 2. Processing of the germinated spelt (*Triticum spelta*) seeds to obtain the bound phenolics through direct purification by solid-phase extraction (SPE) and through liquid-liquid extraction (LLE) without and with SPE purification. For analyses indicated, see Table 1.

2.5. Solid-Phase Extraction

The SPE tubes Strata-X polymeric RP 3 mL/100 mg (Phenomenex, Torrance, CA, USA) were initially preconditioned with 3 mL 99.9% methanol, followed by equilibration with 3 mL water, or water acidified with HCl or formic acid (dependent on solvent used to provide the dried methanolic extracts). The samples that contained the extractable, free, soluble conjugate or bound phenolics were then added onto the SPE columns with the liquid allowed to enter the tube matrix. The tubes were then washed with 4 mL water and vacuum-dried for 2 min. Finally, the target compounds were eluted with 2 mL 70% aqueous methanol. The eluates obtained represented the corresponding purified extractable, free, soluble conjugate and bound phenolics.

2.6. Determination of Total Phenolics Content

The TPC was determined using the Folin–Ciocalteu method, according to the protocol described by Mencin et al. [4]. A standard curve was prepared with Trolox, and the data are expressed as mg Trolox equivalents per g dry weight (mg TE/g DW).

2.7. Liquid Chromatography–Mass Spectrometry Analysis

All the instruments and analysis conditions for the liquid chromatography–mass spectrometry (LC-MS) were the same as previously reported by Mencin et al. [4]. Identification of the individual phenolics was confirmed by comparisons of retention times and spectra with standard databases, and by addition of standards to samples, followed by fragmentation of each component (Supplementary Table S1). The contents of *p*-coumaric acid, *trans*-ferulic acid, caffeic acid derivatives and *p*-hydroxybenzoic acid were calculated from the peak areas of the samples and the corresponding standards and are expressed as µg per g dry weight (µg/g DW) of the germinated spelt seeds.

2.8. HPLC Method Validation

Calibration, limits of detection (LOD), limits of quantification (LOQ), linearity and accuracy were measured for method validation. Linearity was evaluated by analysing mixtures of phenolic acid standard solutions and by spiking samples with known amounts of various analytes. A calibration curve was constructed separately for each compound by plotting peak area vs. concentration. The calibration curve was fitted by linear least-squares regression, and the value obtained for the correlation coefficient showed that the method is linear in the concentration range studied.

Accuracy was evaluated through the percent recovery of the phenolic compounds calculated by spiking the sample with known amounts of the compounds at three different concentrations (low, medium, high).

The calculations for the LOD were calculated using the following equation $LOD = 3.3\ \sigma/S$ and the LOQ were calculated using the equation $LOQ = 10\ \sigma/S$; where σ is the standard deviation of the response and S is the slope of the calibration curve. The calibration equations, correlation coefficient, LOD, LOQ and the average of recovery of each phenolic acid in spiked samples are compiled in Supplementary Materials Table S1.

2.9. DPPH$^{\bullet}$ Scavenging Activity

The antioxidant activities of the phenolics from the germinated spelt seed extracts were determined using DPPH$^{\bullet}$ [22], according to Mencin et al. [4]. The antioxidant activities using DPPH$^{\bullet}$ (referred to as DPPH in Figures and Tables) are expressed as mg TE/g DW.

2.10. ABTS$^{\bullet+}$ Scavenging Activity

The scavenging activities of the phenolics from the spelt extracts were determined using ABTS$^{\bullet+}$, according to the method described by Mencin et al. [4]. The antioxidant activities using ABTS$^{\bullet+}$ (referred to as ABTS in Figures and Tables) are expressed as mg TE/g DW.

2.11. Statistical Analysis

All of the analyses were performed for the data obtained in three parallel runs, on two separate extractions. The data are means ± SD. One-way analysis of variance (ANOVA) was performed using the SPSS programme (Version 22 for Windows). The comparisons of the treatment means were based on Duncan's multiple range tests, for a significance level of 0.05.

3. Results and Discussion

3.1. Effects of Extraction Parameters on Extractable Phenolics

The extraction yields of the phenolics from such plant tissues are affected by several parameters, including extraction technique, time, temperature and solvent-to-sample ratio, and number of repeated extractions and type of solvent [23]. Although the phenolic profiles of the crude samples showed high levels of the phenolic acids analysed here (Table 1), these samples need to be purified before LC-MS analysis. Purification using SPE can prevent impurities from accumulating on the analytical HPLC column. When a column becomes contaminated or blocked up with impurities, the pressure increases and the peaks broaden or split. The food matrix is generally a problem in any analytical technique, so there is the need to remove the contaminants so that they do not block up the column. Additionally, the crude samples showed broader peaks (Figure S1) compared to the purified samples, which indicated that better separation between the peaks can be achieved with the purification step. In the crude sample, the components were present at a much higher concentration, so the peaks begin to overlap the neighbour component at a lower concentration.

