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Impact of the type of microcarrier and agitation modes on the expansion performances of mesenchymal stem cells derived from umbilical cord

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9 modes on the expansion performances of mesenchymal
10 stem cells derived from umbilical cord
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14 C. Loubière^a, I. Chevalot^a, N. De Isla^{b,c}, C. Sion^a, L. Reppel^{b,d,e}, E.
15 Guedon^a, E. Olmos^{a,*}
16

17 ^aLaboratoire Réactions et Génie des Procédés, Université de Lorraine, CNRS, LRGF,
18 F-54000 Nancy, France

19 ^bCNRS, IMoPA, UMR 7365, Vandoeuvre-lès-Nancy, France

20 ^cUniversité de Lorraine, Faculté de Médecine, Vandoeuvre-lès-Nancy, France

21 ^dCHRU de Nancy, Unité de Thérapie cellulaire et Tissus and FR 3209,
22 Vandoeuvre-lès-Nancy, France

23 ^eUniversité de Lorraine, Faculté de Pharmacie, Département de
24 Microbiologie-Immunologie, Nancy, France
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28
29 **Abstract**
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31 The present study proposed to compare the impact of agitation mode (static,
32 orbital and mechanical) on the culture of mesenchymal stem cells extracted
33 from the Wharton's jelly of umbilical cords (WJ-MSC), in a clinical grade
34 culture medium, using human platelet lysate and different xeno-free micro-
35 carriers. Attachment, expansion and detachment performances were charac-
36 terized by a new dedicated tool of microscopic image post-treatment, allowing
37 an in situ cell counting without detachment step. Results showed that perfor-
38 mances in static mode were not necessarily representative of those obtained in
39 dynamic mode. Moreover, impacts on nutrient consumptions and metabolite
40 productions were identified, such as a higher glutamine consumption when
41 Cytodex-1 microcarriers were used. The detachment strategy used was rela-
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tively efficient for Star-Plus, Plastic-Plus and Hillex II, but not sufficient for Cytodex-1. Despite Cytodex-1 presented promising attachment and expansion performances, Star-Plus and Plastic-Plus showed a better compromise, respectively for the orbital and the mechanical agitation modes.

Keywords: Mesenchymal stem cells, Wharton's jelly, microcarrier, agitation, cell counting method

1 Introduction

In recent years, Mesenchymal Stem / Stromal Cells (MSC) are increasingly used in regenerative medicine. According to the U.S. National Institute of Health, the number of completed or ongoing MSC-based studies passed from around 500 in 2016 to 854 in July 2018 (US National Library of Medicine, National Institute of Health). This is mainly due to the promising application of MSC in cell therapies with actions as trophic mediators and displaying bioactive factors secretion [1]. Their immuno-modulatory properties also present a great interest for this medical purpose, allowing either autologous and allogenic treatments. They are notably involved into treatments for type I and II Diabetes, cancers and heart failures in some others [2].

MSC were firstly isolated from bone marrow by Friedenstein *et al.* and identified as a population of anchorage cells with a fibroblast behavior and the capacity to regenerate bone cells [3]. Since then, other parts of the body were identified as MSC sources, such as adipose-tissues [4], synovium [5], lung [6], muscles [7], umbilical-cord blood [8] or umbilical-cord matrix (Wharton's jelly) [9]. In order to establish robust criteria to define MSC,

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8 19 the International Society for Cellular Therapy (ISCT) proposed three mini-
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10 20 malist conditions: (i) the capacity to adhere on plastic in standard culture
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12 21 conditions, (ii) the expression or non-expression of specific surface antigens
13
14 22 (positive for CD105, CD73 and CD90; negative for CD45 CD34, CD14 or
15
16 23 CD11b, CD79a or CD19 and HLA-DR), and the *in vitro* differentiation abil-
17
18 24 ity into osteoblasts, adipocytes and chondrocytes [10].

19 25 These cells are nevertheless present in limited quantities in the body,
20
21 26 which decrease with ageing [11]. *In vitro* expansions are thus necessary to
22
23 27 reach the high cell dose requirements for the medical purposes, between 10^9
24
25 28 and 10^{12} cells/lot [12]. To improve their performance, expansion processes
26
27 29 have been investigated from static planar cultures to stirred tank bioreactors,
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29 30 taking into account both the quantity and the quality of the harvested cells
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31 31 [13, 14, 15].

31 32 Concerning the quality, MSC have the particularity to be the drug itself,
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33 33 directly injected into the patient, meaning that, in addition to the process
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35 34 itself (robust control and monitoring), all components involved in the culture
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37 35 medium must be suitable for clinical applications. This led to the use of
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39 36 chemically defined culture media displaying much higher costs [16], or to
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41 37 the serum replacement by human blood-derived alternatives like the human
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43 38 platelet lysate (hPL) [17]. The use of hPL has notably been validated for
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45 39 MSC cultures, with higher expansion rates than with fetal bovine serum,
46
47 40 making it a good substitute at an affordable cost [18]. It also presents the
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49 41 advantage to be rich in proteins and growth factors, avoiding the need of
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51 42 bFGF addition.

51 43 For the large-scale production of MSC, the technology of microcarriers,
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8 developed by Van Wezel *et al.* in 1967, is today widely used to allow adherent-
9 dependent cell cultures. They consist in micro-spheres from 90 to 400 μm
10 diameter with a density slightly higher than water [19]. Cells adhere on
11 these beads, which are then suspended in a stirred tank bioreactor. Agita-
12 tion allows homogenization of the culture medium, mass transfers and limits
13 concentration gradients. For applications with MSC, it has been demon-
14 strated that cultures on microcarriers provided a higher number of cells than
15 in mono- or multi-layer planar cultures, although they require more efforts
16 in investments and R&D [20, 21]. Recently, MSC cultures have been suc-
17 cessfully conducted at a few liter-scale [22, 23]. A stirred system allows thus
18 to cultivate MSC in a more robust, effective and scalable system, but in-
19 vestigations are still necessary to understand the impact of the agitation on
20 cell biological responses [24, 25]. Studies recently pointed out the lack of
21 robust and transferable methodology for microcarrier selection. As reported
22 by Jung *et al.*, very few studies reported the comparison of growth depending
23 on the type of microcarriers, and studies are difficult to compare one to each
24 other due to various cell sources and culture conditions [26]. For instance, a
25 systematic microcarrier screening was described, involving 13 microcarriers
26 under both static and dynamic conditions [27], but this study was based on
27 bone marrow MSC.
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44 During last years, efforts have been made to perform and study cultures of
45 MSC derived from umbilical cords and more specifically from the Wharton's
46 jelly (WJ-MS). These studies reported some relevant advantages in com-
47 parison to other sources. For instance, cell isolation procedure is non-invasive
48 and provides a higher number of cells with up to $5 \cdot 10^6$ cells / cm of cord [28].
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8 69 This criterion becomes important with the increasing needs of MSC for clin-
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10 70 ical trials and for a potential industrial implementation. Another advantage
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12 71 concerns the higher *in vitro* proliferative and immunomodulatory properties
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14 72 of WJ-MSC in comparison to bone marrow MSC [29, 30, 31]. Moreover, con-
15
16 73 sidering that WJ-MSC have the age of the newborn, less impact due to the
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18 74 donor age is expected. Until now, WJ-MSC have been successfully isolated
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20 75 and cultivated, in culture scales up to 800 mL [32, 33, 34, 35].

21 76 Recently, two studies explored the culture of WJ-MSC on microcarriers
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23 77 in a medium supplemented with hPL, adapted for clinical use [36, 37]. Petry
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25 78 *et al.* compared five different microcarriers (Hillex II, ProNectin F-coated,
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27 79 Plastic, Plastic-Plus and Glass-coated) in static mode and validated the dy-
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29 80 namic culture in Spinner flasks with Plastic-Plus, which gave the best growth
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31 81 and attachment performance in static mode. In parallel, de Soure *et al.* val-
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33 82 idated cell isolations and also dynamic cultures in spinner flasks with Plastic
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35 83 microcarriers coated with a culture medium supplemented with 50 % hPL.
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37 84 To our knowledge, the comparison of microcarriers has only been performed
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39 85 in static mode for WJ-MSC cultures in a hPL-supplemented medium. How-
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41 86 ever, it was previously demonstrated that MSC growth in stirred systems was
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43 87 not necessarily correlated to the growth in static microcarrier culture, and
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45 88 that early cell attachment and spreading on microcarriers was not necessar-
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47 89 ily representative of the efficiency of MSC expansion in agitated microcarrier
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49 90 cultures [38].

50 91 The objective of the present study is to determine a standardized method
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52 92 to cultivate WJ-MSC on microcarriers in a serum-free culture medium, sup-
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54 93 plemented with hPL. To do that, five animal product-free microcarriers were

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8 94 compared, both in static and dynamic modes. Two different agitation modes
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10 95 were studied: Erlenmeyer flasks mixed by an orbital agitation, and spinner
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12 96 flasks involving mechanical agitation. The best microcarrier was determined,
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14 97 regarding three criteria characterizing the different culture steps: (i) the ad-
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16 98 hesion ability, (ii) the growth rate and cell expansion, and (iii) the ease of
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18 99 detachment. In parallel, as MSC can be difficult to detach and as the filtra-
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20 100 tion step, requiring to separate cells from microcarriers, may retain a fraction
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22 101 of cells, significant errors by usual cell counter may occur. Consequently, to
23
24 102 estimate and compare the number of cells per microcarrier *in situ*, and in
25
26 103 a reproducible and objective way, a counting method has been developed,
27
28 104 providing a direct measurement without cell detachment.

105 **Material and methods**

106 *Cell source*

107 WJ-MSC were recovered from a healthy donor. The human umbilical
108 cord was collected after informed mother consent, according to the national
109 legislation regarding human sample collection, manipulation and personal
110 data protection. MSC isolation was performed using the 'explant' method:
111 The umbilical cord was rinsed with PBS, cut into thin slices (around 2 or
112 3 mm thickness), then transferred into cell culture dishes and incubated
113 in MSC expansion medium at 37°C with 5% CO₂. After migration and
114 expansion on the surface, cells were harvested and frozen in a container filled
115 with liquid nitrogen at -196°C. By concern of senescence limitation and stem
116 phenotype alteration, cells used in all cultures of the present study did not
117 exceed 5 passages. Static cell expansions were performed after 3 passages

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8 118 and cultures on microcarriers after 5 passages.

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11 119 *Culture medium*

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13 120 The basal medium used was α -MEM Eagle solution (Lonza), supple-
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15 121 mented with 5 % human platelet lysate (MultiPL'30, Macopharma), 4 mM
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17 122 glutamine (Sigma) and 1 % antibiotic-antimycotic (Gibco). According to the
18
19 123 manufacturer instructions, the hPL derived from screened normal human
20
21 124 donor platelets, was pooled in large batch sizes of 10 L and was sterilized by
22
23 125 filtration of 0.2 μm before filling in closed systems.

24
25 126 Heparin (Sigma-Aldrich) was added to this basal medium for planar static
26
27 127 cultures at a concentration of 2 IU / mL. Concerning dynamic cultures on
28
29 128 microcarriers, first cultures led to the formation of a jelly matrix precipi-
30
31 129 tate, entrapping microcarriers, even with 2 IU / mL heparin in the culture
32
33 130 medium. This gel appeared during the first days and led to high variability
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35 131 of cell adhesion and expansion. The heparin was thus found to be not enough
36
37 132 effective to avoid gel formation. Hence, it was chosen to use a mechanical
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39 133 fibrinogen-depletion instead of the increase of heparin concentration, which
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41 134 may have deleterious effects on cell expansion at higher concentrations [39].
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43 135 Similar fibrinogen-depletion methods have already been described by other
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45 136 authors [40, 41]. For the present study, the culture medium was maintained
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47 137 between 4 and 6 hours at room temperature to allow clot formation. It was
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49 138 then stored at 4°C at least 18 hours. Before fibrin gel removing, the medium
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51 139 was brought to 37°C and submitted to mechanical shocks by agitation. The
52
53 140 biogel was then physically collapsed and the precipitated fibrin was pelleted
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55 141 by centrifugation ($670 \times g$, 10 min, Jouan G 4.11). Finally, the medium was
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57 142 passed through a 0.22 μm filter.

