Evaluation of antioxidant potential on fermented dairy beverages prepared with L*actobacillus acidophilus*: A systematic review

Avalição do potencial antioxidante em bebidas lácteas fermentadas, preparadas com L*actobacillus acidophilus:* Uma revisão sistemática

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ABSTRACT

Taking into account the physiopathology of diseases raised from free radical excess in the body (diabetes, hypertension, obesity, atherosclerosis, among others), some non-pharmacology alternatives has been developed, acting as therapeutic and prophylactic adjuvants, as the probiotic dairy drinks, for instance. In order to gather evidence on the antioxidant aspect about fermented milks in presence of a specific strain (in this case, *Lactobacillus acidophilus)* from *in vitro* experimental trials, this systematic review is developed from the following databases: Medline, Cochrane, Scopus, Science Direct, Scifinder, Web of Science, Scielo and Agricola. The search terms chosen are: "antioxidant activity", "oxidative stress", "Lactobacillus acidophilus", "lactic beverage",

"fermented milk", "yogurt", "in vitro techniques" and "in vivo", associated with the boolean operators "AND" and "OR". The articles which have not filled these conditions were discharged.
Through the search, 1751 articles were retrieved but 624 were discharged due to duplication and 36 had its content read in full. Up from these, eight articles were selected due to fulfillment of acceptance criteria for “in vitro” studies. From these, the composition of 17 samples were selected and used as data base to this research, which constituted three subgroups (A: fermented milk, B: acidophilic milk and C: yoghurt). Their antioxidant profile was deeply studied. Additionally, the DPPH test was the most used to antioxidant evaluation on these selected samples, however ABTS method seems to be preferably in in vitro tests.

Keywords: Yogurt, fermented milk, free radicals, oxidative stress, antioxidant, evidence-health based.

ABSTRACT
Tendo em conta a fisiopatologia das doenças levantadas pelo excesso de radicais livres no corpo (diabetes, hipertensão, obesidade, aterosclerose, entre outras), foram desenvolvidas algumas alternativas não farmacológicas, actuando como adjuvantes terapêuticos e profilácticos, como as bebidas lácteas probióticas, por exemplo. A fim de recolher provas sobre o aspecto antioxidante dos leites fermentados em presença de uma estirpe específica (neste caso, Lactobacillus acidophilus) a partir de ensaios experimentais in vitro, esta revisão sistemática é desenvolvida a partir das seguintes bases de dados: Medline, Cochrane, Scopus, Science Direct, Scifinder, Web of Science, Scielo e Agricola. Os termos de pesquisa escolhidos são: "actividade antioxidante", "stress oxidativo", "Lactobacillus acidophilus", "bebida láctea", "leite fermentado", "iogurte", "técnicas in vitro" e "in vivo", associados aos operadores booleanos "AND" e "OR". Os artigos que não preencheram estas condições foram descarregados.
Através da pesquisa, 1751 artigos foram recuperados, mas 624 foram descarregados devido a duplicação e 36 tiveram o seu conteúdo lido na íntegra. A partir destes, foram seleccionados oito artigos devido ao cumprimento dos critérios de aceitação para estudos "in vitro". Destes, a composição de 17 amostras foi seleccionada e utilizada como base de dados para esta investigação, que constituiu três subgrupos (A: leite fermentado, B: leite acidófilo e C: iogurte). O seu perfil antioxidante foi profundamente estudado. Além disso, o teste DPPH foi o mais utilizado para avaliação antioxidante nestas amostras seleccionadas, no entanto, o método ABTS parece ser de preferência testes in vitro.

Palavras-chave: Iogurte, leite fermentado, radicais livres, stress oxidativo, antioxidante, baseado em provas de saúde.