According to Stalikas [24], very polar phenolic acids cannot be extracted completely with pure organic solvents and mixtures of alcohol-water are instead suggested. Therefore, the extraction efficiency for the extractable phenolics was further examined with different proportions of methanol:water. Here (data not shown), the recovery of all of the analytes was the highest when pure methanol was used, where compared to the extraction with water, extraction with methanol resulted in 4% increase in TPC and 16% increase in the DPPH$^{\bullet}$ scavenging activity of the purified extractable fraction (Table 1). The type of solvent also had an impact on the phenolic profiles of these direct SPE-purified extracts. As can be seen from Table 1 (dataset #2, #3, #4), *p*-hydroxybenzoic acid was the predominant phenolic acid detected in the extractable fraction regardless the extraction solvent. Compared to extraction with the more polar water, extraction with methanol resulted in higher contents of *p*-coumaric acid (10%), *trans*-ferulic acid (14%) and *p*-hydroxybenzoic acid (32%) (Table 1, water 1:9 vs. methanol 1:9; #3 vs. #2). Interestingly, the content of the caffeic acid derivatives (22%) was significantly higher in aqueous extracts than in methanolic extracts (Table 1).

Among these phenolic acids studied here, caffeic acid is the most polar, because its two hydroxyl groups increase its hydrophilicity, which will be why it was better extracted with the more polar solvent, water. *p*-Coumaric acid is more hydrophobic than caffeic acid, followed by ferulic and *p*-hydroxybenzoic acid. This is reflected in the data here, where the impact of solvent polarity on extraction efficiency of individual phenolic acids increased with decreased polarity of the target molecules. According to Terpinc et al. [25], caffeic acid also has significantly greater Folin–Ciocalteu reducing capacity and DPPH$^{\bullet}$ radical scavenging ability than ferulic and *p*-coumaric acids. All of these aspects can partly explain the deviations among the TPC and the antioxidant activities of the individual phenolic acids determined in the present study.

Soluble conjugates are compounds with multiple hydroxyl groups, which increase the hydrophilicity of attached phenolic compounds, especially flavonoids [26]. According to the data shown in Figure 3, the methanolic extracts contained low levels of soluble conjugates. The prevalent free phenolics (within the extractable fraction) have a higher affinity for methanol compared to water. As can be seen from Table 1, TPC was less affected by the extraction solvent than the content of the individual phenolics, which suggests that the aqueous extracts had different compositions, where the phenolic acids were not the main reductants of the phosphomolybdate in the Folin–Ciocalteu reagent. It should be

noted that both crude extracts were dried to dryness, reconstituted in dH_2O and applied to SPE tubes as described in Section 2.2.

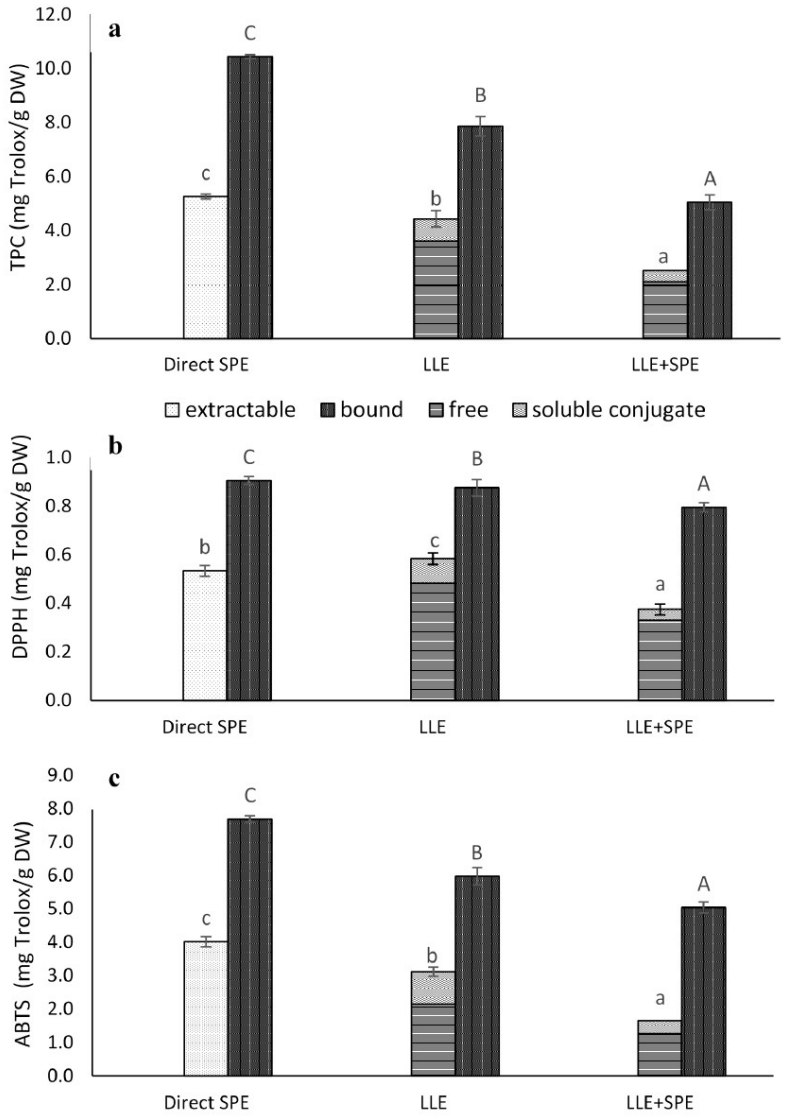

Figure 3. Total phenolics content (TPC) (**a**) and antioxidant activities (DPPH•, ABTS•+ scavenging activities) (**b**,**c**) from the extractions of the germinated spelt (*Triticum spelta*) seeds, as the extractable and bound phenolics from direct solid-phase extraction (SPE), and for the free, soluble conjugate and bound phenolics from the liquid-liquid extraction without (LLE) and with SPE (LLE + SPE) purification. Data are means ± SD. Different letters within the same fractions as extractable and (free + soluble conjugate) or bound indicate significant differences ($p < 0.05$; Duncan's multiple range tests).