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8 *143 Microcarrier preparation*

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10 *144* Five microcarriers were compared, including Cytodex-1 (GE Healthcare),
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12 *145* widely used for adherent dependent cell cultures, and the Pall Starter Kit
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14 *146* composed of Plastic, Plastic-Plus, Hillex II and Star-Plus microcarriers (Pall,
15
16 *147* SoloHill). Microcarriers properties and culture concentrations are presented
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18 *148* in Table 1. The different concentrations of microcarriers were calculated in
19
20 *149* order to display the same available surface for cell expansion, both in static
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22 *150* ($A = 30 \text{ cm}^2$) and dynamic ($A = 250 \text{ cm}^2$) modes . Preliminary cultures
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24 *151* showed that a coating with hPL enhanced cell attachment on microcarriers
25
26 *152* (data not shown). Only Cytodex-1 presented similar cell attachment with
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28 *153* and without hPL coating. In the present work, all microcarriers were thus
29
30 *154* coated with hPL by incubating them in pure hPL at 37°C, 80 % humidity
31
32 *155* and 5 % CO₂ during two hours. Then, microcarriers were washed with fresh
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34 *156* culture medium before cell seeding.

35
36 *157 Cell culture under static conditions*

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38 *158* After quick thawing, cells were seeded at 2000 cells / cm² in T175 flasks
39
40 *159* filled with 25 mL culture medium. The medium was changed every 2 or 3
41
42 *160* days by complete medium removing and addition of the same volume of fresh
43
44 *161* medium. Once 60-70 % of the confluence was reached, the culture medium
45
46 *162* was removed and cells were washed with DPBS before the cell detachment,
47
48 *163* using 5 mL TrypLE (Gibco) per flask. TrypLE is composed of recombinant
49
50 *164* cell-dissociation enzymes that replace trypsin. Incubation did not exceed
51
52 *165* 5 min and the enzymatic digestion was stopped by adding 20 mL of fresh
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54 *166* medium. The suspension was then centrifuged at $130 \times g$ during 5 min,
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56 *167* the supernatant was removed and the pellet was dispersed in fresh culture

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8 168 medium. A filtration step was performed with 40 μm filters, in order to elim-
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10 169 inate potential cell aggregates. 1 mL of the suspension was finally collected
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12 170 to determine the cell number and cell viability.

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14 171 *Cell culture on microcarriers in static mode*

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16 172 Six well plates with ultra-low attachment surface (Corning) were used to
17
18 173 perform static cultures on microcarriers. After addition of the microcarriers
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20 174 at the bottom of the well, cells were seeded at a concentration of 3×10^5 cells
21
22 175 / well in a working volume of 5 mL. After 5 days, the supernatant was
23
24 176 removed and microcarriers were washed with DPBS. Cell detachments were
25
26 177 finally performed by using 1 mL of TrypLE. A complementary agitation can
27
28 178 be applied to promote cell detachment and to reduce the incubation time,
29
30 179 as proposed by Nienow *et al.* [42]. An orbital agitation of 75 rpm (orbital
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32 180 diameter $d_o = 10$ mm) was applied during 5 min, and 5 mL of fresh medium
33
34 181 was then added to stop the enzymatic digestion. Cells were finally separated
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36 182 from microcarriers with a 70 μm filtration step.

37 183 *Cell culture on microcarriers in agitated mode*

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39 184 The following protocol was established from preliminary cultures per-
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41 185 formed using both Cytodex-1 and Star-Plus microcarriers, in order to deter-
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43 186 mine the suitable operating conditions for cell growth comparison on different
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45 187 microcarriers. Two stirred culture systems were used: (i) Erlenmeyer flasks
46
47 188 and (ii) Spinner flasks. Properties of the two systems are presented in Table
48
49 189 2. Cells were recovered from planar cultures at around 70 % of the confluence
50
51 190 and were passed through a 40 μm filter to trap potential aggregates before
52
53 191 seeding. 70 % of the confluence was found to be a good compromise to recover

Table 1: Microcarrier physical properties (based on manufacturer data) and concentrations used in this study.

Name	Manufacturer	Diameter range (μm)	Density (g mL^{-1})	Static culture concentration (g L^{-1})	Dynamic culture concentration (g L^{-1})
Cytodex-1	GE Healthcare	147 - 248	1.03	1.4*	2.35*
Plastic	Pall SoloHill	125 - 212	1.02	15	N/A
Plastic-Plus	Pall SoloHill	125 - 212	1.02	15	25
Star-Plus	Pall SoloHill	125 - 212	1.02	15	25
Hillex II	Pall SoloHill	160 - 200	1.1-1.2	21	34.2

*Cytodex-1 concentrations are given according to the particle dry weight.

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8 192 enough cells while avoiding the formation of aggregates. After counting, cells
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10 193 were inoculated at a concentration of $0.8 \cdot 10^6$ cells / mL, namely $2 \cdot 10^6$ cells
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12 194 per flask. In Erlenmeyer flasks, a gentle agitation was applied to homoge-
13
14 195 nize cell and microcarrier suspension, and cultures were let without agitation
15
16 196 during one hour to allow cell adhesion. Agitation rate was then fixed at the
17
18 197 minimal agitation rate ensuring all particle suspension $N = N_{js} = 75$ rpm
19
20 198 ($d_o = 10$ mm). In Spinner flasks, cultures performed at $N = N_{js}$ led to cell
21
22 199 lysis. It was thus decided to let cultures without agitation during one hour
23
24 200 with gentle agitations at the beginning and at the mid-point, before working
25
26 201 at a low agitation rate $N = 40$ rpm, at which a sufficient number of particles
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28 202 were suspended without major hydromechanical stress [42]. Both cultures
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30 203 performed in Erlenmeyer and spinner flasks were limited to 5 days, with 50
31
32 204 % medium exchange performed at $t_0 + 3d$ and $t_0 + 4d$, in order to ensure a
33
34 205 sufficient glucose concentration for cell expansion and to reduce the lactate
35
36 206 concentration. At the end of the culture, the supernatant was removed and
37
38 207 particles were washed with DPBS. 10 mL of TrypLE were then added with a
39
40 208 complementary agitation during 5 min. Fresh medium was used to stop the
41
42 209 enzymatic digestion and cells were separated from microcarriers with a 70
43
44 210 μm filtration step. Cell number and viability were determined before being
45
46 211 frozen. Microcarriers were recovered from filters and microscopic analyses
47
48 212 were performed to determine the number of cells remaining attached.

213 *Detached cells counting*

214 After each detachment, cell number and cell viability were determined
215 from cell suspensions, using the Vi-CellTM Cell Viability Analyzer Counter
216 (Beckman Counter), based on a Trypan Blue Dye Exclusion method.

Table 2: Stirred systems and agitation conditions chosen for cultures in dynamic mode.

	Bioreactor	
	Erlenmeyer flask	Spinner flask
Manufacturer	Fisherbrand (Fisher Scientific)	DWK Life Sciences Inc. (Wheaton)
Mixing mode	Orbital	Mechanic
Material	Plastic (single-use)	Siliconized glass
Diameter (mm)	66	40
$V_{working}$ (mL)	25	25
N (rpm)	75	40

217 *In situ counting of cells adhered on microcarriers*

218 A dedicated post-treatment was developed to dispose of a robust and
 219 quantitative method to directly count MSC cells attached on microcarriers,
 220 without detachment step. To do that, samples of 200 μL were recovered from
 221 homogeneous particle suspensions. After supernatant removing for metabo-
 222 lite analysis, cells were stained using a DAPI-methanol solution of 1 $\mu\text{g} / \text{mL}$
 223 (DAPI from Sigma) during 15 min at room temperature. Cells were then
 224 washed with DPBS and microscopic analyses were performed. At least 5
 225 photos per operating condition were recorded and loaded in a Matlab script
 226 for post-treatment. First, microcarriers were identified by contour recog-
 227 nition and their coordinates were recorded. Secondly, based on the simple
 228 colour detection by the hue function of Matlab, a mask was applied in or-
 229 der to only keep the blue elements concerning cell nuclei after the DAPI
 230 staining. After contrast adjustments, a binarization was performed and cell

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8 231 coordinates were compared to microcarrier coordinates in order to get the
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10 232 number of cells per microcarrier. Cytodex-1 and Hillex II microcarriers are
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12 233 transparent and were found to allow blue light to pass through the material.
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14 234 In this way, cells adhered in the whole bead surface were counted. On the
15
16 235 contrary, Plastic, Plastic-Plus and Star-Plus microcarriers were found opaque
17
18 236 and only cells present in half of the bead surface were counted. Furthermore,
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20 237 a half-face found empty did not necessarily mean that there was no cell on
21
22 238 the other side. Consequently, in order to predict the number of cells on the
23
24 239 non-visible surface, a Poisson distribution was integrated in the routine. The
25
26 240 routine finally provided the following information: (i) The mean number of
27
28 241 cell per microcarrier $\langle N_{cell/\mu C} \rangle$, (ii) the distribution of the number of cell
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30 242 per microcarrier, (iii) the standard deviation of this distribution σ , and (iv)
31
32 243 the percentage of colonized microcarriers x_{colo} , namely the fraction of parti-
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34 244 cles containing at least one cell. This method based on image analysis was
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36 245 preliminary validated by cell visual counting.

36 246 *Cell morphology investigation*

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38 247 As for cell counting, samples of 200 μL were recovered from a homoge-
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40 248 neous particle suspension. After removing the supernatant, cells were stained
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42 249 using a Live / Dead Cell double staining kit (Sigma) during 30 min at 37°C.
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44 250 It was composed of calcein-AM for living cell membrane staining, and ethid-
45
46 251 ium homodimer for dead cell nuclei staining. Microscopic analyses were then
47
48 252 performed in order to qualitatively determine cell morphology. This oper-
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50 253 ation was performed at $t_0 + 2$ days in order to ensure cell attachment and
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52 254 their final morphology, while no doubling population already occurred. The
53
54 255 objective was to distinguish round from spreaded cells.

256 *Metabolites analysis*

257 Everyday, glucose, lactate, glutamine, ammonia and lactate deshydro-
258 genase (LDH) concentrations were measured from supernatant samples col-
259 lected throughout the cultures, by using the Gallery multiparametric analyzer
260 (Thermo Fisher Scientific). Glucose (GOD-POD), L-Lactic Acid and LDH
261 (SCE) kits from Thermo Fisher Scientific and Glutamine V2 Bio and NH₃
262 Bio kits from Roche were used.

