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PROPRIETES ANTINEOPLASIQUES DES COMPOSANTES MEMBRANAIRES DES BACTERIES PROBIOTIQUES ET DES METABOLITES SECONDAIRES DES PETITS FRUITS

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RÉSUMÉ

Le cancer colorectal (CCR) représente l'une des principales causes de mortalité liées au cancer dans le monde. De nombreux efforts ont été déployés afin de déterminer les facteurs de risques liés à l'étiologie de ce cancer. Bien qu'une composante génétique ait été établie dans la pathogénèse de la maladie, de nombreuses études épidémiologiques suggèrent que 90% des cas de CCR sont sporadiques c'est-à-dire qu'ils se développent chez des individus ne possédant aucune prédisposition génétique. Elles indiquent que les facteurs environnementaux comme le mode de vie et l'alimentation influencent beaucoup plus l'incidence de la maladie. Mis à part les éléments environnementaux, d'autres études ont également démontré l'implication du microbiote intestinal dans le développement de ce cancer. Plusieurs études montrent que les polyphénols présents dans les petits fruits, les plantes médicinales, les végétaux et les métabolites produits par les bactéries probiotiques et leurs composantes cellulaires peuvent agir comme agents chimio préventifs. Toutefois très peu d'études ont jusqu'ici évalués les possibles effets synergiques entre les composantes présentes dans les végétaux et les métabolites produits par les bactéries probiotiques au cours de la fermentation ou même leurs constituants cellulaires. Les objectifs de cette étude sont donc :

a) Évaluer *in vitro* les propriétés antinéoplasiques de différentes classes de polyphénols obtenues par séparation chromatographique (CLHP) d'un jus de canneberges concentré et ceux de calices d'*Hibiscus sabdariffa L.* obtenues suite à une extraction par solvants seuls et combiné avec des composés membranaires extraites d'une biomasse contenant trois bactéries probiotiques : *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2.
b) Déterminer l'effet de différentes doses d'un lait fermenté probiotique spécifique (1.2×10^8 - 1×10^9 UFC) contenant *L. acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2 et ces différentes fractions (composantes membranaires, surnageant: métabolites bactériens, culot : bactéries seules) sur les propriétés antinéoplasiques mesurées dans un modèle animal.

Les résultats obtenus dans cette étude suggèrent que certains composés phénoliques du jus de canneberges (acides phénoliques et flavonols) et de *l'Hibiscus sabdariffa* (anthocyanines et acides phénoliques) sont capables d'inhiber *in vitro* la croissance des cellules cancéreuses et d'induire l'activité de la quinone réductase. De plus, lorsque ces derniers sont combinés aux composantes membranaires l'inhibition au niveau de la prolifération cellulaire est augmentée. Cette étude montre également que la plus forte dose de lait fermenté ($1x10^9$ UFC) et certaines fractions du lait comme par exemple le culot, sont capables de diminuer la formation de cryptes aberrantes, d'induire l'activité d'enzymes détoxifiantes du foie et d'inhiber l'activité d'enzymes fécales. Les résultats de cette étude ont permis de démontrer que les bactéries probiotiques et leurs composantes ainsi que les composés phénoliques pourraient contribuer à diminuer le risque de développer le CCR.

ABSTRACT

Colorectal cancer (CRC) represent one of the principal cause of cancer related-death worldwide. Many efforts have been made to determine risk factors linked to this cancer etiology. Although a genetic predisposition have been linked to the pathogenesis of CRC, numerous epidemiological studies have identified that 90% of the CRC cases are sporadic meaning that they develop in individuals with no genetical predisposition. Those studies indicated that environmental factors such as lifestyle (excessive alcohol consumption, smoking, physical inactivity) and diet influence greatly CRC incidence. Besides the environmental factors, other studies demonstrated the involvement of the intestinal microbiota in CRC carcinogenesis. Many studies showed that natural compounds like phenolic compounds found in berries, in medicinal herbs, in vegetables, the probiotics bacteria and their membrane constituents and their metabolites have chemopreventive effects against colon cancer development. However, only few studies have evaluated the possible synergy between the polyphenols present in berries and plant and the probiotic bacteria constituents and metabolites produced during fermentation. The objectives of this study are:

a) Investigate *in vitro* the anticancer properties of different classes of phenolic compounds obtained by HPLC fractionation of a cranberry concentrate juice and solvents extraction from *Hibiscus sabdariffa L*. dry calyces combined with cells walls obtained from a biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2.

b) Determine *in vivo* the effect of different doses ($1.2x10^{8}$-$1x10^{9}$ CFU) of a specific probiotic fermented milk produced by *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 and its different fractions (cell wall constituents, supernatant: derived-metabolites, pellet: bacteria).

The results obtained in this study suggest that some classes of phenolic compounds from either cranberry concentrate juice (phenolic acids, flavonols) or *Hibiscus sabdariffa L.* calices (anthocyanins, phenolic acids) are able to inhibit cancerous cell growth and

induce an increase of the quinone reductase (QR) activity. Moreover, when combined with the cell wall constituents there is an increase of the inhibitory effect against the colon cancer cell proliferation. This study also shows that the highest dose of a fermented milk ($1x10^9$ CFU) and fractions such as pellet are able to significantly reduce the formation and the multiplicity of precancerous lesions, increase the activity of a detoxifying enzyme and decrease the activity of fecal enzymes. Those results suggest that the fermented milk and its different components, the cranberry concentrate juice and the *Hibiscus sabdariffa* could probably act as chemopreventive substance against CRC development.

TABLE DES MATIÈRES

1. INTRODUCTION 1

2. GÉNÉRALITÉS SUR LE CANCER COLORECTAL (CCR) 2

3. ALIMENTATION, CANCER COLORECTAL ET MICROBIOTE INTESTINAL 3

3.1 ALIMENTATION ET CCR 3

3.1.1 Consommation de viande rouge et transformée 4

3.1.2 Consommation de poisson 5

3.1.3 Consommation de fruits et de légumes 6

3.1.4 Consommation de céréales et de grains entiers 6

3.2 MICROBIOTE INTESTINAL ET CCR 8

3.2.1 Les bactéries « drivers » 9

3.2.2 Les bactéries « passengers » 10

3.2.3 Les probiotiques 11

3.3 ALIMENTATION ET MICROBIOTE INTESTINAL 12

3.3.1 Viande rouge et flore intestinale 13

3.3.2 -Grains entiers et flore intestinale 13

3.3.3 Fruits, légumes et flore intestinale 14

4 PREUVES DE PREVENTION DE CANCER A PARTIR DE PETITS FRUITS, HERBES MEDICINALES ET PROBIOTIQUES 16

A. LES PROBIOTIQUES ..... **ERREUR ! SIGNET NON DEFINI.**

B. LES PETITS FRUITS ET SES COMPOSÉS BIOACTIFS 17

1. Preuves in vitro de l'effet antinéoplasique de petits fruits et de leurs composés bioactifs 18

1. PREUVES IN VIVO DE L'EFFET ANTINÉOPLASIQUE DES PETITS FRUITS ET DE LEURS COMPOSÉS BIOACTIFS 19

2. L'HIBISCUS SABDARIFFA 19

5 QUELQUES MÉCANISMES IMPLIQUÉS DANS LA PRÉVENTION DU CCR 20

5.1 LA MODULATION DES ENZYMES DE PHASE II 21

5.2 L'INHIBITION DE LA PROLIFÉRATION DES CELLULES CANCÉREUSES 22

5.3 LA RÉDUCTION ET/OU L'INHIBITION DE LA FORMATION DES FOYERS DE CRYPTES ABERRANTES 22

5.4 L'ALTÉRATION DE L'ACTIVITÉ ENZYMATIQUE DE LA FLORE INTESTINALE 23

5.5 LA CAPACITÉ ANTIOXYDANTE 23

6 HYPOTHÈSES 25

7 OBJECTIFS 26

8 METHODOLOGIE 27

9. ARTICLE 1 : THE SYNERGISTIC EFFECT OF CELL WALL EXTRACTED FROM PROBIOTIC BIOMASS CONTAINING LACTOBACILLUS ACIDOPHILUS CL1285, L. CASEI LBC80R AND L. RHAMNOSUS CLR2 ON ANTICANCER ACTIVITY OF CRANBERRY JUICE - HPLC FRACTIONS 29

9.1 INTRODUCTION 34
9.2 MATERIALS AND METHODS 36
9.2.1 Chemical reagents 36
9.2.3 HPLC-DAD fractionation of concentrated cranberry juice and total phenol determination. 36
a) Fractionation of concentrated juice 36
b) Total phenol content 37
9.2.4 Probiotic cell walls extraction 38
a) Physical treatment 38
b) Enzymatic treatment 38
9.2.5 Measurement of cell proliferation 39
a) Cell cultures 39
b) Preparation of samples 39
c) Antiproliferative assay 39
9.2.6 Quinone reductase assay 40
a) Assay procedure 40
b) Protein determination 41
9.2.7 Statistical analysis 41
9.3 RESULTS AND DISCUSSION 42
9.3.1 CRANBERRY CONCENTRATE JUICE HPLC FRACTIONNATION 42
9.3.2 Total phenol content 42
9.3.3 Antiproliferative assay 43
a) Antiproliferative activity of cranberry fractions against HT-29. 43
b) Antiproliferative activity of cell wall constituents from a probiotic biomass HT-29. 46
c) Antiproliferative activity of cranberry fractions combined with probiotic cell wall against HT-29 proliferation 47
9.3.4 Quinone reductase assay 49
a) Quinone reductase induction by fractions from cranberry concentrate juice 49
b) Quinone reductase induction by probiotic cell wall constituents 50
c) Quinone reductase induction by cranberry fractions combined with probiotic cell wall constituents. 52

9.4 CONCLUSION 53

10. ARTICLE 2 : CHEMOPREVENTIVE EFFECTS OF EXTRACTS FROM *HIBISCUS SABDARIFFA L.* 59

10.1 INTRODUCTION 63
10.2 MATERIALS AND METHODS 65
10.2.1 Chemicals reagents 65
10.2.2 Raw materials 65
10.2.3 Extraction of phenolic compounds 65
10.2.4 Total phenol content 66
10.2.5 Probiotic cell wall extraction 66
a) Physical treatment 66
b) Enzymatic treatment 66
10.2.6 Cell cultures, antiproliferative assay and quinone reductase assay 67
a) Cell proliferation agent MTT 67
b) Preparation of samples 67
c) Cell lines and culture media 68
d) Antiproliferative assay 68
10.2.7 Quinone reductase assay 68
10.2.8 Free radical scavenging capacity 70
10.2.9 Statistical analysis 70
10.3 RESULTS AND DISCUSSION 71
10.3.1 Total phenol content : 71
10.3.2 Antiproliferative assay 72
a) Antiproliferative activity of Hs extracts against HT-29 proliferation 72
b) Antiproliferative activity of Hs extract combined with cell wall constituents from a probiotic biomass against HT-29 proliferation 74
10.3.3 Quinone reductase assay 75
a) Effect of Hs extracts on quinone reductase activity 75
b) Effect of Hs extracts combined with cell wall constituents on quinone reductase activity. 77
10.3.4 Free radical scavenging capacity 78
10.4 CONCLUSION 80

11. ARTICLE 3 : CANCER PREVENTIVE EFFECT OF A SPECIFIC PROBIOTIC FERMENTED MILK CONTAINING *LACTOBACILLUS ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON MALE F344 RATS TREATED WITH 1,2-DIMETHYLHYDRAZINE 87

11.1 INTRODUCTION 91
11.2 MATERIALS AND METHODS 94

11.2.1 Chemicals *94*
11.2.2 PROBIOTICS 94
11.2.3 ANIMALS, HOUSING AND DIET 94
11.2.4 EXPERIMENTAL DESIGN 95
11.2.5 ABERRANT CRYPT ANALYSIS 96
11.2.6 LIVER AND CAECUM PROCESSING 96
11.2.7 PROTEIN ESTIMATION 96
11.2.8 QUINONE REDUCTASE (QR) ASSAY 97
11.2.9 GLUTATHIONE-S-TRANSFERASE (GST) ASSAY 97
11.2.10 B-GLUCOSIDASE AND B-GLUCURONIDASE ASSAY 98
11.2.11 STATISTICAL ANALYSIS 98
11.3 RESULTS 99
11.3.1 EFFECT OF THE PROBIOTIC FERMENTED MILK ADMINISTRATION ON THE NUMBER OF AC PER COLON INDUCED BY DMH TREATMENT. 99
11.3.2 EFFECT OF THE PROBIOTIC FERMENTED MILK ADMINISTRATION ON THE NUMBER OF ACF PER COLON INDUCED BY DMH TREATMENT. 99
11.3.3 EFFECT OF THE PROBIOTIC FERMENTED MILK ADMINISTRATION ON THE MULTIPLICITY OF AC PER ACF IN F344 RATS TREATED WITH DMH. 100
11.3.4 EFFECT THE ADMINISTRATION OF THE PROBIOTIC FERMENTED MILK ON (QR) AND (GST) ACTIVITIES IN F344 RATS TREATED WITH DMH. 101
11.3.5 EFFECT OF THE ADMINISTRATION OF THE FERMENTED MILK ON B-GLUCOSIDASE AND B-GLUCURONIDASE ACTIVITIES IN F344 RATS TREATED WITH DMH. 103
11.5 CONCLUSION 109
12. ARTICLE 4 : CANCER PREVENTIVE EFFECT OF COMPONENTS FROM A SPECIFIC PROBIOTIC FERMENTED MILK AND CELL WALL CONSTITUENTS FROM A BIOMASS CONTAINING *LACTOBACILLUS ACIDOPHILUS* CL1285, *L. CASEI* LBC80R, *L. RHAMNOSUS* CLR2 ON MALE F344 RATS TREATED WITH 1,2-DIMETHYLHYDRAZINE. 116
12.1 INTRODUCTION 120
12.2 MATERIALS AND METHODS 122
12.2.1 CHEMICALS 122
12.2.2 SAMPLE PREPARATION 122
12.2.3 ANIMALS, HOUSING AND DIET 123
12.2.4 EXPERIMENTAL DESIGN 124
12.2.5 ABERRANT CRYPT ANALYSIS 124
12.2.6 LIVER AND CAECUM PROCESSING 125

12.2.7 PROTEIN ESTIMATION 125
12.2.8 QUINONE REDUCTASE (QR) ASSAY 126
12.2.9 GLUTATHIONE-S-TRANSFERASE (GST) ASSAY 126
12.2.10 B-GLUCOSIDASE AND B-GLUCURONIDASE ASSAY 127
12.2.11 STATISTICAL ANALYSIS 127

12.3 RESULTS 128

12.3.1 EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ADMINISTRATION ON THE NUMBER OF AC PER COLON INDUCED BY DMH TREATMENT 128
12.3.2 EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ADMINISTRATION ON THE NUMBER OF ACF PER COLON INDUCED BY DMH TREATMENT. 128
12.3.3 EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ADMINISTRATION ON THE MULTIPLICITY OF AC PER ACF IN F344 RATS TREATED WITH DMH. 129
12.3.4 EFFECT OF THE ADMINISTRATION OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON (QR) AND (GST) ACTIVITIES IN F344 RATS TREATED WITH DMH 131
12.3.5 EFFECT OF THE ADMINISTRATION OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2ON B-GLUCOSIDASE AND B-GLUCURONIDASE ACTIVITIES IN F344 RATS TREATED WITH DMH. 132

12.4 DISCUSSION 134

13. DISCUSION 144

14. CONCLUSION 148

15. PERSPECTIVES FUTURES 149

16. REFERENCES 150

LISTE DES TABLEAUX

TABLEAU 1: INFLUENCE DE CERTAINS ALIMENTS SUR LE DÉVELOPPEMENT DU CANCER COLORECTAL.................... 7

TABLE 2 : TIME OF ELUTION OF CRANBERRY CONCENTRATE JUICE FRACTIONS OBTAINED BY HPLC-DAD FRACTIONATION. F1: COMPOSED WITH PHENOLIC ACIDS, F2: CONSTITUTED WITH MOSTLY ANTHOCYANINS AND F3: CONTAINED MAINLY FLAVONOLS. 37

TABLE 3 : TOTAL PHENOLIC CONTENT OF CRANBERRY FRACTIONS. F1, F2 AND F3 WERE COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. F1: COMPOSED WITH PHENOLIC ACIDS, F2: CONSTITUTED WITH MOSTLY ANTHOCYANINS AND F3: CONTAINED MAINLY FLAVONOLS. 42

TABLE 4: IC_{50} VALUES OF THE CRANBERRY FRACTIONS FOR THE INHIBITION OF HT-29 GROWTH. HT-29 CELLS WERE INCUBATED WITH DIFFERENT CONCENTRATIONS (156.25-2500MG/ML) OF CRANBERRY FRACTIONS FOR 48 HOURS. F1, F2 AND F3 WERE COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P \leq 0.05$). F1: COMPOSED WITH PHENOLIC ACIDS, F2: CONSTITUTED WITH MOSTLY ANTHOCYANINS AND F3: CONTAINED MAINLY FLAVONOLS. 44

TABLE 5 : EFFECT OF EXTRACTS OF CELL WALL CONSTITUENTS OBTAINED FROM A PROBIOTIC BIOMASS ON HT-29 CELL VIABILITY. HT-29 CELLS WERE EXPOSED TO DIFFERENT CONCENTRATIONS (62.5 TO 1000 μG/ML) OF CELL WALL CONSTITUENTS. CELL WALL WERE EXTRACTED FROM A PROBIOTIC BIOMASS CONTAINING *LACTOBACILLUS ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. CW: CELL WALL CONSTITUENT. 46

TABLE 6 : TOTAL PHENOLIC CONTENT *HIBISCUS SABDARIFFA* (HS) EXTRACTS. FAJ, FBJ AND FCJ WERE EXTRACTED FROM *HIBISCUS SABDARIFFA* DRY CALYCES: FAJ: COMPOSED MAINLY WITH WATER-SOLUBLE PHENOLIC COMPOUND, FBJ: RICH WITH APOLAR PHENOLIC COMPOUND AND FCJ: RICH WITH ANTHOCYANINS. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. 71

TABLE 7 : IC50 VALUES OF THE HIBISCUS SABDARIFFA (HS) EXTRACTS TESTED. HT-29 CELLS WERE INCUBATED WITH DIFFERENT CONCENTRATIONS (1500-5000MG/ML) OF PHENOLIC EXTRACTS FROM HIBISCUS SABDARIFFA DRY CALYCES FOR 48 HOURS. THREE EXTRACTS WERE OBTAINED: FAJ: COMPOSED MAINLY WITH WATER-SOLUBLE PHENOLIC COMPOUND, FBJ: RICH WITH APOLAR PHENOLIC COMPOUND AND FCJ: RICH WITH ANTHOCYANINS. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P \leq 0.05$). 72

TABLE 8: RADICAL SCAVENGING CAPACITY OF *HIBISCUS SABDARIFFA* (HS) EXTRACTS....................................... 78

LISTE DES FIGURES

FIGURE 1 : MODELE EXPERIMENTAL POUR L'EXPERIENCE *IN VIVO*. 28

FIGURE 2: EFFECT OF FRACTIONS FROM CRANBERRY CONCENTRATE JUICE OBTAINED BY HPLC ON HT-29 PROLIFERATION. F1, F2 AND F3 ARE THREE FRACTIONS COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC. F1 IS COMPOSED WITH PHENOLIC ACIDS, F2 IS CONSTITUTED WITH MOSTLY IN ANTHOCYANINS AND F3 CONTAINED MAINLY FLAVONOLS. VALUES ARE MEAN ± STANDARD DEVIATION. 44

FIGURE 3:EFFECT OF THE CRANBERRY FRACTIONS ASSOCIATED WITH PROBIOTIC CELL WALL ON HT-29 PROLIFERATION. HT-29 CELLS WERE EXPOSED TO CRANBERRY FRACTIONS COMBINED WITH EXTRACT OF CELL WALL CONSTITUENTS. F1, F2 AND F3 WERE COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC AND CW CONSTITUENTS WERE EXTRACTED FROM A PROBIOTICS BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285*, L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2. THE SAMPLES ARE TESTED AT DIFFERENT CONCENTRATIONS F1: 625 µG/ML, F2: 2000 µG/ML, F3: 625 µG/ML AND CW: 100 µG/ML. DATA ARE PRESENTS AS MEAN ± STANDARD DEVIATION. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P \leq 0.05$). F1: COMPOSED WITH PHENOLIC ACIDS, F2: CONSTITUTED WITH MOSTLY ANTHOCYANINS AND F3: CONTAINED MAINLY FLAVONOLS AND CW: CELL WALL CONSTITUENTS. 48

FIGURE 4: EFFECT OF FRACTION FROM CRANBERRY CONCENTRATE JUICE OBTAINED BY HPLC THE INDUCTION OF QUINONE REDUCTASE ACTIVITY. MURINE HEPATOMA (HEPA 1C1C7) CELLS WERE EXPOSED TO DIFFERENT CONCENTRATION OF CRANBERRY FRACTIONS (3.91 TO 500 µG/ML). F1, F2 AND F3 WERE COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. F1: COMPOSED WITH PHENOLIC ACIDS, F2: CONSTITUTED WITH MOSTLY ANTHOCYANINS AND F3: CONTAINED MAINLY FLAVONOLS. 49

FIGURE 5: EFFECT OF CELL WALL EXTRACTED FROM A PROBIOTIC BIOMASS ON THE INDUCTION OF QUINONE REDUCTASE ACTIVITY. MURINE HEPATOMA (HEPA 1C1C7) CELLS WERE EXPOSED TO DIFFERENT CONCENTRATION OF EXTRACT OF CELL WALL CONSTITUENTS (3.91 TO 500 µG/ML). CW CONSTITUENTS WERE FROM A PROBIOTICS BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285*, L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2. DATA ARE PRESENTED AS MEAN ± STANDARD DEVIATION. CW: CELL WALL CONSTITUENTS. 51

FIGURE 6: EFFECT OF THE CRANBERRY FRACTIONS ASSOCIATED WITH PROBIOTIC CELLS WALLS ON THE INDUCTION OF QUINONE REDUCTASE ACTIVITY: MURINE HEPATOMA (HEPA 1C1C7) CELLS WERE EXPOSED TO CRANBERRY FRACTIONS COMBINED WITH EXTRACT OF CELL WALL CONSTITUENTS. F1, F2 AND F3 WERE COLLECTED BY FRACTIONATION OF CRANBERRY CONCENTRATED JUICE BY HPLC AND CW CONSTITUENTS WERE EXTRACTED FROM A PROBIOTICS BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285*, L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2. THE SAMPLES ARE TESTED AT A CONCENTRATION OF 100 µG/ML. DATA ARE PRESENTS AS MEAN ± STANDARD DEVIATION. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤

0.05). F1: composed with phenolic acids, F2: constituted with mostly anthocyanins and F3: contained mainly flavonols and CW: cell wall constituents. 52

Figure 7 : Effect of extracts from dried calyces of *Hibiscus sabdariffa* (Hs) on HT-29 proliferation. HT-29 cells were treated with different concentrations (1500-5000mg/ml) of phenolic extracts from *Hibiscus sabdariffa* dry calyces. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces: FaJ: composed mainly with water-soluble phenolic compound, FbJ: rich with apolar phenolic compound and FcJ: rich with anthocyanins. Data are presented as mean ± standard deviation. 72

Figure 8 : Effect of *Hibiscus sabdariffa* (Hs) extracts associated with probiotic cells walls on HT-29 proliferation. HT-29 cells were exposed to *Hibiscus sabdariffa* extracts combined with extract of cell wall constituents. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces and CW constituents were extracted from a probiotic biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. The samples are tested at a concentration less than 50% inhibition rate: FaJ (2500 µg/ml), FbJ (2000 µg/ml), FcJ (500 µg/ml) and CW (100 µg/ml). Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). FaJ: composed mainly with water-soluble phenolic compound, FbJ: rich with apolar phenolic compound FcJ: rich with anthocyanins and CW: cell wall constituents. 74

Figure 9 : Effect of *Hibiscus sabdariffa* (Hs) extracts on the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were treated with different concentrations (3.91-500 µg/ml) of phenolic extracts from *Hibiscus sabdariffa* dry calyces. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces: FaJ: composed mainly with water-soluble phenolic compound, FbJ: rich with apolar phenolic compound and FcJ: rich with anthocyanins. Data are presented as mean ± standard deviation 76

Figure 10 : Effect of *Hibiscus sabdariffa* (Hs) extracts associated with probiotic cells walls on the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were exposed to *Hibiscus sabdariffa* extracts combined with extract of cell wall constituents. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces and CW constituents were extracted from a probiotic biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. All samples are tested at a concentration of 100µg/ml. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). FaJ: composed mainly with water-soluble phenolic compound, FbJ: rich with apolar phenolic compound FcJ: rich with anthocyanins and CW: cell wall constituents 77

Figure 11 :Representation of the experimental design... 95

Figure 12 : Effect of increasing doses of *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk on total number of aberrant crypts. Error bars represent

THE STANDARD DEVIATION OF THE MEAN TOTAL AC/COLON OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK AND PC: DMH ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05). A ≤ 0.05 VS PC GROUP.......................... 99

FIGURE 13 : EFFECT OF INCREASING DOSES OF *L. ACIDOPHILUS* CL1285 AND *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 PROBIOTIC FERMENTED MILK ON TOTAL NUMBER OF ABERRANT CRYPT FOCI INDUCED BY DMH. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN TOTAL ACF/COLON OBTAINED FROM 8 RATS IN EACH GROUP. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05) FROM CARCINOGEN CONTROL. 100

FIGURE 14 : TOPOGRAPHICAL VIEW OF NORMAL AND ABERRANT CRYPTS ON THE COLONIC MUCOSA STAINED WITH METHYLENE BLUE OF RATS TREATED WITH DMH. (A) TOPOGRAPHICAL VIEW OF G4 COLONIC MUCOSA SHOWING SOME NORMAL CRYPT AND ACF WITH 2 CRYPTS (20X). (B) TOPOGRAPHICAL VIEW OF PC COLONIC MUCOSA SHOWING MULTIPLE ACF, TWO WITH FOUR CRYPTS AND ONE WITH TWO CRYPTS (20X). THE ARROWS SHOWS THE CRYPTS (NORMAL AND ABERRANT ONES) IN THE COLON MUCOSA.................... 100

FIGURE 15 : EFFECT OF INCREASING DOSES OF *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR 2 PROBIOTIC FERMENTED MILK ON NUMBER OF ABERRANT CRYPT FOCI (ACF) OF TWO TO FIVE ABERRANT CRYPT INDUCED BY DMH. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN OF THE NUMBER OF AC PER FOCUS OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK AND PC: DMH ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05). A ≤ 0.05 VS PC GROUP. 101

FIGURE 16 : EFFECT OF INCREASING DOSES OF *L ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 PROBIOTIC FERMENTED MILK ON GLUTATHIONE S-TRANSFERASE INDUCTION IN DMH TREATED F344 RATS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF GLUTATHIONE S-TRANSFERASE INDUCTION OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK, PC: DMH ONLY AND NC: SALINE WATER ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05). A ≤ 0.05 VS PC GROUP AND Γ ≤ 0.05 VS NC GROUP. 102

FIGURE 17 : EFFECT OF INCREASING DOSES OF *L ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 PROBIOTIC FERMENTED MILK ON QUINONE REDUCTASE INDUCTION IN DMH TREATED F344 RATS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN OF QUINONE REDUCTASE INDUCTION OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK, PC: DMH ONLY AND NC: SALINE WATER ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05): A ≤ 0.05 VS PC GROUP AND Γ ≤ 0.05 VS NC GROUP. 102

FIGURE 18 : EFFECT OF INCREASING DOSES OF *L ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 PROBIOTIC FERMENTED MILK B-GLUCOSIDASE ACTIVITY IN DMH TREATED F344 RATS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN B-GLUCOSIDASE SPECIFIC ACTIVITY OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK, PC: DMH ONLY AND NC: SALINE WATER ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05): A ≤ 0.05 VS PC GROUP. 103

FIGURE 19 : EFFECT OF INCREASING DOSES OF *L ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 PROBIOTIC MILK OF B-GLUCURONIDASE ACTIVITY IN DMH TREATED F344 RATS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN B-GLUCURONIDASE SPECIFIC ACTIVITY OBTAINED FROM 8 RATS IN EACH GROUP. G3: DMH + 2 ML FERMENTED MILK, G4: DMH + 1.5 ML FERMENTED MILK, G5: DMH + 1 ML FERMENTED MILK, G6: DMH + 0.5 ML FERMENTED MILK, G7: DMH + 0.25 ML FERMENTED MILK, PC: DMH ONLY AND NC: SALINE WATER ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05): A ≤ 0.05 VS PC GROUP AND Γ ≤ 0.05 VS NC GROUP. 104

FIGURE 21 : EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON TOTAL NUMBER OF ABERRANT CRYPTS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN TOTAL AC/COLON OBTAINED FROM 8 RATS IN EACH GROUP. G4: DMH + 2 ML FERMENTED MILK (FM), G5: DMH + FERMENTED MILK SUPERNATANT (S), G6: DMH + FERMENTED MILK PELLET (P), G7: DMH + CELL WALL CONSTITUENTS (CW) PC: DMH ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05). 128

FIGURE 22 : EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON TOTAL COUNT OF ABERRANT CRYPT FOCI. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN TOTAL ACF/COLON OBTAINED FROM 8 RATS IN EACH GROUP. G4: DMH + 2 ML FERMENTED MILK (FM), G5: DMH + FERMENTED MILK SUPERNATANT (S), G6: DMH + FERMENTED MILK PELLET (P), G7: DMH + CELL WALL CONSTITUENTS (CW) PC: DMH ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05)............ 129

FIGURE 23 : EFFECT OF DIFFERENT COMPONENTS FROM A SPECIFIC FERMENTED MILK AND CELL WALL EXTRACTED FROM A BIOMASS CONTAINING *L. ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON NUMBER OF ABERRANT CRYPT FOCI (ACF) OF TWO TO FIVE ABERRANT CRYPT INDUCED BY DMH. ERROR BARS REPRESENT THE STANDARD DEVIATION OF THE MEAN OF THE NUMBER OF AC PER FOCUS OBTAINED FROM 8 RATS IN EACH GROUP. G4: DMH + 2 ML FERMENTED MILK (FM), G5: DMH + FERMENTED MILK SUPERNATANT (S), G6: DMH + FERMENTED MILK PELLET (P), G7: DMH + CELL WALL CONSTITUENTS (CW) PC: DMH ONLY. DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (P ≤ 0.05). 130

Figure 24 : Topographical view of normal and aberrant crypts on the colonic mucosa stained with methylene blue of rats treated with DMH. (A) Topographical view of PC colonic mucosa showing multiple ACF, one with four crypts, one with 2 crypts and the last two one are ACF with a single crypt (20X). (B) Topographical view of G4 colonic mucosa showing multiple ACF, one with two crypts and the last two ones are single crypt ACF (20X). The arrows shows the crypts in the colon mucosa. G4: DMH + 2 ml fermented milk (FM), PC: DMH only. 130

Figure 25 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on glutathione S-transferase activity. Error bars represent the standard deviation of the mean of glutathione S-transferase induction obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control................................. 131

Figure 26 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on quinone reductase. Error bars represent the standard deviation of the mean of quinone reductase induction obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control 132

Figure 27 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on b-glucosidase activity. Error bars represent the standard deviation of the mean of b-glucuronidase specific activity obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control 132

Figure 28 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on b-glucuronidase activity. Error bars represent the standard deviation of the mean of b-glucuronidase specific activity obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control 133

LISTE DES ÉQUATIONS

EQUATION 1:INHIBITION (%) = 100-(OD TREATED CELLS/OD CONTROL CELLS*100) 40
EQUATION 2: SPECIFIC ACTIVITY = (ABSORBANCE OF MTT 595NM/MIN (5MINUTES) X 3247 NMOL/MG)/ (ABSORBANCE OBTAINED FOR DETERMINING PROTEIN AT 595NM). 41
EQUATION 3 :INHIBITION PERCENTAGE = 100-(OD CONTROLS CELLS/OD TREATED CELLS*100)........................ 68
EQUATION 4: SPECIFIC ACTIVITY = (ABSORBANCE OF MTT 595 NM/MIN (5 MINUTES) * 3247 NMOL/MG)/ (ABSORBANCE OBTAINED FOR DETERMINING PROTEIN AT 595 NM). 69
EQUATION 5 : SCAVENGING ACTIVITY (%) = [1-(A_{SAMPLE}/$A_{CONTROL}$)]*100.. 70

LISTE DES ABRÉVIATIONS

5-FU : 5-fluorouracil
AC: aberrant crypt
ACF: aberrant crypt foci
ADN: acide desoxyribonucléique
AOM: azoxymethane
BCA: bicinchoninic acid protein
BSA: bovine serum albumin
BFT: *B. fragilis* toxin
CAC: colitis-associated cancer
CCAC: Canadian Council on Animal Care
CCR: cancer colorectal
CDNB: 1-chloro-2,4-dinitrobenzene
CFU: colony forming unit
CHPL: chromatographie a haute performance liquide
CI_{50} : concentration inhibitrice
CRC: colorectal cancer
DMH: dimethylhydrazine
DTD: DT-diaphorase
FAD: flavin adenine dinucleotide
FAP: familial adenomatous polyposis
FM: fermented milk
GIT: gastrointestinal tract
GST: glutathione-S-transferase
HNPCC: hereditary non polyposis colon cancer
HPLC: high performance liquid chromatography
IBD: inflammatory bowel disease
LAB: lactic acid bacteria
MAM: methylazoxymthanol
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADP: nicotinamide adenine dinucleotide phosphate

PAF: polypose adénomateuse familiale

pks: polyketide synthase

QR: quinone reductase

UDPGD: uridine diphospho glucuronyl transferase

1. INTRODUCTION

Le cancer constitue un problème de santé majeur. Il s'agit d'une des maladies chroniques les plus meurtrières dans le monde, avec 14 millions de cas diagnostiqués dont 8.2 millions de morts pour l'année 2012 OMS (2015).

La carcinogénèse implique une séquence de mutations conduisant à l'initiation et à la progression de la transformation d'une cellule normale à une tumeur bénigne puis en une tumeur maligne (Hanahan *et al.*, 2000). Ces mutations perturbent les voies de signalisations normales impliquées dans le métabolisme, la prolifération, la différenciation, la survie et la mort cellulaire.

Ce processus peut se résumer en trois étapes :

a) L'initiation qui est irréversible caractérisée par l'accumulation de lésions génétiques au niveau de la cellule : des mutations ponctuelles, suppression des gènes ou encore réarrangement des chromosomes.
b) La promotion définie comme la croissance des cellules préalablement endommagées génétiquement parlant et une accumulation d'autres mutations. Cette étape est considérée comme étant réversible.
c) La progression caractérisée par l'instabilité au niveau du caryotype et la croissance de tumeur maligne.

(Pitot, 1993)

Au Canada, le cancer a représenté 30% des décès pour l'année 2009. Selon les statistiques de la Société Canadienne du Cancer, 41% des Canadiennes et 45% des Canadiens seront atteints d'un cancer au cours de leur vie. Une estimation réalisée en 2014 a affirmé que parmi les 97700 canadiens qui ont été diagnostiqué avec un cancer 40000 en mourront et pour ce qui concerne les femmes parmi les 93600 diagnostiqués 36600 vont en mourir (www.cancer.ca).

Le taux de survie lié au cancer a augmenté au cours des dernières années grâce aux progrès réalisés dans le domaine de la détection et des traitements.

En ce qui concerne la détection des tests de dépistages sont effectué de façon régulière afin de déterminer la présence de tumeur avant l'apparition de tous symptômes. Bien que ces tests ne sont pas totalement infaillibles, leur réalisation permet de 1) augmenter le taux de survie, plus tôt le cancer est détecté meilleurs sont les chances que le traitement fonctionnent 2) améliorer la qualité de vie; une détection précoce peut diminuer la durée du traitement et de la convalescence.

2. GÉNÉRALITÉS SUR LE CANCER COLORECTAL (CCR)

Le cancer colorectal (CCR) est le 4ème cancer avec le nombre de décès le plus élevé dans le monde (OMS, 2015). L'incidence de ce cancer est 18% plus élevés dans les régions plus développés par rapport aux régions qui le sont moins. Les régions où l'incidence est la plus élevée sont : l'Europe, l'Amérique du Nord et l'Océanie tandis que les pays de l'Afrique de l'Est présentent le taux d'incidence le plus bas (Globocan, 2012). Au Canada, il représente le 3ème cancer le plus diagnostiqué chez les hommes et le deuxième chez la femme. Pour l'année 2014, 13% des nouveaux cas de cancer diagnostiqués et 12% des décès par cancer étaient dus au CCR (www.cancer.ca).

Le développement du CCR résulte de l'accumulation de nombreux changements génétiques et épigénétiques. Ces changements provoquent la transformation de la muqueuse intestinale normale en un carcinome par l'activation de pro-oncogènes et l'inactivation des gènes suppresseurs de tumeurs; dans un processus appelé séquence adénome-adénocarcinome (Kinzler *et al.*, 1996, Leslie *et al.*, 2002).

La séquence adénome-adénocarcinome commence avec une mutation du gène APC situé sur le chromosome 5 qui provoque une hyperprolifération de l'épithélium de l'intestin (foyers de cryptes aberrantes). L'hyperprolifération de l'épithélium aboutit à la formation des polypes et de petit adénome. Les petits adénomes peuvent se transformer en adénome intermédiaire s'il y a mutation au niveau du gène K-ras. Ces derniers se développent en adénome tardifs lorsqu'une mutation apparait sur le gène DCC situé sur le chromosome 18. Si le gène p53 subit une mutation ou devient inactive l'adénome tardif peut progresser en un carcinome.

Approximativement 5-10% des cas de CCR diagnostiqués surviennent dans un contexte héréditaire. Il s'agit de la polypose adénomateuse familiale (PAF) ou encore le CCR héréditaire sans polypose (HNPCC ou syndrome de Lynch) (Power *et al.*, 2010, Söreide *et al.*, 2006, Söreide *et al.*, 2009).

Parmi les 90% de cas de CCR sporadiques; 3 voies moléculaires distinctes peuvent expliquer la série d'évènements menant au développement de la maladie; une caractérisée par une instabilité chromosomique (environ 65-85%), une autre caractérisée par une hyperméthylation de l'ADN au niveau des ilots CpG des régions promotrice du gène (15-20%) et la dernière caractérisée par une instabilité au niveau des microsatellites (15%) (Al-Sohaily *et al.*, 2012, Goel *et al.*, 2003, Manceau *et al.*, 2012, Pino *et al.*, 2010). Elles ne sont pas mutuellement exclusives, certaines tumeurs peuvent présenter des caractéristiques appartenant à chacune de ces 3 voies (Goel *et al.*, 2003).

Mis à part les facteurs de risques héréditaires, il existe plusieurs autres éléments considérés comme étant un facteur de risque pour le CCR comme l'âge (Regula *et al.*, 2006), le sexe (Gordon *et al.*, 2010), un historique familial de CCR et des facteurs environnementaux comme la diète (Fung *et al.*, 2013) et la consommation excessive d'alcool (Giovannucci *et al.*, 1995).

3. ALIMENTATION, CANCER COLORECTAL ET MICROBIOTE INTESTINAL

3.1 Alimentation et CCR

L'alimentation est considérée comme étant le facteur de risque majeur en ce qui a trait à cette maladie. Des études épidémiologiques ont montré que plus de 80% des cas de CCR ont été attribué à des facteurs reliés à la diète dans les populations occidentales (Bingham, 2000, Theodoratou *et al.*, 2014).

Lors de la carcinogénèse du CCR, certains éléments de l'alimentation peuvent agir soit en tant que pro-carcinogène ou agent anti-carcinogène (Chan *et al.*, 2010). Les nombreuses études expérimentales et cliniques ont permis de distinguer deux types de régime alimentaire :

a) Le premier ou diète occidentale favorisant le développement du CCR. Elle est caractérisée par une consommation élevée en viande rouge et/ou transformée, aliments transformés, glucides raffinés, sucres (Yusof *et al.*, 2012).
b) Le second ou régime alimentaire « santé » ayant un rôle protecteur face au développement du CCR. Elle est caractérisée par une consommation élevée en fruits, végétaux, grains entiers, poissons, céréales et faible en viande rouge et/ou transformée, et sucres (Magalhaes *et al.*, 2012, Yusof *et al.*, 2012).

3.1.1 Consommation de viande rouge et transformée

Le CCR est le seul cancer dont l'incidence a été associée à une consommation élevée de viande rouge (WCRF/AICR, 2011). Les viandes rouges et transformées ont un taux élevé de composés carcinogènes pouvant causer des dommages à l'ADN (Sutandyo, 2010). Ces composés sont :

a) Les amines aromatiques hétérocycliques (AAH) : sont formés lorsque les viandes sont cuites à haute température pendant longtemps.
b) Les hydrocarbures aromatiques polycycliques (HAP) : résultent de la combustion incomplète de matériaux organiques comme le bois et le charbon.
c) Les composés N-nitrosés produits dans la viande ou par des processus métaboliques dû à des réactions redox d'oxyde d'azote, les nitrites et les nitrates avec des amines secondaires.
d) Le fer hémique : Ce composé se retrouve en grande concentration dans les viandes rouges. Il est capable de former des composés N-nitrosés et également d'augmenter la prolifération cellulaire au niveau de la muqueuse intestinale (Baena *et al.*, 2015)

De nombreuses études épidémiologiques ont été réalisées afin d'évaluer le lien entre le CCR et la consommation de viande rouge (Larsson *et al.*, 2006, Norat *et al.*, 2005,

Smolinska *et al.*, 2010). Une étude épidémiologique internationale a démontré que le risque de développer le CCR augmentait de 35% et de 49% chez des sujets consommant plus de 160g/jour de viande rouge et 25g/jour de viande transformée respectivement (Ferguson, 2010). Il a été rapporté que la consommation en très grande quantité de viande rouge (100g/jour) et transformée (50g/jour) était associée à une augmentation significative du risque de développer le CCR (Chan *et al.*, 2011).

Le rapport émis par World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) classe les preuves associant le CCR à la consommation de la viande comme étant convaincantes (WCRF/AICR, 2011).

Il faut garder à l'esprit que la viande contient des nutriments importants comme les protéines, les acides aminés, les vitamines (Lafarga *et al.*, 2014). La consommation de cette dernière en quantité raisonnable fait partie d'une alimentation balancée. Le risque de développer le CCR résulte de la consommation excessive de la viande.

3.1.2 Consommation de poisson

La consommation de poisson possèderait un potentiel protecteur contre le CCR dû à la présence de vitamine D, de sélénium et d'oméga-3 (Vargas *et al.*, 2012).

Des études ont mis en évidence l'effet protecteur des acides gras de la famille des oméga-3 contre le développement du CCR (Villanueva *et al.*, 2011, Wendel *et al.*, 2009).

Une méta-analyse publiée en 2012 a montré une diminution de 12% du risque de CCR avec une alimentation riche en poisson. La conclusion tirée à partir de cette analyse a été que la consommation de poisson était inversement associée au risque de développer le CCR (Wu *et al.*, 2012). Une autre analyse rapporte que les preuves sur l'association de la consommation de poisson et le risque de CCR demeuraient peu concluantes (Song *et al.*, 2014).

Un rapport émis par WCRF/AICR se basant sur la revue de dix-neuf études de cohorte indique la possibilité qu'une grande consommation de poisson réduise le risque de CCR (Vargas *et al.*, 2012).

3.1.3 Consommation de fruits et de légumes

L'effet positif des fruits et des légumes dans la diminution du risque de développer le CCR serait dû à la présence de nombreux métabolites possédant un potentiel protecteur affectant différentes voies biochimiques (Aggarwal *et al.*, 2006).

De nombreuses études épidémiologiques ont investigué le rôle des composés phénoliques, des fibres, des vitamines présents dans les fruits et végétaux par rapport à la réduction des cancers (Gorham *et al.*, 2007, J Tarraga Lopez *et al.*, 2013, Vauzour *et al.*, 2010).

Les résultats concernant la diminution du risque de CCR par la consommation de fruits et de végétaux sont très contradictoires. Le consortium EPIC « European Prospective Investigation into Cancer and Nutrition » en 2012, a indiqué qu'il existait une forte relation entre la consommation de fruits et de légumes chez des participants non-fumeurs et physiquement actifs et la réduction du risque du CCR (Gonzalez *et al.*, 2012, Hooper *et al.*, 2008). Le rapport d'une méta-analyse réalisée en 2011 montre une réduction de 10%, 9% et 8% du risque de développer un CCR avec la consommation de fruits, de légumes et des deux respectivement. Ces résultats montrent que la consommation de fruits et de végétaux n'affecteraient en rien le risque de développer la maladie (Aune *et al.*, 2011b). Une autre étude réalisée en 2007, a montré une diminution de 26% du risque de développer le cancer du côlon au niveau distal de l'organe chez les individus consommant beaucoup de fruits et de légumes par rapport à ceux dont la consommation était au niveau le plus bas. Cette réduction n'a pas été observée au niveau proximal du côlon (Koushik *et al.*, 2007).

En prenant compte de ces différentes études on peut supposer que la consommation de fruits et de légumes serait inversement associée au cancer du côlon principalement au niveau distal ; et que le potentiel protecteur serait effectif chez des individus adoptant un mode de vie sain.

3.1.4 Consommation de céréales et de grains entiers

Les grains entiers sont une source importante de fibres. Ces dernières sont associées à la réduction du cancer par divers mécanismes comme l'augmentation du volume des

selles, la diminution du temps du transit intestinal et la modification de la flore intestinale (Bingham, 1990, Lipkin *et al.*, 1999). Ils contiennent également d'autres éléments possédant un potentiel protecteur comme les vitamines, les acides phénoliques et les phytoestrogènes (Cotterchio *et al.*, 2006, Webb *et al.*, 2005).

L'effet de fibres alimentaires et d'aliments composés de grains entiers sur l'incidence du CCR a été évalué.

Dans une méta-analyse réalisée en 2011, des chercheurs ont démontré que pour chaque 10g/jour de fibres alimentaires et de fibres de céréales ingérés, 10% de diminution dans le risque de développer un CCR a été observé et 20% de diminution pour 20g/jour de grains ingérés a été observé (Aune *et al.*, 2011a).

Cette étude prouve que la consommation de grains entiers, de fibres alimentaire et de céréales est inversement associée au développement de la maladie.

Le tableau suivant résume les effets des différents aliments sur le risque de développer un CCR et leur niveau de confiance accepté de nos jours.

Tableau 1: Influence de certains aliments sur le développement du cancer colorectal.

Aliments	**Effets sur le risque du cancer colorectal**	**Niveau de confidence des preuves**
Viande rouge et/ou transformée	Augmentation	Convaincantes
Fruits et végétaux	Diminution	Limitées et suggestives
Grains entiers	Diminution	Probables
Poissons	Diminution	Suggestives

Tableau adapté de (WCRF/AICR, 2011)

Différents facteurs sont capables d'influencer la carcinogénèse du CCR. Les informations tirées de la littérature montre que l'identification d'un aliment ou d'un élément nutritif et du degré d'influence de ce dernier sur le développement de la maladie est une tâche ardue et complexe. Cependant, les différentes études épidémiologiques et cliniques suggèrent que l'adoption d'un mode de vie sain et d'une alimentation saine peut certainement réduire la mortalité et la morbidité liée à ce cancer.

3.2 Microbiote intestinal et CCR

Le tractus gastro-intestinal contient la plus grande quantité de bactéries comparé au reste du corps humain. Le dénombrement bactérien de la flore intestinale est 10X plus élevés que celui de toutes les cellules somatiques et germinales (Savage, 1977, Turnbaugh *et al.*, 2009a) du corps humain. Selon certaines études la flore intestinale est héritée et considérée comme étant généralement stable. Elle est modulée par l'alimentation et l'exposition à certains médicaments (Azcárate-Peril *et al.*, 2011, Penders *et al.*, 2006). La représentation des embranchements bactériens au niveau du microbiote intestinal est la suivante : *Firmicutes* 30.6-83% (*Clostridium, Rumonicus, Eubacterium*…), *Bacteriodetes* 8-48% (*Bacteroides*), *Actinobacteria* 0.7-16.7% (*Bifidobacterium, BF*) et *Proteobacteria* 0.1-26.6% (*Enterobacteriace*) (Şerban, 2011, Tap *et al.*, 2009).

Ce n'est que récemment que le potentiel pro-carcinogène de certaines bactéries colonisant le colon a été reconnu. Contrairement aux autres types de cancer (estomac, foie, col de l'utérus) où un seul agent infectieux est responsable du développement de la maladie (de Martel et al. 2010), les mécanismes menant au CCR sont le résultat d'une grande variété d'espèces bactériennes (Cuevas-Ramos *et al.*, 2010, Kostic *et al.*, 2013, Wu *et al.*, 2009).

