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CÍNTIA RAQUEL DE FREITAS

IDENTIFICAÇÃO DE MARCADORES MICROSSATÉLITES CORRELACIONADOS À ATIVIDADE ANTIOXIDANTE EM *Lactuca sativa* L.

PATOS DE MINAS – MG
MARÇO DE 2019

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Biotecnologia como requisito parcial para obtenção do título de Mestre em Biotecnologia.

Orientador: Prof. Dr. Luiz Antonio Augusto Gomes
Coorientador (a): Prof.ª Dra. Terezinha Aparecida Teixeira

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BANCA EXAMINADORA

Prof. Dr. Luiz Antonio Augusto Gomes

Prof. Dr. Aulus Estevão Anjos de Deus Barbosa

Prof. Dr. Valter Carvalho de Andrade Júnior

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RESUMO

A alface (*Lactuca sativa* L.) pode ser considerada a principal hortaliça folhosa consumida no Brasil. Está presente no cardápio diário do brasileiro, preferencialmente em forma de salada e seu consumo apresenta grande importância nutricional. A hortaliça constitui uma abundante fonte de nutrientes. É rica em sais minerais, como cálcio e ferro; vitaminas, como A, B1, B2 e C, além de apresentar outros compostos fitoquímicos. Dentre os fitoquímicos presentes na alface, destacam-se os carotenoides (provitamina A) e antocianinas. Estas substâncias possuem ação antioxidante e atuam prevenindo o envelhecimento e controlando o estresse oxidativo relacionado à ação de radicais livres. O presente trabalho teve como objetivo estudar a divergência genética entre 12 cultivares de alface (Gabriela, Luiza, Model, Optima, Raider Plus, Red Star, Salad Bowl, Silvana, Sophia, Thaís, Verônica e Darkland) que apresentam diferentes colorações, com base em 10 pares de primers microssatélites e associar esses resultados à avaliação da atividade antioxidante de cada cultivar realizada pelos métodos de captura dos radicais livres DPPH• e ABTS+•, redução do complexo de Fosfomolibdênio e quantificação dos compostos fenólicos por meio do reagente *Folin-Ciocalteau*. A metodologia constituiu-se de avaliação das características fenotípicas das cultivares, através da verificação da cor de cada cultivar e quantificação da atividade antioxidante; e genotipagem das amostras a partir de 10 pares de primers SSR. Os valores de atividade antioxidante obtidos por todos os métodos foram correlacionados com a coloração das cultivares através da Correlação de Pearson e Teste de Mantel. Buscando avaliar a dissimilaridade entre as cultivares foi gerada uma matriz de distância de Gower. Os locos microssatélites foram comparados com os teores de atividade antioxidante obtidos para cada cultivar mediante os testes ABTS+•, DPPH•, redução do complexo de fosfomolibdênio e teor de fenólicos totais; assim como a coloração das plantas. Os resultados demonstraram que a cultivar Red Star (coloração roxo escura) apresentou maior %AA na maioria dos testes realizados. Os locos KSL-37, KSL-137, KSL-245 apresentaram correlação positiva e estatisticamente significativa com as cultivares de coloração "roxo escuro", Red Star e Gabriela. SML-022 relacionou-se positivamente com os resultados obtidos pelo teste de redução do complexo fosfomolibdênio. Desta maneira, estes marcadores revelaram-se bons indicadores de atividade antioxidante, podendo ser utilizados em estudos futuros que busquem associar esta característica à cultivares de alface.

Palavras chave: alface, marcadores moleculares, SSR, β-caroteno, anticianina, antioxidante

ABSTRACT

Lettuce (*Lactuca sativa* L.) can be considered the main leafy vegetable consumed in Brazil. It is present in the daily menu of the Brazilian, preferably in the form of salad and its consumption presents great nutritional importance. Greenery is an abundant source of nutrients. It is rich in minerals such as calcium and iron; vitamins, such as A, B1, B2 and C, in addition to other phytochemical compounds. Among the phytochemicals present in lettuce, we highlight carotenoids (provitamin A) and anthocyanins. These substances have antioxidant action and act to prevent aging and control the oxidative stress related to the action of free radicals. The present work had the objective of studying the genetic divergence between 12 lettuce cultivars (Gabriela, Luiza, Model, Optima, Raider Plus, Red Star, Salad Bowl, Silvana, Sophia, Thaís, Verônica and Darkland) in 10 pairs of microsatellite primers and to associate these results to the evaluation of the antioxidant activity of each cultivar performed by the DPPH • and ABTS + • free radical capture methods, reduction of the Phosphomolybdenum complex and quantification of the phenolic compounds by means of the *Folin-Ciocalteau* reagent. The methodology consisted of evaluating the phenotypic characteristics of the cultivars, by verifying the color of each cultivar and quantifying the antioxidant activity; and genotyping of samples from 10 pairs of SSR primers. The antioxidant activity values obtained by all methods were correlated with the staining of the cultivars through Pearson's Correlation and Mantel's Test. In order to evaluate the dissimilarity between the cultivars, a distance matrix of Gower was generated. Microsatellite loci were compared with the levels of antioxidant activity obtained for each cultivar using the ABTS + •, DPPH •, phosphomolybdenum complex reduction and total phenol content; as well as the coloring of the plants. The results showed that the cultivar Red Star (dark purple coloring) showed higher% AA in most of the tests performed. The loci KSL-37, KSL-137, KSL-245 showed a positive and statistically significant correlation with the "dark purple" cultivars, Red Star and Gabriela. SML-022 was positively related to the results obtained by the phosphomolybdenum complex reduction test. Thus, these markers have been shown to be good indicators of antioxidant activity, and may be used in future studies that seek to associate this characteristic with lettuce cultivars.

Keywords: lettuce, molecular markers, SSR, β-carotene, anthocyanin, antioxidant

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INTRODUÇÃO

A alface (*Lactuca sativa* L.) pode ser considerada a principal hortaliça folhosa consumida no Brasil (SALA; COSTA, 2012). Está presente no cardápio diário do brasileiro, preferencialmente em forma de salada e seu consumo apresenta grande importância nutricional.

A hortaliça constitui uma abundante fonte de nutrientes. É rica em sais minerais, como cálcio e ferro; vitaminas, como A, B1, B2 e C (FERNANDES, et al., 2002) além de apresentar outros compostos fitoquímicos. Dentre os fitoquímicos presentes na alface, destacam-se os carotenoides (provitamina A) e antocianinas (SILVA et al., 2018). Estas substâncias possuem ação antioxidante e atuam prevenindo o envelhecimento e controlando o estresse oxidativo relacionado à ação de radicais livres, que desencadeiam doenças crônicas não-transmissíveis como aterosclerose, doenças coronárias e algumas neoplasias (FELTRIM et al., 2009).

A vitamina A pode ser consumida diretamente através de alimentos de origem animal, já nos vegetais, como na alface, é obtida através dos carotenoides, substâncias precursoras de vitamina A, sendo o β-caroteno o mais importante e abundante (SILVA; MURA, 2010). A deficiência desta vitamina é um sério problema de saúde pública em todo o mundo, podendo causar aumento do risco de mortalidade, morbidade e cegueira (MILAGRES et al., 2007; RAMALHO et al., 2008).

Atualmente, existe uma grande diversidade de cultivares de alface exibindo diferenças nos formatos, tamanhos e cores das plantas (SUINAGA et al., 2013). A abundância de cores é decorrente de diferentes concentrações de pigmentos vegetais como a clorofila, os carotenoides e as antocianinas (TAIZ, ZEIGER, 2004; VIEIRA et al., 2010). Porém não há na literatura trabalhos que discutam a capacidade antioxidante de diferentes cultivares de alface comercializadas no Brasil, indicando aquelas que apresentem maiores concentrações de compostos antioxidantes, para atender à crescente parcela da população que busca incluir alimentos saudáveis em seu cardápio, bem como aos programas de melhoramento genético da espécie.

Visando associar características genéticas e fenotípicas; comerciais e nutricionais de interesse, tem sido amplamente utilizada a manipulação assistida por marcadores moleculares em programas de melhoramento genético. Esta técnica permite a seleção indireta para características desejáveis em gerações segregantes precoces, reduzindo tempo e recursos,

além de apresentar a possibilidade de detectar polimorfismos de DNA em todo o genoma da planta (CAIXETA, et al., 2016).

Os marcadores moleculares são utilizados para verificar a diversidade genética dentro de uma mesma espécie, através da detecção de variações no genoma (CAIXETA, et al., 2016). Atualmente, existe uma infinidade de marcadores moleculares disponíveis para utilização em pesquisas envolvendo vegetais, entre os mais utilizados encontram-se os marcadores microssatélites.

Estes apresentam elevado polimorfismo, são codominantes, altamente reprodutíveis, requerem pequenas quantidades de DNA e apresentam baixo custo, com alto poder de resolução. A alta diversidade alélica possibilita a detecção de polimorfismo em populações multiparentais e em populações derivadas de híbridos de genótipos relacionados, além de ser eficaz na distinção de genótipos intimamente relacionados (CAIXETA et al., 2016), constituindo parte fundamental do pré-melhoramento genético (PEREIRA et al., 2016).

Marcadores microssatélites para alface tem sido desenvolvidos desde a década de 1990. (VAN DE WIEL et al., 1999; SIMKO, 2009; RAUSCHER, SIMKO, 2013; HONG et al, 2015), e tem demonstrado, em todos os experimentos, resultados extremamente úteis na distinção de cultivares de alface, bem como na triagem de cultivares para programas de melhoramento.

O presente trabalho teve como objetivo geral correlacionar a atividade antioxidante estimada através de quatro diferentes métodos aos locos microssatélites observados em doze cultivares de alface a partir de 10 pares de primers. Concomitantemente objetivou-se relacionar as diferentes cores apresentas pelas cultivares aos percentuais de atividade antioxidante verificados. Os resultados encontrados constituem uma etapa fundamental para o melhoramento assistido por marcadores moleculares, visando obter cultivares com elevada atividade antioxidante na espécie *Lactuca sativa* L.

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

Considerações gerais sobre a cultura da alface

A alface (*Lactuca sativa* L.) pertence à família Asteraceae. É originária de regiões de clima temperado do Sul da Europa e Ásia Ocidental. Há registros de sua existência por volta do ano 4.500 a.C. no antigo Egito e a sua chegada ao Brasil, através dos portugueses, ocorreu no ainda no século XVI. Atualmente pode ser considerada uma das hortaliças mais populares no mundo (SALA; COSTA, 2012).

No Brasil, a alface é considerada a hortaliça folhosa mais comercializada e consumida, devido à sua capacidade de produção durante o ano todo, utilizações culinárias e aceitação cultural (ABCSEM, 2012). No ano de 2016, sua comercialização em atacado superou 288 milhões de reais com produção de 105.207 toneladas (CONAB, 2017), e estima-se que no varejo tenha atingido 8 bilhões de reais, com produção acima de 1,5 milhão de toneladas (ABCSEM, 2012). Seu cultivo é realizado principalmente por produtores familiares e pode gerar até cinco empregos diretos por hectare (SALA et al., 2008).

O ciclo da planta pode ser dividido em quatro fases: germinação, transplante, fase vegetativa (ou formação de cabeça) e fase reprodutiva, sendo o cultivo realizado nos sistemas convencional, orgânico ou hidropônico. Para fins comerciais, a alface é cultivada até a terceira fase (TANAMATI, 2012). É uma planta bianual, floresce melhor sob dias longos e temperaturas elevadas, sendo que condições climáticas opostas favorecem o período vegetativo. A temperatura ideal para o seu desenvolvimento ocorre entre 7 a 24ºC. No campo, o ciclo varia de 65 a 80 dias, da semeadura à colheita. Em estufa, o ciclo é ainda mais reduzido, de 45-50 dias (FILGUEIRA, 2008).

A hortaliça é uma espécie herbácea, apresenta raízes ramificadas e superficiais, abrangendo somente os primeiros 25 cm de solo, até o momento de ser transplantada. Quando ocorre semeadura direta, a raiz pivotante pode atingir até 60 cm de profundidade. Possui caule pequeno ao qual se prendem as folhas, que crescem em forma de roseta, podendo ser lisas ou crespas, e formar ou não uma estrutura em forma de "cabeça", com coloração que varia entre tons de verde, ou de roxo, de acordo com a cultivar (FILGUEIRA, 2013).

Encontram-se disponíveis no mercado diversos tipos de alface, podendo ser classificadas em cinco principais: repolhuda lisa, repolhuda crespa ou americana, solta lisa, solta crespa, solta crespa roxa, e romana. O tipo “repolhuda lisa” apresenta folhas lisas, delicadas e macias, com nervuras pouco aparentes e aspecto oleoso, forma uma cabeça típica e compacta. A “repolhuda crespa ou americana” possui folhas crespas, consistentes e crocantes, cabeça grande e bem compacta. As cultivares do tipo “solta lisa” possuem folhas

lisas e soltas, delicadas, e não há formação de cabeça compacta. Já as dos tipos "solta crespa" e "solta crespa roxa" possuem folhas grandes e crespas, textura macia, mas consistente, sem formação de cabeça; podendo ter coloração verde ou roxa. O tipo "romana" apresenta forma de cone, folhas tipicamente alongadas, duras, com nervuras claras, formando uma cabeça tenra e alongada (HENZ; SUINAGA, 2009)

Nos últimos anos houve uma mudança no tipo de alface consumido preferencialmente pela população brasileira. Anteriormente, havia preferência por alfaces do grupo lisa, mas atualmente o grupo crespa lidera o mercado. Esta mudança ocorreu possivelmente devido ao fato das cultivares deste tipo apresentarem folhas flabeladas, bordas onduladas, folhas flexíveis de coloração verde-clara e serem melhor adaptadas às condições tropicais, pois não formam o miolo que favorece o acúmulo de água nas folhas internas e, consequentemente de fitopatógenos que dificultam o manejo da lavoura (SILVEIRA, 2016; SALA; COSTA, 2012).

Devido à sua alta perecibilidade após a colheita, a produção de alface é concentrada em regiões próximas aos grandes centros consumidores (SANTOS et al., 2001). É utilizada na alimentação preferencialmente *in natura,* como principal ingrediente de saladas, compondo o cardápio diário de milhares de brasileiros.

A alface possui grande importância na nutrição e saúde humana. Uma planta com 350 g apresenta, aproximadamente: 56 kcal, 95,80% de água, 2,3% de hidratos de carbono, 1,20% de proteínas, 0,20% de gorduras, 0,50% de sais minerais (13,3 mg de potássio, 147,0 mg de fósforo, 133,0 mg de cálcio e 3,85 mg de sódio, magnésio e ferro). Contém, ainda, vitamina A, vitaminas do complexo B (B1 e B2) e C. As folhas de coloração verde-escura, principalmente as externas, contêm 30 vezes mais vitamina A que as internas (FRANCO, 1987).