The recoveries of *p*-coumaric acid, *trans*-ferulic acid, caffeic acid derivatives and *p*-hydroxybenzoic acid after the purification step when methanolic extract was applied on the SPE tube were 93.49%, 94.00%, 95.54% and 89.51%, respectively (Supplementary Table S1). These data all indicate better selectivity of methanol than water for the extraction of the phenolic acids from spelt.

In addition, the extraction techniques need to take into account the location of the phenolic acids in the plant tissues. However, methanol can disrupt cell walls and inhibit enzyme activities, and it is a good solvent for most phenolics. The exceptions here are the phenolics that are bound to insoluble carbohydrates and proteins within the plant matrix [23]. However, various reports in the literature have shown that the total phenolics that can be extracted with polar solvents (e.g., water, methanol, ethanol) can vary considerably, depending on the sample matrix used [27].

As the yields of the total and individual phenolics were higher in methanol, the further optimisation was carried out with methanol only. The sample-to-methanol ratios of 1:6 and 1:9 were also tested with methanol extraction. When compared to the sample-to-solvent ratio of 1:6, that of 1:9 resulted in a 36% increase in TPC (Table 1, #2 vs. #4) and 14% increase in DPPH• scavenging activity (Table 1). These data demonstrate that the higher proportion of methanol (i.e., 1:9) was better for increased reduction capacity according to Folin–Ciocalteu reagent and greater DPPH free radical scavenging activity. Increasing the proportion of methanol in the extraction will provide a greater concentration gradient during its diffusion, and thus the analytes will have a greater tendency to leave the matrix and move into the liquid phase [28]. However, too high a sample-to-solvent ratio coincides with higher solvent consumption following the extraction and energy consumption for concentration in a later processing stage [28]. It should be stressed here that the optimisation of these particular parameters was performed to maximise the adsorption of the target molecules (i.e., the phenolics) onto the SPE tube matrix.

As can be seen in Table 1, the higher sample-to-solvent ratio of 1:9 had a negligible impact on the extraction yield of most of the individual phenolic acids, expressed per unit mass of the sample matrix. However, *p*-hydroxybenzoic acid did show a statistically significant positive impact (11%), and so 9 mL of solvent was used per gramme of seeds for the further analyses, with larger sample volumes also needed for further analysis.

3.2. HPLC Method Validation

The calibration data for each standard phenolic acid as well as the LOD and LOQ for the spelt sample spiked with phenolic acid standards are shown in Table S1. As can be seen, the linearity of all compounds is satisfactory with R^2 values > 0.9911.

The proposed method was found to be suitable and reliable for the determination of phenolic compounds as the recoveries ranged from 89.51% to 95.54%.

The LOD determined in spelt seed samples ranged from 0.094 to 0.524 μg/g, with the lowest and the highest values for *trans*-ferulic acid and caffeic acid, respectively. Furthermore, the LOQ values of the studied phenolic acids varied from 0.285 μg/g for *trans*-ferulic acid to 1.586 μg/g for caffeic acid (Supplementary Table S1).

3.3. Effects of the Extraction Parameters on Bound Phenolics

Wholegrain *Triticum* seeds are an excellent source of bound phenolic acids (which represent >90% of the total phenolic acids), followed by bound flavonoids and other phenolics [2,4,7,29–31]. As reported by Balli et al. [32], the hydrolysis conditions can significantly affect the total amounts and profile of the bound phenolics than can be extracted. The type of base/acid, the solid-to-solvent ratio, and the extraction method determine the amounts and type of phenolics released, and the antioxidant activities of the extracts obtained. In general, an increased solvent volume for the hydrolysis increases the extraction efficiency, while decreasing the solvent volume lowers the extraction efficiency due to saturation effects, although this will also decrease the cost. Depending on the method used to release and assess the bound phenolics, the conditions applied might be

destructive or inefficient, thus causing degradation or incomplete release of the bound phenolics [5].

Most of these studies have used alkaline hydrolysis with NaOH for the release of the bound phenolics from *Triticum* seeds, while other studies have obtained higher amounts of phenolics using acid hydrolysis with H_2SO_4 or HCl [9]. In general, esterified phenolic acids (i.e., those linked to the cell wall polysaccharides by ester bonds) are more efficiently liberated by alkaline hydrolysis, while acid hydrolysis is more recommended for the release of phenolic acids from glycosylated forms (i.e., those linked to the solubilising sugars by ether bonds) [23]. While acidic pH and high temperature might result in degradation of some phenolics, on the other hand, after acidic hydrolysis, the extracted bound phenolics can be directly injected into a chromatographic system for further analysis after neutralisation and filtration. In contrast, alkaline hydrolysis requires an additional extraction procedure using diethyl ether or ethyl acetate [5].