Les analyses réalisées au niveau du microbiote de patients atteints du CCR sur les sites tumoraux et non-tumoraux ont amené à une modélisation du rôle de la flore intestinale sur le développement de la maladie. Le modèle porte le nom de : « ***driver-passenger model*** ». Ce modèle suggère que les bactéries « **driver** » ou « **pilotes** » initient le développement du CCR et elles sont ensuite remplacées par les bactéries « **passengers** » ou « **passagères** » qui sont capable de promouvoir le développement du CCR (Tjalsma, 2012).

3.2.1 Les bactéries « drivers »

Les bactéries pilotes ou encore « ***drive**r* » : capables de favoriser la carcinogénèse en déclenchant une inflammation, en augmentant la prolifération des cellules de la muqueuse intestinale ou encore en induisant des dommages à l'ADN (Tjalsma, 2012).

a) *Enterococcus faecalis* : Les souches diffèrent par rapport à leur capacité de produire des espèces réactives à l'oxygène. Ils sont capables d'induire des dommages à l'ADN et des instabilités au niveau du génome (Sears *et al.*, 2014). Des études ont démontré leur rôle en tant que précurseur de la carcinogénèse du CCR (Wang *et al.*, 2008, Yang *et al.*, 2013b).

Le potentiel oncogène de certaines souches d'*E. faecalis* a été démontré. En effet leur capacité de produire de l'anion superoxide a entrainé l'induction de dommages au niveau de l'ADN, des colites et des cancers chez les souris Il^{-10} tandis que celles ne produisant pas l'anion n'ont pas provoqué tous ces dégâts dans la muqueuse intestinale des souris (Wang *et al.*, 2012)

b) *Bacteroides fragilis* : cette bactérie contient une toxine appelée « *B. fragilis toxin* » (BFT). BFT est une toxine dépendante du zinc capable de cliver la protéine E-cadhérine provoquant ainsi une stimulation de la voie de signalisation β-catenine/Wnt nucléaire qui va entrainer une augmentation de la prolifération des cellules des carcinomes de colon et l'expression du pro-oncogène (Petit *et al.*, 2013, Soler *et al.*, 1999). Plusieurs études ont démontré les mécanismes impliqués dans la formation de tumeur intestinale avec la bactérie *B. fragilis* comme précurseur. (Goodwin *et al.*, 2011, Tosolini *et al.*, 2011).

Une étude a rapporté que les souris $Apc^{Min/+}$ colonisées avec la bactérie *B. fragilis* développaient des cas sévères de colite et le nombre de tumeur du colon avait augmenté comparé aux souris colonisées avec la même souche de bactérie mais ne produisant pas la toxine BFT (Wu *et al.*, 2009).

c) *Esherichia coli* : les souches adhérentes-invasives jouent un rôle dans les cas de CCR associés à la colite. Certaines souches portent le gène « *polyketide*

synthase » (pks) encodant une génotoxine : la colibactine (Nougayrède *et al.*, 2006).

Arthur *et al.* (2012) ont montré que chez les souris Il^{-10} traités avec de l'azoxyméthane (AOM) et colonisés avec la souche d'*E. coli* contenant le gène *pks*; il y a augmentation du nombre de tumeurs comparé aux souris du groupe contrôle

3.2.2 Les bactéries « passengers »

Les bactéries passagères ou « ***passengers*** » : qui normalement ne colonisent pas la flore intestinale d'un individu sain ou s'y retrouvent en faible quantité. L'environnement tumoral (présence d'inflammation au niveau du côlon, développement d'un adénome ou d'un carcinome) favorise la croissance de ces dernières. Elles sont considérées comme étant des bactéries opportunistes (Tjalsma, 2012)

a) *Streptococcus gallolyticus* : Il existe plusieurs observations cliniques qui lient la présence intestinale de cette bactérie avec le CCR (Abdulamir *et al.*, 2011, Klein *et al.*, 1977).

Une méta-analyse réalisée en 2012 a révélé que 43% des patients infectés par cette bactérie présentaient des adénomes au niveau du colon et 18% de ces patients avaient des carcinomes. Ces valeurs sont significativement plus élevées comparées à la prévalence retrouvée au niveau de la population générale (Boleij *et al.*, 2012b).

Des études dans un modèle cellulaire ont démontré que comparativement aux autres bactéries de la flore intestinale, la prolifération de *S. gallolyticus* augmente en présence des métabolites excrétés par les cellules tumorales (Boleij *et al.*, 2012a, Hirayama *et al.*, 2009).

Se basant sur les différentes études réalisées une hypothèse a été avancée par les chercheurs. Elle se résume ainsi : la diminution de la fonction de la barrière épithéliale dans des tumeurs intestinales favoriserait la croissance de *S. gallolyticus*. Également ces dernières joueraient un rôle dans l'augmentation de la croissance tumorale par

l'induction de cytokines pro-inflammatoires (ex : IL-8) ou par l'expression de la cyclooxygénase-2 (COX-2) (Abdulamir *et al.*, 2010, Boleij *et al.*, 2013).

b) *Fusobacterium* spp. : Cette bactérie n'a pas été associée à une caractéristique particulière des tumeurs intestinales, mais des études ont démontré qu'elle était beaucoup plus abondante chez des patients atteints du CCR (Ahn *et al.*, 2013, Kostic *et al.*, 2012) et elle serait responsable de la progression de la maladie du stade d'adénome à celui de cancer (Bashir *et al.*, 2015).

Castellarin *et al.* (2012) ont rapporté qu'il y avait une représentation excessive de ces bactéries au niveau des cellules tumorales du CCR et ont également confirmé que ces dernières étaient envahissantes.

Une recherche menée en 2013, a été en mesure d'identifier la capacité de *F. nucleatum* d'accélérer la carcinogénèse dans l'intestin grêle et le côlon chez les souris $Apc^{Min/+}$ (Kostic *et al.*, 2013). Cependant plus d'études sont nécessaires afin de mieux définir le lien entre les bactéries du microbiote et le CCR et également mieux comprendre les mécanismes impliqués dans l'induction de la carcinogénèse du CCR.

La dysbiose de la flore intestinale est un facteur déterminant en ce qui a trait au développement du CCR. La modulation du microbiote intestinal par des bactéries Tout comme certaines espèces bactériennes agissent en tant qu'agent promoteur de la carcinogénèse du CCR (ex : bactéries ***drivers*** ou ***passengers***), d'autres peuvent prévenir ou réduire le risque de développer la maladie. Ces bactéries appelées probiotiques sont définies comme étant des microorganismes qui consommés dans des quantités adéquates ont la capacité de conférer des propriétés bénéfiques pour la santé de l'hôte. Les espèces bactériennes les plus communément comme probiotique sont *Bifidobacterium* et *Lactobacillus* (Hill *et al.*, 2014).

3.2.3 Les probiotiques

La relation spécifique entre la composition microbienne de l'intestin et le développement du CCR n'est pas encore parfaitement comprise, cependant l'hypothèse développée

apres de noubreuses études cliniques est que le microbiote intestinal des patients atteints du CCR différait de celui des patients sains. En effet les comptes de *Fusobacterium*, *Proteobacteria*, *Bacteroides* et certaines souches de *Clostridium* étaient beaucoup plus élevé et le compte de plusieurs souches de *Lactobacillus* de étaient plus bas chez les sujets atteints du CCR par rapport aux sujets sains (Castellarin *et al.*, 2012, Scanlan *et al.*, 2008, Sobhani *et al.*, 2013, Wong *et al.*, 2006).

La capacité des probiotiques de moduler à composition et la diversité de la flore intestinale afin de réduire la présence de bactéries associées au développement du CCR a été démontrée dans de nombreuses études (Zhang *et al.*, 2012b, Zhu *et al.*, 2012).

Par exemple l'administration de *L. grasseri* OLL2716: LG21 chez des patients atteints du CCR a augmenté le compte de *Lactobacillus* et diminué le compte de *Clostridium perfigens* (Ohara *et al.*, 2010) et aussi l'administration combinée de *Lactobacillus rhamnosus* LC705 et de *Propionibacterium freudenreichii* ssp. *shermanii* JS a augmenté de manière significative le compte de lactobacilles et de *Propionibacteria spp* dans les fèces (Hatakka *et al.*, 2008).

3.3 Alimentation et microbiote intestinal

L'alimentation influence grandement la composition et la diversité de la flore intestinale. Les composants alimentaires qui ne sont pas digérés dans le système digestifs servent de substrat pour le métabolisme des bactéries du microbiote intestinal (Graf *et al.*, 2015). Les changements alimentaires sont capables de provoquer un décalage brusque au niveau de la diversité et de la composition du microbiote intestinal. La flore intestinale d'un individu sain est composé principalement de bactéries anaérobies strictes de deux *phyla* differents : *Firmicutes* et *Bacteriodetes* , en plus des *Actinobacteria*, *Proteobacteria* et *Verrucomicrobia* (Eckburg et al., 2005).

De nombreuses études ont observé des modifications dans le microbiote intestinal après un changement au niveau de la diète (Hildebrandt *et al.*, 2009, Turnbaugh *et al.*, 2009b).

De nombreuses études ont évalué l'effet de divers aliments et d'éléments nutritifs sur la composition et la diversité de la flore intestinale.

3.3.1 Viande rouge et flore intestinale1

L'origine et la quantité de gras de la viande rouge peuvent occasionner un changement au niveau de la composition de la flore intestinale qui favoriserait peut-être le développement du CCR (Hildebrandt *et al.*, 2009). Une équipe de chercheur a rapporté que la consommation de la viande rouge favorisait la croissance des bactéries comme *Desulfovibrio*, *Desulfobacter,* et *Desulfobulbus* (Attene-Ramos *et al.*, 2007). Le principal métabolite produit par ces bactéries est le sulfure d'hydrogène (H_2S), ce dernier est considéré comme un produit extrêmement dangereux pour la muqueuse intestinale de par sa capacité génotoxique et inflammatoire. Il est capable de causer spécifiquement des dommages à l'ADN par la production de radicaux libres. L'effet de trois types de viandes (bœuf, poisson et poulet) cuits de deux façons différentes (frit et bouillit) sur la composition du microbiote a été évalué. Il a été démontré que la cuisson influençait la croissance de *Clostridium spp*, la concentration est plus élevée dans les viandes frites comparées à celles qui ont été bouillies. Aussi la concentration de *Bifidobacterium spp*, le groupe de bactéroïdes et aussi le groupe *Coriobacterium/Atopobium* varient selon le type de viande. Ces résultats suggèrent que la méthode de cuisson et le type de viande peuvent influencer la flore intestinale (Shen *et al.*, 2010a).

3.3.2 -Grains entiers et flore intestinale

Les grains entiers sont capable de jouer un rôle important dans le maintien d'une flore intestinale en santé et aussi dans l'augmentation de la diversité bactérienne de cette dernière (Martinez *et al.*, 2013). Il existe plusieurs études qui ont suggéré que la consommation de produits à base de grains entiers favoriserait la croissance de *Bifidobacterium* spp par exemple une étude a démontré que la consommation de céréales à base de son de blé entier (GE) pour le petit déjeuner a significativement augmenté le nombre de *Bifidobacterium* spp. et de *Lactobacillus/Enterococcus* par rapport aux sujets ayant consommé des céréales à base de son de blé raffinés (GR)

(Costabile *et al.*, 2008). Car contrairement aux grains qui ont été raffinés, les grains entiers sont beaucoup plus riches en substances possédant des propriétés chimio préventifs comme les fibres, les composés antioxydants, les composés phytochimiques (Song *et al.*, 2015)

Une autre étude a montré que la consommation de céréales enrichies de mais après 3 semaines avait augmenté le nombre de bifidobactéries dans les fèces des volontaires (Carvalho-Wells *et al.*, 2010).

Les produits composés de grains entiers sont également capables de réduire la fermentation protéique au niveau de l'intestin (Ross *et al.*, 2013).

3.3.3 Fruits, légumes et flore intestinale

Plusieurs études ont démontré l'influence de la consommation des fruits et des légumes sur la composition de la flore intestinale.

Cet effet bénéfique serait dû à la présence de polyphénols et de fibres. Seulement 10% des polyphénols sont absorbés dans l'intestin grêle, et les 90% restant se retrouvent dans le côlon. Les bactéries de la flore intestinale sont capables de convertir les polyphénols en composés bioactifs capables de moduler la population du microbiote intestinal et ainsi affecter la santé de l'hôte (Bosscher *et al.*, 2009, Hollman *et al.*, 1999).

Des études ont démontré que certaines doses de polyphénols pouvaient modifier la composition du microbiote en inhibant la croissance de certaines souches bactériennes néfastes comme *Clostridium perfringens*, *Clostridium difficile*, *Bacteroides* tout en favorisant la croissance d'autres microorganismes bénéfiques comme *Bifidobacterium* spp, *Lactobacillus rhamnosus*, *L. reuteri* et *L. acidophilus* (Dolara *et al.*, 2005, Lee *et al.*, 2006a, Molan *et al.*, 2009, Pozuelo *et al.*, 2012, Yamakoshi *et al.*, 2001).

Il a été démontré que les légumes crucifères comme le brocoli et le chou-fleur avaient la capacité d'altérer la flore intestinale. La consommation des végétaux crucifères a favorisé la croissance des bactéries suivantes *Eubacterium hallii*, *Phascolarctobacterium faecium, Alistipes putredinis*, et *Eggerthella spp* (Li *et al.*, 2009a). La consommation de jus de bleuet augmentait la quantité de *Bifidobacterium*

spp dans les selles des volontaires comparés à ceux ayant bu le placebo (Vendrame *et al.*, 2011).

Bien que de nombreuses études soulignent l'effet bénéfique des composés phénoliques sur la flore intestinale, il existe quand même des limitations avec les résultats obtenus. Plus d'expérience sont nécessaires notamment au niveau clinique afin de mieux comprendre .l'effet spécifique des phénols au niveau de la flore intestinale, également les interactions des métabolites des phénols et des composés produits par certaines bactéries présentes au niveau du microbiome.

4 PREUVES DE PREVENTION DE CANCER A PARTIR DE PETITS FRUITS, HERBES MEDICINALES ET PROBIOTIQUES

A. LES PROBIOTIQUES

Les bactéries probiotiques sont définies comme étant des micro-organismes vivants qui peuvent conférer des effets bénéfiques à l'organisme dit « hôte » lorsqu'ils sont administrés en quantité adéquates (Gobbetti *et al.*, 2010, Sanders *et al.*, 2010). Les effets bénéfiques sont spécifiques à la souche non à l'espèce. Les probiotiques sont consommés habituellement sous forme de produits fermentés ou encore de suppléments de bactéries déshydratées. Les propriétés de ces bactéries sont utiles pour assister le système gastro-intestinal par le maintien d'un pH optimal, la stimulation du système immunitaire, en aidant pour une meilleure digestion etc… (Watson *et al.*, 2015).

a) Preuves in vitro de l'effet antinéoplasique de probiotiques

Tiptiri-Kourpeti *et al.* (2016) ont démontré que la souche de *Lactobacillus casei* ATCC 393 pouvait inhiber la prolifération des cellules murines (CT26) et humaines (HT29) de CCR .Une diminution de la viabilité de ces cellules a été observée lorsqu'elles ont été incubées pendant une journée à une concentration de 10^9UFC/ml de *L.casei* vivantes. La réduction a été de 78% pour les cellules de HT29 et 52% pour CT26.

Orlando *et al.* (2016) a démontré que la présence de *Lactobacillus rhamnosus* GG provoque une inhibition de la prolifération, l'induction de l'apoptose et l'arrêt du cycle cellulaire au niveau des cellules Caco-2, HT-29 and SW480.

Kahouli *et al.* (2016) ont démontré que deux extraits provenant de *L.reuteri* NCIMB 701359 (le surnageant et le milieu conditionné) avaient la capacité de d'inhiber la prolifération et d'induire l'apoptose au niveau des cellules SW-480.

b) *Preuves in vivo de l'effet antinéoplasique de probiotiques*

Kuugbee *et al.* (2016) ont démontré que la consommation d'un cocktail de probiotiques composé d'oligofructose-maltodextrine enrichie de Lactobacillus acidophilus, Bifidobacteria bifidum et Bifidobacteria infantum était capable de moduler le microbiote de la flore intestinale et aussi de réduire le développement du cancer colorectal en diminuant l'incidence, la multiplicité, le compte et le volume de tumeurs.

La souche de *Lactobacillus plantarum* LS/07 consommé par des rats chez qui le CCR a été préalablement induit a augmenté l'induction des enzymes β-galactosidose et β-glucosidase et diminuait l'activité de la β-glucuronidase (Hijová *et al.*, 2016)

La consommation de *Lactobacillus rhamnosus* GG CGMCC 1.2134 peut réduire significativement le compte, la multiplicité et l'incidence des tumeurs. La consommation de cette souche de probiotique est capable de réduire également l'expression de la β-caténine, des protéines inflammatoires et des protéines anti-apoptotiques mais d'augmenter l'expression des protéines pro-apoptotiques (Gamallat *et al.*, 2016).

Shin *et al.* (2016) ont démontré que les souches de *Lactobacillus plantarum* BF-LP284 tuées par la chaleur ont la capacité de diminuer la taille des tumeurs chez des souris BALB/c.

c) *Preuves cliniques de l'effet antinéoplasique de probiotiques*

Une étude clinique réalisée par Yang *et al.* (2016) a démontré que l'administration de probiotiques à des patients atteints de CCR sur le point de subir une opération de résection peut influencer la récupération de la fonction intestinale.

Gianotti *et al.* (2010) ont démontré que l'administration préopératoire de *L. johnsonii* (La1) était beaucoup plus efficace que celle de *B. longum* (BB536). En effet la première souche est capable d'adhérer au niveau de la muqueuse intestinale et ainsi moduler la flore intestinale en diminuant la concentration de bactérie pathogène et aussi en atténuant l'immunité locale.

B. PETITS FRUITS ET SES COMPOSÉS BIOACTIFS

Les petits fruits sont considérés internationalement comme aliments dit « fonctionnels » de par leur richesse en composés bioactifs : vitamines, fibres, minéraux et composés phénoliques. Les composés phénoliques sont des métabolites secondaires produits par les plantes. Les principales classes de composés phénoliques retrouvés au niveau des petits fruits sont : les anthocyanes (malvidine, cyanidine, delphinidine), les acides phénoliques (acide gallique, acide caféique), stillbenoides (reservatrol), les flavonols et les flavanols (Nile *et al.*, 2014, Szajdek *et al.*, 2008). La quantité de composés phénoliques dans les fruits est déterminée par la région, la méthode de culture, les conditions météorologiques, le temps de récolte, le temps et les conditions de stockage (Zhang *et al.*, 2008b). Dans les dernières années les polyphénols ont été soumis à des recherches intensives à cause de leur effet potentiellement bénéfique pour la santé en ce qui a trait à réduction de l'incidence de certains cancers, et des maladies cardio-vasculaires (Paredes-López *et al.*, 2010b). Leurs effets bénéfiques ont principalement été attribués à leur capacité antioxydante (Seeram, 2008)

1. Preuves in vitro de l'effet antinéoplasique de petits fruits et de leurs composés bioactifs

López de las Hazas *et al.* (2016) ont réalisé une étude qui a prouvé que des extraits de raisins et de framboises riches en anthocyanes et leurs métabolites possèdent un effet apoptotique sur les cellules HT-29.

Une étude menée par Kondo *et al.* (2011) a prouvé qu'une fraction riche en acide ursolique provenant de canneberges avait la capacité d'inhiber la croissance des cellules DU145 (cellules de cancer de la prostate).

Un extrait de myrtilles riches en anthocyanes a réduit de façon significative le niveau d'espèces réactives de l'oxygène dans les cellules HT-29 (250µg/ml pendant 24 heures), et dans les cellules Caco-2 (50µg/ml pendant 1 heure).

2. Preuves in vivo de l'effet antinéoplasique des petits fruits et de leurs composés bioactifs

Il a été démontré que la consommation d'une boisson faite de prune diminuait le nombre de foyer de cryptes aberrantes de 48% chez des rats préalablement traités avec l'AOM, et réduisait également la prolifération de cellules mucosales de 24% (Banerjee *et al.*, 2016).

La consommation d'extrait de myrtilles riche en anthocyanes (concentré à 10%),chez des souris Balb/c préalablement induites au CCR par injection de AOM (concentration de 3%) et de DSS (concentration de 5%), a significativement réduit le nombre de tumeurs (Lippert *et al.*, 2017).

3. Preuves cliniques de l'effet antinéoplasique des petits fruits et de leurs composés bioactifs

Une étude réalisée par Wang *et al.* (2010) a prouvé que la consommation de framboises noires peut induire la déméthylation des gènes suppresseurs de tumeurs et de moduler d'autres biomarqueurs liés au développement du CCR.

Dans une autre étude, il a été observé que des framboises noires lyophilisées consommées durant plus de 10 jours par des patients atteints de CCR ont augmenté la concentration de facteur de stimulation des colonies de granulocytes et de macrophages (GM-CSF) et diminué celles des interleukine-8 (Mentor-Marcel *et al.*, 2012).

C. L'HIBISCUS SABDARIFFA

L'hibiscus sabdariffa est une fleur considérée comme étant un aliment populaire et une plante médicinale dans certaines régions comme l'Afrique de l'Est, le Sud de l'Asie et le Mexique (McKay *et al.*, 2010b). Commercialement on la retrouve sous forme de tisane, de confiture ou encore de jus. Dans la médecine traditionelle, elle est utilisée pour la

prévention de maladie cardio vasculaire, de l'hypertension ou encore de l'hépatite (Salah *et al.*, 2002). Les composés phénoliques présents en grande quantité au niveau de cette plante sont les anthocyanes et l'acide ascorbique (Jung *et al.*, 2013).

a) Preuves in vitro de l'effet antinéoplasique de l'hibiscus sabdariffa et de ses composés bioactifs.

Wu *et al.* (2016) ont démontré que les anthocyanines de l'hibiscus sabdariffa l. induisaient une nécrose et une autophagie chez les cellules du cancer du sein MCF-7.

Une étude a démontré qu'un extrait d'hibiscus sabdariffa est capable d'induire l'apoptose chez des cellules du cancer gastrique. (Lin *et al.*, 2007)

b) Preuves in vivo de l'effet antinéoplasique de l'hibiscus sabdariffa et de ses composés biologiques

Sunkara *et al.* (2015) ont démontré que l'administration d'hibiscus sabadarifa sous forme de jus et d'aliments a réduit l'incidence, la multiplicité et la taille des tumeurs chez les rats comparés au contrôle.

Toutefois, à ce jour il n'y a pas d'études cliniques démontrant l'activité néoplasique de cette plante.

5 QUELQUES MÉCANISMES IMPLIQUÉS DANS LA PRÉVENTION DU CCR

L'une des stratégies les plus prometteuses dans la lutte contre le cancer est la chimioprévention. Elle consiste en l'inhibition ou encore le ralentissement du processus de la carcinogénèse par l'utilisation de substances pharmacologiques ou nutritionnelles (Hawk *et al.*, 2005, Surh, 2003). Les interventions chimiopréventives peuvent être appliquer durant les deux premières étapes de la carcinogénèse; dès l'apparition des défauts moléculaires jusqu'à l'accumulation d'aberrations moléculaires, d'histopathologies et cellulaires qui caractérisent la maladie (Surh, 2003).

Les substances chimiopréventives sont divisées en trois catégories dépendamment de la manière qu'elles ont de diminuer ou de protéger la vulnérabilité des tissus par rapport à la carcinogénèse :

- Les inhibiteurs de la formation de substances carcinogènes
- Les agents bloquants (anti-initiation)
- Les agents de suppression (anti-prolifération/anti-progression)

En tenant compte du lien existant entre le développement du CCR, l'alimentation et la flore intestinale, de nombreuses études épidémiologiques et cliniques ont démontré l'effet antinéoplasique de bactéries probiotiques et/ou de leurs composantes et également de composés phénoliques présents au niveau des petits fruits ou des plantes. La modulation des enzymes de phases II, l'inhibition de la prolifération des cellules cancéreuses, la réduction et/ou inhibition de la formation de cryptes aberrantes, l'altération de l'activité enzymatique de la flore intestinale et la capacité antioxydante représentent quelque uns des mécanismes par lesquels les éléments mentionnés ci-dessus exercent leur effet antinéoplasiques.

5.1 La modulation des enzymes de phase II

Les enzymes de phase II encore appelées enzymes détoxifiantes ont la capacité de transformer les substances carcinogènes en métabolites moins toxiques ou encore inactives. Dans cette catégories d'enzymes on retrouve : glutathionne-S-transférase (GST), UDP glucuronyltransferase (UDPGT) et NAD(P)H quinone réductase (ou quinone réductase) (Sheweita *et al.*, 2003).

Les petits fruits (plus précisément les composés phénoliques formant leur profil phytochimique) ainsi que les bactéries probiotiques (et leurs différentes composantes) ont la capacité de moduler l'activité des enzymes détoxifiantes A titre d'exemple Umesalma et al. (2014) ont démontré que l'administration d'acide ellagique, un phénol retrouvé à de très grandes concentrations dans les petits fruits comme la fraise, la canneberge, la framboise et les raisins, chez des rats Winstar mâles souffrant du CCR

augmentait l'activité spécifique d'enzymes de phase II comme la GST, l'UDPGT et la DTD.

5.2 L'inhibition de la prolifération des cellules cancéreuses

Un des faits saillants qui se produit lors de la carcinogénèse est l'habilité qu'acquiert les cellules de proliférer indéfiniment (Evan *et al.*, 2001). Inhiber cette première étape représente une excellente stratégie pour la chimioprévention. Par exemple la delphidine et la malvidine, isolées d'un extrait de bleuets riche en anthocyanines ont démontré leur habilité à inhiber la prolifération de deux lignées de cellules cancéreuses du colon (DLD-1 et COLO205) par induction de l'apoptose (Zu *et al.*, 2010)

Tiptiri-Kourpeti *et al.* (2016) ont démontré que la souche de *Lactobacillus casei* ATCC 33 à une concentration de 10^9UFC/ml a inhibé à 78% la prolifération des cellules HT-29, une lignée cellulaire modèle de CCR, après une incubation de 24 heures.

5.3 La réduction et/ou l'inhibition de la formation des foyers de cryptes aberrantes

Les cryptes aberrantes (CA) sont considérées comme étant des lésions précurseures du CCR et sont utilisées comme biomarqueur. Elles sont caractérisées par des épithélia plus épais et large montrant des ouvertures luminales altérées comparées aux cryptes normales (Gupta *et al.*, 2007). De nombreuses études cliniques ont prouvé que les patients atteints de CCR présentaient un nombre plus élevé de CA comparés ceux n'ayant pas de cancer (Commane *et al.*, 2005). da Silva Almeida *et al.* (2015) ont démontré que l'administration de *Lactobacillus casei* CRL 1014 (10^9UFC/ml) réduit significativement la formation de foyers de cryptes aberrantes (FCA) chez les rats mâles Winstar après 5 mois d'injection de DMH. L'effet d'un extrait de raisin riche en anthocyanines sur les biomarqueurs du cancer colorectal a été étudié. Les chercheurs ont démontré la capacité de ce dernier de diminuer efficacement la formation de FCA induite par injection d'azoxyméthane chez des rats mâles Fisher 344 (Lala *et al.*, 2006).

Boateng et al. (2007) ont démontré que l'administration de jus de canneberges (d'une concentration de 20%) chez des rats souffrant du CCR a réduit de 77% des FCA comparé au contrôle.

5.4 L'altération de l'activité enzymatique de la flore intestinale

Il existe un grand nombre d'enzymes que l'on retrouve principalement dans les suspensions fécales qui peuvent générer des métabolites carcinogènes au niveau de l'intestin. Ces enzymes sont la β-glucuronidase, la β-glucosidase, l'azoréductase et l'uréase. Certaines souches de probiotiques ou encore de produits à base de probiotique sont capables de moduler l'activité de ces enzymes. Une étude menée par Kim *et al.* (2008) a démontré que la bactérie *Bifidobacterium. adolescentis* SPM0212 inhibait l'activité d'enzymes fécales telle que la β-glucosidase, β-glucuronidase lorsqu'elle est ingérée quotidiennement par des rats pendant trois semaines. Des études ont également démontré la capacité des composés phénoliques de réduire et/ou d'inhiber l'activité de ces enzymes : Venkatachalam *et al.* (2016) ont montré que l'acide rosmarinique réduisait significativement l'activité de la β-glucosidase, la β-glucuronidase, β-galactosidase, mucinase, nitroreductase et la sulphatase comparé au contrôle positif.

5.5 La capacité antioxydante

Le stress oxydatif se produit lorsque la production d'espèces réactives à l'oxygène (ROS) est excessive. La cellule se retrouve ainsi dans l'incapacité de les métaboliser ce qui provoque l'accumulation de hauts niveaux de ROS. Une concentration élevée de ROS au niveau de la cellule est associée à divers types de dommages au niveau du matériel génétique comme : les modifications des bases et brisure des brins d'ADN, le réarrangement des chromosomes, l'hyper ou l'hypoméythylation de l'ADN (Lim *et al.*, 2008, Valko *et al.*, 2007, Valko *et al.*, 2006).

Une souche de *Bifidobacterium longum* ATCC 15708 et de *Lactobacillus acidophilus* ATCC 4536 ont affiché une activité antioxydante en inhibant la peroxydation de l'acide

linoléique par 28% et 48% respectivement et également en démontrant leur habileté à piéger un radical libre par 21%-52% respectivement (Baffoni *et al.*, 2012). Rodríguez-Ramiro *et al.* (2011) ont démontré qu'un extrait de polyphénols de cacao et les proacyanidines B2 présent dans le cacao pouvait protéger les cellules en contrecarrant plusieurs types de radicaux libres et également en régulant à la hausse plusieurs défenses antioxydants. Mensah *et al.* (2015) ont démontré qu'un extrait hydro-éthanolique de calices d'*Hibiscus sabdariffa* possédait un potentiel antioxydant important. Une fraction riche en flavonols extraite de produit dérivé de la canneberge présente un plus forte activité antioxydante que les fractions riche en acide phénoliques et en monomères de flavan-3-ol (Rupasinghe *et al.*, 2016).

6 HYPOTHÈSES

Les produits ayant retenu l'attention dans ce projet sont les petits fruits (comme les canneberges), les plantes médicinales (Fleur de Jamaïque) et un lait fermenté produit par 3 souches de lactobacilles (Bio-K+^{MD}).

Les hypothèses suivantes ont été énoncées:

1. Les bactéries probiotiques *Lactobacillus acidophilus* CL1285^{MD} *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} présentes dans le lait fermenté Bio-K+^{MD} et leurs composants membranaires réduisent le développement des foyers de cryptes aberrantes et inhibent la croissance des cellules cancéreuses respectivement.
2. La combinaison des composants membranaires extraits des bactéries *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} avec les composés phénoliques issus des petits fruits et de plantes médicinales agissent en synergie afin d'inhiber la croissance des cellules cancéreuses et d'induire l'activité d'une enzyme détoxifiante du foie.

7 OBJECTIFS

a) Évaluer l'effet des composants membranaires extraits des bactéries probiotiques présentes dans le lait fermente Bio-K+^{MD} et des composés phénoliques extraits de concentré de jus de canneberges et de calices d'*Hibiscus sabdariffa* L. sur :
   - La prolifération des cellules cancéreuses.
   - L'induction de l'activité de la quinone réductase.

b) Déterminer l'effet synergique de la combinaison des composants membranaires et des composés phénoliques sur:
   - La prolifération des cellules cancéreuses.
   - L'induction de la quinone réductase

c) Déterminer l'effet dose-réponse du lait fermenté de Bio-K+^{MD} dans la réduction des lésions précancéreuses (foyers de cryptes aberrantes) et l'activité d'enzymes impliquées dans le CCR telles que les enzymes détoxifiantes du foie (QR et GST) et les enzymes fécales (β-glucosidase et β-glucuronidase).

d) Déterminer l'effet de différentes fractions du lait fermenté de Bio-K+^{MD} et des composantes membranaires extraites des bactéries probiotiques dans ce lait fermenté dans la réduction des lésions précancéreuses (foyers de cryptes aberrantes) et l'activité d'enzymes impliquées dans le CCR tels que les enzymes détoxifiantes du foie (QR et GST) et les enzymes fécales (β-glucosidase et β-glucuronidase).

8 METHODOLOGIE

Les expériences ont été réalisées en utilisant deux types de modèles : un modèle cellulaire et un modèle animal.

Modèle cellulaire : Dans un premier temps les composés phénoliques des calices d'*Hibiscus sabdariffa* L. ont été extraits en utilisant les méthodes utilisées par Seeram *et al.* (2004), Neto *et al.* (2006) et (Wu *et al.*, 2005) et le jus de canneberges concentré a été fractionné par CLHP selon la méthode de Caillet *et al.* (2012b) puis des composés membranaires de bactéries probiotiques ont été extraits d'une biomasse contenant les bactéries *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} utilisant les méthodes de Kim *et al.* (2002) et (Signoretto *et al.*, 1998). Ensuite les effets chimio préventifs (effet antiprolifératif, modulation de l'activité d'une enzyme détoxifiante du foie et effet antioxydant) des fractions de canneberges obtenues par CLHP, des extraits d'*Hibiscus sabdariffa* L. et des composants membranaires ont été déterminés.

M**odèle animal** La méthode utilisée est la suivante : le CCR a été induit chez des rats mâles par injections d'une substance carcinogène (DMH) durant une période de 6 semaines. Ces derniers ont dû être gavés avec soit différentes concentrations du lait fermenté ou de différentes fractions de ce dernier dépendamment de l'expérience durant 12 semaines. La figure suivante représente la modélisation graphique de la procédure expérimentale.

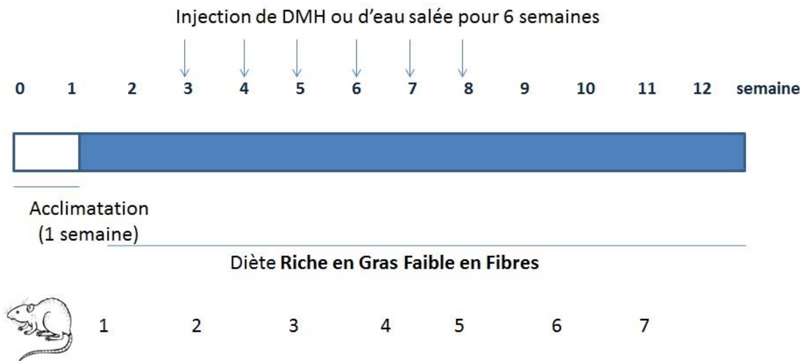

Figure 1 : Modèle expérimental pour l'expérience *in vivo*.

Selon l'ordre de l''expérience (1ère ou 2ème) ; les groupes ont été réparti de la manière suivante :

a) 1ère expérience : **1** : contrôle négatif (eau saline seule), **2** : contrôle positif (DMH seul), **3** : DMH + 2 ml de lait fermenté, **4** : DMH + 1.5 ml de lait fermenté, **5** : DMH + 1 ml de lait fermenté, **6** : DMH + 0.5 ml de lait fermenté, **7** : DMH + 0.25 ml de lait fermenté. L'administration des différentes doses se fait chaque jour.

b) 2ème expérience : **1** : contrôle négatif (eau saline seule), **2** : contrôle probiotique (lait fermenté seul), **3** : contrôle positif (DMH seul), **4** : DMH + lait fermenté, **5** : DMH + surnageant du lait fermenté, **6** : DMH + culot du lait fermenté, **7** : DMH + composants membranaires de *L. acidophilus* CL1285MD, *L. casei* LBC80RMD et *L. rhamnosus* CLR2MD. Le volume de chaque administration est de 2ml et est journalière.

9. ARTICLE 1 : THE SYNERGISTIC EFFECT OF CELL WALL EXTRACTED FROM PROBIOTIC BIOMASS CONTAINING LACTOBACILLUS ACIDOPHILUS CL1285, L. CASEI LBC80R AND L. RHAMNOSUS CLR2 ON ANTICANCER ACTIVITY OF CRANBERRY JUICE - HPLC FRACTIONS

Kerlynn Desrouillères[1], Mathieu Millette[2], Behnoush Maherani[1], Majid Jamshidian[1], Monique Lacroix [1*]

Effets synergiques des composantes membranaires extraites d'une biomasse de bactéries probiotiques contenant *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2 sur les activités antinéoplasiques des fractions de concentré de jus de canneberges obtenues par CLHP

Soumis au Journal of Functional Foods le 20 janvier 2015, cet article a été refusé. En tenant compte des commentaires de l'éditeur du journal, des corrections ont été apportées et l'article a été soumis au Journal of Nutrition and Cancer le 27 octobre 2015. Ce journal a également rejeté l'article, après avoir réalisé les corrections, il sera soumis à un autre journal.

<u>Contribution des auteurs</u> :

J'ai réalisé les expériences, rédigé l'article et participé à l'élaboration des protocoles. Dr. Millette m'a supervisé tout au long des expériences, corrigé

l'article et participé aux discussions concernant le projet. Dr. Behnoush et Dr. Jamshidian ont participé à la correction de l'article. Dr. Lacroix est la responsable du projet, a participé à toutes les prises de décisions, aux discussions concernant le projet et aux corrections de l'article.

Résumé :

Les effets antinéoplasiques de fractions de polyphénols obtenues par une séparation chromatographique (CLHP) d'un jus de canneberges concentré et de composantes membranaires extraites d'une biomasse contenant trois souches de lactobacilles (*Lactobacillus acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2) ont été évalués. Trois fractions ont été obtenues à partir du jus. Les échantillons ont été testés à différentes concentrations pour vérifier leur capacité antiproliférative et leur habilité d'induire l'activité de la quinone réductase (QR). Pour le test antiprolifératif les cellules utilisées sont les HT-29 et pour la QR les cellules utilisées sont les cellules de l'hépatome murin Hepa 1c1c7. Seule la fraction 1 (F1) contenant principalement des acides phénoliques at été en mesure d'inhiber la prolifération des cellules cancéreuses avec une concentration inhibitrice (CI_{50}) variant de 11.00 µg EAG/ml. Il a été démontré seule F3 possédait le potentiel d'induire l'activité de la QR. Les résultats ont également démontré que, combinées aux extraits de composantes membranaires, toutes les fractions de canneberges ont un potentiel inhibiteur plus élevé que lorsqu'ils ont été testés seuls, ceci indiquerait un possible effet de synergie entre les deux composés.

Mots clés : jus de canneberge, prolifération, cancer du côlon, fractionnement par CHPL, induction de QR, composant membranaire de probiotique

The synergistic effect of cell wall extracted from probiotic biomass containing Lactobacillus acidophilus CL1285, L. casei LBC80R and L. rhamnosus CLR2 on anticancer activity of cranberry juice - HPLC fractions

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ABSTRACT

Anticancer effects were evaluated on HPLC fractions obtained from a cranberry concentrated juice and cell wall constituents extracted from a probiotic biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2. Three fractions were obtained from the cranberry concentrated juice. The samples were tested at increasing concentrations for the anti-proliferative assay using HT-29 cells line and for the quinone reductase (QR) assay using Hepa 1c1c7 murine hepatoma cells. Only fraction 1 (F1) which is highly concentrated with phenolic acids was able to inhibit the growth of the HT-29 cells line with IC_{50} values of 11.00 µg Gallic acid equivalent (GAE)/ml. It was also observed that only fraction F3 witch is highly concentrated in flavonols had potency as QR inducer. Furthermore, the results showed that all cranberry fractions combined with cell wall constituents extracted from the probiotic biomass were more effective at inhibiting the growth of HT-29 as compared to the cranberry fractions tested alone which indicating a possible synergy effect between these bio-functional compounds.

Keywords: cranberry juice, cell proliferation, colon cancer, HPLC fractionation, QR induction, probiotic cell wall, *Lactobacillus acidophilus* CL1285, *Lactobacillus casei* LBC80R and *Lactobacillus rhamnosus* CLR2

9.1 INTRODUCTION

Colorectal cancer (CRC) is the third most predominant cancer in both men and women (Haggar *et al.*, 2009). CRC is strongly related to dietary habits, many epidemiological and clinical studies attributes its high incidence to obesity, diet low in fruits, vegetables and whole grains but rich in fat, red and processed meat (Rasool *et al.*, 2013, Siegel *et al.*, 2014). Besides the diet, there's also a link between CRC and the intestinal microbiota (Hope *et al.*, 2005, Rowland, 2009). Some studies reported a difference between the microbiome of healthy subjects and patients with colon cancer (Shen *et al.*, 2010b, Sobhani *et al.*, 2011) while others showed that the presence of some commensal bacteria in the gut could led to inflammation which can subsequently generate colonic cancer cells (Petit *et al.*, 2013, Yang *et al.*, 2013a). Taking into account those information, an emphasis have been made on using dietary agents like berries or probiotics to prevent mutagenesis and carcinogenesis of CRC.

Cranberry belongs to the genus *Vaccinium*, fam. *Ericaceae* and are commonly consumed as part of a Western diet in fresh or processed form (Brown *et al.*, 2012, Stoner *et al.*, 2008). They are known for their beneficial health effects towards bacterial infection involving the urinary tract disorders, dental decay, as well as stomach ulcers and chronic disease like cancer and diabetes (Heinonen, 2007, Howell *et al.*, 2010, Zhang *et al.*, 2005). Those biological activities are mainly due to the presence of various phenolic compounds, including anthocyanins, flavonols, flavanols, phenolic acids and proanthocyanidins (Paredes-López *et al.*, 2010a, Seeram, 2008). Several *in vitro* studies have shown the anticancer effects of berries/and or berries extracts by strongly inhibiting cellular growth, inducing apoptosis, or enzyme metabolism (Bomser *et al.*, 1996, Flis *et al.*, 2012, Seeram *et al.*, 2006).

Probiotics are defined as live microorganisms which when consumed in adequate number exert beneficial effects in the host (Hill *et al.*, 2014). There is a numerous health benefits that have been attributed to probiotics such as protection against gastrointestinal pathogens, modulation of the immune system,

reduction of high level of cholesterol and blood pressure and anticarcinogenic capacity (D'Aimmo *et al.*, 2007, Malago *et al.*, 2011).

Their inhibitory effects against several colon cancer cell lines growth have been demonstrated in many studies (Choi *et al.*, 2006, Saikali *et al.*, 2004). Besides the whole bacterial structure, others bioactive components commonly found onto the probiotics such as cell walls (or it's different components peptidoglycan, lipoteichoic acids), proteins or secreted by them such as exopolysaccharides, bacteriocins, organic acids, responsible for their health effects and other less soluble factors (Saad *et al.*, 2013). Among all the mechanism involved in the chemopreventive strategy, xenobiotic-detoxification is considered one of the major mechanisms. The enzymes responsible of the detoxification are phase II-xenobiotic enzyme such as glutathione-S-transferase (GST), uridine diphosphate-glucuronosyl transferase (UGT) and quinone reductase (QR) (Hong *et al.*, 1997, Talalay, 2000). QR catalyses the reduction of two electrons of toxic oxygen metabolites which lead to the deactivation of these reactive metabolites that could protect the exposed tissue from oxidative stress (Chen *et al.*, 2004). It also have the ability to reduce oxidative stress (Kang *et al.*, 2004a). This enzyme is as key member of a panel of *in vitro* assays to evaluate chemopreventive properties of diverse biomolecules/bioactive components/chemical substances (Su *et al.*, 2004)

Thus the aims of this study were to evaluate: 1) the effect of fractions from a cranberry concentrated juice and cell walls extracted from a probiotic biomass on the *in vitro* antiproliferative properties against one colon cancer cell line, 2) their ability to induce the phase II detoxifying enzymes QR and 3) the effect of the combination of cranberry fractions with the probiotic cells walls on the growth of colon cancer cells and the ability of liver cells to induce QR activity.

9.2 MATERIALS AND METHODS

9.2.1 Chemical reagents

Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin-Ciocalteu, menadione, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), flavin adenine dinucleotide (FAD), digitonin, bovine serum albumin (BSA), β-naphtoflavone (BNF), sodium dodecyl sulphate (SDS) and Tween 20, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Cancer cell culture media (DMEM-Ham's F12 and MEMEBSS), foetal bovine serum (FBS) and other supplements were obtained from Fisher Scientific Limited (Ottawa, ON, Canada).

9.2.2 Raw materials

The cranberry concentrate juice (50°Brix) was graciously provided by Atoka Cranberries Inc. (Manseau, QC, Canada) and the probiotic biomass by Bio-K Plus International Inc. (Laval, QC, Canada).

9.2.3 HPLC-DAD fractionation of concentrated cranberry juice and total phenol determination.

a) Fractionation of concentrated juice

The HPLC analyses were performed on a ProStar 230 from Varian Canada Inc. (Mississauga, ON, Canada), equipped with a ternary pump delivery system, a Rheodyne injection valve and a ProStar 330 diode-array UV-Vis detector from Varian Canada Inc. integration and data elaboration was performed using Star Chromatography Workstation software. A Zorbax SB-C18, 5 µm, 9.4×250 mm column from Agilent Technologies Canada Inc. (Mississauga, ON, Canada) was used. All solvents were filtered with a 0.20 µm Whatman paper from Millipore Canada Ltd. (Etobicoke, ON, Canada). An elution gradient was carried out using the following solvent system: mobile phase A, MilliQ water/formic acid (97/3, v/v);

mobile phase B methanol only (100 v/v). The linear gradient elution was 85% A - 15% B. A volume of 1ml of each sample was injected after filtration through a 0.2µm filter disk. The flow rate was 3 mL/min and the detection was achieved by photodiode array (250-550nm). Three fractions were recovered (Table 2). The solvent was then removed by evaporation using the SpeedVac automatic evaporation system (Savant System, Holbrook, NY, USA). Finally, the dry matter was obtained by freeze drying the fractions for at least 48 hours using a Labconco freeze-drying system (Kansas City, MO, USA) and stored at -20° until used (Caillet *et al.*, 2012b).

Table 2 : Time of elution of cranberry concentrate juice fractions obtained by HPLC-DAD fractionation. F1: composed with phenolic acids**, F2:** constituted with mostly anthocyanins **and F3:** contained mainly flavonols.

Fraction	Time of elution (min)
F1	0-12
F2	13-28
F3	29-45

b) Total phenol content

Total phenol compound content in each cranberry fraction was determined by spectrophotometry according to the Folin-Ciocalteu method (Singleton *et al.*, 1965). The total phenolic content was estimated using a calibration curve (r^2 = 0.9994) by plotting known solution of gallic acid (7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml). The total phenolic content of each fractions was expressed as microgram gallic acid equivalent (GAE) per mg powder (µg GAE/mg of powder).

9.2.4 Probiotic cell walls extraction

a) Physical treatment

Concentrated biomass of probiotic *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 (Bio-K Plus International Inc.) fermented in MRS broth were centrifuged at 9 000 x g for 30 minutes at 4°C. The resulting pellets were washed with phosphate buffer 10mM pH 7.2 then suspended in the same buffer at a ratio of 1:4 (w/v). The suspension was heated at 65°C for 40 minutes, then submitted to ultrasound in 500 W, 20 KHz (Q500, QSONICA, Newtown, CT, USA) using 1 s^{-1} pulse on and 1 s^{-1} pulse off with 40% acoustic power (amplitude) for 30 minutes at 4°C. The sonicated samples were centrifuged at 800xg for 30 minutes; the supernatant was recovered and subjected to ultracentrifugation at 50 000xg for 30 minutes. The pellet was collected and suspended in 10 ml of 100mM Tris-HCl buffer pH 7.5 (Kim *et al.*, 2002, Signoretto *et al.*, 1998).

b) Enzymatic treatment

The pellet obtained in the previous step was firstly incubated under agitation with 50µl of α-amylase (20mg/ml) for 2 hours at 37°C. Then, 50µl of $MgSO_4$ (1M), 100µl of DNase (1mg/ml), 100µl of RNase (50mg/ml) were added and the solution was incubated at 37°C under agitation for 2 hrs. Finally, 100µl of trypsin (10mg/ml) and 10µl of 1M $CaCl_2$ were added and the samples was incubated overnight under agitation. After the incubation, 3.5ml of 4% SDS (w/v) was added in the 10ml of samples and heated at 100°C for 15 minutes. The samples were centrifuged at 30 000xg for 30 minutes. The new obtained pellet was washed with distilled water (4X), and then with different solution: LiCl (8M), EDTA (100mM) and acetone. Each solution was used twice to wash the pellet. The washed pellets were mixed with 10ml of $(NH_4)_2CO_3$ and 250µl of alkaline phosphatase (1mg/ml) and incubated overnight at 37°C under agitation. Then the sample was heated at 100°C for 5 minutes and centrifuged at 30 000xg for 45 minutes. The pellet was washed with distilled water (2X), suspended in water

and lyophilized. The powder was weighed and kept at -20°C until used (Signoretto *et al.*, 1998).