Em 100 g de folhas verdes a alface atinge mais de 4.000 Unidades Internacionais (UI) de provitamina A, valor quatro vezes maior do que o apresentado pelo tomate; porém, as folhas internas, de coloração mais branca, possuem menores quantidades (CAETANO et al., 2001).

Devido à sua grande importância na alimentação, nutrição e economia, constantes esforços são movidos para o desenvolvimento de cultivares cada vez mais adaptadas ao clima de cada região, resistentes a doenças e para incorporar características nutricionais. Para isso, são desenvolvidos diversos programas de melhoramento genético da espécie, onde se busca alcançar esses objetivos levando inúmeros benefícios para produtores e consumidores.

Melhoramento genético da alface

O melhoramento genético da alface iniciou-se em seu local de origem por meio de seleções feitas por habitantes da região do Mediterrâneo. As seleções iniciais resultaram num grande pool gênico de *Lactuca serriola*, seguidas por introgressões de genes de outras espécies do gênero *Lactuca* (LINDQUIST, 1960). Ao ser introduzida na Europa Ocidental, no século XV já haviam sido descritos alguns tipos de alface, como a lisa, batávia e romana (SALA; COSTA, 2012). No Brasil, a introdução foi feita pelos portugueses, em 1650 (SALA, 2011).

Até a década de 1980 o consumo era preferencialmente de alface do tipo repolhuda lisa ou "manteiga" representada pelas cultivar centenária "White Boston", suscetível ao *Lettuce mosaic virus* (LMV) e cuja produção de sementes é praticamente inviável no país, devido às condições climáticas, que favorecem o desenvolvimento do vírus. No início da década de 1990 a alface lisa ainda liderava o mercado com um volume correspondente a 51% do total comercializado em São Paulo (SALA; COSTA, 2012).

O melhoramento genético da alface no Brasil foi iniciado pelo Professor Marcílio de Souza Dias na ESALQ em 1953, que possibilitou o lançamento da cultivar 'Gorga' oriunda do cruzamento entre as cultivares 'Great Lakes' e 'Batávia Blonde'. A cultivar foi a primeira a combinar espessura foliar, arquitetura de planta aberta, tolerância ao pendoamento precoce e adaptação ao verão chuvoso, sem formação de cabeça. O fator que dificultou seu cultivo foi sua suscetibilidade ao *tip burn* (deficiência na translocação de cálcio causando a queima de bordas das folhas externas) e má formação de cabeça no cultivo de verão (SALA; COSTA, 2016).

O clima era um desafio a ser enfrentado pelos alfacicultores da região sudeste durante o verão. As cultivares do tipo repolhuda eram importadas da Europa e Estados Unidos e nesta região, as temperaturas elevadas associadas à alta pluviosidade levavam o alfacicultor a perdas de até 60% em decorrência da maior umidade relativa que favorecia o ataque de fungos e bactérias. O pendoamento precoce, que é induzido pelas altas temperaturas, também agravava o problema, reduzindo a oferta do produto durante o verão (SALA; COSTA, 2012).

Em 1968 Iroshi Nagai iniciou seus trabalhos em melhoramento de alface, visando à obtenção de cultivares do tipo manteiga resistentes ao vírus-do-mosaico da alface e ao calor, surgindo a cultivar 'Brasil 48' em 1973. Posteriormente, outras cultivares denominadas Brasil 202, Brasil 221, Brasil 303 e Brasil 311, obtidas por Nagai, também obtiveram sucesso, assim como suas versões comerciais (MELO; MELO, 2003).

Na década de 1990, Nagai desenvolveu a série Brasil 500, com genótipos de folhas crespas, resistente a viroses e com tolerância ao florescimento precoce (MELO; MELO, 2003). O desenvolvimento desta série permitiu que o país se tornasse mais independente das empresas internacionais na produção de sementes (GOMES; MALUF; CAMPOS, 2000).

Outra importante contribuição para o setor foi o surgimento da cultivar "Regina" desenvolvida pelo Dr. Cyro Paulino da Costa na USP-ESALQ. Esta cultivar permitiu ampliar o período de cultivo da alface no verão, devido ao formato aberto e sem formação de cabeça, possibilitando o cultivo em muitas regiões, uma vez que não permite o acúmulo de água nas folhas e consequentemente, reduz as perdas; além de ser resistente ao LMV (SALA; COSTA, 2012).

A aceitação do conceito de alface aberta permitiu o aprimoramento da alface do tipo crespa 'Grand Rapids', mais adaptada ao cultivo verão. A partir da década de 1990 esse tipo de alface ganhou importância no país (SALA; COSTA, 2016). O lançamento das cultivares 'Verônica' e 'Vera' (tipo crespa 'Grand Rapids') pela equipe liderada pelo Dr. Paulo T. Della Vecchia da Agroflora S.A (DELLA VECCHIA et al., 1999) permitiu o cultivo de alface no verão em todo o Brasil. Essas cultivares foram a sustentabilidade da alfacicultura por décadas, devido ao pendoamento lento, não formação de cabeça, resistente as chuvas de verão, fácil manuseio e transporte e ciclo curto (CABRAL, 2016).

Em 2008 os pesquisadores Ciro Paulino da Costa e Fernando César Sala lançaram a cultivar 'Gloriosa', selecionada a partir de variantes da cultivar Lucy Brown. 'Gloriosa' é do tipo americana tropicalizada e resistente a *Thielaviopsis basicola,* apresenta tolerância ao pendoamento precoce e adaptação ao cultivo de verão. (SALA; COSTA, 2008). Esta cultivar visa a preencher a demanda de cultivares tropicalizadas e adaptadas para a alfacicultura brasileira, podendo contribuir para um mercado de folhosas higienizadas e com embalagens apropriadas nos grandes centros consumidores (SALA, 2011).

Outro objetivo do melhoramento da alface é a agregação de características importantes para a nutrição humana. Trabalhos relacionados à presença de compostos bioativos na alface, tem sido realizados por diversos pesquisadores. Mou (2005), avaliando 44 cultivares de alface de diferentes tipos, observou que existem diferenças discrepantes entre os teores de β-caroteno para essas cultivares. De forma semelhante Cassetari et al. (2014) avaliaram a variabilidade genética da clorofila e do β-caroteno na alface, verificando a correlação entre estas características. Os autores observaram também uma grande diferença dos teores de β-caroteno entre as cultivares, principalmente para a cultivar "Salinas 88" que obteve um elevado valor de β-caroteno na suas folhas.

Estudos realizados por Gazula et al. (2007) mostraram a grande variedade de teores de antocianinas em diferentes cultivares de alface. As antocianinas são pigmentos vegetais responsáveis pelas cores azul, roxo e todas as tonalidades do vermelho encontradas em flores, frutos, folhas e caules de plantas (MARÇO; POPPI; SCARMÕNIO, 2008). Segundo Ryder (1999) a cor e a intensidade vermelha da folha da alface variam com o teor de clorofila e teor de antocianina.

Buscando obter cultivares com elevados teores de carotenoides, substância precursora da vitamina A, Kerr e colaboradores através de programa de melhoramento, obtiveram a cultivar Uberlândia 10.000. As linhagens foram selecionadas baseando-se em caracteres morfológicos, como por exemplo coloração das folhas, resistência ao pendoamento precoce, sabor doce e adaptação a variações no pH do solo de 4 a 8 (SOUSA et al., 2007).

A cultivar Uberlândia 10.000 originou-se de seleções a partir do cruzamento de 'Maioba' e 'Salad Bowl-Mimosa', que deu origem à cultivar 'Moreninha de Uberlândia' que, apesar do alto teor de vitamina A, tinha características inadequadas às exigências do consumidor, sendo, então, cruzada com a cultivar 'Vitória de Santo Antão', que resultou, finalmente, na cultivar Uberlândia 10.000 (SOUSA et al., 2007).

A importância de compostos naturais com capacidade antioxidante para a medicina preventiva vem sendo amplamente reconhecida nos últimos anos. Desta maneira, o interesse por compostos naturais com propriedades antioxidantes, considerados por muito tempo como não nutritivos, vem aumentando e ganhando destaque nos programas de melhoramento que contam, atualmente, com diversos recursos biotecnológicos para o desenvolvimento de cultivares que contenham características nutricionais e comerciais satisfatórias.

As tendências atuais e futuras são atrelar as técnicas clássicas de melhoramento às biotecnológicas, visando a otimização dos resultados obtidos no melhoramento genético da alface. Esse emprego pode contribuir significativamente para o conhecimento básico da cultura e do caráter estudado, além da geração e do desenvolvimento de cultivares melhoradas (SOUSA et al., 2007).

Melhoramento assistido por marcadores moleculares

Marcadores são caracteres cujo padrão de herança pode ser observado nos níveis morfológico, bioquímico ou molecular. Eles são assim chamados porque podem ser usados para extrair, ainda que indiretamente, informações sobre a herança de características desejáveis. Um marcador deve ser polimórfico, ou seja, existir em diferentes formas, de modo

que o cromossomo portador do gene mutante possa ser distinguido do marcador que ele também carrega. Este polimorfismo pode ser detectado em três diferentes níveis: fenotípico (marcadores morfológicos); proteico (marcadores bioquímicos) ou através de diferenças na sequência de DNA (marcadores moleculares) (CHAWLA, 2009).

Até a década de 1960 os estudos genéticos eram realizados exclusivamente através de marcadores morfológicos. Estes são de fácil identificação nos organismos e geralmente são controlados por somente um gene. Apesar de sua grande contribuição para o estabelecimento dos princípios de mapeamento genético e ligação gênica, devido ao número reduzido de marcadores morfológicos em uma mesma linhagem, há a redução de associações significativas entre esses marcadores e características de interesse (BORÉM, 1999). Além disso, Paterson e colaboradores (1991) destacam que os marcadores morfológicos podem ser afetados pela dominância gênica, efeitos ambientais, pleiotropias ou epistasias.

Buscando resolver esses problemas iniciou-se o uso de marcadores proteicos, especialmente as isoenzimas para análise de produtos da expressão de genes. As isoenzimas, também denominadas marcadores bioquímicos, fazem parte de um conjunto de técnicas que possibilitam a caracterização da variabilidade genética de populações naturais e cultivadas, estudos em evolução e dispersão de espécies e análises filogenéticas. Porém, ainda se trata de um método indireto de estudar os genes; apresenta importantes limitações, como a reduzida cobertura que é feita nos genomas investigados devido ao pequeno número de locos que podem ser identificados, e o baixo nível de polimorfismo identificado por loco (CAIXETA et al. 2016).

Técnicas mais modernas em biologia molecular utilizam o DNA como foco de estudo e desenvolvimento de marcadores. Marcadores ligados a diferentes características de plantas têm sido desenvolvidos e permitem a seleção indireta de fatores desejáveis em gerações segregantes precoces (CAIXETA et al. 2016). No melhoramento de plantas é crescente o número de trabalhos a utilizar a manipulação assistida por marcadores moleculares, visando maior eficácia na transferência de genes.

De acordo com Ferreira e Grattapaglia (1998) um marcador molecular é qualquer fenótipo molecular oriundo de um gene expresso ou de um segmento específico de DNA. Quando este marcador apresenta-se na progênie de acordo com as leis básicas de herança mendeliana pode ser também denominado “marcador genético”.

Os marcadores genético-moleculares podem ser associados a uma infinidade de informações sobre o organismo estudado. Essas informações podem ser utilizadas em estratégias de conservação de recursos genéticos, complementação de informações ecológicas,

direcionamento do enriquecimento da base genética, análise da diversidade e pureza genética dentro de uma mesma espécie, facilitação do planejamento de cruzamentos e para seleção de genótipos com características desejadas em programas de melhoramento genético (FALEIRO, 2007).

A Seleção Assistida por Marcadores (SAM) é comumente utilizada em importantes empresas de melhoramento de sementes, com o objetivo de acelerar e otimizar o processo de desenvolvimento de cultivares. Através da SAM, a seleção de uma característica de interesse ocorre de forma indireta, por meio de marcadores moleculares ligados à característica. O método é indicado, principalmente nas seguintes situações: quando a seleção fenotípica é mais cara ou demorada; quando a expressão dos genes que controlam o caráter requer condições específicas que não podem ser reproduzidas; quando a hereditariedade da característica é baixa e a seleção fenotípica é, dessa forma, pouco eficiente; e quando se deseja selecionar várias características ou vários genes simultaneamente (SAKIYAMA et al., 2014).

De acordo com Caixeta (et al. 2016) as principais vantagens dos marcadores moleculares sobre as observações fenotípicas encontram-se na possibilidade de comparar materiais genéticos amostrados em diferentes ambientes, tipos de tecido ou estágio de desenvolvimento; e ainda detectar polimorfismos de DNA em todo o genoma.

Diversas técnicas moleculares estão disponíveis atualmente para detectar polimorfismos no DNA. A escolha do método a ser utilizado depende do objetivo do estudo, da infraestrutura disponível, dos recursos financeiros, da disponibilidade de recursos humanos com treinamento apropriado e do nível de conhecimento da genética molecular da espécie a ser estudada (FALEIRO, 2007).

Os primeiros marcadores genéticos baseados em DNA foram os marcadores RFLP (*Restriction Fragment Lenght Polymorphism* – Polimorfismo de restrição de DNA) descritos por Grodzicker e colaboradores (1974). Estes marcadores surgiram logo após a descoberta das enzimas de restrição por Linn e Arber (1968) e Meselson e Yuan (1968). Os polimorfismos detectados através desta técnica decorrem da criação ou eliminação de sítios de restrição, devido à mutação ou rearranjo dos segmentos de DNA através de deleções, inserções, inversões ou translocações ocorridas nas fitas de DNA (CAIXETA et al. 2016). Através deles foram construídos os primeiros mapas de ligação (HELENTJARIS et al., 1986) e identificado o primeiro QTL (*Quantitative trait loci* – Locos controladores de características quantitativas) (EDWARDS et al., 1987; PETERSON et al., 1988). Porém, o seu elevado custo, dificuldade de manter instalações apropriadas para utilização e descarte do material radioativo e o tempo

gasto para obtenção destes marcadores limitaram o seu uso (FERREIRA; GRATTAPAGLIA, 1998)

Posteriormente à descoberta da reação em cadeia da polimerase (PCR), que possibilitou a visualização de seguimentos específicos do DNA após a sua amplificação, houve o surgimento de uma nova geração de marcadores moleculares. O primeiro a ser publicado baseando-se nesta técnica foi o RAPD (*Randomly Amplified Polymorphic DNA*; DNA Polimórfico Amplificado ao Acaso); desenvolvido por dois grupos de pesquisadores que trabalharam de maneira independente (WILLIAMS et al., 1990; WELSH; MCCLELLAND, 1990). Nesta técnica são amplificados seguimentos de DNA distribuídos ao acaso no genoma, sem a necessidade do conhecimento prévio da sequência. A detecção dos produtos da amplificação é realizada em gel de agarose (CAIXETA et al. 2016). Por se tratar de um marcador dominante, sua principal limitação está no baixo conteúdo informativo por loco (FERREIRA; GRATTAPAGLIA, 1998).