263 *Kinetic parameters calculation*

264 Based on the previous experimental measurements, different parameters
265 were calculated to characterize cell culture kinetics. An expansion factor
266 F^{exp} , expressed in Eq. (1), used data from cell counting by image analysis
267 and compared the number of cell produced between $t_0 + 2$ days and $t_0 + 5$
268 days. Also based on image analysis results, the distribution of the number
269 of cell per microcarrier was determined and the corresponding coefficient of
270 variation c_v was calculated according to Eq. (2), with σ the standard devia-
271 tion presented in Eq. (3). In this last parameter, $N_{cell/\mu C}(i)$ corresponds to
272 the number of cell counted on the i^{th} microcarrier and n represents the total
273 number of microcarriers treated by image analysis. Two parameters based
274 on metabolite concentrations were finally calculated. In one hand, Y_{Glu} yield
275 was defined according to the Eq. (4), and described the concentration of
276 glucose consumed on the cell concentration production, from $t_0 + 2$ days to
277 $t_0 + 5$ days, with $\Delta_{2 \rightarrow 5} C_{Glucose}^{consumption}$ corresponding to the cumulative glucose con-
278 sumption between $t_0 + 2$ days and $t_0 + 5$ days, $\Delta_{2 \rightarrow 5} \langle N_{cell/\mu C} \rangle$ for the mean
279 number of cell produced between $t_0 + 2$ days and $t_0 + 5$ days, and $C_{\mu C/L}$ the
280 microcarrier concentration in the system. In the other hand, the Lac/Glu

ratio was defined as the cumulative lactate production on the cumulative glucose consumption during the whole culture.

$$F^{exp} = \frac{\langle N_{cell/\mu C} \rangle_{t_0+5d} - \langle N_{cell/\mu C} \rangle_{t_0+2d}}{\langle N_{cell/\mu C} \rangle_{t_0+2d}} \quad (1)$$

$$c_v = \frac{\sigma}{\langle N_{cell/\mu C} \rangle} \cdot 100 \quad (2)$$

$$\sigma = \sqrt{\frac{1}{n-1} \sum_{i=1}^n (N_{cell/\mu C}(i) - \langle N_{cell/\mu C} \rangle)^2} \quad (3)$$

$$Y_{Glu} = \frac{\Delta_{2 \rightarrow 5} C_{Glucose}^{consumption}}{\Delta_{2 \rightarrow 5} \langle N_{cell/\mu C} \rangle \cdot C_{\mu C/L}} \quad (4)$$

Phenotypic analysis

After trypsinization, cells were frozen before the flow cytometry analyses. They were then quickly thawed with fresh culture medium, pre-warmed at 37°. After centrifugation, cells were re-suspended in DPBS and their viability was validated using a Live / Dead kit assay. Cells were then incubated into DPBS-BSA 0.5 % (Gibco, Sigma-Aldrich) during 15 minutes and double marks were realized using antibodies targeting the specific antigen coupled with Phycoerythrin (PE) or Fluorescein isothiocyanate (FITC) during 45 minutes, at room temperature and out of direct light. Table 3 summarizes the antibodies used in this study. Cells were finally washed in DPBS and suspended in 300 μ L DPBS, before the analysis using the flow cytometer Gallios (Beckman Coulter, Brea, CA, USA). The number of positive cells was obtained using the Kaluza software (Beckman Coulter, Brea, CA, USA).

Table 3: List of antibodies used for the flow cytometry analyses.

Antigen	Fluorophore	Manufacturer	Reference	Species	Isotype
Control isotype	FITC	DAKO	X0927	Mouse	IgG1 κ
Control isotype	PE	DAKO	X0928	Mouse	IgG1 κ
CD 90	FITC	Beckman Coulter	IM1838U	Mouse	IgG2a
CD 34	PE BD	Pharmingen	555882	Mouse	IgG1 κ
HLA-DR	FITC	Beckman Coulter	IM0463U	Mouse	IgG2b
CD 73	PE BD	Pharmingen	550257	Mouse	IgG1 κ
CD 45	FITC	DakoCytomation	F0861	Mouse	IgG1 κ
CD 166	PE	Beckman Coulter	A22361	Mouse	IgG1
CD 44	FITC	Beckman Coulter	IM1219U	Mouse	IgG1

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8 296 *Multilineage differentiation studies*

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10 297 Cell multipotency was validated by using StemPro Differentiation Kits
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12 298 (Gibco).

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14 299 *Adipogenesis differentiation*

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16 300 As recommended by the manufacturer, cells were seeded at 10,000 cells
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18 301 / cm² on 24-well plates with 1 mL of culture medium. After 24 h, culture
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20 302 medium was replaced with pre-warmed Complete Adipogenesis Differentia-
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22 303 tion Medium and re-feed every 3 days. After at least 40 days of culture, cells
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24 304 were washed with DPBS and fixed in a 4 % formaldehyde solution during
25
26 305 30 min. Thereafter, cells were twice washed with distilled water and incu-
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28 306 bated with Oil Red-O solution (0.5 % in isopropanol) at room temperature
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30 307 for 15 min. Cells were finally rinsed with distilled water before microscopic
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32 308 visualization.

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34 309 *Osteogenesis differentiation*

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36 310 As recommended by the manufacturer, cells were seeded at 5,000 cells
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38 311 / cm² on 24-well plates with 1 mL of culture medium. After 24 h, culture
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40 312 medium was replaced with pre-warmed Complete Osteogenesis Differentia-
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42 313 tion Medium and re-feed every 3 days. After at least 40 days of culture, cells
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44 314 were washed with DPBS and fixed in a 4 % formaldehyde solution during
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46 315 30 min. Thereafter, cells were twice washed with distilled water and stained
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48 316 with 2 % Alizirin Red S solution at room temperature for 2-3 min. Cells
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50 317 were finally rinsed with distilled water before microscopic visualization.

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8 318 *Chondrogenesis differentiation*

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10 319 Micromass cultures were performed in 24-well plates by seeding 5 μL
11 320 droplets of a cell solution of 1.6×10^7 viable cells / mL. After 1 night in high
12 321 humidity conditions, warmed chondrogenesis media was added and replaced
13 322 every 3 days. After at least 40 days of culture, cells were washed with DPBS
14 323 and fixed in a 4 % formaldehyde solution during 30 min. Thereafter, cells
15 324 were twice washed with distilled water and incubated with 1 % Alcian Blue
16 325 solution prepared in 0.1 HCl at room temperature for 30 min. Cells were
17 326 finally rinsed three times with 0.1 HCl and distilled water before microscopic
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27 328 *Data statistical analysis*

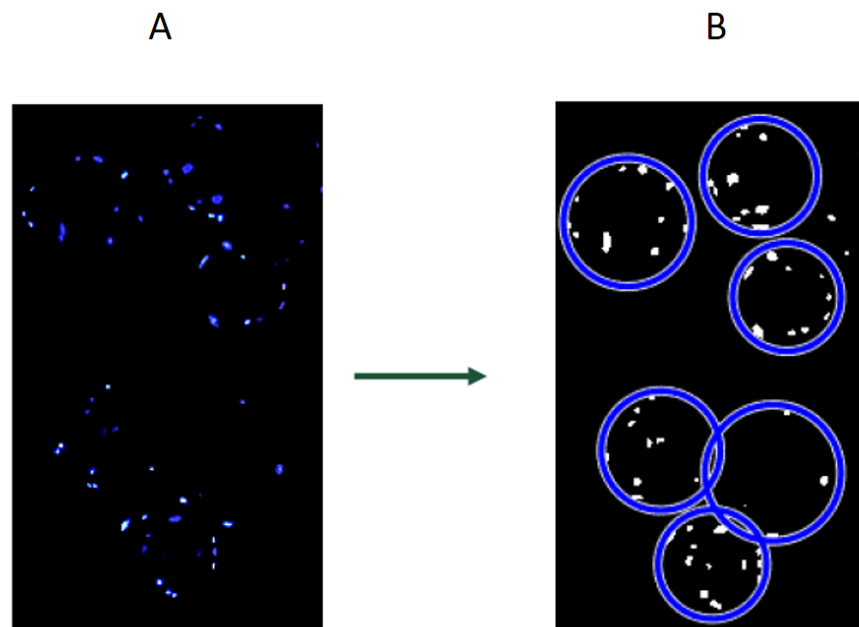
28
29 329 A one-way analysis of variance (ANOVA) test was used with a Fisher's
30 330 Least Significant Difference (LSD) test admitting significance at *p-value* <
31 331 0.05 to determine if the different groups were significantly different. For
32 332 static cultures, standard deviations were calculated according to data from
33 333 cultures performed in parallel ($n=3$). Concerning dynamic cultures, standard
34 334 deviations were calculated according to data of different cultures ($n=2$).
35 335 Higher variability may be thus expected than with cultures performed in the
36 336 same time, with the same inoculum.

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45 337 **Results and discussion**

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47 338 *In situ cell counting on microcarriers validation*

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49 339 Our original method to count cells *in situ* on microcarriers, without pre-
50 340 liminary detachment, was first validated. Based on image analyses, cell nuclei

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8 341 emission from DAPI staining was recorded to distinguish and count cells on
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10 342 584 microcarriers (18 photos processing). Thresholds for blue colour detec-
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12 343 tion were validated with no interference, for each microcarriers, as shown in
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14 344 Fig. 1. Cell detection and counting were validated with 5 % mean deviation
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16 345 with the human visual counting, as presented in Fig 2. The method provided
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18 346 robust results up to 15 cells per microcarrier, which occurred in the photo
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20 347 displaying a mean number of 10 cells per microcarriers.



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Figure 1: Example of photo treatment by image analysis after DAPI staining and microcarriers recognition: Contrast adjustment and blue color isolation (A), and microcarrier detection and cell binarization and counting (B).

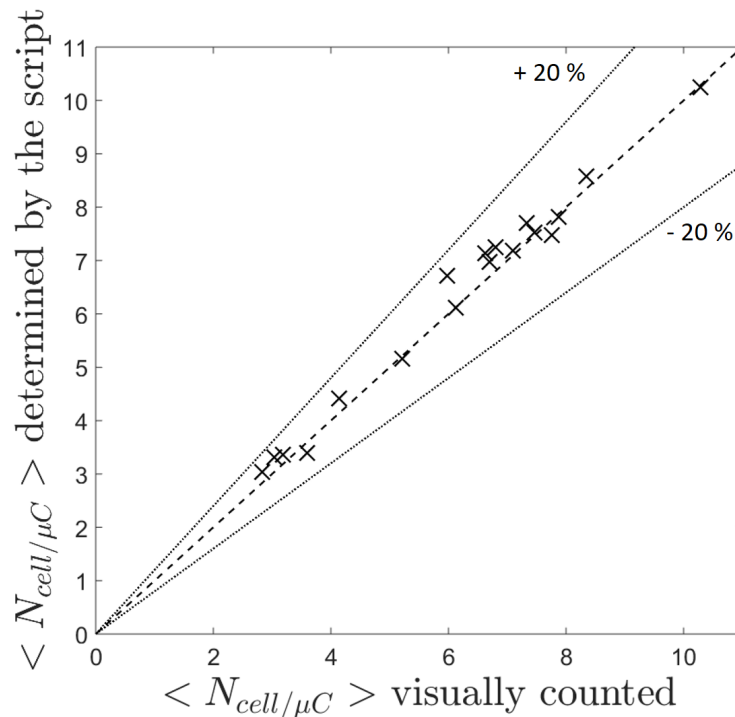


Figure 2: Comparison of the mean cell number per microcarrier determined by automatic post-treatment and by visual counting.