9.2.5 Measurement of cell proliferation

a) Cell cultures

The HT-29 cells (ATCC HTB-38) and the Hepa 1c1c7 murine hepatoma cells (ATCC CRL-2026) were obtained from America Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in complete DMEM-Ham's F12 and MEM-EBSS medium, respectively containing 10% of FBS. They were maintained in a humidified atmosphere incubator at 37°C with 5% CO_2.

b) Preparation of samples

Evaluation of cell viability assay and QR induction assay was done, measured for cranberry fractions and cell wall extract. These compounds were serially two-folded diluted using 10% (v/v) DMSO solution to have serial concentrations. The concentrations of cranberry concentrate juice fractions are 20, 39, 78, 155, 310, 625, 1250 and 2500 µg/ml while concentrations of 8, 16, 32, 63, 125, 250 and 1000 µg/ml were considered for cell wall extract. For the QR assay, the concentrations tested were 8, 16, 32, 63, 125, 250 and 500 µg/ml for both samples

c) Antiproliferative assay

The antiproliferative effect of our samples was measured using MTT color assay. The HT-29 cells were seeded at 2 x 10^4 cells in 200 µl of complete growth medium in 96 wells microplate Corning® CELLBIND®Surface microplate (Costar, Corning Inc., Corning, NY, USA). After 24 hours of incubation at 37°C in 5% CO_2, the medium was replaced with 90 µl of fresh medium containing 10 µl of serial concentration of samples. Control wells received the same amount of medium with 10 µl of DMSO 10% and blank wells received 100 µl of medium with no cells. After 48 hrs of incubation, the growth medium containing the sample is

decanted and replaced with 200 µl of new growth medium containing 25 µl of MTT. The microplate were incubated for 4 hours. Then, 200 µl of DMSO with 25 µl of Sorensen buffer were added to the microplate. Absorbance was measured at 562nm with a microplate reader (EL800 BioTek Winooski, VT, USA). The absorbance of the control samples was set at 100% of cell proliferation. At least five replications for each sample were used to determine the cell proliferation. The cell proliferation was determined by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals (Vistica *et al.*, 1991). The inhibition rate was calculated as follows:

Equation 1: Inhibition (%) = 100-(OD treated cells/OD control cells*100)

IC_{50} is the fraction concentration under which the proliferation of 50% of the cells is inhibited.

9.2.6 Quinone reductase assay

a) Assay procedure

Hepa 1c1c7 cells were seeded in 96 well microplate in 200 µl of MEM/EBSS medium at a concentration of 1 x 10^4cells. After 24 hours of incubation at 37° C in 5% CO_2 in humidified incubator the medium was decanted and replaced with 190 µl of fresh medium containing 10 µl of serial concentrations of samples. After 48 hours of incubation the medium was decanted, and the cells were incubated for 10 minutes with 50 µl of 0.8% digitonin and 2 mM EDTA solution, then the microplates were agitated for 10 minutes in an orbital shaker and 200 µl of a reaction mixture were added in each well. A stock solution was prepared as described for each series of assays: 20.5 ml distilled water, 1.1 ml of 500 mM Tris-HCl buffer pH 7.4, 14.7 mg of bovine serum albumin, 146.7 µl of 1.5% (w/v) Tween 20 solution, 14.7 µl of 7.5 mM FAD^+, 146.7 µl of 150 mM Glucose 6-phosphate solution, 13.2 µl of 50 mM $NADP^+$ solution, 44 µl of Glucose 6-phosphate dehydrogenase solution, 6.6 mg MTT and 22 µl of 50 mM menadione solution. The microplates were incubated for 5 min at room temperature then the absorbance was read at 595 nm with a microplate reader.

b) Protein determination

The protein quantification was performed using BCA assay kit from Fisher Scientific Limited according to the manufacture's specifications. A 20 µl aliquot of lysed cells was transferred to a new microplate and 300 µl BCA were added to each well and incubated for 30 min at humidified incubator under 5% CO_2 at 37°C. Then, absorbance was read at 595 nm with a microplate reader. The specific activity of QR is defined as nM MTT formazan formed per min per mg protein. The specific activity was calculated as follows:

Equation 2: Specific activity = (absorbance of MTT 595nm/min (5minutes) x 3247 nmol/mg)/ (absorbance obtained for determining protein at 595nm**).**

Induction of QR of treated samples was calculated by dividing specific QR activity of the treated sample to specific QR of the control samples (cells + DMSO 10%).

9.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate the differences between the samples tested SPSS Base 16.0 software (Stat-Packets statistical analysis software, SPSS Inc., Chicago IL, USA). Significant differences ($p \leq 0.05$) were evaluated using Duncan's comparisons test.

9.3 RESULTS AND DISCUSSION

9.3.1 Cranberry concentrate juice HPLC fractionnation

The HPLC analysis of the cranberry concentrate juice allowed the separation of the phenolic compounds present in the juice according to their polarity. The first compounds to elute are the most polar ones with the smallest molecular weight. In a previous study conducted in our laboratory, standard phenolic compounds were analysed through HPLC and their retention time was recorded (Caillet *et al.*, 2012b). Based on those results we could hypothesized the phenolic content of each fraction as follows: fraction 1 (F1) contained mainly phenolic acid while fraction 2 (F2) is constituted mainly of anthocyanins and fraction 3 (F3) is composed mostly with flavonols.

9.3.2 Total phenol content

The concentration of phenolic compound in each fractions obtained from the cranberry concentrate juice was analysed. The results are presented in Table 3. We observed that the highest phenolic content was obtained in F3, the fraction enriched with flavonols. Those results coincide with the ones obtained by Vu *et al.* (2012) when using the extracts obtained from cranberries fruits, puree, depectinised puree and pomace. The authors found that the extracts containing the apolar phenolic compounds (flavonols, flavan-3-ols and proanthocyanidins) for each samples tested were the ones with the highest phenolic content.

Table 3 : Total phenolic content of cranberry fractions. F1, F2 and F3 were collected by fractionation of cranberry concentrated juice by HPLC. Data are presented as mean ± standard deviation. **F1**: composed with phenolic acids, **F2:** constituted with mostly anthocyanins and **F3**: contained mainly flavonols.

Fractions	Phenolic composition	µg GAE/mg of powder
F1	Phenolic acid	8.17±5.59
F2	Anthocyanins	70.75±3.55
F3	Flavonols	136.41±8.94

9.3.3 Antiproliferative assay

“Normal cells” are sensitive to environmental growth inhibitors unlike “cancer cells”, they do not respond to the inhibitory growth signals that are able to grow without the stimulatory growth factors indispensable for normal cell proliferation (Karp, 2009). In view of the ability of cancer cells to grow indefinitely, using substances (natural and/or chemical) to inhibit their growth is one of the chemoprevention strategy against cancer.

a) Antiproliferative activity of cranberry fractions against HT-29.

The effect of cranberry fractions on HT-29 growth and the IC50 values are presented in Figure. 2 and Table 4 respectively. The IC50 represent the concentration required to inhibit the growth of 50% of the cancer cells and the lower the value is the more efficient the fraction is to act as an antiproliferative agent against cancer cells.

A concentration-dependant response was observed for all three fractions concerning the inhibitory effect against HT-29 cells.

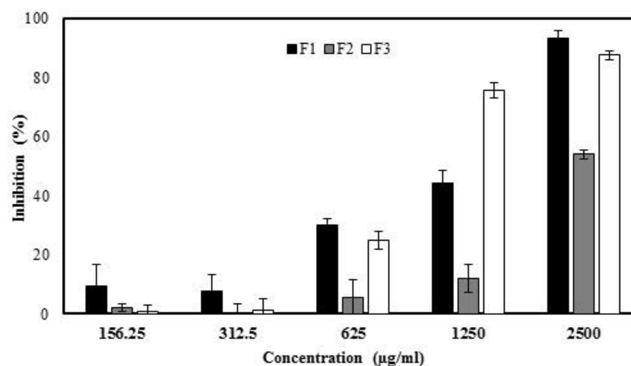

Figure 2: **Effect of fractions from cranberry concentrate juice obtained by HPLC on HT-29 proliferation**. F1, F2 and F3 are three fractions collected by fractionation of cranberry concentrated juice by HPLC. F1 is composed with phenolic acids, F2 is constituted with mostly in anthocyanins and F3 contained mainly flavonols. Values are mean ± standard deviation.

Table 4: IC_{50} values of the cranberry fractions for the inhibition of HT-29 growth. HT-29 cells were incubated with different concentrations (156.25-2500µg/ml) of cranberry fractions for 48 hours. F1, F2 and F3 were collected by fractionation of cranberry concentrated juice by HPLC. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). **F1**: composed with phenolic acids, **F2:** constituted with mostly anthocyanins and **F3**: contained mainly flavonols.

Fractions	IC_{50} (µg GAE/ml)
F1	11.00±2.36[a]
F2	194.88±1.58[c]
F3	129.30±2.18[b]

The fraction 1 (F1); which contains mainly phenolic acids, has an IC_{50} value of 11.00±2.36 µg GAE/ml. This fraction is the more effective at inhibiting HT-29 cells growth. The phenolic acids usually found in cranberry are p-coumaric, sinapic, caffeic and ferulic acids (Zuo *et al.*, 2002). Those compounds have demonstrated their antiproliferative effects on cancer cells in *in vitro* studies. For example ferulic and p-coumaric acids present in whole grains cereals bran were able to inhibit the growth of Caco-2 cells lines by 43 and 57% respectively compared to control after 2-3 days treatment at a concentration of 1500 µM (Janicke *et al.*, 2005). Murad *et al.* (2015) evaluated the effect of caffeic acid on

HT-29 viability at different concentration (1.25 to 80.0 μM) from 0.5 to 96 hours. They found that caffeic acid had inhibitory effect against HT-29 growth.

Therefore the results obtained in our study concerning the effect of phenolic acids on colon cancer cells proliferation corresponds with previous cited studies.

Fraction 2 (F2), mainly composed of anthocyanins, presents the highest IC_{50} values. At the concentrations tested, F2 was not able to inhibit the growth of 50% of the cells. Anthocyanins are colored pigments especially abundant in berries such as blueberries, blackberries, strawberries, black currants, grapes, cranberries (da Silva *et al.*, 2007). The anthocyanins available in cranberry are mostly cyanidin 3-galactoside, cyanidin 3-arabinoside, peonidin (Côté *et al.*, 2010). Many studies demonstrated the inhibitory effect of anthocyanins from different fruits against cancer cells. For example, Zhang *et al.* (2008b) demonstrated that anthocyanins fractions from strawberry had an inhibitory effect on HT-29 growth of 60% compared to control at a concentration of 0.25 mg/ml. However anthocyanins from cranberry seems to have little to no effect on cancer cells growth. For instance Murphy *et al.* (2003) tested purified cyanind-3-galactoside on eight tumors cells lines growth. They found that even at the highest concentration, the inhibition rate was less than 50% for all the cancer cells. And also Seeram *et al.* (2004) tested anthocyanins sub-fractions from cranberry against several tumour cell lines from prostate, colon and oral. The authors found that the anthocyanins were able to limit prostate cancer cells growth by 50-70% but not oral and colon tumour cells proliferation. Our results are in agreements with these previous studies. Further investigations are needed to evaluate the antioxidant capacity of F2 and/or the antiangiogenic effect. Also further analyses are needed to determine the compounds present in F2 and whether the subsequent bioactivity is due to a single or all the compounds.

Fraction 3 (F3) is mainly composed of flavonols and presents also a high inhibitory effect with an IC_{50} of 129.30± 2.18 μg GAE/ml. It is known that the main flavonols in cranberry are quercetin, kaempferol and myricetin. *In vitro* and

in vivo studies demonstrated a number of potential role for quercetin and kaempferol in cancer prevention (Yang *et al.*, 2000, Zhang *et al.*, 2008a). A study conducted by Li *et al.* (2009b) demonstrated the ability of kaempferol to inhibit the proliferation of HCT116 cells in a dose dependent manner. The antiproliferative activity was stronger for the cell line wild type p53. Another study designed by Zhang *et al.* (2012a) to compare the anticancer activity of quercetin and quercetin-5', 8-disulfonate against colorectal cancer cell lines LoVo. The authors found that both compounds were able to inhibit the growth of the cells in a concentration dependant manner with IC_{50} values of 40.02 and 20.08 µM respectively. Those results suggest that quercetin-5',8-disulfonate is more effective against cancer cell proliferation. Our results are in agreement with these previous studies. Further investigation should be realised to determine if the inhibitory effect is mainly due by a single compounds in each fraction or it's rather the combination of all the compounds and also what is the mechanism by which phenolic compounds distraught the proliferation of colon cancer cells.

b) Antiproliferative activity of cell wall constituents from a probiotic biomass HT-29.

As shown in Table 5, HT-29 growth was not inhibited by the cell wall extracted from a probiotic biomass containing *L. acidophilus* CL1285, *L. casei* LB80CR and *L. rhamnosus* CLR2.

Table 5 : Effect of extracts of cell wall constituents obtained from a probiotic biomass on HT-29 cell viability. HT-29 cells were exposed to different concentrations (62.5 to 1000 µg/ml) of cell wall constituents. Cell wall were extracted from a probiotic biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2. Data are presented as mean ± standard deviation. **CW:** cell wall constituent.

Concentration (µg/ml)	Cell viability (%)
0	100.00±1.15
62.5	114.26±8.81
125	117.11±9.54
250	122.53±5.51

500	114.18±6.70
1000	104.47±9.78

Some studies showed the inhibitory effect of probiotic cell wall on colon cancer cells proliferation. Liu *et al.* (2011) found that crude cell wall extracted from *Lactobacillus casei* 01 inhibit the growth of HT-29 cells by nearly 9 % and Wang *et al.* (2014) tested the cells walls extracted from four lactobacilli strains (X11, X12, M5 and K14) for antiproliferative activity. The authors found that the inhibition varied between 8.48 and 36.59 %.

Our results does not coincide with the ones obtained by previous studies. The antiproliferative activity of the probiotics and their components might be strain specific which could perhaps explain the disagreement between our results and the ones obtained in previous studies. The cell wall extracted in this study came from a probiotic biomass containing three different probiotics strains, the hypothesis made was that cell wall of each of those bacteria possess an inhibitory effect against colon cancer cells proliferation when use as a single bioactive compounds but when they are combined together there might be an antagonist effect. Further investigations need to be realised to determine the effect of the probiotic cell wall of each probiotic alone on colon cancer cell proliferation.

c) *Antiproliferative activity of cranberry fractions combined with probiotic cell wall against HT-29 proliferation.*

The effect of the combination of cranberry fraction and probiotic cell wall extracts on HT-29 growth are presented in Fig. 3.

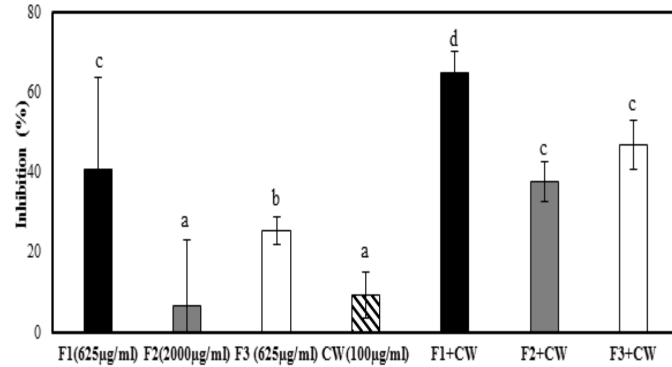

Figure 3:Effect of the cranberry fractions associated with probiotic cell wall on HT-29 proliferation. HT-29 cells were exposed to cranberry fractions combined with extract of cell wall constituents. F1, F2 and F3 were collected by fractionation of cranberry concentrated juice by HPLC and CW constituents were extracted from a probiotics biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. The samples are tested at different concentrations **F1**: 625 µg/ml, **F2**: 2000 µg/ml, **F3**: 625 µg/ml and **CW**: 100 µg/ml. Data are presents as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). **F1**: composed with phenolic acids, **F2:** constituted with mostly anthocyanins and **F3**: contained mainly flavonols and **CW**: cell wall constituents.

When combining the cranberry fractions with the probiotic cell wall, we observed an increase of the inhibitory effect of each sample against HT-29 proliferation as compared to the fractions alone. F1 and F3 alone inhibit HT-29 by 40.82 ± 23.00% and 25.22 ± 3.5 % respectively at 625 µg/ml but when combined with the cell walls extracts (100 µg/ml); the inhibitory effect increase by nearly 20%. F2 alone at a concentration of 2000 µg/ml inhibit the proliferation of by 6.60 ± 16.57%, but with the cells wall there's an increase of nearly 30% of the inhibition rate. Studies showed that phenolic compounds from berries could act as antimicrobial substances against food pathogens such as *E. coli* (Caillet *et al.*, 2012a, Lacombe *et al.*, 2010) and it was found by Millette *et al.* (2007) that a soluble fraction from a fermented milk produced by *L. acidophilus* and *L. casei* was also able to inhibit the growth of food pathogens. Those studies showed that phenolic compounds from berries and compounds from or produced by probiotic bacteria have the same antimicrobial properties and others studies also demonstrated that phenolic compounds present in berries could act as growth promoting factors for probiotics. They are able activate bacterial growth but also increase the bacterial count in a culture medium (Ávila *et al.*, 2009, Tabasco *et*

al., 2011). We can hypothesise that probiotic bacteria and phenolic compounds can act as complementary bioactive compounds. The increase of the inhibition rate when combining the two compounds could be explained by an interaction between the phenolic compounds and the component of the probiotic cell wall (peptidoglycan, teichoic acid and cell-wall associated polysaccharides). This interaction may have an effect on the cancer cell proliferation by either modifying the cell morphology or affecting some growth factors that could lead to the cell death. Further studies will have to be realised to determine the nature of the link between phenolic compounds and cell wall and the precise mechanism by which that link affect cancer cells growth.

9.3.4 Quinone reductase assay

a) Quinone reductase induction by fractions from cranberry concentrate juice

The effect of the cranberry fractions on the induction of QR activity are presented in Fig. 4.

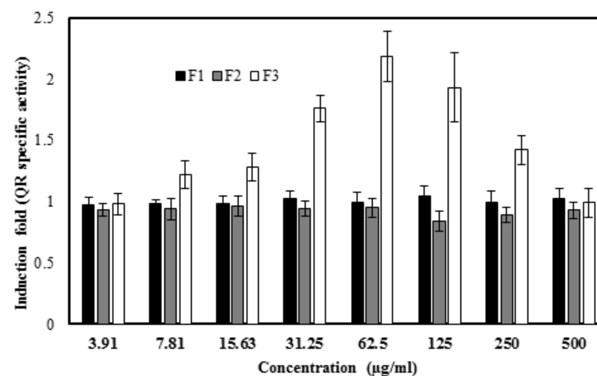

Figure 4: Effect of fraction from cranberry concentrate juice obtained by HPLC the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were exposed to different concentration of cranberry fractions (3.91 to 500

µg/ml). F1, F2 and F3 were collected by fractionation of cranberry concentrated juice by HPLC. Data are presented as mean ± standard deviation. **F1**: composed with phenolic acids, **F2:** constituted with mostly anthocyanins and **F3**: contained mainly flavonols.

Berries are known for their antioxidant properties, which explain their ability to help the organism to protect itself from free radicals that tend to provoke damages in DNA. β-naphtoflavone (BNF) was used as a positive control at a concentration of 20 µM have an induction of 2.45 ± 0.1 folds (results not shown). F1 and F2 did not have any effect on the induction of QR activity. The results concerning F1 are consistent with previous studies. Phenolic acids like caffeic and ferulic acids were found to be weak inducer of QR activity in some *in vitro* studies (Fahey *et al.*, 2002, Yang *et al.*, 2009). Studies on the potency of anthocyanins to induce QR activity have been contradictory. For instance Shih *et al.* (2006) have identified the capacity of anthocyanins to induce QR activity while Srivastava *et al.* (2007) informed that QR activity was not induce by natural extracts of anthocyanins. This conflicting results make it difficult to hypothesize about the results obtained for F2.

F3, mainly composed of flavonols is the only fraction able to induce QR activity. The greater induction was observed at 62.5 µg/ml and it was of 2.18 ± 0.206 folds. Previous studies showed that flavonols like quercetin, myricetin and kaempherol were effective QR inducers with maximal induction within the range 1.6 to 3.6 (Uda *et al.*, 1997). Our results seems to coincide with studies that have been conducted before. Further investigations are required to identify whether the capacity to induce QR activity of F3 are due to one compound or the combination of compounds and also the effect of the cranberry fraction on the inhibitory activity of phase I enzymes presents in the liver.

b) Quinone reductase induction by probiotic cell wall constituents

The effect of cell wall extracted from the probiotic biomass on QR activity are presented in Fig. 5.

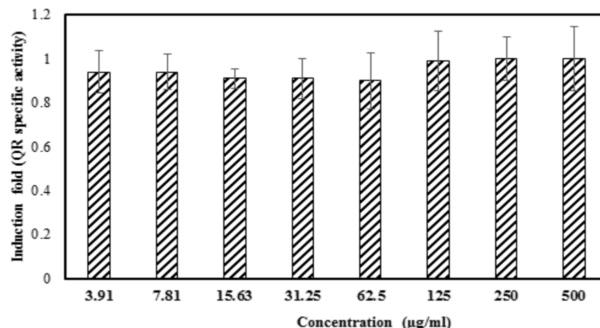

Figure 5: Effect of cell wall extracted from a probiotic biomass on the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were exposed to different concentration of extract of cell wall constituents (3.91 to 500 µg/ml). CW constituents were from a probiotics biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. Data are presented as mean ± standard deviation. **CW**: cell wall constituents.

Probiotic bacteria have the ability to inhibit colon cancer using diverse mechanisms like enhancement of the host immune response, alteration of intestinal microbiota, production of beneficial compounds (Ewaschuk *et al.*, 2006, Goldin *et al.*, 1984, Hirayama *et al.*, 2000). Since the induction of phase II enzyme like QR is considered one of the major mechanism to inhibit the first stage of colon carcinogenesis therefore we investigated the effect of mixed cells walls from *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2 on QR activity. Unfortunately our results showed that the cell wall did not have any effect on QR activity at the concentration tested. A study conducted by Chang *et al.* (2010) demonstrated that cell extracts from four strains of lactobacilli (*L. acidophilus* KFRI342, *L. casei* KFRI707, *L. acidophilus* LA72712 and *L. casei* KFRI809) were able to induce QR activity, and KFRI342 being the strain with the greater activity. Our results do not coincide with this study, further investigations are needed to determine whether increasing the concentration tested could eventually lead to an induction of QR activity or testing the cell wall of each probiotic strain would have an effect on QR induction.

c) Quinone reductase induction by cranberry fractions combined with probiotic cell wall constituents.

The effect of the combination of cell wall and cranberry fractions on QR activity are presented in Fig. 6.

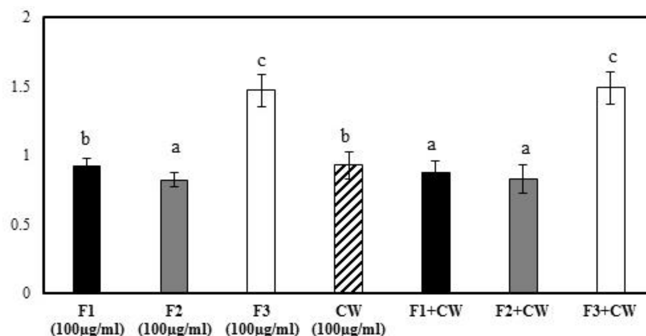

Figure 6: Effect of the cranberry fractions associated with probiotic cells walls on the induction of quinone reductase activity: Murine Hepatoma (Hepa 1c1c7) cells were exposed to cranberry fractions combined with extract of cell wall constituents. F1, F2 and F3 were collected by fractionation of cranberry concentrated juice by HPLC and CW constituents were extracted from a probiotics biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. The samples are tested at a concentration of 100 µg/ml. Data are presents as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). **F1**: composed with phenolic acids, **F2:** constituted with mostly anthocyanins and **F3**: contained mainly flavonols and **CW**: cell wall constituents.

The induction fold of QR activity by cranberry fraction did not change when they were combined with the extracted cell wall from the probiotic biomass. Unlike the antiproliferative effect, combining the cell wall extracts with the cranberry fractions did not result into an enhancement of QR activity. The chemical interaction between the probiotic cell wall components and the phenolic compounds which favor an increase in the antiproliferative effect when combing the two compounds did not affect the QR activity either positively nor negatively.

9.4 CONCLUSION

It was found that fractions 1 and 3 purified from cranberry concentrate were able to inhibit the growth of HT-29 cell line with F3 being the most effective one. Anthocyanin-rich fraction 2 was the fraction the least effective at inhibiting the growth of HT-29. Cell wall extracted from a probiotic biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2 alone did not affect the growth of colon cancer cells but when combined with the cranberry fractions, a significant increase in the inhibition rate compared to the fractions alone was observed. Only flavonol-rich fraction 3 was able to induce QR activity. The concentration of phenolic compounds did not have a direct correlation with the antiproliferative effect of the fractions. The results obtained in this study confirm the potential of cranberry as a chemopreventive agent against cancer but also prove that probiotics components and berries could act as in activating or potentiating manner to prevent colon cancer cells growth.

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REFERENCES

1. Haggar FA, Boushey RP (2009) Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in colon and rectal surgery* **22**, 191-197.
2. Rasool S, Kadla S, Rasool V *et al.* (2013) A comparative overview of general risk factors associated with the incidence of colorectal cancer. *Tumor Biol* **34**, 2469-2476.
3. Siegel R, DeSantis C, Jemal A (2014) Colorectal cancer statistics, 2014. *CA: A Cancer Journal for Clinicians* **64**, 104-117.
4. Rowland IR (2009) The Role of the Gastrointestinal Microbiota in Colorectal Cancer. *Current Pharmaceutical Design* **15**, 1524-1527.
5. Hope ME, Hold GL, Kain R *et al.* (2005) Sporadic colorectal cancer – role of the commensal microbiota. *FEMS Microbiology Letters* **244**, 1-7.
6. Sobhani I, Tap J, Roudot-Thoraval F *et al.* (2011) Microbial dysbiosis in colorectal cancer (CRC) patients. *PloS one* **6**, e16393.
7. Shen XJ, Rawls JF, Randall TA *et al.* (2010) Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut Microbes* **1**, 138-147.
8. Petit C, Oswald E, Nougayrede J-P (2013) Rôle des génotoxines produites par des bactéries du microbiote dans le cancer colorectal. *Revue Francophone des Laboratoires* **2013**, 77-82.
9. Yang T, Owen JL, Lightfoot YL *et al.* (2013) Microbiota impact on the epigenetic regulation of colorectal cancer. *Trends in Molecular Medicine* **19**, 714-725.
10. Stoner GD, Wang L-S, Casto BC (2008) Laboratory and clinical studies of cancer chemoprevention by antioxidants in berries. *Carcinogenesis* **29**, 1665-1674.
11. Brown EM, Gill CI, McDougall GJ *et al.* (2012) Mechanisms underlying the anti-proliferative effects of berry components in in vitro models of colon cancer. *Current pharmaceutical biotechnology* **13**, 200-209.
12. Howell A, Botto H, Combescure C *et al.* (2010) Dosage effect on uropathogenic Escherichia coli anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study. *BMC Infectious Diseases* **10**, 94.
13. Zhang L, Ma J, Pan K *et al.* (2005) Efficacy of Cranberry Juice on Helicobacter pylori Infection: a Double-Blind, Randomized Placebo-Controlled Trial. *Helicobacter* **10**, 139-145.
14. Heinonen M (2007) Antioxidant activity and antimicrobial effect of berry phenolics – a Finnish perspective. *Molecular Nutrition & Food Research* **51**, 684-691.
15. Paredes-López O, Cervantes-Ceja M, Vigna-Pérez M *et al.* (2010) Berries: Improving Human Health and Healthy Aging, and Promoting Quality Life—A Review. *Plant Foods Hum Nutr* **65**, 299-308.

16. Seeram NP (2008) Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of Their Intake on Human Health, Performance, and Disease. *Journal of Agricultural and Food Chemistry* **56**, 627-629.
17. Bomser J, Madhavi D, Singletary K *et al.* (1996) In vitro anticancer activity of fruit extracts from Vaccinium species. *Planta medica* **62**, 212-216.
18. Seeram NP, Adams LS, Zhang Y *et al.* (2006) Blackberry, Black Raspberry, Blueberry, Cranberry, Red Raspberry, and Strawberry Extracts Inhibit Growth and Stimulate Apoptosis of Human Cancer Cells In Vitro. *Journal of Agricultural and Food Chemistry* **54**, 9329-9339.
19. Flis S, Jastrzebski Z, Namiesnik J *et al.* (2012) Evaluation of inhibition of cancer cell proliferation in vitro with different berries and correlation with their antioxidant levels by advanced analytical methods. *Journal of Pharmaceutical and Biomedical Analysis* **62**, 68-78.
20. Hill C, Guarner F, Reid G *et al.* (2014) Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**, 506-514.
21. D'Aimmo MR, Modesto M, Biavati B (2007) Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology* **115**, 35-42.
22. Malago J, Koninkx JJG (2011) Modulation of Immune System by Probiotics to Protect Against Enteric Disorders. In *Probiotic Bacteria and Enteric Infections*, pp. 263-286 [JJ Malago, JFJG Koninkx and R Marinsek-Logar, editors]: Springer Netherlands.
23. Saikali J, Picard C, Freitas M *et al.* (2004) Fermented Milks, Probiotic Cultures, and Colon Cancer. *Nutrition and Cancer* **49**, 14-24.
24. Choi SS, Kim Y, Han KS *et al.* (2006) Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. *Letters in Applied Microbiology* **42**, 452-458.
25. Saad N, Delattre C, Urdaci M *et al.* (2013) An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology* **50**, 1-16.
26. Hong WK, Sporn MB (1997) Recent Advances in Chemoprevention of Cancer. *Science* **278**, 1073-1077.
27. Talalay P (2000) Chemoprotection against cancer by induction of phase 2 enzymes. *Biofactors* **12**, 5-11.
28. Chen X-L, Kunsch C (2004) Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. *Current pharmaceutical design* **10**, 879-891.
29. Kang Y-H, Pezzuto JM (2004) Induction of Quinone Reductase as a Primary Screen for Natural Product Anticarcinogens. In *Methods in Enzymology*, vol. Volume 382, pp. 380-414 [S Helmut and P Lester, editors]: Academic Press.
30. Su B-N, Gu J-Q, Kang Y-H *et al.* (2004) Induction of the Phase II Enzyme, Quinone Reductase, by Withanolides and Norwithanolides from Solanaceous Species. *Mini-Reviews in Organic Chemistry* **1**, 115-123.
31. Caillet S, Lorenzo G, Côté J *et al.* (2012) Cancer chemopreventive effect of fractions from cranberry products. *Food Research International* **45**, 320-330.

32. Singleton VL, Rossi JA (1965) Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture* **16**, 144-158.
33. Kim J, Woo H, Kim Y-S *et al.* (2002) Screening for antiproliferative effects of cellular components from lactic acid bacteria against human cancer cell lines. *Biotechnol Lett* **24**, 1431-1436.
34. Signoretto C, Boaretti M, Canepari P (1998) Peptidoglycan synthesis by Enterococcus faecalis penicillin binding protein 5. *Arch Microbiol* **170**, 185-190.
35. Vistica DT, Skehan P, Scudiero D *et al.* (1991) Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production. *Cancer Research* **51**, 2515-2520.
36. Vu KD, Carlettini H, Bouvet J *et al.* (2012) Effect of different cranberry extracts and juices during cranberry juice processing on the antiproliferative activity against two colon cancer cell lines. *Food Chemistry* **132**, 959-967.
37. Karp G (2009) *Cell and Molecular Biology: Concepts and Experiments*: John Wiley & Sons.
38. Zuo Y, Wang C, Zhan J (2002) Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS. *Journal of agricultural and food chemistry* **50**, 3789-3794.
39. Janicke B, Önning G, Oredsson SM (2005) Differential Effects of Ferulic Acid and p-Coumaric Acid on S Phase Distribution and Length of S Phase in the Human Colonic Cell Line Caco-2. *Journal of Agricultural and Food Chemistry* **53**, 6658-6665.
40. Murad LD, Soares NdCP, Brand C *et al.* (2015) Effects of Caffeic and 5-Caffeoylquinic Acids on Cell Viability and Cellular Uptake in Human Colon Adenocarcinoma Cells. *Nutrition and Cancer* **67**, 532-542.
41. da Silva FL, Escribano-Bailón MT, Pérez Alonso JJ *et al.* (2007) Anthocyanin pigments in strawberry. *LWT - Food Science and Technology* **40**, 374-382.
42. Côté J, Caillet S, Doyon G *et al.* (2010) Bioactive Compounds in Cranberries and their Biological Properties. *Critical Reviews in Food Science and Nutrition* **50**, 666-679.
43. Zhang Y, Seeram NP, Lee R *et al.* (2008) Isolation and Identification of Strawberry Phenolics with Antioxidant and Human Cancer Cell Antiproliferative Properties. *Journal of Agricultural and Food Chemistry* **56**, 670-675.
44. Murphy BT, MacKinnon SL, Yan X *et al.* (2003) Identification of Triterpene Hydroxycinnamates with in Vitro Antitumor Activity from Whole Cranberry Fruit (Vaccinium macrocarpon). *Journal of Agricultural and Food Chemistry* **51**, 3541-3545.
45. Seeram NP, Adams LS, Hardy ML *et al.* (2004) Total Cranberry Extract versus Its Phytochemical Constituents: Antiproliferative and Synergistic Effects against Human Tumor Cell Lines. *Journal of Agricultural and Food Chemistry* **52**, 2512-2517.
46. Yang K, Lamprecht SA, Liu Y *et al.* (2000) Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. *Carcinogenesis* **21**, 1655-1660.

47. Zhang Y, Chen AY, Li M *et al.* (2008) Ginkgo biloba Extract Kaempferol Inhibits Cell Proliferation and Induces Apoptosis in Pancreatic Cancer Cells. *Journal of Surgical Research* **148**, 17-23.
48. Li W, Du B, Wang T *et al.* (2009) Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. *Chemico-Biological Interactions* **177**, 121-127.
49. Zhang H, Zhang M, Yu L *et al.* (2012) Antitumor activities of quercetin and quercetin-5′,8-disulfonate in human colon and breast cancer cell lines. *Food and Chemical Toxicology* **50**, 1589-1599.
50. Liu C-T, Chu F-J, Chou C-C *et al.* (2011) Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from Lactobacillus casei 01. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **721**, 157-162.
51. Wang S-M, Zhang L-W, Fan R-B *et al.* (2014) Induction of HT-29 cells apoptosis by lactobacilli isolated from fermented products. *Research in Microbiology* **165**, 202-214.
52. Lacombe A, Wu VCH, Tyler S *et al.* (2010) Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7. *International Journal of Food Microbiology* **139**, 102-107.
53. Caillet S, Côté J, Sylvain J-F *et al.* (2012) Antimicrobial effects of fractions from cranberry products on the growth of seven pathogenic bacteria. *Food Control* **23**, 419-428.
54. Millette M, Luquet FM, Lacroix M (2007) In vitro growth control of selected pathogens by Lactobacillus acidophilus- and Lactobacillus casei-fermented milk. *Letters in Applied Microbiology* **44**, 314-319.
55. Tabasco R, Sánchez-Patán F, Monagas M *et al.* (2011) Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. *Food Microbiology* **28**, 1345-1352.
56. Ávila M, Hidalgo M, Sánchez-Moreno C *et al.* (2009) Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. *Food Research International* **42**, 1453-1461.
57. Fahey JW, Stephenson KK (2002) Pinostrobin from Honey and Thai Ginger (Boesenbergia pandurata): A Potent Flavonoid Inducer of Mammalian Phase 2 Chemoprotective and Antioxidant Enzymes. *Journal of Agricultural and Food Chemistry* **50**, 7472-7476.
58. Yang J, Liu RH (2009) Induction of phase II enzyme, quinone reductase, in murine hepatoma cells in vitro by grape extracts and selected phytochemicals. *Food Chemistry* **114**, 898-904.
59. Shih C-H, Siu, Ng R *et al.* (2006) Quantitative Analysis of Anticancer 3-Deoxyanthocyanidins in Infected Sorghum Seedlings. *Journal of Agricultural and Food Chemistry* **55**, 254-259.
60. Srivastava A, Akoh CC, Fischer J *et al.* (2007) Effect of Anthocyanin Fractions from Selected Cultivars of Georgia-Grown Blueberries on Apoptosis

and Phase II Enzymes. *Journal of Agricultural and Food Chemistry* **55**, 3180-3185.
61. Uda Y, Price KR, Williamson G *et al.* (1997) Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. *Cancer Letters* **120**, 213-216.
62. Goldin BR, Gorbach SL (1984) The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *The American Journal of Clinical Nutrition* **39**, 756-761.
63. Ewaschuk JB, Walker JW, Diaz H *et al.* (2006) Bioproduction of Conjugated Linoleic Acid by Probiotic Bacteria Occurs In Vitro and *In Vivo* in Mice. *The Journal of Nutrition* **136**, 1483-1487.
64. Hirayama K, Rafter J (2000) The role of probiotic bacteria in cancer prevention. *Microbes and infection* **2**, 681-686.
65. Chang JH, Shim YY, Cha SK *et al.* (2010) Probiotic characteristics of lactic acid bacteria isolated from kimchi. *Journal of Applied Microbiology* **109**, 220-230
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10. ARTICLE 2 : CHEMOPREVENTIVE EFFECTS OF EXTRACTS FROM *HIBISCUS SABDARIFFA L.*

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Effets chimiopréventifs des extraits obtenus à partir des calices séchées *d'Hibiscus sabdariffa L.*

Soumis au journal : Nutrition and Cancer le 28 octobre 2015. Cet article a été refusé. Il sera corrigé en tenant compte des remarques des correcteurs puis soumis à un autre journal.

Contribution des auteurs :

J'ai réalisé les expériences, rédigé l'article et participé à l'élaboration des protocoles. Dr. Millette m'a supervisé tout au long des expériences, corrigé l'article et participé aux discussions concernant le projet. Dr Khanh Dang Vu a participé aux discussions concernant le projet, élaboré les protocoles et corrigé l'article. Dr. Lacroix est la responsable du projet, a participé à toutes les prises de décisions, aux discussions concernant le projet et aux corrections de l'article.

Résumé :

Les propriétés chimiopréventives et antioxydantes d'extraits de polyphénols (FaJ-FbJ-FcJ) obtenus à partir de calices séchés d'*Hibiscus sabdariffa* (Hs) ont été évaluées. Les effets des extraits de Hs seuls et combinés aux constituants des composantes membranaires (CW) extraits d'une biomasse probiotique contenant les souches de lactobacilles suivantes : *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2 sur la prolifération de cellules cancéreuses du colon et l'activité de la quinone réductase (QR) ont été déterminé. Les extraits de polyphénols sont constitués en composés phénoliques solubles dans l'eau (FaJ), composés phénoliques apolaires (FbJ) et anthocyanines (FcJ). Tous les extraits testés ont été capable d'inhiber la croissance des cellules HT-29 avec des valeurs de CI_{50} variant de 82.41 à 226.18 µg EAG/ml. Les résultats ont aussi montré que combinés aux CW, une augmentation significative de l'effet inhibiteur de tous les extraits de Hs a été observée au niveau de la prolifération des cellules HT-29 ($p \leq 0.05$). Également l'activité de la quinone réductase a augmenté significativement ($p \leq 0.05$) lorsque FcJ a été combiné avec CW. La capacité antioxydante des extraits de Hs a été étudiée également en utilisant *N, N*-diethyl-*p*-phenylenediamine et les résultats obtenus montrent que l'extrait FcJ possédait la plus grande capacité pour piéger les radicaux libres ($p \leq 0.05$). En se basant sur les résultats obtenus, il est possible d'affirmer que l'*Hibiscus sabdarifa L.* possèderait un grand potentiel chimiopréventif seul et aussi combiné avec les composantes membranaires de probiotiques.

Mots clés : activité antiproliférative, quinone réductase, extraits de composés phénoliques, activité antioxydante, effet chimiopréventif, *Hibiscus sabdariffa* L.

Chemopreventive effects of extracts from Hibiscus sabdariffa

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ABSTRACT

Cancer chemopreventive and antioxidant properties of phenolic extracts (FaJ-FbJ-FcJ) obtained from *Hibiscus sabdariffa Linne* (Hs) dry calyces were evaluated. The effect of Hs phenolic extracts alone and combined with cell wall constituents (CW) extracted from a biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on the activity of quinone reductase and the growth of colon cancer cells were determined. The phenolic extracts consisted of water-soluble phenolic compounds (FaJ), apolar phenolic compounds (FbJ) and anthocyanins (FcJ). All extracts were able to inhibit the proliferation of HT-29 cells showing IC_{50} ranging from 82.41 to 226.18 µg GAE/ml. The results also showed that when combined with the CW, a significant increase in the inhibitory effects of all Hs extracts against HT-29 proliferation ($p \leq 0.05$) is observed. The activity of quinone reductase (QR) increases significantly ($p \leq 0.05$) when FcJ is combined with CW. The antioxidant capacity of the Hs phenolic extracts was investigated using *N, N*-diethyl-*p*-phenylenediamine and it was found that FcJ s was the extract showing the highest free-radical scavenging activity ($p \leq 0.05$). According to those results *Hibiscus sabdariffa* L. has a great potential as a chemopreventive substance either alone or combined with probiotic cell wall.

Keywords: Antiproliferative activity, quinone reductase, phenolic extracts, antioxidant activity, cancer chemopreventive effect, *Hibiscus sabdariffa* L.

10.1 INTRODUCTION

Cancer is the leading cause of death in developed countries and the second cause in developing countries (Jemal *et al.*, 2010) (National Cancer Statistics). Colorectal cancer represents the third most common diagnosed cancer for men and the second one for women in the western countries (Jemal *et al.*, 2011). Epidemiological studies have identified that diet plays a major role in CRC incidence (Willett, 2000); higher intakes of red and processed meat, processed food, sweets and lower intake of fruits, vegetables, dietary fiber, grains is associated with an increased CRC risk (Fung *et al.*, 2013, Miller *et al.*, 2010, Randi *et al.*, 2010).

The term chemoprevention is defined as the use of natural or synthetic substances to prevent, suppress or reverse the carcinogenic process (Hong *et al.*, 1997). Over the years there has been an increasing interest in the potential anticancer activities of plant-derived phytochemicals (Galati *et al.*, 2004, Ullah *et al.*, 2013) and many of those compounds has proven effective in the reduction of cancer risk by scavenging reactive oxygen species (ROS) (Gangwar *et al.*, 2014), deregulating cancer cell proliferation , inducing apoptosis or cycle arrest and inhibiting/inducing the activity of some enzymes.

Hibiscus sabdariffa L. (Hs) belongs to the *Malvaceae* family. It is described as a perennially tropical wild plant with a height up to 2 meter with large red to purple flowers. Depending on the geographical distribution, Hs goes by the name of “roselle”, “flor de Jamaica”, “Jamaica sorrel”, “lozey”, “l’oseille”. The calyces and the flower pods from this plant are frequently used to produce jelly, jam, tea, juice, wine and syrup (Bako *et al.*, 2009, Bolade *et al.*, 2009). Ethnobotanical information concerning Hs reveals that in traditional medicine it has been used as diuretic, mild laxative, sedative, antitussive, gastrointestinal disorder treatment (Akindahunsi *et al.*, 2003, Monroy-Ortíz *et al.*, 2007). Those therapeutic roles are mainly due to Hs rich phytochemical profile, the main constituents are organic acids, anthocyanins, polysaccharides, flavonoids and phenolic acids (Da-Costa-Rocha *et al.*, 2014, Ramirez-Rodrigues *et al.*, 2011). Previous studies have

demonstrated the antioxidant capacity (Ajiboye *et al.*, 2011, Ramakrishna *et al.*, 2008), the anti-obesity effect (Alarcon-Aguilar *et al.*, 2007, Carvajal-Zarrabal *et al.*, 2009) and the immunomodulatory activity (Fakeye *et al.*, 2008, Okoko *et al.*, 2012) of phenolic fractions or extracts obtained from *Hibiscus sabdariffa* L. (Hs).

Thus the objectives of this study to evaluate 1) the antiproliferative properties of Hs extracts alone and combined with probiotic cells walls on HT-29 proliferation, 2) the effect of those extracts when tested alone and combined on the activity of quinone reductase and 3) the antioxidant capacity of Hs extract.

10.2 MATERIALS AND METHODS

10.2.1 Chemicals reagents

Chemicals such as gallic acid, Folin-Ciocalteu reagent, methanol, dimethylsulfoxide, menadione, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, EDTA, FAD^+, digitonin, BSA, β-naphtoflavone, SDS, Tween 20, *N,N*-diethyl-*p*-phenylenediamine were purchased from Sigma (Oakville, ON, Canada). Cancer cell culture media (DME F'12, MEM-EBSS), FBS and other supplements were obtained from Fisher Scientific Limited (Ottawa, ON, Canada). Acetone and acetic acid were purchased from Laboratoires Mat (Québec, QC, Canada).

10.2.2 Raw materials

Dried calyces of *Hibiscus sabdariffa* bought from Mexico were used to determine the antiproliferative activity, antiradical properties and the ability to induce the activity of quinone reductase of the flower. They were sealed under vacuum and stored at -20°C until used.

10.2.3 Extraction of phenolic compounds

The extraction was conducted in a mild condition to avoid oxidation, thermal degradation and other chemical and biochemical change in the sample. Three different solvents solutions were used to obtain three different fractions. The most water soluble phenolic compounds were extracted with water/methanol solvent (85:15, v/v) (Seeram *et al.*, 2004), the most apolar phenolic compounds such as flavonols, flavan-3-ols and proanthocyanidins extracted with acetone/methanol/water solvent (40/40/20, v/v) (Neto *et al.*, 2006), and the anthocyanins were extracted with methanol/water/acetic acid solvent (85:15:0.5, v/v/v) (Wu *et al.*, 2005)

10.2.4 Total phenol content

Total phenol compound content in each cranberry fraction was determined by spectrophotometry according to the Folin-Ciocalteu method (Singleton *et al.*, 1965). The total phenolic content was estimated using a calibration curve (r^2 = 0.9994) by plotting known solution of gallic acid (7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml). The total phenolic content of each fractions was expressed as microgram gallic acid equivalent (GAE) per mg powder (µg GAE/mg of powder).

10.2.5 Probiotic cell wall extraction

a) Physical treatment

Concentrated biomass of probiotic *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 (Bio-K Plus International Inc.) fermented in MRS broth were centrifuged at 9 000 x g for 30 min at 4°C. The resulting pellets were washed with phosphate buffer 10mM pH 7.2 then suspended in the same buffer at a ratio of 1:4 (w/v). The suspension was heated at 65°C for 40 min, then submitted to ultrasound in 500 W, 20 KHz (Q500, QSONICA, Newtown, CT, USA) using 1 s^{-1} pulse on and 1 s^{-1} pulse off with 40% acoustic power (amplitude) for 30 min at 4°C. The sonicated samples were centrifuged at 800xg for 30 min; the supernatant was recovered and subjected to ultracentrifugation at 50 000xg for 30 min. The pellet was collected and suspended in 10 ml of 100mM Tris-HCl buffer pH 7.5 (Kim *et al.*, 2002, Signoretto *et al.*, 1998).

b) Enzymatic treatment

The pellet obtained in the previous step was firstly incubated under agitation with 50µl of α-amylase (20mg/ml) for 2 hrs at 37°C. Then, 50µl of $MgSO_4$ (1M), 100µl of DNase (1mg/ml), 100µl of RNase (50mg/ml) were added and the solution was incubated at 37°C under agitation for 2 hrs. Finally, 100µl of trypsin (10mg/ml) and 10µl of 1M $CaCl_2$ were added and the samples was incubated overnight under agitation. After the incubation, 3.5ml of 4% SDS (w/v) was added in the

10ml of samples and heated at 100°C for 15 min. The samples were centrifuged at 30 000xg for 30 min. The new obtained pellet was washed with distilled water (4X), and then with different solution: LiCl (8M), EDTA (100mM) and acetone. Each solution was used twice to wash the pellet. The washed pellets were mixed with 10ml of $(NH_4)_2CO_3$ and 250µl of alkaline phosphatase (1mg/ml) and incubated overnight at 37°C under agitation. Then the sample was heated at 100°C for 5 min and centrifuged at 30 000xg for 45 min. The pellet was washed with distilled water (2X), suspended in water and lyophilized. The powder was weighed and kept at -20°C until used (Signoretto *et al.*, 1998).