O AFLP (*Amplified Fragment Lenght Polymorphism* – Polimorfismo de Comprimento de Fragmentos Amplificados) foi proposto por Vos e colaboradores em 1995. Esta técnica associa as metodologias exploradas pelos métodos RFLP e RAPD, combinando a distribuição aleatória de sítios de restrição entre genomas e à amplificação aleatória de fragmentos empregando-se primers de sequencias arbitrarias. Sua característica principal é a capacidade de indicar simultaneamente diversas regiões diferentes distribuídas de maneira aleatória no genoma (MULLER; WOLFENBARGER, 1999). É utilizado principalmente para construção de mapas de ligação e clonagem de genes de interesse. A grade desvantagem do método consiste na dificuldade de identificar variantes alélicos para um loco específico, o que tem levado à utilização da técnica, como marcador dominante (CAIXETA et al. 2016).

Outra técnica baseada em PCR bastante utilizada é a SSR (Simple Sequence Repeat – Repetições de Sequências Simples), também conhecida como marcadores microssatélites.

Marcadores Microssatélites

Os marcadores microssatélites são compostos de sequências simples repetidas de dois a cinco nucleotídeos em *tandem* na sequência de DNA, tais como (CA)n, (AGAT)n e (ATT)n. O polimorfismo nestes locos é resultado de variações do número de repetições das sequências de nucleotídeos (AKKAYA et al., 1992).

Cada microssatélite constitui um loco genético altamente variável, multialélico e de grande capacidade informativa. Ao ser individualmente amplificado, utilizando-se um par de primers complementar às sequências que o flanqueiam, podem ser observadas diversas bandas com tamanhos diferentes. A variação no tamanho das bandas geradas a partir da PCR e visualizadas após a eletroforese ocorre devido a diferentes números de unidades repetitivas dentro da estrutura do microssatélite em cada amostra de DNA. A variação no número de repetições pode ocorrer devido ao *crossing-over* desigual, erro da DNA polimerase durante a replicação (*slippage*) ou retrotransposição (KALIA et al., 2011).

Os microssatélites podem estar presentes em todas as regiões do genoma, sejam codantes ou não codantes. A maioria destas regiões repetitivas é encontrada no DNA, mas também podem ser observadas nos cloroplastos e mitocôndrias. Estas regiões podem ser divididas em quatro grupos distintos: (1) repetições perfeitas, quando não há interrupções; (2) repetições imperfeitas, quando há bases não repetidas interrompendo a sequência; (3) repetições compostas, quando duas ou mais classes de microssatélites estão dispostas de maneira adjacente; e (4) repetição simples, quando o microssatélite é formado por apenas uma classe de repetição (CAIXETA et al. 2016).

A obtenção dos marcadores é realizada pela amplificação do DNA via PCR, com o uso de primers específicos (desenvolvidos para a própria espécie a ser analisada, ou de espécies correlacionadas) para as regiões que flanqueiam os microssatélites. Os primers são desenhados a partir do sequenciamento de fragmentos de DNA, obtido de bibliotecas genômicas, onde os microssatélites foram previamente localizados. Após a amplificação, a separação ocorre através da técnica de eletroforese em géis de poliacrilamida ou agarose e a identificação do polimorfismo pode ocorrer por auto-radiografia, coloração com prata ou fluorescência (géis de poliacrilamida), por coloração com brometo de etídio sob luz ultravioleta (FALEIRO, 2007), ou ainda por meio do sequenciamento do DNA (ZEINALABEDINI et al.; 2014).

A natureza altamente informativa, aliada à alta especificidade e rapidez da tecnologia de PCR, faz dos marcadores microssatélites uma importante ferramenta para estudos de genomas eucarióticos. Em alface os primeiros locos microssatélites foram obtidos por Van de Wiel (et al.,1999). Neste estudo, os autores avaliaram 28 pares de primers, dos quais 26 apresentaram polimorfismos perante seis variedades de alface testadas, apresentando em média 3,5 alelos por loco. Observou-se ainda que praticamente todos os conjuntos de primers com amplificação de microssatélites produziram alelos no parente mais próximo da alface,

Lactuca serriola, mas apenas metade dos conjuntos de primers produziu alelos nas espécies mais distantes *L. saligna* e *L. virosa*.

Rauscher e Simko (2013) desenvolveram 97 marcadores microssatélites e realizaram testes em 36 acessos de *Lactuca* (sendo 33 de *Lactuca sativa*, 1 de *Lactuca serriola*, 1 de *Lactuca saligna* e 1 de *Lactuca virosa*). Testes revelaram que qualquer combinação de 32 marcadores foi capaz de distinguir os genótipos de todos os 36 acessos.

Hong (et al. 2015) obteve variabilidade suficientemente capaz de diferenciar 92 cultivares de alface a partir de 58 pares de primers. Foram observados entre dois e oito alelos por loco, obtendo uma média de três alelos por loco. O Conteúdo de Informação Polimórfica (PIC) observado foi de 0,425. Estes resultados comprovam que os marcadores utilizados poderiam ser usados para selecionar cultivares similares de maneira eficiente.

Zhang et al. (2016) identificaram trinta e quatro genes estruturais relacionados às principais vias de antocianina em alface, além de 12 genes considerados regulatórios dos teores de antocianina. Seu estudo também identificou através de análises *in silico* 3.607 marcadores microssatélites associados a estes genes.

Microssatélites associados a teores de β-caroteno já foram desenvolvidos e validados para batata doce (*Ipomoea batatas* L.) por Zhang e colaboradores (2016). Neste trabalho, foram desenvolvidos um total de 214 marcadores, sendo que 44 mostraram associação significativa com os teores de β-caroteno encontrados nos 239 genótipos de batata doce avaliados.

Radicais livres e mecanismos de proteção antioxidante

Um radical livre é uma molécula que possui um elétron desemparelhado isolado em um orbital, sendo altamente reativo, pois inicia reações em cadeia extraindo elétrons de moléculas em sua proximidade para completar seu próprio orbital (SMITH; MARKS; LIEBERMAN, 2007). Estas espécies reativas constituem três classes de compostos: espécies reativas de oxigênio (EROs), espécies reativas de enxofre (EREs) e espécies reativas de nitrogênio (ERNs) (MARTELLI; NUNES, 2014).

A geração de radicais livres faz parte de um processo contínuo em cumprimento de funções biológicas, possibilitando a geração de adenosina-trifosfato (ATP) por meio da cadeia transportadora de elétrons (BARBOSA et al., 2010). Sua produção pode ser ainda acentuada por fatores genéticos ou ambientais. Entre os fatores ambientais encontram-se o fumo, a

poluição, uso de medicamentos, a dieta, a radiação e o estresse (MORAES-DE-SOUZA, 2007).

As espécies reativas de oxigênio podem se apresentar como o radical ânion superóxido (O2•-), formado a partir da redução do oxigênio molecular por um elétron, o peróxido de hidrogênio (H2O2), gerado pela redução do oxigênio molecular por dois elétrons, e pelo radical hidroxil (OH•), proveniente da redução do oxigênio molecular por três elétrons (RIBEIRO et al., 2005).

A produção contínua de ERO durante os processos metabólicos levou ao desenvolvimento de muitos mecanismos endógenos de defesa antioxidante, para limitar os níveis intracelulares e impedir a indução de danos. Os antioxidantes são agentes responsáveis pela inibição e redução das lesões causadas pelos radicais livres nas células (VASCONCELOS, 2015). Sies e Stahl (1995) definiram antioxidante como "qualquer substância que, presente em baixas concentrações quando comparada a do substrato oxidável, atrasa ou inibe a oxidação deste substrato de maneira eficaz".

Os compostos antioxidantes podem atuar de diferentes maneiras no organismo: 1) impedindo a formação de radicais livres, principalmente pela inibição das reações em cadeia com o cobre e o ferro; 2) interceptando os radicais livres gerados pelo metabolismo, impedindo, dessa forma que os tecidos sejam atingidos; 3) ou ainda reparando as lesões causadas (ALFIERI; LEUNG; GRACE, 1998).

O complexo de defesas antioxidantes é constituído por grupos de defesa enzimática e não enzimática, que operam de maneira colaborativa e regulada no organismo. Dentre os antioxidantes enzimáticos se encontram as enzimas superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT). O sistema de defesa não enzimático é constituído por glutationa, carotenoides (provitamina A), tocoferóis, vitamina C, e flavonoides, dentre outros (BIANCHI; ANTUNES, 1999; OROIAN; ESCRICHE, 2015).

Normalmente há um equilíbrio entre a produção de radicais livres e sua posterior neutralização pelos sistemas antioxidantes. Entretanto, quando há uma produção excessiva desses compostos ou uma deficiência dos sistemas antioxidantes, ocorre o estresse oxidativo; situação que é prejudicial aos componentes celulares e indivíduos de maneira geral. O estresse oxidativo provoca diversas lesões aos constituintes celulares, como a peroxidação dos lipídeos de membrana; oxidação de receptores hormonais e enzimas; além de lesões no material genético, oxidando de bases do DNA que podem culminar em processos mutagênicos e tumorais (MARTELLI; NUNES, 2014).

Visando evitar os efeitos deletérios causados pelo estresse oxidativo, tem sido crescente a busca por alimentos que apresentem propriedades antioxidantes. Estudos comprovam que algumas substâncias presentes na alface, como carotenoides (STAHL; SIES, 2013; VIGNOLINI, et al. 2018) e antocianinas (SKROVANKOVA et al. 2015; DIACONEASA, 2015), apresentam elevado potencial antioxidante. Esses compostos absorvem radicais livres e inibem o início da reação em cadeia ou interrompem a propagação de reações em cadeia das reações oxidativas promovidas pelos radicais (PODSEDEK, 2007).

Os carotenoides são compostos que apresentam ampla distribuição na natureza, possui diversas estruturas químicas e inúmeras funções. Apesar de serem micronutrientes, presentes em níveis muito baixos (microgramas por grama), os carotenoides estão entre os constituintes alimentícios de maior importância. São pigmentos naturais responsáveis pelas cores de amarelo a laranja ou vermelho de muitas frutas, hortaliças, gema de ovo, crustáceos cozidos e alguns peixes (RODRIGUEZ-AMAYA et al., 2008). Os principais carotenoides são o licopeno (SHAMI; MORAIS, 2004) e o β-caroteno (RICE-EVANS; MILLER; PAGANGA, 1996).

As antocianinas são compostos fenólicos pertencentes a classe dos flavonoides (ROBARDS et al., 1999). Esses compostos apresentam vasta distribuição na natureza e tem como principais funções: atração aos insetos, fotoproteção, modulação da fotoinibição, potencialização da fotossíntese, além de atuarem como antioxidantes (MALACRIDA; MOTTA, 2006). São responsáveis pelas cores azul, roxo e todas as tonalidades do vermelho encontradas em flores, frutos, folhas e caules de plantas (MARÇO; POPPI; SCARMINIO, 2008). Estudos indicam que esta substância bioativa retarda o envelhecimento, prolonga a vida das células, aumenta as defesas imunitárias, propicia uma melhor circulação sanguínea, e protege o organismo contra o acúmulo de lipídeos nas artérias (ROGEZ, 2000).

A importância de compostos naturais com capacidade antioxidante para a medicina preventiva vem sendo amplamente reconhecida nos últimos anos. Acredita-se que alguns tipos de câncer, doenças cardiovasculares e cerebrovasculares, bem como diabetes e doenças reumáticas sejam causados ou acelerados por estresse oxidativo (WEISBURGER & WILLIAMS, 2000). Desta maneira, a busca por compostos naturais com propriedades antioxidantes, tem aumentado consideravelmente.

PRINCÍPIOS DE ALGUNS MÉTODOS UTILIZADOS PARA DETERMINAR A ATIVIDADE ANTIOXIDANTE

O presente trabalho utiliza os métodos de captura dos radicais livres DPPH• e ABTS+•, redução do complexo de Fosfomolibdênio e quantificação dos compostos fenólicos por meio do reagente *Folin-Ciocalteau*. Essas são metodologias comumente empregadas na avaliação da atividade antioxidante de diversos compostos, principalmente os oriundos de fontes naturais.

Método de avaliação da atividade antioxidante pela captura do radical DPPH•

O procedimento que emprega o radical DPPH• é um ensaio químico utilizado para determinar a atividade antioxidante de uma substância com base na captura desse radical livre. Esse método é um dos mais utilizados, pois é considerado um teste rápido, prático, preciso, simples, econômico, com boa estabilidade e muito sensível (OLIVEIRA, 2015; SUCUPIRA et al., 2012). Inicialmente, os radicais livres de DPPH• possuem coloração roxa devido ao elétron livre que ele possui. Esta cor é perdida quando um radical de hidrogênio fornecido por um composto antioxidante entra em ressonância com a molécula de DPPH•, reduzindo-se assim a sua absorbância. (SANTOS et al., 2007). A redução do radical DPPH• é acompanhada pela diminuição da absorbância ao decorrer da reação (HUANG et al., 2005; BRAND-WILLIANS et al., 1995).

Método de avaliação da atividade antioxidante pela captura do radical ABTS+•

O método tem como princípio o sequestro do cátion radical ABTS+•, que necessita de preparo por vias químicas ou enzimáticas, apresentando uma cor azul esverdeado, por meio da mistura do ABTS com perssulfato de potássio (K2S2O8) que tem absorção máxima em 645,734 e 815 nm. Na presença de um antioxidante, o radical ABTS+• é reduzido a ABTS, provocando a perda de coloração da mistura. O ensaio é aplicado em estudos com extratos vegetais, compostos puros e antioxidantes lipossolúveis e hidrossolúveis (RE et al., 1999).

Método da redução do complexo de Fosfomolibdênio

A metodologia do complexo de fosfomolibdênio é simples e muito usada para avaliar a atividade antioxidante total de uma mistura de compostos, como os extratos vegetais e suas frações. Este método, elaborado por PRIETO et al., (1999), baseia-se na redução do molibdênio (VI) a molibdênio (V). Quando esse complexo reage com determinados compostos antioxidantes, ocorre a formação de um complexo entre fosfato/molibdênio (V) em pH ácido, o qual é determinado utilizando o espectrofotômetro. Esse complexo possui cor amarela, alterando-se a coloração do meio reacional para verde à medida que é reduzido (BALESTRIN, 2006), sendo mais intensa a cor verde quanto maior for o poder antioxidante da amostra (BORA et al., 2005).