1 INTRODUCTION:

The antioxidant activity of a chemical compound is the intrinsic ability to combat formation and action of free radicals, highly reactive chemical species responsible for attacking biomolecules and which can lead to oxidative stress (GEBICKI, 2016), which is able of generate serious effects on life, if they alter cellular DNA and RNA (BARREIROS et al., 2006). As an alternative on combating formation of these radicals, substances of human metabolism are constantly formed, as well as the possibility of extra

protection obtained by the consumption of chemically synthesized foods or substances (RAMALHO et al., 2006).

It is known that microorganisms are considered a strong ally in the fight against free radicals due to the formation of antioxidant substances from their metabolism. *Lactobacillus acidophilus* is one of the main responsible for the functional aspect of the food in which it is inserted (AMARETII et al., 2013; EJTAHED et al., 2012; KIM et al., 2006; MENKOVSKA et al., 2017) The use of probiotics on preparation of dairy foods has been an important bet of industries and large research centers, as they improve the physical, chemical, sensorial and functional characteristics of foods, especially the antioxidant aspect (JANKOVIC et al., 2010; LI et al., 2019; MUTAMED et al., 2018; RIJKERS et al., 2011; VANDENPLAS et al., 2015), which justifies continuous researches concerning this kind of food and its functional aspects.

In this sense, the main goal of the study was to gather evidence regarding the antioxidant potential of fermented dairy beverages prepared with probiotic *Lactobacillus acidophilus*. For this, the principles of evidence-based health (Systematic Review) were used, a type of study which is structured based on a selection rationale of scientific evidence in a certain area of interest, as a way to find the conclusions (EL DIB, 2007), being considered the best level of evidence for decision making (GALVÃO et al., 2014).

2 MATERIALS AND METHODS:

2.1. SEARCH DELINEATION

The research design, based on the recommendations of the "Preferred Reporting Items for Systematic Reviews and Meta-Analysis - PRISMA" (LIBERATI aet al., 2009), took into account the PICOS strategy, in which the population (P) considered in the study were the samples of fermented milks obtained by the uses of at least *Lactobacillus acidophilus (L.A.)*; The intervention (I) considered as mandatory condition the presence of L.A in the fermentation of milk; The control (S) was based on results obtained from scientific literature for unfermented or fermented milks with similar microbiological constitution in front of the selected samples; The outcome (O) was defined by the antioxidant activity of the samples prepared by fermentation in the presence of interested probiotic; The kind of study (S) selected for the constitution of the research, including those with in vitro analytical design, and also, retrospectives. Remark: The results obtained from *in vivo* experiments were not considered for this report.

2.2 SYSTEMATIC SEARCH

The systematic search for scientific articles is carried out in the databases Medline, Cochrane Central Register of Controlled Trials, Scopus, Science Direct, Scifinder, Web of Science, Scielo and Agricultural, on February 2016, updated on March 2019, with no restrictions about language or publication date. The search strategies involved terms such as "antioxidant activity", "oxidative stress", "*Lactobacillus acidophilus*", "fermented milk", "lactic beverage", "yogurt", "in vitro techniques" and "in vivo". Boolean operators "AND" and "OR", with different combinations depending on the need of each database.

In addition to the chosen databases, the search was also performed through manual screening based on the bibliographic references available by the articles that met the inclusion criteria.

2.3 SELECTION CRITERIA

The studies retrieved in the scientific literature had their titles and summaries evaluated, and were included in the research if they met some eligibility criteria, being:

- Fermented milk has used the probiotic *L.A.*, and eventually other species of lactic acid bacteria associated with them;
- Studies that presented the evaluation of the antioxidant activity in fermented milks obtained by uses of *L.A.*, considering only *in vitro* tests.

Additionally, some exclusion criteria were established as well:

- Studies that evaluated samples with the probiotic associated with yeasts, prebiotics and / or compounds of vegetable origin;
- Samples that had antioxidant activity evaluated in the bacterial culture, and not in the dairy beverage obtained after fermentation;
- Samples that had antioxidant activity evaluated by *in vivo* experiments;
- Systematic reviews, meta-analysis, book chapters, abstracts or expanded abstracts.