10.2.6 Cell cultures, antiproliferative assay and quinone reductase assay

a) Cell proliferation agent MTT

MTT, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide is a tetrazolium salt that have been widely used for the assessment of cytotoxicity, cell viability and proliferation studies in cell biology (Kiernan, 2001, van Meerloo *et al.*, 2011). The assay is based on the reduction of the yellow tetrazolium salt into purple formazan crystals by dehydrogenase and reducing agents present in metabolically active cells. The amount of formazan is measured using a spectrophotometer to determine the viable cells since the amount of formazan is proportional with the number of living cells (van Meerloo *et al.*, 2011).

b) Preparation of samples

The stock of hibiscus extract were diluted using 10% (v/v) dimethylsulfoxyde (DMSO) (Sigma-Aldrich, Oakville, ON, Canada). The concentrations tested were 15, 20, 25, 30, 35, 40, 45 and 50 mg/ml for the antiproliferative assay and for the quinone reductase assay, the concentration tested were 5. 2.5, 1.25, 0.63, 0.32, 0.16 and 0.08 mg/ml.

c) *Cell lines and culture media*

Hepa 1c1c7 murine hepatoma cells (ATCC CRL-2026) and HT-29, human colon adenocarcinoma (ATCC HTB-38) were purchased from the American Type Culture Collection, ATCC, Rockville, MD, USA. The HT-29 and Hepa 1c1c7 cells were maintained in complete DMEM-Ham's F12 and MEM EBSS growth medium respectively containing 10% of FBS at 37°C in 5% CO_2 atmosphere.

d) *Antiproliferative assay*

The antiproliferative effect of our samples was measured using MTT color assay. The HT-29 were seeded at 2 x 10^4 cells in 200µl of complete growth medium in 96 wells microplate Corning® CELLBIND®Surface microplate (Costar, Corning Inc., Corning, NY, USA). After 24 hrs of incubation at 37°C in 5% CO_2, the medium was replaced with 90µl of fresh medium containing 10µl of serial concentration of samples. Control wells received the same amount of medium with 10µl of DMSO 10% and blank wells received 100µl of medium with no cells. After 48 hrs of incubation, the growth medium containing the sample is decanted and replaced with 200µl of new growth medium containing 25µl of MTT. The microplate were incubated for 4 hours. Then, 200µl of DMSO with 25µl of Sorensen buffer were added to the microplate. Absorbance was measured at 562nm for each concentration compared to the control. At least five replications for each sample were used to determine the cell proliferation.

Equation 3 : Inhibition percentage = 100-(OD controls cells/OD treated cells*100)

IC_{50} is the fraction concentration under which the proliferation of 50% of the cells is inhibited.

10.2.7 Quinone reductase assay

8.1.1.1 Assay procedure:

Hepa 1c1c7 cells were seeded in 96 well microplate in 200µl of MEM/EBSS medium at a concentration of 1 X 10^4cells. After 24 hours of incubation at 37° C

in 5% CO_2, humidified incubator; the medium was decanted and replaced with 190µl of fresh medium containing 10µl of serial concentrations of samples. After 48 hours of incubation the medium was decanted, and the cells were incubated for 10 minutes with 50µl of 0.8% digitonin and 2mM EDTA solution, then the microplates were agitated for 10 min in an orbital shaker and 200µl of a reaction mixture were added in each well. (22 ml of solution were prepared for each set of assays: 20.5 ml distilled water, 1.1 ml of 500mM Tris-HCl buffer pH 7.4, 14.7 mg of bovine serum albumin, 146.7µl of 1.5% (w/v) Tween 20 solution, 14.7µl of 7.5mM FAD^+, 146.7µl 0f 150mM Glucose 6-phosphate solution, 13.2µl of 50mM $NADP^+$ solution, 44µl of Glucose 6-phosphate dehydrogenase solution, 6.6 mg MTT and 22µl of 50mM menadione solution). The microplate was incubated for 5 minutes at room temperature then the absorbance was read at 595nm using a microplate reader (ELISA reader, CLX800-BioTek Instruments).

8.1.1.2 Protein estimation:

The protein quantification was performed using BCA assay kit (Thermo Scientific) according to the manufacture's specifications. A 20µl aliquot of lysed cells was transferred to a new microplate and 300µl BCA were added to each. Then, the microplate was incubated for 30 min in the humidified incubator in 5 % CO_2 at 37°C and after the incubation, absorbance was read at 595 nm with a spectrophotometer (ELISA reader, CLX800-BioTek Instruments).

The specific activity of quinone reductase is defined as nM MTT formazan formed per min per mg protein.

Equation 4: Specific activity = (absorbance of MTT 595 nm/min (5 minutes) * 3247 nmol/mg)/ (absorbance obtained for determining protein at 595 nm).

Induction of QR of treated samples was calculated by dividing specific QR activity of the treated sample to specific QR of the control samples (cells + DMSO 10 %).

10.2.8 Free radical scavenging capacity

Antioxidant properties of hibiscus extracts (1 mg/ml) were evaluated following a modified procedure of the DPPD colorimetric method by (Caillet *et al.*, 2006). 200 µl of each extracts were added to an electrolytic cell containing 3 ml of 0.15 M NaCl and submitted to electrolysis for 1min (10 mA direct current (DC), 400V) using a power supply from Bio-Rad, model 1000/500 (Mississauga, ON, Canada). After the electrolysis an aliquot of 200 µl was added to 2 ml of DPD solution (2.5 % p/v) and mixed thoroughly. The generated oxidative species react instantly with DPD producing a red coloration that can be measured at 515 nm using a spectrophotometer from Varian Canada Inc. (Mississauga, ON, Canada). The scavenging percentage was calculated according to the following equation:

Equation 5 : Scavenging activity (%) = $[1-(A_{sample}/A_{control})]*100$

10.2.9 Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate the differences between the samples tested with SPSS Base 16.0 software (Stat-Packets statistical analysis software, SPSS Inc., Chicago IL). Significant differences ($p \leq 0.05$) were evaluated using Duncan's comparisons test.

10.3 RESULTS AND DISCUSSION

10.3.1 Total phenol content :

To quantify the contribution of the phenolic compounds to the antioxidant capacity and the antiproliferative effects of each extract, the concentration of polyphenols was analysed. The results are presented in Table 6. The phenolic content varies from 30 to 70 µg GAE/mg of powder. The flavonoids rich extract (FbJ) and the anthocyanin-rich extract (FcJ) present the highest phenolic concentration as compared to the polyphenol rich extract (FaJ).

Table 6 : Total phenolic content *Hibiscus sabdariffa* (Hs) extracts. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces: **FaJ**: composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound and **FcJ**: rich with anthocyanins. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$).

Fractions	**Phenolic Compounds**	**µg GAE/mg of powder**
FaJ	Water-soluble	30.84±0.23[a]
FbJ	Apolar	67.60±1.69[b]
FcJ	Anthocyanins	69.63±0.49[b]

The results showed that fractions FbJ and FcJ rich an apolar phenolic compounds and anthocyanins respectively have a higher concentration of phenolic contents. Previous studies showed that the most abundant polyphenols in *Hibiscus sabdariffa* L. are mainly anthocyanins and flavonoids. Phenolic acids only represent 11% of the phenolic profile of Hs (Borrás-Linares *et al.*, 2015, Carvajal-Zarrabal *et al.*, 2012).

10.3.2 Antiproliferative assay

a) Antiproliferative activity of Hs extracts against HT-29 proliferation

The effect of Hs extracts on HT-29 growth and the IC_{50} values of each extract are shown in Fig. 8 and Table 6 respectively.

A concentration-dependant response was observed for two extracts FaJ; the extracts rich with water-soluble phenolic compounds (polyphenols-rich extract) and FbJ which contained mainly apolar phenolic compounds. Those extracts have IC_{50} values statistically similar.

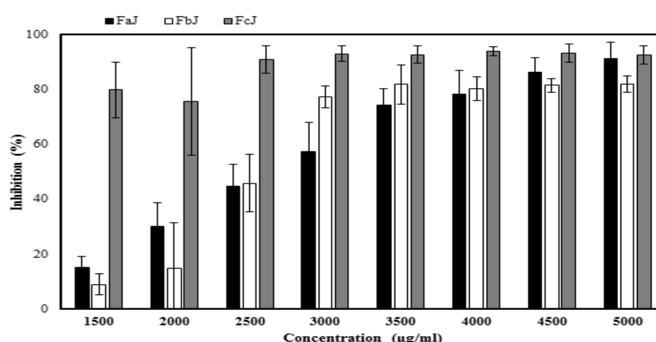

Figure 7 : Effect of extracts from dried calyces of *Hibiscus sabdariffa* (Hs) on HT-29 proliferation. HT-29 cells were treated with different concentrations (1500-5000µg/ml) of phenolic extracts from *Hibiscus sabdariffa* dry calyces. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces: **FaJ**: composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound and **FcJ**: rich with anthocyanins. Data are presented as mean ± standard deviation.

Table 7 : IC50 values of the Hibiscus sabdariffa (Hs) extracts tested. 200 µl of phenolic extracts from *Hibiscus sabdariffa* dry calyces were added to an electrolytic cell containing 3 ml of 0.15 M NaCl and submitted to electrolysis for 1min. **FaJ:** composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound and **FcJ**: rich with anthocyanins. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$).

Fractions	µg GAE/mg of powder
FaJ	104.42 ± 12.28^{a}
FbJ	226.18 ± 25.10^{b}
FcJ	82.41 ± 8.98^{a}

The water-soluble phenolic compounds found in Hs are mostly phenolic acids like protocatechuic, chlorogenic, gallic acids, anthocyanins such as delphinidine-3-sambubioside and cyaniding-3-sambubioside and finally some flavonoids like quercetin, myrecitin, kaemperol, rutin (Beltrán-Debón *et al.*, 2010, Peng *et al.*, 2011).

Many studies have investigated and demonstrated the anticancer properties of those compounds (Hou *et al.*, 2005, Reddy *et al.*, 2012, Yang *et al.*, 2008, Yin *et al.*, 2009). Thurow *et al.* (2012) investigated the effect of chlorogenic acid on Caco-2 cells proliferation. They found that at a concentration of 150, 300 and 500 µmol chlorogenic acid was able to decrease by 63.7, 90.1 and 85.7 % respectively Caco-2 cell proliferation. They also observed the change in cells morphology when exposed to cholorogenic acid. The cytotoxicity effect of rutin against SW480 cells was evaluated by Alonso-Castro *et al.* (2013). The authors found that rutin had a high cytotoxicity effect against the cells (IC_{50} = 125 µM) when tested at a concentration of 20 mg/kg.

FbJ, flavonols-rich extract has an IC_{50} of 226.18±25.10 µg GAE/mg of powder. This extract is mainly composed of with flavonoids like quercetin, luteoloin, hibiscin-3-glucoside, sabdartrin, gossypitrin and eugenol (McKay *et al.*, 2010a, Williamson *et al.*, 2009). *In vitro* and *in vivo* studies have shown the chemopreventive effects of those compounds (Kim *et al.*, 2003, Lee *et al.*, 2006b, Meraiyebu *et al.*, 2013).

Jaganathan *et al.* (2011) investigated the antiproliferative effect of eugenol on two colon cancer cell lines: HT-29 and HCT-15. Results showed that the growth of both cells was inhibited by eugenol and also HCT-15 was more sensitive (IC_{50} = 300 µM) to eugenol than HT-29 cells (IC_{50} = 500 µM).

FcJ (anthocyanin-rich extract) present an IC_{50} value of 82.41±8.98 µg GAE/mg of powder. The anthocyanins mainly found in *Hibiscus sabdariffa* are delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside and cyanidin-3-glucoside (Williamson *et al.*, 2009). Anthocyanins are a large group polyphenols widely disturbed in our diet (Hertog *et al.*, 1993). They have been extensively

investigated because of their health promoting effects such as their antioxidant capacity (Rahman *et al.*, 2006), protective effect against DNA damage (Esselen *et al.*, 2009), and role in cancer prevention (Bunea *et al.*, 2013).

A study conducted by Shin *et al.* (2009) demonstrated the ability of anthocyanins isolated from *Vitis coignetiae* to induce apoptosis and inhibit cell viability of HCT-116 cells in a dose dependant manner. Jing *et al.* (2008) investigated the effect of anthocyanin-rich fraction from different sources on HT-29 proliferation. All samples were able to inhibit the growth of the cancer cells but at different rates.

Our results are in agreement with previous cited studies concerning all extracts. Further investigation should be performed to determine by which mechanism the compounds affect the cancer cell growth (apoptosis, regulating the anti-inflammatory response) and also whether the antiproliferative capacity of each extract is a due to a synergetic or an additive effect of all the compounds.

b) Antiproliferative activity of Hs extract combined with cell wall constituents from a probiotic biomass against HT-29 proliferation

In a previous study conducted by Desrouillères K. *et al.* (2015), it was reported that the cells walls from the probiotic biomass did not affect the growth of HT-29 cells. The effect of the combination of Hs extracts and probiotic cell wall extracts on HT-29 growth are presented in Fig. 8.

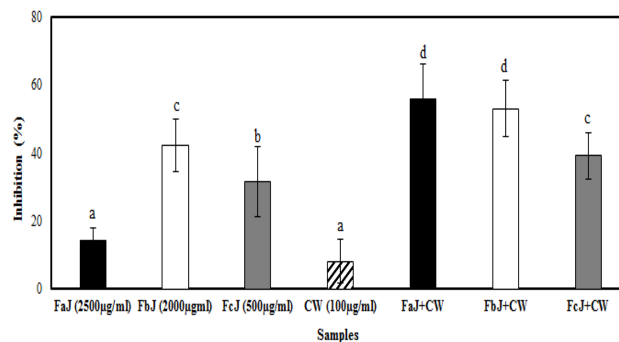

Figure 8 : Effect of *Hibiscus sabdariffa* (Hs) extracts associated with probiotic cells walls on HT-29 proliferation. HT-29 cells were exposed to *Hibiscus sabdariffa* extracts combined with extract of cell wall constituents. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces and CW constituents were extracted from a probiotic biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. The samples are tested at a concentration

less than 50% inhibition rate: FaJ (2500 µg/ml), FbJ (2000 µg/ml), FcJ (500 µg/ml) and CW (100 µg/ml). Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). **FaJ**: composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound **FcJ**: rich with anthocyanins and **CW**: cell wall constituents**.**

When combining Hs extract with the probiotic cell wall, we observed an increase of the inhibitory effect of each sample against HT-29 proliferation as compared to the fractions alone. FaJ alone inhibit HT-29 proliferation by 14.06 ± 3.71 % at a concentration of 2500 µg/ml but when combined with the cell wall extracts (100 µg/ml), the inhibitory effect increase by nearly 40 %. FbJ alone at a concentration of 2000 µg/ml inhibit the proliferation of by 42.35 ± 7.85 %, but with the cell wall there is an increase of nearly 10 % of the inhibition rate and FcJ combined with the cell wall extract inhibit HT-29 proliferation by 39.14 ± 6.66 % which represent an increase of 8 % compared to the inhibition rate of the phenolic extract when tested alone. Those results suggest that the cell wall combined with FaJ work in a synergetic manner to inhibit the cancer cell proliferation while FbJ and FcJ with the cell wall have an additive effect or a potentiating. The increase of the inhibition rate when combining the two compounds could be explained by an interaction between the phenolic compounds and the component of the probiotic cell wall (peptidoglycan, teichoic acid and cell-wall associated polysaccharides). This interaction may have an effect on the cancer cell proliferation by either modifying the cell morphology or affecting some growth factors that could lead to cell death. Further studies are required to determine the nature of the link between phenolic compounds and cell wall and the precise mechanism by which that link affect cancer cells growth.

10.3.3 Quinone reductase assay

a) Effect of Hs extracts on quinone reductase activity

QR activity of cells treated with Hs extract are shown in Fig. 9.

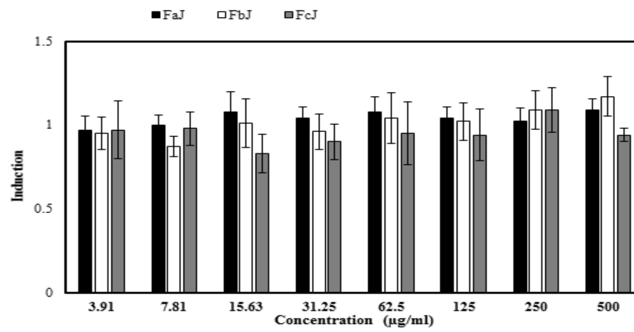

Figure 9 : Effect of *Hibiscus sabdariffa* (Hs) extracts on the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were treated with different concentrations (3.91-500 µg/ml) of phenolic extracts from *Hibiscus sabdariffa* dry calyces. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces: **FaJ**: composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound and **FcJ**: rich with anthocyanins. Data are presented as mean ± standard deviation

None of the fraction were able to induce the enzyme activity. Those results are in disagreement with the ones reported in previous studies (Adaramoye *et al.*, 2008, Wang *et al.*, 2000). The key antioxidant compounds in Hs are the anthocyanins and protocatechuic acid and chlorogenic acid derivatives (Mohd-Esa *et al.*, 2010).

In a study conducted by Li *et al.* (2012), it was shown that three compounds isolated from black chokeberry fruits were able to double the induction of QR at a concentration lower than 20 µM. Those compounds were protocatechuic acid, neochlorogenic methyl ester and quercetin. The only extract that may contain Hs phenolic acid is FaJ; besides the phenolic acids it also contained the anthocyanins and some flavonol and flavanol. It could be speculated that the presence of all those compounds have an antagonist effect upon the ability of chlorogenic and protocatechuic acids to induce QR activity.

As for the anthocyanin-rich extract (FcJ); a study conducted by Ajiboye *et al.* (2011) showed that anthocyanin extract from *Hibiscus* when fed to rats, has the ability to increase the activity of phase II detoxifying such as glutathione S-transferase, quinone reductase, and uridyl diphosphoglucuronosyl transferase by 65%, 45%, and 57%, respectively in rats. In our study there is no induction, but some studies showed that a compound can have little to no potential at inducing

QR activity when tested *in vitro* but tested in an animal model gived different results. They suggested that an intermediate of the compound was produced within a metabolic process that occur *in vivo*, and that intermediate was a strong QR inducer (Bolling *et al.*, 2011, Kawabata *et al.*, 2000, Yang *et al.*, 2009).

Other fact that could explain the discrepancies between our results and the one obtained in previous studies is the use of different extraction procedure (Sindi *et al.*, 2014). Further investigation are needed to determine the effect of the phenolic acids and the anthocyanins from *Hibiscus* on the activity of the enzyme such as a fractionation by HPLC of FaJ to obtain enriched fractions of each classes of polyphenols and also the use of an animal model.

b) Effect of Hs extracts combined with cell wall constituents on quinone reductase activity.

In a previous study conducted in our laboratory it as reported that the cell wall extracted from the probiotic biomass containing three strains of lactobacillus had no effect on QR activity. The effect of the combination of Hs extracts and cell walls are presented in Fig 10.

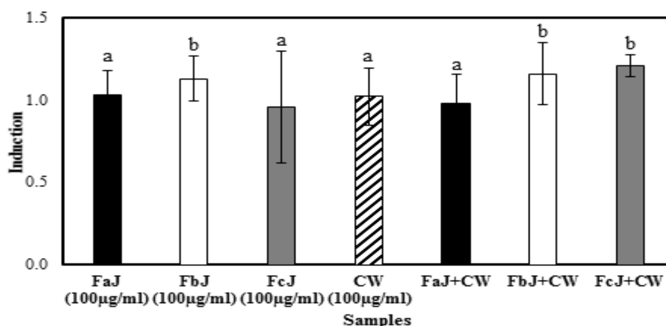

Figure 10 : Effect of *Hibiscus sabdariffa* (Hs) extracts associated with probiotic cells walls on the induction of quinone reductase activity. Murine hepatoma (Hepa 1c1c7) cells were exposed to *Hibiscus sabdariffa* extracts combined with extract of cell wall constituents. FaJ, FbJ and FcJ were extracted from *Hibiscus sabdariffa* dry calyces and CW constituents were extracted from a probiotic biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2. All samples are tested at a concentration of 100µg/ml. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq 0.05$). **FaJ**: composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound **FcJ**: rich with anthocyanins and **CW**: cell wall constituents

Although for FaJ and FbJ; the cell wall did not affect their inducer rate; for FcJ in the other the hand; the addition of cell wall significantly increase ($p \leq 0.05$) his inducer rate. It is possible that a link between the anthocyanins and the compounds present in the cell wall formed a new molecule that is able to increase the QR activity. Further studies should be realised to investigate the nature of the interaction between anthocyanins and cell wall and also to determine if the effect of anthocyanin-rich extract combined with cell wall on QR activity is a dose-dependent response.

10.3.4 Free radical scavenging capacity

Reactive oxygen species (ROS) are produced in a normal cellular metabolism either by the mitochondrial respiratory chain or during endogenous cell reaction. They are vital in the stimulation of signaling pathways. Oxidative stress occurs when there is an imbalance between the formation of free radicals and the elimination of ROS.

Table 8: Radical scavenging capacity of *Hibiscus sabdariffa* (Hs) extracts HT-29 cells were incubated with different concentrations (1500-5000µg/ml) of phenolic extracts from Hibiscus sabdariffa dry calyces for 48 hours. Three extracts were obtained: **FaJ:** composed mainly with water-soluble phenolic compound, **FbJ**: rich with apolar phenolic compound and **FcJ**: rich with anthocyanins. Data are presented as mean ± standard deviation. Different letters are significantly different

Fractions	**TE/mg of powder**
FaJ	1.33±0.85[a]
FbJ	1.34±0.65[a]
FcJ	2.54±0.60[b]

The free radical scavenging capacity of Hs extracts is presented in Table 8. The anthocyanin-rich extract have the highest scavenging activity. Some study suggest that the strong antioxidant activity of Hs is partly due to their scavenging capacity of reactive oxygen species and free radicals (Mohd-Esa *et al.*, 2010, Sáyago-Ayerdi *et al.*, 2007).

Ajiboye *et al.* (2011) showed that *Hibiscus sabdariffa* anthocyanin extract has the ability to scavenge efficiently DPPH radical, superoxide ion and hydrogen peroxide. At a concentration of 2 mg/ml the extract produce a 92% scavenging effect of DPPH radical and at 1 mg/ml a 69% and 90% scavenging effect against superoxide ion and hydrogen peroxide respectively. Our results are in agreement with previous studies.

10.4 CONCLUSION

This study showed that all extracts from *Hibiscus sabdariffa* were able to inhibit HT-29 proliferation. The extract with the greatest efficacy was FcJ, the anthocyanin-rich extract. It was also demonstrated that when combining cell wall and *Hibiscus sabdariffa* extracts the inhibition rate of each extract increases. None of the extracts was able to induce QR activity and only the anthocyanin-rich fraction showed a significant increase when combined with the cell wall. That extract also showed the strongest capacity to scavenge free radicals as compared to the other two. Those results confirm the chemopreventive potential of *Hibiscus sabdariffa* and Hs extract combined with cell wall constituents could have either act either in synergetic manner or a potentiating manner depending on the extracts against colon cancer cell proliferation

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REFERENCES

Adaramoye, Oluwatosin, Bayo Ogungbenro, Oluchi Anyaegbu, and Michael Fafunso. 2008. "Protective Effects of Extracts of Vernonia amygdalina, Hibiscus sabdariffa and Vitamin C against Radiation-induced Liver Damage in Rats." *Journal of Radiation Research* 49 (2):123-131. doi: 10.1269/jrr.07062.

Ajiboye, Taofeek O., Nasir A. Salawu, Musa T. Yakubu, Adenike T. Oladiji, Musbau A. Akanji, and Joseph I. Okogun. 2011. "Antioxidant and drug detoxification potentials of Hibiscus sabdariffa anthocyanin extract." *Drug and Chemical Toxicology* 34 (2):109-115. doi: doi:10.3109/01480545.2010.536767.

Akindahunsi, A. A., and M. T. Olaleye. 2003. "Toxicological investigation of aqueous-methanolic extract of the calyces of Hibiscus sabdariffa L." *Journal of Ethnopharmacology* 89 (1):161-164. doi: http://dx.doi.org/10.1016/S0378-8741(03)00276-9.

Alarcon-Aguilar, Francisco J., Alejandro Zamilpa, Ma Dolores Perez-Garcia, Julio C. Almanza-Perez, Eunice Romero-Nuñez, Efrain A. Campos-Sepulveda, Laura I. Vazquez-Carrillo, and Ruben Roman-Ramos. 2007. "Effect of Hibiscus sabdariffa on obesity in MSG mice." *Journal of Ethnopharmacology* 114 (1):66-71. doi: http://dx.doi.org/10.1016/j.jep.2007.07.020.

Alonso-Castro, Angel Josabad, Fabiola Domínguez, and Alejandro García-Carrancá. 2013. "Rutin Exerts Antitumor Effects on Nude Mice Bearing SW480 Tumor." *Archives of Medical Research* 44 (5):346-351. doi: 10.1016/j.arcmed.2013.06.002.

Bako, IG, MA Mabrouk, and A Abubakar. 2009. "Antioxidant effect of ethanolic seed extract of hibiscus sabdariffa linn (Malvaceae) alleviate the toxicity induced by chronic administration of sodium nitrate on some haematological parameters in wistars rats." *Advance Journal of Food Science and Technology* 1 (1):39-42.

Beltrán-Debón, R., C. Alonso-Villaverde, G. Aragonès, I. Rodríguez-Medina, A. Rull, V. Micol, A. Segura-Carretero, A. Fernández-Gutiérrez, J. Camps, and J. Joven. 2010. "The aqueous extract of Hibiscus sabdariffa calices modulates the production of monocyte chemoattractant protein-1 in humans." *Phytomedicine* 17 (3–4):186-191. doi: http://dx.doi.org/10.1016/j.phymed.2009.08.006.

Bolade, MK, IB Oluwalana, and O Ojo. 2009. "Commercial practice of roselle (Hibiscus sabdariffa L.) beverage production: Optimization of hot water extraction and sweetness level." *World Journal of Agricultural Sciences* 5 (1):126-131.

Bolling, Bradley W., Li Li Ji, Chen-Hsien Lee, and Kirk L. Parkin. 2011. "Dietary supplementation of ferulic acid and ferulic acid ethyl ester induces quinone reductase and glutathione-S-transferase in rats." *Food Chemistry* 124 (1):1-6. doi: http://dx.doi.org/10.1016/j.foodchem.2010.05.093.

Bunea, Andrea, Dumitriţa Rugină, Zoriţa Sconţa, Raluca M. Pop, Adela Pintea, Carmen Socaciu, Flaviu Tăbăran, Charlotte Grootaert, Karin Struijs, and John VanCamp. 2013. "Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells." *Phytochemistry* 95 (0):436-444. doi: http://dx.doi.org/10.1016/j.phytochem.2013.06.018.

Caillet, Stéphane, Stéphane Salmiéri, and Monique Lacroix. 2006. "Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric method." *Food Chemistry* 95 (1):1-8. doi: http://dx.doi.org/10.1016/j.foodchem.2004.12.011.

Carvajal-Zarrabal, O, PM Hayward-Jones, Z Orta-Flores, C Nolasco-Hipólito, DM Barradas-Dermitz, MG Aguilar-Uscanga, and MF Pedroza-Hernández. 2009. "Effect of Hibiscus sabdariffa L. dried calyx ethanol extract on fat absorption-excretion, and body weight implication in rats." *BioMed Research International* 2009.

Da-Costa-Rocha, Inês, Bernd Bonnlaender, Hartwig Sievers, Ivo Pischel, and Michael Heinrich. 2014. "Hibiscus sabdariffa L. – A phytochemical and pharmacological review." *Food Chemistry* 165 (0):424-443. doi: http://dx.doi.org/10.1016/j.foodchem.2014.05.002.

Esselen, Melanie, Jessica Fritz, Melanie Hutter, and Doris Marko. 2009. "Delphinidin Modulates the DNA-Damaging Properties of Topoisomerase II Poisons." *Chemical Research in Toxicology* 22 (3):554-564. doi: 10.1021/tx800293v.

Fakeye, Titilayo O., Anirban Pal, D. U. Bawankule, and S. P. S. Khanuja. 2008. "Immunomodulatory effect of extracts of Hibiscus sabdariffa L. (Family Malvaceae) in a mouse model." *Phytotherapy Research* 22 (5):664-668. doi: 10.1002/ptr.2370.

Fung, Teresa T., and Lisa S. Brown. 2013. "Dietary Patterns and the Risk of Colorectal Cancer." *Current nutrition reports* 2 (1):48-55. doi: 10.1007/s13668-012-0031-1.

Galati, Giuseppe, and Peter J. O'Brien. 2004. "Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties." *Free Radical Biology and Medicine* 37 (3):287-303. doi: http://dx.doi.org/10.1016/j.freeradbiomed.2004.04.034.

Gangwar, Mayank, Manish Kumar Gautam, Amit Kumar Sharma, Yamini B. Tripathi, R. K. Goel, and Gopal Nath. 2014. "Antioxidant Capacity and Radical Scavenging Effect of Polyphenol Rich Mallotus philippenensis Fruit Extract on Human Erythrocytes: An In Vitro Study." *The Scientific World Journal* 2014:12. doi: 10.1155/2014/279451.

Hanahan, Douglas, and Robert A. Weinberg. 2000. "The Hallmarks of Cancer." *Cell* 100 (1):57-70. doi: 10.1016/S0092-8674(00)81683-9.

Hertog, Michaël G. L., Peter C. H. Hollman, Martijn B. Katan, and Daan Kromhout. 1993. "Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands." *Nutrition and Cancer* 20 (1):21-29. doi: 10.1080/01635589309514267.

Hong, Waun Ki, and Michael B. Sporn. 1997. "Recent Advances in Chemoprevention of Cancer." *Science* 278 (5340):1073-1077. doi: 10.1126/science.278.5340.1073.

Hou, De-Xing, Xuhui Tong, Norihiko Terahara, Dong Luo, and Makoto Fujii. 2005. "Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway." *Archives of Biochemistry and Biophysics* 440 (1):101-109. doi: http://dx.doi.org/10.1016/j.abb.2005.06.002.

Jaganathan, Saravana Kumar, Abhijit Mazumdar, Dilip Mondhe, and Mahitosh Mandal. 2011. "Apoptotic effect of eugenol in human colon cancer cell lines." *Cell Biology International* 35 (6):607-615. doi: 10.1042/CBI20100118.

Jemal, A., R. Siegel, J. Xu, and E. Ward. 2010. "Cancer Statistics, 2010." *CA: A Cancer Journal for Clinicians* 60 (5):277-300. doi: 10.3322/caac.20073.

Jemal, Ahmedin, Freddie Bray, Melissa M. Center, Jacques Ferlay, Elizabeth Ward, and David Forman. 2011. "Global cancer statistics." *CA: A Cancer Journal for Clinicians* 61 (2):69-90. doi: 10.3322/caac.20107.

Jing, Pu, Joshua A. Bomser, Steven J. Schwartz, Jian He, Bernadene A. Magnuson, and M. Mónica Giusti. 2008. "Structure−Function Relationships of Anthocyanins from Various Anthocyanin-Rich Extracts on the Inhibition of Colon Cancer Cell Growth." *Journal of Agricultural and Food Chemistry* 56 (20):9391-9398. doi: 10.1021/jf8005917.

Kawabata, Kunihiro, Tomohiro Yamamoto, Akira Hara, Masahito Shimizu, Yasuhiro Yamada, Kengo Matsunaga, Takuji Tanaka, and Hideki Mori. 2000. "Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats." *Cancer Letters* 157 (1):15-21. doi: http://dx.doi.org/10.1016/S0304-3835(00)00461-4.

Kiernan, JA. 2001. "Classification and naming of dyes, stains and fluorochromes." *Biotechnic & histochemistry* 76 (5-6):261-278.

Kim, JiYeon, HeeJong Woo, Young-Suk Kim, and HyongJoo Lee. 2002. "Screening for antiproliferative effects of cellular components from lactic acid bacteria against human cancer cell lines." *Biotechnology Letters* 24 (17):1431-1436. doi: 10.1023/A:1019875204323.

Kim, Sun Suk, O. Jin Oh, Hye-Young Min, Eun-Jung Park, Youngleem Kim, Hyen Joo Park, Yong Nam Han, and Sang Kook Lee. 2003. "Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells." *Life Sciences* 73 (3):337-348. doi: http://dx.doi.org/10.1016/S0024-3205(03)00288-1.

Lee, Wei-Jiunn, Lan-Feng Wu, Wen-Kang Chen, Chau-Jong Wang, and Tsui-Hwa Tseng. 2006. "Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K–Akt pathways." *Chemico-Biological Interactions* 160 (2):123-133. doi: http://dx.doi.org/10.1016/j.cbi.2006.01.002.

Li, Jie, Ye Deng, Chunhua Yuan, Li Pan, Heebyung Chai, William J. Keller, and A. Douglas Kinghorn. 2012. "Antioxidant and Quinone Reductase-Inducing Constituents of Black Chokeberry (Aronia melanocarpa) Fruits." *Journal of Agricultural and Food Chemistry* 60 (46):11551-11559. doi: 10.1021/jf303712e.

McKay, Diane L., C-Y. Oliver Chen, Edward Saltzman, and Jeffrey B. Blumberg. 2010. "Hibiscus Sabdariffa L. Tea (Tisane) Lowers Blood Pressure in Prehypertensive and Mildly Hypertensive Adults." *The Journal of Nutrition* 140 (2):298-303. doi: 10.3945/jn.109.115097.

Meraiyebu, AB, OT Olaniyan, C Eneze, YD Anjorin, and JB Dare. 2013. "Anti-inflammatory Activity of Methanolic Extract of Hibiscus sabdariffa on Carrageenan Induced Inflammation in Wistar Rat."

Miller, Paige E., Samuel M. Lesko, Joshua E. Muscat, Philip Lazarus, and Terryl J. Hartman. 2010. "Dietary Patterns and Colorectal Adenoma and Cancer Risk: A Review of the Epidemiological Evidence." *Nutrition and Cancer* 62 (4):413-424. doi: 10.1080/01635580903407114.

Mohd-Esa, Norhaizan, Fong Shin Hern, Amin Ismail, and Chew Lye Yee. 2010. "Antioxidant activity in different parts of roselle (Hibiscus sabdariffa L.) extracts and potential exploitation of the seeds." *Food Chemistry* 122 (4):1055-1060. doi: http://dx.doi.org/10.1016/j.foodchem.2010.03.074.

Monroy-Ortíz, C., and P.C. España. 2007. *Plantas medicinales utilizadas en el estado de Morelos*: Universidad Autónoma del Estado de Morelos.

Neto, Catherine C., Christian G. Krueger, Toni L. Lamoureaux, Miwako Kondo, Abraham J. Vaisberg, Robert A. R. Hurta, Shannon Curtis, Michael D. Matchett, Horace Yeung, Marva I. Sweeney, and Jess D. Reed. 2006. "MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit (Vaccinium macrocarpon) that inhibit tumor cell growth and matrix metalloproteinase expression in vitro." *Journal of the Science of Food and Agriculture* 86 (1):18-25. doi: 10.1002/jsfa.2347.

Okoko, Tebekeme, and Diepreye Ere. 2012. "Hibiscus sabdariffa extractivities on cadmium—mediated alterations of human U937 cell viability and activation." *Asian Pacific Journal of Tropical Medicine* 5 (1):33-36. doi: http://dx.doi.org/10.1016/S1995-7645(11)60241-1.

Peng, Chiung-Huei, Charng-Cherng Chyau, Kuei-Chuan Chan, Tsung-Hsien Chan, Chau-Jong Wang, and Chien-Ning Huang. 2011. "Hibiscus sabdariffa Polyphenolic Extract Inhibits Hyperglycemia, Hyperlipidemia, and Glycation-Oxidative Stress while Improving Insulin Resistance." *Journal of Agricultural and Food Chemistry* 59 (18):9901-9909. doi: 10.1021/jf2022379.

Rahman, M. Mamunur, Takashi Ichiyanagi, Tadazumi Komiyama, Yoshihiko Hatano, and Tetsuya Konishi. 2006. "Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism." *Free Radical Research* 40 (9):993-1002. doi: doi:10.1080/10715760600815322.

Ramakrishna, BV, GK Jayaprakasha, BS Jena, and RP Singh. 2008. "Antioxidant activitiesof roselle (Hibiscus sabdariffa) calyces and fruit extracts." *Journal of Food Science and Technology* 45 (3):223-227.

Ramirez-Rodrigues, Milena M., Maria L. Plaza, Alberto Azeredo, Murat O. Balaban, and Maurice R. Marshall. 2011. "Physicochemical and Phytochemical Properties of Cold and Hot Water Extraction from Hibiscus sabdariffa." *Journal of Food Science* 76 (3):C428-C435. doi: 10.1111/j.1750-3841.2011.02091.x.

Randi, Giorgia, Valeria Edefonti, Monica Ferraroni, Carlo La Vecchia, and Adriano Decarli. 2010. "Dietary patterns and the risk of colorectal cancer and adenomas." *Nutrition reviews* 68 (7):389-408.

Reddy, T. C., D. B. Reddy, A. Aparna, K. M. Arunasree, G. Gupta, C. Achari, G. V. Reddy, V. Lakshmipathi, A. Subramanyam, and P. Reddanna. 2012. "Anti-leukemic effects of gallic acid on human leukemia K562 cells: Downregulation of COX-2, inhibition of BCR/ABL kinase and NF-κB inactivation." *Toxicology in Vitro* 26 (3):396-405. doi: http://dx.doi.org/10.1016/j.tiv.2011.12.018.

Sáyago-Ayerdi, Sonia G., Sara Arranz, José Serrano, and Isabel Goñi. 2007. "Dietary Fiber Content and Associated Antioxidant Compounds in Roselle Flower (Hibiscus sabdariffa L.) Beverage." *Journal of Agricultural and Food Chemistry* 55 (19):7886-7890. doi: 10.1021/jf070485b.

Seeram, N. P., L. S. Adams, M. L. Hardy, and D. Heber. 2004. "Total Cranberry Extract versus Its Phytochemical Constituents: Antiproliferative and Synergistic Effects against Human Tumor Cell Lines." *Journal of Agricultural and Food Chemistry* 52 (9):2512-2517. doi: 10.1021/jf0352778.

Shih, Ping-Hsiao, Chi-Tai Yeh, and Gow-Chin Yen. 2007. "Anthocyanins Induce the Activation of Phase II Enzymes through the Antioxidant Response Element Pathway against Oxidative Stress-Induced Apoptosis." *Journal of Agricultural and Food Chemistry* 55 (23):9427-9435. doi: 10.1021/jf071933i.

Shin, D.Y., W.S. Lee, J.N. Lu, M.H. Kang, C.H. Ryu, G.Y. Kim, and Y.H. Choi. 2009. "Induction of apoptosis in human colon cancer HCT-116 cells by anthocyanins through suppression of Akt and activation of p38-MAPK." *International Journal of Oncology* 35:1499-1504.

Signoretto, Caterina, Marzia Boaretti, and P. Canepari. 1998. "Peptidoglycan synthesis by Enterococcus faecalis penicillin binding protein 5." *Archives of Microbiology* 170 (3):185-190. doi: 10.1007/s002030050632.

Sindi, Heba A., Lisa J. Marshall, and Michael R. A. Morgan. 2014. "Comparative chemical and biochemical analysis of extracts of Hibiscus sabdariffa." *Food Chemistry* 164 (0):23-29. doi: http://dx.doi.org/10.1016/j.foodchem.2014.04.097.

Singleton, V. L., and Joseph A. Rossi. 1965. "Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents." *American Journal of Enology and Viticulture* 16 (3):144-158.

Thurow, Taylor, and Sun-Ok Lee. 2012. "Effect of chlorogenic acid and neochlorogenic acid on human colon cancer cells." *Disc J* 13:86-93.

Ullah, M. F., Aamir Ahmad, HusainY Khan, H. Zubair, FazlulH Sarkar, and S. M. Hadi. 2013. "The Prooxidant Action of Dietary Antioxidants Leading to Cellular DNA Breakage and Anticancer Effects: Implications for Chemotherapeutic Action Against Cancer." *Cell Biochemistry and Biophysics* 67 (2):431-438. doi: 10.1007/s12013-011-9303-4.

van Meerloo, Johan, GertjanJ L. Kaspers, and Jacqueline Cloos. 2011. "Cell Sensitivity Assays: The MTT Assay." In *Cancer Cell Culture*, edited by Ian A. Cree, 237-245. Humana Press.

Wang, Chau-Jong, Jin-Ming Wang, Wea-Lung Lin, Chia-Yih Chu, Fen-Pi Chou, and Tsui-Hwa Tseng. 2000. "Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats." *Food and Chemical Toxicology* 38 (5):411-416. doi: http://dx.doi.org/10.1016/S0278-6915(00)00011-9.

Willett, Walter C. 2000. "Diet and cancer." *The oncologist* 5 (5):393-404.

Williamson, E.M., S. Driver, and K. Baxter. 2009. *Stockley's Herbal Medicines Interactions: A Guide to the Interactions of Herbal Medicines, Dietary Supplements and Nutraceuticals with Conventional Medicines*: Pharmaceutical Press.

Wu, Xianli, and Ronald L. Prior. 2005. "Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries." *Journal of Agricultural and Food Chemistry* 53 (7):2589-2599. doi: 10.1021/jf048068b.

Yang, J., J. Guo, and J. Yuan. 2008. "In vitro antioxidant properties of rutin." *LWT - Food Science and Technology* 41 (6):1060-1066. doi: http://dx.doi.org/10.1016/j.lwt.2007.06.010.

Yang, Jun, and Rui Hai Liu. 2009. "Induction of phase II enzyme, quinone reductase, in murine hepatoma cells in vitro by grape extracts and selected phytochemicals." *Food Chemistry* 114 (3):898-904. doi: http://dx.doi.org/10.1016/j.foodchem.2008.10.045.

Yin, Mei-Chin, Chun-Che Lin, Hsi-Chin Wu, Shih-Ming Tsao, and Cheng-Kuang Hsu. 2009. "Apoptotic Effects of Protocatechuic Acid in Human Breast, Lung, Liver, Cervix, and Prostate Cancer Cells: Potential Mechanisms of Action." *Journal of Agricultural and Food Chemistry* 57 (14):6468-6473. doi: 10.1021/jf9004466.

11. ARTICLE 3 : CANCER PREVENTIVE EFFECT OF A SPECIFIC PROBIOTIC FERMENTED MILK CONTAINING *LACTOBACILLUS ACIDOPHILUS* CL1285, *L. CASEI* LBC80R AND *L. RHAMNOSUS* CLR2 ON MALE F344 RATS TREATED WITH 1,2-DIMETHYLHYDRAZINE

Kerlynn Desrouillères[1], Mathieu Millette[2], Khanh Dang Vu[1], Romain Touja[1] and Monique Lacroix[1, *]

Effets antinéoplasiques des bactéries *Lactobacillus. acidophilus* CL1285, *L. casei* LB80CR et *L. rhamnosus* CLR2 sur des rats traités avec le 1,2-diméthylhydrazine

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Contribution des auteurs :

J'ai réalisé les expériences, rédigé l'article et participé à l'élaboration des protocoles. Dr. Millette m'a supervisé tout au long des expériences, corrigé l'article et participé aux discussions concernant le projet. Dr Khanh Dang Vu a participé aux discussions concernant le projet, élaboré les protocoles et corrigé l'article. Romain Touja a aidé a réaliser les expériences. Dr. Lacroix est la responsable du projet, a participé à toutes les prises de décisions, aux discussions concernant le projet et aux corrections de l'article

Résumé :

L'effet antinéoplasique d'un lait fermenté (FM) contenant trois souches de probiotiques (*Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2) a été étudié chez des rats traités avec le 1,2-diméthylhydrazine (DMH). Les rats ont été divisés en 7 groupes de 8 animaux. Les rats ont été alimenté avec une diète riche en gras et faible en fibres, le groupe 1 a servi de contrôle négatif tandis que les rats des groupes 2 à 7 ont reçu du DMH (30mg/kg s.c.) une fois par semaine durant six semaines. Les rats des groupes 3 à 7 ont été gavés avec 2, 1.5, 1, 0.5, 0.25 ml de FM respectivement chaque jour. Après 12 semaines, les rats ont été sacrifiés et leurs côlons, caecum et foie ont été prélevés. La présence de foyers de cryptes aberrantes (FCA) au niveau du colon a été déterminée par observation au microscope. L'activité des enzymes détoxifiantes du foie comme la quinone réductase (QR) et la glutathionne-S-transférase (GST) et fécales comme la β-glucuronidase et la β-glucosidase a été évaluée par spectrométrie. Les rats nourris avec les 3 doses les plus élevées ont significativement ($p \leq 0.05$) moins de cryptes aberrantes (CA), tandis que ceux nourris avec 1.5 et 2 ml de FM ont significativement moins de FCA comparé aux rats du groupe 2 ($p \leq 0.05$). Les rats nourris avec les plus fortes doses de lait fermenté ont une activité de GSTsignificativement plus élevée, tandis que seulement ceux nourris avec 2 ml ont eu significativement moins d'activité de la β-glucuronidase comparé aux rats du groupe 2 ($p \leq 0.05$). Ces résultats indiquent que FM peut avoir un rôle potentiel dans la prévention du cancer colorectal.

Mots clés : bactéries probiotiques, cancer colorectal, foyers de cryptes aberrantes, lait fermenté, antinéoplasique.

Cancer preventive effect of a specific probiotic fermented milk containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on male F344 rats treated with 1,2-dimethyhydrazine.

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ABSTRACT

The effect of fermented milk (FM) consisting of three probiotic strains (*Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2) on colon cancer prevention in rats treated with dimethylhydrazine (DMH) was investigated. The rats were divided into 7 groups of 8 animals. Rats were fed with a high fat low fibre diet; group 1 was a negative control, while groups 2 to 7 were injected with DMH (30 mg/kg s.c) once a week for six weeks. Groups 3 to 7 were gavaged respectively with 2, 1.5, 1, 0.5 and 0.25 ml of FM every day. After 12 weeks, the rats were sacrificed and the colon, caecum and liver were collected. Aberrant crypt foci (ACF) in the colon were determined using a microscope. Detoxifying enzymes like quinone reductase (QR) and glutathione-S-transferase (GST) and faecal enzymes such as β-glucosidase and β-glucuronidase were evaluated by spectrophotometry. The rats fed with the three highest doses significantly lowered aberrant crypt (AC) count, while those supplemented with 1.5 and 2ml FM lowered significantly ACF count compared to group 2 ($p \leq 0.05$). Highest doses fed-rats significantly induced GST activity, while only rats fed with 2 ml reduced significantly β-glucuronidase activity compared to group 2 ($p \leq 0.05$). These results indicate that the FM could have a potential role in colorectal cancer prevention

Keywords: probiotic bacteria, colorectal cancer, aberrant crypt foci, fermented milk, anticancer.

11.1 INTRODUCTION

Colorectal cancer (CRC) is the third most common and dangerous cancer in both men and women after breast, prostate and lung cancer(2014b). According to a recent report of the American Cancer Society an estimated of 96 830 cases of colon and 40 000 cases of rectal cancers are expected to occur in 2014 although the incidence rate has been decreasing for most of the past two decades(2014a). In 2014, The Canadian Society of Cancer estimated that in Canada, 24 400 Canadians will be diagnosed with CRC, this represents 13% of all new cases of cancer(2014b). Epidemiological studies have identified multiple risk factors for CRC: hereditary such as hereditary non polyposis colon cancer (HNPCC), familial adenomatous polyposis (FAP) and medical conditions such as inflammatory bowel disease (IBD) and colitis associated cancer (CAC) (Haggar *et al.*, 2009, Rowley, 2005, Stevens *et al.*, 2007). Moreover, many environmental factors like smoking, alcohol consumption, obesity, physical inactivity, diet rich in saturated fat and red meat along with an inadequate consumption of vegetable and fruits have been linked with CRC.

The development of CRC is a multistage process caused by a series of genetic and epigenetics alterations (Fearon *et al.*, 1990). It begins with the formation of a dysplastic aberrant crypt foci (ACF) which subsequently progress to early adenomas then late adenomas and ultimately to colonic carcinoma (Markowitz *et al.*, 2009, Takayama *et al.*, 1998). ACF are identified pre-cancerous lesions. Biochemical, genetic and morphological studies showed that they share similar alterations with colonic tumours (Bird *et al.*, 2000, Mori *et al.*, 2005, Rudolph *et al.*, 2005). Aberrant crypts (AC) appear as a single or group of crypts. They may represent early lesions capable of progression to CRC and/or be predictive markers of future risk. Therefore, they proved their usefulness in chemoprevention studies (Bird, 1995, Stevens *et al.*, 2007). Furthermore, animal models with DMH inducing colon cancer have been widely used as a model to screen agent with potentially chemopreventive properties (Chen *et al.*, 2013, O'Neill *et al.*, 1997, Onoue *et al.*, 1997).

Some studies reported that colon tumours induced by this alkylating agent present the many cell kinetics, molecular characteristics and pathological features associated with human colon carcinoma (Ma *et al.*, 1996, Shamsuddin *et al.*, 1981). DMH is metabolized into azoxymethane (AOM) and methylazoxymethanol (MAM) in the liver, and then is transported to the colon via blood and bile. In the intestine, colonic cells transform MAM into methyldiazoniums ions and subsequently into carboniums ions which will later generate oxidative stress and DNA damage leading to cancer (Sengottuvelan *et al.*, 2009).