Quantificação de compostos fenólicos utilizando o reagente de Folin-Ciocalteau

As metodologias existentes para análise de compostos fenólicos podem ser divididas em quantificação de compostos fenólicos totais, quantificação individual ou quantificação de um grupo ou classe de fenólicos (ANGELO; JORGE, 2007). Vários testes espectrofotométricos baseados em diferentes fundamentos foram desenvolvidos para análise de substâncias fenólicas, o mais utilizado recentemente tem sido o ensaio de redução do reagente Folin-Ciocalteau, devido a sua sensibilidade à redução (ANGELO; JORGE, 2007; SOUSA et al., 2007). O reagente Folin-Ciocalteau é constituído pelos ácidos fosfomolibídico e fosfotunguístico e nessas substâncias o tungstênio e o molibdênio apresentam um estado de oxidação +6. Quando em contato com substâncias fenólicas esses compostos são reduzidos a um estado de oxidação +5 exibindo coloração azulada característica que possibilita a quantificação do teor dos agentes redutores (SOUSA et al., 2007).

Capítulo II

IDENTIFICATION OF MICROSSATELLITE MARKERS CORRELATED TO ANTIOXIDANT ACTIVITY IN *Lactuca sativa* L.

Este capítulo foi escrito de acordo com as normas da revista Frontiers in Plant Science

IDENTIFICATION OF MICROSSATELLITE MARKERS CORRELATED TO ANTIOXIDANT ACTIVITY IN *Lactuca sativa* L.

Cíntia Raquel de Freitas[1], Joana D'Arc Mendes Vieira[3], Alexandre Henrique de Oliveira Mendes[2], Matheus de Souza Gomes[1], Marcos de Souza Gomes[2], Terezinha Aparecida Teixeira[1], Luiz Antônio Augusto Gomes[1]

[1] Laboratório de Genética Molecular, Instituto de Biotecnologia, Universidade Federal de Uberlândia, City Patos de Minas, State Minas Gerais, Country Brazil

[2] Laboratório de Bioquímica e Biologia Molecular, Instituto de Biotecnologia, Universidade Federal de Uberlândia, City Patos de Minas, State Minas Gerais, Country Brazil

[3] Departamento de Agricultura, Universidade Federal de Lavras, City Lavras, State Minas Gerais, Country Brazil.*

Correspondence:

Cíntia Raquel de Freitas

cintia.r.freitas@hotmail.com

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Abstract

Lettuce (*Lactuca sativa* L.) can be considered the main leafy vegetable consumed in Brazil. It is rich in minerals such as calcium and iron; vitamins such as A, B1, B2 and C; as well as carotenoids (provitamin A) and anthocyanins. These substances have antioxidant action acting to prevent aging and controlling oxidative stress. The present work had the objective of studying the genetic divergence between 12 lettuce cultivars (Gabriela, Luiza, Model, Optima, Raider Plus, Red Star, Salad Bowl, Silvana, Sophia, Thaís, Verônica and Darkland) in 10 pairs of microsatellite primers and to associate these results to the evaluation of the antioxidant activity of each cultivar performed by the DPPH • and ABTS + • free radical capture methods, reduction of the Phosphomolybdenum complex and quantification of the phenolic compounds by means of the *Folin-Ciocalteau* reagent. The methodology consisted of verification of the color of each cultivar, quantification of the antioxidant activity and genotyping of the samples. The antioxidant activity values obtained by all methods were correlated with the staining of the cultivars through Pearson's Correlation and Mantel's Test. Microsatellite loci were compared with the levels of antioxidant activity obtained for each cultivar using the ABTS + •, DPPH •, phosphomolybdenum complex reduction and total phenol content; and also with the coloring of the plants. The results showed that the cultivar Red Star (dark purple color) presented higher % AA in most of the tests performed. The loci KSL-37, KSL-137, KSL-245 showed a positive and statistically significant correlation with the "dark purple" coloration. SML-022 was positively related to the results obtained by the phosphomolybdenum complex reduction test. Thus, these markers have been shown to be good indicators of antioxidant activity, and may be used in future studies that seek to associate this characteristic with lettuce cultivars.

1 Introduction

Lettuce (*Lactuca sativa* L.) can be considered the main leafy vegetable consumed in Brazil (SALA, COSTA, 2012). It is present in the daily menu of the Brazilian, preferably in the form of salad and its consumption presents great nutritional importance.

Greenery is an abundant source of nutrients. It is rich in minerals such as calcium and iron; vitamins, such as B1, B2 and C (FERNANDES et al., 2002) in addition to presenting other phytochemical compounds. Among the phytochemicals present in lettuce, we highlight carotenoids (provitamin A) and anthocyanins (SILVA et al., 2018). These substances have an antioxidant action and act to prevent aging and control oxidative stresses related to the action of free radicals, which trigger chronic non-transmissible diseases such as atherosclerosis, coronary diseases and some neoplasms (FELTRIM et al., 2005).

Currently, there is a great diversity of lettuce cultivars displaying differences in the shapes, sizes and colors of the plants (SUINAGA et al., 2013). It can be classified into five main types based on the formation of head and leaf type: smooth cabbage, cabbage or American crisp, loose smooth, loose curly, loose purple, and Roman curly (HENZ; SUINAGA, 2009).

The differences in color are due to variations in the concentrations of plant pigments such as chlorophyll, carotenoids and anthocyanins (TAIZ, ZEIGER, 2004; VIEIRA et al., 2010). However, there are no studies in the literature that discuss the antioxidant capacity of different cultivars commercialized in Brazil, indicating lettuce cultivars that present higher concentrations of antioxidant compounds, to attend to the growing population that seeks to include healthy foods in their menu, as well as to the programs of genetic improvement of the species.

Aiming to associate genetic and phenotypic characteristics; commercial and nutritional aspects of interest, the manipulation assisted by molecular markers in genetic breeding programs has been widely used. This technique allows indirect selection for desirable traits in early segregating generations, reducing time and resources, as well as the possibility of detecting DNA polymorphisms throughout the genome of the plant (CAIXETA et al., 2016).

Molecular markers are used to verify genetic diversity within the same species by detecting variations in the genome (CAIXETA et al., 2016). Currently, there are a multitude of molecular markers available for use in research involving plants, among the most used are the microsatellite markers.

These are high polymorphism, are codominant, highly reproducible, require small amounts of DNA and present low cost with high resolution power. The high allelic diversity allows the detection of polymorphism in multiparent populations and in populations derived from hybrids of related genotypes, besides being effective in distinguishing closely related genotypes (CAIXETA et al., 2016), being a fundamental part of genetic pre-breeding (PEREIRA et al., 2016).

Microsatellite markers for lettuce have been developed since the 1990s. (VAN DE WIEL et al., 1999, SIMKO, 2009, RAUSCHER, SIMKO, 2013, HONG et al, 2015). And it has demonstrated in all the experiments extremely useful results in the distinction of lettuce cultivars, as well as in the selection of cultivars for breeding programs of the species.

The present work had as general objective to correlate the estimated antioxidant activity through four different methods to the microsatellite loci obtained from 10 pairs of primers observed in twelve lettuce cultivars. Concomitantly, it was aimed to relate the different colors presented by the cultivars to the percentages of antioxidant activity verified. The results found constitute a fundamental step for the improvement assisted by molecular markers, aiming to obtain cultivars with high antioxidant activity in the species Lactuca sativa L.

2 Materials and methods

2.1 Collection of samples

The seedlings of the lettuce cultivars used (Table 1) were obtained from sowing in trays of expanded polystyrene and were then transplanted in pots containing soil, organic matter and chemical fertilizer in the quantities suitable for lettuce cultivation. Irrigation was performed by drip irrigation. The seedlings were obtained and kept in the Department of Agriculture of the Federal University of Lavras, until the plants reached the commercial stage, when the samples were collected for the analyzes. The analyzes were carried out in the Biochemistry and Molecular Biology Laboratory and in the Laboratory of Molecular Genetics, both at the Biotechnology Institute of the Federal University of Uberlândia, Campus de Patos de Minas.

Samples of the leaves of each cultivar were collected by collecting about 40 grams of fresh leaves from each plant. Before the chemical and molecular analyzes the leaves were cleaned, which were washed with running water to remove the impurities. Afterwards, the leaves were placed in plastic bags and identified again to be stored in the freezer at -80 º C. After a minimum of 24 hours in the ultra freezer the leaves were dehydrated by freeze drying.

2.2 Evaluation of cultivar color and antioxidant activity

Cultivars with five different color patterns were used: light green, medium green, dark green, medium purple and dark purple. Each sample was classified according to the predominant color in its leaves, so that "1" represents the presence of a certain color and "0" represents absence.

The samples of the lettuce leaves after freeze-dried were ground manually to obtain smaller fragments. Subsequently, 0.05 g of the dried plant material was weighed into a beaker and 2.5 mL of the extraction solvent (ethanol) was added, shaking for 30 seconds. Soon after, using a funnel and a piece of cotton, the mixture was filtered in a new beaker. After that, serial dilutions of 20 $mg.mL^{-1}$, 10 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$ for use in the antioxidant activity tests were performed.

The tests to obtain the antioxidant activities of the cultivars were: evaluation of the capacity of radical sequestration DPPH • (LOPES-LUTZ et al 2008), ABTS + • (RE et al., 1999; GOMES et al. (SINGLETON and ROSSI, 1965). The results were expressed as percentages of antioxidant activity (% AA) presented by each cultivar and were compared with the value of the antioxidant activity of the butylhydroxytoluene (BHT) standard (for the phosphomolybdenum complex reduction test) and the gallic acid standard (for the quantification test of the total phenolic content). The analyzes were performed in duplicate of the triplicates, that is, for each of the three replicates of the 12 cultivars, two replicates were made in each concentration used.

2.3 Identification of microsatellite loci

The DNA of leaf samples from each lettuce cultivar was extracted separately according to the protocol of Doyle and Doyle (1990). After extraction, quantification and qualification, each individual's DNA was stored in a freezer at -20°C for use in all subsequent molecular analyzes.

Microsatellite markers published by Hong et al. (2015) were used: KSL 026, KSL 037, KSL 051, KSL 123, KSL 137, KSL 173, KSL 245, SML 001, SML 007 and SML 022 (Table 2). Reactions of DNA amplification and resolution reactions on polyacrylamide gels were performed. PCR amplification reactions had a final volume of 10 µL containing 5 ng of DNA, 0.2 µM of each primer, 0.25 mM dNTP, 1.5 mM MgCl2, 1x PCR buffer, 1 unit of the enzyme Taq DNA polymerase and 3 µl vaseline. The thermocycler programming for the amplifications consisted of: a) an initial cycle of 94 ° C for 4min, followed 40 cycles at 94 ° C for 30s, 55 ° C for 30s and 72 ° C for 45s and one final cycle at 72 ° C for 10min.

After the PCR reaction, DNA denaturation was performed. 4 µl of 98% Formamide denaturing buffer (10 mM EDTA, pH 8.0, 1 mg / mL Xylene Cyanol and 1 mg / mL Bromofhenol blue) was added to the sample, followed by complete denaturation at 94°C for 5 min in thermal cycler.

The amplification products were separated on 8% polyacrylamide gel [acrylamide / bisacrylamide (19: 1), 7.5 M urea and 5x TBE buffer], prepared on 16-well capacity sandwich glass plate. The glass plates were cleaned with the aid of a tissue paper soaked in ethanol. The large (gel adhesion) plate was cleaned with 550 µl of a solution containing 95% Ethanol + 0.5% Acetic Acid and 1 µl Bind silane. This solution was spread across the surface of the plate with the help of a tissue. The small plate (gel repulsion) was treated in the same manner with ethanol and Sigmacote.

8 µl of the denatured PCR reaction was applied to the 8% polyacrylamide gel, the electrophoresis run being carried out for a period of approximately 3 h with constant power of 60 W. Molecular weight marker Ladder 50 bp was loaded on the lateral ends of each gel. The gels were stained with silver nitrate, according to the procedure described by Creste et al. (2001).

The base pair (bp) size estimate for each allele was obtained by the regression-based reverse mobility of products of known size of the 50 bp molecular marker applied to the polyacrylamide gel. The molecular markers information was annotated for each cultivar evaluated, in the form of genotypic representation, given as a function of the number of alleles per locus.

2.4 Statistical analyzes

For the tests of DPPH • and ABTS + • analyzes of variance, followed by Test F, were performed to verify if there was a significant difference for the effect of the concentrations on the antioxidant activity of the 12 cultivars, as well as for differences between cultivars. For both tests, a completely randomized design was used in a factorial scheme (12 X 3), with 12 lettuce cultivars analyzed and three concentrations (20; 10 and 5 mg mL-1) with three replicates. The analyzes were performed using the Sisvar program (FERREIRA, 2011).

In relation to the tests Reduction of the Phosphomolybdenum complex and Quantification of the phenolic compounds, analyzes of variance were also performed, followed by the F test, to

verify if there was a significant difference between the samples, according to the application of each methodology used. For the tests cited above, a completely randomized design was used, with three replications. The analyzes were performed using the Sisvar program (FERREIRA, 2011). The values obtained for each cultivar, in the different concentrations, in both tests, were compared by the Skott-Knott averages test, 5%.

The antioxidant activity values obtained by all methods were also correlated with the color of the cultivars through Pearson's Correlation and Mantel's Test. To evaluate the dissimilarity between the cultivars, a Gower distance matrix was generated from which the grouping according to the UPGMA method (Unweighted pair group method using arithmetic averages) could be obtained with the aid of the Genes program (CRUZ, 2013).

After obtaining the molecular results the values of Polimorphic Information Content (PIC) were calculated for all evaluated loci. Microsatellite loci were compared with the levels of antioxidant activity obtained for each cultivar using the ABTS + •, DPPH •, phosphomolybdenum complex reduction and total phenol content; as well as the staining of the plants, through the Pearson correlation and Mantel test. A dissimilarity matrix was generated based on the codominant markers obtained, which was correlated, through the Mantel Test, to the matrix of dissimilarity based on the antioxidant activity and staining of the cultivars. All analyzes were performed using the Genes program (CRUZ, 2013).

3 Results

3.1 Correlation between cultivar staining and antioxidant activity

The results obtained for ABTS +, DPPH •, phosphomolybdenum complex reduction and quantification of total phenolic content can be observed in Supplementary Materials 1, 2, 3 and 4, respectively.