2.4 SORTING AND DATA EXTRACTION

The collected data included the general characteristics of the studies, details on the samples and their preparation, fermentation time and temperature, as well as the samples microbiological constitution. However, the critical analysis disseminated by this systematic review has been considered only the information related to antioxidant activity

evaluation, and the recovered results were quantified based on times number they were used in the evaluations of the selected samples, regarding both the frequency of use of the analytical method and the average results obtained, provided there are sufficient data for comparisons.

Meanwhile, the data are compared with the information available in the scientific literature, both for unfermented milks, for control samples (in the case of availability of information), and for fermented milks by other lactic acid bacteria, as well as results from samples with the same microbiological profile as samples here considered (L.A.).

3 RESULTS:

In total, 1751 articles were retrieved from the databases searched: Medline (n=534), Cochrane (n=3), Scopus (n=780), Science Direct (n=329), Scifinder (n=64), Web of Science (n= 9), Scielo (n=0) and Agricola (n=32), and no article was retrieved through manual screening. From this, 624 articles were excluded due to duplication, and then, 1127 studies had their titles and abstracts evaluated according to the inclusion and exclusion criteria settled down. From these, 1091 articles were excluded, and so, 36 articles were selected for reading of their full contents, regarding studies with *in vitro* experimental design, resulting in a total of eight articles included in the research.

Regarding the samples, the total of 83 kinds of fermented milks made up the eight selected studies, of which about 80% of them (n=66) were disregarded due to non-compliance on inclusion criteria settled down in the research. Thus, only 20.48% of the total available samples (n=17) in the eight articles were used as data to base the research.

Moreover, based on the descriptive analysis of the included samples, different final products were obtained according to the microbiological profile of the samples, including fermented milks, acidophilic milks and yoghurts, which were separated into subgroups according to said classification in order to compare them with precision and fidelity, being: Subgroup A (fermented milks, n=3), subgroup B (acidophilic milk, n=8) and subgroup C (yoghurts, n= 6).

3.1 ANTIOXIDANT CHARACTERIZATION

In order to know the antioxidant profile of the subgroups related to the fermented milks here studied, it was necessary to evaluate the results described from the methods used in the characterization of each set of samples. Therefore, the possible comparison for subgroup A, was related to the ABTS radical elimination ability test, applied by

66.67% among the samples constituted this group (n = 2), with the result described in Frame 1.

Frame 1 Characterization of antioxidant profile for the samples from subgroup A

Subgroup A			
Test	**Adherence to the method**	**Average result**	**Scientific literature** **
ABTS radical elimination ability	66.67% (n=2)*	23.30%	20.90% (*LcB*; Ll) (VIRTANEN et al., 2007)

*n: number of samples
***LcB*: *Leuconostoc cremoris B; Ll: Lactococcus lactis*

The research of VIRTANEN et al., 2007 showed the evaluation of fermented milk in presence of an association about lactic bacteria, with a lower result (20.90%) for the eliminatory capacity of the ABTS radical, compared to a sample prepared just on *L.A.* (23.30%).

Concerning subgroup B, antioxidant activity characterization contemplated methods for evaluation of DPPH radical eliminatory capacity, ABTS radical, reducing power and metallic ions chelating capacity. The mean results obtained compared to data get from the literature can be viewed in the Table 1.

Table 1 Antioxidant characterization of the subgroup B samples compared to data from scientific literature

Subgroup B			
Test	**Adherence of the method**	**Average result**	**Scientific literature****
DPPH radical eliminatory capacity	71.43% (n=5)	55.09%	12.50% and 17.50% (Fermented milks with *Lc* and *Lb*, respectively) (ZHANG et al., 2011)
ABTS radical eliminatory capacity	25.00% (n=2)	34.50%	15.70% (Unfermented milk) (VIRTANEN et al., 2007)
Reduction powder	25.00% (n=2)	0.388	0.138 and 0.451 (Fermented milk by *La* and *Lh*, respectively) (LI et al., 2015)
Chelating capacity of metallic ions	28.57% (n=2)	36.26%	28.19% (Fermented milk by *La*) (LI et al., 2015)