348 *Cell attachment and expansion on microcarriers*

349 The number of cells attached at the beginning of the culture is closely
 350 linked to the cell expansion, since cell expansion latency may occur if a too
 351 low number of cells is adhered. However, a microcarrier presenting lower
 352 attachment capability than another may present a better expansion during
 353 the culture, and *vice versa*. Consequently, attachment and expansion are
 354 complementary. In the present study, cell adherence was described accord-
 355 ing to four parameters calculated during the first days of the cultures when
 356 possible: (i) the mean number of cells per microcarrier $\langle N_{cell/\mu C} \rangle$, (ii)

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8 357 the percentage of inoculated cells who adhered, (*iii*) the coefficient of vari-
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10 358 ation c_v characterizing the cell number distribution (Eq. (2)) and (*iv*) the
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12 359 percentage of colonized microcarriers x_{colo} . Four additional parameters were
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14 360 also calculated to describe cell expansion: (*i*) Y_{Glu} (Eq. (4)), (*ii*) F^{exp} (Eq.
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16 361 (1)), (*iii*) LDH concentration kinetics, and (*iv*) Lac/Glu ratios. However,
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18 362 probably related to a lack of place, MSC tended to gather together till some
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20 363 aggregates formation during longer cultivation durations. In some cases,
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22 364 microcarriers previously colonized were recovered empty, whereas a minor-
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24 365 ity presented over-colonizations, as already reported by Ferrari *et al.* with
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26 366 porcine bone marrow MSC [43]. Moreover, in dynamic mode, cell attach-
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28 367 ment was observed at $t_0 + 48$ h in order to ensure cell adhesion and final
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30 368 morphology. During this duration, cell division was indeed negligible, with
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32 369 no population doubling, allowing to discuss on cell adhesion at the beginning
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34 370 of the cultures.

371 *Cell attachment and expansion comparison in static mode*

372 Without agitation, cells adhered on microcarriers may be found close one
373 to each other with cell aggregates in some cases, making impossible the use
374 of the *in situ* cell counting method. Consequently, microscopic analyses pro-
375 vided qualitative data and kinetics of metabolite concentration were used
376 to quantitatively compare expansion performances. Different cell population
377 behaviours and distributions on microcarriers were observed according to the
378 type of microcarrier. Concerning Cytodex-1 and Plastic-Plus, cell bridges
379 formed between microcarriers, as shown in Fig. 3 (A) and (D). Hence, het-
380 erogeneous cell distributions were observed at the end of the culture, with
381 some microcarriers full of cells and others without cells in the case of Plastic-

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8 382 Plus, or with only one or two cells in the case of Cytodex-1. All Cytodex-1
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10 383 were nevertheless colonized with at least one cell. Some cell colonies were
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12 384 observed on Plastic microcarriers, but the number of cells on microcarriers
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14 385 was significantly lower, as presented in Fig. 3 (C). Star-Plus and Hillex II
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16 386 qualitatively presented more homogeneous distributions without aggregates
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18 387 (Fig. 3 (D) and (E)).

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19 388 Glucose consumption and lactate production kinetics showed that Cytodex-
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21 389 1, Star-Plus and Hillex II microcarriers led to higher cell expansions (Fig. 4).
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23 390 These microcarriers presented better fractions of colonized particles, which
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25 391 seemed to globally impact the cell expansion. On the contrary, Plastic mi-
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27 392 crocarriers were notably unsuitable for MSC culture in comparison to other
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29 393 microcarriers regarding the low cell expansion after 5 days of culture and the
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31 394 LDH concentration. LDH concentration was indeed higher for the culture
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33 395 using Plastic microcarriers than with others, despite a lower number of cells
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35 396 observed on particles. This confirmed a lower cell viability in comparison to
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37 397 other microcarriers. Only Cytodex-1, Star-Plus, Plastic-Plus and Hillex II
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39 398 microcarriers were thus selected for the dynamic cultures.

399 *Cell attachment and expansion comparison with an orbital agitation*

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41 400 Cultures in Erlenmeyer flasks were performed without agitation during
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43 401 the first two hours. Then, a continuous orbital agitation was applied to en-
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45 402 sure particle suspension, but gathering most of particles inward. They led
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47 403 to different cell attachments depending on the type of microcarrier used.
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49 404 As presented in Fig. 5, Cytodex-1 presented the best capability with 59
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51 405 % of inoculated cells adhered on microcarriers surfaces, whereas Star-Plus
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53 406 and Plastic-Plus presented lower values with 20 % and 37 % respectively.

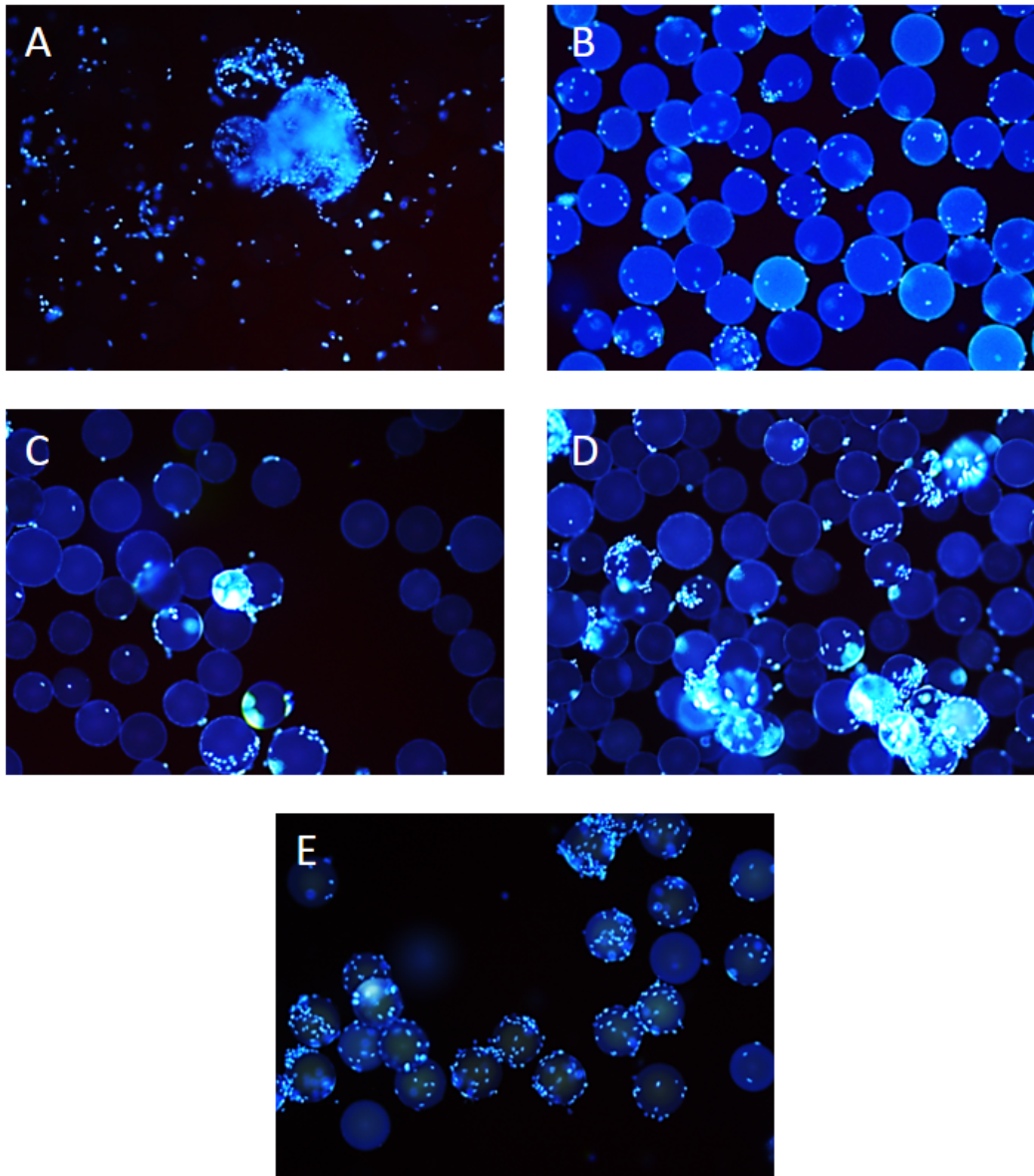


Figure 3: Cell nuclei DAPI coloration at $t_0 + 5$ days in static mode: Cytodex-1 (A), Star-Plus (B), Plastic (C), Plastic-Plus (D) and Hillex II (E) microcarriers.

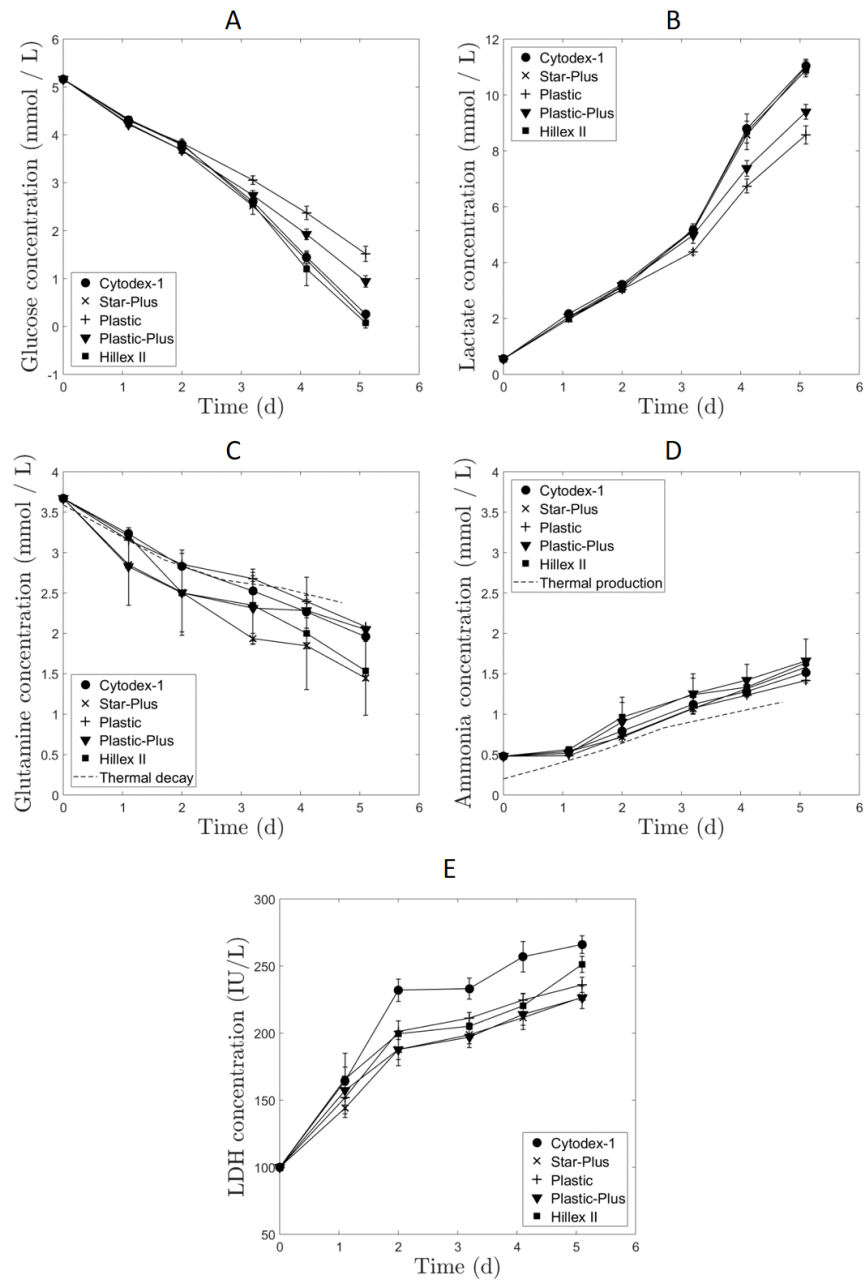


Figure 4: Kinetics of metabolite concentrations over time in static mode and according to the type of microcarrier used: Glucose (A), lactate (B), glutamine (C), ammonia (D) and LDH (E) ($n = 3$).