In the ABTS + and DPPH tests, there were significant differences between cultivars and between the concentrations used. It was possible to observe that, in most cultivars, there is an increasing relation between the antioxidant activity of the samples and the concentration of each extract, and as the concentration of the extract increases, an increase in antioxidant activity occurs simultaneously.

It was verified the correlation between the results of the tests of evaluation of the antioxidant activity, in all the concentrations and the coloring of the leaves of the cultivars. The results (Table 3) revealed that the "dark purple" color was positively related to the antioxidant activity assessed by the ABTS + • method at all concentrations tested, presenting a significance level of 1% according to the T test and the Mantel Test in the concentrations of 5 and 10 mg.mL-1, and 5% in both tests at the concentration of 20 mg.mL-1.

Another color that stood out for the oxidant activity and showed a significant correlation between the characteristics was the "average purple", having been associated positively with the total phenolic content at 1% of significance according to the Mantel Test. The "dark green" presented negative correlation at 5% of significance according to the Mantel Test, with the total phenolic content, revealing that in the presence of this color there was a decrease in the antioxidant activity evaluated by this test.

The results obtained in all tests of % AA together with the colors observed for each cultivar made it possible to obtain measures of dissimilarity through Gower distance. It can be verified that the greatest genetic distance between the cultivars evaluated occurs between Red Star and

Darkland (0.69), and the shortest distance (0,09) between Thaís and Verônica. The grouping (Figure 1) was generated using the unweighted pair group method using arithmetic averages (UPGMA). The cut made at the similarity level of 73% reveals the formation of three distinct groups. Group I agglomerates the cultivars Thaís, Verônica, Sophia, Salad Bowl, Raider Plus, Darkland, Optima and Silvana; represents all stains evaluated except "dark purple", and includes those cultivars that presented values of antioxidant activity below those present in groups II and III in some of the tests evaluated. Group I also showed the cultivars that presented the highest level of similarity among the evaluated ones, Thaís and Verônica, both presenting light green coloration and also associating the cultivars Raider Plus and Darkland, that presented dark green coloration and resulted in low antioxidant activity in comparison with the others, mainly in relation to the DPPH • and ABTS + • tests.

Group II presented the cultivars Luíza (dark green) and Model (light green) that stood out for the antioxidant activity revealed by the DPPH •, ABTS + • and Phosphomolibdenium tests. And the group III showed the cultivars Gabriela and Red Star, both dark purple and showed high antioxidant activity in all tests, especially the Red Star cultivar, which presented higher antioxidant activity values than the other cultivars in three tests (DPPH • , ABTS + and Phosphomolybdenum).

3.2 Microsatellite markers

The 10 microsatellite markers used in the genotyping of the 12 lettuce accesses showed 100% polymorphism, amplifying a total of 32 alleles ranging from 2 (KSL-26, KSL-137, KSL-245, SML-007, SML-022) to 6 (SML-001) alleles per locus, with a mean of 3.2. The mean value obtained for the PIC was 0.473, ranging from 0.141 (SML-007) to 0.726 (KSL-37). These results are available in Table 2.

The polymorphic loci revealed greater genetic distance between the cultivars Gabriela and Model (0.71) and the smaller between the cultivars Thais and Verônica (0.34), through the estimation of dissimilarity. The results based on the markers confirm what was revealed by the phenotypic evaluation, showing a greater distance between cultivars of dark purple and light green, and greater proximity between those with light green coloration.

3.3 Correlation between phenotypic and molecular characteristics

Dissimilarity matrices elaborated from the phenotypic and genotypic data were correlated through the Mantel Test and the Test T. A positive and statistically significant correlation was verified between the data at the 5% level of significance.

In order to correlate the microsatellite markers individually with the antioxidant activity tests in all concentrations, Pearson's correlation and the Mantel test were performed, the results of which can be observed in Table 4.

The analysis showed a positive and statistically significant correlation between the "dark green" cultivars and the KSL-26 and SML-001 loci. These markers, therefore, should not be used to identify lettuce cultivars with high antioxidant activity, since the accesses with this characteristic showed the smallest antioxidant activities in the different tests performed.

The KSL-37, KSL-137 and KSL-245 loci had a positive and statistically significant correlation with the "dark purple" color, presented by the cultivars Red Star and Gabriela. These cultivars showed high antioxidant activity, and thus the loci can be used in future studies to identify potential antioxidant activity in different lettuce cultivars.

KSL-137 also showed to be directly related to the antioxidant activity revealed by the DPPH test at all concentrations tested (5, 10 and 20 mg mL -1) and with Total Phenolic contents. KSL-245 is positively related to the results obtained for DPPH • (10 mg mL-1) and SML-022 with the phosphomolybdenum complex reduction test.

4 Discussion

4.1 Correlation between cultivar staining and antioxidant activity

Carotenoids are remarkable compounds because they have wide distribution in nature, diverse chemical structures and varied functions. Although micronutrients present at very low levels (micrograms per gram), carotenoids are among the most important food constituents. They are natural pigments responsible for the colors of yellow to orange or red of many fruits, vegetables, egg yolk, cooked crustaceans and some fish (RODRIGUEZ-AMAYA et al., 2008). Since large amounts of these pigments can be found in green leafy vegetables (OLSON, 1991). Tests performed by Maiani et al. (2009) show that lettuce, spinach and carrots are plants with considerable concentrations of β-carotene (870-2960, 3100-4810 and 4350-8840 µg 100g-1).

Mou (2005), evaluating 44 lettuce cultivars of different types, observed that there are discrepant differences between the β-carotene contents for these cultivars. Similarly, Cassetari et al. (2015) evaluated the genetic variability for chlorophyll and β-carotene contents in lettuce, verifying the correlation between these characteristics. The authors also observed a large difference in β-carotene levels among cultivars, especially for the "Salinas 88" cultivar, which obtained a high β-carotene value in its leaves.

The lettuce leaf color is also affected by the chlorophyll content, which governs the degree of green color and anthocyanin content, controlling the red color pattern (CASSETARI, 2015). According to Ryder (1999) a pair of complementary genes controls the presence or absence of anthocyanin (CcGg) and a multiple allele system controls the model and color distribution. If the dominant allele for each complementary gene is present, the red coloration will occur. Other combinations result in green coloration. The R allele gives a general distribution of red on the leaf surface; Rs allows red-spotting on the leaves and R = induces a reddish on the leaf margin.

Both compounds, β-carotene and anthocyanin, present antioxidant activity (STAHL; SIES, 2005; ROGEZ, 2000). Thus, it is possible to infer that the high antioxidant activity of the cultivars Red Star, Model and Gabriela, observed in the tests of antioxidant activity can be associated to the levels of β-carotene and / or anthocyanins present in the leaves of the plants, however, each cultivar presents , possibly a different allelic pattern that results in distinct colorations.

Another factor that may have contributed to the percentages of antioxidant activity presented in this study is the type of cultivar. Lettuces of the American and Roman type, which have leaves that overlap to different degrees, eventually forming a compact head in some American lettuce cultivars, may eventually present discrepant results, when sampling is made from younger leaves, more which are less exposed to light and therefore have lower levels of chlorophyll and carotenoids.

In the present study, the cultivar Red Star presented a high percentage of antioxidant activity in all tests performed. This fact may be directly associated with the leaf color of this cultivar

(dark purple), suggesting the presence of phytochemical antioxidants β-carotene and anthocyanins. However, the Darkland cultivar, despite dark green coloration and high β-carotene content (Mou et al., 2005) presented low antioxidant activity in all analyzes. Eventually, this may have happened due to the sampling, and some more internal leaves, with lighter green coloration, could be collected, presenting a lower concentration of these compounds. This explanation may also be valid for the American-type cultivar Raider Plus. Considering the work of Mou et al., 2005 and Cassetari et al., 2015, there is a discrepancy in β-carotene values for Salinas 88, which corroborates this assertion, since in the sampling, internal leaves were collected in the work of Mou et al., 2005 and external leaves in the work of Cassetari et al., 2015.

4.2 Microsatellite markers

PIC results reflect the discriminatory power of the markers because they considered the number of alleles per locus and the relative frequency of these alleles, being considered polymorphic, loci with values of PIC≥0.1 and highly polymorphic when PIC≥0, 7 (CRUZ, 2008). The maximum value of expected PIC for biallelic markers is 0.5, being considered more informative the primers that present values in a range between 0.45 and 0.5 (TATIKONDA et al., 2009). The mean value obtained for the PIC was 0.473, ranging from 0.141 (SML-007) to 0.726 (KSL-37). As the PIC provides an estimate of the discriminatory power of a marker (WEIR, 1996), it can be inferred that the markers used were efficient in distinguishing the hits.

Several studies describe the found values of PIC in analysis of microsatellite markers in lettuce. The result obtained in this work is above that published by Simko (2009) of 0.32 and Hong (et al. 2015) of 0.425, but is smaller than that found by Van de Wiel (1999) of 0.55 and by Rauscher and Simko (2013) of 0.56.

4.3 Correlation between phenotypic and molecular characteristics

In the case of lettuce, recent studies seek to develop microsatellite markers aiming to optimize the diversity analysis (HONG et al., 2013, HONG et al., 2015, WANG et al., 2017, ZHOU, 2019). However, few studies aim to associate characteristics of interest to the identified loci.

In coffee, Pereira (2015), in studies developed to add resistance to *Meloidogyne exigua*, verified, through polymorphic allele grouping analyzes, the formation of three distinct groups, which separated as the reaction to the nematoid. Allowing the identification of three markers linked to the resistance trait in F5 progenies.

Rosa (2015) identified a SNP molecular marker associated with the Rpp4 gene of resistance to soybean Asian rust. Asian soybean rust is the most destructive disease that attacks the crop and can cause losses of more than 80% in tropical and subtropical regions.

Brazilian wheat genotypes obtained validation of molecular markers for resistance to giberela carried out by Scherloski and collaborators (2015). Seven markers significantly associated to the resistance of wheat to giberela were validated, with alleles of resistance and susceptibility being identified.

Microsatellites associated with β-carotene contents have already been developed and validated for sweet potato (*Ipomoea batatas* L.) by Zhang et al. (2016). In this work, a total of 214 markers were developed, and 44 showed a significant association with the β-carotene levels found in the 239 sweet potato genotypes evaluated.

Zhang et al. (2016) identified thirty-four structural genes related to anthocyanin main pathways in lettuce, in addition to 12 genes considered to be regulatory anthocyanin levels. Their study also identified through in silico analyzes 3,607 microsatellite markers associated with these genes. The genes related to anthocyanin biosynthesis and SSRs detected, along with the results of the present work provide useful tools for lettuce breeding programs, aiming to potentiate the antioxidant activity of the vegetable.

Since in this study, the cultivar Red Star presented high values for the antioxidant activity in all the tests carried out, a fact that can be directly associated with the color of the leaves (dark purple) and with the presence of anthocyanin; and the KSL-37, KSL-137, KSL-245 and SML-022 loci proved to be good indicators of antioxidant activity, and studies could be used in studies that seek to associate this trait with lettuce cultivars.

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Table 1 - Cultivars of lettuces used in the experiment.

Name of cultivar	Type	Color
Gabriela	Red leaf	Dark Purple
Luiza	Romaine	Dark Green
Model	Green leaf	Light Green
Optima	Romaine	Medium Green
Raider Plus	Iceberg	Dark Green
Red Star	Red leaf	Dark Purple
Salad Bowl	Red leaf	Medium Purple
Silvana	Iceberg	Medium Green
Sophia	Romaine	Light Green
Thaís	Green leaf	Light Green
Verônica	Green leaf	Light Green
Darkland	Romaine	Dark Green

Table 2: Description of the markers used in this study and their respective PIC values

Number	Primer	Primer sequence (5'-3')		Allele	PIC
1	KSL-26	F: GGGCTTTCTCTCCTTTCCTTT	R: AATTTGGATCCTGTCGAGGG	2	0.345
2	KSL-37	F: TCTCTTGCTCCAATACCCGA	R: GTATCGGGCTCATGTCCCTT	5	0.726
3	KSL-51	F: CCCCTACCACCACCAAAGTC	R: TACCAAATGACATGCACCCC	3	0.535
4	KSL-123	F: ATTGTAACTTCTGCGGGCCT	R: GCCTCACATGTTCTTCCCCT	4	0.599
5	KSL-137	F: TTCTCTGAGCTTCACAAGAGGG	R:TCATCACCATCATCATTTCCC	2	0.373
6	KSL-173	F: ATAGTCACGACTCACGCCCA	R: CCATTTTCCTCTTTCTGCGA	4	0.687
7	KSL-245	F: CTTCACCTCCGGAATCCTGT	R: GAGGCACGACTGCCATTTAG	2	0.304
8	SML-001	F: CCATGGATCCTGTGTGAAGA	R: CACCATGTTCCACTTCCACTT	6	0.712
9	SML-007	F: ACACTTGCCGATTCCTTCAC	R: ACCCGTGTTGAAAATGGAGA	2	0.141
10	SML-022	F: GGGCCTCAAATCCTCTCTG	R: TGTTCTTCCCCTCTTTGGAA	2	0.304

Table 3: Correlation between antioxidant activity and staining of cultivars

	Tests					
Coloring	ABTS+• 5 mg.mL^{-1}	ABTS+• 10 mg.mL^{-1}	ABTS+• 20 mg.mL^{-1}	DPPH• 10 mg.mL^{-1}	DPPH• 20 mg.mL^{-1}	PHENOLICS
Dark green	ns	ns	ns	ns	ns	-0,57^{+}
Medium purple	ns	ns	ns	ns	ns	0,49^{++}
Dark purple	0,83^{**++}	0,87^{**++}	0,63^{*+}	0,39^{+}	0,55^{+}	0,47^{+}

** *: Significant at 1 and 5% probability by t test

++ +: Significant at 1 and 5% probability by the Mantel test based on 5000 simulations

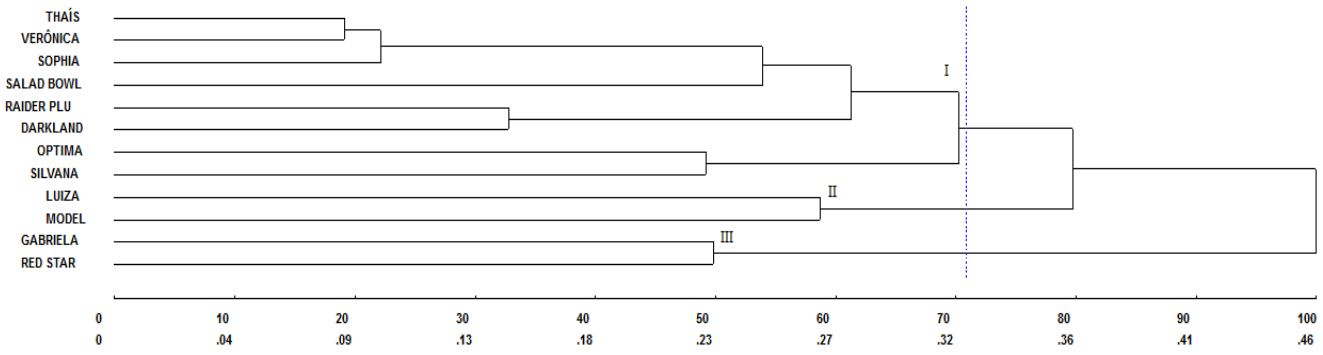
ns: not significant

Table 4: Correlation between phenotypic characteristics and microsatellite loci.