*n: number of samples
***Lc: Lactobacillus casei; Lb: Lactobacillus bulgaricus; La: Lactobacillus acidophilus; Lh: Lactobacillus helveticus*

By means of set results presented, some cases should be highlighted, such as the one obtained for the DPPH method, for instance, when compared to a sample fermented by a probiotic specie (*Lactobacillus casei*), the mean result for the subgroup B (fermented only by *L.A.*), is four times bigger. Another highlight is for the ABTS method, compared to an unfermented milk, characterizing twice the antioxidant capacity when in the presence of the probiotic target of the research.

Some cases like the result obtained for the reducing power method are also common on *in vitro* evaluations, in which samples evaluated by the same method and composed by the same microbiological constitution, present divergent results, demonstrating the fragility of the method.

In the case of subgroup C, the tests used in the number of samples sufficient for comparison were: Elimination ability of DPPH radical (usual method and by IC50 method), ABTS radical (IC50 method), reducing power and metal ion chelating capacity. The set of results are shown on Table 2.

Table 2 Antioxidant characterization for subgroup C samples, compared to data from the scientific literature

Subgroup C			
Test	**Adherence of the method**	**Average result**	**Scientific literature****
DPPH radical eliminatory capacity (IC50)	66.67% (n=4)*	1.78 mg/mL	2.23 mg/mL (*St* + *Lb*) and 1.51 mg/mL (*St, Lb, La, Lc and Lpc)* (SAH et al., 2014)
DPPH radical eliminatory capacity (%)	33.33% (n=2)*	48.71%	71.20% (*St* + *Lb*) and 81.90% (*St, Lb* and *Lf*) (MADHU et al., 2012)
ABTS radical eliminatory capacity (IC50)	66.67% (n=4)*	1.86 mg/mL	1.63 mg/mL (S*t, Lb, La, Lc* and *Lp*) (SAH et al., 2014)
Reduction powder (700 nm)	33.33% (n=2)*	0.6429	0.6494 (*St, Lb and La*) (ZHANG et al., 2015)
Metal ion chelating capacity	33.33% (n=2)*	38.47%	52.56% (*St* + *Lb*) (LI et al., 2015) , 54.69% (*St* + *Lb* + *Bl*) and 80.15% (*St* + *Lb* + *Bl* + WPC*** (UNAL et al., 2013)

*n: number of samples
***Bl: Bacillus lactis La: Lactobacillus acidophilus, Lb: Lactobacillus bulgaricus, Lc: Lactobacillus casei, Lf: Lactobacillus fermentum, Lpc: Lactobacillus paracasei, St: Streptococcus thermophylus.*
****Whey protein concentrate*