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8 407 Concerning Hillex II, cell adhesion was found to be very low with only 9
9 408 % of adhered cells. The percentage of colonized microcarriers x_{colo} , repre-
10 409 sented in Fig. 6, was found to be related to the number of cells, with higher
11 410 x_{colo} obtained with cultures displaying more attached cells. Fig. 7 showed
12 411 that despite different mean cell numbers per microcarriers, the coefficients
13 412 of variation of the cell distributions c_v remained similar for Cytodex-1, Star-
14 413 Plus and Plastic-Plus, displaying tighter distributions than for Hillex II. Cell
15 414 spreading was notably found larger and similar to fibroblasts for cells at-
16 415 tached on Cytodex-1, as presented in Fig. 8. On the contrary, a significant
17 416 fraction of cells adhered on the other microcarriers displayed round mor-
18 417 phologies. Cell-microcarrier interactions are dependent on the microcarrier
19 418 material and should be strong enough to maintain cells attached when sub-
20 419 jected to hydromechanical stress.

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31 420 After 5 days of culture, a higher number of cells was observed on Cytodex-
32 421 1, as shown in Fig. 5, with at least 2.4 more cells than on other microcarri-
33 422 ers. No cell expansion was observed using Plastic-Plus and Hillex II, making
34 423 them unsuitable for cultures in Erlenmeyer flasks. In order to quantitatively
35 424 compare expansion performances, the expansion factor F^{exp} , the cumulative
36 425 glucose consumption Y_{Glu} (Eq. (4)) and the Lac/Glu ratio were calculated
37 426 for each microcarrier and presented in Table 4. A higher expansion factor
38 427 was found with Star-Plus. Hence, despite Cytodex-1 presented the best cell
39 428 attachment capability, cell expansion performance was found better using
40 429 Star-Plus microcarriers. As cell expansion is indeed limited by the free sur-
41 430 face on microcarriers, a fraction of Cytodex-1 may already contain a too high
42 431 number of cells from the beginning of the culture, that may reduce the sub-

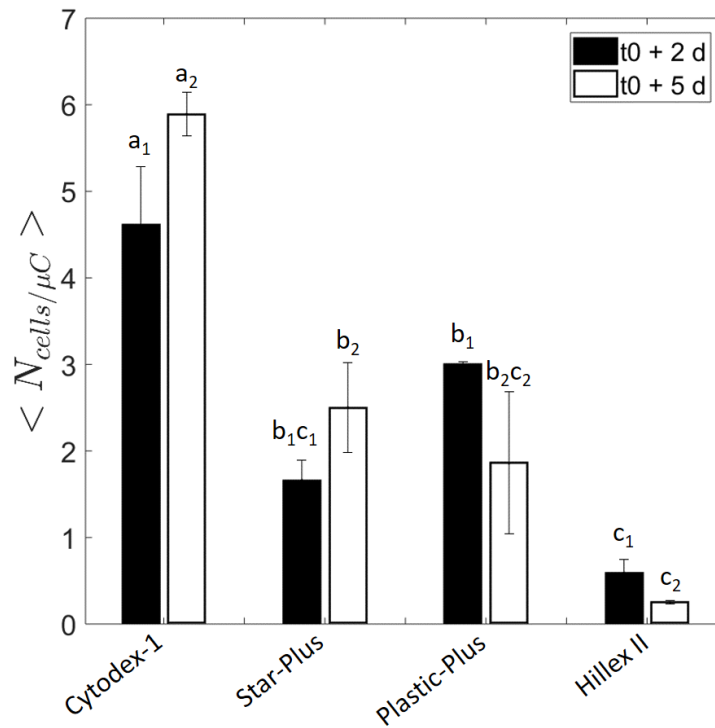


Figure 5: Mean number of cells per microcarrier cultivated with an orbital agitation and according to the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

strate kinetics due to an early confluence.

The mean number of cells may also be related to the metabolite / substrate kinetics, presented in Fig. 9. As expected with a low cell adhesion, Hillex II presented low expansion performances. The mean number of cells per microcarrier, as well as the metabolite kinetics, declined, traducing cell death or detachment and limited cell proliferation. Regarding the LDH concentration, significantly more LDH was produced using Hillex II with 186 IU L⁻¹, in comparison to 169, 161 and 162 IU L⁻¹ for respectively Cytodex-1, Star-Plus and Plastic-Plus, before the first culture medium exchange

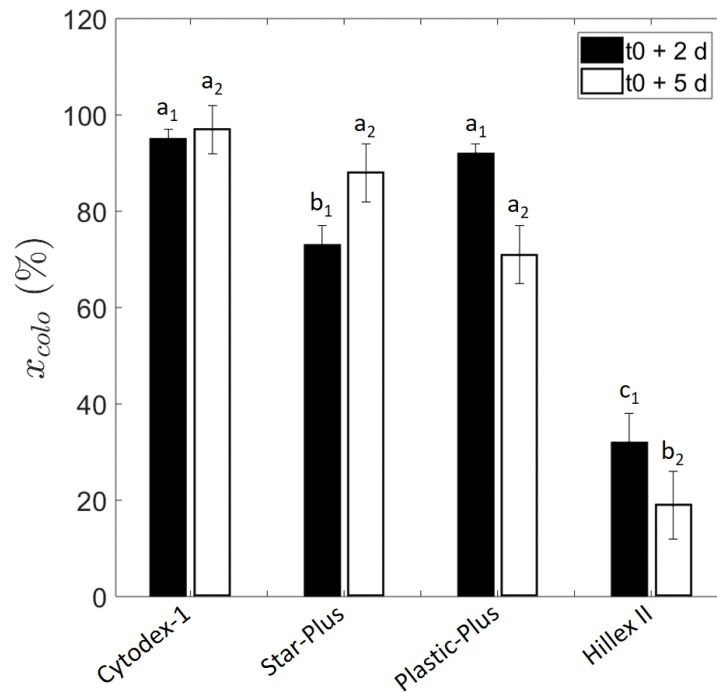


Figure 6: Fraction of microcarriers containing at least one cell in culture with an orbital agitation and according to the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

(Fig. 9 (E)), whereas the total number of cells was found to be the lowest. It may be thus assumed that cell viability declined during the first days of the cultures. On the other hand, Cytodex-1 presented the highest Y_{Glu} yield of $2.2 \cdot 10^{-10} \text{ mol cell}^{-1}$ in comparison to $1.4 \cdot 10^{-10} \text{ mol cell}^{-1}$ calculated with Star-Plus. Due to a higher number of cells, the LDH concentrations was notably expected to be higher after 5 days of cultures (Fig. 9 (E)). Star-Plus presented a higher number of cells than on Plastic-Plus at $t_0 + 2$ days and thus led to a higher metabolic activity during the first days. However, at $t_0 + 5$ days, the mean number of cells on Plastic-Plus declined despite a metabolic

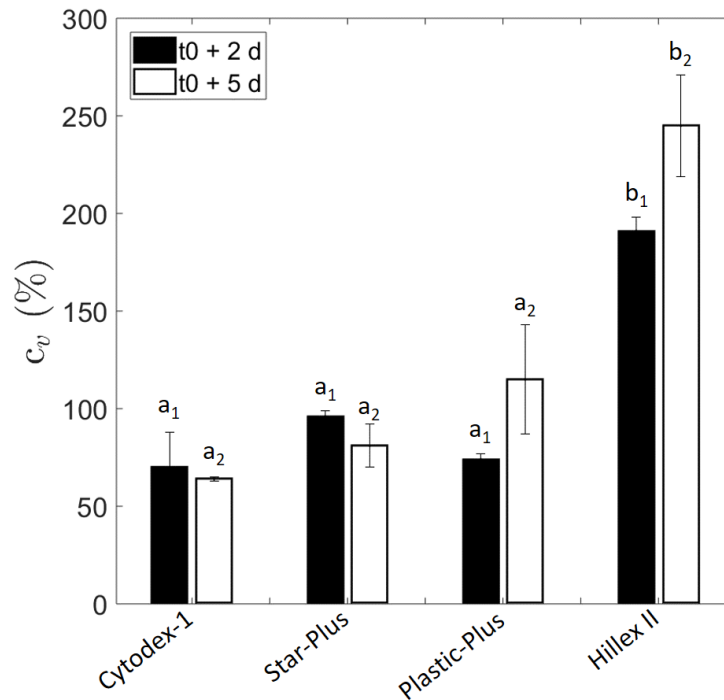
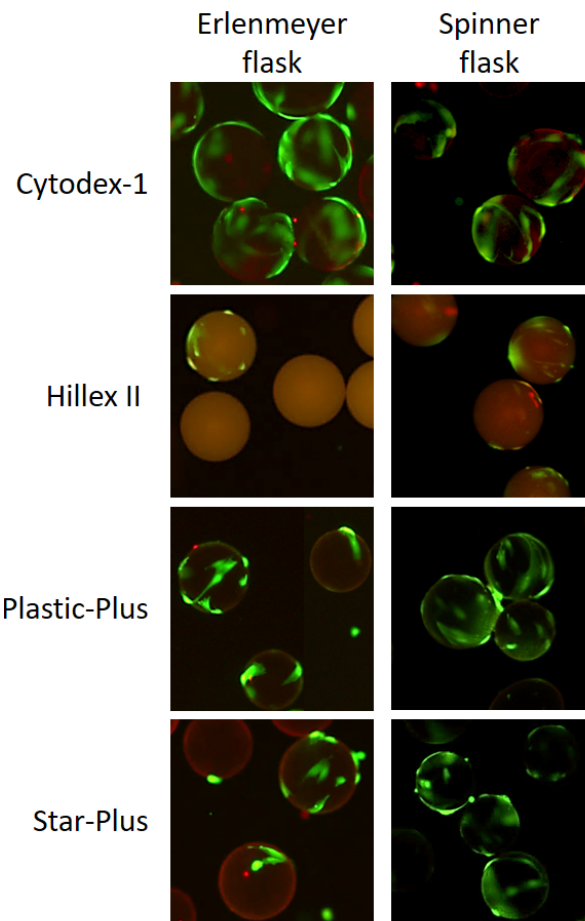


Figure 7: Coefficient of variation of the distribution characterizing the number of cell per microcarrier with an orbital agitation and according the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

450 activity. It may be supposed that a fraction of viable cell remained in sus-
 451 pension, without attachment possibility on microcarriers. However, cells in
 452 suspension would not be taken into account by the *in situ* counting method.
 453 Cell distribution on microcarriers at $t_0 + 2$ days and $t_0 + 5$ days are presented
 454 in Fig. 7. c_v remained similar over time, except for Hillex II. In this case,
 455 the deviation became significantly higher and may be related to the decrease
 456 of the colonized microcarrier fraction (Fig. 6).
 457 Interesting results were obtained with *Lac/Glu* ratios, presented in Table 4.
 458 Values over 2 indicated the use of another carbon source than glucose into the



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Figure 8: Cell morphology grown on microcarriers at $t_0 + 2$ days according to the type of microcarrier and the agitation mode. Green staining for living cell membranes and red staining for dead cell nuclei.

44 different metabolic pathways, such as glutamine that may be a second source
45 of carbon and energy. In static cultures performed without glutamine, cells
46 exhibited round morphologies and finally detached after a few days, without
47 proliferation (data not shown). It was thus observed that glutamine was nec-
48 essary for cell expansion. Hence, glutamine may be used by cells, even in low
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464 concentration and with limited cell respiratory activity. Such results were
 465 already reported in consistent other studies for Madin Darby Canine Kidney
 466 (MDCK) cells and Vero cells [44, 45]. So, the same metabolic adaptation
 467 may occur with WJ-MSA.

Table 4: Kinetic parameters calculated for cultures involving an orbital agitation and according to the type of microcarrier used ($n = 2$).