Various animal studies have used ACF as biological markers to demonstrate the efficiency of dietary interventions against the development of CRC (Boateng *et al.*, 2007, Narushima *et al.*, 2010, Zhong *et al.*, 2012). Probiotics are defined as live microorganisms which when consumed in adequate number exert beneficial effects in the host (Hill *et al.*, 2014). Most of the probiotics available on the market are lactic acid bacteria (LAB) like lactobacilli and streptococci but it is possible to find non-LAB probiotics such as bifidobacteria.

The use of LAB as functional foods components is of high interest because they are natural residents of the human gastrointestinal tract (GIT). Furthermore, other mechanisms could explain the high interest in the use of probiotics as a potential preventive food against CRC, such as competitive exclusion of pathogenic intestinal flora (Rafter, 2003), alteration of intestinal flora enzyme activity (Goldin *et al.*, 1984), reduction of carcinogenic secondary bile acids (De Boever *et al.*, 2000), binding of carcinogens (Fotiadis *et al.*, 2008), production of anti-tumorigenic or anti-mutagenic compounds (Zhao *et al.*, 2013).

Sivieri *et al.* (2008) demonstrated that the oral administration of *Enterococcus faecium* CRL 183 resulted in a significant reduction of DMH-induced ACF formation in male Wistar rats. The consumption of *ENT. faecium* CRL 183 also leads to a reduced average number of tumours. They suggested that those effects were likely exerted by stimulating the immune system.

Kumar *et al.* (2012) showed that the supplementation of *Lactobacillus plantarum* AS1 in male Wistar rats either before initiation or during and selection/promotion phases of

colon carcinogenesis was to alter antioxidant enzyme activities. The probiotic treatment was also able to decrease significantly the mean volume diameter and total number of tumours in rats that have been pre-and post-treated with DMH.

A particular fermented milk made from *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 can reduce the incidence, the duration of antibiotic-associated diarrhoea and *Clostridium difficile* infections in randomized placebo controlled (Beausoleil *et al.*, 2007, Gao *et al.*, 2010, Sampalis *et al.*, 2010). These probiotic bacteria demonstrated antimicrobial capacity (Millette *et al.*, 2007) and are able to survive the gastrointestinal transit and modulate the intestinal microbiota of mice (Millette *et al.*, 2008). Moreover, Baldwin *et al.* (2010) observed a synergistic effect of probiotic formulation with a chemotherapeutic agent, the 5-fluorouracil (5-FU). The probiotic bacteria were able to increase the apoptosis-induction capacity of 5-FU by activating the caspase 3 and reducing the p21 expression. Therefore, it would be interesting to evaluate its effect on the prevention of colon carcinogenesis.

Besides the reduction and/or inhibition of the formation of ACF, others mechanisms are investigated concerning the chemopreventive effects of dietary agents against colorectal carcinogenesis. The mechanisms involved may be the modulation of the activity of enzyme responsible for the detoxification and excretion of carcinogens like the xenobiotic metabolizing phase II enzymes like quinone reductase or glutathione-S-transferase for example (Sheweita *et al.*, 2003) or the modulation of bacterial enzymes which are able to catalyse the conversion of procarcinogenic substances into carcinogenic substances such as β-glucosidase and β-glucuronidase (Simon *et al.*, 1986).

Thus, the aim of this study was to investigate the effect of a probiotic fermented milk made of *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on colon carcinogenesis in male F344 rats treated with DMH. The effect of the probiotic product on hepatic detoxifying enzymes (QR and GST) and faecal enzymes (β-glucosidase and β-glucuronidase) has been evaluated

11.2 MATERIALS AND METHODS

11.2.1 Chemicals

N,N-Dimethylhydrazine, 1-chloro-2,4-dinitrobenzene (CDNB), methylene blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), menadione, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), flavin adenine dinucleotide (FAD), bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate (NADP), Tween 20, 4-nitrophenol, L-glutathione reduced, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-glucuronide were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

11.2.2 Probiotics

A fermented milk graciously provided by Bio-K Plus International Inc. (Laval, QC, Canada) stored at 4°C and containing at least 50 x 10^9 colony forming units (CFU)/g of three (3) strains of a proprietary probiotics formulation: *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2 was used. Five doses of FM was prepared: 2 ml (1 x 10^9 CFU), 1.5 ml (7.4 x 10^8 CFU), 1 ml (4.9 x 10^8 CFU), 0.5 ml (2.5 x 10^8 CFU) and 0.25 ml (1.2 x 10^8 CFU) to be administrate to the rats.

11.2.3 Animals, housing and diet

Fifty-six F344 male rats aged five weeks and weighing an average of 122.60 ± 11.07 g were obtained from Charles River Laboratories (Laval, QC, Canada) and housed in stainless wire cages (3 rats / cage). The temperature and the relative humidity were maintained at 22°C and 55% respectively. The light-dark cycles were 12 hour each. All the animals had free access to water and rat chow commercial pellets provided by Harlan (Madison, WI, USA). The composition of the high fat low fibre diet (Harlan) was 39.8% corn starch, 20% casein, 10% malto-dextrin, 10% corn oil, 12% lard, 2% cellulose, 4.375% mineral mixture, 1.25% vitamin mixture, 0.3% choline bitartrate, 0.3%

L-cystine and 0.0044% TBHQ (www.harlan.com). All experimental animal care and treatment followed the guidelines set by the Canadian Council on Animal Care (CCAC).

11.2.4 Experimental design

The rats underwent a one week period of acclimation. After that period, they were randomly divided into 7 groups of 8 animals. All rats were fed with the high fat low fibre diet (Harlan). Group 1 (NC) served as negative control and received 0.85% saline water by gavage. Rats in groups 2 to 7 were injected with DMH dissolved in saline solution (30 mg/kg subcutaneously.) once a week for six weeks. Group 2 (PC) served as carcinogen control. In addition to the diet, rats in group 3 to 7 (G3 to G7) were supplemented by gavage with different doses of fermented milk containing *Lactobacillus acidophilus / L. casei /L. rhamnosus*. The doses supplemented for each group were 2, 1.5, 1, 0.5 and 0.25 ml, respectively, for G3, G4, G5, G6 and G7. Animals were monitored daily for general health and body weights were recorded twice a week for the duration of the study. After twelve weeks of experimental period, rats were sacrificed in a CO_2 chamber. The entire colon segment was collected for AC analysis while the liver and caecum were removed and stored at -80°C for subsequent enzymatic assays. The experimental design is described in Fig. 11.

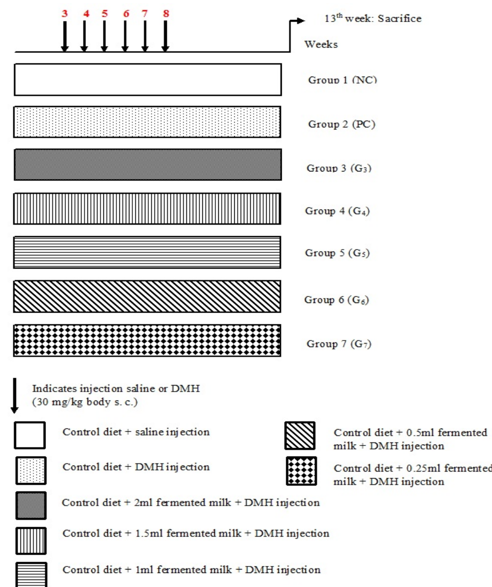

Figure 11 :Representation of the experimental design

11.2.5 Aberrant crypt analysis

The entire colon of each animal was removed and washed thoroughly with saline water (0.85% NaCl). They were cut longitudinally and laid flat on Whatman paper in a Petri dish after they were fixed in 10% buffered formalin solution for at least 24 hours. Then, the colon was stained with 0.2% methylene blue for 3 min and placed in saline water overnight for the removal of excess staining. The mucosal side was placed on a glass slide and examined under microscope using 20X objective for assessment of ACF as well as the number of AC per focus (Bird, 1995)

11.2.6 Liver and caecum processing

Rats livers from different groups were weighed and samples (1-2 g) were cut from each liver. A volume of 5 ml of sucrose solution (0.25 M) was added into each sample and homogenization process was conducted using a tissue homogenizer purchased from Fisher Scientific (Ottawa, ON, Canada). The homogenized samples were centrifuged at 5 000 x g for 30 min at 4°C and the obtained supernatants were mixed with 0.2 volume of CaCl2 solution (0.1 M) and were centrifuged at 27 000 x g for 20 min at 4°C. The supernatants obtained (cytosol) were filtered through a 0.2 µm filter membrane and were used for estimation of protein for GST and QR activities in rat liver.

Rats caecal contents from different treatment groups were weighed and then mixed with 10 ml of 100 mM phosphate buffered saline and finally homogenized. The homogenization process was conducted using a tissue homogenizer. The homogenized samples were then centrifuged at 1 508 x g for 15 min at 4 °C and the obtained supernatants were centrifuged again at 10 000 x g for 15 min at 4°C. The clear obtained supernatants were filtered throughout a 0.2 µm filter membrane and used for total protein content and β-glucosidase and β-glucuronidase activities.

11.2.7 Protein estimation

Supernatants of rat liver and caecum were used for determination of total protein content using Pierce BCA protein assay kit purchased from Fisher Scientific (Ottawa,

ON, Canada) according to the manufacturer's protocol. Briefly 25 µl of each sample were added into each well of a 96 wells microplate. Then, 200 µl of BCA solution was added to the samples wells. For the blank, wells were filled with 225 µl BCA solution. All microplates were incubated for 30 minutes at 37°C. Absorbance was measured at 562 nm using a microplate reader (EL800 BioTek Winooski, VT, USA). Protein concentration was determined using BSA as standard.

11.2.8 **Quinone reductase (QR) assay**

Quinone reductase (EC. 1.6.99.2) assay was conducted using a microplate following a method described by Kang *et al.* (2004b) for *in vitro* assay with some modifications. Briefly, 30 µl of samples from liver supernatants were deposited into 96-wells microplate. Distilled water (30 µl) was used a negative control. Then, 200 µl of reaction mixture were added into each well. 22 ml of reaction mixture was prepared for the assays, it contains 20.5 ml distilled water, 1.1 ml 0.5 M Tris-HCl buffer pH 7.5, 14.7 mg BSA, 146.7µl 1.5% Tween 20, 14.7 µl 7.5 mM FAD^+, 146.7 µl of 150 mM glucose 6-phosphate, 13.2 µl of 50 mM $NADP^+$, 44 µl of glucose 6-phosphate dehydrogenase, 6.6 mg de tetrazolium blue bromide and 22 µl of 50 mM menadione. The microplate was incubated for 5 min at room temperature and the optical density (OD) values were measured in a microplate reader at 595 nm. Specific activity was calculated and expressed as nM MTT formazan formed per min per mg of protein. The induction of the activity of QR enzyme for the treated groups was calculated by dividing the enzyme specific activity of the treated group to the enzyme specific activity for the control.

11.2.9 **Glutathione-S-transferase (GST) assay**

Glutathione-S-transferase (EC. 2.5.1.18) assay was conducted using the method described by Sharma *et al.* (1997) for microplate with some modifications. Briefly, 100 µl of samples from liver supernatants were added into a cuvette. Distilled water (100 µl) was used as a negative control. A quantity of 1.9 ml of reaction mix (100 µl of 20 mM CDNB, 100 µl of 20 mM reduced glutathione, 1.7 ml of reagent buffer) were added into each cuvette. The absorbance was monitored at 340 nm for 5 min using a

spectrophotometer purchased from Varian Canada Inc. (Mississauga, ON, Canada). Values of delta O.D were used to calculate the activity of GST. Specific GST activity was calculated and expressed as nM CDNB conjugate formed per min per mg protein. Induction of GST enzyme for the treated groups was calculated by dividing the enzyme specific activity of the treated group to the enzyme specific activity for the control.

11.2.10 **β-glucosidase and β-glucuronidase assay**

β-glucosidase (EC. 3.2.1.21) and β-glucuronidase (EC. 3.2.1.31) assays were conducted using respectively *p*-nitrophenyl β-D-glucopyranoside and *p*- nitrophenyl β-D-glucuronide as substrate, respectively. Briefly, 10 μl of samples from faecal supernatants were added into a 96-wells microplate. Distilled water (10 μl) was used as negative control. A volume of 40 μl of reaction mixture were added into each well then the microplate were incubated at 37°C for 15 min. A 12 ml solution of reaction mixture containing 3 ml of 5 mM of respective substrate, 3 ml of 100 mM phosphate buffered saline and 6 ml of distilled water was prepared. The reaction was stopped by adding 250 μl a solution of 0.01 M of NaOH. OD values were measured in a microplate reader at 405 nm. The activities of both enzymes were calculated based on the standard curve of p-nitrophenol (0-70 μM). One activity unit is defined as the quantity of enzyme required to hydrolyse substrate into one μM of *p*-nitrophenol per minute. Specific β-glucosidase and β-glucuronidase activities were calculated and expressed as μM of *p*-nitrophenol formed per min per mg protein.

11.2.11 **Statistical analysis**

Data were analysed by one-way analysis of variance (ANOVA), using the SPSS Base 16.0 software (Stat-Packets statistical analysis software, SPSS Inc., Chicago IL, USA) and expressed as means ± standard deviation. The differences among the groups were analysed with a post hoc Duncan's test. Significance was considered at $p \leq 0.05$.

11.3 RESULTS

11.3.1 Effect of the probiotic fermented milk administration on the number of AC per colon induced by DMH treatment.

The effect of the administration of different doses of fermented milk on the formation of AC is shown in Fig. 13. Animals in G3 reduced by 47% the total count of AC found in the colon compared to the PC group. Rats in G4 and G5 were able to reduce by 28 and 35%, respectively, the count of AC compared to the PC group. Rats in G6 showed a 16% reduction of the total count of AC but the results were not statistically different ($p \geq 0.05$) when compared to the PC group.

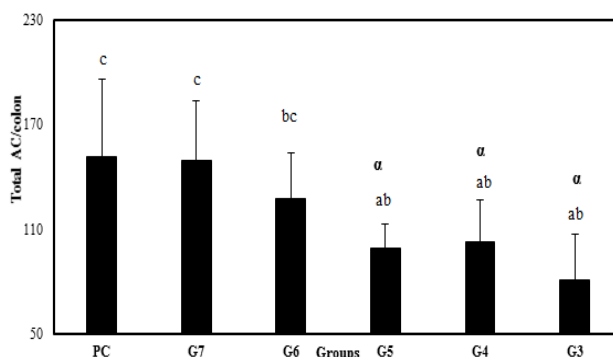

Figure 12 : Effect of increasing doses of *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk on total number of aberrant crypts. Error bars represent the standard deviation of the mean total AC/colon obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk and PC: DMH only. Different letters are significantly different ($p \leq 0.05$). $\alpha \leq 0.05$ vs PC group

11.3.2 Effect of the probiotic fermented milk administration on the number of ACF per colon induced by DMH treatment.

The incidence of ACF is shown in Fig. 14. All rats treated with DMH developed ACF. Rats in the groups fed with 2 and 1.5 ml of fermented milk (G3 and G4) were able to reduce significantly ($p \leq 0.05$) total number of ACF by 28% as compared to the carcinogen control group (PC). Fig. 15

shows the topographical view of ACF in experimental animals. The histopathological observations of the colon segment of G4 rats revealed the presence of more normal crypts than aberrant ones as compared to the segment of rats treated with DMH (PC) where it is possible to observe at least 3 foci with at least 2 aberrant crypts (Fig. 15b).

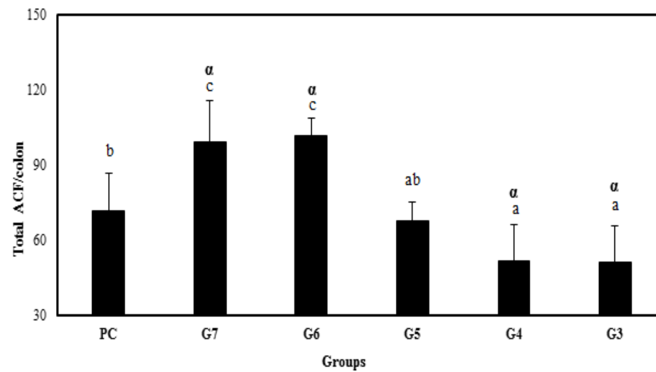

Figure 13 : Effect of increasing doses of *L. acidophilus* CL1285 and *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk on total number of aberrant crypt foci induced by DMH. Error bars represent the standard deviation of the mean total ACF/colon obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control.

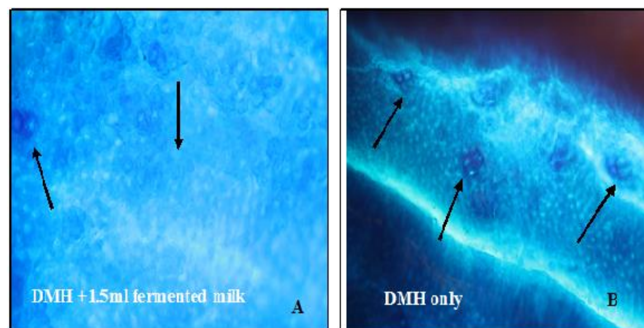

Figure 14 : Topographical view of normal and aberrant crypts on the colonic mucosa stained with methylene blue of rats treated with DMH. (A) Topographical view of G4 colonic mucosa showing some normal crypt and ACF with 2 crypts (20X). (B) Topographical view of PC colonic mucosa showing multiple ACF, two with four crypts and one with two crypts (20X). The arrows shows the crypts (normal and aberrant ones) in the colon mucosa

11.3.3 **Effect of the probiotic fermented milk administration on the multiplicity of AC per ACF in F344 rats treated with DMH.**

The number of AC per focus was also evaluated because there is a direct correlation between the ACF size and the probability of the colon carcinoma development. The mean number of crypts per foci is presented in Fig. 16. The results were divided into

two groups: one presenting foci with 2 or 3 aberrant crypts (2-3AC/ACF) and the other showing foci with 4 or 5 aberrant crypts (4-5AC/ACF). Results showed that all rats supplemented with the fermented milk were able to reduce significantly ($p \leq 0.05$) the number of foci containing 4 or 5 aberrant crypts. The rats in groups G3 and G5 were able to eliminate completely ($p \leq 0.05$) the number of foci containing 4 or 5 aberrant crypts. Rats in groups G4, G6 and G7 showed a significant reduction ($p \leq 0.05$) in the number of AC per focus in the group 4-5 AC/ACF. A respective reduction of 90, 96 and 72% was observed compared to rats in PC group. For the 2-3 AC/ACF, our results showed that the rats in G3 group were able to lower significantly ($p \leq 0.05$) the count of AC per focus by 42% as compared to PC.

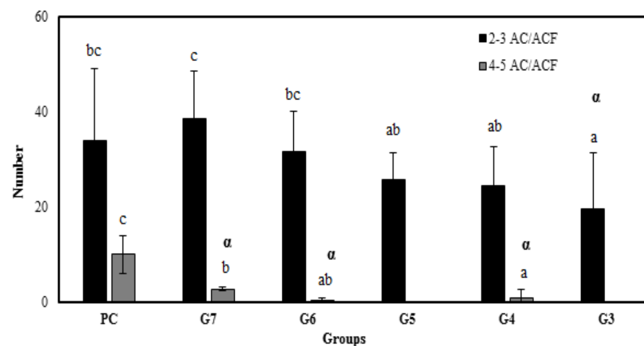

Figure 15 : Effect of increasing doses of *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR 2 probiotic fermented milk on number of aberrant crypt foci (ACF) of two to five aberrant crypt induced by DMH. Error bars represent the standard deviation of the mean of the number of AC per focus obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk and PC: DMH only. Different letters are significantly different ($p \leq 0.05$). $\alpha \leq 0.05$ vs PC group.

11.3.4 **Effect the administration of the probiotic fermented milk on (QR) and (GST) activities in F344 rats treated with DMH.**

The effect of the fermented milk on the activity of two detoxifying enzymes are presented in Fig 17 and Fig. 18. Results showed that rats in group G4 and G5 were able to induce significantly GST activity ($p \leq 0.05$). An increase of 154 and 109% of the enzyme activity was observed, respectively, as compared to the negative control group (NC). Rats in G3, G4 and G5 were able to induce significantly GST activity as

compared to the carcinogen control group (PC) ($p \leq 0.05$). As for QR, results showed that all rats treated with DMH had a significant reduction ($p \leq 0.05$) of the enzyme activity as compared to NC group.

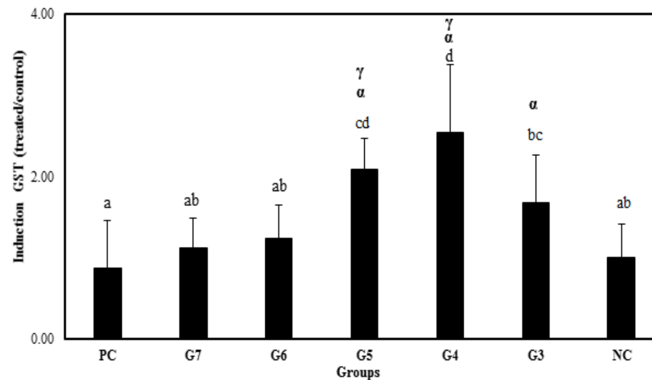

Figure 16 : Effect of increasing doses of *L acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk on glutathione S-transferase induction in DMH treated F344 rats. Error bars represent the standard deviation of glutathione S-transferase induction obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk, PC: DMH only and NC: saline water only. Different letters are significantly different ($p \leq 0.05$). $\alpha \leq 0.05$ vs PC group and $\gamma \leq 0.05$ vs NC group.

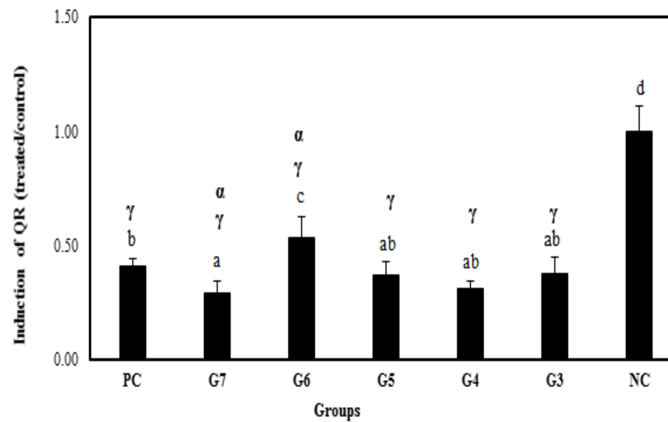

Figure 17 : Effect of increasing doses of *L acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk on quinone reductase induction in DMH treated F344 rats. Error bars represent the standard deviation of the mean of quinone reductase induction obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk, PC: DMH only and NC: saline water only. Different letters are significantly different ($p \leq 0.05$): $\alpha \leq 0.05$ vs PC group and $\gamma \leq 0.05$ vs NC group.

11.3.5 **Effect of the administration of the fermented milk on β-glucosidase and β-glucuronidase activities in F344 rats treated with DMH.**

The activities of β-glucosidase and β-glucuronidase are shown in Fig. 20 and Fig. 21. The results showed that the activity of β-glucosidase in the carcinogen control (PC) compared to the negative control (NC) was not different statistically ($p > 0.05$). Moreover, supplementation of the highest doses of fermented milk (2, 1.5 and 1ml) did not affect the activity of β-glucosidase as compared to NC. But for the rats fed with 0.5 and 0.25 ml fermented milk, the enzyme activity increased significantly ($p \leq 0.05$) as compared to both controls groups (NC and PC). The activity of β-glucuronidase was reduced significantly ($p \leq 0.05$) in rats from G3. The enzymatic activity was reduced by 49% as compared to NC.

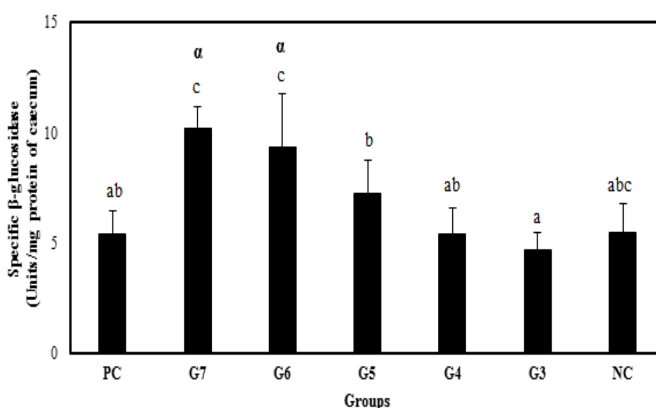

Figure 18 : Effect of increasing doses of *L acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic fermented milk β-glucosidase activity in DMH treated F344 rats. Error bars represent the standard deviation of the mean β-glucosidase specific activity obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk, PC: DMH only and NC: saline water only. Different letters are significantly different ($p \leq 0.05$): $\alpha \leq 0.05$ vs PC group.

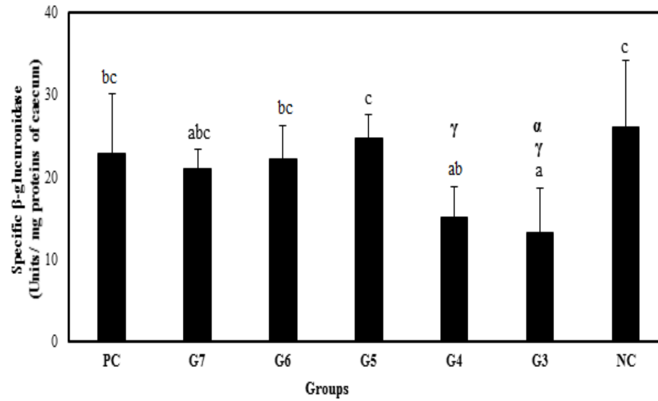

Figure 19 : Effect of increasing doses of *L acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 probiotic milk of β-glucuronidase activity in DMH treated F344 rats. Error bars represent the standard deviation of the mean β-glucuronidase specific activity obtained from 8 rats in each group. G3: DMH + 2 ml fermented milk, G4: DMH + 1.5 ml fermented milk, G5: DMH + 1 ml fermented milk, G6: DMH + 0.5 ml fermented milk, G7: DMH + 0.25 ml fermented milk, PC: DMH only and NC: saline water only. Different letters are significantly different ($p \leq 0.05$): $\alpha \leq 0.05$ vs PC group and $\gamma \leq 0.05$ vs NC group.

11.4 DISCUSSION

In this study, the objectives were to determine the effect of the consumption of a probiotic fermented milk containing by *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on the formation of DMH induced ACF in male F344 rats. Then, the effect of the probiotic formula on two hepatic detoxifying and two faecal enzymes were evaluated.

The results suggest that the probiotic formula was able to reduce the initial step of CRC development. Several studies showed the ability of numerous strains belonging to species *L. acidophilus, L .casei* and *L. rhamnosus* to either prevent or inhibit ACF formation (Rao *et al.*, 1999, Verma *et al.*, 2013b). For instance Chang *et al.* (2012) showed that *Lactobacillus acidophilus* KFRI342 (2 x 10^9 CFU) was able to reduce significantly the number of ACF in DMH-induced F344 rats, while Yamazaki *et al.* (2000) showed that short and long term oral administration of *L. casei* Shirota lowered the number of large ACF.

Although the precise mechanism by which *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 inhibits the formation of ACF is still unknown, we believe that this effect may proceed through diverse mechanisms including alteration of intestinal microbiota enzymes (Goldin *et al.*, 1984) enhancement of host immune response (Lee *et al.*, 2004) and inactivation of carcinogenic compounds (Orrhage *et al.*, 2002).

The phase II detoxifying enzymes such as QR and GST are widely induced in cells exposed to xenobiotic. They are able to convert a pro-carcinogen into its inactive form that could be easily excreted (Wang *et al.*, 1995). An enhancement of those enzymatic activities are considered to be beneficial for the health since it means that the carcinogen excretion is also enhanced. This effect is correlated with a protection against chemically induced tumours as it is demonstrated in many studies (Li *et al.*, 2014, Sangeetha *et al.*, 2012). For example, Vinothkumar *et al.* (2014) showed that the supplementation of increasing doses of troxerutin ranging from 12.5 to 50 mg/kg was able to induce significantly ($p < 0.05$) the activities of phase II enzymes such as GST, DTD and UDPGD in DMH treated rats.

GSTs are a family of phase II enzyme that have an important role in the early stages of detoxification by eliminating various endogenous and exogenous chemicals that may have a carcinogenic potential by catalysing the conjugation of glutathione. When an induction of GST activity is observed in an organism it usually correlates with improved detoxification potential against harmful compounds (Perez *et al.*, 2010). In fact, an induction of GST expression by dietary nutrients or drugs enhances the toxicological defence of normal colon tissue thus reducing their exposure to carcinogens. Our results are in agreement with previous studies. Muthu *et al.* (2013) showed that the GST activity of rats treated with DMH alone was significantly decreased compared to GST activity from control rats (no DMH). They also demonstrated that umbelliferone treatment during initiation, post-initiation and the entire experimental period significantly increased the activity of GST as compared to rats treated with DMH alone.

QR is able to catalyse the reduction of two electron of quinine to protect cells against mutagenicity and carcinogenicity from free radicals and reactive oxygen species that can be generated by one electron reduction (Horning *et al.*, 1978). Our results differed from those obtained in previous studies. For example, Khan *et al.* (2011) demonstrated that rats supplemented with different doses of farnesol (50 and 100 mg/kg) significantly increased ($p < 0.01$) QR- activity compared to rats treated with DMH only. GST- is considered to be the major detoxifying enzyme, an increase in its activity is the primary defence against xenobiotic compounds (Coles *et al.*, 2003). The induction of GST activity by the highest doses of probiotics favours the elimination of carcinogenic compounds. Taking into account these information, it could be hypothesized that the organism was detoxified by GST activity enhancement explaining the fact that the supplementation of probiotics did not have any effect on QR activity.

β-glucosidase and β-glucuronidase, two faecal enzymes are capable of deconjugating toxins and/or carcinogens that have been previously detoxified in the liver by glucuronide conjugation. A reduction in the activity of those enzymes can lead to a reduced exposure to carcinogenic substance thus a reduction in the incidence of CRC (Goldin *et al.*, 1984, Verma *et al.*, 2013a). β-glucuronidase catalyse the hydrolysis of glucuronide conjugate which generate toxic and carcinogenic substance (Chipman, 1982) and β-glucosidase catalyse the hydrolysis of terminal non-reducing in β-D-

glucosides which induced the release of toxic colonic mutagens and carcinogens. Various studies using animal models showed the ability of other probiotic preparations to decrease the activity of those enzymes (Chang *et al.*, 2012, Verma *et al.*, 2013b) and some clinical studies also demonstrated the same inhibitory effects (De Preter *et al.*, 2007, Goldin *et al.*, 1984). A study conducted by Asha *et al.* (2012) demonstrated that mice fed with *L. fermentum* (2 x 10^8 CFU/g diet), *L. plantarum* (2 x 10^8 CFU/g diet) and vincristine alone decreased the activity of β-glucosidase by 70.4, 46.68 and 70.94% respectively. The authors also showed that when fed with *L. fermentum* (2 x 10^8 CFU/g diet), *L. plantarum* (2 x 10^8 CFU/g diet) and vincristine alone, the activity of β-glucuronidase in mice decrease significantly by 43.55, 35.49 and 54% respectively. Concerning β-glucosidase, our results disagree with previous studies unlike the results obtained for β-glucuronidase activity. The secretion of those faecal enzymes in large amount imply an unbalanced intestinal microbiota that could lead to the development of CRC. Those enzymes are mostly produced by many., *Peptostreptococcus* sp., *Staphylococcus* sp., *Clostridium* sp. (*Cl. paraputrificum*, *Cl. clostridioforme*, *Cl. perfringens*), *Bacteroides* sp. (*B. vulgatis*, *B. uniforme*, *B. fragilis*), *Enterococcus* sp *Ruminococcus* (*gnavus*), *Eubacterium* sp. and *Escherichia coli* (Dabek *et al.*, 2008, de Moreno de LeBlanc *et al.*, 2005, Nakamura *et al.*, 2002). Since probiotics can modify the microbiota by favouring the growth of lactobacilli and bifidobacteria it could be hypothesized that the administration of the fermented milk at the highest dose inhibit the growth of those bacteria in the rat colon leading to a reduction of the production of β-glucuronidase thus a reduction on its enzymatic activity.

As for β-glucosidase, it's not only synthesized by anaerobic bacteria but also by some lactobacilli strains. It is possible that high doses of fermented milk inhibit the growth of the anaerobic bacteria. This result in the reduction of β-glucosidase produced, while the presence of the lactobacilli strains in the milk contribute to the production of this enzyme. However, the lowest doses inhibit the growth of the anaerobic bacteria thus reducing the production of β-glucosidase. However lactobacilli can also produce that enzyme an enhancement of the enzymatic activity might be possible.

Some of the previous studies realised have already highlighted important properties of the probiotics present in this specific fermented milk like their antimicrobial effect, their

ability to modulate the microbiota, to act in a synergetic manner with fluorouracil inducing apoptosis in colon cancer cells, their reducing effect on antibiotic-associated diarrhoea and *Clostridium difficile* infections incidence (Baldwin *et al.*, 2010, Gao *et al.*, 2010, Millette *et al.*, 2007). This one showed that they also have a great potential as chemopreventive agent against CRC. When taking all this information into account, it is possible to suggest the consumption of a fermented milk containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 as part of a diet to either maintain or establish a heathy environment in the colon.

11.5 CONCLUSION

This study demonstrated that a specific probiotic fermented milk containing *L. acidophilus* CL1285 *L. casei* LBC80R and *L. rhamnosus* CLR2 could have a preventive effect against colon carcinogenesis by decreasing the total number of ACF in DMH-treated rats. Treatments consisting of the highest doses significantly enhanced the hepatic GST activity as compared to the control. It could also be concluded that the induction of hepatic detoxifying enzymes and the reduction of the faecal bacterial enzymes could represent some mechanisms of action in prevention of ACF formation in DMH-treated rats. Further investigation are undergoing to determine which component from the fermented milk is responsible for inducing or inhibiting the enzymatic activities involved in the carcinogenesis of CRC and other are needed to determine whether the carrier nature affect the ability of the probiotic to reduce the early stages of colorectal carcinogenesis.

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REFERENCES

American Cancer Society. (2014, October 31). *Cancer Facts & Figures 2014*. Retrieved from American Cancer Society: http://www.cancer.org/acs/groups/content/@research/documents/webcontent/acspc-042151.pdf

Asha, A., & Gayathri, D. (2012). Synergistic impact of Lactobacillus fermentum, Lactobacillus plantarum and vincristine on 1, 2-dimethylhydrazine-induced colorectal carcinogenesis in mice. *Experimental and therapeutic medicine, 3*(6), 1049-1054. doi: http://dx.doi.org/10.3892/etm.2012.536

Baldwin, C., Millette, M., Oth, D., Ruiz, M. T., Luquet, F. M., & Lacroix, M. (2010). Probiotic Lactobacillus Acidophilus and L. Casei Mix Sensitize Colorectal Tumoral Cells to 5-Fluorouracil-Induced Apoptosis. *Nutrition and Cancer, 62*(3), 371-378. doi: http://dx.doi.org/10.1080/01635580903407197

Beausoleil, M., Fortier, N., Guénette, S., L'Ecuyer, A., Savoie, M., Franco, M., . . . Weiss, K. (2007). Effect of a fermented milk combining Lactobacillus acidophilus Cl1285 and Lactobacillus casei in the prevention of antibiotic-associated diarrhea: a randomized, double-blind, placebo-controlled trial. *Canadian Journal of Gastroenterology, 21*(11), 732-736.

Bird, R. P. (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Letters, 93*(1), 55-71. doi: http://dx.doi.org/10.1016/0304-3835(95)03788-X

Bird, R. P., & Good, C. K. (2000). The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicology Letters, 112–113*(0), 395-402. doi: http://dx.doi.org/10.1016/S0378-4274(99)00261-1

Boateng, J., Verghese, M., Shackelford, L., Walker, L. T., Khatiwada, J., Ogutu, S., . . . Chawan, C. B. (2007). Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. *Food and Chemical Toxicology, 45*(5), 725-732. doi: http://dx.doi.org/10.1016/j.fct.2006.10.019

Canadian Cancer Society's Advisory Committee . (2014, October 31). *Colorectal Cancer Statistics*. Retrieved from Candian Cancer Society: http://www.cancer.ca/~/media/cancer.ca/CW/cancer%20information/cancer%20101/Canadian%20cancer%20statistics/Canadian-Cancer-Statistics-2014--EN.pdf

Chang, J.-H., Shim, Y. Y., Cha, S.-K., Reaney, M. J., & Chee, K. M. (2012). Effect of Lactobacillus acidophilus KFRI342 on the development of chemically induced precancerous growths in the rat colon. *Journal of medical microbiology, 61*(3), 361-368.

Chen, Q., Liang, Y., Zhu, C., Yan, Y., & Pang, G. (2013). Effects of casein glycomacropeptide on the early development of primary colorectal cancer in rats. *Food Science and Human Wellness, 2*(3–4), 113-118. doi: http://dx.doi.org/10.1016/j.fshw.2013.09.001

Chipman, J. K. (1982). Bile as a source of potential reactive metabolites. *Toxicology, 25*(2–3), 99-111. doi: http://dx.doi.org/10.1016/0300-483X(82)90022-1

Coles, B. F., & Kadlubar, F. F. (2003). Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *Biofactors, 17*(1), 115-130. doi: http://dx.doi.org/10.1002/biof.5520170112

Dabek, M., McCrae, S. I., Stevens, V. J., Duncan, S. H., & Louis, P. (2008). Distribution of β-glucosidase and β-glucuronidase activity and of β-glucuronidase gene gus in human colonic bacteria. *FEMS Microbiology Ecology, 66*(3), 487-495. doi: http://dx.doi.org/10.1111/j.1574-6941.2008.00520.x

De Boever, P., Wouters, R., Verschaeve, L., Berckmans, P., Schoeters, G., & Verstraete, W. (2000). Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity. *Applied microbiology and biotechnology, 53*(6), 709-714. doi: http://dx.doi.org/10.1007/s002530000330

de Moreno de LeBlanc, A., & Perdigón, G. (2005). Reduction of beta-glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. *Biocell, 29*, 15-24.

De Preter, V., Raemen, H., Cloetens, L., Houben, E., Rutgeerts, P., & Verbeke, K. (2007). Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. *Eur J Clin Nutr, 62*(2), 225-231. doi: http://dx.doi.org/10.1038/sj.ejcn.1602706

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell, 61*(5), 759-767. doi: http://dx.doi.org/10.1016/0092-8674(90)90186-I

Fotiadis, C. I., Stoidis, C. N., Spyropoulos, B. G., & Zografos, E. D. (2008). Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer. *World journal of gastroenterology : WJG, 14*(42), 6453-6457. doi: http://dx.doi.org/10.3748/wjg.14.6453

Gao, X. W., Mubasher, M., Fang, C. Y., Reifer, C., & Miller, L. E. (2010). Dose–Response Efficacy of a Proprietary Probiotic Formula of Lactobacillus acidophilus CL1285 and Lactobacillus casei LBC80R for Antibiotic-Associated Diarrhea and Clostridium difficile-Associated Diarrhea Prophylaxis in Adult Patients. *The American journal of gastroenterology., 105*(7), 1636-1641. doi: http://dx.doi.org/10.1038/ajg.2010.11

Goldin, B. R., & Gorbach, S. L. (1984). The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *The American Journal of Clinical Nutrition, 39*(5), 756-761.

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in colon and rectal surgery, 22*(4), 191-197. doi: http://dx.doi.org/10.1055/s-0029-1242458

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., . . . Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol, 11*(8), 506-514. doi: http://dx.doi.org/10.1038/nrgastro.2014.66

Horning, E. C., Thenot, J. P., & Helton, E. D. (1978). Toxic agents resulting from the oxidative metabolism of steroid hormones and drugs. *Journal of Toxicology and Environmental Health, 4*(2-3), 341-361. doi: http://dx.doi.org/10.1080/15287397809529665

Kang, Y. H., & Pezzuto, J. M. (2004). Induction of Quinone Reductase as a Primary Screen for Natural Product Anticarcinogens. *Methods in enzymology, 382*, 380-414. doi: http://dx.doi.org/10.1016/S0076-6879(04)82021-4

Khan, R., & Sultana, S. (2011). Farnesol attenuates 1,2-dimethylhydrazine induced oxidative stress, inflammation and apoptotic responses in the colon of Wistar rats. *Chemico-biological interactions, 192*(3), 193-200. doi: http://dx.doi.org/10.1016/j.cbi.2011.03.009

Kumar, R. S., Kanmani, P., Yuvaraj, N., Paari, K. A., Pattukumar, V., Thirunavukkarasu, C., & Arul, V. (2012). Lactobacillus plantarum AS1 Isolated from South Indian Fermented Food Kallappam Suppress 1,2-Dimethyl Hydrazine (DMH)-Induced Colorectal Cancer in Male Wistar Rats. *Applied Biochemistry and Biotechnology, 166*(3), 620-631. doi: http://dx.doi.org/10.1007/s12010-011-9453-2

Lee, J. W., Kim, E. H., Yim, I. B., & Joo, H. G. (2004). Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *Journal of Veterinary Science, 5*(1), 41-48.

Li, X., Xu, X., Ji, T., Liu, Z., Gu, M., Hoang, B. H., & Zi, X. (2014). Dietary feeding of flavokawain A, a Kava chalcone, exhibits a satisfactory safety profile and its association with enhancement of phase II enzymes in mice. *Toxicology Reports, 1*, 2-11. doi: http://dx.doi.org/10.1016/j.toxrep.2014.02.002

Ma, Q., Hoper, M., Anderson, N., & Rowlands, B. J. (1996). Effect of supplemental L-arginine in a chemical-induced model of colorectal cancer. *World journal of surgery, 20*(8), 1087-1091.

Markowitz, S. D., & Bertagnolli, M. M. (2009). Molecular Basis of Colorectal Cancer. *New England Journal of Medicine, 361*(25), 2449-2460. doi: http://dx.doi.org/10.1056/NEJMra0804588

Millette, M., Luquet, F. M., & Lacroix, M. (2007). In vitro growth control of selected pathogens by Lactobacillus acidophilus- and Lactobacillus casei-fermented milk. *Letters in Applied Microbiology, 44*(3), 314-319. doi: http://dx.doi.org/10.1111/j.1472-765X.2006.02060.x

Millette, M., Luquet, F. M., Ruiz, M. T., & Lacroix, M. (2008). Characterization of probiotic properties of Lactobacillus strains. *Dairy Science and Technology, 88*(6), 695-705. doi: http://dx.doi.org/10.1051/dst:2008018

Mori, H., Hata, K., Yamada, Y., Kuno, T., & Hara, A. (2005). Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chemico-biological interactions, 155*(1–2), 1-9. doi: http://dx.doi.org/10.1016/j.cbi.2005.04.005

Muthu, R., & Vaiyapuri, M. (2013). Synergistic and individual effects of umbelliferone with 5-fluorouracil on tumor markers and antioxidant status of rat treated with 1,2-dimethylhydrazine. *Biomedicine & Aging Pathology, 3*(4), 219-227. doi: http://dx.doi.org/10.1016/j.biomag.2013.08.007

Nakamura, J., Kubota, Y., Miyaoka, M., Saitoh, T., Mizuno, F., & Benno, Y. (2002). Comparison of four microbial enzymes in Clostridia and Bacteroides isolated from human feces. *Microbiology and immunology, 46*(7), 487-490. doi: http://dx.doi.org/10.1111/j.1348-0421.2002.tb02723.x

Narushima, S., Sakata, T., Hioki, K., Itoh, T., Nomura, T., & Itoh, K. (2010). Inhibitory Effect of Yogurt on Aberrant Crypt Foci Formation in the Rat Colon and

Colorectal Tumorigenesis in RasH2 Mice. *Experimental Animals, 59*(4), 487-494. doi: http://dx.doi.org/10.1538/expanim.59.487

O'Neill, I. K., Loktionov, A., Manson, M. M., Ball, H., Bandaletova, T., & Bingham, S. A. (1997). Comparison of metabolic effects of vegetables and teas with colorectal proliferation and with tumour development in DMH-treated F344 rats. *Cancer Letters, 114*(1–2), 287-291. doi: http://dx.doi.org/10.1016/S0304-3835(97)04682-X

Onoue, M., Kado, S., Sakaitani, Y., Uchida, K., & Morotomi, M. (1997). Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. *Cancer Letters, 113*(1–2), 179-186. doi: http://dx.doi.org/10.1016/S0304-3835(97)04698-3

Orrhage, K., Annas, A., Nord, C., Brittebo, E., & Rafter, J. (2002). Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice. *Scandinavian journal of gastroenterology, 37*(2), 215-221.

Perez, J., Jayaprakasha, G., Cadena, A., Martinez, E., Ahmad, H., & Patil, B. (2010). In vivo induction of phase II detoxifying enzymes, glutathione transferase and quinone reductase by citrus triterpenoids. *BMC Complementary and Alternative Medicine, 10*(1), 51. doi: http://dx.doi.org/10.1186/1472-6882-10-51

Rafter, J. (2003). Probiotics and colon cancer. *Best Practice & Research Clinical Gastroenterology, 17*(5), 849-859.

Rao, C., Sanders, M., Indranie, C., Simi, B., & Reddy, B. (1999). Prevention of colonic preneoplastic lesions by the probiotic Lactobacillus acidophilus NCFMTM in F344 rats. *International Journal of Oncology, 14*(5), 939-983. doi: http://dx.doi.org/10.3892/ijo.14.5.939

Rowley, P. T. (2005). Inherited Susceptibility to Colorectal Cancer. *Annual Review of Medicine, 56*(1), 539-554. doi: http://dx.doi.org/10.1146/annurev.med.56.061704.135235

Rudolph, R. E., Dominitz, J. A., Lampe, J. W., Levy, L., Qu, P., Li, S., . . . Potter, J. D. (2005). Risk Factors for Colorectal Cancer in Relation to Number and Size of Aberrant Crypt Foci in Humans. *Cancer Epidemiology Biomarkers & Prevention, 14*(3), 605-608. doi: http://dx.doi.org/10.1158/1055-9965.EPI-04-0058

Sampalis, J., Psaradellis, E., & Rampakakis, E. (2010). Efficacy of BIO K+ CL1285® in the reduction of antibiotic-associated diarrhea–a placebo controlled double-blind randomized, multi-center study. *Arch Med Sci, 6*, 56-64. doi: http://dx.doi.org/10.5114/aoms.2010.13508

Sangeetha, N., Viswanathan, P., Balasubramanian, T., & Nalini, N. (2012). Colon cancer chemopreventive efficacy of silibinin through perturbation of xenobiotic metabolizing enzymes in experimental rats. *European Journal of Pharmacology, 674*(2–3), 430-438. doi: http://dx.doi.org/10.1016/j.ejphar.2011.11.008

Sengottuvelan, M., Deeptha, K., & Nalini, N. (2009). Resveratrol ameliorates DNA damage, prooxidant and antioxidant imbalance in 1,2-dimethylhydrazine induced rat colon carcinogenesis. *Chemico-biological interactions, 181*(2), 193-201. doi: http://dx.doi.org/10.1016/j.cbi.2009.06.004

Shamsuddin, A., & Phillips, R. (1981). Preneoplastic and neoplastic changes in colonic mucosa in Crohn's disease. *Archives of pathology & laboratory medicine, 105*(6), 283-286.

Sharma, S., Nemecz, S., Zhu, S., & Steele, V. (1997). Identification of chemopreventive agents by screening for induction of glutathione-S-transferase as a biomarker. *Methods in Cell Science, 19*(1), 49-52. doi: http://dx.doi.org/10.1023/A%3A1009750705969

Sheweita, S., & Tilmisany, A. (2003). Cancer and phase II drug-metabolizing enzymes. *Current drug metabolism, 4*(1), 45-58. doi: http://dx.doi.org/10.2174/1389200033336919

Simon, G., & Gorbach, S. (1986). The human intestinal microflora. *Digestive Diseases and Sciences, 31*(9), 147-162. doi: http://dx.doi.org/10.1007/BF01295996

Sivieri, K., Spinardi-Barbisan, A. L. T., Barbisan, L. F., Bedani, R., Pauly, N. D., Carlos, I. Z., . . . Rossi, E. A. (2008). Probiotic Enterococcus faecium CRL 183 inhibit chemically induced colon cancer in male Wistar rats. *European Food Research and Technology, 228*(2), 231-237. doi: http://dx.doi.org/10.1007/s00217-008-0927-6

Stevens, R. G., Swede, H., & Rosenberg, D. W. (2007). Epidemiology of colonic aberrant crypt foci: Review and analysis of existing studies. *Cancer Letters, 252*(2), 171-183. doi: http://dx.doi.org/10.1016/j.canlet.2006.11.009

Takayama, T., Katsuki, S., Takahashi, Y., Ohi, M., Nojiri, S., Sakamaki, S., . . . Niitsu, Y. (1998). Aberrant Crypt Foci of the Colon as Precursors of Adenoma and Cancer. *New England Journal of Medicine, 339*(18), 1277-1284. doi: http://dx.doi.org/10.1056/NEJM199810293391803

Verma, A., & Shukla, G. (2013a). Administration of prebiotic inulin suppresses 1,2 dimethylhydrazine dihydrochloride induced procarcinogenic biomarkers fecal enzymes and preneoplastic lesions in early colon carcinogenesis in Sprague Dawley rats. *Journal of Functional Foods, 5*(2), 991-996. doi: http://dx.doi.org/10.1016/j.jff.2013.02.006

Verma, A., & Shukla, G. (2013b). Probiotics Lactobacillus rhamnosus GG, Lactobacillus acidophilus Suppresses DMH-Induced Procarcinogenic Fecal Enzymes and Preneoplastic Aberrant Crypt Foci in Early Colon Carcinogenesis in Sprague Dawley Rats. *Nutrition and Cancer, 65*(1), 84-91. doi: http://dx.doi.org/10.1080/01635581.2013.741746

Vinothkumar, R., Vinoth Kumar, R., Sudha, M., Viswanathan, P., Balasubramanian, T., & Nalini, N. (2014). Modulatory effect of troxerutin on biotransforming enzymes and preneoplasic lesions induced by 1,2-dimethylhydrazine in rat colon carcinogenesis. *Experimental and Molecular Pathology, 96*(1), 15-26. doi: http://dx.doi.org/10.1016/j.yexmp.2013.10.009

Wang, W., & Higuchi, C. M. (1995). Induction of NAD(P)H: quinone reductase by vitamins A, E and C in Colo205 colon cancer cells. *Cancer Letters, 98*(1), 63-69. doi: http://dx.doi.org/10.1016/S0304-3835(06)80011-X

www.harlan.com.