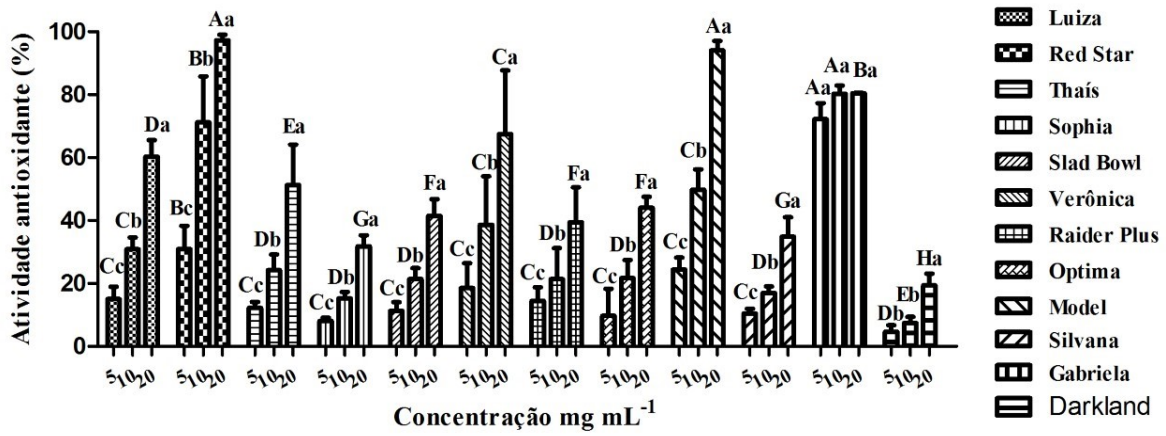
TESTS	KSL-26	KSL-37	KSL-137	KSL-245	SML-001	SML-022
DPPH• 5 mg.mL^{-1}	ns	ns	0.43^{+}	ns	ns	ns
DPPH• 10 mg mL^{-1}	ns	ns	0.48^{+}	0.48^{+}	ns	ns
DPPH• 20 mg.mL^{-1}	ns	ns	0.55^{+}	ns	ns	ns
RED. OF THE FOSFO COMPLEX. 20 mg.mL^{-1}	ns	ns	ns	ns	ns	0.63^{*+}
TOTAL PHENOLICS 20 mg.mL^{-1}	ns	ns	0.51^{+}	ns	ns	ns
COLORS						
DARK GREEN	0.41^{+}	ns	ns	ns	0.66^{*++}	ns
MEDIUM PURPLE	ns	ns	ns	ns	ns	ns
DARK PURPLE	ns	0.45^{+}	0.45^{++}	0.26^{+}	ns	ns

** *: Significant at 1 and 5% probability by t-test.
++ +: Significant at 1 and 5% probability by the Mantel test based on 5000 simulations;
ns: not significant.

Figure 1: Dendrogram obtained by the UPGMA method, based on the cultivar staining and antioxidant activity observed in the DPPH, ABTS, Phosphomolibdenium and Total Phenolic tests

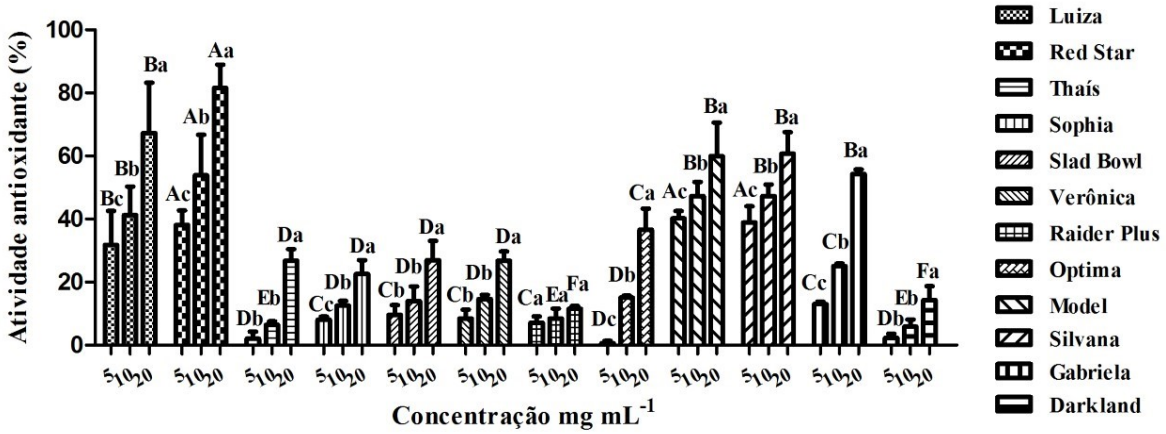


Supplementary Material 1



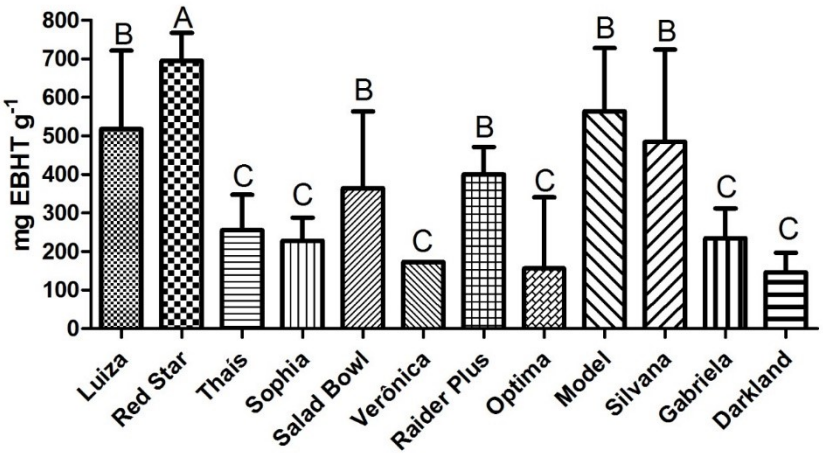
Supplementary Material 1: Evaluation of the antioxidant activity by means of the ABTS radical methodology. (5, 10 and 20 mg mL-1) of the cultivars (Luiza, Red Star, Thais, Salad Bowl, Veronica, Raider Plus, Optima, Model, Silvana, Gabriela and Darkland). Means followed by the same letter, upper case to compare the concentration between cultivars and lowercase to compare the concentration within each cultivar, did not differ significantly at 5% probability by the Scott-Knott test.

Supplementary Material 2



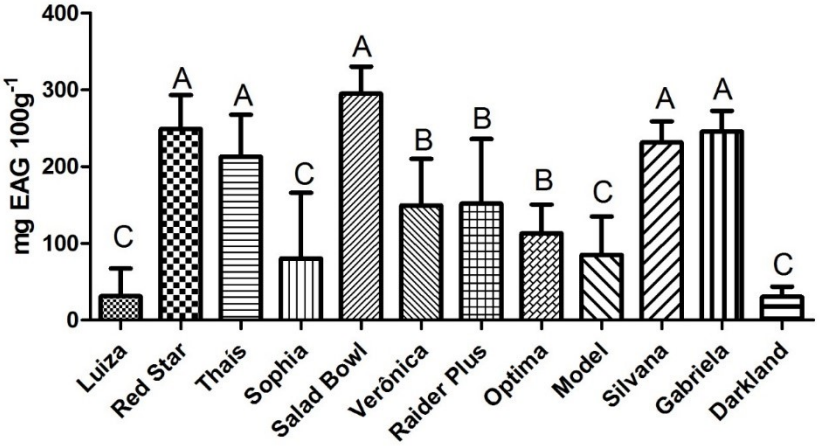
Supplementary Material 2: Evaluation of the antioxidant activity by means of the DPPH radical test. (5, 10 and 20 mg mL) of the cultivars (Luiza, Red Star, Thais, Salad Bowl, Veronica, Raider Plus, Optima, Model, Silvana, Gabriela and Darkland). Means followed by the same letter, upper case to compare the concentration between cultivars and lowercase to compare the concentration within each cultivar, did not differ significantly at 5% probability by the Scott-Knott test.

Supplementary Material 3



Supplementary Material 3: Evaluation of the antioxidant activity by means of the reduction method of the Phosphomolybdenum complex. Antioxidant activity of the varieties (Luiza, Red Star, Thais, Sophia, Salad Bowl, Veronica, Raider Plus, Optima, Model, Silvana, Gabriela and Darkland) expressed in mg EBHT g-1 fresh weight. Averages followed by the same letter, upper case to compare the concentration between the cultivars. They do not differ significantly at 5% probability by the Scott-Knott test.

Supplementary Material 4



Supplementary Material 4: Evaluation of antioxidant activity by quantification of total phenolic content by the Folin-Ciocalteau assay. Total phenolic content of the varieties (Luiza, Red Star, Thais, Sophia, Salad Bowl, Veronica, Raider Plus, Optima, Model, Silvana, Gabriela and Darkland) expressed in mg EAG 100g-1 fresh weight. Averages followed by the same letter, upper case to compare the concentration between the cultivars. They do not differ significantly at 5% probability by the Scott-Knott test.

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CONCLUSÃO

Com base nos diferentes métodos utilizados para avaliar a atividade antioxidante, pode-se concluir que dentre as cultivares de alface investigadas, a que mais se destacou foi a cultivar Red Star, apresentando para os métodos sequestro do radical DPPH•, sequestro do radical ABTS•+ e redução do complexo de Fosfomolibdênio altos valores para a atividade antioxidante. Isso pode estar diretamente associado com o fator coloração das folhas e com a presença da antocianina, já que a cultivar Red Star apresenta coloração roxa escura. Esta cor relacionou-se positivamente com a atividade antioxidante avaliada pelos métodos ABTS+• e DPPH•.

As cultivares que apresentam coloração "verde escura" apresentaram correlação negativa com o teor de fenólicos totais, revelando que na presença desta coloração houve uma diminuição na atividade antioxidante avaliada mediante este teste.

Os locos KSL-37, KSL-137 e KSL-245 apresentaram correlação positiva e estatisticamente significativa com a coloração "roxa escura". SML-022 relacionou-se positivamente com os resultados obtidos pelo teste de redução do complexo fosfomolibdênio. Desta maneira, estes marcadores revelaram-se bons indicadores de atividade antioxidante, podendo ser utilizados em estudos futuros que busquem associar esta característica às cultivares de alface.

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ANEXO

Anexo I: Author Guidelines "Frontiers in Plant Science"

1. Summary Table

Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript (max. length)	Peer review	Author fees	Submitted to PubMed Central or other indexing databases
Original Research	350 words	✔	15	12'000 words	✔	✔	✔
Review	350 words	✔	15	12'000 words	✔	✔	✔
Book Review	✘	✘	1	1'000 words	✔	✘	✔
Brief Research Report	250 words	✔	4	4'000 words	✔	✔	✔
Classification	250 words	✔	10	2'000 words	✔	✔	✔
Case Report	350 words	✔	4	3'000 words	✔	✔	✔
Clinical Study Protocol	350 words	✔	15	12'000 words	✔	✔	✔
Clinical Trial	350 words	✔	15	12'000 words	✔	✔	✔
Code	250 words	✔	3	3'000 words	✔	✔	✔
Community Case Study	350 words	✔	5	5'000 words	✔	✔	✔
Conceptual Analysis	350 words	✔	10	8'000 words	✔	✔	✔
CPC	250 words	✔	6	2'500 words	✔	✔	✔
Curriculum, Instruction, and Pedagogy	350 words	✔	5	5'000 words	✔	✔	✔
Data Report	✘	✔	2	3'000 words	✔	✔	✔
Editorial	✘	✘	0	1'000 words*	✔	✘	✔
Empirical Study	350 words	✔	10	8'000 words	✔	✔	✔
Evaluation	350 words	✔	5	6'000 words	✔	✔	✔
Field Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Focused Review [1]	350 words	✔	5	5'000 words	✔	✘	✔
Frontiers Commentary [2]	✘	✘	1	1'000 words	✔	✘	✔
General Commentary	✘	✘	1	1'000 words	✔	✔	✔
Hypothesis and Theory	350 words	✔	15	12'000 words	✔	✔	✔
Methods	350 words	✔	15	12'000 words	✔	✔	✔
Mini Review	250 words	✔	2	3'000 words	✔	✔	✔
Opinion	✘	✔	1	2'000 words	✔	✔	✔
Policy & Practice Reviews	350 words	✔	15	12'000 words	✔	✔	✔
Policy Briefs	125 words	✔	5	3'000 words	✔	✔	✔
Protocols	350 words	✔	15	12'000 words	✔	✔	✔
Perspective	250 words	✔	2	3'000 words	✔	✔	✔
Registered Report	350 words	✔	15	12,000 words	✔	✔	✔
Research Snapshot	50 words	✔	1	500 words	✔	✔	✔
Specialty Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Systematic Reviews	350 words	✔	15	12'000 words	✔	✔	✔
Technology Report	350 words	✔	15	12'000 words	✔	✔	✔

(1) Tier 2 article - field level article reserved to authors of selected Tier 1 articles.

* Editorials for Research Topics with 5 to 10 published articles have a maximum of 1'000 words, for Research Topics with more than 10 published articles the following applies: 1'100 words for 11 articles, 1'200 for 12 articles, 1'300 for 13 articles etc. up to maximum 5'000 words, for 50 or more papers.

Appendices and footnotes will be considered in the total length and word count of the article.

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2.3. Manuscript Requirements and Style Guide

2.3.1. General standards

Word Files
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2.3.1.5. Search Engine Optimization (SEO)

There are a few simple ways to maximize your article's discoverability. Follow the steps below to improve search results of your article:

Include a few of your article's keywords in the title of the article;
Do not use long article titles;
Pick 5 to 8 keywords using a mix of generic and more specific terms on the article subject(s);
Use the maximum amount of keywords in the first 2 sentences of the abstract;
Use some of the keywords in level 1 headings.