In continuing this study, we next determined how the solid (residue)-to-NaOH ratio affected the efficiency of the later phenol binding to the SPE columns. The range of solid-to-liquid ratio selected for the alkaline hydrolysis in the present study was based on frequently documented literature data. Here, greater accessibility of the analyte to NaOH should result in increased levels of phenolics released. At the same time, we investigated what a highly concentrated (*versus* more dilute) hydrolysate obtained under the same experimental conditions would mean for the binding and elution efficiency of the phenolics obtained once applied to the SPE purification. Therefore, the seed residues from the methanol extraction had different volumes of 2 M NaOH added (10 mL, 15 mL, 20 mL; Table 1, dataset #11, #12, #13), to determine the effects of the added alkali on the levels of bound total and individual phenolics, and on the antioxidant activities of the extracts obtained.

As can be seen in Table 1, the SPE purified samples with higher bound TPCs also expressed higher antioxidant activities. Comparing the volumes of alkali added, as 10 mL vs. 20 mL NaOH, the larger volume resulted in 23% higher TPC and 16% greater $DPPH^{\bullet}$ scavenging activity. However, there were no significant differences between 15 mL and 20 mL NaOH for TPC, although 15 mL NaOH provided 9% greater $DPPH^{\bullet}$ scavenging activity compared to 20 mL NaOH, which reached statistical significance. In agreement with previous reports [6,24], different phenolic acids were extracted in different proportions depending on these hydrolysis conditions. Indeed, here the different seed residue-to-NaOH ratio (*w/v*; under the same purification conditions) had a large influence on the contents of the phenolic acids per unit mass of sample matrix. As can be seen in Table 1, comparison of the addition of 20 mL NaOH with that of 10 mL and 15 mL NaOH (respectively) resulted in the higher levels of *p*-coumaric acid (40%, 19%), *trans*-ferulic acid (70%, 35%), caffeic acid derivatives (209%, 67%) and *p*-hydroxybenzoic acid (61%, 21%). All of these phenolic acids increased linearly with increased volume of NaOH ($R^2 \geq 0.98$), which suggested a significant impact of solid-to-liquid ratio on the alkaline lability of these phenolic acids.

Thus, an increase in the sample-to-solvent ratio will lead to a greater concentration gradient during the extraction, which will promote the movement of the analytes from the matrix into the liquid phase [28]. The addition of 20 mL NaOH was therefore the optimal choice for all of the phenolic acids after SPE purification (expressed in DW), and was therefore incorporated into the protocol.

3.4. Optimisation of Solid-Phase Extraction Conditions for Extractable Phenolics

One of the aims of this study was to develop and optimise a direct SPE method for rapid and selective separation of the extractable phenolics prior to analysis by LC-MS. In the first step, the influence of sample preparation on the interactions between analyte and sorbent was investigated. When an analyte is extracted from a matrix by a sorbent, the selective removal of impurities can often be achieved by changing the polarity of the solvent [10]. Based on our preliminary data (data not shown), it was necessary to reduce the amount of methanol in the extracts obtained to optimised the binding capacity of the phenolics applied directly to the SPE tubes that contained Strata X-RP. Our first approach

was dilution of the methanolic extract with water to avoid potential losses from evaporation and reconstitution. As shown in Table 1 (#5), application of 30 mL extract at 10% methanol resulted in lower yields of the total and individual phenolic acids compared to the more standard reconstituted sample (Table 1, #5 vs. #6). This suggested that when the 30 mL sample was added directly to the SPE tube, part of the phenolic acids flowed through the column without binding to the matrix, potentially due to a combination of excessive solvent application and too high a proportion of methanol.

Consequently, the second approach was that after preparation of the extractable phenolics, instead of dilution of the methanol, it was evaporated off and the dry residues were re-dissolved (i.e., reconstituted) in 5 mL water. In this step, if required, pH adjustment based on the analyte pKa is also recommended, to improve the liquid extraction efficiency. With the pKa values of the target phenolic acids here from 4.54 to 4.65, reconstitution was carried out with three different solvents: water, at pH 7; and acidic water, at pH 3 with formic acid or at pH 2 with HCl (Table 1, dataset #6, #7, #8). The same solvent in which the sample was dissolved was used to equilibrate the SPE column. The pH of the water used for the reconstruction had negligible effects on TPC and on the antioxidant activities (Table 1). Acidification of the water to pH 2 with HCl compared to water at pH 7 improved the TPC (9%) and compared to water at pH 7 and acidic water at pH 3, also increased the antioxidant activity (6%, 4%, respectively). There were no significant differences between TPCs of acidic water pH 2 and pH 3. According to these data, the pH of the reconstitution water affected only the extractable *p*-hydroxybenzoic acid content (Table 1). Lower yields in the SPE eluate were observed (expressed per unit mass of sample matrix) when the water reconstitution at pH 7 was applied directly to the SPE column, compared to the acidic water at pH 3 or pH 2 (27%, 29%, respectively). It would thus appear that acidification of the water and the subsequent protonation of the carboxylic groups is important only for efficient π-π interactions between the SPE tube matrix of the Strata X-RP sorbent and *p*-hydroxybenzoic acid (which has the shortest chain among these phenolic acids). Further, for the successful binding of other phenolic acids to the SPE sorbent, acidification of the water is not necessary.