Microcarrier	F^{exp} (-)	Y_{Glu} $\times 10^{-10}$ (mol cell $^{-1}$)	Lac/Glu (g gl $^{-1}$)
Cytodex-1	0.28	2.2	2.0
Star-Plus	0.51	1.4	2.0
Plastic-Plus	-0.38	N/A	2.1
Hillex II	-0.58	N/A	1.7

468 *Cell attachment and expansion comparison with a mechanical agitation*

469 Cell cultures in spinner flasks started with one hour without agitation,
 470 except a gentle agitation at mid-time to promote cell homogenization on mi-
 471 crocarriers. Particles were then dispersed by a mechanical agitation. In this
 472 system, cell attachment was similar for Cytodex-1, Star-Plus and Plastic-
 473 Plus microcarriers with 3.6 to 3.7 cells per microcarrier at $t_0 + 2$ days, as
 474 presented in Fig. 10, and cell displayed similar fibroblast-like morphologies,
 475 as shown in Fig. 8. On the contrary, only 1.8 cells per microcarrier were
 476 counted on Hillex II microcarriers, with 30 % empty microcarriers as pre-
 477 sented in Fig. 11. As a consequence, cells on Hillex II presented a more
 478 heterogeneous distribution than on Cytodex-1 and Plastic-Plus, with $c_v =$
 479 105 %, whereas 68 to 80 % were obtained with the other microcarriers (Fig.

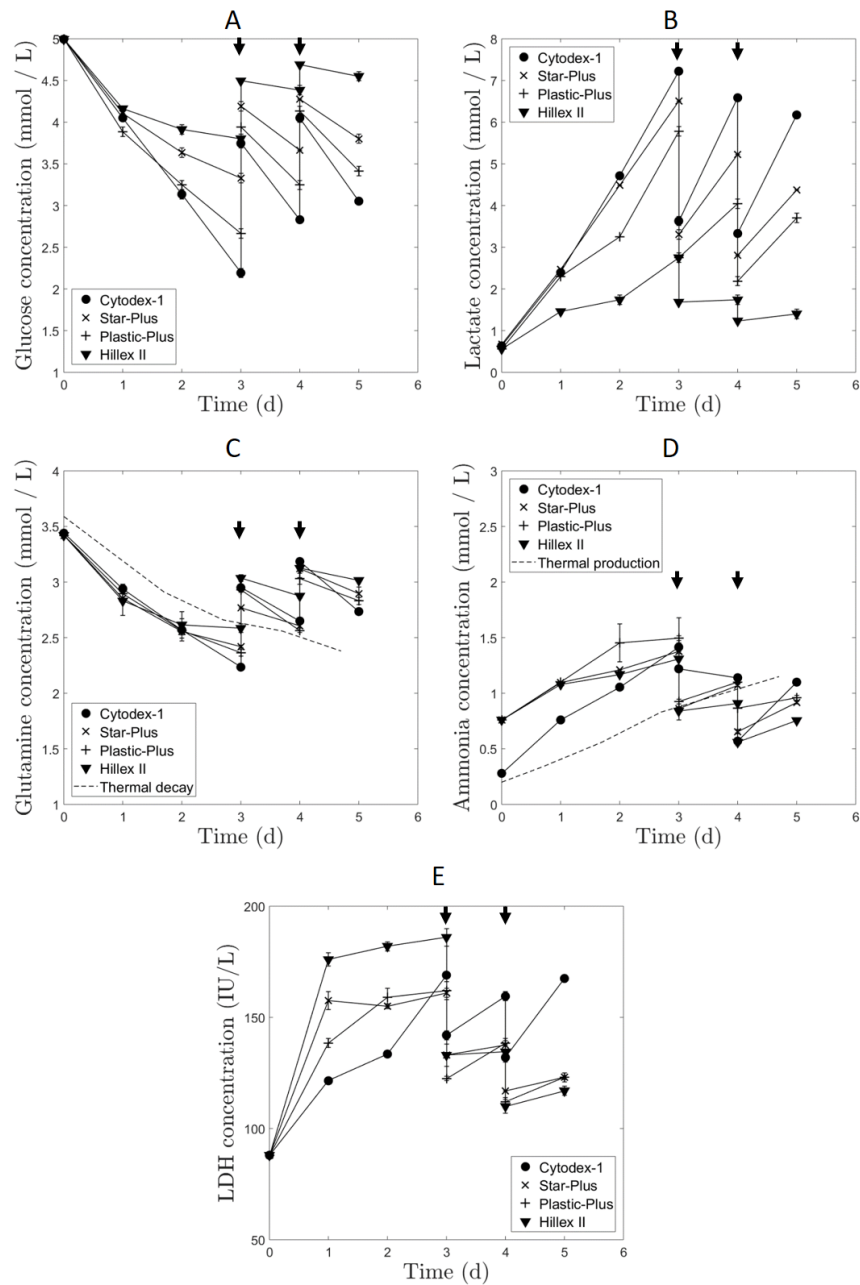


Figure 9: Kinetics of metabolite concentrations during cultures stirred with an orbital agitation and according to the type of microcarrier used: Glucose (A), lactate (B), glutamine (C), ammonia (D) and LDH (E). Vertical arrows correspond to an exchange of 50 % culture medium ($n = 2$).

480 12).

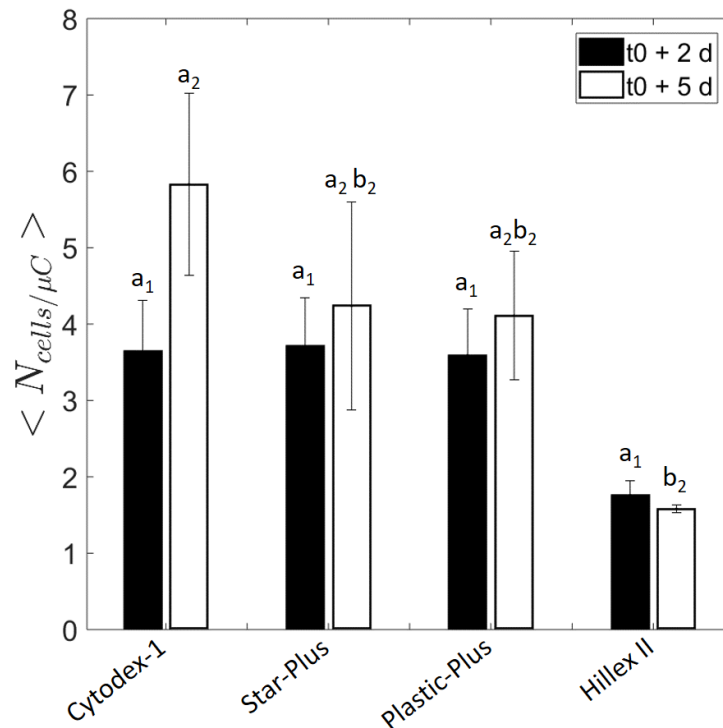


Figure 10: Mean number of cells per microcarrier cultivated with a mechanical agitation and according to the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

481 In addition to the low cell attachment observed on Hillex II, no cell ex-
 482 pansion was noticed at $t_0 + 5$ days on Fig. 10. Cells adhered on Hillex II
 483 notably displayed more round morphologies than on other microcarriers, as
 484 shown in Fig. 8. Concerning the three other microcarriers, similar mean
 485 numbers of cells per microcarrier at $t_0 + 5$ days were observed. However, the
 486 kinetics of metabolite concentrations, showed that cells used more glucose in
 487 cultures with Plastic-Plus microcarriers and more glutamine in cultures with

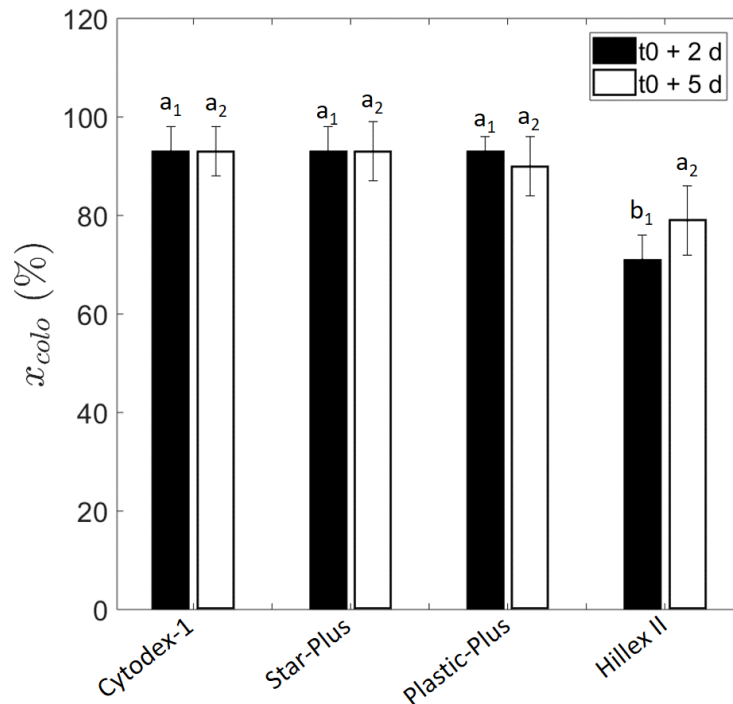


Figure 11: Fraction of microcarriers containing at least one cell in culture with a mechanical agitation and according to the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

488 Cytodex-1 (Fig. 13), despite no significant difference was observed on the
 489 mean number of cells per microcarriers on Cytodex-1, Star-Plus and Plastic-
 490 Plus at $t_0 + 2$ days and $t_0 + 5$ days. Hence, the metabolic activity, related to
 491 viable cells, was higher for cultures carried out with these two microcarriers,
 492 but with different metabolic pathways, involving different carbon sources for
 493 cell expansion. As for cultures in Erlenmeyer flasks, *Lac/Glu* ratios, and
 494 displaying values over 2, led to the hypothesis that glutamine was used by
 495 cells and led to the production of more lactate (Table 5). Here, this assump-
 496 tion was notably supported by the results on glutamine consumption with

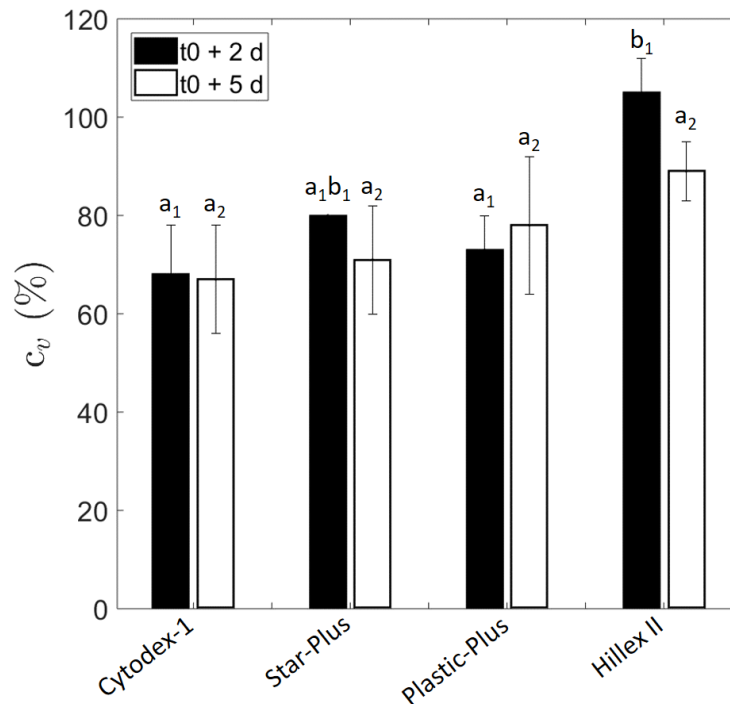


Figure 12: Coefficient of variation of the distribution characterizing the number of cell per microcarrier with a mechanical agitation and according to the type of microcarrier at $t_0 + 2$ days (black bars) and $t_0 + 5$ days (white bars) ($n = 2$).