Yamazaki, K., Tsunoda, A., Sibusawa, M., Tsunoda, Y., Kusano, M., Fukuchi, K., . . . Morotomi, M. (2000). The effect of an oral administration of Lactobacillus casei strain shirota on azoxymethane-induced colonic aberrant crypt foci and colon cancer in the rat. *Oncology Reports, 9*(7), 977-1059. doi: http://dx.doi.org/10.3892/or.7.5.977

Zhao, H., Zhou, F., Qi, Y., Dziugan, P., Bai, F., Walczak, P., & Zhang, B. (2013). Screening of Lactobacillus strains for their ability to bind Benzo(a)pyrene and the mechanism of the process. *Food and Chemical Toxicology, 59*(0), 67-71. doi: http://dx.doi.org/10.1016/j.fct.2013.05.040

Zhong, Y., Chiou, Y.-S., Pan, M.-H., Ho, C.-T., & Shahidi, F. (2012). Protective effects of epigallocatechin gallate (EGCG) derivatives on azoxymethane-induced colonic carcinogenesis in mice. *Journal of Functional Foods, 4*(1), 323-330. doi: http://dx.doi.org/10.1016/j.jff.2011.12.01

12. ARTICLE 4 : CANCER PREVENTIVE EFFECT OF COMPONENTS FROM A SPECIFIC PROBIOTIC FERMENTED MILK AND CELL WALL CONSTITUENTS FROM A BIOMASS CONTAINING *LACTOBACILLUS ACIDOPHILUS* CL1285, *L. CASEI* LBC80R, *L. RHAMNOSUS* CLR2 ON MALE F344 RATS TREATED WITH 1,2-DIMETHYLHYDRAZINE.

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Effets antinéoplasiques des fractions obtenus à partir du lait fermenté et des composantes membranaires extraites d'une biomasse contenant des bactéries *Lactobacillus acidophilus* CL1285*, L. casei* LB80CR et *L. rhamnosus* CLR2 sur des rats traités avec le 1,2-dimethylhydrazine.

<u>Contribution des auteurs</u> :

J'ai réalisé les expériences, rédigé l'article et participé à l'élaboration des protocoles. Dr. Millette m'a supervisé tout au long des expériences, corrigé l'article et participé aux discussions concernant le projet. Olivier Fortin a aidé à réaliser les expériences. Dr Khanh Dang Vu a participé aux discussions concernant le projet, élaboré les protocoles et corrigé l'article. Dr. Lacroix est la responsable du projet, a participé à toutes les prises de décisions, aux discussions concernant le projet et aux corrections de l'article. Majid Jamshidian et Behnoush Maherani ont participé aux corrections de l'article.

Résumé :

L'effet de fractions obtenues à partir d'un lait fermenté et des composantes membranaires extraits d'une biomasse contenant *L. acidophilus* CL1285, *L. casei* LBC80R et *L. rhamnosus* CLR2 sur la prévention du cancer du côlon chez des rats traités avec le diméthylhydrazine (DMH) a été étudié. Les rats ont été divisés en sept groupes de huit. Tous ont été nourris avec une diète riche en gras et faible en fibres, le groupe 1 a servi de contrôle négatif et le groupe 2 de contrôle probiotique. Les rats des groupes 3 à 7 ont été injectés avec le DMH (30mg/kg s.c) une fois par jour durant six semaines. Le groupe 3 est considéré comme étant le contrôle positif. Les rats des groupes 4 à 7 ont été gavé quotidiennement avec le surnageant et le culot du lait fermenté et les composants membranaires respectivement. Après 12 semaines, les rats ont été sacrifiés et leurs côlons, caecum et foies ont été prélevé. La présence de foyers de cryptes aberrantes (FCA) au niveau du colon a été déterminée par observation au microscope. L'activité des enzymes détoxifiantes du foie comme la quinone réductase (QR) et la glutathionne-S-transférase (GST) et fécales comme la β-glucuronidase et la β-glucosidase a été évalué par spectrométrie. Les rats des groupes 5 à 7 ont significativement ($p \leq 0.05$) moins de cryptes aberrantes (CA) and FCA comparé au groupe 3. Les rats nourris avec le culot ont une activité de QR significativement ($p \leq 0.05$) plus faible comparé au groupe 3. L'administration du culot a également diminué significativement ($p \leq 0.05$) l'activité de la β-glucuronidase. Ces résultats indiquent que l'administration du FM et des métabolites relâchés durant la fermentation du lait pourrait jouer un rôle dans la prévention du cancer colorectal.

Mots clés : bactéries probiotiques, cancer colorectal, foyers de cryptes aberrantes, fractions de lait fermenté, antinéoplasiques, composants membranaires

Cancer preventive effect of components from a specific probiotic fermented milk and cell wall constituents from a biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R, *L. rhamnosus* CLR2 on male F344 rats treated with 1,2-dimethylhydrazine.

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ASTRACT

The effect of fractions obtained from a specific probiotic fermented milk (FM) and cell wall constituents (CW) extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on colon carcinogenesis in male F344 rats treated with 1,2-dimethylhydrazine (DMH) was investigated. The rats were divided into seven groups of eight. All were fed with a high fat low fiber diet; group 1 (NC) served as negative control while group 2 (FMC) served as probiotic control. Groups 3 to 7were injected with DMH (30 mg/kg s.c) weekly for six weeks. Group 3 served as positive control (PC). In addition to the DMH, groups 4 to 7 received respectively (FM), fermented milk supernatant (S), fermented milk pellet (P) and (CW) through gavage. After 12 weeks, the rats were sacrificed and the colon, caecum and liver were collected. Number of aberrant crypt foci (ACF) observed on the mucosa of the collected colon were determined using a microscope. Detoxifying enzymes like quinone reductase (QR) and glutathione-S-transferase (GST) and faecal enzymes such as β-glucosidase and β-glucuronidase were evaluated by spectrophotometry. Rats in group 5 to 7 had significantly lowered AC and ACF count as compared to the PC ($p \leq 0.05$). Rats administrated with P were able to induce QR activity compared to PC ($p \leq 0.05$). Also along with rats supplemented with FM they were able to significantly reduce β-glucuronidase activity ($p \leq 0.05$). These results indicated that supplementaion of FM containing probiotic bacteria and their metabolites released during the fermentation could prevent the development of colorectal carcinogenesis

Keywords: probiotic, colorectal cancer, aberrant crypt foci (ACF), fractions od fermented milk, cell wall, anticancer.

12.1 INTRODUCTION

According to GLOBOCAN, colorectal cancer (CRC) is the third most common cancer in men (746,000 cases) and the second in women (614,000 cases). There's a high variability within the incidence of CRC worldwide and 55% of cases occur in more developed countries (Globocan, 2012). Numerous epidemiological studies associate the high incidence of CRC to a "western pattern diet". Western diet mean a diet with higher intake of red and/or processed meat, processed food, sweets intake and low intake of fruits, vegetables, dietary fiber. Those studies suggest that modulating the eating habits could either decrease or prevent the risk of developing CRC (Miller *et al.*, 2010, Randi *et al.*, 2010). Besides the diet, there's also a strong link between CRC and the colon microflora since the presence of some bacteria like *E. coli*, *Bacilus fragilis* increase the risk of the disease (Dove *et al.*, 1997, Kado *et al.*, 2001).

Probiotics are defined as live microorganism which when consumed in adequate amounts exert beneficial effect with the host health (Hill *et al.*, 2014). Although they are consumed as dietary supplements, probiotics can also be included in fermented or unfermented foods (Forssten *et al.*, 2011). The carrier widely use are fermented dairy products like yogurt and yogurt type drinks (McDonough *et al.*, 1987). Furthermore, probiotic can be included in food system or food products like cheese (Ibrahim *et al.*, 2010) or soy based foods (Rekha *et al.*, 2010). Studies showed that fermented dairy products have many health promoting effects such as improvement of lactose metabolism, regulate the body mass, reduction of serum cholesterol and reduction of cancer risk (Ebringer *et al.*, 2008, Malik *et al.*, 2003, Vasiljevic *et al.*, 2008). The bioactive properties of the fermented dairy products are attributed to the presence of probiotic bacteria (the whole microorganism and the cell wall components) and their metabolites released during milk fermentation like bioactive peptides sequences and extracellular polysaccharides (Korhonen *et al.*, 2003, Vinderola *et al.*, 2006).

In a previous study conducted by Desrouillères *et al.* (2015) it was reported 2 ml of a specific fermented milk containing *Lactobacillus acidophilus* CL1285, *L. case*i LBC80R

and *L. rhamnosus* CLR2 was able to significantly reduce the formation and the multiplicity of aberrant crypts (Acs), the activity of β-glucuronidase and also induce the activity of gluthatione S-transferase (GST).

Thus the aim of this study was to investigate whether the cancer preventive effect of the fermented milk was due to the presence of probiotic *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 (pellet of fermented milk), the peptides and the exopolysaccharides release during the fermentation process (supernatant of fermented milk) or the cell wall constituents (CW) of the three strains of probiotic extracted from a biomass individually or the combination of all the components..

12.2 MATERIALS AND METHODS

12.2.1 Chemicals

N,N dimethylhydrazine, 1-chloro-2,4-dinitrobenzene, Methylene blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), menadione, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, EDTA, FAD^+, Bovine Serum Albumine (BSA), Tween 20, 4-nitrophenol, L Glutathione reduced, p-nitrophenol, p-nitrophenyl β-D-glucopyranoside, p-nitrophenyl β-D-glucuronide were purchased from Sigma Chemical Co (Sigma-Aldrich, Oakville, ON, Canada).

12.2.2 Sample preparation

a) Preparation of the components from the fermented milk

A fermented milk graciously provided by Bio-K Plus International Inc. was stored at 4°C containing at least 50 x 10^9 colony forming units (CFU) of three (3) strains of a proprietary probiotics formulation: *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2 was used in this study. The fermented milk (FM) was centrifuged at 9000xg for 30 minutes at 4°C, the pellet (P) and supernatant (S) obtained were kept for the rest of the experimentation.

b) Cell wall constituents extraction

i. Physical treatment

A biomass containing *L. acidophilus* CL1285*, L. casei* LBC80R and *L. rhamnosus* CLR2 was used in this study for cell wall extraction (CW).

The first step consisted in a centrifugation of the biomass at 9 000 xg for 30 minutes at 4°C. The obtained pellets were washed with phosphate buffer 10 mM (pH 7.2) then suspended in the same buffer at a ratio of 1:4 (w/v). The obtained suspension was firstly heated at a temperature of 65°C for 40 minutes, then submitted to ultrasound in 500 W, 20 KHz (Q500, QSONICA, Newtown, CT, USA) using 1 s^{-1} pulse on and 1 s^{-1} pulse off with 40% acoustic power (amplitude) for 30 minutes at 4°C. The ultrasonic samples were centrifuged at 8 00xg for 30 minutes; the supernatant was recovered and

subjected to ultracentrifugation at 50000g for 30 minutes. The pellet was collected and suspended in 10 ml of 100mM Tris-HCl buffer pH 7.5 (Kim *et al.*, 2002, Signoretto *et al.*, 1998)

ii. Enzymatic treatment

The pellet obtained in the previous step was firstly incubated under agitation with 50µl of α-amylase (20mg/ml) for 2 hours at 37°C. Then, 50µl of $MgSO_4$ (1M), 100µl of DNase (1mg/ml), 100µl of RNase (50mg/ml) were added and the solution was incubated at 37°C under agitation for 2 hours. Finally, 100µl of trypsin (10mg/ml) and 10µl of 1M $CaCl_2$ were added and the samples was incubated overnight under agitation. After the incubation, 3.5ml of 4% SDS (w/v) was added in the 10ml of samples and heated at 100°C for 15 minutes. The samples were centrifuged at 30000g for 30 minutes. The new obtained pellet was washed with distilled water (4X), and then with different solution: LiCl (8M), EDTA (100mM) and acetone. Each solution was used twice to wash the pellet. The washed pellets were mixed with 10ml of $(NH_4)_2CO_3$ and 250µl of alkaline phosphatase (1mg/ml) and incubated overnight at 37°C under agitation. Then the sample was heated at 100°C for 5 minutes and centrifuged at 30000g for 45 minutes. The obtained pellet was washed with distilled water (2X), suspended in water and lyophilized. The obtained powder were weighed and kept at -20°C until used (Signoretto *et al.*, 1998).

12.2.3 Animals, housing and diet

Fifty-six F344 male rats aged between five and six weeks and weighing an average of 104.5 ± 4.73g were obtained from Charles River Laboratories (Laval, Québec, Canada) and housed in stainless wire cages (3 rats / cage). The temperature and the humidity were maintained at 22°C and 55% respectively. The light-dark cycles were 12 hour each. All the animals had free access to water and rat chow commercial pellets provided by Harlan (Madison, WI, USA). The composition of the high fat low fiber diet (Harlan) was 39.8% corn starch, 20% casein, 10% malto-dextrin, 10% corn oil, 12% lard, 2% cellulose, 4.375% mineral mixture, 1.25% vitamin mixture, 0.3% choline

bitartrate, 0.3% L-cystine and 0.0044% TBHQ (www.harlan.com). All experimental animal care and treatment followed the guidelines set by the Canadian Council on Animal Care (CCAC).

12.2.4 Experimental design

The rats underwent a one week period of acclimation. After that period, they were randomly divided into 7 groups of 8 animals. All rats were fed with the high fat low fiber diet (Harlan). Group 1 (NC) served as negative control and received 0.85% saline water by gavage. Rats in group 2 (FMC) served as probiotics control. Rats in groups 3 to 7 were injected with DMH dissolved in saline solution (30mg/kg subcutaneously) once a week for six weeks. Group 3 (PC) served as carcinogen control. In addition to the diet, rats in group 4 to 7 (G4 to G7) were supplemented by gavage with different probiotic treatments The components supplemented were: the whole fermented milk (FM), fermented milk supernatant (S), pellet of the fermented milk (P) and cells walls (CW) respectively for G4, G5, G6 and G7. Animals were monitored daily for general health and body weights were recorded twice a week for the duration of the study. After twelve weeks of the experimental period, the rats were sacrificed in a CO_2 chamber. The colon were collected for AC analysis while the liver and caecum were removed and stored at -80°C for subsequent enzymatic assays..

12.2.5 Aberrant crypt analysis

The entire colon for each animal was removed and washed thoroughly with saline water (0.85% NaCl). They were cut longitudinally and laid flat on Whatman paper in a Petri dish after they were fixed in 10% buffered formalin solution for at least 24 hours. Then, the colon was stained with 0.2% methylene blue for 3 min and placed in saline water overnight for the removal of excess staining. The mucosal side was placed on a glass slide and examined under microscope using 20X objective for assessment of ACF as well as the number of aberrant crypt per focus (AC) (Bird, 1995).

12.2.6 Liver and caecum processing

Rat livers from different groups were weighed and samples (1-2g) were cut from each liver. A volume of 5ml of sucrose solution (0.25M) was added into each sample and homogenization process was conducted using a tissue homogenizer (Fisher Scientific Tissuemiser homogenizer, Canada). The homogenized samples were centrifuged at 5000xg for 30 min at 4°C and the obtained supernatants were mixed with 0.2 volume of $CaCl_2$ solution (0.1M) and were centrifuged at 27000xg for 20 min at 4°C. The supernatants obtained (cytosol) were filtered through a 0.2µm filter membrane and were used for estimation of protein in rat liver and glutathione-S-transferase (GST) and quinone reductase (QR) activities.

Rat caecal contents from different treatment groups were weighed and then mixed with 10ml of 100mM phosphate buffered saline and finally homogenized. The homogenization process was conducted using a tissue homogenizer. The homogenized samples were then centrifuged at 3500 rpm for 15 min at 4 °C and the obtained supernatants were centrifuged again at 10000xg for 15 min at 4°C. The clear obtained supernatants were filtered throughout a 0.2µm filter membrane and used for total protein content and β-glucosidase and β-glucuronidase activities.

12.2.7 Protein estimation

Supernatants of rat liver and caecum were used for determination of protein content using BCA kit (Pierce BCA protein assay, Fisher Scientific, Canada) according to the manufacturer's protocol. Briefly 25 µl of each sample were added into each well of a 96 wells microplate. Then, 200 µl of BCA solution was added to the samples wells. For the blank, wells were filled with 225 µl BCA solution. All microplate were incubated for 30 minutes at 37°C. Absorbance was measure at 562 nm using a microplate reader (EL800 BioTek Instruments Inc.). Protein concentration was determined using bovine serum albumin (BSA) as standard.

12.2.8 Quinone reductase (QR) assay

Quinone reductase (EC. 1.6.99.2) assay was conducted using a microplate following a method described by (Kang *et al.*, 2004b) for *in vitro* assay with some modifications. Briefly, 30µl of samples from liver supernatants were deposited into 96-wells microplate. Distilled water (30µl) was used a negative control. Then, 200µl of reaction mixture containing 20.5ml distilled water, 1.1ml 0.5M Tris-HCl buffer pH 7.5, 14.7 mg BSA, 146.7µl 1.5% Tween 20, 14.7µl 7.5mM FAD^+, 146.7µl of 150mM glucose 6-phosphate, 13.2µl of 50mM $NADP^+$, 44µl of glucose 6-phosphate dehydrogenase, 6.6mg de tetrazolium blue bromide and 22µl of 50mM menadione was added into each well. The microplate was incubated for 5 min at room temperature and the optical density (OD) values were measured in a microplate reader (EL800, BioTek Instruments Inc.) at 595nm. Specific activity was calculated and expressed as nM MTT formazan formed per min per mg of protein. The induction of the activity of QR enzyme for the treated groups was calculated by dividing the enzyme specific activity of the treated group to the enzyme specific activity for the control.

12.2.9 Glutathione-S-transferase (GST) assay

Glutathione -S-transferase (EC. 2.5.1.18) assay was conducted using the method described by (Sharma *et al.*, 1997) for microplate with some modifications. Briefly, 100µl of samples from liver supernatants were added into a cuvette. Distilled water (100µl) was used as a negative control. A quantity of 2ml of reaction mix (100µl of 20mM 1-chloro-2, 4-dinitrobenzene [CDNB], 100µl of 20mM reduced glutathione, 1.7ml of reagent buffer) were added into each cuvette. The absorbance was monitored at 340 nm for 5min using a spectrophotometer (Varian Inc.). Values of delta O.D were used to calculate the activity of GST. Specific GST activity was calculated and expressed as nM CDNB conjugate formed per min per mg protein. Induction of GST enzyme for the treated groups was calculated by dividing the enzyme specific activity of the treated group to the enzyme specific activity for the control.

12.2.10 β-glucosidase and β-glucuronidase assay

β-glucosidase (EC. 3.2.1.21) and β-glucuronidase (EC. 3.2.1.31) assays were conducted using respectively p-nitrophenyl β-D-glucopyranoside and p- nitrophenyl β-D-glucuronide as substrate respectively. Briefly, 10 µl of samples from caecal supernatants were added into a 96-wells microplate. Distilled water (10 µl) was used as negative control. A volume of 40 µl of reaction mixture (A stock solution (12 ml) was prepared, it contained :3 ml of 5 mM of respective substrate, 3 ml of 100 mM phosphate buffered saline and 6 ml of distilled water) were added into each well then the microplate were incubated at 37°C for 15 min. The reaction was stopped by adding 250 µl of a solution of 0.01 M of NaOH. OD values were measured in a microplate reader at 405 nm. The activities of both enzymes were calculated based on the standard curve of p-nitrophenol (0-70 µM). One activity unit is defined as the quantity of enzyme required to hydrolyse substrate into one µM of p-nitrophenol per minute. Specific β-glucosidase and β-glucuronidase activities were calculated and expressed as µM of p-nitrophenol formed per min per mg protein.

12.2.11 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA), using the SPSS Base 16.0 software (Stat-Packets statistical analysis software, SPSS Inc., Chicago IL) and expressed as means ± standard deviation. The differences among the groups were analysed with a post hoc Duncan's test. Significance was declared at $p \leq 0.05$.

12.3 RESULTS

12.3.1 Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 administration on the number of AC per colon induced by DMH treatment

The effect of the administration of the fermented milk (FM) and its different components on the formation of AC is shown in Fig. 2. Results showed that all rats administrated with the FM or its different fractions have significantly lowered the count of AC compared to the carcinogen control group (PC). Animals in group G4 (FM) showed a count of AC significantly lower compared to the positive control group. by 43% the total count of AC found in the colon compared to the PC group. Rats in G5 (S) and G6 (P) were able to reduce by more than 50% the count of AC compared to the PC group. The reduction was by 58 and 57% respectively. Rats in G7 (CW) showed a 34% reduction of the total count of AC ($p \leq 0.05$) when compared to the PC group.

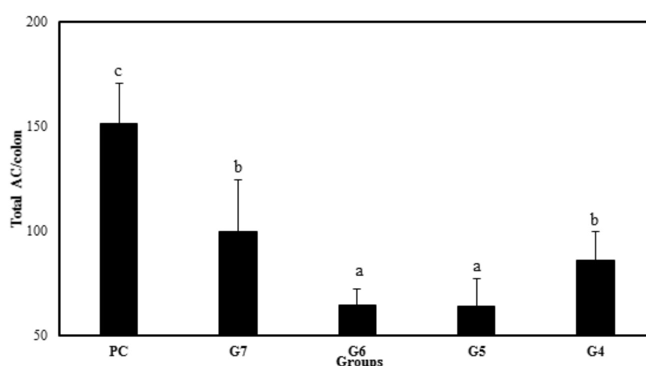

Figure 20 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on total number of aberrant crypts. Error bars represent the standard deviation of the mean total AC/colon obtained from 8 rats in each group. **G4**: DMH + 2 ml fermented milk (FM), **G5**: DMH + fermented milk supernatant (S), **G6**: DMH + fermented milk pellet (P), **G7**: DMH + cell wall constituents (CW) **PC**: DMH only. Different letters are significantly different ($p \leq 0.05$).

12.3.2 Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L.*

***acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 administration on the number of ACF per colon induced by DMH treatment.**

The incidence of ACF is shown in Fig. 22. All rats treated with DMH developed ACF. Rats in the groups fed with FM (G4) were able to reduce significantly ($p \leq 0.05$) the total number of ACF by 43% as compared to the carcinogen control group (PC). Rats fed with S (G5), P (G6) and CW (G7) were also able to significantly ($p \leq 0.05$) reduce the number of ACF by 52%, 51% and 20% respectively.

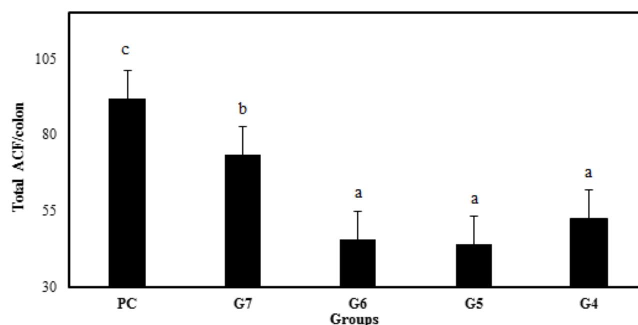

Figure 21 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on total count of aberrant crypt foci. Error bars represent the standard deviation of the mean total ACF/colon obtained from 8 rats in each group. **G4**: DMH + 2 ml fermented milk (FM), **G5**: DMH + fermented milk supernatant (S), **G6**: DMH + fermented milk pellet (P), **G7**: DMH + cell wall constituents (CW) **PC**: DMH only. Different letters are significantly different ($p \leq 0.05$).

12.3.3 Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 administration on the multiplicity of AC per ACF in F344 rats treated with DMH.

A foci can be constituted by more than one aberrant crypt (AC) and when it present 2 or more AC, the probability of that foci to become a carcinoma is higher. The number of AC/ACF was also evaluated and the mean number of crypts per foci is presented in Fig.23. The results were divided into two groups: one presenting foci with 2 or 3 aberrant crypts (2-3AC/ACF) and the other one showing foci with 4 or 5 aberrant crypts (4-5AC/ACF). Results showed that all rats supplemented with the fermented milk or its

components were able to reduce significantly the crypts number per foci crypts. Rats in G4 (FM) and G6 (P) were able to eliminate the number of foci containing 4 or 5 aberrant crypts. Rats in G5 (S) and G7 (CW) were able to reduce the number crypt per foci in the group 4 or 5 aberrant crypts by 88% and 91% respectively ($p \leq 0.05$). For the 2-3AC/ACF, groups G4, G5, G6 and G7 lowered the count of AC per focus by 34%, 59%, 58% and 45% respectively ($p \leq 0.05$).

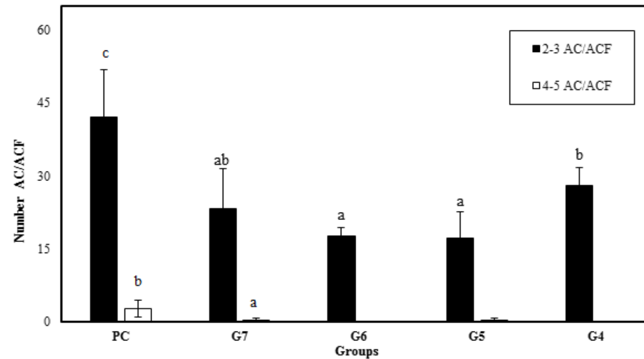

Figure 22 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on number of aberrant crypt foci (ACF) of two to five aberrant crypt induced by DMH. Error bars represent the standard deviation of the mean of the number of AC per focus obtained from 8 rats in each group. **G4**: DMH + 2 ml fermented milk (FM), **G5**: DMH + fermented milk supernatant (S), **G6**: DMH + fermented milk pellet (P), **G7**: DMH + cell wall constituents (CW) **PC**: DMH only. Different letters are significantly different ($p \leq 0.05$).

Figure 23 : Topographical view of normal and aberrant crypts on the colonic mucosa stained with methylene blue of rats treated with DMH. (A) Topographical view of PC colonic mucosa showing multiple ACF, one with four crypts, one with 2 crypts and the last two one are ACF with a single crypt (20X). (B) Topographical view of G4 colonic mucosa showing multiple ACF, one with two crypts and the last two ones are single crypt ACF

(20X). The arrows shows the crypts in the colon mucosa. **G4**: DMH + 2 ml fermented milk (FM), **PC**: DMH only.

12.3.4 Effect of the administration of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on (QR) and (GST) activities in F344 rats treated with DMH

The effect of the fermented milk on the activity of two detoxifying enzymes are presented in Fig 25 and Fig. 26. Results showed that only rats in G2 were able to induce significantly GST activity ($p \le 0.05$) by 53% as compared to the negative control group (NC). Rats in G3, G4 and G6 were not significantly different when compared to the NC group ($p \ge 0.05$). As for QR, results showed that only rats in G6 were able to significantly increase the activity of the enzyme ($p \le 0.05$) by compared to the PC group.

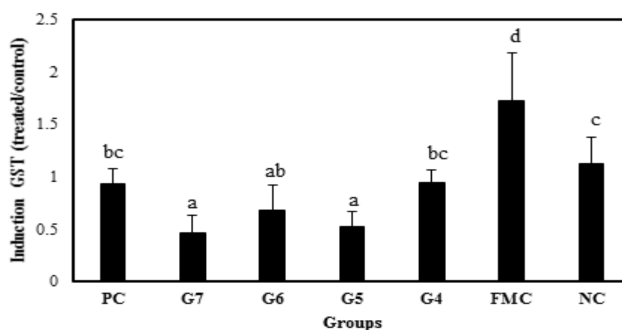

Figure 24 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on glutathione S-transferase activity. Error bars represent the standard deviation of the mean of glutathione S-transferase induction obtained from 8 rats in each group. Different letters are significantly different ($p \le 0.05$) from carcinogen control.

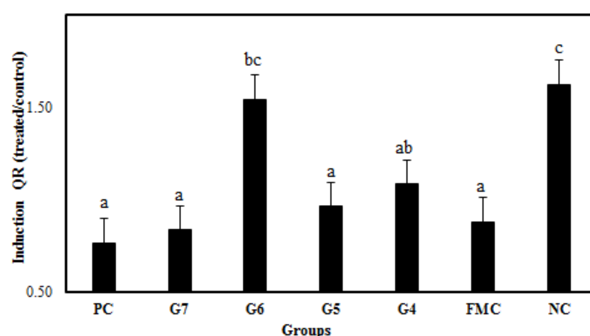

Figure 25 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on quinone reductase. Error bars represent the standard deviation of the mean of quinone reductase induction obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control

12.3.5 Effect of the administration of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2on β-glucosidase and β-glucuronidase activities in F344 rats treated with DMH.

The activities of β-glucosidase and β-glucuronidase are shown in Fig. 27 and Fig. 28. The results showed that the activity of β-glucosidase in the carcinogen control (PC) compared to the negative control (NC) was different statistically ($p \leq 0.05$). Moreover, only rats in probiotic control group (FMC) were able to significantly reduce ($p \leq 0.05$) the activity of β-glucosidase compared to both negative and positive controls groups. The reduction rate was 40% when comparted to NC group. The activity of β-glucuronidase was reduce significantly ($p \leq 0.05$) by rats in G4 (FM) and G6 (P). The enzymatic activity was reduced by 36% and 31% as compared to NC respectively. While rats in G3 and G7 were able to significantly increase the activity

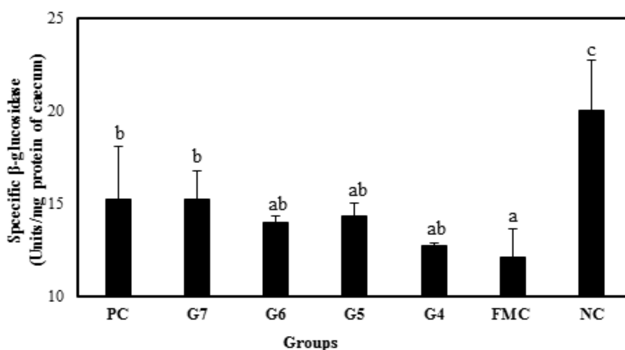

Figure 26 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on β-glucosidase activity. Error bars represent the standard deviation of the mean of β-glucuronidase specific activity obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control

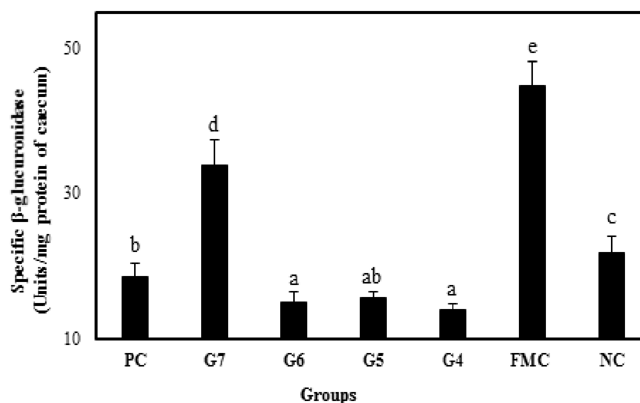

Figure 27 : Effect of different components from a specific fermented milk and cell wall extracted from a biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2 on β-glucuronidase activity. Error bars represent the standard deviation of the mean of β-glucuronidase specific activity obtained from 8 rats in each group. Different letters are significantly different ($p \leq 0.05$) from carcinogen control

12.4 DISCUSSION

The aim of this was firstly to determine the effect of the consumption of the different fractions (S and P) of a specific probiotic FM containing a mixture of bacteria (*L. acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2) and the CW constituents extracted from a biomass containing those bacteria on the formation of DMH-induced ACF in male F344 rats. Then, the effect of those dietary supplements on two hepatic detoxifying and two caecal enzymes were evaluated. The fractions were obtained by centrifugation of the fermented milk. It was able to concentrate different components either in the pellet or the supernatant depending on the molecular weight. The pellet will probably contain the probiotic bacteria and proteins while the supernatant will contain mostly derived-peptides from casein and/or whey proteins and it may also have some exopolysaccharides.

ACF are abnormal crypt found in the colon mucosa, they differ from normal crypt by a number of properties such as: altered luminal openings, thickened epithelial and larger surface. They are considered as the precancerous lesions to colorectal adenomas and biomarker for colorectal carcinoma (Yokota *et al.*, 1997).

Our results showed a significant ($p \leq 0.05$) reduction of the ACF count and the number of AC per colon in all rats that were administrated with the dietary supplements as compared to rats in PC group. Apart from the total number of AC, the number of AC per foci also decreased. Rats fed with S (G5) and P (G6) have a significant ($p \leq 0.05$) lower count of AC and AC per focus compared to rat fed with the whole fermented milk (G4). Those result suggest that the pellet and the supernatant have a greater inhibitory effect on AC formation than whole FM.

The mechanism involve in the inhibition of the ACF formation is still not fully known but it's believed that this effect may proceed through the alteration of microbial enzymes (Goldin *et al.*, 1984), enhancement of the immune system (Lee *et al.*, 2004), inactivation of

carcinogenic compounds (Orrhage *et al.*, 2002), alteration of the pH colonic environment (Wollowski *et al.*, 2001) and the microbial community (Bertkova *et al.*, 2010).

Some studies showed that the consumption of probiotic via FM could re-establish the balance of the intestinal microbiota (Lourens-Hattingh *et al.*, 2001). For instance, after consumption of FM containing *Bifidobacterium*., this bacteria tend to become a part of the intestinal microbiota and also after consumption of FM containing *L. casei*, there's a remodelling of the gut microbiome by influencing the existing microbial community (Gogineni *et al.*, 2013). While other studies showed the effect of FM on the immune system, Nanno *et al.* (2011) demonstrated that the daily administration of a FM containing *L. casei* Shirota during 3 weeks restored the activity of natural killer (NK) cells in healthy subject, also an induction of macrophages activation and TNF-α production was observed in rats fed with *L. rhamnosus* GC *B. adolesentis* bacterial extracts (Bertkova *et al.*, 2010).

Numerous studies have demonstrated the immunomodulatory effects of peptides-derived from casein and whey and exopolysaccharides (Nishimura-Uemura *et al.*, 2003, Vinderola *et al.*, 2007). They are able to enhance the immune system by the stimulation and regulation of cytokine production, increasing macrophage production (Ciszek-Lenda *et al.*, 2011, Medrano *et al.*, 2011, Ng *et al.*, 2002). Bermudez-Brito *et al.* (2013) investigated the immunomodulatory effect of *Bifidobacterium breve* CNCM I-4035 and its cell free supernatant in human like dendritic cells (DC); they found that both live bacteria and the supernatant were able to modify the release of cytokines by different pathway; the supernatant decrease the pro-inflammatory cytokines while *B. breve* induce their release.

The molecules in the supernatant (cell or bacterial-free fraction) of a FM have also proved their antiproliferative property (Cousin *et al.*, 2012, Escamilla *et al.*, 2012). Wan *et al.* (2014) demonstrated that supernatant from *L. debrurckii* was able to efficiently inhibit the proliferation and induce apoptosis in colon cancer cells.

As for the probiotic cell wall, some studies showed their ability to modify the immune system (Kolling *et al.*, 2015, Sun *et al.*, 2005b) while other demonstrated their capacity to bind mutagenic compounds thus preventing gut mutagenicity and ultimately prevent colon cancer (Sreekumar *et al.*, 1998, Zhang *et al.*, 1990). Our results are in agreement with

previous studies since it seems that all the dietary supplements have a potential to exert one of the mechanism by which the formation of ACF is inhibited.

The effect of the probiotic dietary supplements on the activity of two detoxifying enzymes: quinone reductase (QR) and glutathione S-transferase (GST) were assayed. The induction of these two enzymes an important mechanism of chemoprevention (Goyal, 2012). QR facilitate the excretion of quinones resulting from the metabolism of hydrocarbon by the reduction of two electron (Benson *et al.*, 1980) and GST is involved in the detoxification of xenobiotic agents by catalyzing the conjugation of GSH, it's also implicated in the elimination of peroxide that are formed during the metabolism (Shih *et al.*, 2007).

None of the rats treated with DMH that receive the dietary treatment was able to induce GST activity. As for QR, rats fed with the pellet induce significantly ($p \leq 0.05$) the enzyme activity compared to PC group.

GST is considered to be the primary enzyme to detoxify the organism (Coles *et al.*, 2003), the administration of the probiotic supplements didn't affect the activity of this enzyme meaning that the carcinogenic compounds weren't eliminated. The induction of QR helped detoxify the enzyme. We could propose a hypothesis that the action of this two enzymes may be connected; if one is able to detoxify a toxin efficiently the other one may not be recquired to do it. However, this hypothesis need to be further studied.

Rats that were fed with cell wall constituents and supernatant present the lowest activity for both enzyme, it is possible that the concentration tested, of both samples is too low to induce the expression of these enzymes. Further investigation may be require to determine whether the effect of cell wall, supernatant and pellet are dose dependant.

The effect of the daily administration of probiotic supplements on two caecal enzymes activity was also evaluated. β-glucuronidase is able to convert innocuous compounds into carcinogenic metabolites. After being detoxify in the liver, various carcinogen are conjugated to glucuronic acid by β-glucuronidase excreted them into the small intestine (Humblot et al., 2007; Rowland, 2009). β-glucosidase catalyse the hydrolysis of the glycosidic bound which result in the removal of the glucopyranosyl residues from the non-reducing end of β-glucosides (JR. & A., 2010).

Some studies showed the ability of probiotic to reduce the activity of those enzymes (Bertkova et al., 2010; Kekkonen et al., 2011).

Our results showed that only rats fed with P (G6) and FM (G4) reduced significantly ($p \leq 0.05$) the activity of β-glucuronidase as compared to carcinogen control group. Rad fed with S (G5) didn't affect the activity of the enzyme while the one that were administrated CW (G7) significantly ($p \leq 0.05$) increase the enzyme activity.

In a study conducted by de Moreno et al. (de Moreno de LeBlanc & Perdigón, 2005), it was demonstrated that the mice fed with yogurt were able to reduce β-glucuronidase activity compared to the DMH control group, while the rats fed with the supernatant did not reduce the activity of the enzyme.

Concerning β-glucosidase, the rats fed with FM, P and S reduce β-glucosidase activity but compared to rats in positive control group, the reduction wasn't statistically different ($p > 0.05$). Those results suggest that a higher concentration of those dietary supplements might have that reductive effect

12.5 CONCLUSION

In summary, this study suggest that the whey and casein-derived peptide, exopolysaccharides, the probiotics and their cells walls present in the specific fermented milk could individually exert a cancer preventing effect by reducing and eventually inhibiting the formation of ACF. We speculate that higher concentration of P, CW and S should present a greater effect on the liver and fecal enzymes activities than the fermented milk. Our findings also reveal an interesting effect of the consumption of the fermented milk on healthy subjects. Still further studies will be needed to identify the role and the nature of the specific peptides and/or exopolysaccharides from the fermented milk and determine the mechanisms involved in all the dietary supplements as cancer prevention.

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REFERENCES

Benson, A. M., Hunkeler, M. J., & Talalay, P. (1980). Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proceedings of the National Academy of Sciences, 77*(9), 5216-5220. Retrieved from http://www.pnas.org/content/77/9/5216.abstract

Bermudez-Brito, M., Muñoz-Quezada, S., Gomez-Llorente, C., Matencio, E., Bernal, M. J., Romero, F., & Gil, A. (2013). Cell-Free Culture Supernatant of *Bifidobacterium breve* CNCM I-4035 Decreases Pro-Inflammatory Cytokines in Human Dendritic Cells Challenged with *Salmonella typhi* through TLR Activation. *PLoS ONE, 8*(3), e59370. doi:10.1371/journal.pone.0059370

Bertkova, I., Hijova, E., Chmelarova, A., Mojzisova, G., Petrasova, D., Strojny, L., . . . Zitnan, R. (2010). The effect of probiotic microorganisms and bioactive compounds on chemically induced carcinogenesis in rats. *Neoplasma, 57*(5), 422-428. doi:10.4149/neo_2010_05_422

Bird, R. P. (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Letters, 93*(1), 55-71. doi:http://dx.doi.org/10.1016/0304-3835(95)03788-X

Ciszek-Lenda, M., Nowak, B., Śróttek, M., Gamian, A., & Marcinkiewicz, J. (2011). Immunoregulatory potential of exopolysaccharide from Lactobacillus rhamnosus KL37. Effects on the production of inflammatory mediators by mouse macrophages. *International Journal of Experimental Pathology, 92*(6), 382-391. doi:10.1111/j.1365-2613.2011.00788.x

Coles, B. F., & Kadlubar, F. F. (2003). Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *Biofactors, 17*(1), 115-130. doi:http://dx.doi.org/10.1002/biof.5520170112

Cousin, F. J., Jouan-Lanhouet, S., Dimanche-Boitrel, M.-T., Corcos, L., & Jan, G. (2012). Milk Fermented by <italic>Propionibacterium freudenreichii</italic> Induces Apoptosis of HGT-1 Human Gastric Cancer Cells. *PLoS ONE, 7*(3), e31892. doi:10.1371/journal.pone.0031892

de Moreno de LeBlanc, A., & Perdigón, G. (2005). Reduction of beta-glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. *Biocell, 29*, 15-24. Retrieved from http://www.scielo.org.ar/scielo.php?script=sci_arttext&pid=S0327-95452005000100003&nrm=iso

Desrouillères, K., Millette, M., Vu, K. D., Touja, R., & Lacroix, M. (2015). Cancer preventive effects of a specific probiotic fermented milk containing Lactobacillus acidophilus CL1285, L. casei LBC80R and L. rhamnosus CLR2 on male F344 rats treated with 1,2-dimethylhydrazine. *Journal of Functional Foods, 17*(0), 816-827. doi:http://dx.doi.org/10.1016/j.jff.2015.06.035

Dove, W. F., Clipson, L., Gould, K. A., Luongo, C., Marshall, D. J., Moser, A. R., . . . Jacoby, R. F. (1997). Intestinal Neoplasia in the ApcMin Mouse: Independence from the Microbial and Natural Killer (beige Locus) Status. *Cancer Research, 57*(5), 812-814. Retrieved from http://cancerres.aacrjournals.org/content/57/5/812.abstract

Ebringer, L., Ferenčík, M., & Krajčovič, J. (2008). Beneficial health effects of milk and fermented dairy products — Review. *Folia Microbiologica, 53*(5), 378-394. doi:10.1007/s12223-008-0059-1

Escamilla, J., Lane, M. A., & Maitin, V. (2012). Cell-Free Supernatants from Probiotic Lactobacillus casei and Lactobacillus rhamnosus GG Decrease Colon Cancer Cell Invasion In Vitro. *Nutrition and Cancer, 64*(6), 871-878. doi:10.1080/01635581.2012.700758

Forssten, S. D., Sindelar, C. W., & Ouwehand, A. C. (2011). Probiotics from an industrial perspective. *Anaerobe, 17*(6), 410-413.

Globocan. (2012). http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

Gogineni, V., Morrow, L., & Malesker, M. (2013). Probiotics: mechanisms of action and clinical applications. *Journal of Probiotics and Health, 1*, 101.

Goldin, B. R., & Gorbach, S. L. (1984). The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *The American Journal of Clinical Nutrition, 39*(5), 756-761. Retrieved from http://ajcn.nutrition.org/content/39/5/756.abstract

Goyal, P. (2012). Cancer chemoprevention by natural products: current & future prospects. *J Integr Oncol, 1*, e101.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., . . . Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol, 11*(8), 506-514. doi:http://dx.doi.org/10.1038/nrgastro.2014.66

Humblot, C., Murkovic, M., Rigottier-Gois, L., Bensaada, M., Bouclet, A., Andrieux, C., . . . Rabot, S. (2007). β-Glucuronidase in human intestinal microbiota is necessary for the colonic genotoxicity of the food-borne carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline in rats. *Carcinogenesis, 28*(11), 2419-2425. doi:10.1093/carcin/bgm170

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2010). *Probiotics and immunosenescence: cheese as a carrier* (Vol. 59).

JR., C. K., & A., E. (2010). β-Glucosidases. *Cellular and Molecular Life Sciences, 67*(20), 3389-3405. doi:http://dx.doi.org/10.1007/s00018-010-0399-2

Kado, S., Uchida, K., Funabashi, H., Iwata, S., Nagata, Y., Ando, M., . . . Morotomi, M. (2001). Intestinal Microflora Are Necessary for Development of Spontaneous Adenocarcinoma of the Large Intestine in T-Cell Receptor β Chain and p53 Double-Knockout Mice. *Cancer Research, 61*(6), 2395-2398. Retrieved from http://cancerres.aacrjournals.org/content/61/6/2395.abstract

Kang, Y. H., & Pezzuto, J. M. (2004). Induction of Quinone Reductase as a Primary Screen for Natural Product Anticarcinogens. *Methods in enzymology, 382*, 380-414. doi:http://dx.doi.org/10.1016/S0076-6879(04)82021-4

Kekkonen, R. A., Holma, R., Hatakka, K., Suomalainen, T., Poussa, T., Adlercreutz, H., & Korpela, R. (2011). A Probiotic Mixture Including Galactooligosaccharides Decreases Fecal β-Glucosidase Activity but Does Not Affect Serum Enterolactone Concentration in Men during a Two-Week Intervention. *The Journal of Nutrition, 141*(5), 870-876. doi:10.3945/jn.110.137703

Kim, J., Woo, H., Kim, Y.-S., & Lee, H. (2002). Screening for antiproliferative effects of cellular components from lactic acid bacteria against human cancer cell lines. *Biotechnology Letters, 24*(17), 1431-1436. doi:10.1023/A:1019875204323

Kolling, Y., Salva, S., Villena, J., Marranzino, G., & Alvarez, S. (2015). Non-viable immunobiotic Lactobacillus rhamnosus CRL1505 and its peptidoglycan improve systemic and respiratory innate immune response during recovery of immunocompromised-malnourished mice. *International immunopharmacology*.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current Pharmaceutical Design, 9*(16), 1297-1308.

Lee, J. W., Kim, E. H., Yim, I. B., & Joo, H. G. (2004). Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *Journal of Veterinary Science, 5*(1), 41-48.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1-17. doi:http://dx.doi.org/10.1016/S0958-6946(01)00036-X

McDonough, F. E., Hitchins, A. D., Wong, N. P., Wells, P., & Bodwell, C. E. (1987). Modification of sweet acidophilus milk to improve utilization by lactose-intolerant persons. *The American Journal of Clinical Nutrition, 45*(3), 570-574. Retrieved from http://ajcn.nutrition.org/content/45/3/570.abstract

Medrano, M., Racedo, S. M., Rolny, I. S., Abraham, A. G., & Pérez, P. F. (2011). Oral Administration of Kefiran Induces Changes in the Balance of Immune Cells in a Murine Model. *Journal of Agricultural and Food Chemistry, 59*(10), 5299-5304. doi:10.1021/jf1049968

Miller, P. E., Lesko, S. M., Muscat, J. E., Lazarus, P., & Hartman, T. J. (2010). Dietary Patterns and Colorectal Adenoma and Cancer Risk: A Review of the Epidemiological Evidence. *Nutrition and Cancer, 62*(4), 413-424. doi:10.1080/01635580903407114

Nanno, M., Kato, I., Kobayashi, T., & Shida, K. (2011). Biological effects of probiotics: what impact does Lactobacillus casei shirota have on us? *International journal of immunopathology and pharmacology, 24*(1 Suppl), 45S.

Ng, K.-Y., & Griffiths, M. (2002). Enhancement of macrophage cytokine release by cell-free fractions of fermented milk. *Milchwissenschaft, 57*(2), 66-70.