Therefore, reconstruction in 5 mL water (pH 7) was selected as the standard reconstitution step here. This is not in agreement with Buszewski and Szultka [33], who reported that pH should be two units lower than the pKa of the analytes. A similar approach was adopted by Irakli et al. [11], who evaporated off their extraction solvent (70% methanol) from 2 mL of extract, followed by addition of 2 mL acidified water (1% (*v*/*v*) acetic acid, pH 2.6). The extraction efficiency of phenolic acids was further examined at different pHs of their loading solution (pH 2.6, 5.1). Here, they reported that the recovery of all of their analytes was increased by acidification of the loading solution to pH 2.6 when compared to pH 5.1, due to the suppression of the dissociation of the phenolic acids and to their more effective adsorption onto the tube sorbent.

Next for the reconstruction of these extractable phenolic acids, the effects of the loading volume on the extraction efficiency were studied. Here, with the extract reconstituted in 5 mL water (pH 7), two different volumes were then applied directly to the SPE tube: 3 mL and 5 mL (Table 1, dataset #9, #10). According to the data obtained, the higher SPE loading volume resulted in slightly, but significantly, higher TPC (Table 1, 4%) and DPPH$^{\bullet}$ scavenging activity (Table 1; 3%) of the SPE purified extracts. The extraction efficiency per unit mass of sample matrix also increased with the increase in the amount of sample loading for each of the extractable phenolic acids, *p*-coumaric acid (8%), *trans*-ferulic acid (13%), caffeic acid derivatives (9%) and *p*-hydroxybenzoic acid (11%). Therefore, a 5 mL loading volume was adopted as the isolation and purification procedure for these extractable phenolics.

To summarise to this point, this study has confirmed that the efficiency of the recoveries through the direct SPE purification is affected by the sample volume and the solvent composition (i.e., 10% methanol, water, acidic water) in which the sample is applied to the tube. According to these data, we believe that among these extractable antioxidants there

is a large proportion of very polar antioxidants, which re-dissolve best in the neutral water, but which must first be fully liberated from the cells (with the methanol extraction).

Compared to the crude extract here, there were some losses in terms of the *p*-coumaric acid (37%), *trans*-ferulic acid (24%), caffeic acid derivatives (47%) and *p*-hydroxybenzoic acid (63%) from the direct SPE purification under this optimised sample processing: i.e., extraction with 99.9% methanol; sample-to-solvent ratio 1:9; reconstitution with water (pH 7); and 5 mL loading sample directly onto the SPE tube. We can conclude therefore that the sample preparation (i.e., evaporation of methanol, reconstitution in suitable solvent) before the direct SPE purification had a significant effect on the loss of these phenolic acids.

It can be stressed here that during the few hours of solvent evaporation, the most thermolabile compounds might undergo decomposition and lose their antioxidant activities. Moreover, some of the compounds successfully extracted from the germinated spelt might remain on the walls of the test tubes, and therefore incomplete dissolution might also contribute to poorer recovery. The various intermediate steps, such as centrifugation and filtration, will also affect the final recovery rates. These findings in the present study can be used as a basis for future studies, with a view to further optimisation of this protocol.

3.5. Optimisation of Solid-Phase Extraction Conditions for Bound Phenolics

Sample hydrolysis is a key step during the treatment of these samples, as this breaks down and releases the bound phenolics. In most studies, the extraction of the bound phenolics is completed with ethyl acetate or diethyl ether extraction after their hydrolysis (Figure 2). One of the main sources of errors during such analytical procedures is the sample preparation. It is crucial to shorten the procedures and to enhance the accuracy and selectivity for the identification of the majority of the chemical components here [34].

The principle of SPE is similar to that of LLE, in terms of the partitioning of the solutes between two phases (Figure 2) [10]. Generally, during the extraction process, an aqueous sample passes through an immobilised phase, and is afterwards extracted (or released) using a suitable organic solvent [33]. Therefore, instead of further solvent extraction here, the NaOH hydrolysate was applied to SPE to increase the yields of the bound phenolics in the extracts (Figure 2). The main objective of the SPE here was thus for removal of interfering matrix components, for concentration and isolation of the bound phenolics, and for changing the matrix of the analyte as needed for subsequent analysis.

In the present case, the phenolic acids must have a greater affinity for the sorbent that makes up the solid phase than for the sample (liquid) matrix. The relationships between the target compounds and the sorption during SPE include hydrophobic interactions (e.g., van der Waals forces) and hydrophilic interactions (e.g., dipole-dipole, induced dipole-dipole, hydrogen bonding, $\pi-\pi$ interactions). Additionally, there are electrostatic attractions between the charged groups on the analyte of interest and the sorbent surface, along with molecular recognition mechanisms [33].

Separation of the analytes and the interfering compounds (i.e., impurities) using SPE can be realized by selective washing, starting with the compound of interest and the impurities being retained on the sorbent bed when the sample passes through. The impurities can then be rinsed off the sorbent bed with wash solutions that are strong enough to remove them, but weak enough to leave the compounds of interest behind [10]. As reported by Rodríguez et al. [35], the strategy to concentrate phenolics using a polystyrene–divinylbenzene sorbent include sorbent activation, concentration of the water sample (pH 2–3), drying of the sorbent bed, and elution using methanol or other solvents. As the liberated (alkaline hydrolysed) phenolics in the present study were already transferred into acidified water, no additional pretreatment appeared necessary. In Section 3.4. we also demonstrated that the reconstitution and equilibration of the extractable phenolic acids using water or acidified water showed negligible differences in the levels of total and individual phenolic acids following SPE.