497 Cytodex-1.

498 It should nevertheless be noted that after 5 days, some broken Cytodex-1
 499 were observed on microscopic analyses. The fraction of broken microcarriers
 500 remained low but may be expected to increase for longer cultures, and should
 501 thus be taken into account for cell quality. No broken particles were found in
 502 cultures performed with Star-Plus, Plastic-Plus and Hillex II, showing that
 503 these microcarriers are more robust in stirred systems than Cytodex-1.

504 To summarize the results related to the cell attachment and expansion
 505 criteria, the agitation mode used was found to have a significant impact

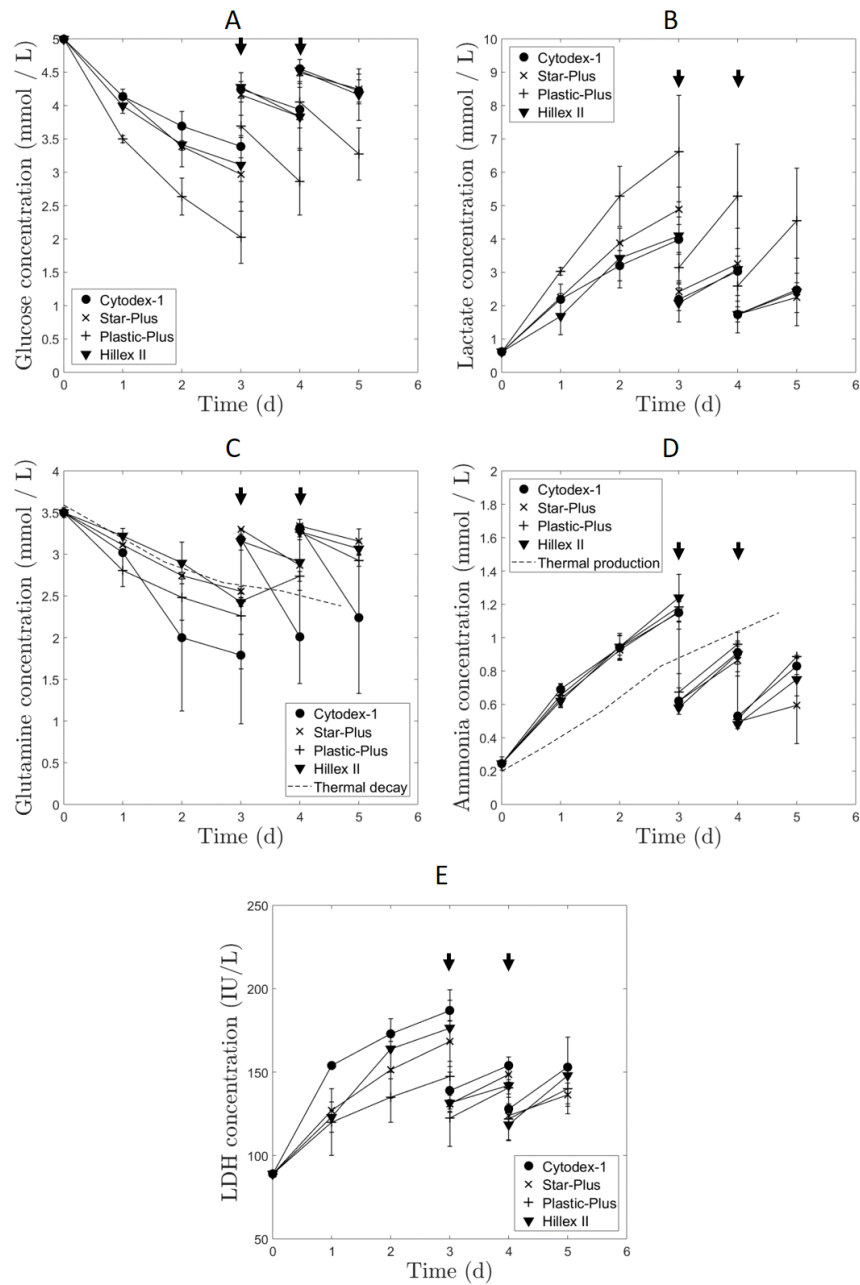


Figure 13: Kinetics of metabolite concentrations during cultures stirred with a mechanical agitation and according to the type of microcarrier used: Glucose (A), lactate (B), glutamine (C), ammonia (D) and LDH (E). Vertical arrows correspond to 50 % culture medium exchange ($n = 2$).

Table 5: Kinetic parameters calculated for cultures involving a mechanical agitation and according to the type of microcarrier used ($n = 2$).

Microcarrier	F^{exp} (-)	Y_{Glu} $\times 10^{-10}$ (mol cell $^{-1}$)	Lac/Glu (g gl $^{-1}$)
Cytodex-1*	0.60	0.4	2.2
Star-Plus	0.14	1.7	2.1
Plastic-Plus	0.15	3.7	2.2
Hillex II	-0.10	N/A	1.9

on microcarrier performances. Static cultures of cells attached on microcarriers were found not representative of what could occur during dynamic cultures, in particular concerning Hillex II microcarriers. In the case of the orbital agitation, cells presented the best adhesion capability on Cytodex-1, the highest expansion rate on Star-Plus, and Plastic-Plus and Hillex II were found unsuitable for MSC cultures in Erlenmeyer flasks. By using a mechanical agitation, similar number of cells were obtained with Cytodex-1, Star-Plus and Plastic-Plus, but cell expansion seemed to involve different carbon sources with a higher glutamine consumption for cells on Cytodex-1 than with others. Hillex II was still found unsuitable with this agitation mode. As a result, microcarrier selection in static mode for applications in dynamic mode may be questionable, considering that performances in this condition could not be necessarily transposed to agitated cultures.

Cell detachment from microcarriers

The last criterion to compare microcarriers adequacy for MSC cultures is the ease of detachment. Results of the microscopic analysis performed on

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8 522 microcarriers recovered after filtration used for the cell-particles separation
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10 523 are presented in Fig. 14. Cells presented good detachments from Star-Plus,
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12 524 Plastic-Plus and Hillex II, with almost no cells observed on microcarriers.
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14 525 On the contrary, many cells remained attached on Cytodex-1.
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16 526 Cell number and viability were controlled after cell detachment and filtra-
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18 527 tion (Table. 6 and 7). In static mode, the cell viability was similar for all
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20 528 microcarriers, between 81 and 85 %. However, cells on Cytodex-1 presented
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22 529 lower viabilities of 83 % and 73 % after cultures with respectively both or-
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24 530 bital and mechanical agitations, than those on Star-Plus and Plastic-Plus
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26 531 microcarriers. Concerning Hillex II, in stirred cultures, the detachment step
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28 532 damaged cells and led to cell viabilities of 61 % (orbital agitation) and 62
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30 533 % (mechanical agitation), explaining the poor number of cells counted on
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32 534 microcarriers of only 0.5 to 0.6 10^5 cells.

32 Table 6: Cell number counted after detachment according to the type of microcarrier (\times
33 10^5 , $n = 2$).
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35 Microcarrier	36 Static	37 Orbital agitation	38 Mechanical agitation
39 Cytodex-1	40 1.3 ± 0.2	41 3.4 ± 1.8	42 0.5 ± 0.1
43 Star-Plus	44 1.9 ± 0.9	45 2.8 ± 0.6	46 1.2 ± 1.0
47 Plastic	48 1.1 ± 0.3	49 N/A	50 N/A
51 Plastic-Plus	52 1.3 ± 0.5	53 3.5 ± 0.9	54 2.0 ± 1.3
55 Hillex II	56 1.7 ± 1.1	57 0.5 ± 0.3	58 0.6 ± 0.6

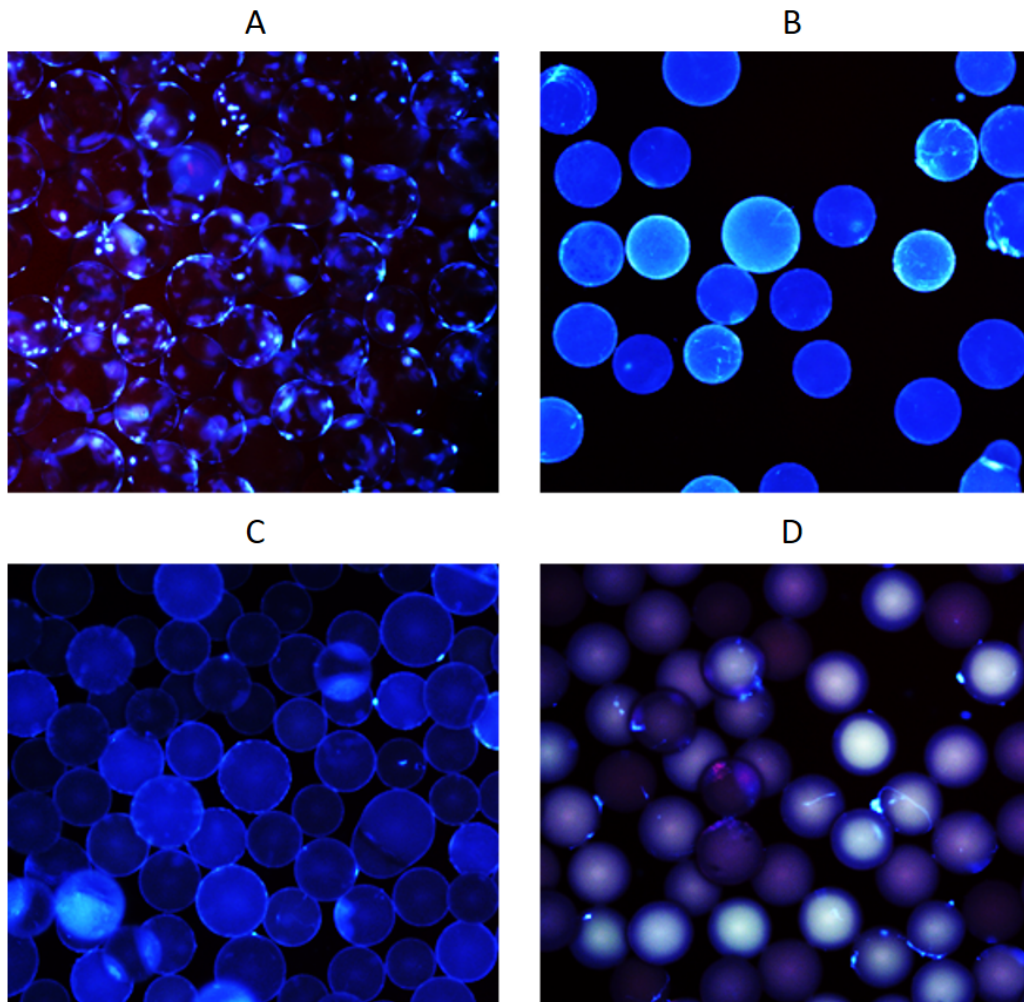


Figure 14: DAPI cell nuclei staining after cell detachment and filtration: Cytodex-1 (A), Star-Plus (B), Plastic-Plus (C) and Hillex II (D).

535 *MSC identity validation*

536 *Surface antigen expression*

537 WJ-*MSC* immunophenotype was characterized for various operating con-
538 ditions in dynamic mode, involving the four microcarriers (Cytodex-1, Plastic-

Table 7: Cell viability after detachment according to the type of microcarrier ($n = 2$).