2.3.1.6. Title

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

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Authors should try to avoid, if possible:

Titles that are a mere question without giving the answer.
Unambitious titles, for example starting with "Towards", "A description of", "A characterization of", "Preliminary study on".
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For article types requiring it, the running title should be a maximum of 5 words in length. (see Summary Table)

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Except for special names (e.g. GABAergic), capitalize only the first letter of headings and subheadings. Headings and subheadings need to be defined in Times New Roman, 12, bold. You may insert up to 5 heading levels into your manuscript (not more than for example: 3.2.2.1.2 Heading title).

2.3.1.10. Abstract

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references. The text of the abstract section should be in 12 point normal Times New Roman. See Summary Table for abstract requirement and length according to article type.

For Clinical Trial article types, please include the Unique Identifier and the URL of the publicly accessible website on which the trial is registered.

2.3.1.11. Keywords

All article types: you may provide up to 8 keywords; at least 5 are mandatory.

2.3.1.12. Text

The entire document should be single-spaced and must contain page and line numbers in order to facilitate the review process. Your manuscript should be written using either LaTeX or MS-Word.

Templates are available (see above)

2.3.1.13. Nomenclature

The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the Acknowledgments.
Equations should be inserted in editable format from the equation editor.
Italicize Gene symbols and use the approved gene nomenclature where it is available. For human genes, please refer to the HUGO Gene Nomenclature Committee (HGNC). New gene

symbols should be submitted here. Common Alternative gene aliases may also be reported, but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here. Protein products are not italicized.
We encourage the use of Standard International Units in all manuscripts.
Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the recommendations by IUPAC.
Astronomical objects should be referred to using the nomenclature given by the International Astronomical Union provided here.
Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format:
urn:lsid:::[:]
For more information on LSIDs please see Inclusion of Zoological Nomenclature section.

2.3.1.14. Sections

Your manuscript is organized by headings and subheadings. For Original Research Articles, Clinical Trial Articles, and Technology Reports the section headings should be those appropriate for your field and the research itself.

For Original Research Articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field:

Introduction
Succinct, with no subheadings.

Materials and Methods
This section may be divided by subheadings. This section should contain sufficient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval statement should be included in this section (for further information, see section Materials and Data Policies)

Results
This section may be divided by subheadings. Footnotes should not be used and have to be transferred into the main text.

Discussion
This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior art related to the subject so to place the novelty of the discovery in the appropriate context; discuss the potential short-comings and limitations on their interpretations; discuss their integration into the current understanding of the problem and how this advances the current views; speculate on the future direction of the research and freely postulate theories that could be tested in the future.

For further information, please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Case Reports and Data Reports amongst others or you can check the descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page.

2.3.1.15. Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

2.3.1.16. Author Contributions Statement

The Author Contributions Statement is mandatory and should represent all the authors. It can be up to several sentences long and should briefly describe the tasks of individual authors. Please list only 2 initials for each author, without full stops, but separated by commas (e.g. JC, JS). In the case of two authors with the same initials, please use their middle initial to differentiate between them (e.g. REW, RSW). The Author Contributions Statement should be included at the end of the manuscript before the References.

2.3.1.17. Conflict of Interest Statement

A Conflict of Interest Statement needs to be included at the end of the manuscript before the references. Here, the authors need to declare whether or not the submitted work was carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest. For more information on conflicts of interest, see our Editorial Policies.

2.3.1.18. Contribution to the Field Statement

When you submit your manuscript, you will be required to briefly summarize in 200 words your manuscript's contribution to, and position in, the existing literature of your field. This should be written avoiding any technical language or non-standard acronyms. The aim should be to convey the meaning and importance of this research to a non-expert. While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your statement should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings - whether positive or negative - contribute to progress in your research discipline. This will assist the Chief Editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed statement will also facilitate the identification of the Editors and Reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

Example Statement on: Markram K and Markram H (2010) The Intense World Theory – a unifying theory of the neurobiology of autism. Front. Hum. Neurosci. 4:224. doi: 10.3389/fnhum.2010.00224

Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics,

toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

2.3.2. References

All citations in the text, figures or tables must be in the reference list and vice-versa. The references should only include articles that are published or accepted. Data sets that have been deposited to an online repository should be included in the reference list, include the version and unique identifier when available. For accepted but unpublished works use "in press" instead of page numbers. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for the article types that allow such inclusions. Personal communications should be documented by a letter of permission. Website urls should be included as footnotes. Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source. Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal publication for the same preprint exists, the official journal publication is the preferred source.

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

SCIENCE, ENGINEERING, and HUMANITIES: For articles submitted in the domains of SCIENCE, ENGINEERING and HUMANITIES please apply Author-Year system for in-text citations.
Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be called according to the surname of the first author, followed by the year. For works by 2 authors include both surnames, followed by the year. For works by more than 2 authors include only the surname of the first author, followed by et al., followed by the year. For Humanities and Social Sciences articles please include page numbers in the in-text citations.

Article in a print journal:
Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal:
Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:
Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book:
Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract:
Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent:
Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data:
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:
Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint:
Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style.

Frontiers Science Endnote Style

Frontiers Science, Engineering and Humanities Bibstyle

HEALTH, PHYSICS AND MATHEMATICS: For articles submitted in the domain of HEALTH or the journal Frontiers in Physics and Frontiers in Applied Mathematics and Statistics please apply the Vancouver system for in-text citations.
Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis for Health articles, and in square brackets for Physics and Mathematics articles.

Reference examples

Article in a print journal:

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.
Article in an online journal:
Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:
Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book:
Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract:
Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Patent:
Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data:
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:
Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master’s thesis]. [Chicago (IL)]: University of Chicago

Preprint:
Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to Citing Medicine.

Frontiers Health Endnote Style

Frontiers Health and Physics Bibstyle

2.3.3. Disclaimer

Any necessary disclaimers which must be included in the published article should be clearly indicated in the manuscript.

2.3.4. Supplementary Material

Frontiers journals do not support pushing important results and information into supplementary sections. However, data that are not of primary importance to the text, or which cannot be included in the article because it is too large or the current format does not permit it (such as movies, raw data traces, power point presentations, etc.) can be uploaded during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to FigShare for permanent storage, during the publication stage of the article, and receive a DOI.

The Supplementary Material can be uploaded as Data Sheet (word, excel, csv, cdx, fasta, pdf or zip files), Presentation (power point, pdf or zip files), Supplementary Image (cdx, eps, jpeg, pdf, png or tif), Supplementary Table (word, excel, csv or pdf), Audio (mp3, wav or wma) or Video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv).

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files. For Supplementary Material templates (LaTex and Word) see Supplementary Material for Frontiers.

Suggested Fonts

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

Headings and subheadings need to be defined in Times New Roman, 12, bold.

The text of the abstract section should be in 12 point normal Times New Roman.

The body text is in 12 point normal Times New Roman.

2.3.5. File Requirements

For Latex Files, when submitting your article please ensure to upload all relevant manuscript files including:

tex file
PDF
.bib file (if the bibliography is not already included in the .tex file)
Figures should be included in the provided pdf. In case of acceptance, our Production Office might require high resolution files of the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file, and upload them as ‘Supplementary Material Presentation’.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using 'Track Changes'. Editors and Reviewers can only download the PDF file of the submitted manuscript .

2.3.6. Additional Requirements per article types

2.3.6.1. CrossMark Policy

CrossMark is a multi-publisher initiative to provide a standard way for readers to locate the current version of a piece of content. By applying the CrossMark logo Frontiers is committing to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the complete citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response: Commentary: Title of original article".

2.3.6.3. Book Reviews

The title of a book review needs to follow the format "Book Review: Title of book". For book Reviews, you must also provide the full book details at the beginning of the article in this format: "Book Review: Full book reference"

2.3.6.4. Focused Reviews

For Tier 2 invited Focused Reviews, to shape the paper on the importance of the research to the field, we recommend structuring the Review to discuss the paper's Introduction, Materials and Methods, Results and Discussion. In addition the authors must submit a short biography of the corresponding author(s). This short biography has a maximum of 600 characters, including spaces

A picture (5 x 5 cm, in *.tif or *.jpg, min 300 dpi) must be submitted along with the biography in the manuscript and separately during figure upload.

Focused Reviews highlight and explain key concepts of your work. Please highlight a minimum of four and a maximum of ten key concepts in bold in your manuscript and provide the definitions/explanations at the end of your manuscript under "Key Concepts". Each definition has a maximum of 400 characters, including spaces.

2.3.6.5 Systematic Reviews

For Systematic Reviews, the following article structure applies.

Title: include systematic review/meta-synthesis/meta-analysis as appropriate in the title
Each of the sections should include specific sub-sections as follows

Abstract

Background
Methods
Results
Conclusions
Introduction

Rationale
Objectives
Research question
Methods

Study design
Participants, interventions, comparators
Systematic review protocol
Search strategy
Data sources, studies sections and data extraction
Data analysis
Results

Provide a flow diagram of the studies retrieved for the review
Study selection and characteristics
Synthesized findings
Risk of bias
Discussion

Summary of main findings
Limitations
Conclusions

2.3.6.6. Data Reports

For Data Reports, please make sure to follow these additional specific guidelines.

1. The data sets (defined as a collection of data that contains individual data units organized in a standardized reusable format, including pre-processed or raw data) must be deposited in a public repository for long-term data preservation prior to submission of the Data Report. The data set(s) is to be fixed and made publicly available upon publication of the Data Report.

2. Our data sharing policy also requires that the dataset be made available to the Frontiers editors and reviewers during the review process of the manuscript. Prior to submission of your Data Report manuscript, please ensure that the repository you have selected supports confidential peer-review. If it does not, we recommend that the authors deposit the datasets to figshare or Dryad Digital Repository for the peer-review process. The data set(s) can then be

transferred to another relevant repository before final publication, should the article be accepted for publication at Frontiers.

Note that it is the authors' responsibility to maintain the data sets after publication of the Data Report. Any published Frontiers Data Report article will be considered for retraction should the data be removed from the final selected repository after publication or the access become restricted.

3. The submitted manuscript must include the following details:

Detailed statement of contribution of the data report to the field
Name of the data set
Name of the database/repository where the data set has been submitted
Link to the data set for confidential peer-review (which can be updated after acceptance, prior to publication once the data is made public)
Description of how the data was acquired, data collection period
Filters applied to the data
Overview of the data files and their formats
Reference to and/or description of the protocols or methods used to collect the data
Information on how readers may interpret the data set and reuse the data
All these elements will be peer-reviewed and are required for the publication of the Data Report.

Any future updates to the data set(s) should be deposited as independent versions in a repository and the relevant information may be published as General Commentaries linked on the Frontiers website to the initial Data Report.

Any detailed analyses or new scientific insights relating to the Data Report can be submitted as independent research articles which can also be linked on the Frontiers website to the Data Report article. The protocols and methodology used to collect the data can also be submitted as Methods articles.

2.3.6.7. Case Reports

Case Reports should include the following:

Background

Case Presentation

For human patients: age, sex and occupation of the patient, presenting symptoms, the patient's history and any relevant family or social history, and relevant clinical findings

For animal patients: age, sex, and breed of the animal, presenting problems, the animal's history, and relevant clinical findings.

Description of laboratory investigations and diagnostic tests.

Discussion of the underlying pathophysiology and the novelty or significance of the case. Authors are required to obtain written informed consent from the patients (or their legal representatives) for the publication.

2.3.6.8. Policy & Practice Reviews

For Policy and Practice Reviews, the following article structure applies:

Abstract
Introduction
Sections on assessment of policy/guidelines options and implications
Actionable Recommendations and Conclusions

2.3.6.9. Policy Briefs

For Policy Briefs, the following article structure applies:

Abstract (bullet point format)
Introduction
Sections on Policy Options and Implications
Section on Actionable Recommendations
Conclusions

2.3.6.10. Protocols

For Protocols articles, please make sure to follow these additional specific guidelines.

The submitted manuscript must include the following sections:

An Abstract.

An Introduction outlining the protocol and summarizing its possible applications.

A Materials and Equipment section providing a list of reagents or other materials and/or equipment required to carry out the protocol. For basic-science protocols, the formulation of any solutions, e.g. buffers, should be clearly indicated in the Materials and Equipment section.

A Stepwise Procedures section listing, stepwise, the stages of the protocol. The timing of each step or related series of steps should be indicated, as should points at which it is possible to pause or halt the procedure without adversely influencing the outcome. For steps requiring repeated measurements, details of precision and accuracy should be presented. Limits of detection or quantification should also be stipulated where appropriate.

An Anticipated Results section describing, and illustrating with figures, where possible, the expected outcome of the protocol. Any analytical software or methods should be presented in detail in this section, as should possible pitfalls and artifacts of the procedure and any troubleshooting measures to counteract them. These last may also be described in an optional Notes section.

Code or training data sets referenced by the protocol and useful in its execution should be hosted in an online repository; their accession numbers or other stable identifiers should be referenced in the Anticipated Results.

The significance of the protocol and any advance represented by the method compared with other, similar methods should be presented in the contribution to the field statement accompanying your manuscript.

2.3.6.11. Code

The code should be novel and presented in human-readable format, adhere to the standard conventions of the language used (variable names, indentation, style and grammar), be well documented (comments in source), be provided with an example data set to show efficacy, be compilable or executable free of errors (stating configuration of system used).

The code should only call standard (freely accessible) libraries or include required libraries, and include a detailed description of the use-scenarios, expected outcomes from the code and known limitations of the code.

Please therefore make sure to provide access to the following upon submission:

Abstract explicitly including the language of code

Keywords including the language of the code in the following format:"code:language"" e.g.: "code:matlab"

Contribution to the field statement including the utility of the code and its language

Main Text including:

code description
application and utility of the code
link to an accessible online code repository where the most recent source code version is stored and curated (with an associated DOI for retrieval after review)
access to test data and readme files
methods used
example of use
known issues
licensing information (Open Source licenses recommended)
Compressed Archive (.zip) of the reviewed version of the code as supplementary material (.zip archives are currently available under the “Presentation” dropdown menu).

2.3.6.12. Registered Report

Registered Reports are empirical research articles outlining a proposed methodology and analyses which are peer-reviewed and pre-registered before data collection. Registered Reports should include an Introduction, Methods and preliminary results from any pilot experiments (if applicable). If the Registered Report is endorsed following peer-review and the research is conducted according to the approved methodology, the manuscript will be given In Principle Acceptance. Following data collection, the authors should submit a

complete manuscript containing the peer-reviewed sections included in the Registered Report, as well as the Results and Discussion sections. If the Results include unregistered analysis, these should be indicated separately as 'Exploratory Analysis'. Authors have 1 year after their registered report is accepted to submit a full manuscript. The format is appropriate for any hypothesis-driven research, including both original studies and replications.