Nishimura-Uemura, J., Kitazawa, H., Kawai, Y., Itoh, T., Oda, M., & Saito, T. (2003). Functional alteration of murine macrophages stimulated with extracellular polysaccharides from Lactobacillus delbrueckii ssp. bulgaricus OLL1073R-1. *Food Microbiology, 20*(3), 267-273. doi:http://dx.doi.org/10.1016/S0740-0020(02)00177-6

Orrhage, K., Annas, A., Nord, C., Brittebo, E., & Rafter, J. (2002). Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice. *Scandinavian journal of gastroenterology, 37*(2), 215-221.

Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology, 100*(6), 1171-1185. doi:10.1111/j.1365-2672.2006.02963.x

Randi, G., Edefonti, V., Ferraroni, M., La Vecchia, C., & Decarli, A. (2010). Dietary patterns and the risk of colorectal cancer and adenomas. *Nutrition reviews, 68*(7), 389-408.

Rekha, C. R., & Vijayalakshmi, G. (2010). Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *Journal of Applied Microbiology, 109*(4), 1198-1208. doi:10.1111/j.1365-2672.2010.04745.x

Rowland, I. R. (2009). The Role of the Gastrointestinal Microbiota in Colorectal Cancer. *Current Pharmaceutical Design, 15*(13), 1524-1527. doi:10.2174/138161209788168191

Sharma, S., Nemecz, S., Zhu, S., & Steele, V. (1997). Identification of chemopreventive agents by screening for induction of glutathione-S-transferase as a biomarker. *Methods in Cell Science, 19*(1), 49-52. doi:http://dx.doi.org/10.1023/A%3A1009750705969

Shih, P.-H., Yeh, C.-T., & Yen, G.-C. (2007). Anthocyanins Induce the Activation of Phase II Enzymes through the Antioxidant Response Element Pathway against Oxidative Stress-Induced Apoptosis. *Journal of Agricultural and Food Chemistry, 55*(23), 9427-9435. doi:10.1021/jf071933i

Signoretto, C., Boaretti, M., & Canepari, P. (1998). Peptidoglycan synthesis by Enterococcus faecalis penicillin binding protein 5. *Archives of Microbiology, 170*(3), 185-190. doi:10.1007/s002030050632

Sreekumar, O., & Hosono, A. (1998). Antimutagenicity and the Influence of Physical Factors in Binding Lactobacillus gasseri and Bifidobacterium longum Cells to Amino Acid Pyrolysates. *Journal of Dairy Science, 81*(6), 1508-1516. doi:http://dx/doi.org/10.3168/jds.S0022-0302(98)75716-9

Sun, J., Shi, Y.-H., Le, G.-W., & Ma, X.-Y. (2005). Distinct immune response induced by peptidoglycan derived from Lactobacillus sp. *World journal of gastroenterology : WJG, 11*(40), 6330-6337. doi:10.3748/wjg.v11.i40.6330

Vasiljevic, T., & Shah, N. P. (2008). Probiotics—From Metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714-728. doi:http://dx.doi.org/10.1016/j.idairyj.2008.03.004

Vinderola, G., Matar, C., Palacios, J., & Perdigón, G. (2007). Mucosal immunomodulation by the non-bacterial fraction of milk fermented by Lactobacillus helveticus R389. *International Journal of Food Microbiology, 115*(2), 180-186. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2006.10.020

Vinderola, G., Perdigón, G., Duarte, J., Farnworth, E., & Matar, C. (2006). Effects of the oral administration of the exopolysaccharide produced by Lactobacillus kefiranofaciens on the gut mucosal immunity. *Cytokine, 36*(5–6), 254-260. doi:http://dx.doi.org/10.1016/j.cyto.2007.01.003

Wan, Y., Xin, Y. I., Zhang, C., Wu, D., Ding, D., Tang, L. I., . . . Li, W. (2014). Fermentation supernatants of Lactobacillus delbrueckii inhibit growth of human colon cancer cells and induce apoptosis through a caspase 3-dependent pathway. *Oncology Letters, 7*(5), 1738-1742. doi:10.3892/ol.2014.1959

Wollowski, I., Rechkemmer, G., & Pool-Zobel, B. L. (2001). Protective role of probiotics and prebiotics in colon cancer. *The American Journal of Clinical Nutrition, 73*(2), 451s-455s. Retrieved from http://ajcn.nutrition.org/content/73/2/451s.abstract

www.harlan.com.

Yokota, T., Sugano, K., Kondo, H., Saito, D., Sugihara, K., Fukayama, N., . . . Yoshida, S. (1997). Detection of aberrant crypt foci by magnifying colonoscopy. *Gastrointestinal Endoscopy, 46*(1), 61-65.

Zhang, B. X., & Ohta, Y. (1990). Binding of Mutagens by Fractions of the Cell Wall Skeleton of Lactic Acid Bacteria on Mutagens. *Journal of Dairy Science, 74*(5), 1477-1481. doi:http://dx.doi.org/10.3168/jds.S0022-0302(91)78306-9

13. DISCUSION

Le cancer colorectal dont les facteurs de risques principaux sont l'alimentation et la composition du microbiote intestinal constitue une des maladies chroniques avec le taux le plus élevé de mortalité et de morbidité. La prévention de la maladie ou tout au plus la diminution du risque de développer la maladie peut être réalisée par l'adoption d'une alimentation saine qui modulerait la composition et la diversité de la flore intestinale, favorisant ainsi la croissance des « bonnes » bactéries. Due à la présence de nombreuses molécules possédant plusieurs effets bénéfiques pour la santé les aliments comme les baies, les plantes médicinales, les laits fermentés sont considérés comme étant des aliments pouvant avoir une influence sur l'incidence et au développement de la maladie.

Ce travail a montré que les composés phénoliques contenus dans le jus concentré de canneberge et les calices séchés de la fleur d'hibiscus sont capables d'inhiber la prolifération des cellules cancéreuses (**Chapitres 8** et **9**). Cette inhibition peut avoir été réalisée soit par arrêt du cycle cellulaire ou encore par une voie existante pour la mort cellulaire (l'apoptose, la nécrose ou encore l'autophagie).

Des études ont montré que les composés phénoliques présents dans les petits fruits (comme la canneberge) et dans l'hibiscus pouvaient inhiber la prolifération des cellules cancéreuses par un des mécanismes cités ci-dessus (Chiu *et al.*, 2015, Neto *et al.*, 2014, Prasain *et al.*, 2016, Wu *et al.*, 2016).

Les composants membranaires extraits d'une biomasse contenant *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} ne présentent aucun effet inhibiteur sur la croissance des cellules cancéreuses (Chapitre **8**). Cependant ces dernières ont réduit la formation des cryptes (Chapitre **11**). Des études ont démontré l'effet inhibiteur des composantes membranaires extraites de probiotiques au niveau des cellules cancéreuses de colon (Sun *et al.*, 2005a) Certaines suggèrent que les mécanismes impliqués dans cette réduction est dû à une augmentation de la capacité

de la réponse immune des souris dû à la présence des peptidoglycanes tandis que d'autres impliquent que les composants membranaires des probiotiques étaient responsable de leur capacité de se lier avec des substances carcinogènes (ex : mycotoxines ou encore des amines aromatiques hétérocycliques). Cette liaison a tendance à provoquer l'exclusion de ces substances au niveau de la paroi intestinale et ainsi réduire le taux d'exposition à ces substances toxiques (Hernandez-Mendoza *et al.*, 2009, Lahtinen § *et al.*, 2004, Peltonen *et al.*, 2001).

Cette étude a montré que combinés aux composés phénoliques (obtenu soit à partir du de canneberges concentré ou des calices de la fleur d'hibiscus), les composants membranaires extraits des probiotiques de BioK+ ont exercé une certaine inhibition de la croissance des cellules du cancer du côlon. Une augmentation significative de l'effet inhibiteur des fractions/extraits de composés phénolique a été observée. Pour les trois fractions de canneberges et un extrait provenant des calices de la fleur d'hibiscus (FaJ) l'augmentation du pouvoir inhibiteur révèle un effet synergique tandis que pour les deux autres extraits de l'hibiscus (FbJ et FcJ), il s'agit en réalité d'un effet additif.

L'induction de l'activité de la quinone réductase (QR) est l'un des mécanismes utilisé afin de combattre la carcinogénèse. Alors que la fraction contenant des flavonols (F3) obtenue à partir du jus de canneberge concentré induit l'activité de l'enzyme (QR), aucun des extraits de la fleur d'hibiscus n'a été en mesure de le faire. Les composants membranaires également n'ont présenté aucun effet inducteur au niveau de l'activité de l'enzyme aux concentrations testées. Lorsque les composés phénoliques ont été combinés aux extraits des composants membranaires des probiotiques de BioK en ce qui concerne les fractions de canneberges, aucun changement par rapport à l'induction a été observé mais en ce qui a trait des extraits de fleur de Jamaïque plus précisément l'extrait FcJ (riche en anthocyanes) une augmentation significative de l'induction de la QR a été observé.

Nos études ont également montrées que la fraction riche en anthocyanes possède une plus grande efficacité à piéger les radicaux libres. Des études réalisées au sein de notre laboratoire ont également démontré la capacité antioxydant des canneberges (Caillet *et al.*, 2011).

En comparant les composés phénoliques de même nature obtenus à partir du jus de canneberge et de l'*hibiscus* sabdariffa, on est capable d'observer une nette différence en ce qui concerne leur potentiel chimio préventif. Ces différences peuvent être expliquées par le fait que la canneberge et l'hibiscus sabdariffa possède chacun un mélange de composés phénoliques différents en taille moléculaire, polarité, solubilité affectant la distribution et les propriétés de chacun d'eux. Il faut également tenir compte du fait que la transformation thermiques (séchage au soleil des calices de l'hibiscus sabdariffa) et chimiques (processus pour obtenir du jus concentré à partir de la canneberge) affectent également la distribution, la disponibilité biologique et les propriétés des composés phénoliques. Cependant malgré les différences existantes au niveau de leur efficacité, cette étude a permis de mettre en évidence leur potentiel chimiopréventif. Une approche intéressante serait de combiner ces deux substances afin de déterminer si le potentiel chimiopréventif diminuerait ou augmenterait. Et si augmentation il y a serait-elle due à un effet additif ou plutôt synergique.

Ce travail a également démontré que :

1) La dose de 2 ml du lait fermenté contenant les bactéries : *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} a pu réduire significativement la formation de lésions précancéreuses, d'induire l'activité de la glutathionne S-transférase et également de réduire l'activité enzymatique de la glucuronidase sur des rats traités au DMH (Chapitre **10**)
2) Dans les 2 ml de lait fermenté, l'effet anticancer est principalement dû à la présence des bactéries : *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD} (retrouvé au niveau du culot du lait fermenté). En effet, il s'agit de la seule fraction en plus d'avoir réduit de manière significative les lésions précancéreuse a été capable d'induire l'activité d'une enzyme détoxifiante du foie (en l'occurrence la QR) et de réduire celle de la β-glucuronidase (Chapitre **11**).

Parmi les avantages du lait BioK+, les plus intéressants sont : <u>la présence de 50 milliards de bactéries probiotiques actives prêtes à agir dès la mise en bouche</u>, <u>le fait que les bactéries fermentent dans le pot et continue une fois ingérée</u>.

L'une des implications de ces avantages est qu'une fois nourris avec le lait, 50 milliards de bactéries probiotiques se retrouvent au niveau de la flore intestinale des rats, bactéries capable de s'accumuler au niveau la paroi intestinale et croitre de façon exponentielle. La croissance de ces dernières rend difficile sinon impossible la croissance de bactéries pathogènes et/ou liées au développement du cancer colorectal par le simple fait qu'il faudrait compétitionner afin d'utiliser les ressources leur permettant de se multiplier mais également parce que la présence des probiotiques rend la paroi intestinale hostile à la croissance de certains pathogènes à cause des changements de pH.

La capacité de fermentation continuelle après ingestion des trois souches de probiotiques de la formulation BioK suggère une altération au niveau du métabolisme de la flore intestinale du pH et également de la concentration d'acides gras à courte chaîne. La production d'acide gras résulte de la fermentation de carbohydrates non-digérés par des bactéries, les principaux acides gras à courte chaîne sont l'acétate ou encore le butyrate. Ces derniers sont connus pour leur habilité à induire l'apoptose chez les cellules cancéreuses du colon, également comme source d'énergie pour les colonocytes. Certaines études suggèrent qu'ils jouent un rôle important dans le maintien de l'homéostasie au niveau de la paroi intestinale (Kahouli *et al.*, 2015, Puertollano *et al.*, 2014, Zeng *et al.*, 2014).

14. CONCLUSION

En résumé ce travail a pu démontrer que les composés phénoliques présents dans les canneberges et la fleur d'hibiscus possèdent des propriétés chimiopréventives. La capacité d'inhiber la prolifération des cellules cancéreuses, la modulation de l'activité de la quinone réductase et la capacité antioxydante. Aussi le lait fermenté BioK+ (et certaines de ses composantes) réduisent la formation de lésions prénéoplasiques, modulaient l'activité des enzymes détoxifiantes et fécales liées au développement du cancer. Cette étude a permis d'observer une possible synergie entre les composés phénoliques présents dans les canneberges et les calyces de l'hibiscus et les composants membranaires extraits de la biomasse contenant *L. acidophilus* CL1285^{MD}, *L. casei* LBC80R^{MD} et *L. rhamnosus* CLR2^{MD}. Cette étude a clairement démontré que ces substances peuvent être utilisées afin de diminuer le risque de développer le CCR mais aussi utiliser de concert avec les traitements de chimiothérapie et les médicaments existants sur le marché peut augmenter les chances de survie des patients déjà atteints de la maladie.

15. PERSPECTIVES FUTURES

Les résultats obtenus dans ce travail représente une ouverture sur la capacité chimiopréventive des substances testées, d'autres investigations sont nécessaires pour extrapoler les résultats obtenus *in vitro* dans des expériences *in vivo* pour finalement proposer une recherche clinique. Ci-dessous sont présentées quelques-unes des expériences qui pourraient être réalisées dans un futur proche.

1) L'identification des composés phénoliques présent dans les fractions les plus efficaces obtenus à partir du concentré de jus de canneberge et des calices de la fleur d'hibiscus par CHPL et ensuite déterminer si leur activité chimiopréventive est due à la présence de l'ensemble des molécules ou de préférence à une seule.
2) L'évaluation des capacités anti-inflammatoires et d'induire les réponses immunitaires des composés phénoliques et des extraits des composés membranaires d'abord *in vitro* et ensuite *in vivo* (après avoir préalablement déterminé le rapport entre la quantité de tisane ou de jus concentré de canneberges ingérée et quantité de phénols retrouvés au niveau du colon).
3) Démontrer comment le lait fermenté BioK+(et ses composantes) modifient la flore intestinale favorisant ainsi la diminution des lésions prénéoplasiques.
4) Déterminer *in vivo* si le lait fermenté BioK+ combiné soit avec le jus de canneberges soit ou une tisane faite à partir d'*Hibiscus sabdariffa* présentent des propriétés antinéoplasiques.

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16. REFERENCES

ACS (2014a) Cancer Facts & Figures 2014. in *American Cancer Society*).

CCSsAC (2014b) Colorectal Cancer Statistics. in *Candian Cancer Society*).

Abdulamir A, Hafidh R & Bakar F (2010) Molecular detection, quantification, and isolation of Streptococcus gallolyticus bacteria colonizing colorectal tumors: inflammation-driven potential of carcinogenesis via IL-1, COX-2, and IL-8. *Molecular Cancer* 9(1):249.

Abdulamir A, Hafidh R & Bakar F (2011) The association of Streptococcus bovis/gallolyticus with colorectal tumors: The nature and the underlying mechanisms of its etiological role. *Journal of Experimental & Clinical Cancer Research* 30(1):11.

Adaramoye O, Ogungbenro B, Anyaegbu O & Fafunso M (2008) Protective Effects of Extracts of Vernonia amygdalina, Hibiscus sabdariffa and Vitamin C against Radiation-induced Liver Damage in Rats. *Journal of Radiation Research* 49(2):123-131.

Aggarwal BB & Shishodia S (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology* 71(10):1397-1421.

Ahn J, Sinha R, Pei Z, Dominianni C, Wu J, Shi J, Goedert JJ, Hayes RB & Yang L (2013) Human Gut Microbiome and Risk for Colorectal Cancer. *Journal of the National Cancer Institute* 105(24):1907-1911.

Ajiboye TO, Salawu NA, Yakubu MT, Oladiji AT, Akanji MA & Okogun JI (2011) Antioxidant and drug detoxification potentials of Hibiscus sabdariffa anthocyanin extract. *Drug and Chemical Toxicology* 34(2):109-115.

Akindahunsi AA & Olaleye MT (2003) Toxicological investigation of aqueous-methanolic extract of the calyces of Hibiscus sabdariffa L. *Journal of Ethnopharmacology* 89(1):161-164.

Al-Sohaily S, Biankin A, Leong R, Kohonen-Corish M & Warusavitarne J (2012) Molecular pathways in colorectal cancer. *Journal of Gastroenterology and Hepatology* 27(9):1423-1431.

Alarcon-Aguilar FJ, Zamilpa A, Perez-Garcia MD, Almanza-Perez JC, Romero-Nuñez E, Campos-Sepulveda EA, Vazquez-Carrillo LI & Roman-Ramos R (2007) Effect of Hibiscus sabdariffa on obesity in MSG mice. *Journal of Ethnopharmacology* 114(1):66-71.

Alonso-Castro AJ, Domínguez F & García-Carrancá A (2013) Rutin Exerts Antitumor Effects on Nude Mice Bearing SW480 Tumor. *Archives of Medical Research* 44(5):346-351.

Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA & Jobin C (2012) Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science (New York, N.Y.)* 338(6103):120-123.

Asha A & Gayathri D (2012) Synergistic impact of Lactobacillus fermentum, Lactobacillus plantarum and vincristine on 1, 2-dimethylhydrazine-induced colorectal carcinogenesis in mice. *Experimental and therapeutic medicine* 3(6):1049-1054.

Attene-Ramos MS, Wagner ED, Gaskins HR & Plewa MJ (2007) Hydrogen Sulfide Induces Direct Radical-Associated DNA Damage. *Molecular Cancer Research* 5(5):455-459.

Aune D, Chan DSM, Lau R, Vieira R, Greenwood DC, Kampman E & Norat T (2011a) *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* http://www.bmj.com/bmj/343/bmj.d6617.full.pdf

Aune D, Lau R, Chan DSM, Vieira R, Greenwood DC, Kampman E & Norat T (2011b) Nonlinear Reduction in Risk for Colorectal Cancer by Fruit and Vegetable Intake Based on Meta-analysis of Prospective Studies. *Gastroenterology* 141(1):106-118.

Ávila M, Hidalgo M, Sánchez-Moreno C, Pelaez C, Requena T & Pascual-Teresa Sd (2009) Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. *Food Research International* 42(10):1453-1461.

Azcárate-Peril MA, Sikes M & Bruno-Bárcena JM (2011) *The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer?* G401-G424 p. http://ajpgi.physiology.org/ajpgi/301/3/G401.full.pdf

Baena R & Salinas P (2015) Diet and colorectal cancer. *Maturitas* 80(3):258-264.

Baffoni L, Gaggìa F, Gioia D & Biavati B (2012) Role of intestinal microbiota in colon cancer prevention. *Ann Microbiol* 62(1):15-30.

Bako I, Mabrouk M & Abubakar A (2009) Antioxidant effect of ethanolic seed extract of hibiscus sabdariffa linn (Malvaceae) alleviate the toxicity induced by chronic administration of sodium nitrate on some haematological parameters in wistars rats. *Advance Journal of Food Science and Technology* 1(1):39-42.

Baldwin C, Millette M, Oth D, Ruiz MT, Luquet FM & Lacroix M (2010) Probiotic Lactobacillus Acidophilus and L. Casei Mix Sensitize Colorectal Tumoral Cells to 5-Fluorouracil-Induced Apoptosis. *Nutrition and Cancer* 62(3):371-378.

Banerjee N, Kim H, Talcott ST, Turner ND, Byrne DH & Mertens-Talcott SU (2016) Plum polyphenols inhibit colorectal aberrant crypt foci formation in rats: potential role of the miR-143/protein kinase B/mammalian target of rapamycin axis. *Nutrition Research* 36(10):1105-1113.

Bashir A, Miskeen AY, Bhat A, Fazili KM & Ganai BA (2015) Fusobacterium nucleatum: an emerging bug in colorectal tumorigenesis. *European Journal of Cancer Prevention* 24(5):373-385.

Beausoleil M, Fortier N, Guénette S, L'Ecuyer A, Savoie M, Franco M, Lachaine J & Weiss K (2007) Effect of a fermented milk combining Lactobacillus acidophilus Cl1285 and Lactobacillus casei in the prevention of antibiotic-associated diarrhea: a randomized, double-blind, placebo-controlled trial. *Canadian Journal of Gastroenterology* 21(11):732-736.

Beltrán-Debón R, Alonso-Villaverde C, Aragonès G, Rodríguez-Medina I, Rull A, Micol V, Segura-Carretero A, Fernández-Gutiérrez A, Camps J & Joven J (2010) The

aqueous extract of Hibiscus sabdariffa calices modulates the production of monocyte chemoattractant protein-1 in humans. *Phytomedicine* 17(3–4):186-191.

Benson AM, Hunkeler MJ & Talalay P (1980) Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proceedings of the National Academy of Sciences* 77(9):5216-5220.

Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F & Gil A (2013) Cell-Free Culture Supernatant of *Bifidobacterium breve* CNCM I-4035 Decreases Pro-Inflammatory Cytokines in Human Dendritic Cells Challenged with *Salmonella typhi* through TLR Activation. *PLoS ONE* 8(3):e59370.

Bertkova I, Hijova E, Chmelarova A, Mojzisova G, Petrasova D, Strojny L, Bomba A & Zitnan R (2010) The effect of probiotic microorganisms and bioactive compounds on chemically induced carcinogenesis in rats. *Neoplasma* 57(5):422-428.

Bingham SA (1990) Mechanisms and experimental and epidemiological evidence relating dietary fibre (non-starch polysaccharides) and starch to protection against large bowel cancer. *Proceedings of the Nutrition Society* 49(02):153-171.

Bingham SA (2000) Diet and colorectal cancer prevention. *Biochem Soc Trans* 28(2):12-16.

Bird RP (1995) Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer letters* 93(1):55-71.

Bird RP & Good CK (2000) The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicology Letters* 112–113(0):395-402.

Boateng J, Verghese M, Shackelford L, Walker LT, Khatiwada J, Ogutu S, Williams DS, Jones J, Guyton M, Asiamah D, Henderson F, Grant L, DeBruce M, Johnson A, Washington S & Chawan CB (2007) Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. *Food and Chemical Toxicology* 45(5):725-732.

Bolade M, Oluwalana I & Ojo O (2009) Commercial practice of roselle (Hibiscus sabdariffa L.) beverage production: Optimization of hot water extraction and sweetness level. *World Journal of Agricultural Sciences* 5(1):126-131.

Boleij A, Dutilh BE, Kortman GAM, Roelofs R, Laarakkers CM, Engelke UF & Tjalsma H (2012a) Bacterial Responses to a Simulated Colon Tumor Microenvironment. *Molecular & Cellular Proteomics* 11(10):851-862.

Boleij A, Roelofs R, Danne C, Bellais S, Dramsi S, Kato I & Tjalsma H (2012b) Selective Antibody Response to Streptococcus gallolyticus Pilus Proteins in Colorectal Cancer Patients. *Cancer Prevention Research* 5(2):260-265.

Boleij A & Tjalsma H (2013) The itinerary of Streptococcus gallolyticus infection in patients with colonic malignant disease. *The Lancet Infectious Diseases* 13(8):719-724.

Bolling BW, Ji LL, Lee C-H & Parkin KL (2011) Dietary supplementation of ferulic acid and ferulic acid ethyl ester induces quinone reductase and glutathione-S-transferase in rats. *Food Chemistry* 124(1):1-6.

Bomser J, Madhavi D, Singletary K & Smith M (1996) In vitro anticancer activity of fruit extracts from Vaccinium species. *Planta medica* 62(03):212-216.

Borrás-Linares I, Herranz-López M, Barrajón-Catalán E, Arráez-Román D,
Gonzálezlvarez I, Bermejo M, Gutiérrez AF, Micol V & Segura-Carretero A

(2015) Permeability Study of Polyphenols Derived from a Phenolic-Enriched Hibiscus sabdariffa Extract by UHPLC-ESI-UHR-Qq-TOF-MS. *International journal of molecular sciences* 16(8):18396-18411.
Bosscher D, Breynaert A, Pieters L & Hermans N (2009) Food-based strategies to modulate the composition of the intestinal microbiota and their associated health effects. *J Physiol Pharmacol* 60(Suppl 6):5-11.
Brown EM, Gill CI, McDougall GJ & Stewart D (2012) Mechanisms underlying the anti-proliferative effects of berry components in in vitro models of colon cancer. *Current pharmaceutical biotechnology* 13(1):200-209.
Bunea A, Rugină D, Sconţa Z, Pop RM, Pintea A, Socaciu C, Tăbăran F, Grootaert C, Struijs K & VanCamp J (2013) Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells. *Phytochemistry* 95(0):436-444.
Caillet S, Côté J, Doyon G, Sylvain JF & Lacroix M (2011) Antioxidant and antiradical properties of cranberry juice and extracts. *Food Research International* 44(5):1408-1413.
Caillet S, Côté J, Sylvain J-F & Lacroix M (2012a) Antimicrobial effects of fractions from cranberry products on the growth of seven pathogenic bacteria. *Food Control* 23(2):419-428.
Caillet S, Lorenzo G, Côté J, Doyon G, Sylvain JF & Lacroix M (2012b) Cancer chemopreventive effect of fractions from cranberry products. *Food Research International* 45(1):320-330.
Caillet S, Salmiéri S & Lacroix M (2006) Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric method. *Food Chemistry* 95(1):1-8.
Carvajal-Zarrabal O, Barradas-Dermitz D, Orta-Flores Z, Hayward-Jones P, Nolasco-Hipolito C, Aguilar-Uscanga M, Miranda-Medina A & Bin Bujang K (2012) Hibiscus sabdariffa L., roselle calyx, from ethnobotany to pharmacology. *Journal of Experimental Pharmacology* 4:25-39.
Carvajal-Zarrabal O, Hayward-Jones P, Orta-Flores Z, Nolasco-Hipólito C, Barradas-Dermitz D, Aguilar-Uscanga M & Pedroza-Hernández M (2009) Effect of Hibiscus sabdariffa L. dried calyx ethanol extract on fat absorption-excretion, and body weight implication in rats. *BioMed Research International* 2009.
Carvalho-Wells AL, Helmolz K, Nodet C, Molzer C, Leonard C, McKevith B, Thielecke F, Jackson KG & Tuohy KM (2010) Determination of the in vivo prebiotic potential of a maize-based whole grain breakfast cereal: a human feeding study. *British Journal of Nutrition* 104(09):1353-1356.
Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA & Holt RA (2012) Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Research* 22(2):299-306.
Chan A & Giovannucci E (2010) Primary Prevention of Colorectal Cancer. *Gastroenterology* 138(6):2029-2043.e2010.
Chan DSM, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E & Norat T (2011) Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE* 6(6):e20456.

Chang J-H, Shim YY, Cha S-K, Reaney MJ & Chee KM (2012) Effect of Lactobacillus acidophilus KFRI342 on the development of chemically induced precancerous growths in the rat colon. *Journal of medical microbiology* 61(3):361-368.

Chang JH, Shim YY, Cha SK & Chee KM (2010) Probiotic characteristics of lactic acid bacteria isolated from kimchi. *Journal of Applied Microbiology* 109(1):220-230.

Chen Q, Liang Y, Zhu C, Yan Y & Pang G (2013) Effects of casein glycomacropeptide on the early development of primary colorectal cancer in rats. *Food Science and Human Wellness* 2(3–4):113-118.

Chen X-L & Kunsch C (2004) Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. *Current pharmaceutical design* 10(8):879-891.

Chipman JK (1982) Bile as a source of potential reactive metabolites. *Toxicology* 25(2–3):99-111.

Chiu CT, Hsuan SW, Lin HH, Hsu CC, Chou FP & Chen JH (2015) Hibiscus sabdariffa leaf polyphenolic extract induces human melanoma cell death, apoptosis, and autophagy. *Journal of food science* 80(3).

Choi SS, Kim Y, Han KS, You S, Oh S & Kim SH (2006) Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. *Letters in Applied Microbiology* 42(5):452-458.

Ciszek-Lenda M, Nowak B, Śróttek M, Gamian A & Marcinkiewicz J (2011) Immunoregulatory potential of exopolysaccharide from Lactobacillus rhamnosus KL37. Effects on the production of inflammatory mediators by mouse macrophages. *International Journal of Experimental Pathology* 92(6):382-391.

Coles BF & Kadlubar FF (2003) Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *Biofactors* 17(1):115-130.

Commane D, Hughes R, Shortt C & Rowland I (2005) The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 591(1–2):276-289.

Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR & Tuohy KM (2008) Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. *British Journal of Nutrition* 99(01):110-120.

Côté J, Caillet S, Doyon G, Sylvain JF & Lacroix M (2010) Bioactive Compounds in Cranberries and their Biological Properties. *Critical Reviews in Food Science and Nutrition* 50(7):666-679.

Cotterchio M, Boucher BA, Manno M, Gallinger S, Okey A & Harper P (2006) Dietary Phytoestrogen Intake Is Associated with Reduced Colorectal Cancer Risk. *The Journal of Nutrition* 136(12):3046-3053.

Cousin FJ, Jouan-Lanhouet S, Dimanche-Boitrel M-T, Corcos L & Jan G (2012) Milk Fermented by <italic>Propionibacterium freudenreichii</italic> Induces Apoptosis of HGT-1 Human Gastric Cancer Cells. *PLoS ONE* 7(3):e31892.

Cuevas-Ramos G, Petit CR, Marcq I, Boury M, Oswald E & Nougayrède J-P (2010) Escherichia coli induces DNA damage in vivo and triggers genomic instability in

mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* 107(25):11537-11542.
D'Aimmo MR, Modesto M & Biavati B (2007) Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology* 115(1):35-42.
Da-Costa-Rocha I, Bonnlaender B, Sievers H, Pischel I & Heinrich M (2014) Hibiscus sabdariffa L. – A phytochemical and pharmacological review. *Food Chemistry* 165(0):424-443.
da Silva Almeida AP, Avi CM, Barbisan LF, de Moura NA, Caetano BFR, Romualdo GR & Sivieri K (2015) Yacon (Smallanthus sonchifolius) and Lactobacillus acidophilus CRL 1014 reduce the early phases of colon carcinogenesis in male Wistar rats. *Food Research International* 74:48-54.
da Silva FL, Escribano-Bailón MT, Pérez Alonso JJ, Rivas-Gonzalo JC & Santos-Buelga C (2007) Anthocyanin pigments in strawberry. *LWT - Food Science and Technology* 40(2):374-382.
Dabek M, McCrae SI, Stevens VJ, Duncan SH & Louis P (2008) Distribution of β-glucosidase and β-glucuronidase activity and of β-glucuronidase gene gus in human colonic bacteria. *FEMS Microbiology Ecology* 66(3):487-495.
De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G & Verstraete W (2000) Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity. *Applied microbiology and biotechnology* 53(6):709-714.
de Moreno de LeBlanc A & Perdigón G (2005) Reduction of beta-glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. *Biocell* 29:15-24.
De Preter V, Raemen H, Cloetens L, Houben E, Rutgeerts P & Verbeke K (2007) Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. *Eur J Clin Nutr* 62(2):225-231.
Desrouillères K, Millette M, Vu KD, Touja R & Lacroix M (2015) Cancer preventive effects of a specific probiotic fermented milk containing Lactobacillus acidophilus CL1285, L. casei LBC80R and L. rhamnosus CLR2 on male F344 rats treated with 1,2-dimethylhydrazine. *Journal of Functional Foods* 17(0):816-827.
Desrouillères K., Millette M., Dang Khanh V. & M. L (2015) Anticancer properties of HPLC fractions from a concentrated cranberry juice and cell wall extracted from a probiotic biomass containing Lactobacillus acidophilus CL1285, L. casei LBC80R and L. rhamnosus CLR2. *Journal of Functionnal Food* <submitted manuscript>.
Dolara P, Luceri C, Filippo CD, Femia AP, Giovannelli L, Caderni G, Cecchini C, Silvi S, Orpianesi C & Cresci A (2005) Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 591(1–2):237-246.
Dove WF, Clipson L, Gould KA, Luongo C, Marshall DJ, Moser AR, Newton MA & Jacoby RF (1997) Intestinal Neoplasia in the ApcMin Mouse: Independence from the Microbial and Natural Killer (beige Locus) Status. *Cancer Research* 57(5):812-814.

Ebringer L, Ferenčík M & Krajčovič J (2008) Beneficial health effects of milk and fermented dairy products — Review. *Folia Microbiol* 53(5):378-394.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE & Relman DA (2005) Diversity of the Human Intestinal Microbial Flora. *Science (New York, N.Y.)* 308(5728):1635-1638.

Escamilla J, Lane MA & Maitin V (2012) Cell-Free Supernatants from Probiotic Lactobacillus casei and Lactobacillus rhamnosus GG Decrease Colon Cancer Cell Invasion In Vitro. *Nutrition and Cancer* 64(6):871-878.

Esselen M, Fritz J, Hutter M & Marko D (2009) Delphinidin Modulates the DNA-Damaging Properties of Topoisomerase II Poisons. *Chemical Research in Toxicology* 22(3):554-564.

Evan GI & Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* 411(6835):342-348.

Ewaschuk JB, Walker JW, Diaz H & Madsen KL (2006) Bioproduction of Conjugated Linoleic Acid by Probiotic Bacteria Occurs In Vitro and In Vivo in Mice. *The Journal of Nutrition* 136(6):1483-1487.

Fahey JW & Stephenson KK (2002) Pinostrobin from Honey and Thai Ginger (Boesenbergia pandurata): A Potent Flavonoid Inducer of Mammalian Phase 2 Chemoprotective and Antioxidant Enzymes. *Journal of Agricultural and Food Chemistry* 50(25):7472-7476.

Fakeye TO, Pal A, Bawankule DU & Khanuja SPS (2008) Immunomodulatory effect of extracts of Hibiscus sabdariffa L. (Family Malvaceae) in a mouse model. *Phytotherapy Research* 22(5):664-668.

Fearon ER & Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61(5):759-767.

Ferguson LR (2010) Meat and cancer. *Meat science* 84(2):308-313.

Flis S, Jastrzebski Z, Namiesnik J, Arancibia-Avila P, Toledo F, Leontowicz H, Leontowicz M, Suhaj M, Trakhtenberg S & Gorinstein S (2012) Evaluation of inhibition of cancer cell proliferation in vitro with different berries and correlation with their antioxidant levels by advanced analytical methods. *Journal of Pharmaceutical and Biomedical Analysis* 62(0):68-78.

Forssten SD, Sindelar CW & Ouwehand AC (2011) Probiotics from an industrial perspective. *Anaerobe* 17(6):410-413.

Fotiadis CI, Stoidis CN, Spyropoulos BG & Zografos ED (2008) Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer. *World journal of gastroenterology : WJG* 14(42):6453-6457.

Fung TT & Brown LS (2013) Dietary Patterns and the Risk of Colorectal Cancer. *Current nutrition reports* 2(1):48-55.

Galati G & O'Brien PJ (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine* 37(3):287-303.

Gamallat Y, Meyiah A, Kuugbee ED, Hago AM, Chiwala G, Awadasseid A, Bamba D, Zhang X, Shang X, Luo F & Xin Y (2016) Lactobacillus rhamnosus induced epithelial cell apoptosis, ameliorates inflammation and prevents colon cancer development in an animal model. *Biomedicine & Pharmacotherapy* 83:536-541.

Gangwar M, Gautam MK, Sharma AK, Tripathi YB, Goel RK & Nath G (2014) Antioxidant Capacity and Radical Scavenging Effect of Polyphenol Rich Mallotus philippenensis Fruit Extract on Human Erythrocytes: An In Vitro Study. *The Scientific World Journal* 2014:12.

Gao XW, Mubasher M, Fang CY, Reifer C & Miller LE (2010) Dose–Response Efficacy of a Proprietary Probiotic Formula of Lactobacillus acidophilus CL1285 and Lactobacillus casei LBC80R for Antibiotic-Associated Diarrhea and Clostridium difficile-Associated Diarrhea Prophylaxis in Adult Patients. *The American journal of gastroenterology.* 105(7):1636-1641.

Gianotti L, Morelli L, Galbiati F, Rocchetti S, Coppola S, Beneduce A, Gilardini C, Zonenschain D, Nespoli A & Braga M (2010) A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients. *World Journal of Gastroenterology : WJG* 16(2):167-175.

Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA & Willett WC (1995) Alcohol, Low-Methionine-Low-Folate Diets, and Risk of Colon Cancer in Men. *Journal of the National Cancer Institute* 87(4):265-273.

Globocan (2012) *http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx*.), (Consulté le février)

Gobbetti M, Cagno RD & De Angelis M (2010) Functional Microorganisms for Functional Food Quality. *Critical Reviews in Food Science and Nutrition* 50(8):716-727.

Goel A, Arnold CN, Niedzwiecki D, Chang DK, Ricciardiello L, Carethers JM, Dowell JM, Wasserman L, Compton C, Mayer RJ, Bertagnolli MM & Boland CR (2003) Characterization of Sporadic Colon Cancer by Patterns of Genomic Instability. *Cancer Research* 63(7):1608-1614.

Gogineni V, Morrow L & Malesker M (2013) Probiotics: mechanisms of action and clinical applications. *Journal of Probiotics and Health* 1:101.

Goldin BR & Gorbach SL (1984) The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *The American Journal of Clinical Nutrition* 39(5):756-761.

Gonzalez CA, Lujan-Barroso L, Bueno-de-Mesquita HB, Jenab M, Duell EJ, Agudo A, Tjønneland A, Boutron-Ruault MC, Clavel-Chapelon F, Touillaud M, Teucher B, Kaaks R, Boeing H, Steffen A, Trichopoulou A, Roukos D, Karapetyan T, Palli D, Tagliabue G, Mattiello A, Tumino R, Ricceri F, Siersema PD, Numans ME, Peeters PPH, Parr CL, Skeie G, Lund E, Quirós JR, Sánchez-Cantalejo E, Navarro C, Barricarte A, Dorronsoro M, Ehrnström R, Regner S, Khaw K-T, Wareham N, Key TJ, Crowe FL, Blaker H, Romieu I & Riboli E (2012) Fruit and vegetable intake and the risk of gastric adenocarcinoma: A reanalysis of the european prospective investigation into cancer and nutrition (EPIC-EURGAST) study after a longer follow-up. *International Journal of Cancer* 131(12):2910-2919.

Goodwin AC, Shields CED, Wu S, Huso DL, Wu X, Murray-Stewart TR, Hacker-Prietz A, Rabizadeh S, Woster PM, Sears CL & Casero RA (2011) Polyamine catabolism contributes to enterotoxigenic Bacteroides fragilis-induced colon tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 108(37):15354-15359.

Gordon MA, Zhang W, Yang D, Iqbal S, El-Khouiery A, Nagashima F, Lurje G, Labonte M, Wilson P, Sherrod A, Ladner RD & Lenz H-J (2010) Gender-specific genomic profiling in metastatic colorectal cancer patients treated with 5-fluorouracil and oxaliplatin. *Pharmacogenomics* 12(1):27-39.
Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, Newmark HL, Giovannucci E, Wei M & Holick MF (2007) Optimal Vitamin D Status for Colorectal Cancer Prevention: A Quantitative Meta Analysis. *American Journal of Preventive Medicine* 32(3):210-216.
Goyal P (2012) Cancer chemoprevention by natural products: current & future prospects. *J Integr Oncol* 1:e101.
Graf D, Di Cagno R, Fåk F, Flint HJ, Nyman M, Saarela M & Watzl B (2015) Contribution of diet to the composition of the human gut microbiota. *Microbial Ecology in Health and Disease* 26:10.3402/mehd.v3426.26164.
Gupta AK, Pretlow TP & Schoen RE (2007) Aberrant Crypt Foci: What We Know and What We Need to Know. *Clinical Gastroenterology and Hepatology* 5(5):526-533.
Haggar FA & Boushey RP (2009) Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in colon and rectal surgery* 22(4):191-197.
Hanahan D & Weinberg RA (2000) The Hallmarks of Cancer. *Cell* 100(1):57-70.
Hatakka K, Holma R, El-Nezami H, Suomalainen T, Kuisma M, Saxelin M, Poussa T, Mykkänen H & Korpela R (2008) The influence of Lactobacillus rhamnosus LC705 together with Propionibacterium freudenreichii ssp. shermanii JS on potentially carcinogenic bacterial activity in human colon. *International Journal of Food Microbiology* 128(2):406-410.
Hawk ET & Levin B (2005) Colorectal Cancer Prevention. *Journal of Clinical Oncology* 23(2):378-391.
Heinonen M (2007) Antioxidant activity and antimicrobial effect of berry phenolics – a Finnish perspective. *Molecular Nutrition & Food Research* 51(6):684-691.
Hernandez-Mendoza A, Garcia H & Steele J (2009) Screening of Lactobacillus casei strains for their ability to bind aflatoxin B 1. *Food and chemical toxicology* 47(6):1064-1068.
Hertog MGL, Hollman PCH, Katan MB & Kromhout D (1993) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutrition and Cancer* 20(1):21-29.
Hijová E, Kuzma J, Strojný L, Bomba A, Bertková I, Chmelárová A, Hertelyová Z, Kuliková L, Štofilová J & Ambro Ľ (2016) Effect of Lactobacillus plantarum LS/07 on Intestinal Bacterial Enzyme Activities in the Prevention of Cancer, Atherosclerosis and Dysbiosis. in *Acta Veterinaria*), p 294.
Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F & Wu GD (2009) High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. *Gastroenterology* 137(5):1716-1724.e1712.
Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC & Sanders ME (2014) Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 11(8):506-514.

Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, Kinoshita T, Saito N, Ochiai A, Tomita M, Esumi H & Soga T (2009) Quantitative Metabolome Profiling of Colon and Stomach Cancer Microenvironment by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. *Cancer Research* 69(11):4918-4925.

Hirayama K & Rafter J (2000) The role of probiotic bacteria in cancer prevention. *Microbes and infection* 2(6):681-686.

Hollman PCH & Katan MB (1999) Dietary Flavonoids: Intake, Health Effects and Bioavailability. *Food and Chemical Toxicology* 37(9–10):937-942.

Hong WK & Sporn MB (1997) Recent Advances in Chemoprevention of Cancer. *Science* 278(5340):1073-1077.

Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, Ryder JJ, Hall WL & Cassidy A (2008) Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition* 88(1):38-50.

Hope ME, Hold GL, Kain R & El-Omar EM (2005) Sporadic colorectal cancer – role of the commensal microbiota. *FEMS Microbiology Letters* 244(1):1-7.

Horning EC, Thenot JP & Helton ED (1978) Toxic agents resulting from the oxidative metabolism of steroid hormones and drugs. *Journal of Toxicology and Environmental Health* 4(2-3):341-361.

Hou D-X, Tong X, Terahara N, Luo D & Fujii M (2005) Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Archives of Biochemistry and Biophysics* 440(1):101-109.

Howell A, Botto H, Combescure C, Blanc-Potard A-B, Gausa L, Matsumoto T, Tenke P, Sotto A & Lavigne J-P (2010) Dosage effect on uropathogenic Escherichia coli anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study. *BMC Infectious Diseases* 10(1):94.

Ibrahim F, Ruvio S, Granlund L, Salminen S, Viitanen M & Ouwehand AC (2010) *Probiotics and immunosenescence: cheese as a carrier.* 53-59 p. http://femsim.oxfordjournals.org/femsim/59/1/53.full.pdf

J Tarraga Lopez P, Solera Albero J & Antonio Rodriguez-Montes J (2013) Is it Possible to Reduce the Incident of Colorectal Cancer by Modifying Diet and Lifestyle? *Current Cancer Therapy Reviews* 9(3):157-163.

Jaganathan SK, Mazumdar A, Mondhe D & Mandal M (2011) Apoptotic effect of eugenol in human colon cancer cell lines. *Cell Biology International* 35(6):607-615.

Janicke B, Önning G & Oredsson SM (2005) Differential Effects of Ferulic Acid and p-Coumaric Acid on S Phase Distribution and Length of S Phase in the Human Colonic Cell Line Caco-2. *Journal of Agricultural and Food Chemistry* 53(17):6658-6665.

Jemal A, Bray F, Center MM, Ferlay J, Ward E & Forman D (2011) Global cancer statistics. *CA: A Cancer Journal for Clinicians* 61(2):69-90.

Jemal A, Siegel R, Xu J & Ward E (2010) Cancer Statistics, 2010. *CA: A Cancer Journal for Clinicians* 60(5):277-300.

Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA & Giusti MM (2008) Structure−Function Relationships of Anthocyanins from Various Anthocyanin-Rich Extracts on the Inhibition of Colon Cancer Cell Growth. *Journal of Agricultural and Food Chemistry* 56(20):9391-9398.

Jung E, Kim Y & Joo N (2013) Physicochemical properties and antimicrobial activity of Roselle (Hibiscus sabdariffa L.). *Journal of the Science of Food and Agriculture $V 93* (15):3769-3776.

Kado S, Uchida K, Funabashi H, Iwata S, Nagata Y, Ando M, Onoue M, Matsuoka Y, Ohwaki M & Morotomi M (2001) Intestinal Microflora Are Necessary for Development of Spontaneous Adenocarcinoma of the Large Intestine in T-Cell Receptor β Chain and p53 Double-Knockout Mice. *Cancer Research* 61(6):2395-2398.

Kahouli I, Handiri N, Malhotra M, Riahi A & Alaoui-Jamali M (2016) Characterization of L. reuteri NCIMB 701359 Probiotic Features for Potential Use as a Colorectal Cancer Biotherapeutic by Identifying Fatty Acid Profile and Anti-Proliferative Action against Colorectal Cancer Cells. *Drug Des* 5(131):2169-0138.100013.

Kahouli I, Malhotra M, Tomaro-Duchesneau C, Rodes LS, Aloui-Jamali MA & Prakash S (2015) Identification of Lactobacillus Fermentum Strains with Potential against Colorectal Cancer by Characterizing Short Chain Fatty Acids Production, Anti-Proliferative Activity and Survival in an Intestinal Fluid: In Vitro Analysis. *Journal of Bioanalysis & Biomedicine* 7(4):104.

Kang Y-H & Pezzuto JM (2004a) Induction of Quinone Reductase as a Primary Screen for Natural Product Anticarcinogens. *Methods in Enzymology*, Helmut S & Lester P (Édit.) Academic Press, Vol Volume 382. p 380-414.

Kang YH & Pezzuto JM (2004b) Induction of Quinone Reductase as a Primary Screen for Natural Product Anticarcinogens. *Methods in Enzymology* 382:380-414.

Karp G (2009) *Cell and Molecular Biology: Concepts and Experiments.* John Wiley & Sons. http://books.google.ca/books?id=arRGYE0GxRQC

Kawabata K, Yamamoto T, Hara A, Shimizu M, Yamada Y, Matsunaga K, Tanaka T & Mori H (2000) Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Letters* 157(1):15-21.

Khan R & Sultana S (2011) Farnesol attenuates 1,2-dimethylhydrazine induced oxidative stress, inflammation and apoptotic responses in the colon of Wistar rats. *Chemico-Biological Interactions* 192(3):193-200.

Kiernan J (2001) Classification and naming of dyes, stains and fluorochromes. *Biotechnic & histochemistry* 76(5-6):261-278.

Kim J, Woo H, Kim Y-S & Lee H (2002) Screening for antiproliferative effects of cellular components from lactic acid bacteria against human cancer cell lines. *Biotechnol Lett* 24(17):1431-1436.

Kim SS, Oh OJ, Min H-Y, Park E-J, Kim Y, Park HJ, Nam Han Y & Lee SK (2003) Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells. *Life Sciences* 73(3):337-348.

Kim Y, Lee D, Kim D, Cho J, Yang J, Chung M, Kim K & Ha N (2008) Inhibition of proliferation in colon cancer cell lines and harmful enzyme activity of colon bacteria by Bifidobacterium adolescentis SPM0212. *Archives of pharmacal research* 31(4):468-473.

Kinzler KW & Vogelstein B (1996) Lessons from Hereditary Colorectal Cancer. *Cell* 87(2):159-170.

Klein RS, Recco RA, Catalano MT, Edberg SC, Casey JI & Steigbigel NH (1977) Association of Streptococcus bovis with Carcinoma of the Colon. *New England Journal of Medicine* 297(15):800-802.

Kolling Y, Salva S, Villena J, Marranzino G & Alvarez S (2015) Non-viable immunobiotic Lactobacillus rhamnosus CRL1505 and its peptidoglycan improve systemic and respiratory innate immune response during recovery of immunocompromised-malnourished mice. *International immunopharmacology*.