Microcarrier	Static	Orbital agitation	Mechanical agitation
Cytodex-1	82 % \pm 7	83 % \pm 1	73 % \pm 4
Star-Plus	81 % \pm 10	95 % \pm 0	87 % \pm 5
Plastic	78 % \pm 5	N/A	N/A
Plastic-Plus	83 % \pm 16	94 % \pm 1	84 % \pm 8
Hillex II	82 % \pm 7	61 % \pm 17	62 % \pm 13

Plus, Star-Plus and Hillex II) and the two agitation modes (orbital and mechanical) studied. Fig. 15 presented the percentage of expression obtained from MSC-specific cell surface markers. In all cases, less than 5 % expressed HLA-DR, CD45 and CD34. Concerning CD44, CD166, CD90 and CD73, more than 90 % cells expressed these markers, except in the cases of cultures on Cytodex-1 with an orbital agitation and on Star-Plus with a mechanical agitation, where CD90 markers were respectively expressed by 78 % and 75 % of the cell populations. Even that the role of CD90 in MSC remains unknown, its expression has been related to the maintain of the undifferentiated state [46] and the immunopressive properties of MSC [47]. Moreover, it has been reported a decrease on CD90 expression on MSC submitted to mechanical stimulation [48]. Consequently, the decrease on CD90 expression found in this study could indicate a shift in the stemness state of MSC towards a state more susceptible to differentiation. Nevertheless, the percent of CD90 expression remains higher than 75 %. All these values allow to validate the cell immunophenotype for stem cell characterization.

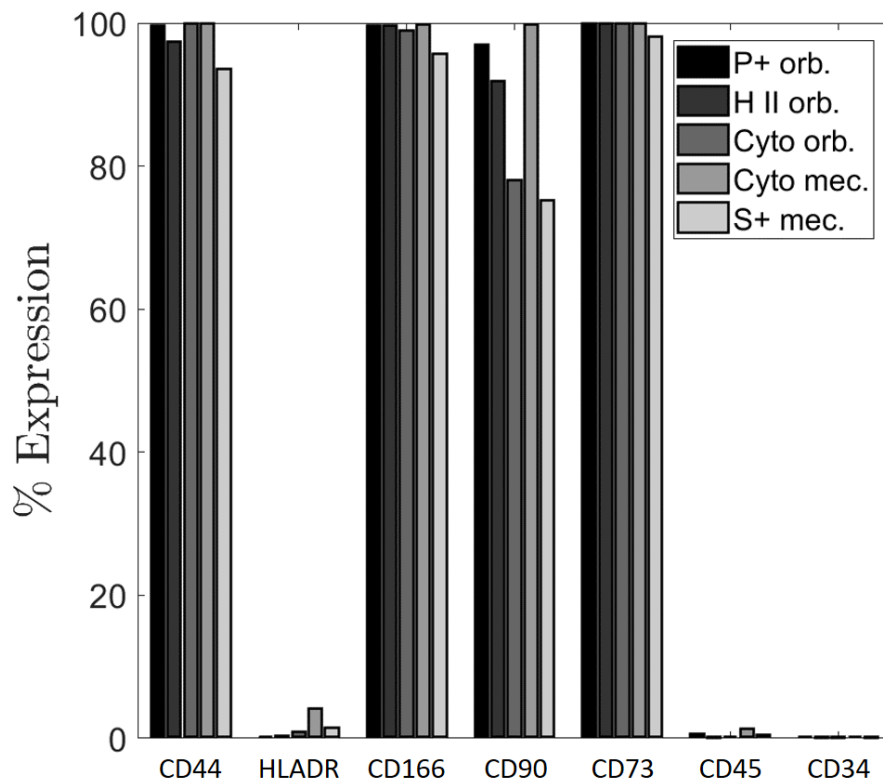


Figure 15: Characterization of GW-MSC immunophenotype, expanded under various dynamic conditions, by flow cytometry: Cells cultivated with an orbital agitation on Plastic-Plus (P+ orb.), Hillex II (H II orb.) and Cytodex-1 (Cyto orb.), and with a mechanical agitation on Cytodex-1 (Cyto mec.) and Star-Plus (S+ mec.).

555 *Cell differentiation validation*

556 Differentiated cells into adipocytes, osteocytes and chondrocytes were
 557 observed after cell staining, as shown in Fig. 16. The type of microcarrier
 558 used and the agitation mode did not impact cell multipotency. However,
 559 in all cases the number of adipocyte differentiated cells was low, as already
 560 reported in the literature [49]. MSC from Wharton's jelly seemed to require
 561 more time than other MSC for adipocyte differentiation.

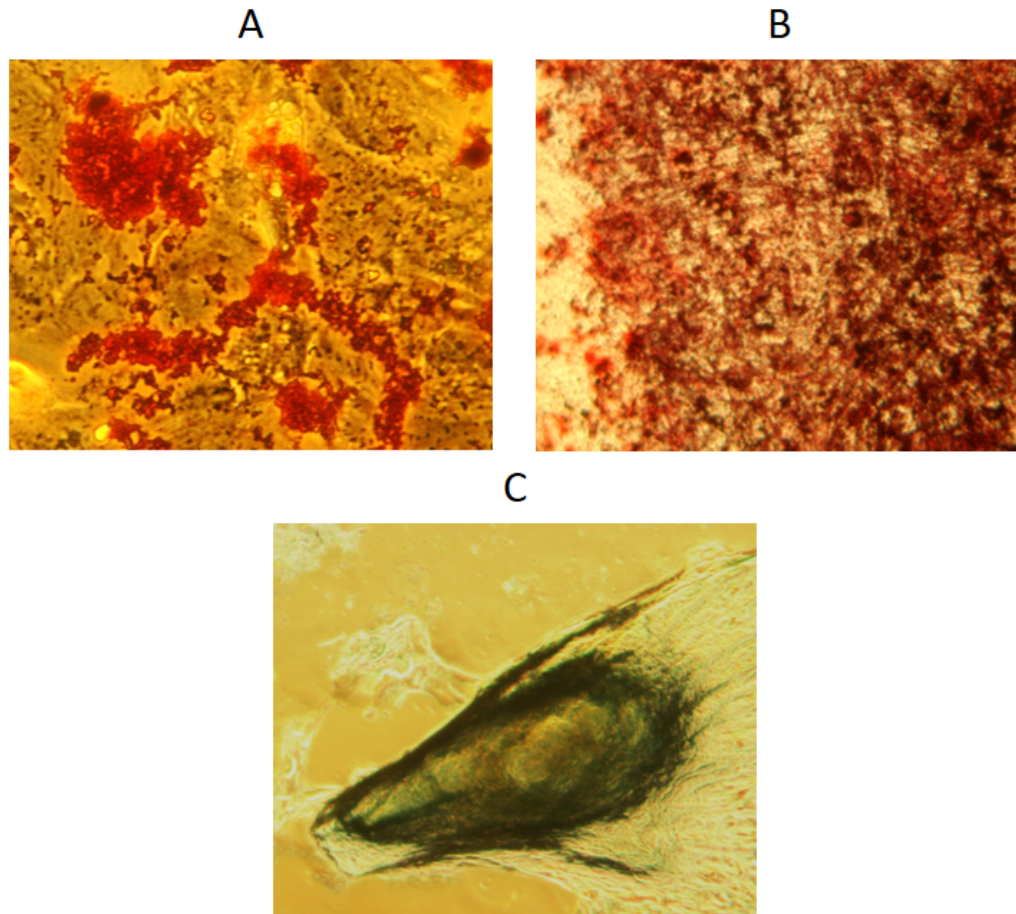


Figure 16: Differentiated WJ-MSC: (A) adipocytes stained with Oil Red, (B) osteocytes stained with Alizarin Red, (C) chondrocytes stained with Alcian blue.

562 *Microcarriers performances comparison*

563 Microcarriers were finally compared according to the three criteria con-
564 cerning cell attachment, expansion and ease of detachment. Each agitation
565 mode led to the results summarized in Table 8. In the present study, the
566 growth performance comparison was done relatively to the different micro-
567 carriers used, for each agitation mode. In static mode, Cytodex-1, Star-Plus,

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8 568 and Hillex II presented the best attachment and expansion performances,
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10 569 but Cytodex-1 was found to be unsuitable for the cell detachment step. A
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12 570 significant fraction of cells remained attached, leading to cell loss at the end
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14 571 of the culture process. Using an orbital agitation, Cytodex-1 was preferred
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16 572 for attachment and expansion performances, but as for static cultures, the
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18 573 detachment step remained difficult. Consequently, Star-Plus microcarriers
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20 574 seemed to be the best compromise considering the three criteria of the study.
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22 575 In dynamic mode using mechanical agitation, Cytodex-1, and Plastic-Plus
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24 576 presented similar attachment and expansion performances, but the detach-
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26 577 ment step was still difficult for Cytodex-1, making Plastic-Plus more reliable.
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28 578 In both dynamic cases, Hillex II presented the lowest attachment and expan-
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30 579 sion performances, despite promising results in static mode.

31 580 **Conclusion**

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33 581 Microcarriers were compared for three different agitation modes, namely
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35 582 static mode, orbital and mechanical agitation. Three culture steps were
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37 583 investigated, involving the attachment efficiency, the cell proliferation and
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39 584 the ease of detachment. It was assumed that all these three criteria were
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41 585 equally important for the cultivation process improvement. Results showed
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43 586 that performances obtained in static mode were not necessarily representa-
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45 587 tive of culture performances in dynamic mode. They confirmed the need to
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47 588 already transpose the investigated agitation mode for microcarrier screening
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49 589 and selection before scale-up. Cell aggregates were notably observed in static
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51 590 cultures for Cytodex-1 and Plastic-Plus, but were found to be well dispersed
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53 591 in agitated mode.

Table 8: Microcarriers performances summary according to the agitation mode.

Microcarrier	Cytodex-1	Star-Plus	Plastic	Plastic-Plus	Hillex II
Static mode					
Cell attachment on microcarriers	++	++	-	++	++
Cell distribution and microcarrier occupancy	-	++	-	-	++
Cell expansion	++	++	-	+	++
Ease of detachment	-	++	-	++	++
Orbital agitation					
Cell attachment on microcarriers	++	+	N/A	+	-
Cell distribution and microcarrier occupancy	++	++	N/A	++	-
Cell expansion	+	++	N/A	+	-
Ease of detachment	-	++	N/A	++	+
Mechanical agitation					
Cell attachment on microcarriers	++	++	N/A	++	-
Cell distribution and microcarrier occupancy	++	++	N/A	++	-
Cell expansion	++	+	N/A	++	-
Ease of detachment	-	++	N/A	++	+

-: Lowest performances observed in the study.

+: Intermediate performances.

++: Best performances observed in the study.

N/A: Not applicable.

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8 592 This study demonstrated that the detachment step is still a challenge for
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10 593 the development of a robust process, especially when Cytodex-1 were used.
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12 594 Star-Plus and Plastic-Plus were found to be better compromises, respectively
13
14 595 for the orbital and the mechanical agitation modes.
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601 **Nomenclature***Abbreviations*

FBS	Foetal Bovine Serum
hPL	human Platelet Lysate
MSC	Mesenchymal Stem / Stromal Cell
WJ	Wharton's jelly

Variables

c_v	Coefficient of variation of the cell number per microcarrier distribution	(%)
F^{exp}	Cell expansion factor	(d ⁻¹)
Lac/Glu	Lactate concentration on glucose concentration ratio	(-)
n	Number of microcarrier	(-)
$\langle N_{cell/\mu C} \rangle$	Mean number of cell per microcarrier	cell / μC
N	Agitation rate	(rpm)
Y_{Glu}	Glucose consumption yield	(-)
$V_{working}$	Working volume	(mL)
x_{colo}	Colonized microcarrier fraction	(%)
s	Standard deviation of the number of cell per microcarrier distribution	cell / μC

Subscript

js	particle just-suspended state
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