Registered Reports have a maximum word count of 3,000 and may include 2 Figures/Tables. Following data collection, the completed version of the manuscript should follow the guidelines for an Original Research article with a maximum word count of 12,000. Registered Reports incur a A-type article fee, charged after the acceptance of the completed manuscript.

2.4. Figure and Table Guidelines

2.4.1. CC-BY Licence

All figures, tables, and images will be published under a Creative Commons CC-BY licence and permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.2. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

Permissions may be necessary in the following scenarios:

Republishing
Modifying/adapting
Partial Figures
It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.3. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.4. Figure and Table Requirements

Legends
Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image Size
Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

All articles are prepared using the 2 column layout: 2 column articles can contain images 85 mm or 180 mm wide.

2.4.5. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.

JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode
Images must be submitted in the color mode RGB.

Resolution Requirements
All images must be uploaded separately in the submission procedure and have a resolution of 300 dpi at final size. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Chemical Structures
Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use Frontiers ChemDraw Template, if working with another program please follow the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility
Figures must be legible. Check the following:

The smallest visible text is no less than 8 points in height, when viewed at actual size.
Solid lines are not broken up.
Image areas are not pixilated or stair stepped.
Text is legible and of high quality.
Any lines in the graphic are no smaller than 2 points width.

2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies, including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

2.6. Materials and Data Policies

Frontiers is committed to open science and open data, and we strongly encourage authors to maximize the availability of data included in their articles by making generated data publicly available where possible, and ensuring that published data sets are cited in accordance with our data citation guidelines. We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published articles.

Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the Transparency and Openness (TOP) guidelines, and the joint declaration of data citation principles produced by FORCE 11.

2.6.1. Availability of Materials

Authors are strongly encouraged to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

Resource Identification Initiative
Authors wishing to participate in the Resource Identification Initiative should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here.

2.6.2. Availability of Data

Frontiers requires that authors make all data relevant to the conclusions of the manuscript available to editors and reviewers during peer-review to enable complete and objective evaluation of the work described.

We strongly encourage authors to make the raw data supporting the conclusions of the manuscript available in publicly accessible repositories. To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability office at datapolicy@frontiersin.org prior to submission with any queries concerning data reporting.

2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the references list to maximize future usability. The following format should be used:

[Dataset] Author names. (year) Data Title. Repository name. Version. Persistant identifier

2.6.4. Data Availability Statements

Data availability statements are required for all manuscripts published with Frontiers. During the submission process, authors will be asked to detail the location of the raw data underlying the conclusions made in the manuscript, and whether it will be made available to other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analysed in the manuscript. These datasets should be cited in accordance with our data citation guidelines.

A statement will be automatically generated using the information provided in the submission form; however, manuscripts containing incomplete or incorrect statements will be prevented from entering the review process.

Examples of acceptable statements
Datasets are in a publicly accessible repository:

The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

Datasets are available on request:

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

All relevant data is contained within the manuscript:

All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

Restrictions apply to the datasets:

The datasets for this manuscript are not publicly available because: [VALID REASON]. Requests to access the datasets should be directed to [NAME, EMAIL].

Data has been obtained from a third party:

The data analyzed in this study was obtained from [SOURCE], the following licenses/restrictions apply [RESTRICTIONS]. Requests to access these datasets should be directed to [NAME, EMAIL].

No datasets were generated for this study

2.6.5. Recommended and Required Repositories

Authors are required to deposit the following data-types in public, community-supported repositories, such as those listed below, prior to publication of an associated Frontiers manuscript:

Data-type	Recommended Repositories	Metadata Standard
Genetic and genomic sequence (DNA/ RNA)^	GenBank DNA Data Bank of Japan (DDBJ) European Nucleotide Archive (ENA)	MiXS
Metagenomic sequence	EBI Metagenomics	MiXS
DNA and RNA trace or short-read sequencing data	NCBI Trace Archive NCBI Sequence Read Archive	MiXS
Genetic polymorphism data, including SNP and CNV data	dbSNP dbVar European Variation Archive DGVa	MiXS
Gene expression data; chromatin immunoprecipitation data (deep-sequencing or microarray)	ArrayExpress Gene Expression Omnibus (GEO)	MIAME / MINSEQE
Data linking genotype to phenotype	dbGaP	
Protein sequence data	UniProt	
Proteome profiling data	PRIDE PeptideAtlas ProteomeXchange	MIAPE
Small molecule, protein, protein complex data structural data	Crystallography Open Database Cambridge Structural Database wwPDB (Protein DataBank) Electron Microscopy Databank	CIF
Taxonomy data	Zoobank	

^ Genetic sequence variants should be annotated according to the guidelines established by the Human Variome Project.

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

Data-type	Recommended Repositories	Metadata Standard
Protein-protein interaction data	Database of Interacting Proteins (DIP)	MIMIx
Metabolite and metabolome profiling data	MetaboLights Human Metabolome Database	MSI
Small-molecule screening data, chemical compound data	PubChem	CIF
Flow cytometry data	Flow Repository	
Brain Imaging data / Neuroimaging data	OpenNeuro INDI NITRC NeuroVault [Statistical maps]	BIDS
Trait data	TRY database	
Phenology data	National Phenology Network	
Any data	FigShare Dryad Digital Repository	None

2.6.6. Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code, allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

2.6.7. Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) or European Nucleotide Archive (ENA). Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare or dryad.

2.6.8 Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyse data, false discovery rates (FDR) for large-scale studies and threshold or cut-off rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification,

miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

2.7. Statistics

Frontiers requires that all statements concerning quantitative differences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be sufficiently detailed to be reproduced.

3. Editorial Policies and Publication Ethics

Frontiers' ethical policies are a fundamental element of our commitment to the scholarly community. These policies apply to all the Frontiers in journal series. Frontiers has been a member of the Committee of Publication Ethics since January 2015 and follows COPE guidelines where applicable.

3.1. Authorship and Author Responsibilities

Frontiers follows the International Committee of Medical Journal Editors guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;
Drafting the work or revising it critically for important intellectual content;
Provide approval for publication of the content;

Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:

AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

The corresponding author takes primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The corresponding author should also be available post-publication to respond to any queries or critiques.

Requests to modify the authors list after submission should be made to the editorial office using the authorship changes form.

3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

Original content and duplicate publication: Frontiers only publishes original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.
Redundant publication: Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.
Fabrication and falsification: Frontiers opposes both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).
Plagiarism: Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see here.

We reserve the right to contact the affiliated institutions of authors, who have not acted according to good research and publication practices.

3.3. Translations

Frontiers accepts manuscript submissions that are exact translations of previously published work. This should be clearly stated in the manuscript upon submission. Permission from the original publisher and authors needs to be sought and also stated in the manuscript, and the relevant documents should be provided as supplementary data for verification by the Editor and the editorial office. The original work from which the manuscript has been translated should be clearly referenced.

"This is a (‘language’) language translation/reprint of (‘insert title here’) originally published in (‘insert name here’). (‘Insert name here’) prepared this translation with support from (insert name of funding source, if any). Permission was granted by (‘Insert name here’).”
Please note that Frontiers may request copies of related publications if there are any concerns about overlap or possible redundancy.

3.4. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with COPE guidelines, we expect that “original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations.” This condition also applies to an author’s own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author’s thesis so long as this is the only form in which it has appeared, is in line with the author’s university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list. For some examples, please check our in Manuscript Requirements and Style Guide at 2.3.1

Conferences, Proceedings and Abstracts

Manuscripts that first appeared as conference papers must be expanded upon if they are to be considered as original work. You are required to add a substantial amount of original content in the form of new raw material (experiments, data) or new treatment of old data sets which lead to original discussion and/or conclusions, providing value that significantly exceeds the original conference version. As a rule of thumb, at least 30% of content must be original. Authors submitting such work are required to:

- Seek permission for reuse of the published conference paper if the author does not hold the copyright (proof of permission should be submitted as supplementary material or sent to editorial.office@frontiersin.org with the manuscript ID upon submission).

- Cite the conference in the Acknowledgements section, or the references section if applicable.

Blogs

Although permissible, extended manuscript content which previously appeared online in non-academic media, e.g. blogs, should be declared at the time of submission in the acknowledgements section of the manuscript.

3.5. Image Manipulation

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (eg. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique must be declared in the methods section of the manuscript. Image grouping and splicing must be clearly stated in the manuscript and the figure text. Any concerns raised over undeclared image modifications will be investigated and the authors will be asked to provide the original images.

3.6. Conflicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional affiliations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment.

The Frontiers review system is designed to guarantee the most transparent and objective editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent.

Failure to declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

What Should I Disclose?

As an author, disclosure of any potential conflicts of interest should be done during the submission process. Consider the following questions and make sure you disclose any positive answers:

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work?
Do you have financial relationships with entities that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work?
Do you have any patents and copyrights, whether pending, issued, licensed and/or receiving royalties related to the research?
Do you have other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?
If you failed to disclose any of the potential conflicts of interest above during submission, or in case of doubt, please contact as soon as possible the Frontiers Editorial Office at editorial.office@frontiersin.org with the details of the potential conflicts.

Example statement: "Author xxx was employed by company xxxx. All other authors declare no competing interests."

The handling editors and reviewers will be asked to consider the following potential conflicts of interest before accepting any editing or review assignment:

FAMILY	1. Are any of the authors a spouse or significant other, a member of the same family or a very close personal friend? Review Editors should also not be a member of the same family as the handling editor.
COLLABORATIONS	2. Are you currently hosting or have hosted a Frontiers Research Topic with any of the authors within the past 2 years? Are you currently hosting a Frontiers Research Topic with the Editor? 3. Are you currently collaborating or have you collaborated on a research project or a publication with any of the authors within the past 2 years? 4. Are you currently collaborating or have you collaborated with any of the authors as an advisor or in any other direct supervisory capacity in the past five years? 5. Are you currently collaborating or have you collaborated with any of the authors as a student or in any other direct subordinate capacity in the past five years? Note: Review Editors should not accept assignments if they have a close professional relationship with the handling editor, which in their view could affect the objectivity of the review.
AFFILIATION	6. Are you affiliated with the same institution as the editor? Are you affiliated with the same institution as any of the authors? If so, has this resulted in interactions, collaborations, or mutual interests with the authors that would compromise your impartiality in conducting this review? 7. Are you a current member of a committee or department that coincides with an affiliation with the editor or any of the authors?
FINANCIAL	8. Do you have a business or professional partnership with any author? 9. Do you have financial interests or business relations with any organization involved in this research or in the preparation of the manuscript? 10. Do you have any financial interest or competing interests in the content of the manuscript that might affect your ability to perform an objective review?

3.7. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

3.7.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations. Frontiers follows International Association of Veterinary Editors guidelines for publication of studies including animal research. Approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the

ethics committee that approved the study. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research.

Humane Endpoints
All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

3.7.2. Studies involving human subjects

Research involving human subjects is expected to have been conducted in accordance with the World Medical Association's Declaration of Helsinki. Studies involving human participants must be performed in accordance with relevant institutional and national guidelines, with the appropriate institutional ethics committee's prior approval and informed written consent from all human subjects involved in the study including for publication of the results. Conformation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work and confirm that study subjects (or when appropriate, parent or guardian) have given written informed consent. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee]. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. In order to protect subject anonymity, identifying information should not be included in the manuscript unless such information is absolutely necessary for scientific purposes AND explicit approval has been granted by the subjects.

3.7.3. Inclusion of identifiable human data

Frontiers follows the ICMJE recommendations on the protection of research participants, which state that patients have a right to privacy that should not be violated without informed consent. We require non-essential identifiable details to be omitted from all manuscripts, and written informed consent will be required if there is any doubt that anonymity can be maintained.

It is the responsibility of the researchers and authors to ensure that these principles are complied with, including the obtaining of written, informed consent.

Written informed consent can be documented on a form provided by an institution or ethics committee, and it must clearly state how the identifiable data will be used. Frontiers also makes available its own form , which may be used for this purpose, but use of the Frontiers form is not required if a suitable alternative form of consent, meeting the ICMJE recommendations, is used. We consider it to be the author' duty to encourage participants or patients whose consent for publication is required to read and understand the ICMJE guidelines, for their information prior to completing the consent form. Participants should also be encouraged to ask any questions and to ensure they are comfortable before they sign the consent form.

The completed consent forms should be stored by authors or their respective institutions, in accordance with institutional policies. Frontiers does not need to view the completed form, and this should not be included with the submission. The completed form should be made available on request from the editor or editorial office, both during the review process and post-publication.

The determination of what constitutes identifiable data lies with our editors and editorial office staff, and manuscripts may be rejected if the required consent documents cannot be provided. Please note that written informed consent for publication is required for all case report articles where the patient or subject is identified or identifiable.

3.7.4. Clinical Trials

The World Health Organization defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." In accordance with the Clinical Trial Registration Statement from the International Committee of Medical Journal Editors (ICMEJ), all clinical trials must be registered in a public trials registry at or before the onset of participant enrolment. This requirement applies to all clinical trials that begin enrolment after July 1, 2005. To meet the requirements of the ICMJE, and Frontiers', clinical trials can be registered with any Primary Registry in the WHO Registry Network or an ICMJE approved registry.

Clinical trial reports should be compliant with the Consolidated Standards of Reporting Trials (CONSORT) both in terms of including a flow diagram presenting the enrolment, intervention allocation, follow-up, and data analysis with number of subjects for each and taking into account the CONSORT Checklist of items to include when reporting a randomized clinical trial.

The information on the clinical trial registration (Unique Identifier and URL) must be included in the abstract.

3.8. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a

Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed using the below template. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabeled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet authorship criteria has been included. The contribution to the field statement should be used to clearly state the reason for the Correction. Please note, a correction is not intended to replace the original manuscript.

The title of the submission should have the following format: "Corrigendum: Title of original article". It is advised to use the corrigendum Word and LaTeX templates.

If the error was introduced during the publishing process, the Frontiers Production Office should be contacted.

3.9. Retractions

As a member of the Committee on Publication Ethics (COPE), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

Retractions are not about punishing authors.
Retraction statements should be public and linked to the original, retracted article.
While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)
Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)
Major plagiarism
The reporting of unethical research, the publication of an article that did not have the required ethics committee approval
Legal issues pertaining to the content of the article e.g. libellous content
Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold (‘gift’) authorship
Politically-motivated articles where objectivity is a serious concern
The singling out of individuals or organizations for attack
Faith issues (e.g. intelligent design)
Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)
Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy here

3.10. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at support@frontiersin.org.

For any ethical concerns, please contact us at editorial.office@frontiersin.org.