Therefore, the reconstitution of the bound phenolics in non-acidified water would result in greater losses due to various intermediate steps (i.e., drying, reconstitution,

filtration). As the liberated phenolics will have higher affinity towards the non-polar stationary phase due to their protonation, they will be adsorbed easily from an aqueous environment onto the non-polar polymeric sorbent. Finally, elution would be achieved by solvents with lower polarity. The SPE optimisation also involved the selection of a suitable washing and elution solution, which would provide the highest recovery rates, although this was not included in current study.

According to Rodríguez et al. [35], retention of phenolics is the result of a reversed-phase mechanism and π-π interactions among electrons from the aromatic rings in the sorbent and in the phenol molecules. This latter depends strongly on the properties of the crude extract. The correct choice of the reconstitution media (i.e., composition, pH) is therefore essential to obtain quantitative retention of phenolics.

Solvents with acidic pH are usually used for neutralisation of extracts after hydrolysis, as the phenolics are generally more stable at lower pH. Therefore, the degradation of the bound phenolics is lower at lower pH, compared to higher pH [30]. For extraction into organic solvents, low pH causes the phenolic group equilibrium to be shifted to fully protonated, and thus less polar, and more soluble in less polar solvents. This depends on other functional groups near the phenol group, in terms of whether they are electron withdrawing or donating, which can change the pKa of the phenolic group. In addition, other groups on the phenolic molecules might have their own pKa, which would change the solubility at different pHs. As already mentioned, if reversed-phase SPE is used for acidic analytes, the pH is usually adjusted to two units below the pKa of the target molecules.

In the present study, the influence of hydrolysate neutralisation with HCl or formic acid on TPC, antioxidant activity and the content of the individual phenolic acids were investigated. The results will define the preferred pH of the aqueous phase to achieve higher extraction yields and antioxidant activities. For hydrolysate neutralisation, we used 6 M HCl to adjust to pH 2 or concentrated formic acid to adjust to pH 3. As can be seen in Table 1, compared to pH 3, at pH 2 the TPC and $DPPH^{\bullet}$ scavenging activity decreased (29%, 66%, respectively), which indicated that pH influenced the protonation of the –COOH group and the electron-donating capacity of the phenolics. In addition, compared to pH 2, with the neutralised hydrolysate at pH 3 there were significantly increased levels of bound *p*-coumaric acid (25%), *trans*-ferulic acid (47%), caffeic acid derivatives (29%) and *p*-hydroxybenzoic acid (60%) (Table 1, dataset #14, #15). The increase in the concentration of some of the phenolics in the milder acidic medium might have been due to their higher stability at pH 3 than pH 2 [23]. Although the samples here were not subjected to acid hydrolysis, the alkali hydrolysates were neutralised with an appropriate amount of HCl or formic acid and stored overnight at 4 °C (until LC-MS analysis). Due to prolonged exposure, the most acid-labile phenolics might have degraded to some extent.

Our suggestion here is not consistent with Arranz and Saura Calixto [6], who reported that hydroxybenzoic, caffeic, cinammic, ferulic and protocatechuic acids were the main constituents of the sulphuric acid hydrolysate of wheat (methanol/H_2SO_4; 90:10 (*v/v*) at 85 °C for 20 h). In addition, they showed significantly higher levels of total phenolic acids for strong acidic hydrolysis than for alkali hydrolysis.

In the present study, for the hydrolysates neutralised with HCl and formic acid (respectively) the losses for *p*-coumaric (34%, 28%), *trans*-ferulic (32%, 21%) and *p*-hydroxybenzoic acids (28%, 18%) were higher than for the caffeic acid derivatives (42%, 45%). It is known that caffeic acid can undergo significant oxidative degradation during alkaline hydrolysis [36]. The recoveries for TPC with the hydrolysate at pH 2 were 53%, and at pH 3 they were 62%. The lower losses for individual compounds in comparison with TPC can be explained by the higher content of non-phenolic reductants and chelating agents in the relevant purified extracts. Although Folin–Ciocalteu reagent provides very useful assay, it might be non-specific and therefore not reliable. This would support Verma et al. [31], who reported that alkali hydrolysis liberated nearly twice the amounts of phenolics compared to acid hydrolysis, as determined by Folin–Ciocalteu assays. However, according to their HPLC-UV, the difference between the two protocols in terms of identifiable phenolic acids

was only 21%. Those deviations were confirmed also by Irakli et al. [11], who reported that the bound phenolics content (using the Folin–Ciocalteu method; and with different cereals) were three-fold those measured using HPLC with diode-array detection.

From these results, we can conclude that with the hydrolysate adjusted to pH 3, not only were there more total and individual phenolics obtained, but also that the extract showed better scavenging activities for free radicals and better recovery of phenolics. Last, but not least, the use of formic acid is more appropriate than the use of HCl, as chlorides can corrode stainless steel flow paths, which can lead to ion contamination and pitting of the LC flow path.

To validate the influence of the sample volume loaded onto the SPE tubes on the extraction recoveries, three different loading volumes were tried (3 mL, 5 mL, 8 mL; Table 1, dataset #16, #17, #18). From Table 1, it can be seen that compared to the loaded hydrolysates of 3 mL and 5 mL (respectively), that of 8 mL showed increased TPC (77%, 79%). The DPPH$^{\bullet}$ scavenging activity also increased with the increase in the sample volume loaded onto the SPE tubes. In addition, and again compared to 3 mL and 5 mL (respectively), the 8 mL loaded hydrolysate showed small increases in the extracted bound *p*-coumaric acid (20%, 8%), *trans*-ferulic acid (6%, 2%) and *p*-hydroxybenzoic acid (11%, 15%). However, the content of caffeic acid derivatives with 3 mL was 16% higher than that with 8 mL (Table 1, #16 vs. #18).