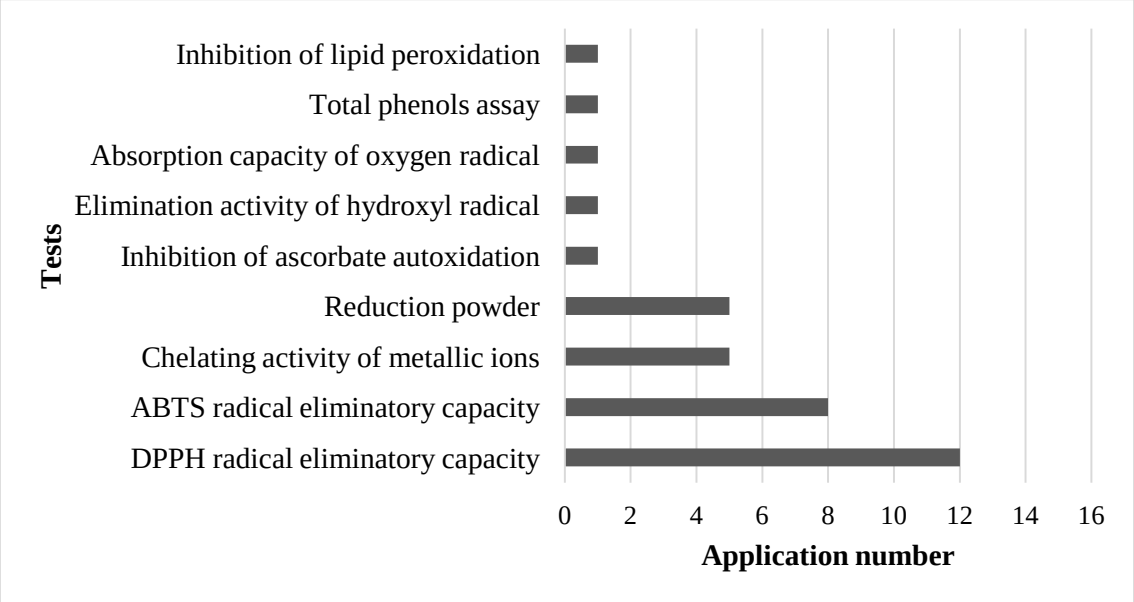
The DPPH method by IC50 should be inversely proportional interpreted, and thus it is possible to confirm that subgroup C samples has an antioxidant capacity higher than the probiotic-free yoghurt sample, but lower than the sample prepared with a set of them (14), as shown in the table. Still, the usual method of DPPH again demonstrates its fragility, once different from subgroup B, the subgroup C samples present a lower antioxidant capacity compared to the yogurt prepared without probiotic microorganisms. In addition, when associated with several microorganisms, probiotics and non-probiotics, besides *L.A.*, antioxidant activity was pronounced (SAH et al., 2014).

The same pattern is get concerning ABTS method by IC50, on which the sample constituted by several probiotics has shown better antioxidant aspect rather than the samples of this subgroup C.

A significant aspect was observed by the metal ion chelating ability method, which showed a relevant result for the sample added of whey protein concentrate (UNAL et al., 2013), reinforcing the own antioxidant capacity from proteins and amino acids, as presented in the study of (AJIBOLA et al., 2011).

Regarding the general aspect for the analytical methods, Figure 1 gathers information on both kinds of methods and the frequency which they are used for evaluation of the samples constituting the research.

Figure 1 Tests and application number of antioxidant methods were used to evaluate cellular extracts of fermented milks, acidophilic milks and yoghurts, by the 17 samples from eight recovered articles, after reaching the inclusion criteria about the present review



Taking into account the Figure 1 and in addition to the previously described data, the test of DPPH radical eliminatory capacity was generally the most often used among the evaluations of whole samples studied, used in a frequency of 70.59%, followed by the test of elimination capacity of ABTS radical (47.06%) and the chelating activity of the metal ions method, which reached the same level of approach when compared to the test of reducing power (29.41%).

From what has been verified, the wide use of DPPH method occurred due to being one usual method, low cost and which has a simple procedure for antioxidant activity evaluation, according to conclusions presented by (BOLIGON et al., 2014; KEDARE et al., 2011), as well. Despite of this, it is recognized as a fragile method, able to conferring considerably variable results according to the interferences that may occur during the preparation, which may justify the fact that there are divergent results for samples of the same microbiological constitution and that were evaluated by the same analytical method, as the results here obtained.

Thus, taking into account the methodological characteristics known for the DPPH method, it is suggested that the ABTS method is preferably used for *in vitro* evaluations, as it is also characterized by a simple methodological procedure, but present less analytical variation, and thereby indicating the achievement of more reliable results. These findings were also presented by (FLOEGEL et al., 2011), when comparing the ABTS and DPPH methods in the evaluation of antioxidant capacity of popular American foods, rich in antioxidants, and concluded the antioxidant profile was better reproduced by the ABTS method upon to the DPPH method. The similar findings were also presented by (THAIPONG et al., 2006).