Kondo M, MacKinnon SL, Craft CC, Matchett MD, Hurta RAR & Neto CC (2011) Ursolic acid and its esters: occurrence in cranberries and other Vaccinium fruit and effects on matrix metalloproteinase activity in DU145 prostate tumor cells. *Journal of the Science of Food and Agriculture* 91(5):789-796.

Korhonen H & Pihlanto A (2003) Food-derived bioactive peptides-opportunities for designing future foods. *Current pharmaceutical design* 9(16):1297-1308.

Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M & Garrett WS (2013) Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. *Cell Host & Microbe* 14(2):207-215.

Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS & Meyerson M (2012) Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Research* 22(2):292-298.

Koushik A, Hunter DJ, Spiegelman D, Beeson WL, van den Brandt PA, Buring JE, Calle EE, Cho E, Fraser GE, Freudenheim JL, Fuchs CS, Giovannucci EL, Goldbohm RA, Harnack L, Jacobs DR, Kato I, Krogh V, Larsson SC, Leitzmann MF, Marshall JR, McCullough ML, Miller AB, Pietinen P, Rohan TE, Schatzkin A, Sieri S, Virtanen MJ, Wolk A, Zeleniuch-Jacquotte A, Zhang SM & Smith-Warner SA (2007) Fruits, Vegetables, and Colon Cancer Risk in a Pooled Analysis of 14 Cohort Studies. *Journal of the National Cancer Institute* 99(19):1471-1483.

Kumar RS, Kanmani P, Yuvaraj N, Paari KA, Pattukumar V, Thirunavukkarasu C & Arul V (2012) Lactobacillus plantarum AS1 Isolated from South Indian Fermented Food Kallappam Suppress 1,2-Dimethyl Hydrazine (DMH)-Induced Colorectal Cancer in Male Wistar Rats. *Appl Biochem Biotechnol* 166(3):620-631.

Kuugbee ED, Shang X, Gamallat Y, Bamba D, Awadasseid A, Suliman MA, Zang S, Ma Y, Chiwala G, Xin Y & Shang D (2016) Structural Change in Microbiota by a Probiotic Cocktail Enhances the Gut Barrier and Reduces Cancer via TLR2 Signaling in a Rat Model of Colon Cancer. *Digest Dis Sci* 61(10):2908-2920.

Lacombe A, Wu VCH, Tyler S & Edwards K (2010) Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7. *International Journal of Food Microbiology* 139(1–2):102-107.

Lafarga T & Hayes M (2014) Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients. *Meat science* 98(2):227-239.

Lahtinen § S, Haskard¶ C, Ouwehand § A, Salminen § S & Ahokas J (2004) Binding of aflatoxin B1 to cell wall components of Lactobacillus rhamnosus strain GG. *Food additives and contaminants* 21(2):158-164.

Lala G, Malik M, Zhao C, He J, Kwon Y, Giusti MM & Magnuson BA (2006) Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats. *Nutrition and cancer* 54(1):84-93.

Larsson SC & Wolk A (2006) Meat consumption and risk of colorectal cancer: A meta-analysis of prospective studies. *International Journal of Cancer* 119(11):2657-2664.

Lee HC, Jenner AM, Low CS & Lee YK (2006a) Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in microbiology* 157(9):876-884.

Lee JW, Kim EH, Yim IB & Joo HG (2004) Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *Journal of Veterinary Science* 5(1):41-48.

Lee W-J, Wu L-F, Chen W-K, Wang C-J & Tseng T-H (2006b) Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K–Akt pathways. *Chemico-Biological Interactions* 160(2):123-133.

Leslie A, Carey FA, Pratt NR & Steele RJC (2002) The colorectal adenoma–carcinoma sequence. *British Journal of Surgery* 89(7):845-860.

Li F, Hullar MAJ, Schwarz Y & Lampe JW (2009a) Human Gut Bacterial Communities Are Altered by Addition of Cruciferous Vegetables to a Controlled Fruit- and Vegetable-Free Diet. *The Journal of Nutrition* 139(9):1685-1691.

Li J, Deng Y, Yuan C, Pan L, Chai H, Keller WJ & Kinghorn AD (2012) Antioxidant and Quinone Reductase-Inducing Constituents of Black Chokeberry (Aronia melanocarpa) Fruits. *Journal of Agricultural and Food Chemistry* 60(46):11551-11559.

Li W, Du B, Wang T, Wang S & Zhang J (2009b) Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. *Chemico-Biological Interactions* 177(2):121-127.

Li X, Xu X, Ji T, Liu Z, Gu M, Hoang BH & Zi X (2014) Dietary feeding of flavokawain A, a Kava chalcone, exhibits a satisfactory safety profile and its association with enhancement of phase II enzymes in mice. *Toxicology Reports* 1:2-11.

Lim S-O, Gu J-M, Kim MS, Kim H-S, Park YN, Park CK, Cho JW, Park YM & Jung G (2008) Epigenetic Changes Induced by Reactive Oxygen Species in Hepatocellular Carcinoma: Methylation of the E-cadherin Promoter. *Gastroenterology* 135(6):2128-2140.e2128.

Lin H-H, Chen J-H, Kuo W-H & Wang C-J (2007) Chemopreventive properties of Hibiscus sabdariffa L. on human gastric carcinoma cells through apoptosis induction and JNK/p38 MAPK signaling activation. *Chemico-Biological Interactions* 165(1):59-75.

Lipkin M, Reddy B, Newmark H & Lamprecht SA (1999) DIETARY FACTORS IN HUMAN COLORECTAL CANCER. *Annual Review of Nutrition* 19(1):545-586.
Lippert E, Ruemmele P, Obermeier F, Goelder S, Kunst C, Rogler G, Dunger N, Messmann H, Hartmann A & Endlicher E (2017) Anthocyanins Prevent Colorectal Cancer Development in a Mouse Model. *Digestion* 95(4):275-280.
Liu C-T, Chu F-J, Chou C-C & Yu R-C (2011) Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from Lactobacillus casei 01. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 721(2):157-162.
López de las Hazas M-C, Mosele JI, Macià A, Ludwig IA & Motilva M-J (2016) Exploring the Colonic Metabolism of Grape and Strawberry Anthocyanins and Their in Vitro Apoptotic Effects in HT-29 Colon Cancer Cells. *Journal of Agricultural and Food Chemistry* 10.1021/acs.jafc.6b04096.
Lourens-Hattingh A & Viljoen BC (2001) Yogurt as probiotic carrier food. *International Dairy Journal* 11(1–2):1-17.
Ma Q, Hoper M, Anderson N & Rowlands BJ (1996) Effect of supplemental L-arginine in a chemical-induced model of colorectal cancer. *World journal of surgery* 20(8):1087-1091.
Magalhaes B, Peleteiro B & Lunet N (2012) Dietary patterns and colorectal cancer: systematic review and meta-analysis. *European journal of cancer prevention* 21(1):15-23.
Malago J & Koninkx JJG (2011) Modulation of Immune System by Probiotics to Protect Against Enteric Disorders. *Probiotic Bacteria and Enteric Infections*, Malago JJ, Koninkx JFJG & Marinsek-Logar R (Édit.) Springer Netherlands, 10.1007/978-94-007-0386-5_12. p 263-286.
Malik M, Zhao C, Schoene N, Guisti MM, Moyer MP & Magnuson BA (2003) Anthocyanin-Rich Extract From Aronia meloncarpa E. Induces a Cell Cycle Block in Colon Cancer but Not Normal Colonic Cells. *Nutrition and Cancer* 46(2):186-196.
Manceau G, Laurent-Puig P & Blons H (2012) Biologie moléculaire et prise en charge des cancers colorectaux. *Oncologie* 14(1):45-51.
Markowitz SD & Bertagnolli MM (2009) Molecular Basis of Colorectal Cancer. *New England Journal of Medicine* 361(25):2449-2460.
Martinez I, Lattimer JM, Hubach KL, Case JA, Yang J, Weber CG, Louk JA, Rose DJ, Kyureghian G, Peterson DA, Haub MD & Walter J (2013) Gut microbiome composition is linked to whole grain-induced immunological improvements. *ISME J* 7(2):269-280.
McDonough FE, Hitchins AD, Wong NP, Wells P & Bodwell CE (1987) Modification of sweet acidophilus milk to improve utilization by lactose-intolerant persons. *The American Journal of Clinical Nutrition* 45(3):570-574.
McKay DL, Chen C-YO, Saltzman E & Blumberg JB (2010a) Hibiscus Sabdariffa L. Tea (Tisane) Lowers Blood Pressure in Prehypertensive and Mildly Hypertensive Adults. *The Journal of Nutrition* 140(2):298-303.
McKay DL, Chen CO, Saltzman E & Blumberg JB (2010b) Hibiscus sabdariffa L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. *The Journal of nutrition* 140(2):298-303.

Medrano M, Racedo SM, Rolny IS, Abraham AG & Pérez PF (2011) Oral Administration of Kefiran Induces Changes in the Balance of Immune Cells in a Murine Model. *Journal of Agricultural and Food Chemistry* 59(10):5299-5304.

Mensah JK & Golomeke D (2015) Antioxidant and antimicrobial activities of the extracts of the Calyx of Hibiscus Sabdariffa Linn. *Current Science Perspectives* 1(2):69-76.

Mentor-Marcel RA, Bobe G, Sardo C, Wang L-S, Kuo C-T, Stoner G & Colburn NH (2012) Plasma Cytokines as Potential Response Indicators to Dietary Freeze-Dried Black Raspberries in Colorectal Cancer Patients. *Nutrition and Cancer* 64(6):820-825.

Meraiyebu A, Olaniyan O, Eneze C, Anjorin Y & Dare J (2013) Anti-inflammatory Activity of Methanolic Extract of Hibiscus sabdariffa on Carrageenan Induced Inflammation in Wistar Rat.

Miller PE, Lesko SM, Muscat JE, Lazarus P & Hartman TJ (2010) Dietary Patterns and Colorectal Adenoma and Cancer Risk: A Review of the Epidemiological Evidence. *Nutrition and Cancer* 62(4):413-424.

Millette M, Luquet FM & Lacroix M (2007) In vitro growth control of selected pathogens by Lactobacillus acidophilus- and Lactobacillus casei-fermented milk. *Letters in Applied Microbiology* 44(3):314-319.

Millette M, Luquet FM, Ruiz MT & Lacroix M (2008) Characterization of probiotic properties of Lactobacillus strains. *Dairy Science and Technology* 88(6):695-705.

Mohd-Esa N, Hern FS, Ismail A & Yee CL (2010) Antioxidant activity in different parts of roselle (Hibiscus sabdariffa L.) extracts and potential exploitation of the seeds. *Food Chemistry* 122(4):1055-1060.

Molan AL, Lila MA, Mawson J & De S (2009) In vitro and in vivo evaluation of the prebiotic activity of water-soluble blueberry extracts. *World Journal of Microbiology and Biotechnology* 25(7):1243-1249.

Monroy-Ortíz C & España PC (2007) *Plantas medicinales utilizadas en el estado de Morelos.* Universidad Autónoma del Estado de Morelos. http://books.google.ca/books?id=0VKdQwAACAAJ

Mori H, Hata K, Yamada Y, Kuno T & Hara A (2005) Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chemico-Biological Interactions* 155(1–2):1-9.

Murad LD, Soares NdCP, Brand C, Monteiro MC & Teodoro AJ (2015) Effects of Caffeic and 5-Caffeoylquinic Acids on Cell Viability and Cellular Uptake in Human Colon Adenocarcinoma Cells. *Nutrition and Cancer* 67(3):532-542.

Murphy BT, MacKinnon SL, Yan X, Hammond GB, Vaisberg AJ & Neto CC (2003) Identification of Triterpene Hydroxycinnamates with in Vitro Antitumor Activity from Whole Cranberry Fruit (Vaccinium macrocarpon). *Journal of Agricultural and Food Chemistry* 51(12):3541-3545.

Muthu R & Vaiyapuri M (2013) Synergistic and individual effects of umbelliferone with 5-fluorouracil on tumor markers and antioxidant status of rat treated with 1,2-dimethylhydrazine. *Biomedicine & Aging Pathology* 3(4):219-227.

Nakamura J, Kubota Y, Miyaoka M, Saitoh T, Mizuno F & Benno Y (2002) Comparison of four microbial enzymes in Clostridia and Bacteroides isolated from human feces. *Microbiology and immunology* 46(7):487-490.

Nanno M, Kato I, Kobayashi T & Shida K (2011) Biological effects of probiotics: what impact does Lactobacillus casei shirota have on us? *International journal of immunopathology and pharmacology* 24(1 Suppl):45S.

Narushima S, Sakata T, Hioki K, Itoh T, Nomura T & Itoh K (2010) Inhibitory Effect of Yogurt on Aberrant Crypt Foci Formation in the Rat Colon and Colorectal Tumorigenesis in RasH2 Mice. *Experimental Animals* 59(4):487-494.

Neto C, Liberty A, Frade S, Tata A, Ferreira T, Song M, Wu X & Xiao H (2014) Inhibition of Colon Cancer by Polyphenols from Whole Cranberry.

Neto CC, Krueger CG, Lamoureaux TL, Kondo M, Vaisberg AJ, Hurta RAR, Curtis S, Matchett MD, Yeung H, Sweeney MI & Reed JD (2006) MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit (Vaccinium macrocarpon) that inhibit tumor cell growth and matrix metalloproteinase expression in vitro. *Journal of the Science of Food and Agriculture* 86(1):18-25.

Ng K-Y & Griffiths M (2002) Enhancement of macrophage cytokine release by cell-free fractions of fermented milk. *Milchwissenschaft* 57(2):66-70.

Nile SH & Park SW (2014) Edible berries: Bioactive components and their effect on human health. *Nutrition* 30(2):134-144.

Nishimura-Uemura J, Kitazawa H, Kawai Y, Itoh T, Oda M & Saito T (2003) Functional alteration of murine macrophages stimulated with extracellular polysaccharides from Lactobacillus delbrueckii ssp. bulgaricus OLL1073R-1. *Food Microbiology* 20(3):267-273.

Norat T, Bingham S, Ferrari P, Slimani N, Jenab M, Mazuir M, Overvad K, Olsen A, Tjønneland A, Clavel F, Boutron-Ruault M-C, Kesse E, Boeing H, Bergmann MM, Nieters A, Linseisen J, Trichopoulou A, Trichopoulos D, Tountas Y, Berrino F, Palli D, Panico S, Tumino R, Vineis P, Bueno-de-Mesquita HB, Peeters PHM, Engeset D, Lund E, Skeie G, Ardanaz E, González C, Navarro C, Quirós JR, Sanchez M-J, Berglund G, Mattisson I, Hallmans G, Palmqvist R, Day NE, Khaw K-T, Key TJ, San Joaquin M, Hémon B, Saracci R, Kaaks R & Riboli E (2005) Meat, Fish, and Colorectal Cancer Risk: The European Prospective Investigation into Cancer and Nutrition. *Journal of the National Cancer Institute* 97(12):906-916.

Nougayrède J-P, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, Buchrieser C, Hacker J, Dobrindt U & Oswald E (2006) Escherichia coli Induces DNA Double-Strand Breaks in Eukaryotic Cells. *Science* 313(5788):848-851.

O'Neill IK, Loktionov A, Manson MM, Ball H, Bandaletova T & Bingham SA (1997) Comparison of metabolic effects of vegetables and teas with colorectal proliferation and with tumour development in DMH-treated F344 rats. *Cancer Letters* 114(1–2):287-291.

Ohara T, Yoshino K & Kitajima M (2010) Possibility of preventing colorectal carcinogenesis with probiotics. *Hepatogastroenterology* 57(104):1411-1415.

Okoko T & Ere D (2012) Hibiscus sabdariffa extractivities on cadmium—mediated alterations of human U937 cell viability and activation. *Asian Pacific Journal of Tropical Medicine* 5(1):33-36.

OMS (2015) http://www.who.int/mediacentre/factsheets/fs297/en/.),

Onoue M, Kado S, Sakaitani Y, Uchida K & Morotomi M (1997) Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. *Cancer Letters* 113(1–2):179-186.

Orlando A, Linsalata M & Russo F (2016) Antiproliferative effects on colon adenocarcinoma cells induced by co-administration of vitamin K1 and Lactobacillus rhamnosus GG. *International Journal of Oncology* 48:9.

Orrhage K, Annas A, Nord C, Brittebo E & Rafter J (2002) Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice. *Scandinavian journal of gastroenterology* 37(2):215-221.

Paredes-López O, Cervantes-Ceja M, Vigna-Pérez M & Hernández-Pérez T (2010a) Berries: Improving Human Health and Healthy Aging, and Promoting Quality Life—A Review. *Plant Foods Hum Nutr* 65(3):299-308.

Paredes-López O, Cervantes-Ceja ML, Vigna-Pérez M & Hernández-Pérez T (2010b) Berries: improving human health and healthy aging, and promoting quality life—a review. *Plant Foods Hum Nutr* 65(3):299-308.

Peltonen K, El-Nezami H, Haskard C, Ahokas J & Salminen S (2001) Aflatoxin B 1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *Journal of dairy science* 84(10):2152-2156.

Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA & Stobberingh EE (2006) Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy. *Pediatrics* 118(2):511-521.

Peng C-H, Chyau C-C, Chan K-C, Chan T-H, Wang C-J & Huang C-N (2011) Hibiscus sabdariffa Polyphenolic Extract Inhibits Hyperglycemia, Hyperlipidemia, and Glycation-Oxidative Stress while Improving Insulin Resistance. *Journal of Agricultural and Food Chemistry* 59(18):9901-9909.

Perez J, Jayaprakasha G, Cadena A, Martinez E, Ahmad H & Patil B (2010) In vivo induction of phase II detoxifying enzymes, glutathione transferase and quinone reductase by citrus triterpenoids. *BMC Complementary and Alternative Medicine* 10(1):51.

Petit C, Oswald E & Nougayrede J-P (2013) Rôle des génotoxines produites par des bactéries du microbiote dans le cancer colorectal. *Revue Francophone des Laboratoires* 2013(456):77-82.

Pino MS & Chung DC (2010) The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology* 138(6):2059-2072.

Pitot HC (1993) The molecular biology of carcinogenesis. *Cancer* 72(S3):962-970.

Power DG, Gloglowski E & Lipkin SM (2010) Clinical Genetics of Hereditary Colorectal Cancer. *Hematology/Oncology Clinics of North America* 24(5):837-859.

Pozuelo MJ, Agis-Torres A, Hervert-Hernández D, Elvira López-Oliva M, Muñoz-Martínez E, Rotger R & Goni I (2012) Grape antioxidant dietary fiber stimulates Lactobacillus growth in rat cecum. *Journal of food science* 77(2).

Prasain JK, Rajbhandari R, Keeton AB, Piazza GA & Barnes S (2016) Metabolism and growth inhibitory activity of cranberry derived flavonoids in bladder cancer cells. *Food & function* 7(9):4012-4019.

Puertollano E, Kolida S & Yaqoob P (2014) Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. *Current Opinion in Clinical Nutrition & Metabolic Care* 17(2):139-144.

Rafter J (2003) Probiotics and colon cancer. *Best Practice & Research Clinical Gastroenterology* 17(5):849-859.
Rahman MM, Ichiyanagi T, Komiyama T, Hatano Y & Konishi T (2006) Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism. *Free Radical Research* 40(9):993-1002.
Ramakrishna B, Jayaprakasha G, Jena B & Singh R (2008) Antioxidant activitiesof roselle (Hibiscus sabdariffa) calyces and fruit extracts. *Journal of Food Science and Technology* 45(3):223-227.
Ramirez-Rodrigues MM, Plaza ML, Azeredo A, Balaban MO & Marshall MR (2011) Physicochemical and Phytochemical Properties of Cold and Hot Water Extraction from Hibiscus sabdariffa. *Journal of Food Science* 76(3):C428-C435.
Randi G, Edefonti V, Ferraroni M, La Vecchia C & Decarli A (2010) Dietary patterns and the risk of colorectal cancer and adenomas. *Nutrition reviews* 68(7):389-408.
Rao C, Sanders M, Indranie C, Simi B & Reddy B (1999) Prevention of colonic preneoplastic lesions by the probiotic Lactobacillus acidophilus NCFMTM in F344 rats. *International Journal of Oncology* 14(5):939-983.
Rasool S, Kadla S, Rasool V & Ganai B (2013) A comparative overview of general risk factors associated with the incidence of colorectal cancer. *Tumor Biol.* 34(5):2469-2476.
Reddy TC, Reddy DB, Aparna A, Arunasree KM, Gupta G, Achari C, Reddy GV, Lakshmipathi V, Subramanyam A & Reddanna P (2012) Anti-leukemic effects of gallic acid on human leukemia K562 cells: Downregulation of COX-2, inhibition of BCR/ABL kinase and NF-κB inactivation. *Toxicology in Vitro* 26(3):396-405.
Regula J, Rupinski M, Kraszewska E, Polkowski M, Pachlewski J, Orlowska J, Nowacki MP & Butruk E (2006) Colonoscopy in Colorectal-Cancer Screening for Detection of Advanced Neoplasia. *New England Journal of Medicine* 355(18):1863-1872.
Rekha CR & Vijayalakshmi G (2010) Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *Journal of Applied Microbiology* 109(4):1198-1208.
Rodríguez-Ramiro I, Ramos S, Bravo L, Goya L & Martín MÁ (2011) Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. *The Journal of nutritional biochemistry* 22(12):1186-1194.
Ross AB, Pere-Trépat E, Montoliu I, Martin F-PJ, Collino S, Moco S, Godin J-P, Cléroux M, Guy PA & Breton I (2013) A whole-grain–rich diet reduces urinary excretion of markers of protein catabolism and gut microbiota metabolism in healthy men after one week. *The Journal of nutrition* 143(6):766-773.
Rowland IR (2009) The Role of the Gastrointestinal Microbiota in Colorectal Cancer. *Current Pharmaceutical Design* 15(13):1524-1527.
Rowley PT (2005) Inherited Susceptibility to Colorectal Cancer. *Annual Review of Medicine* 56(1):539-554.
Rudolph RE, Dominitz JA, Lampe JW, Levy L, Qu P, Li S, Lampe PD, Bronner MP & Potter JD (2005) Risk Factors for Colorectal Cancer in Relation to Number and Size of Aberrant Crypt Foci in Humans. *Cancer Epidemiology Biomarkers & Prevention* 14(3):605-608.

Rupasinghe V, Neir SV & Parmar I (2016) Polyphenol characterization, anti-oxidant, anti-proliferation and anti-tyrosinase activity of cranberry pomace. *Functional Foods in Health and Disease* 6(11):754-768.
Saad N, Delattre C, Urdaci M, Schmitter JM & Bressollier P (2013) An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology* 50(1):1-16.
Saikali J, Picard C, Freitas M & Holt P (2004) Fermented Milks, Probiotic Cultures, and Colon Cancer. *Nutrition and Cancer* 49(1):14-24.
Salah AM, Gathumbi J & Vierling W (2002) Inhibition of intestinal motility by methanol extracts of Hibiscus sabdariffa L. (Malvaceae) in rats. *Phytotherapy Research $V 16* (3):283-285.
Sampalis J, Psaradellis E & Rampakakis E (2010) Efficacy of BIO K+ CL1285® in the reduction of antibiotic-associated diarrhea–a placebo controlled double-blind randomized, multi-center study. *Arch Med Sci* 6:56-64.
Sanders ME & Marco ML (2010) Food formats for effective delivery of probiotics. *Food Science and Technology* 1.
Sangeetha N, Viswanathan P, Balasubramanian T & Nalini N (2012) Colon cancer chemopreventive efficacy of silibinin through perturbation of xenobiotic metabolizing enzymes in experimental rats. *European Journal of Pharmacology* 674(2–3):430-438.
Savage DC (1977) Microbial Ecology of the Gastrointestinal Tract. *Annual Review of Microbiology* 31(1):107-133.
Sáyago-Ayerdi SG, Arranz S, Serrano J & Goñi I (2007) Dietary Fiber Content and Associated Antioxidant Compounds in Roselle Flower (Hibiscus sabdariffa L.) Beverage. *Journal of Agricultural and Food Chemistry* 55(19):7886-7890.
Scanlan PD, Shanahan F, Clune Y, Collins JK, O'Sullivan GC, O'Riordan M, Holmes E, Wang Y & Marchesi JR (2008) Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environmental Microbiology* 10(3):789-798.
Sears Cynthia L & Garrett Wendy S (2014) Microbes, Microbiota, and Colon Cancer. *Cell Host & Microbe* 15(3):317-328.
Seeram NP (2008) Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of Their Intake on Human Health, Performance, and Disease. *Journal of Agricultural and Food Chemistry* 56(3):627-629.
Seeram NP, Adams LS, Hardy ML & Heber D (2004) Total Cranberry Extract versus Its Phytochemical Constituents: Antiproliferative and Synergistic Effects against Human Tumor Cell Lines. *Journal of Agricultural and Food Chemistry* 52(9):2512-2517.
Seeram NP, Adams LS, Zhang Y, Lee R, Sand D, Scheuller HS & Heber D (2006) Blackberry, Black Raspberry, Blueberry, Cranberry, Red Raspberry, and Strawberry Extracts Inhibit Growth and Stimulate Apoptosis of Human Cancer Cells In Vitro. *Journal of Agricultural and Food Chemistry* 54(25):9329-9339.
Sengottuvelan M, Deeptha K & Nalini N (2009) Resveratrol ameliorates DNA damage, prooxidant and antioxidant imbalance in 1,2-dimethylhydrazine induced rat colon carcinogenesis. *Chemico-Biological Interactions* 181(2):193-201.

Şerban DE (2011) The gut microbiota in the metagenomics era: sometimes a friend, sometimes a foe. *ROMANIAN ARCHIVES* 10:134.
Shamsuddin A & Phillips R (1981) Preneoplastic and neoplastic changes in colonic mucosa in Crohn's disease. *Archives of pathology & laboratory medicine* 105(6):283-286.
Sharma S, Nemecz S, Zhu S & Steele V (1997) Identification of chemopreventive agents by screening for induction of glutathione-S-transferase as a biomarker. *Methods Cell Sci* 19(1):49-52.
Shen Q, Chen YA & Tuohy KM (2010a) A comparative in vitro investigation into the effects of cooked meats on the human faecal microbiota. *Anaerobe* 16(6):572-577.
Shen XJ, Rawls JF, Randall TA, Burcall L, Mpande C, Jenkins N, Jovov B, Abdo Z, Sandler RS & Keku TO (2010b) Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut Microbes* 1(3):138-147.
Sheweita S & Tilmisany A (2003) Cancer and phase II drug-metabolizing enzymes. *Current drug metabolism* 4(1):45-58.
Shih C-H, Siu, Ng R, Wong E, Chiu LCM, Chu IK & Lo C (2006) Quantitative Analysis of Anticancer 3-Deoxyanthocyanidins in Infected Sorghum Seedlings. *Journal of Agricultural and Food Chemistry* 55(2):254-259.
Shih P-H, Yeh C-T & Yen G-C (2007) Anthocyanins Induce the Activation of Phase II Enzymes through the Antioxidant Response Element Pathway against Oxidative Stress-Induced Apoptosis. *Journal of Agricultural and Food Chemistry* 55(23):9427-9435.
Shin DY, Lee WS, Lu JN, Kang MH, Ryu CH, Kim GY & Choi YH (2009) Induction of apoptosis in human colon cancer HCT-116 cells by anthocyanins through suppression of Akt and activation of p38-MAPK. *International Journal of Oncology* 35:1499-1504.
Shin R, Itoh Y, Kataoka M, Iino-Miura S, Miura R, Mizutani T & Fujisawa T (2016) Anti-tumor activity of heat-killed Lactobacillus plantarum BF-LP284 on Meth-A tumor cells in BALB/c mice. *International Journal of Food Sciences and Nutrition* 67(6):641-649.
Siegel R, DeSantis C & Jemal A (2014) Colorectal cancer statistics, 2014. *CA: A Cancer Journal for Clinicians* 64(2):104-117.
Signoretto C, Boaretti M & Canepari P (1998) Peptidoglycan synthesis by Enterococcus faecalis penicillin binding protein 5. *Arch Microbiol* 170(3):185-190.
Simon G & Gorbach S (1986) The human intestinal microflora. *Digest Dis Sci* 31(9):147-162.
Sindi HA, Marshall LJ & Morgan MRA (2014) Comparative chemical and biochemical analysis of extracts of Hibiscus sabdariffa. *Food Chemistry* 164(0):23-29.
Singleton VL & Rossi JA (1965) Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture* 16(3):144-158.
Sivieri K, Spinardi-Barbisan ALT, Barbisan LF, Bedani R, Pauly ND, Carlos IZ, Benzatti F, Vendramini RC & Rossi EA (2008) Probiotic Enterococcus faecium CRL 183 inhibit chemically induced colon cancer in male Wistar rats. *Eur Food Res Technol* 228(2):231-237.

Smolinska K & Paluszkiewicz P (2010) Risk of colorectal cancer in relation to frequency and total amount of red meat consumption. Systematic review and meta-analysis. *Archives of Medical Science* 6(4):605-610.
Sobhani I, Amiot A, Le Baleur Y, Levy M, Auriault M-L, Van Nhieu JT & Delchier JC (2013) Microbial dysbiosis and colon carcinogenesis: could colon cancer be considered a bacteria-related disease? *Therapeutic Advances in Gastroenterology* 6(3):215-229.
Sobhani I, Tap J, Roudot-Thoraval F, Roperch JP, Letulle S, Langella P, Corthier G, Van Nhieu JT & Furet JP (2011) Microbial dysbiosis in colorectal cancer (CRC) patients. *PloS one* 6(1):e16393.
Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM & Mullin JM (1999) Increased tight junctional permeability is associated with the development of colon cancer. *Carcinogenesis* 20(8):1425-1432.
Song M, Chan AT, Fuchs CS, Ogino S, Hu FB, Mozaffarian D, Ma J, Willett WC, Giovannucci EL & Wu K (2014) Dietary intake of fish, ω-3 and ω-6 fatty acids and risk of colorectal cancer: A prospective study in U.S. men and women. *International Journal of Cancer* 135(10):2413-2423.
Song M, Garrett WS & Chan AT (2015) Nutrients, Foods, and Colorectal Cancer Prevention. *Gastroenterology* 148(6):1244-1260.e1216.
Söreide K, Janssen EAM, Söiland H, Körner H & Baak JPA (2006) Microsatellite instability in colorectal cancer. *British Journal of Surgery* 93(4):395-406.
Söreide K, Nedrebø BS, Knapp J-C, Glomsaker TB, Søreide JA & Kørner H (2009) Evolving molecular classification by genomic and proteomic biomarkers in colorectal cancer: Potential implications for the surgical oncologist. *Surgical Oncology* 18(1):31-50.
Sreekumar O & Hosono A (1998) Antimutagenicity and the Influence of Physical Factors in Binding Lactobacillus gasseri and Bifidobacterium longum Cells to Amino Acid Pyrolysates. *Journal of Dairy Science* 81(6):1508-1516.
Srivastava A, Akoh CC, Fischer J & Krewer G (2007) Effect of Anthocyanin Fractions from Selected Cultivars of Georgia-Grown Blueberries on Apoptosis and Phase II Enzymes. *Journal of Agricultural and Food Chemistry* 55(8):3180-3185.
Stevens RG, Swede H & Rosenberg DW (2007) Epidemiology of colonic aberrant crypt foci: Review and analysis of existing studies. *Cancer Letters* 252(2):171-183.
Stoner GD, Wang L-S & Casto BC (2008) Laboratory and clinical studies of cancer chemoprevention by antioxidants in berries. *Carcinogenesis* 29(9):1665-1674.
Su B-N, Gu J-Q, Kang Y-H, Park E-J, Pezzuto JM & Kinghorn AD (2004) Induction of the Phase II Enzyme, Quinone Reductase, by Withanolides and Norwithanolides from Solanaceous Species. *Mini-Reviews in Organic Chemistry* 1(1):115-123.
Sun J, Shi Y-H, Le G-W & Ma X-Y (2005a) Distinct immune response induced by peptidoglycan derived from Lactobacillus sp. *World journal of gastroenterology* 11(40):6330.
Sun J, Shi Y-H, Le G-W & Ma X-Y (2005b) Distinct immune response induced by peptidoglycan derived from Lactobacillus sp. *World Journal of Gastroenterology : WJG* 11(40):6330-6337.

Sunkara R, Shackelford L, Walker L & Verghese M (2015) Inhibition of Chemically-Induced Colon Cancer by Dietary Treatment of Hibiscus sabdariffa L. Dried Calyx in Rats. *Food and Nutrition Sciences* 6(12):1174.

Surh Y-J (2003) Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer* 3(10):768-780.

Sutandyo N (2010) Nutritional carcinogenesis. *Acta medica Indonesiana* 23.

Szajdek A & Borowska E (2008) Bioactive compounds and health-promoting properties of berry fruits: a review. *Plant Foods Hum Nutr* 63(4):147-156.

Tabasco R, Sánchez-Patán F, Monagas M, Bartolomé B, Victoria Moreno-Arribas M, Peláez C & Requena T (2011) Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. *Food Microbiology* 28(7):1345-1352.

Takayama T, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, Kato J, Kogawa K, Miyake H & Niitsu Y (1998) Aberrant Crypt Foci of the Colon as Precursors of Adenoma and Cancer. *New England Journal of Medicine* 339(18):1277-1284.

Talalay P (2000) Chemoprotection against cancer by induction of phase 2 enzymes. *Biofactors* 12(1):5-11.

Tap J, Mondot S, Levenez F, Pelletier E, Caron C, Furet JP, Ugarte E, Muñoz-Tamayo R, Paslier DL & Nalin R (2009) Towards the human intestinal microbiota phylogenetic core. *Environmental microbiology* 11(10):2574-2584.

Theodoratou E, Farrington SM, Tenesa A, McNeill G, Cetnarskyj R, Korakakis E, Din FVN, Porteous ME, Dunlop MG & Campbell H (2014) Associations between dietary and lifestyle risk factors and colorectal cancer in the Scottish population. *European Journal of Cancer Prevention* 23(1):8-17.

Thurow T & Lee S-O (2012) Effect of chlorogenic acid and neochlorogenic acid on human colon cancer cells. *Disc J* 13:86-93.

Tiptiri-Kourpeti A, Spyridopoulou K, Santarmaki V, Aindelis G, Tompoulidou E, Lamprianidou EE, Saxami G, Ypsilantis P, Lampri ES & Simopoulos C (2016) Lactobacillus casei Exerts Anti-Proliferative Effects Accompanied by Apoptotic Cell Death and Up-Regulation of TRAIL in Colon Carcinoma Cells. *PloS one* 11(2):e0147960.

Tjalsma H (2012) What can gut bacteria tell us about colorectal cancer? *Colorectal Cancer* 1(4):265-268.

Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman W-H, Pagès F & Galon J (2011) Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer. *Cancer Research* 71(4):1263-1271.

Turnbaugh PJ & Gordon JI (2009a) The core gut microbiome, energy balance and obesity. *The Journal of Physiology* 587(Pt 17):4153-4158.

Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R & Gordon JI (2009b) The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Science translational medicine* 1(6):6ra14-16ra14.

Uda Y, Price KR, Williamson G & Rhodes MJC (1997) Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. *Cancer Letters* 120(2):213-216.

Ullah MF, Ahmad A, Khan H, Zubair H, Sarkar F & Hadi SM (2013) The Prooxidant Action of Dietary Antioxidants Leading to Cellular DNA Breakage and Anticancer Effects: Implications for Chemotherapeutic Action Against Cancer. *Cell Biochem Biophys* 67(2):431-438.

Umesalma S, Nagendraprabhu P & Sudhandiran G (2014) Antiproliferative and apoptotic-inducing potential of ellagic acid against 1,2-dimethyl hydrazine-induced colon tumorigenesis in Wistar rats. *Mol Cell Biochem* 388(1):157-172.

Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M & Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology* 39(1):44-84.

Valko M, Rhodes C, Moncol J, Izakovic M & Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions* 160(1):1-40.

van Meerloo J, Kaspers GL & Cloos J (2011) Cell Sensitivity Assays: The MTT Assay. *Cancer Cell Culture,* (Methods in Molecular Biology, Cree IA (Édit.) Humana Press, Vol 731. p 237-245.

Vargas AJ & Thompson PA (2012) Diet and nutrient factors in colorectal cancer risk. *Nutrition in Clinical Practice* 27(5):613-623.

Vasiljevic T & Shah NP (2008) Probiotics—From Metchnikoff to bioactives. *International Dairy Journal* 18(7):714-728.

Vauzour D, Rodriguez-Mateos A, Corona G, Oruna-Concha MJ & Spencer JPE (2010) Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients* 2(11):1106-1131.

Vendrame S, Guglielmetti S, Riso P, Arioli S, Klimis-Zacas D & Porrini M (2011) Six-Week Consumption of a Wild Blueberry Powder Drink Increases Bifidobacteria in the Human Gut. *Journal of Agricultural and Food Chemistry* 59(24):12815-12820.

Venkatachalam K, Gunasekaran S & Namasivayam N (2016) Biochemical and molecular mechanisms underlying the chemopreventive efficacy of rosmarinic acid in a rat colon cancer. *European Journal of Pharmacology* 791:37-50.

Verma A & Shukla G (2013a) Administration of prebiotic inulin suppresses 1,2 dimethylhydrazine dihydrochloride induced procarcinogenic biomarkers fecal enzymes and preneoplastic lesions in early colon carcinogenesis in Sprague Dawley rats. *Journal of Functional Foods* 5(2):991-996.

Verma A & Shukla G (2013b) Probiotics Lactobacillus rhamnosus GG, Lactobacillus acidophilus Suppresses DMH-Induced Procarcinogenic Fecal Enzymes and Preneoplastic Aberrant Crypt Foci in Early Colon Carcinogenesis in Sprague Dawley Rats. *Nutrition and Cancer* 65(1):84-91.

Villanueva X & Mach N (2011) Papel de los ácidos grasos omega 3 en la prevención del cáncer de colon. *Revista Española de Nutrición Humana y Dietética* 15(2):47-55.

Vinderola G, Matar C, Palacios J & Perdigón G (2007) Mucosal immunomodulation by the non-bacterial fraction of milk fermented by Lactobacillus helveticus R389. *International Journal of Food Microbiology* 115(2):180-186.

Vinderola G, Perdigón G, Duarte J, Farnworth E & Matar C (2006) Effects of the oral administration of the exopolysaccharide produced by Lactobacillus kefiranofaciens on the gut mucosal immunity. *Cytokine* 36(5–6):254-260.

Vinothkumar R, Vinoth Kumar R, Sudha M, Viswanathan P, Balasubramanian T & Nalini N (2014) Modulatory effect of troxerutin on biotransforming enzymes and preneoplasic lesions induced by 1,2-dimethylhydrazine in rat colon carcinogenesis. *Experimental and Molecular Pathology* 96(1):15-26.

Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A & Boyd MR (1991) Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production. *Cancer Research* 51(10):2515-2520.

Vu KD, Carlettini H, Bouvet J, Côté J, Doyon G, Sylvain J-F & Lacroix M (2012) Effect of different cranberry extracts and juices during cranberry juice processing on the antiproliferative activity against two colon cancer cell lines. *Food Chemistry* 132(2):959-967.

Wan Y, Xin YI, Zhang C, Wu D, Ding D, Tang LI, Owusu L, Bai J & Li W (2014) Fermentation supernatants of Lactobacillus delbrueckii inhibit growth of human colon cancer cells and induce apoptosis through a caspase 3-dependent pathway. *Oncology Letters* 7(5):1738-1742.

Wang C-J, Wang J-M, Lin W-L, Chu C-Y, Chou F-P & Tseng T-H (2000) Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. *Food and Chemical Toxicology* 38(5):411-416.

Wang L-S, Arnold M, Huang Y-W, Sardo C, Seguin C, Martin EW, Huang TH-M, Riedl K, Schwartz SJ & Frankel WL (2010) Modulation of genetic and epigenetic biomarkers of colorectal cancer in humans by black raspberries: a phase I pilot study. *Clinical Cancer Research*:clincanres. 1260.2010.

Wang S-M, Zhang L-W, Fan R-B, Han X, Yi H-X, Zhang L-L, Xue C-H, Li H-B, Zhang Y-H & Shigwedha N (2014) Induction of HT-29 cells apoptosis by lactobacilli isolated from fermented products. *Research in Microbiology* 165(3):202-214.

Wang W & Higuchi CM (1995) Induction of NAD(P)H: quinone reductase by vitamins A, E and C in Colo205 colon cancer cells. *Cancer Letters* 98(1):63-69.

Wang X, Allen TD, May RJ, Lightfoot S, Houchen CW & Huycke MM (2008) Enterococcus faecalis Induces Aneuploidy and Tetraploidy in Colonic Epithelial Cells through a Bystander Effect. *Cancer Research* 68(23):9909-9917.

Wang X, Yang Y, Moore DR, Nimmo SL, Lightfoot SA & Huycke MM (2012) 4-Hydroxy-2-Nonenal Mediates Genotoxicity and Bystander Effects Caused by Enterococcus faecalis–Infected Macrophages. *Gastroenterology* 142(3):543-551.e547.

Watson RR & Preedy VR (2015) *Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion.* Elsevier Science. https://books.google.ca/books?id=p1eZBQAAQBAJ

WCRF/AICR (2011) Colorectal Cancer 2011 Report Food, Nutrition, Physical Activity, and the Prevention of Colorectal Cancer.).

Webb AL & McCullough ML (2005) Dietary Lignans: Potential Role in Cancer Prevention. *Nutrition and Cancer* 51(2):117-131.

Wendel M & Heller AR (2009) Anticancer Actions of Omega-3 Fatty Acids - Current State and Future Perspectives. *Anti-Cancer Agents in Medicinal Chemistry- Anti-Cancer Agents)* 9(4):457-470.

Willett WC (2000) Diet and cancer. *The oncologist* 5(5):393-404.

Williamson EM, Driver S & Baxter K (2009) *Stockley's Herbal Medicines Interactions: A Guide to the Interactions of Herbal Medicines, Dietary Supplements and Nutraceuticals with Conventional Medicines.* Pharmaceutical Press. https://books.google.ca/books?id=NVvaPAAACAAJ

Wollowski I, Rechkemmer G & Pool-Zobel BL (2001) Protective role of probiotics and prebiotics in colon cancer. *The American Journal of Clinical Nutrition* 73(2):451s-455s.

Wong JMW, de Souza R, Kendall CWC, Emam A & Jenkins DJA (2006) Colonic Health: Fermentation and Short Chain Fatty Acids. *Journal of Clinical Gastroenterology* 40(3):235-243.

Wu C-H, Huang C-C, Hung C-H, Yao F-Y, Wang C-J & Chang Y-C (2016) Delphinidin-rich extracts of Hibiscus sabdariffa L. trigger mitochondria-derived autophagy and necrosis through reactive oxygen species in human breast cancer cells. *Journal of Functional Foods* 25:279-290.

Wu S, Feng B, Li K, Zhu X, Liang S, Liu X, Han S, Wang B, Wu K, Miao D, Liang J & Fan D (2012) Fish Consumption and Colorectal Cancer Risk in Humans: A Systematic Review and Meta-analysis. *The American Journal of Medicine* 125(6):551-559.e555.

Wu S, Rhee K-J, Albesiano E, Rabizadeh S, Wu X, Yen H-R, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM & Sears CL (2009) A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* 15(9):1016-1022.

Wu X & Prior RL (2005) Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries. *Journal of Agricultural and Food Chemistry* 53(7):2589-2599.

www.cancer.ca (Toronto, ON),

www.harlan.com,

Yamakoshi J, Tokutake S, Kikuchi M, Konishi H & Mitsuoka T (2001) Effect of Proanthocyanidin-Rich Extract from Grape Seeds on Human Fecal Flora and Fecal Odor. *Microbial Ecology in Health and Disease* 13(1):25-31.

Yamazaki K, Tsunoda A, Sibusawa M, Tsunoda Y, Kusano M, Fukuchi K, Yamanaka M, Kushima M, Nomoto K & Morotomi M (2000) The effect of an oral administration of Lactobacillus casei strain shirota on azoxymethane-induced colonic aberrant crypt foci and colon cancer in the rat. *Oncology Reports* 9(7):977-1059.

Yang J, Guo J & Yuan J (2008) In vitro antioxidant properties of rutin. *LWT - Food Science and Technology* 41(6):1060-1066.

Yang J & Liu RH (2009) Induction of phase II enzyme, quinone reductase, in murine hepatoma cells in vitro by grape extracts and selected phytochemicals. *Food Chemistry* 114(3):898-904.

Yang K, Lamprecht SA, Liu Y, Shinozaki H, Fan K, Leung D, Newmark H, Steele VE, Kelloff GJ & Lipkin M (2000) Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. *Carcinogenesis* 21(9):1655-1660.

Yang T, Owen JL, Lightfoot YL, Kladde MP & Mohamadzadeh M (2013a) Microbiota impact on the epigenetic regulation of colorectal cancer. *Trends in Molecular Medicine* 19(12):714-725.

Yang Y, Wang X, Huycke T, Moore DR, Lightfoot SA & Huycke MM (2013b) Colon Macrophages Polarized by Commensal Bacteria Cause Colitis and Cancer through the Bystander Effect. *Translational Oncology* 6(5):596-IN598.

Yang Y, Xia Y, Chen H, Hong L, Feng J, Yang J, Yang Z, Shi C, Wu W, Gao R, Wei Q, Qin H & Ma Y (2016) The effect of perioperative probiotics treatment for colorectal cancer: short-term outcomes of a randomized controlled trial. *Oncotarget* 7(7):8432-8440.

Yin M-C, Lin C-C, Wu H-C, Tsao S-M & Hsu C-K (2009) Apoptotic Effects of Protocatechuic Acid in Human Breast, Lung, Liver, Cervix, and Prostate Cancer Cells: Potential Mechanisms of Action. *Journal of Agricultural and Food Chemistry* 57(14):6468-6473.

Yokota T, Sugano K, Kondo H, Saito D, Sugihara K, Fukayama N, Ohkura H, Ochiai A & Yoshida S (1997) Detection of aberrant crypt foci by magnifying colonoscopy. *Gastrointestinal endoscopy* 46(1):61-65.

Yusof AS, Isa ZM & Shah SA (2012) Dietary patterns and risk of colorectal cancer: a systematic review of cohort studies (2000-2011). *Asian Pac J Cancer Prev* 13(9):4713-4717.

Zeng H, Lazarova DL & Bordonaro M (2014) Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention. *World J Gastrointest Oncol* 6(2):41-51.

Zhang BX & Ohta Y (1990) Binding of Mutagens by Fractions of the Cell Wall Skeleton of Lactic Acid Bacteria on Mutagens. *Journal of Dairy Science* 74(5):1477-1481.

Zhang H, Zhang M, Yu L, Zhao Y, He N & Yang X (2012a) Antitumor activities of quercetin and quercetin-5′,8-disulfonate in human colon and breast cancer cell lines. *Food and Chemical Toxicology* 50(5):1589-1599.

Zhang J-W, Du P, Gao J, Yang B-R, Fang W-J & Ying C-M (2012b) Preoperative Probiotics Decrease Postoperative Infectious Complications of Colorectal Cancer. *The American Journal of the Medical Sciences* 343(3):199-205.

Zhang L, Ma J, Pan K, Go VLW, Chen J & You W-c (2005) Efficacy of Cranberry Juice on Helicobacter pylori Infection: a Double-Blind, Randomized Placebo-Controlled Trial. *Helicobacter* 10(2):139-145.

Zhang Y, Chen AY, Li M, Chen C & Yao Q (2008a) Ginkgo biloba Extract Kaempferol Inhibits Cell Proliferation and Induces Apoptosis in Pancreatic Cancer Cells. *Journal of Surgical Research* 148(1):17-23.

Zhang Y, Seeram NP, Lee R, Feng L & Heber D (2008b) Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *Journal of agricultural and food chemistry* 56(3):670-675.

Zhao H, Zhou F, Qi Y, Dziugan P, Bai F, Walczak P & Zhang B (2013) Screening of Lactobacillus strains for their ability to bind Benzo(a)pyrene and the mechanism of the process. *Food and Chemical Toxicology* 59(0):67-71.

Zhong Y, Chiou Y-S, Pan M-H, Ho C-T & Shahidi F (2012) Protective effects of epigallocatechin gallate (EGCG) derivatives on azoxymethane-induced colonic carcinogenesis in mice. *Journal of Functional Foods* 4(1):323-330.

Zhu D, Chen X, Wu J, Ju Y, Feng J, Lu G, Ouyang M, Ren B & Li Y (2012) Effect of perioperative intestinal probiotics on intestinal flora and immune function in patients with colorectal cancer. *Journal of Southern Medical University* 32(8):1190-1193.

Zu X-y, Zhang Z-Y, Zhang X-W, Yoshioka M, Yang Y-N & Li J (2010) Anthocyanins extracted from Chinese blueberry (Vaccinium uliginosum L.) and its anticancer effects on DLD-1 and COLO205 cells. *Chinese medical journal* 123(19):2714-2719.

Zuo Y, Wang C & Zhan J (2002) Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS. *Journal of agricultural and food chemistry* 50(13):3789-3794.