Formation of hydrogen bonds between sorbent and phenolics can lead to irreversible sorption and extra difficulties in tube elution [35]. In comparison to their crude forms, it is evident that the lowest losses were seen for the 8 mL loaded samples (18%). As the volume of the loaded sample increases, the proportion of irreversibly adsorbed phenolics remains the same, consequently a higher concentration of phenolics was eluted. Therefore, the 8 mL volume was chosen as the volume for loading onto the SPE for the bound phenolic extracts.

3.6. Liquid-Liquid Extraction vs. Solid-Phase Extraction

Liquid-liquid extraction is the most commonly used technique for extraction of phenolic acids in cereal samples [12]. The whole LLE procedure provides three fractions, as free phenolics, soluble conjugates (combined as extractable phenolics), and bound phenolics. Instead, the direct SPE procedure provides the extractable and bound phenolics fractions (Figures 1 and 2).

For increasingly sensitive chromatographic analyses, good sample preparation is essential, because it protects the chromatographic columns, and allows greater sensitivity through the removal of interfering matrix components. Selective and specific sample preparation is thus a prerequisite for reasonable, economical and sensitive analyses.

In the present study, when the extracts obtained by LLE were compared to those obtained by direct SPE purification they showed 19% lower extractable (i.e., free plus conjugated) phenolics, and 32% lower bound TPC (Figure 3a).

According to Obied et al. [37], SPE provides greater recovery of pomace phenolics than LLE. They also reported that with SPE, higher recoveries were obtained by elution with methanol than by elution with diethyl ether or ethyl acetate.

A different trend was observed in the present study for antioxidant activity, as direct SPE purification provided extracts with higher ABTS$^{\bullet+}$ (extractable, 29%; bound, 29%) and DPPH$^{\bullet}$ scavenging (bound, 3%) activities, but at the same time, the extractable phenolics were less efficient than LLE against DPPH$^{\bullet}$ free radicals (9%) (Figure 3b,c).

Deviations across these different tests are in agreement with Abramovič et al. [38], who reported that the number of exchanged electrons varies greatly with solvent and type of assay, whereby the majority of the compounds exchange more electrons in the Folin–Ciocalteu assays than in the ABTS and DPPH assays. According to their data, in reactions with chromogenic radicals, the numbers of electrons exchanged are higher in buffer at pH 7.4 than in MeOH (by the DPPH assay) and in water (by the ABTS assay).

In the present study, when compared to direct SPE, LLE with ethyl acetate showed lower levels of extractable (i.e., free plus soluble conjugates) *p*-coumaric acid (81%), caffeic

acid derivatives (169%) and *p*-hydroxybenzoic acid (48%), but there were no significant differences in content of *trans*-ferulic acid (Figure 4). LLE compared to direct SPE also showed lower contents of bound *p*-coumaric acid (9%), *trans*-ferulic acid (10%), caffeic acid derivatives (38%) and *p*-hydroxybenzoic acid (144%) (Figure 4). Thus it needs to be taken into consideration that LLE is limited to partition equilibriums in the liquid phase, and requires an additional evaporation and reconstitution step. On the other hand, when using SPE, different interactions can be involved simultaneously. According to the supplier (Phenomenex) specifications, phenolics adsorption onto the SPE Strata X-RP tubes relies on three mechanisms of retention: $\pi-\pi$ bonding, hydrogen bonding and hydrophobic interactions.