The remaining methodologies were applied less frequently, not representing great potential of using on evaluation of the selected samples, being considered as additional test in studies of antioxidant activity proposes, not for a first choice. On this scenario, the procedures were also classified according to the frequency which they were used in each subgroup, as can be seen by Table 3.

Table 3 Antioxidant activity evaluation through different methods and subgroups, as well as the frequency each procedure is used

Antioxidant activity methods	**Subgroups**		
	A	**B**	**C**
DPPH radical eliminatory capacity	25%	33,3%	43%

ABTS radical eliminatory capacity	50%	13,3%	28,5%
Chelating activity of metallic ions	-	13,3%	14,3%
Reduction powder	-	13,3%	14,3%
Inhibition of ascorbate autoxidation	-	6,7%	-
Elimination activity of hydroxyl radical	-	6,7%	-
Absorption capacity of oxygen radical	-	6,7%	-
Total phenols assay	-	6,7%	-
Inhibition of lipid peroxidation	25%	-	-
Total	4 (100%)	15 (100%)	14 (100%)

Taking into account the data described so far, it is possible to infer there are relevant information must be clarified regarding the antioxidant activity on fermented dairy drinks, and that the analytical methodologies, in turn, can behave in a variable way.

In order to determine the effect of *L.A.*, a lot of studies have already presented and ensured their functionalities (ALMEIDA et al., 2015; ANKOLEKAR et al., 2012; DESROUILLÉRES et al., 2015, GHANY, 2014, MENKOVSKA et al., 2017, OGAWA et al., 2015), but few researches are devoted to evaluate the actual antioxidant potential of this type of bacteria in the final product, after fermentation process. Often, it is made in the cultivation of bacteria (probiotic or not) in the preparation of the analyte from this starting culture, and the application of analytical tests to evaluate its antioxidant activity. Thus, such results cannot be extrapolated for the general characterization of the food, since parallel evaluation of both pure culture and the ready-made food is necessary, once the growth and microorganism's viability during the processing of the food can be influenced by several factors, then, trigger changes in the antioxidant profile of the final product.

Another point few explored by the researchers is the dissemination of the results of antioxidant activity performed during the monitoring in the shelf life proposed for the product, as a relevant way to highlight the functional aspect of the food, not only after the final preparation, but while it keeps under the expire date.

Finally, based on presented information, it was verified when the related probiotic is in the constitution of the fermented milk, the antioxidant activity usually is increased, leading us to perceive that only presence of *L.A* is able to provide some antioxidant benefit, and then, in a first moment none negative effect was taken on this sense. But of course, additional studies must to be performed in order to understand the real antioxidant profile of the products contained of *L.A.* during its lifecycle, searching for this kind of specific effect.

About the methodologies evaluated, they can behave in a variable way according to the sample's preparation, or even by the variations during analytical procedures, performed although the methodologies employed are similar. The literature demonstrates the results are better reproduced by ABTS instead of DPPH technique, but both of them are widely used in analytical evaluations, which does not discharge the need of continuous studies about it.

4 CONCLUSION:

By the sample's classification, when *L.A.* is present in the dairy fermented beverages, the potential antioxidant activity was relevant in almost all the cases. Therefore, unless other characteristics of interest are sought, only the presence of this bacterial species seems to get some antioxidant protection. Also, by the presence of other bacteria (probiotic or not), the antioxidant potential despite being improved, does not seem to occur proportionally.

Therefore, fermented beverages in presence of *L.A.* may be consumed searching for antioxidant protection, mainly by wide population, including those ones affected by diseases from physiopathology related with oxidative stress, but not replacing the drug official treatment.

Concerning the analytical methodologies, it is highlighted the importance of association between the available techniques for determination of antioxidant activity, since when more than one test is applied, better accurate and reliable are the results and conclusion of the search. As a methodological emphasis, due to consistent results, the best analytical support and cost-effective, can be preferably suggest using the ABTS method in evaluations *in vitro* antioxidant activity.

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