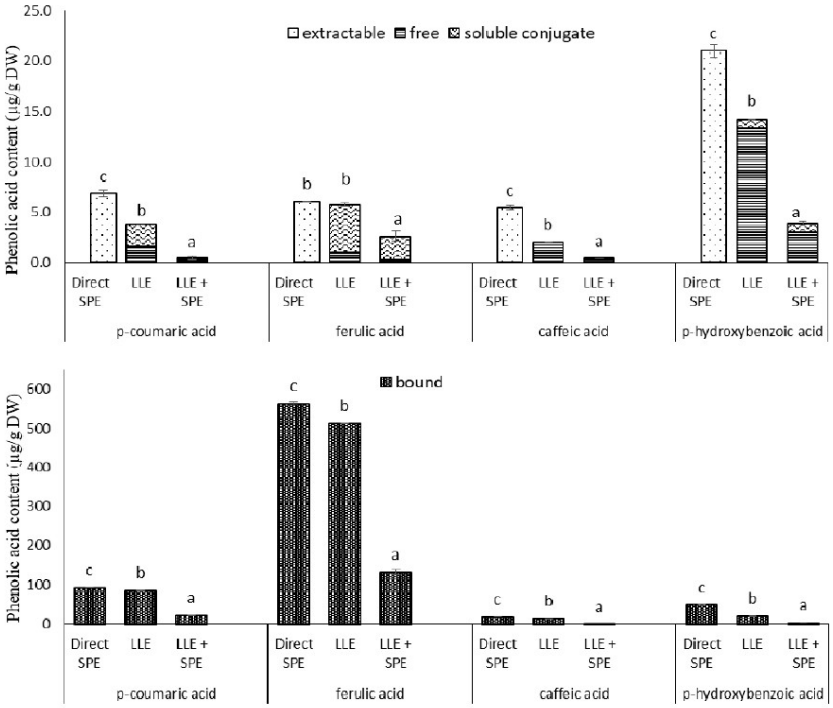

Figure 4. Individual phenolic acid contents from the extractions of the germinated spelt (*Triticum spelta*) seeds, as the extractable and bound phenolics from direct solid-phase extraction (SPE), and for the free, soluble conjugate and bound phenolics from the liquid-liquid extraction without (LLE) and with SPE (LLE + SPE) purification. Data are means ± SD. Different letters within the same phenolic acid as extractable and (free + soluble conjugate) or bound indicate significant differences ($p < 0.05$; Duncan's multiple range tests).

Terpinc et al. [39] showed LC chromatograms of sugars and organic acids in camelina cake extracts prior to and after passage through an SPE Strata X tube. There was also some loss of phenolics during the purification process, but their average recoveries were about 90% when a standard phenolic compound was applied to the SPE tube.

Therefore, to determine whether satisfactory purities of the extracts had already been achieved by LLE, the extracts obtained in the present study were subjected to a further purification step, by SPE (referred here to as LLE + SPE). It can be seen from Figure 4 that LLE + SPE yielded the lowest contents of the extractable and bound total and individual phenolics, as compared to the direct SPE and LLE alone procedures. Similarly, LLE + SPE

yielded extracts with the lowest DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities (Figure 3b,c). Chromatograms of LLE extracts prior to and after passage through SPE are presented in Supplementary Figures S2 and S3. Here, it can be seen that with SPE as the final purification stage following LLE (i.e., for LLE + SPE), better peak shapes can be obtained, with better separation of the different analytes from each other. Thus, LLE without this added SPE results in broader peaks and peaks with shoulders, which causes problems in the precise and accurate integration of these data.

Therefore, compared to LLE and LLE + SPE, the protocol here with direct SPE purification is the most reproducible method, and provides higher recoveries for the extractable and bound phenolics.

4. Conclusions

In the present study, the optimal extraction and purification techniques were determined and the efficiency of two isolation and purification methods (SPE, LLE) were compared. The optimal pretreatment conditions prior to purification of the extractable phenolics directly through SPE were the use of 99.9% methanol as the solvent, a sample-to-methanol ratio of 1:9 (*w/v*), and reconstruction in water at pH 7. The optimal extraction conditions for the bound phenolics from these germinated spelt seeds were hydrolysis with 20 mL 2 M NaOH, neutralisation with formic acid to pH 3, and purification by SPE without preliminary reconstruction. SPE was a better extraction method than LLE, as it provided greater yields of total and individual phenolics and greater antioxidant activities for both the extractable and bound fractions. Except for the removal of the matrix components, the isolation and concentration of these analytes by direct SPE was achieved in only one step. Direct SPE has emerged as an alternative to the more traditional sample preparation with LLE, particularly for the analysis of the bound phenolics. This is due to its simplicity, ease of automation, time savings, reduced use of highly toxic solvents, higher analyte recoveries, higher analyte concentrations, highly purified extracts, medium exchange when needed for subsequent analyses, and energy saving. Optimisation of sample pretreatment prior to direct SPE and optimisation of loading volumes for these SPE tubes also provides a more selective and simplified approach and reductions for the risk of errors. This purification method is recommended for use in various laboratories performing routine analyses of phenolic acids in cereal seeds. Our protocol can serve as a basis for any extraction of phenolics from different cereals, depending on the cereal matrix the protocol can be adapted. The results of the present study will contribute to the determination of bound phenolics, which are often overlooked. Future research may consider the application of different types of SPE columns.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10071085/s1, Supplementary Table S1: Characteristics of phenolic acids from germinated spelt seeds by liquid chromatography–mass spectrometry in negative ion mode; Supplementary Figure S1: Representative HPLC chromatograms of the extractable phenolics of germinated spelt seeds in the crude extract without purification (Table 1, #1) and with purification through solid-phase extraction (Table 1, #2) (detected at 310 nm). Below: Peak times for purified sample corresponding to the defined phenolic acids determined in the present study; Supplementary Figure S2: Representative HPLC chromatograms of free phenolics of germinated spelt seeds after liquid-liquid extraction without (LLE) and with solid phase extraction (LLE + SPE) (detected at 310 nm); Supplementary Figure S3: Representative HPLC chromatograms of bound phenolics of germinated spelt seeds after liquid-liquid extraction without (LLE) and with solid phase extraction (LLE + SPE) (detected at 310 nm).

Author Contributions: Conceptualization, M.M. and P.T.; methodology, M.M., M.M.-P., R.V. and P.T.; software, M.M.-P.; validation, M.M., M.M.-P. and P.T.; formal analysis, M.M. and M.M.-P.; investigation, M.M. and P.T.; resources, P.T.; data curation, M.M. and P.T.; writing—original draft preparation, M.M. and P.T.; writing—review and editing, M.M., M.M.-P., R.V. and P.T.; visualization, M.M. and P.T.; supervision, P.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Slovene Research Agency, through research programmes P4-0234 and P4-0013.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained in this article and supplementary materials.

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

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